

Electron Paramagnetic Resonance

Volume 25

A Review of the Recent Literature.

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Preface

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In recent years, the field of Electron Paramagnetic Resonance (EPR) spectroscopy has witnessed considerable developments in instrumentation that have provided greater resolution or higher sensitivity for the study of system bearing unpaired electrons. Noted developments in high field EPR instrumentation or improvements in smaller bench-top spectrometers, have ensured that the technique is finding wider applications in fields as diverse as quality control and advanced materials. The information that EPR can provide is not simply based on the detection of a free radical or paramagnetic ion, but detailed information on structure, conformation and dynamics on length/time scales not easily accessible by other techniques, can be readily obtained. Both pulsed and continuous wave (CW) EPR measurements contribute to this bountiful abundance of information that can be extracted on any paramagnetic species. Moreover, the utility of the technique can be enhanced, from a chemical perspective, by the use of ancillary spin probes, labels or traps that provide vital additional information on the spin system.

Therefore in this volume, we have drawn together a number of experts in the field of EPR to present the key trends, themes and topics of current relevance and interests in the field. Notably the use of nitroxides as spin labels in EPR to study the structure and dynamics of complex molecular systems remains extremely important, so we present a number of Chapters focussing on nitroxides as probes, labels or guests. In the field of advanced materials and quantum physics, the spin qubit is becoming more and more topical, so we also present the reader with a timely Chapter on the contributions of EPR to this growing field of research. We have also continued our coverage on the role of computational and theoretical methods to provide key insights into the analysis of EPR spectra.

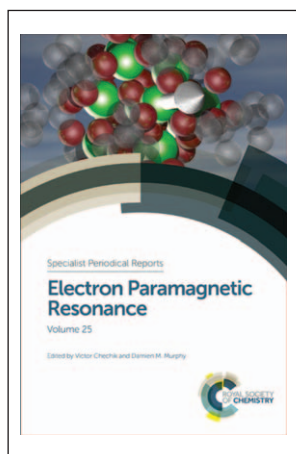
The use of nitroxide spin labels continues to be widespread in EPR spectroscopy, and Lovett *et al.*, presents a timely review Chapter demonstrating how the properties of these labels can be tuned and how they can be incorporated into biomolecules for structural investigations. The theme of spin labels is continued in the Chapter by Krumkacheva and Bagryanskaya, with a focus on the use of trityl radicals. Whilst these radicals have been used for oxymetry and tomography in the past, their use as spin labels for determining the structure of proteins and nucleic acid is relatively new, and these authors provide a comprehensive overview of the latest developments in this field. The area of site directed spin labelling for studying the conformational changes in the catalytic cycle of GTP-hydrolyzing proteins is then treated in detail in the Chapter by Klare. In this Chapter, the ability of EPR to analyse the conformational equilibrium dynamics and characterization of conformational changes in the functions of a protein is covered. The field of host–guest chemistry is then treated in the Chapter by Bagryanskaya and Marque, where the

authors consider the EPR analysis of nitroxides as guests within several different hosts including cyclodextrins, calixarenes and cucurbiturils, providing a comprehensive review of the literature over the past seven years.

On a very different topic, the Chapter by Sproules highlights the recent developments in the design and testing of new spin qubits in the challenging field of quantum computing, and provides illustrative examples of how EPR spectroscopy is crucial for understanding these molecular systems. Barone *et al.*, also offers the reader a broad perspective on the status of a virtual multi-frequency spectrometer for EPR analysis. Unquestionably theoretical calculations are vital to the modern EPR spectroscopist in order to compute reliable magnetic parameters, and even potentially simulate the entire spectrum from first principles, and this Chapter provides a glimpse into the latest exciting developments and implementations underpinning the 'virtual molecular spectrometer', focusing on nitroxide radicals as the exemplar spin system.

As always, we hope that both the expert EPR reader and novice practitioner will value these timely reviews, offering a broad perspective on the latest developments in the field. Finally, we would also like to thank all of our reporters for their expert, prompt and efficient cooperation in the production of these Chapters and the staff at the Royal Society of Chemistry for their editorial support and patience.

Victor Chechik (York) and Damien M. Murphy (Cardiff)



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Tuning the properties of nitroxide spin labels for use in electron paramagnetic resonance spectroscopy through chemical modification of the nitroxide framework

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and Janet E. Lovett^{*b}

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Spin labels containing nitroxyl radicals possess many properties that render them useful for electron paramagnetic resonance (EPR) spectroscopy. This review describes the relationships between the structure and properties of nitroxide spin labels, methods for their synthesis, advances in methods for their incorporation into biomolecules, and selected examples of applications in biomolecule structural investigations.

1 Introduction

Within the field of electron paramagnetic resonance (EPR) spectroscopy, 'spin labelling' describes the attachment of a radical or paramagnetic centre (*i.e.* a molecule containing at least one unpaired electron spin) onto a material of interest, which enables its investigation using paramagnetic resonance spectroscopy. For such applications, spin labels should ideally fulfil several criteria: the framework of the label must stabilise the radical against redox processes; the radical must possess desirable properties for the magnetic resonance experiment (such as chemical stability and spin coherence persistence); and, the label must be readily (and site-specifically) attached without structural distortion of the system under study.

By far the largest family of spin labels are those based on the nitroxyl (N-O[•]) radical, which are called nitroxide spin labels. These are typically five- or six-membered heterocyclic derivatives of piperidine, pyrrolidine, isoindoline, and other heterocycles containing two heteroatoms; importantly, the nitroxyl radical is flanked by two quaternary carbon atoms. The 'classic' nitroxide is the piperidine-based 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO, **1**, Fig. 1), which has found use in many chemical and materials applications. This radical, in which the unpaired electron is located mainly on the nitrogen and oxygen atoms, is stabilised by the steric screening imparted by its four adjacent methyl groups, which protect the radical from reduction or other processes. The lack of α -protons also prevents the decomposition of the nitroxyl to the corresponding nitron. Some other examples of common nitroxide families (2–7) are illustrated.

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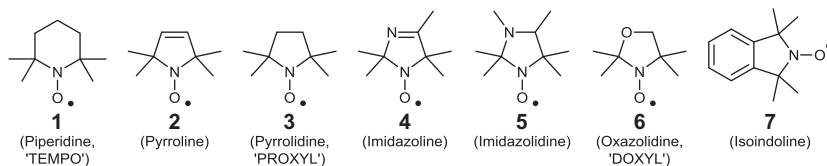


Fig. 1 Structures of parent nitroxides.

A 'spin label' can be defined as a derivative of the parent nitroxide in which the core ring system (or its substituents) is modified to enable its incorporation into a larger framework, and thus to be used as a probe. The framework of interest may be a polymer, a surface, or a biomolecule such as a protein, nucleic acid, sugar or lipid.

Spin labels are commonly used to measure interspin distances (*i.e.* the distance between two stable free radicals) using continuous wave (CW) or pulsed techniques, which for nitroxides have an effective range of 0.5 to >10 nm.^{1–3} They can also be used to probe the local environment of the label, such as its accessibility and dynamic mobility.⁴ Nitroxides are also employed as paramagnetic relaxation enhancers in nuclear magnetic resonance (NMR) spectroscopy, and as polarisation/contrast agents in dynamic nuclear polarisation (DNP) or magnetic resonance imaging (MRI) experiments.^{5–7}

Modification of the basic structure of the nitroxide can lead to dramatic changes in the properties of the spin label, and it is for this reason that a myriad of spin labels have been designed.^{8–18} Essential considerations centre on the structure of the nitroxide around the nitroxyl radical itself, and the functionality used to enable spin labelling. This chapter discusses these aspects, along with recent advances in the synthesis and applications of nitroxide spin labels in EPR spectroscopy.

2 The nitroxide spin label as a probe in EPR spectroscopy

2.1 Magnetic properties

The Zeeman splitting for the nitroxide spin labels is anisotropic and typically $g_{xx} > g_{yy} > g_{zz}$ with g_{xx} and g_{yy} close in value and greater than the free electron g -value. The g_{zz} axis is roughly coincident with the π orbital, approximated as a linear combination of the $2p_z$ orbitals of oxygen and nitrogen. Therefore, in planar systems such as pyrrolinoxyl spin labels, the g_{zz} is perpendicular to the plane of the ring. The x -axis is taken as coincident with the NO bond.¹⁹

The unpaired electron is considered to reside in the π orbital. The spin density is on the nitrogen and oxygen with almost no delocalisation over the rest of the framework (for adjacent alkyl groups).^{20,21} The hyperfine coupling constant, A_{iso} , for nitroxide spin labels is typically in the region of 40 to 47 MHz. Due to the relative spin localisation the hyperfine splitting of the Zeeman levels is dominated by the nitrogen of the NO group (^{16}O nuclei have zero spin and the predominant isotope of nitrogen is ^{14}N with a nuclear spin, I , of 1). The hyperfine axes approximately follow the g -tensor principal axes with $A_{xx} \approx A_{yy} < A_{zz}$ where A_{zz} is

typically about 100 MHz. Both g and A_{iso} are weakly sensitive to solvent polarity and proticity, with A_{iso} increasing and g -values decreasing in increasingly polar/protic solvents.^{22,23} This property has been used to map membrane protein channels, and to probe changes in solvent behaviour associated with the glass-transition temperature in water/glycerol mixtures.^{23,24}

Coupling of proximal nuclei ($I \neq 0$) to the electron gives rise to the characteristic appearance of an EPR spectrum. Usually, the greatest splitting is caused by coupling to the nitroxyl nitrogen atom, with much smaller splitting from other ring substituents. However, this super-hyperfine splitting can afford additional information, such as the extent of protonation of imidazolinyll and imidazolidinyl spin labels.^{25–29} The nature of the substituents flanking the nitroxyl radical can also significantly affect signal linewidth.³⁰ Substituent effects on the ring conformation can be influential: the faster dynamic averaging of the hyperfine interaction of the nitroxyl with the methyl protons in 4-oxo-TEMPO (**8**, Fig. 2) compared to 4-hydroxy-TEMPO (**9**) can be explained by a higher barrier to conformational ring flip in the latter, where the ring framework is fully sp^3 -hybridised (aside from the nitroxyl).³¹ Isotopic labelling such as perdeuteration or ^{15}N -substitution, can also lead to line narrowing, and therefore improve the sensitivity of the spin label.³² This property has been used to improve the precision of measurement of tumbling rates, and in oximetry.^{33–35}

The positioning of certain spin-active nuclei directly adjacent to the nitroxyl can cause more significant splitting. For example, the phosphorus nucleus in PROXYL spin label **10** (Fig. 2) has $I = 1/2$, with the largest hyperfine coupling at 140 MHz.³⁶ The resultant two-line spectrum is then split by the nitrogen atom to give a six-line pattern. It was shown that this spin label is a sensitive probe of dynamics, which suggests that it may be possible to simultaneously label a molecule of interest at two sites, with this label and a standard nitroxide, to enable simultaneous but distinguishable measurements.

The anisotropy in the g and A tensors are such that when the spin labels can rotate rapidly, as might be the case in low-viscosity organic solvents at room temperature, the CW EPR spectrum is motionally narrowed and reveals only g_{iso} and A_{iso} . However, if the tumbling dynamics of the system are slowed, through increasing viscosity, decreasing temperature, or tethering to a larger, more slowly diffusing molecule, the measured line shape of the nitroxide alters (Fig. 3). At X-band microwave frequencies the changes in the lineshape are sensitive to rotational correlation times (τ_c) in the ns region, with particular sensitivity to changes in

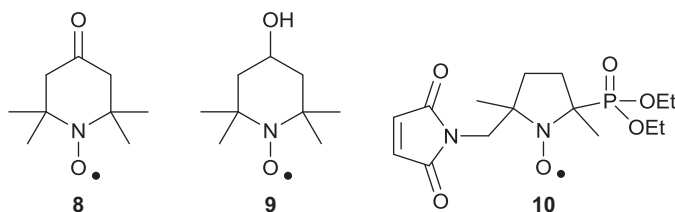


Fig. 2 Structures of TEMPO derivatives and a phosphonate-substituted nitroxide.

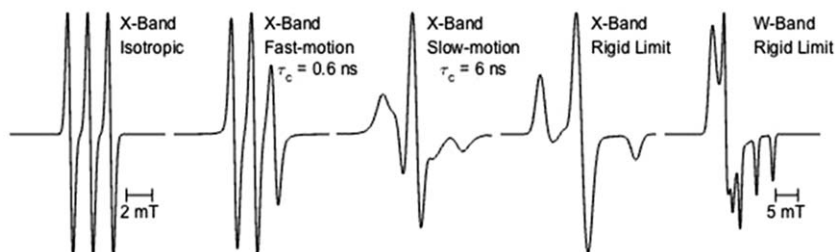
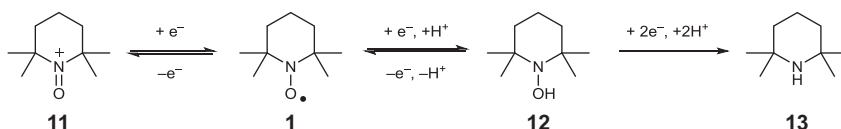


Fig. 3 Representation of the link between τ_c and CW lineshape at X-band microwave frequencies. A comparative W-band CW spectrum shows increased spectral anisotropy. Spectra were simulated with EasySpin using typical parameter values.^{37,47}



Scheme 1 The nitroxyl radical is susceptible to redox reactions.

dynamics when $\tau_c \sim 1$ ns. Altering the microwave frequency will alter the timescales measured and higher frequencies will offer enhanced angular resolution.^{37,38} There are many reports of using this property to map global and local dynamics in spin-labelled proteins.^{38–46}

2.2 Chemical stability of the radical

The nitroxyl radical **1** can undergo redox processes to produce the oxoammonium cation **11** (Scheme 1), hydroxylamine **12** or secondary amine **13**.⁷ From the viewpoint of maintaining the radical on the spin label (*e.g.* to enable EPR studies in living cells), protection against reduction to the hydroxylamine is important. The stability of a given nitroxyl radical to reduction is not only determined by its electrochemical reduction stability,^{48,49} but also by the cyclic framework in which it is contained, and the nature (charge, size, *etc.*) of the associated substituents.^{50,51}

The stability of nitroxyl radicals to reduction is often assessed using the biologically-relevant ascorbic acid as a reducing agent, albeit this is only a simple model for the various processes and differing reducing environments that may be encountered in cells.^{51–53} Through comparison of susceptibility to ascorbate, some general properties of nitroxides have been identified which improve the resilience of the radical in reducing environments. The first of these is that five-membered ring nitroxides are significantly more stable towards reduction than six-membered.^{50,54,55} This is likely a consequence of the change in hybridisation of the nitrogen atom that occurs on reduction, where torsional strain is relieved on going from sp^2 to sp^3 hybridisation for six-membered rings, but increases for five-membered rings. Inductive effects of substituents on the ring can also play a role, particularly for substituted pyrrolinoxyl radicals.^{48,50,56} An approximate order of stability is illustrated in Fig. 4.

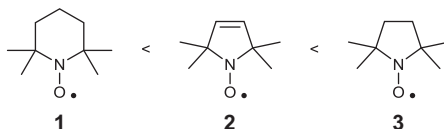


Fig. 4 The stability of nitroxyl radicals towards reduction correlates with the size and nature of the nitroxyl-bearing ring.

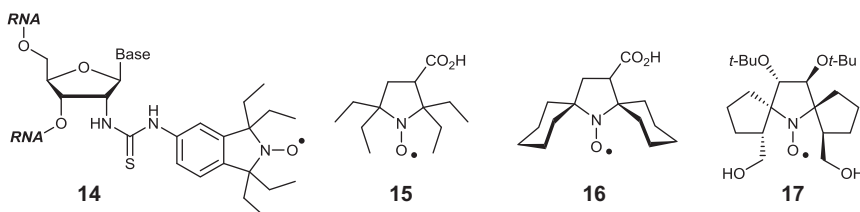


Fig. 5 Nitroxide frameworks shown to be particularly stable towards reduction.

The second general stabilising effect is that of increasing the steric bulk of the flanking alkyl substituents which shield the radical from reduction, or stabilise it relative to the hydroxylamine where an equilibrium exists.⁵⁶ For example, tetraethyl-substituted isoindolinyl nitroxides have been shown to be highly resistant to ascorbic acid reduction, and its stability was further enhanced when bound to the ribose of RNA *via* a thiourea linker (**14**, Fig. 5).⁵⁷ The enhanced stability of the PROXYL framework can be combined with such steric protection to give particularly stable radicals: the tetraethyl-flanked PROXYL (**15**) remains ~90% intact after two hours exposure to ascorbate or frog oocyte cells/cell extract.^{51,58} Bis(spirocyclohexyl) groups flanking the nitroxyl (**16**) also confer stability against bioreduction, although to a lesser extent than tetraethyl substituents. This is likely due to less effective steric shielding for a cyclohexane ring compared to the more mobile ethyl groups.^{52,56,58,59} However, some spirocyclic systems can confer remarkable stability: the fully substituted PROXYL (**17**) was found to be exceptionally resistant to ascorbic acid reduction.⁶⁰

One caveat in label design is that many of these extended alkyl chains increase label hydrophobicity, which may cause problems for some labelling strategies, or unwanted sample aggregation.⁶¹ The electronic influence of substituents can also affect stability, and a careful consideration of both steric and electronic effects is therefore required when designing labels.⁴⁹

2.3 Use of nitroxides in DEER

The pulsed EPR experiment known as double electron–electron resonance (DEER), or pulsed electron double resonance (PELDOR), has become a useful method for measuring nanometre distances between pairs of nitroxides.^{2,3,62–64} The popularity of this experiment in bis-nitroxide systems originates from its use of two microwave frequencies, which allows much of the nitroxide spectrum to be measured under common hardware limitations. The 4-pulse DEER experiment measures

the dipolar coupling frequency as a modulation on a refocused echo.^{65,66} The DEER measurement needs to be set up such that the dipolar frequency can be measured accurately, which means that the echo must have a good signal-to-noise ratio (SNR) with the appropriate DEER inter-pulse delays (*i.e.* time window). The SNR can be improved by repeating the experiment and averaging results. The repetition rate is optimal when the echo is approximately more than 70% recovered, at $1.2 \times T_1$ (longitudinal relaxation time), where T_1 values are typically on the order of one millisecond.⁶⁷ The time window is dependent upon the spin coherence time (T_m) of the spin label. It is often shortest for samples with high local or global concentration, as has been explored for spin-labelled proteins in lipid membranes.^{62,68} Relaxation due to instantaneous diffusion can be reduced by working at as low concentrations as feasible and through careful choice of pulse lengths in experiments such as DEER.⁶² For the 5- and 6-membered nitroxides with four methyl groups flanking the radical, this balance between the T_1 and T_m relaxation times with the Boltzmann distribution is often optimal at around 50 K.⁶²

A typical soluble spin-labelled biomolecule would have a T_m time of 2–3 μ s and this would allow for a time window of *ca.* 3 μ s which corresponds to the accurate measurement of a 3.5 nm distance. However, it has been found that using deuterated solvent and cryoprotectant significantly lengthens the T_m time since the ^2H nuclei have a lower magnetic moment than ^1H , and this reduces the rate of relaxation through spin diffusion.^{69–71} The loss through spin diffusion can also be reduced by using more advanced DEER pulse sequences, *e.g.* 5-pulse DEER.⁷² Further, Norman and co-workers have shown that deuteration of the molecule the spin label is attached to, in their case a protein, reduces the contribution from this relaxation mechanism to such an extent that distances over 14 nm can be measured accurately.^{2,73,74} Interestingly, isotope substitution of the protons on the *gem*-dimethyl substituents does not extend the relaxation time.⁶² Conversely, in CW EPR where distances over 1 nm are assessed by the dipole–dipole broadening on the spectral linewidth, it has been shown that deuteration of the label increases the upper measurable distance from 2 nm to about 2.5 nm.⁷⁵ The alternative isotope substitution of ^{15}N at the nitroxyl moiety has been applied to allow orthogonal labelling using two, or more, nitroxide spin labels: this makes use of the two microwave frequencies used in DEER, and indeed its CW predecessor ELDOR, and the only partial spectral overlap between the ^{15}N ($I = 1/2$) and ^{14}N ($I = 1$) EPR lineshapes.^{76,77}

The lower distance limit in the DEER experiment is determined by the requirement that the bandwidth of both sets of pulses can excite the full dipolar lineshape. In practice this has set the lower limit at approximately 1.5 nm.^{3,78} The DEER experiment itself only requires that the labelled molecule does not tumble fast enough to average out the dipole–dipole coupling between the spin labels.⁷⁹ Thus, if the spin labels can be optimised such that their relaxation rates are favourable for measurement at higher temperatures, then tethered or otherwise immobilised molecules could be used. This would open up the possibility of measurement at or near physiological temperatures.^{80,81}

The DEER technique is capable of extracting distances and their distributions with nanometre accuracy, and also orientational information between the labels when the nitroxide spin labels have a well-defined, narrow distribution of conformations with respect to one another.^{82–86} The likelihood of this occurring is increased if the label is conformationally restricted through either steric bulk, or linker restriction. However, it is important that disruptions to the material are minimal, and that the label can be used in a facile manner through simple and efficient labelling procedures.

The conformations and dynamics of some spin labels attached to biomolecules, particularly methanethiosulfonate (MTS, **18**, Fig. 6), have been investigated computationally and experimentally through EPR analysis and crystallography.^{87–99} Importantly, there is software freely available to enable users to label their target *in silico* and calculate the most probable conformations, since the conformation of the spin label tether must also be considered when interpreting DEER measurements.^{100–102}

2.4 Spin relaxation rates

The DEER experiment relies on measuring the dipolar coupling frequency between the spin labels, which increases as r^{-3} where r is the distance between the radicals. Hence, the measurement of relatively long distances requires the spin labels to have long spin coherence times, which also increases the concentration sensitivity of the experiment for a given dipolar frequency. Nitroxide spin label measurements are usually carried out at 50 K, and enhancing spin coherence times could enable measurements at higher temperatures (*i.e.* liquid nitrogen, rather than the expensive liquid helium currently required by laboratories not equipped with a closed-circuit cryostat). Ultimately, measuring DEER at higher temperatures, perhaps even physiological temperatures, would provide valuable structural information for many systems.

At 50 K, tetramethylpyrrolinoyl spin labels such as MTS (**18**, Fig. 6), have optimal relaxation times for the DEER experiment; other nitroxide frameworks either match these relaxation times, or compare unfavourably to the pyrroline structure. However, for methyl-flanked nitroxyl radicals, as temperatures increase above ~ 70 K, the adjacent methyl groups begin to rotate at a rate comparable to the hyperfine anisotropy, which provides a route for dephasing (Fig. 7). Higher aliphatic groups (such as ethyl) are expected to provide a similar relaxation mechanism, albeit with a different energy barrier.¹⁰³ However, adjacent cyclic groups (*i.e.* bis(spirocyclic) nitroxides) cannot undergo this rapid rotation and

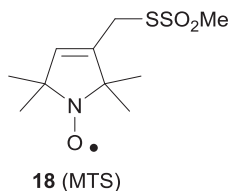


Fig. 6 Structure of the methanethiosulfonate (MTS) nitroxide spin label.

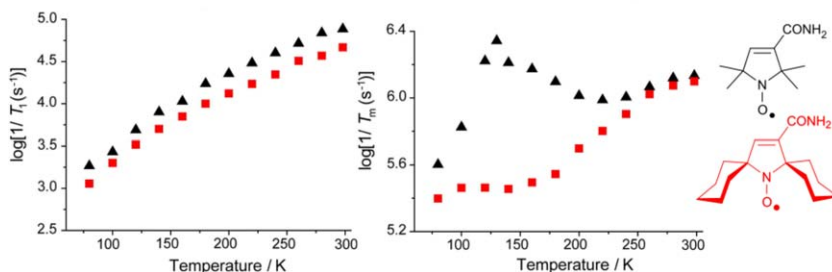


Fig. 7 The temperature dependence of T_1^{-1} and T_m^{-1} for tetramethyl- (triangles) and bis(spirocyclohexyl)-substituted (squares) pyrrolinoxyls. Image modified and reprinted from ref. 81 with permission from Elsevier.

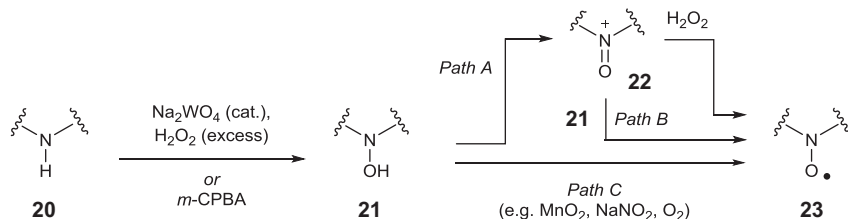
consequent modulation of the field; the T_m for these nitroxides decreases much more slowly with increasing temperature, as other motions are able to modulate the g and A anisotropy.^{104,105} As the glassy solvent matrix softens and eventually melts, the echo dephasing is dominated by motional processes.¹⁰³ Increasing the temperature from 50 K also decreases the T_1 (Fig. 7), and this is dependent upon the structure of the spin label, the degree of spin-orbit coupling and the nature of the solvent.¹⁰³

Despite the above properties, spirocyclic groups do not appreciably increase T_m relative to *gem*-dimethyl-containing nitroxides when measured at ambient temperatures in a trehalose (disaccharide) matrix (Fig. 7).⁸¹ However, a spirocyclohexyl-iodoacetamido-TEMPO label (see **19**, Fig. 9) attached to T4 lysozyme (T4L) allowed for an ambient temperature DEER measurement of 3.2 nm to be taken, whereas MTS (**18**) did not.⁸⁰ A similar effect was found for labelled DNA immobilised in trehalose.⁸¹ Therefore, just as for free labels, the interaction between the label and its environment is important for determining T_m .¹⁰⁵ This effect will be crucial if DEER experiments are to be made at physiological temperatures.

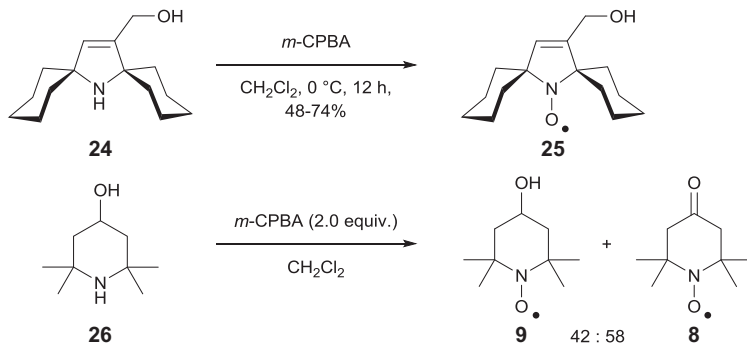
3 Synthetic routes to nitroxide spin labels

3.1 Synthesis of nitroxyl radicals

A prerequisite for the synthesis of any spin label is the installation of the nitroxyl functionality itself. This is most commonly achieved through oxidation of the corresponding secondary amine, for which a variety of oxidants can be used (Scheme 2). The most popular method involves treatment of the amine **20** with excess H_2O_2 and a catalytic amount (typically 15–20 mol%) of Na_2WO_4 .⁹ Although frequently employed, the high polarity of the solvent needed to solubilise the catalyst (usually water/ethanol mixtures) can lead to poor substrate solubility for lipophilic spin labels, and consequent long reaction times and/or low conversion.¹⁰⁶ The mechanism of this process involves oxidation of **20** to the hydroxylamine **21**, which is further oxidised by the relatively strong tungsten(vi) oxidant to oxoammonium salt **22** (Path A). This salt is in turn able to oxidise H_2O_2 to O_2 , itself being reduced to the nitroxyl **23**, or can react with residual hydroxylamine **21** to form two molecules of nitroxyl radical **23** (Path B).¹⁰⁷ Pre-formed hydroxylamines may also be oxidised to



Scheme 2 Methods for the preparation of nitroxyls.



Scheme 3 *N*-Oxidation by *m*-CPBA, and functional group tolerance.

the nitroxyl by mild oxidants such as MnO_2 or NaNO_2 (Path C);^{58,108} this latter oxidation can even proceed spontaneously in the presence of atmospheric oxygen under neutral or basic conditions.⁹

An alternative method for amine oxidation employs 1.5–2 equivalents of *m*-CPBA.^{109,110} This reagent often results in a rapid and high-yielding oxidation, which likely proceeds *via* path B in Scheme 2.¹¹¹ It is tolerant of other functional groups: for example, the double bond of pyrroline **24** (Scheme 3) was unaffected during nitroxyl formation (**25**).¹¹² One cautionary note in all of these oxidations is that the oxoammonium salt **22** (formed in both the $\text{Na}_2\text{WO}_4/\text{H}_2\text{O}_2$ and *m*-CPBA methodologies) has been observed to effect oxidation of alcohols, as in the oxidation of aminoalcohol **26** to a mixture of nitroxyl alcohol **9** and ketone **8**.^{109,110,113}

Due to the potential susceptibility of nitroxyls to reduction or oxidation during chemical synthesis or under biological conditions, several interesting protection strategies have been employed that allow a late-stage deprotection of the nitroxyl itself. This includes the use of an *O*-methyl hydroxylamine derivative **27** (Fig. 8), a robust functionality from which the nitroxyl can be revealed on treatment with *m*-CPBA.¹¹⁴ An elegant photolabile protecting group strategy has also been developed, where irradiation of **28** at 405 nm delivers the nitroxyl in high yield (92%).¹¹⁵ This latter chemistry has been applied to a masked nitroxyl attached to a DNA oligonucleotide at cytidine.

3.2 Synthesis of piperidinyl (TEMPO) nitroxides

2,2,6,6-Tetramethyl-piperidone (**29**, Scheme 4) is ubiquitous as a starting material for the synthesis of the frequently used TEMPO

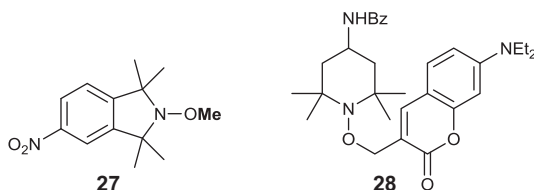
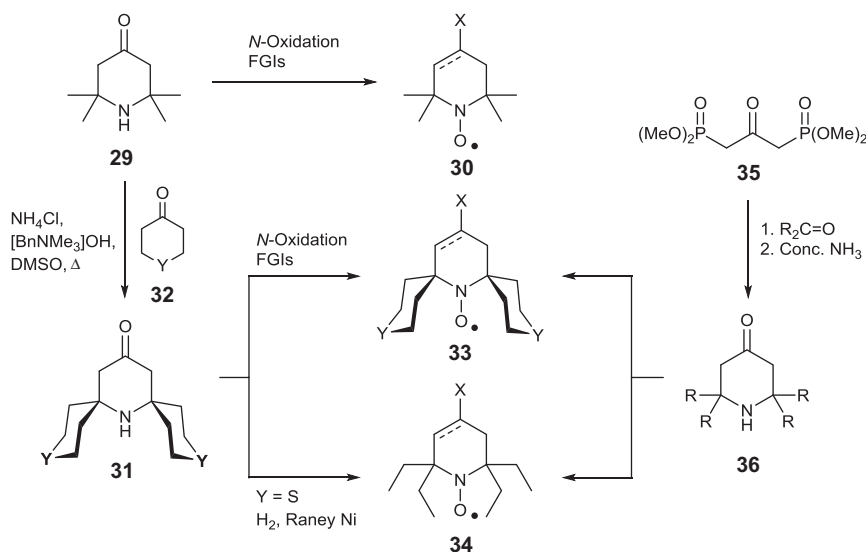


Fig. 8 Masked nitroxyl radicals.



Scheme 4 General synthetic routes to 6-membered ring piperidiny spin labels.

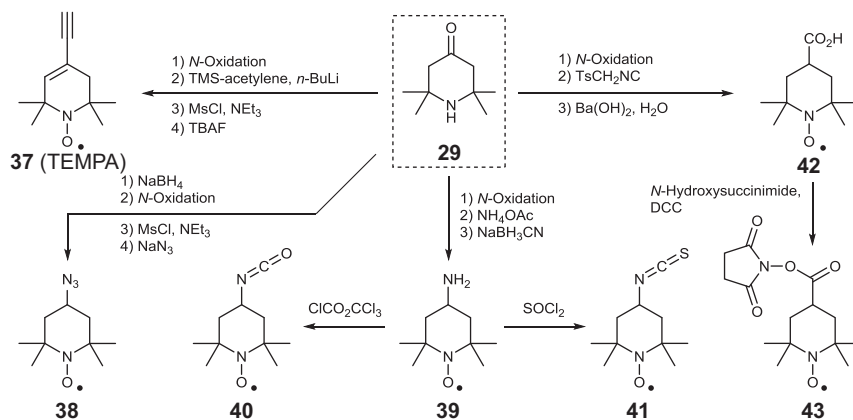
spin labels.⁹ Straightforward functional group interconversions (FGIs) lead to a wide variety of functionalised 2,2,6,6-tetramethylpiperidines (**30**, see following discussion).

An important and convenient method to transform **29** into 2,6-bis(spirocyclic) piperidones **31** ($Y = \text{CH}_2$, O, C=O, SO_2 , NAC, *etc.*) uses a phase-transfer catalyst to effect a formal exchange of acetone for ketone **32**.¹¹⁶ This chemistry provides access to spirocyclic piperidine spin labels **33**, many of which have been found to exhibit enhanced phase memory times in EPR experiments due to the restricted rotational freedom imparted by the spirocyclic ring system.¹⁰⁴ When $Y = \text{S}$, reductive cleavage of the C–S bonds with Raney nickel leads to the tetraethyl-substituted spin label **34**, which offers improved steric shielding of the nitroxyl.^{58,116}

Whilst this ketone exchange methodology is procedurally simple and highly effective for the synthesis of spirocycle-containing spin labels, an alternative ‘*de novo*’ route to piperidine-derived nitroxides involves a double Horner–Wadsworth–Emmons reaction of bisphosphonate **35** to generate an intermediate dienone, which is a substrate for double aza-Michael addition of ammonia leading to piperidone **36**. This method has been used to prepare tetraethyl-substituted ketone and alcohol nitroxides (**34**, $X = \text{O}$ or OH, respectively).¹¹⁷ A similar route has been used to prepare a bis(spirocyclohexyl)ketone (**33**, $X = \text{O}$, $Y = \text{CH}_2$).¹¹⁸

The substituent X at the 4-position of the piperidine ring in **30**, **33** and **34** is easily functionalised to prepare the spin label for attachment to target systems of interest (Scheme 5). *N*-Oxidation of ketone **29**, followed by addition of trimethylsilyl acetylide, and subsequent dehydration and desilylation, leads to enyne nitroxide **37** which is primed for CuAAC click reactions¹¹⁹ or Sonogashira cross-coupling.^{120–123} Alternatively, reduction of the ketone to an alcohol, conversion to a leaving group, and nucleophilic substitution by azide affords the complementary CuAAC azide partner **38**.¹²⁴ Amine **39**, which is suitable for target labelling by methods such as amide formation¹²⁵ or alkylation,^{123,126–130} can be installed by reductive amination with ammonia.¹³¹ **39** can also be converted to isocyanate **40** or thioisocyanate **41** on treatment with diphosgene or thionyl chloride, respectively;^{132,133} these spin labels undergo reaction with amines to give (thio)urea-linked spin labels.^{132,134} Ketone **29** may also be transformed into acid **42** on treatment with tosylmethyl isocyanide (TosMIC), followed by hydrolysis of the intermediate nitrile.¹³⁵ **42** in turn can be used directly as labelling agent, or converted into an activated carboxylate such as the *N*-hydroxysuccinimide ester **43**.¹³⁶

For tetraethyl- or bis(spirocyclohexyl)-substituted piperidinyll nitroxides, comparatively few labelling functionalities (X) have been reported (Fig. 9). Although tetraethyl derivatives such as **44**⁵¹ and spirocyclic amine **45**^{6,137,138} have been synthesised, they have not to date been used for spin labelling. Iodoacetamide **19** has recently been used to spin label T4 lysozyme (T4L) and perform DEER measurements at room temperature.⁸⁰



Scheme 5 Structures and syntheses of commonly used piperidinyll spin labels.

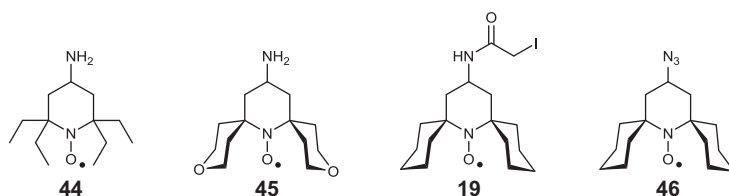


Fig. 9 Tetraethyl- and bis(spirocyclohexyl)-substituted TEMPO derivatives.

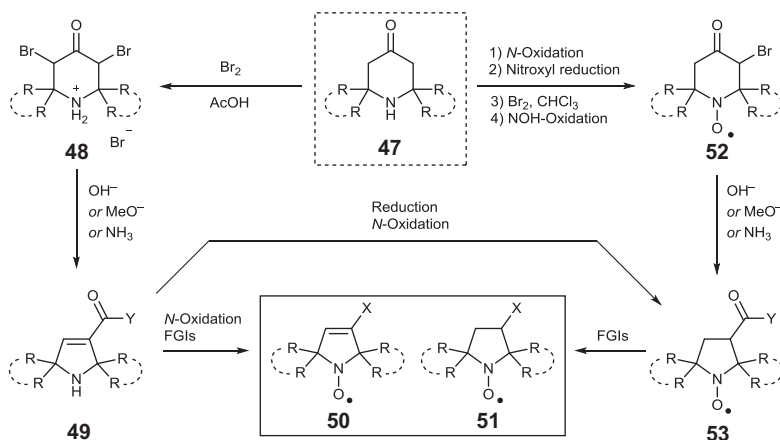
Azide **46** was recently reported by our groups, and was used to label nucleic acids in a CuAAC spin-labelling strategy.¹³⁹

3.3 Synthesis of pyrrolinyl and pyrrolidinyl (PROXYL) nitroxides

5-Membered ring 2,2,5,5-tetrasubstituted pyrrolinyl and pyrrolidinyl (PROXYL) nitroxides can, as mentioned, benefit from improved stability towards reducing conditions compared to their 6-membered ring cousins, and as such enjoy much popularity. However, it is worth noting that substituted PROXYLs are generally synthesised as racemates, and thus are likely to give rise to a mixture of diastereomers on labelling biomolecules.

The unsaturated 3-pyrroline scaffold is most conveniently accessed from the corresponding piperidones (**47**, Scheme 6) *via* an initial double (α,α') bromination.¹⁴⁰ To avoid decomposition pathways such as trans-annular nucleophilic substitution to give an aziridine, the intermediate dibromide **48** is typically isolated as a hydrobromide salt. Exposure to basic conditions (*e.g.* hydroxide, methoxide, or ammonia) effects a Favorskii rearrangement, followed by *in situ* elimination of HBr, to give the 3-substituted pyrroline carboxylic acid derivatives **49**.¹⁴¹ Oxidation to the nitroxyl can be followed by any necessary functional group manipulations to convert these Favorskii-derived carboxylic acid derivatives to the various spin labels of the pyrroline family (**50**, see following text for examples).

The related saturated pyrrolidine nitroxides (often nicknamed 'PROXYLs') **51** (are synthesised *via* a similar route, this time through monobrominated ketone **52**.^{56,58,108} Here, the secondary amine is first 'protected' as a hydroxylamine, which prevents the aforementioned aziridine formation. However, as oxidation is difficult to stop at the hydroxylamine stage, a nitroxyl radical is first formed, which is then reduced to the hydroxylamine. After Favorskii rearrangement, the pyrrolidine nitroxide derivatives **53** can be functionalised as desired (**51**, see following text for examples). Pyrrolidine carboxylic acid derivatives **53** have also been formed by reduction of the corresponding pyrrolines **49** with H₂ and Pd/C.¹⁴¹ As the nitroxyl radical is also at risk of reduction in this step,

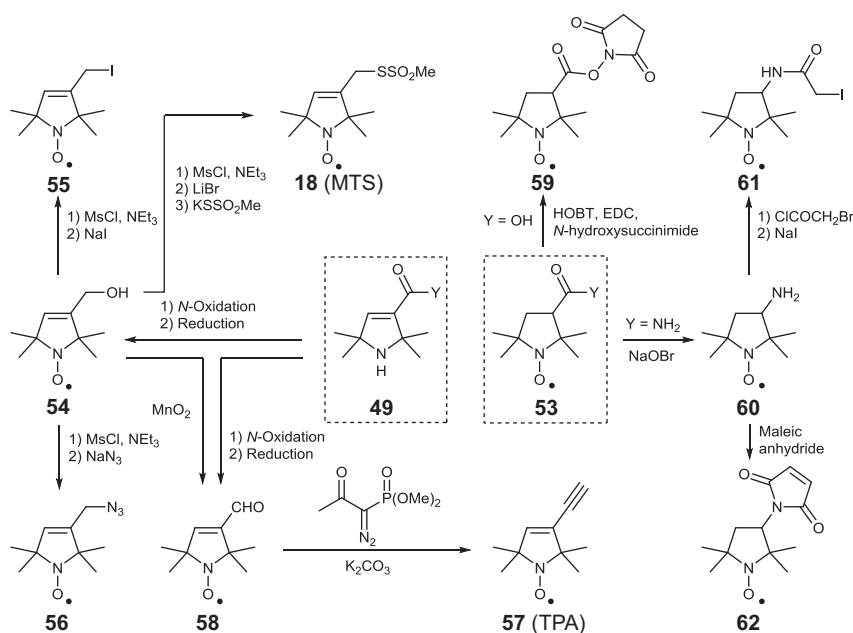


Scheme 6 General synthetic routes to pyrrolinyl and pyrrolidinyl nitroxides.

it is generally performed before *N*-oxidation (however, LiBH₄ has recently been shown to reduce the double bond of an amide pyrrolinyl nitroxide without affecting the radical or carbonyl).³⁰

Syntheses of some commonly used 5-membered nitroxide spin labels are shown in Scheme 7. Several derivatives are prepared from initial reduction of the carboxylic acid sidechain (**49**, Y = OH) to allylic alcohol **54**, a key intermediate that has been transformed into many labelling groups. If carried out after *N*-oxidation, carboxylic acid reduction has been found to be challenging,¹⁴² as competing conjugate reduction and/or reduction of the nitroxyl can be observed. Our groups have recently found that *N*-oxidation and subsequent reduction of the methyl ester (**49**, Y = OMe) with DIBALH at –50 °C effects a clean and high-yielding reduction to **54**, avoiding these side reactions.¹³⁹ Alternatively, acid derivatives **49** may be reduced before *N*-oxidation, the latter process being tolerant of the resultant allylic alcohol.¹¹²

From alcohol **54**, iodide **55** is prepared by mesylation and Finkelstein reaction.¹⁴³ Alcohol **54** has also been converted to methanethiosulfonate MTS (**18**), a popular spin label for the functionalisation of cysteine residues in peptides (see following text for applications).¹⁴⁴ Alternatively, **54** can be transformed into azide **56** by mesylation and substitution.¹⁴⁵ Enyne **57** (known as TPA) is another popular spin label which is readily prepared from aldehyde **58**^{140,146} by Ohira-Bestmann alkynylation,¹⁴⁷ or other alkyne-forming methods.¹⁴⁸ The saturated carboxylic acid **53** (or derivatives) also serve as useful precursors to labelling functionalities. For example, *N*-hydroxysuccinimide ester **59** has been synthesised from **53** (Y = OH) by diimide-mediated esterification.¹⁴⁹ Amine **60** is available



Scheme 7 Structures and syntheses of commonly used pyrrolinyl and pyrrolidinyl spin labels.

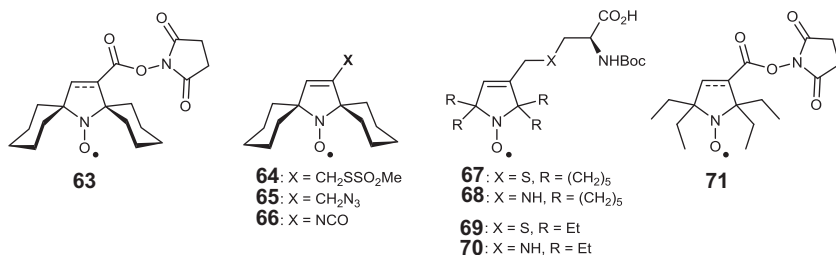


Fig. 10 Bis(spirocyclohexyl)- and tetraethyl-substituted pyrrolinyl and pyrrolidinyl nitroxides.

via a Hofmann rearrangement of the amide **53** (Y = NH₂);⁹ this spin label can be further derivatised to labels such as iodoacetamide **61** (by acylation and iodination)¹⁵⁰ or the Michael-acceptor maleimide **62**.¹⁵¹

A number of bis(spirocyclohexyl)-substituted pyrrolinyl and pyrrolidinyl labels have been prepared (Fig. 10). For the spirocyclic nitroxides, *N*-hydroxysuccinimide esters are known for both the saturated¹⁵² and unsaturated^{30,112} 5-membered rings (**63**), prepared in analogy to their tetramethyl relatives (see **59**, Scheme 7), as have the methanethiosulfonate **64**³⁰ and azide **65**.¹³⁹ Other variants include isocyanate **66**, accessed by treatment of the unsaturated ester **63** with NaN₃ followed by a Curtius rearrangement,¹⁵³ and two spirocyclic amino acid derivatives (**67** and **68**).¹¹² Several tetraethyl-substituted pyrrolinyl and pyrrolidinyl nitroxides, which have been found to be highly stable to reduction, are also known: examples include amino acid derivatives **69** and **70**, and *N*-hydroxysuccinimide esters **71**, prepared in analogy to their spirocyclic counterparts.¹¹²

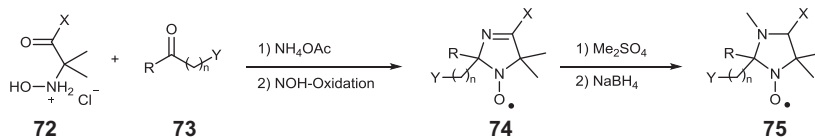
3.4 Synthesis of imidazoliny, imidazolidinyl and oxazolidinyl nitroxides

Spin labels containing additional heteroatoms in the nitroxyl-bearing ring have been synthesised through various modular *de novo* ring-forming routes (Scheme 8). These nitroxides have the benefit of being readily adaptable to contain functional groups for label attachment (substituents X, Y). Synthetically, the hydrochloride salts of α -hydroxylamine ketones (**72**) can be condensed with ketones **73** in the presence of ammonium acetate to obtain unsaturated imidazoline nitroxides **74** after oxidation of the intermediate hydroxylamine.⁸¹ These have been further *N*-alkylated at the imine nitrogen atom, and the resulting iminium ion reduced to form the saturated imidazolidine nitroxides (**75**).⁸¹ This chemistry has been particularly exploited in the field of protein spin labelling, due to the suitability of attachment to proteins *via* the residual sidechain functionalities in the spin labels.

A selection of imidazoliny and imidazolidinyl nitroxides is shown in Fig. 11, which illustrate the diversity of spin-labelling functionalities that can be installed (**76–79**).²⁶ Related oxazolidine nitroxides (often termed ‘DOXYLS’) have also been described (**80**), prepared from condensation of a ketone and an aminoalcohol.¹⁵⁴

3.5 Synthesis of isoindoliny nitroxides

The final major structural class of nitroxide spin label in common usage is the isoindoline-derived scaffold. The most common starting point for



Scheme 8 Routes to spin labels containing additional heteroatoms in the nitroxyl-bearing ring.

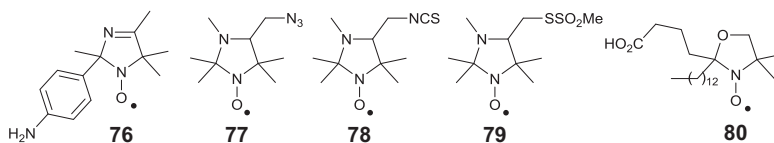
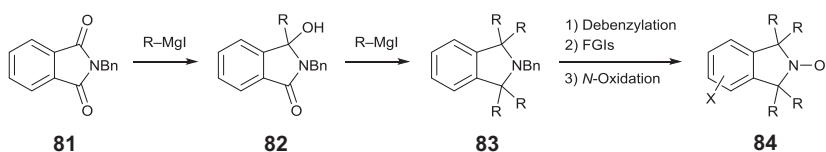


Fig. 11 Structures of selected imidazolinyl, imidazolidinyl and oxazolinyl nitroxides.

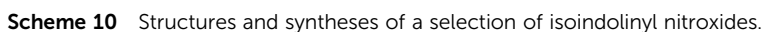


Scheme 9 General route to isoindoline-derived nitroxides.

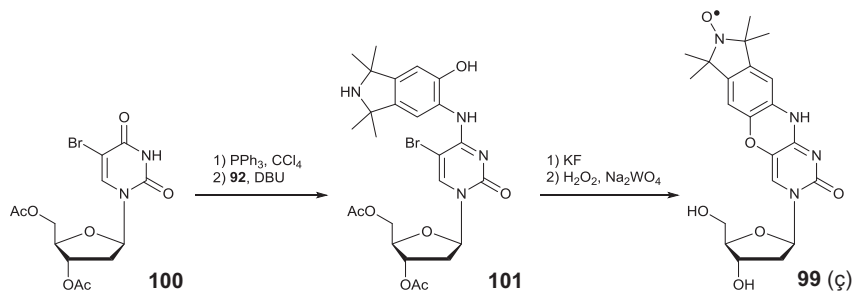
the synthesis of these derivatives is *N*-benzylphthalimide (**81**, Scheme 9), from which the four α -alkyl substituents are installed *via* a challenging quadruple Grignard addition.¹⁵⁵ An improved methodology towards these substrates was recently reported,¹⁵⁶ where the easily formed hemiaminal **82** was isolated from a single addition of Grignard reagent, and then resubmitted to a six-fold excess of organomagnesium compound to reach the fourfold adduct. High-temperature reflux for several days in the latter step afforded tetrasubstituted isoindolines **83** in respectable yields; it is not clear why this procedure offers advantages over the 'one-pot' approach. Following the quadruple alkylation, hydrogenolysis of the amine benzyl group and subsequent straightforward functionalisations of the aromatic ring and *N*-oxidation, affords isoindoline spin label derivatives **84**. The aromatic substituents can be tuned to impart aqueous solubility on the otherwise relatively lipophilic isoindoline skeleton,^{157,158} and/or provide a handle for covalent attachment of the spin label.

An overview of synthetic routes toward some common or recently reported isoindolinyl nitroxides is shown in Scheme 10. For the purpose of clarity, these transformations are illustrated with the parent amine, but it should be noted that *N*-oxidation can be carried out at several different points in these synthetic sequences; the *N*-oxidation step is therefore not specified in the reaction scheme. *N*-Oxidation is in general performed as the last synthetic step, as long as the oxidation conditions are tolerated by other functional groups.

Functionalisation of the parent isoindoline **85** is easily achieved through aromatic substitution chemistry. For example, arene carboxylation to **86** can be effected by bromination^{159,160} followed by cyanation and



Doubly-functionalised isoindolines have been used to create rigidified symmetrical spin-labelling systems. Aminoalcohol **92** has been prepared from amine **87** by diazotisation, hydrolysis to the phenol, *ortho*-nitration, and nitro reduction.¹⁶⁶ Oxidative condensation of **92** with 5-formyluridine followed by *N*-oxidation provides the conformationally restricted spin-labelled uridine derivative **93**.¹⁶⁷ The related benzimidazole-tethered



Scheme 11 Synthesis of cytidine analogue Ç.

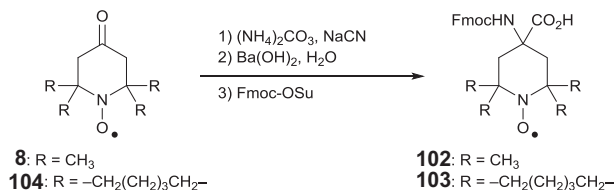
nitroxide uridine derivative **94** was obtained from triamine **95**,¹⁶⁷ the latter prepared by nitration of **85**, then amination through vicarious nucleophilic substitution, and nitro reduction.¹⁶⁷ Triamine **95** has also been employed for the synthesis of **96** from oxidative condensation with the corresponding benzaldehyde.¹⁶⁸ A further approach uses iodophenyl phthalimide **97**, which is prepared from **85** by a double bromination/cyanation/hydrolysis sequence,^{157,159} then condensation with *p*-iodoaniline. A Sonogashira coupling strategy leads to uridine derivative **98**.¹⁶⁹

One further notable example is the cytidine analogue **99** (Scheme 11), also known as Ç, which gives highly rigid systems in the EPR spectroscopic investigation of nucleic acids.¹⁶⁶ **99** was synthesised from isoindoline *o*-aminophenol derivative **92** (Scheme 10) by reaction with 5-bromouridine (**100**) and cyclisation of intermediate bromide **101**. After conversion into a phosphoramidite building block, nucleoside derivative **99** was incorporated into nucleic acids by solid-phase synthesis under modified conditions. A free nucleobase analogue of **99** (known as ç) has been developed using equivalent chemistry on 1-benzyl-5-bromouracil.^{170,171}

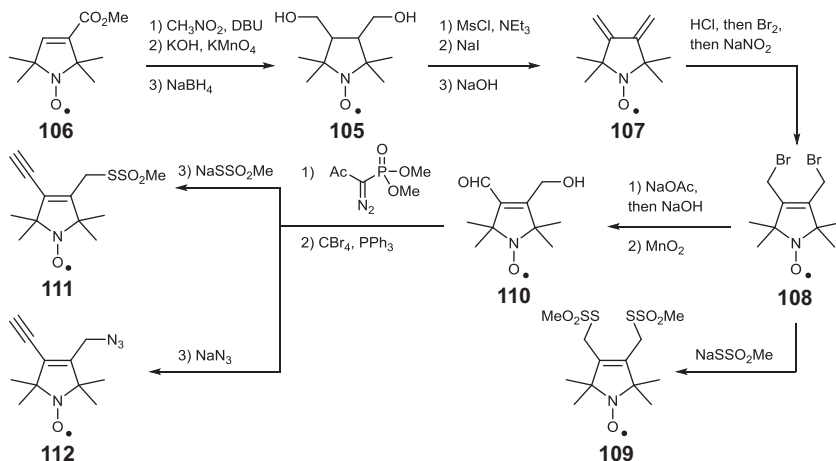
3.6 Bifunctional nitroxides for two-point attachment

Nitroxide spin labels equipped with two functional groups for two-point label attachment offer opportunities to construct particularly rigid systems. The two functional groups can be identical, or different, the latter potentially enabling attachment of each group to a biomolecule of interest under orthogonal reaction conditions. A simple example is amino acid derivative **102** (Scheme 12), which is commonly used to label peptides. It is prepared by a Bucherer–Bergs reaction from the TEMPO ketone **8**, with hydrolysis of the intermediate hydantoin and carbamoylation delivering the Fmoc-protected amino acid.¹⁷² A 2,6-bis(spirocyclohexyl) version of this spin label (**103**) was recently prepared from the corresponding piperidone nitroxide **104** *via* a similar route.¹⁰⁵

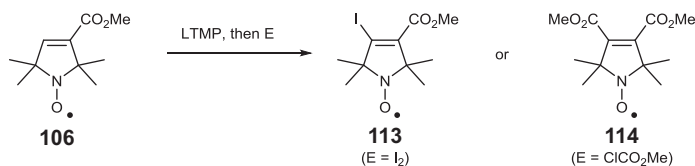
3,4-Bifunctionalised pyrroline spin labels are also available (Scheme 13). Many of these are synthesised from diol **105**, itself prepared by Michael addition of nitromethane to enoate **106**, followed by a Nef reaction and reduction of the intermediate β -carboxyaldehyde.¹⁴⁸ Double iodination/elimination of **105** affords diene nitroxide **107**, which has itself been used as a starting point for numerous nitroxide derivatives (including an alternative route to the isoindoline framework).¹⁷³ **107** can be converted to



Scheme 12 Amino acid derivatives for two-point attachment of piperidiny spin labels.



Scheme 13 Bifunctional spin labels for two-point attachment strategies.



Scheme 14 Synthesis of 3,4-disubstituted pyrroline nitroxides.

1,4-dibromide **108** on treatment with Br_2 , albeit this transformation requires temporary reduction of the nitroxyl to the hydroxylamine, and re-oxidation after bromination. Dibromide **108** can then be transformed into a selection of bifunctional spin labels: treatment with $\text{NaSSO}_2\text{CH}_3$ affords the symmetrical bis(methanethiosulfonate) **109**,¹⁴⁸ whilst hydrolysis and mono-oxidation gives aldehyde **110**, which can in turn be submitted to Ohira-Bestmann alkynylation and Appel bromination, followed by conversion into functionalities such as a methanethiosulfonate (**111**) or azide (**112**).¹⁴⁷

The syntheses of the above bifunctional spin labels are rather lengthy. An alternative route to 3,4-disubstituted pyrroline nitroxides involves deprotonation of the pyrrolinyl ester **106** (Scheme 14) at the β -position with LTMP, followed by trapping with electrophiles (E) such as iodine or a chloroformate, leading to iodide **113** and diester **114** respectively.¹⁷⁴

Improved access to such compounds would lead to shorter and higher-yielding routes to bifunctional nitroxides.

4 Recent advances in applications of spin labels

The site-directed spin labelling using nitroxides at specific sites within a molecule can be achieved either through non-covalent interactions or, more usually, through covalent bond formation. Although spin labelling can be applied in many contexts (such as soft matter and surfaces), discussion in this chapter is limited to biomolecule spin labelling (proteins, oligonucleotides, lipids and sugars).

4.1 Spin labelling of proteins

Of the 20 universally genetically encoded amino acids, the thiol group of cysteine arguably has the most possibilities for orthogonal covalent spin labelling. For SDSL, cysteines are therefore engineered into recombinant proteins at sites of interest. The most popular label for attachment to cysteines is MTS (see **18**, Scheme 7), which attaches to the target by formation of a mixed disulfide with a cysteine thiol.¹⁴⁴ Once bound to a cysteine, the complete modified disulfide-linked side chain is often referred to as R1. It provides a CW EPR spectrum that is sensitive to conformations, and may give narrow distributions in DEER due to the reasonably short linker with its restricted conformational freedom.^{40,90,97,100–102,175}

MTS spin labelling has recently been applied to the intact *E. coli* cobalamin transporter BtuB outer membrane protein, which was labelled at cysteine residues external to the cell.^{176,177} Distances between R1 labels and R1 to TEMPO-labelled cyanocobalamin (TEMPO-CNCbl, **115**, Fig. 12), which binds to BtuB with high affinity, were measured by DEER. It was shown that conformational changes upon ligand binding could be followed to characterise the structure of an extracellular loop.

The conformational freedom of R1 can be further restricted through the addition of substituents:^{148,174,178,179} for example, the 4-pyridyl analogue (**116**, Fig. 13) demonstrates restricted internal motion when attached to a protein, compared to R1.¹⁸⁰ The choice of a pyridine substituent (rather than phenyl) improves label solubility, and reduces protein aggregation post-labelling. Reaction of the disulfide IDSL (**117**) installs an imidazolinoyl side chain (known as V1) upon reaction with

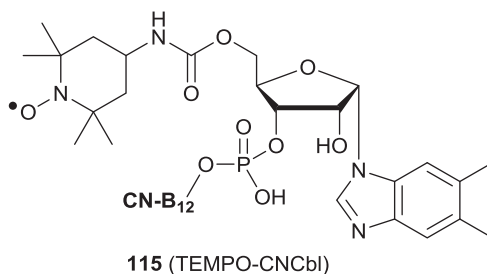


Fig. 12 TEMPO-labelled cyanocobalamin.

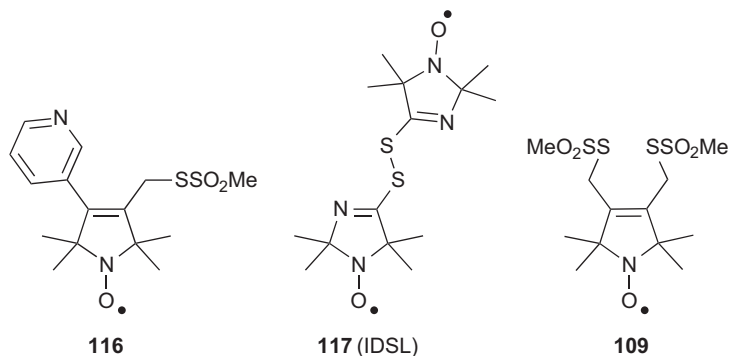


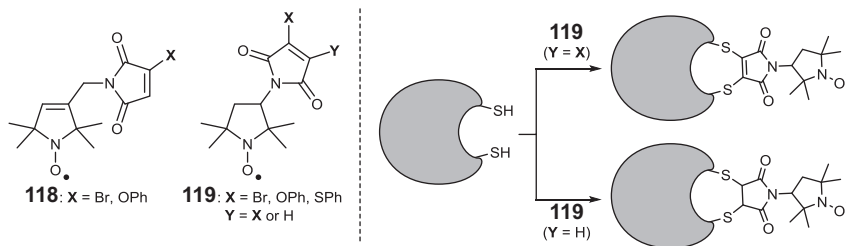
Fig. 13 Structures of various disulfide bond-forming spin labels.

cysteine.^{98,181} A study found this label to have strongly hindered internal motion; the authors suggest that this is due to van der Waals interactions between the disulfide and the nitrogen atom at the 3-position of the ring, but may also be due to minimization of steric interactions.

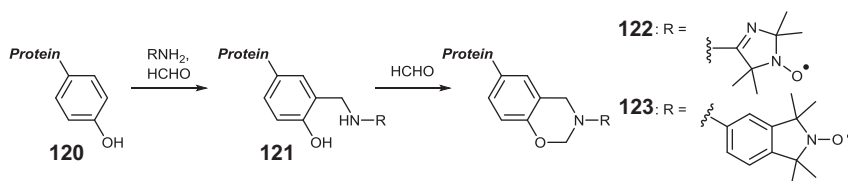
An interesting method to reduce conformational freedom is to covalently attach the spin label to two amino acids. This two-point binding strategy has been achieved using the 'double MTS' label **109** (known as Rx once attached to a pair of proximal cysteines).^{94,148} Due to the rigidity of the spin label, Rx reduces the distance distribution in DEER.^{85,94,182} More than one well-defined conformation may be adopted depending upon local environment and tethering sites, and this can render orientation-selective DEER challenging.⁸⁵ The reaction to form the bridged product involves titration of substoichiometric amounts of **109** to the buffered protein, and subsequent dialysis – often extensive – to remove unbound label.

'Next generation' maleimides such as **118** and **119** (Scheme 15, synthesised in analogy to **62**, Scheme 7, using mono- or dibromomaleic anhydride) may also prove useful for binding to pairs of cysteines.¹⁸³ For a dibromomaleimide (**119**, X, Y = Br), a pair of proximal cysteines can substitute for both, giving a 'cystine mimic' which features an unsaturated bridge; consequently, the dynamics of the spin label are small. This kind of label has been used in 'spinostics' to show that antigens binding to labelled antibody fragments can be detected through changes in the solution-state CW EPR lineshape.¹⁸⁴ Reaction of a monobromomaleimide (**118** or **119**, Y = H) with a single cysteine results in a maleimide linked label. However, if a pair of cysteines binds through a further conjugate addition reaction, then a succinimide bridge will be formed.¹⁸⁵ The reactivity of the maleimide labelling agent can be tuned by replacing the bromide leaving group(s) with *e.g.* phenoxy groups, which changes the labelling kinetics or other properties.¹⁸⁶

Other amino acids can also be targeted for labelling. Ideally, these would be labelled through orthogonal linker chemistry, and be naturally sparse. One candidate is the phenol found in tyrosine, where a three-component Mannich-type condensation between a spin label, the tyrosine-containing protein (**120**, Scheme 16) and formaldehyde give either the Mannich product (**121**) or the benzoxazine rings (**122** and **123**) through



Scheme 15 'Next-generation' maleimide approach to labelling of cysteines.



Scheme 16 Spin labelling of proteins at tyrosine residues.

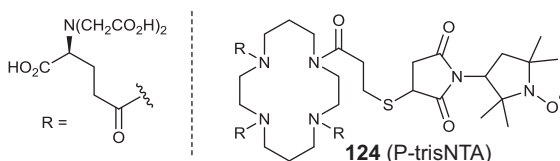


Fig. 14 Spin labelling of the His₆-tag by binding of P-trisNTA.

further reaction with formaldehyde. 4-Amino-imidazolinoyl and amino-isouindoline labels have been employed in this chemistry, and differences in their abilities to measure dynamics characterised.^{187,188}

Polyhistidine motifs such as the His₆-tag are commonly introduced at the N- or C-terminus of recombinant proteins for the purpose of purification with nickel affinity columns. PROXYL-tris-nitrilotriacetic acid (P-trisNTA, **124**, Fig. 14) has a cyclam scaffold with three NTA groups which bind to the polyhistidine-complexed nickel(II) ions, and a linker to a PROXYL nitroxide.¹⁸⁹ The T_m of the nickel-bound P-trisNTA is shorter than that of a standard nitroxide spin label; however, it was successfully used in a DEER experiment to investigate conformational changes in the maltose-binding protein MalE on substrate binding, or the formation of protein–protein complexes. The DEER-derived distance distributions were fairly broad, but useful information could be extracted. The label also successfully bound to the His₆-tagged MalE in cell lysate, which is promising for future work in live cells.

Unnatural amino acids may be incorporated into proteins for the purpose of spin labelling.¹⁸ This strategy can be useful to enable single (orthogonal) labelling of cysteine rich proteins, or to avoid labelling of other natural amino acids. This could eventually allow labelling of proteins in cells or the incorporation of different types of label. A large

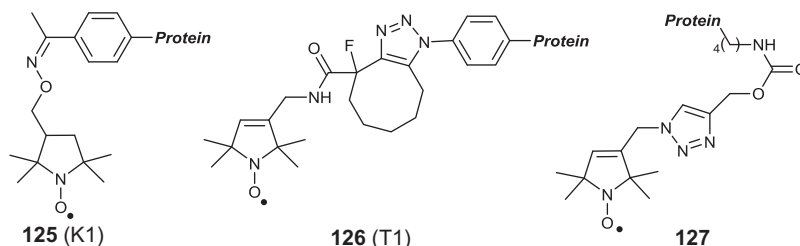


Fig. 15 Spin labelling through functionalisation of unnatural amino acids.

number of unnatural amino acids have been incorporated into proteins using expanded genetic code methodologies for a variety of purposes.¹⁹⁰

For EPR, *p*-acetylphenylalanine has been labelled *via* an oxime to give the K1 side chain (**125**, Fig. 15). Good yields of the unnatural amino acid-containing T4L protein were obtained in *E. coli*, however relatively harsh labelling conditions were required (pH 4, 37 °C for 12 hours). The resultant label possessed a large degree of conformational freedom, but has generally proved useful for labelling proteins where cysteines cannot be easily incorporated.^{191,192} The reaction conditions have since been improved through the use of *p*-methoxyaniline as a catalyst, which presumably forms an intermediate (and more electrophilic) ketimine from the acetophenone.^{193,194} *p*-Azidophenylalanine has also been incorporated into T4L for click cycloadditions: The T1 side chain (**126**) was created through copper-free, strain promoted click with a cyclooctyne-containing spin label synthesised from the corresponding cyclooctyne carboxylic acid and the amine-containing nitroxide.^{13,195} Propargyloxycarbonyl lysine (PocLys) has been incorporated into proteins for click reactions.^{196–198} In work carried out by our groups, two positions of sperm whale myoglobin were mutated to contain PocLys using the expression system of Chin and co-workers.¹⁹⁶ This was labelled for DEER experiments with azido nitroxide **56** (Scheme 7) *via* copper-catalysed click conditions (**127**). However, the results showed some loss of radical and a flexible linker.^{197,199}

There are many advantages to the strategy of incorporating the nitroxide spin label directly into the protein structure. Amino acid nitroxides such as TOAC (**102**, Fig. 16) and TOPP (**128**), can be incorporated into small peptides and proteins during solid-phase synthesis.^{200,201} There has also been a report of a pyrrolinoxyl amino acid (**129**)-containing peptide being chemically ligated through reaction of a C-terminal thioester of the protein and an N-terminal cysteine of the peptide and rearrangement to give a native peptide bond.^{202,203}

Arguably the most flexible way of incorporating the nitroxide is through genetic code expansion methodology. Early work necessarily required chemical aminoacylation of the tRNA with the labelled amino acid (cysteine- or tyrosine-based); while successful incorporation was observed, yields were low.^{204,205} Recently, a highly efficient incorporation of lysine derivative SLK-1 (**130**) into thioredoxin has been reported, using *E. coli* Top10 and RF1 knockout JX33 cells (with higher expression from the latter).⁵³ The radical was found to be partially reduced in the cells,

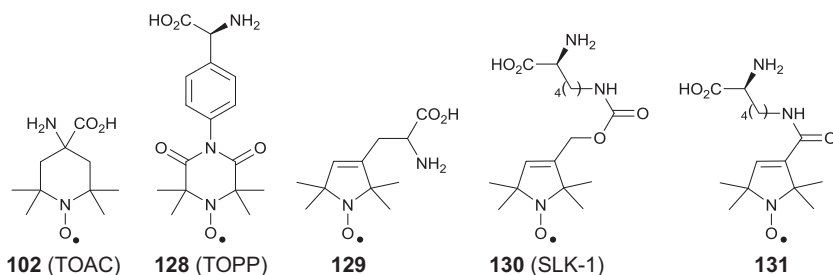


Fig. 16 Spin labelling through direct incorporation of nitroxide amino acids.

although active levels as high as 70% were possible. Similar to the method used to introduce PocLys, the orthogonal tRNA^{Pyl}/pyrrolysyl-tRNA-synthetase (PylRS) pair was evolved to accept the SLK-1. The shorter, amide linked, version **131** did not produce a PylRS mutant.^{53,206} Rotamer libraries of the SLK-1 side chain have been produced to aid interpretation of the DEER results. DEER experiments were performed on isolated double SLK-1 mutant thioredoxin.²⁰⁶

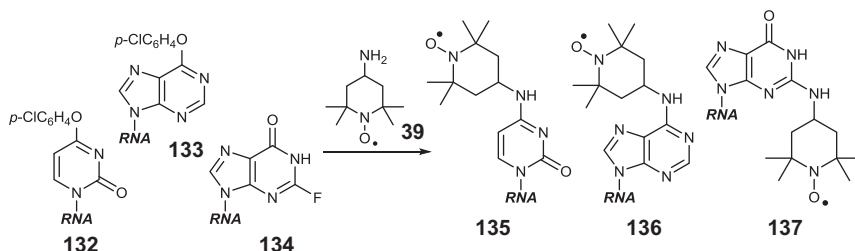
4.2 Spin labelling of nucleic acids

The nucleotides used in DNA and RNA offer a number of potential positions for chemical modification/spin labelling: the pyrimidine and purine rings, the sugar or the phosphodiester. Labelling is most usefully carried out post-oligonucleotide synthesis, which prevents exposure of the nitroxyl to incompatible reaction conditions during solid-phase synthesis (such as TCA or I₂). Modified DNA synthesis conditions can alternatively be employed to circumvent this issue, and allow incorporation of spin labels through the solid-phase DNA synthesis. A brief overview of some recent advances in this area will be undertaken here to demonstrate the scope of covalent SDSL in oligonucleotides.²⁰⁷

Höbartner, Bennati and co-workers have used 4-amino-TEMPO (**39**, Scheme 17) with nucleotide precursors carrying leaving groups (**132–134**) to give C, A and G bases labelled at their exocyclic nitrogen (**135–137**).^{129,130} This was shown to cause little disruption to RNA conformations, and to provide useful distance distributions in DEER experiments.¹³⁰

The 3,4-di(bromomethyl) pyrrolinyl nitroxide **108** (Fig. 17) has been linked to phosphorothioate groups at adjacent nucleotides in DNA or RNA to give the cyclic R5c label **138**, an analogue of the Rx label used in proteins.²⁰⁸ The 2'-position of the ribose may be functionalised in several ways; our groups have recently showed that U, C and A can be alkynylated at this position, which enables a variety of different azide-containing spin labels to be attached to duplex DNA *via* CuAAC (**139**).¹³⁹

It is also possible to spin label oligonucleotides which contain abasic sites. For example, the polyaromatic spin label ζ (**140**) hydrogen bonds to guanine at an opposing abasic site engineered into a DNA duplex.^{170,209} The labelling efficiency of this compound is high at low temperatures. To enhance binding efficiency, further modifications at N3 have been explored.¹⁷¹ The ζ label was found to bind rigidly enough for distance



Scheme 17 Nucleophilic installation of a TEMPO spin label on RNA, prior to strand deprotection.

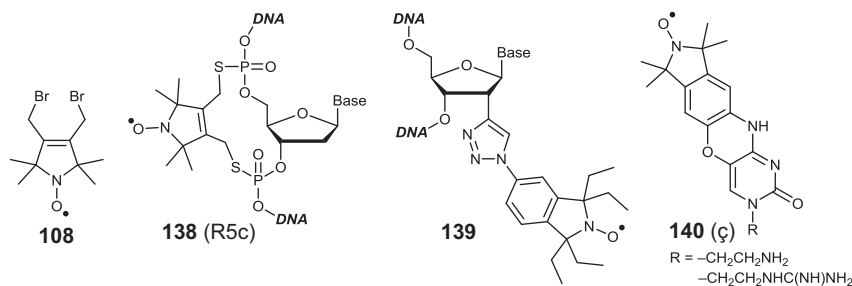
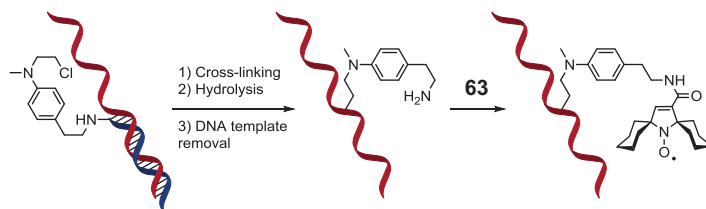


Fig. 17 Spin labelling of the phosphodiester or ribose backbone of DNA, as well as a cytosine analogue for noncovalent SDSL of abasic sites.

and orientation data to be determined from DEER signals. In this study, DNA duplexes of the LacI operator sequence containing one abasic site per strand were doubly labelled with ζ and then either measured alone or bound to the Lac repressor protein which induces duplex bending.²¹⁰

Compared to the chemical synthesis of DNA, the reduced coupling efficiency and chemical stability of RNA imparts an upper limit of around 50 ribonucleotides to synthetic RNA, thus rendering most natural (longer) RNAs inaccessible for SDSL.²¹¹ Two approaches to spin labelling longer RNA targets have been employed: the first uses enzymatic ligation, where short RNA strands containing a 4-thiouridine are spin labelled with iodoacetamido-PROXYL (**61**, Scheme 7) and ligated together with the unmodified RNA fragments using splinted T4 DNA ligation, without reducing agent DTT, and followed by HPLC purification.²¹² Ligation efficiency was 20–40% but the final isolated product was estimated to be close to 100% labelled. The resulting doubly labelled 72-mer nucleotides from the non-coding RNA RsmZ were measured by DEER, together with NMR, experiments to investigate the mechanism for protein sequestration of RsmE in gene expression regulation.²¹³

An alternative strategy (Scheme 18) involves binding of [4-(*N*-2-chloroethyl-*N*-methylamino)benzyl]-phosphoramidate short synthetic DNA sequences to a target sequence of RNA, which then leads to alkylation of the opposing nucleobase. Subsequent hydrolysis of the phosphoramidate bond detaches the short DNA sequence, and leaves the RNA chemically modified at a specific position. The amino group that has now been introduced is then labelled with an *N*-hydroxysuccinimide ester nitroxide (e.g. **63**, Fig. 10).²¹⁴ This approach has been used to doubly label the 341



Scheme 18 DNA-templated RNA spin labelling strategy.

nucleotide IRES site of hepatitis C genomic RNA. Despite a fairly broad distribution of distances in DEER, these results demonstrate exciting new possibilities for structural studies of long RNAs.²¹⁵

Advances have been made to improve the applicability of nitroxide labels for DEER measurements in cells. We have already seen that MTS (**18**) and TEMPO-CNCbl (**115**, Fig. 12) allowed for measurements on the outer membrane of *E. coli*, and that SLK-1 (**130**, Fig. 16) can be biosynthetically incorporated into proteins.^{177,206,216} DEER measurements within cells have so far focused on frog oocytes, in which DEER experiments have been carried out by loading labelled proteins and oligonucleotides by microinjection, and then flash freezing. For example, ubiquitin was double labelled with 3-maleimido-PROXYL (**62**, Scheme 7), and found to have an approximate radical half-life of 50 minutes.²¹⁷ The distances measured did not vary much from the *in vitro* results, although with incubation time the SNR for the DEER deteriorated, which manifested as an apparent broadening of the distance distribution. A 7-mer DNA duplex with TEMPA (**37**, Scheme 5) incorporated at the 5' end of each strand demonstrated fast bioreduction and a broader distance distribution when in the cell.²¹⁸ This is possibly due to the DNA denaturing or undergoing processes within the cells. In another study, two RNA structures and a 12-mer DNA duplex labelled with pyrrolinoxyl TPA (**57**, Scheme 7) have been measured.⁵⁴ Good measurements were possible after 70 minutes incubation time. In this instance the DNA measurements indicated some assembling, possibly end-to-end stacking of the duplexes inside the cell, whereas the RNA structures were seen to compare well with *in vitro* measurements.

4.3 Spin labelling of other biomolecules

Other types of biomolecules are also suitable for spin labelling, but have been less widely exploited. For example, in the field of carbohydrates, the highly-sulfated polysaccharide heparin has been partially labelled at the uronic acid or glycosamine residues using 4-amino-TEMPO (**39**, Scheme 5), or the activated TEMPO ester **43**, to give heparin-polynitroxide derivatives for use in diagnostic and therapeutic applications.¹²⁵ Lipids have also been targeted: the interaction of DOXYL stearic acid (labelled at the 5 and 16 positions from the headgroup, **80**, Fig. 11) with human and bovine serum albumin has recently been used to probe a variety of structural and dynamics properties.²¹⁹ By utilising the 2-position of the nitroxide as an attachment point it is possible to benefit from a reliable

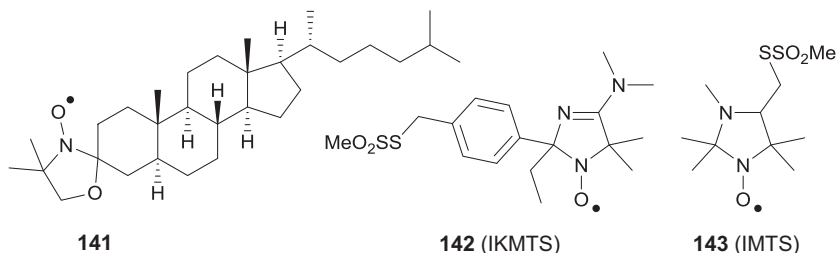


Fig. 18 Spin labels used for the investigation of lipids.

alignment between the magnetic frame of the nitroxide and the labelled substances.³⁸ For example, 3β-DOXYL-5α-cholestane (**141**, Fig. 18) is an analogue of cholesterol and used to probe the structure of membranes.^{34,38} Recent work in this area has investigated the heterogeneities of membrane structures using electron spin echo decay curves to measure local concentrations.²²⁰

Imidazolinoyl (and related imidazolidinoyl) radicals have been used as pH sensitive probes in biomolecules, and have been structurally tuned to exhibit a useful range of pK_a values.^{17,25,28,29} For example, the 1,2-dipalmitoyl-*sn*-glycero-3-phosphothioethanol (PTE) adduct of IKMTS (**142**) has a pK_a of ~ 6 , whilst that of IMTS (**143**) has a pK_a of ~ 3.3 .²⁸ Applications of this chemistry have been limited to the labelling of lipids in different environments to explore effects on pK_a values,²⁸ and to the measurement of electrostatic properties in the lipid bilayer.²⁹ This so far minor class of spin labels may become significantly more important in the future.

5 Summary

Advances in the synthesis of nitroxide spin labels, and associated labelling methods, have broadened the scope of applications of EPR spectroscopy to the study of biomolecules. An intimate knowledge and understanding of the physical aspects that determine relaxation times and radical stability in different environments is fundamental to progressing this wide range of uses. These advances have begun to enable the use of nitroxide spin labels to probe the structure of biomolecules under physiological conditions, a field that looks set to see further developments and discoveries over the coming years.

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Trityl radicals as spin labels

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During the last 30 years, tetrathiatriarylmethyl (TAM) radicals have been widely used as spin probes for oxymetry in electron paramagnetic resonance (EPR) spectroscopy and EPR tomography because of the narrow EPR linewidth and high stability in living systems. Recently, some researchers proposed to use TAMs as spin labels for studies on the structure of proteins and nucleic acids using site-directed spin labeling (SDSL) and pulsed dipolar EPR spectroscopy. In this chapter, the peculiarities of applying TAMs as spin labels, including the synthesis of TAMs, approaches to TAM spin labeling of proteins and nucleic acids, the methods for distance measurement using TAM spin labels, electron spin relaxation time, room temperature measurements, and advantages of orthogonal spin labeling are reviewed. Examples of applications of TAMs to research on the structure and functions of biopolymers are presented from recently published papers.

1 Introduction

A spin label means a paramagnetic molecule (or a stable radical) that is linked to the molecules of systems being studied and allows investigators to obtain information about the system under study using electron paramagnetic resonance (EPR) spectroscopy.^{1–4} Usually, as spin labels, researchers use stable radicals (nitroxides and tetrathiatriarylmethyl [TAM] radicals) that are covalently or noncovalently linked to the analyzed molecules or complexes of transition metals [*e.g.*, Mn(II), Gd(III)].^{5,6}

Pulsed dipolar EPR (PD EPR) spectroscopy combined with site-directed spin labeling (SDSL) is a versatile tool for studying structure and function of proteins and nucleic acids and their complexes when crystallographic data are lacking.^{1–5,7} In most cases, SDSL involves cysteine substitution mutagenesis or genetically encoded unnatural amino acids to introduce a pair of paramagnetic spin labels at certain sites of a biomacromolecule.^{8–10} PD EPR enables measuring a distance and distribution of distances between these spin labels in the range of 20–100 nm and includes various approaches, in particular pulsed electron double resonance (PELDOR)¹¹ or double electron–electron resonance (DEER),¹² double quantum coherence (DQC) EPR,^{13,14} Single-Frequency TEchnique for Refocusing dipolar couplings (SIFTER),¹⁵ or relaxation enhancement (RE).^{16,17} These methods complement other techniques of structural biology and help not only to establish tertiary structure of complex biopolymers but also to study their dynamics. In contrast to X-ray analysis, PD EPR distance measurements do not require obtaining a crystal

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form of the sample and extraction of the molecules or complexes being analyzed.

It should be noted that during the last years, great progress was made in the development of a variety of new approaches to PD EPR spectroscopy both in methods (*e.g.*, arbitrary wave generators, microwave amplifiers, and highly sensitive microresonators) and in the design of new pulse sequences, approaches to combination of SDSL-EPR and protein structure modelling,¹⁸ and the synthesis of spin labels with improved functional properties.^{8,10} The traditionally and most widely used spin labels are based on nitroxide radicals, in particular *S*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)methyl methanesulfonothioate MTSL. In some cases, however, their use has limitations for EPR-based distance measurements because of rapid relaxation in solution and limited stability within live cells.

In the last 30 years, TAM radicals have been widely used as spin probes for oxymetry in EPR and EPR tomography owing to the narrow EPR linewidth and high stability in living systems. Recently, some researchers proposed to use TAMs as spin labels for studies on the structure of proteins and nucleic acids using SDSL and PD EPR spectroscopy.¹⁹ This chapter overviews the special features of applications of TAMs as spin labels, including the synthesis of TAMs, approaches to TAM spin labeling of proteins and nucleic acids, techniques for distance measurement using TAM spin labels, electron spin relaxation time, room temperature measurements, and advantages of orthogonal spin labeling.

2 TAM radicals: structure and properties

A sterically substituted TAM was developed for the first time by Nycomed Innovation AB as a spin-polarizing agent for nuclear magnetic resonance (NMR) imaging combined with the Overhauser effect.^{20–23} The structures of the most widely used Finland trityl and OXO63 are shown in Fig. 1. EPR spectra of these radicals consist of a narrow singlet with the linewidth of 0.01 mT. Due to the very narrow linewidth, TAM radicals are sensitive to oxygen, with the broadening of the EPR line of 0.05 mT per

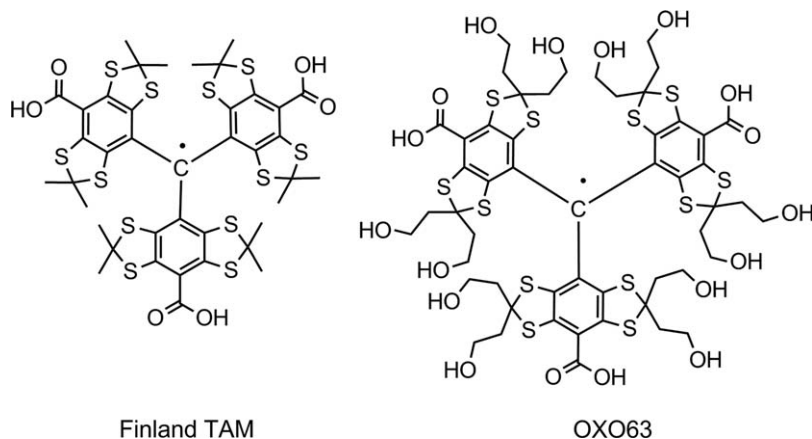


Fig. 1 Chemical structure of the most widely used Finland TAM and OXO63.

1 mM of oxygen.^{24,25} At the same time, concentration broadening of the EPR line is only 0.01 mT mM⁻¹. Due to these properties, TAMs are the best probes for oxygen and are widely used in EPR tomography *in vivo* and *in vitro*.^{26–31}

The key feature of TAM radicals is their relatively high stability in living systems. The half-decay of the TAM radical OXO63 *in vivo* in the kidneys and blood of small animals is in the range of 12 to 21 min according to various experiments.^{32,33} It should be noted that the decay rate decreases with the decreasing oxygen concentration, and in the absence of oxygen, time decay is lengthy. For a TAM radical perdeuterated at exocyclic methyl groups, decay time is longer than 24 h, whereas in a deoxygenated water solution, the decay time of this TAM is longer than 1 year.³⁴ A reaction with the superoxide radical O₂^{•-} or its protonated form HO₂ is the main reason for TAM decay in living systems.³⁵ The OXO63 radical shows low toxicity, with a half-lethal dose (LD₅₀) in mice equal to 8 mM kg⁻¹. The radicals of the Finland series have much higher toxicity.³⁶

Detailed research into the electronic and physical structure of trityl radicals in solution was conducted by several authors^{37–39} and advanced the understanding of TAMs' properties. These advances may facilitate future development of improved trityl radicals for various purposes. For the first time, the conclusion concerning the propeller-shaped structure of a TAM was drawn by Bowman *et al.*³⁸ Those authors carried out an accurate and detailed study using EPR, ENDOR, and quantum-chemical analysis of two simple symmetric trityl radicals (Fig. 2). It was reported that in both liquid and frozen solutions, the same structure is observed

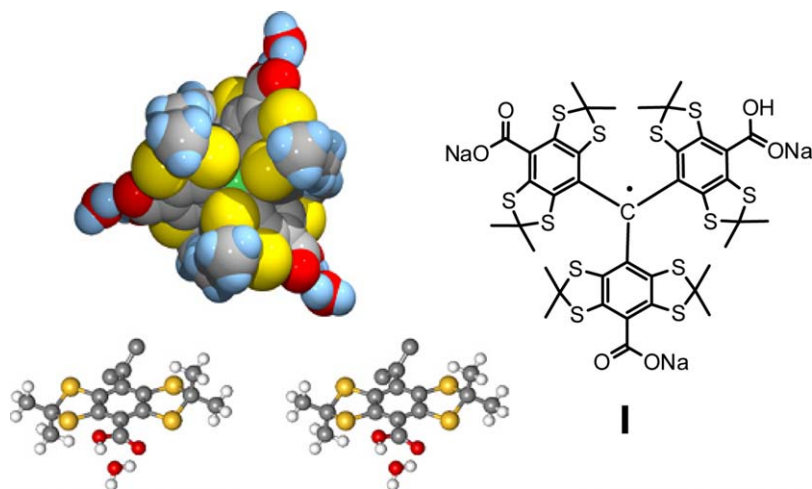


Fig. 2 (Top) The optimized structure of compound I viewed approximately along the threefold axis. The central methyl carbon is colored light green to make it visible through the opening between three sulfur atoms around the threefold axis. (Bottom) A stereoview of one of the aryl groups showing its conformation. The view also includes the central methyl group and the attached carbons from the two other aryl groups, illustrating the “propeller” twist of the aryl groups. The structure was optimized by means of Gaussian 98 with the B3LYP functional and the 6–31G basis set. Adapted from M. K. Bowman, C. Mailer and H. J. Halpern, The solution conformation of triarylmethyl radicals, *J. Magn. Reson.*, 172, 254, Copyright © 2005, with permission from Elsevier.³⁸

where the three phenyl rings are nearly mutually orthogonally twisted by 51° out of plane, so that the rings are almost perpendicular mutually, forming an angle of 84.5° between one another. Most of the unpaired electron spin density is located on the central carbon atom and is completely shielded from the solvent because of this conformation. The peculiarity of TAM structure is responsible for high resistance of TAMs to reduction as well as the slow electron spin relaxation. The propeller-shaped structure is characterized by effective shielding of the central carbon. Polar (carboxylate) groups are located at the ends of blades, while the other part of the molecule is lipophilic. For this reason, Finland TAMs can bind *via* the side surface to lipophilic moieties of biomolecules and membranes. After addition of a Finland TAM to 5% human albumin in a buffered solution (0.9% NaCl and 0.05 M phosphate pH 7), 80% of the TAM forms aggregates with albumin. Aggregation is the reason for toxicity of Finland TAMs.

TAM radicals are chiral molecules at room temperature. The chiral properties of TAM radicals were studied by Driesschaert *et al.*³⁷ They synthesized the right- and left-handed helices separately and evaluated their conformational stability in solution. It was shown that the two stereoisomers differ in their helicity and are configurationally stable enough to be separated and stored independently. The measurements of the kinetics of racemization of the right- and left-handed helices revealed that at room temperature, the half time of isomerization is ~ 1 month, and the two enantiomers of TAM can be separately stored for months in a freezer.

Later, it was shown that a triarylmethanols – the direct precursors of persistent trityl radicals^{40,41} are also propeller-shaped molecules as confirmed by ^1H and ^{13}C NMR signals in the spectra recorded at room temperature; these data indicate formation of two diastereoisomeric solvation complexes.⁴⁰ The interconversion of helicity does not proceed rapidly at the NMR time scale at 25°C , and the half-life of room-temperature racemization varies between 8.4 h and 1.32 years.

Recently, density functional theory (DFT) calculations for a wide range of TAMs were done by Alcona and Bromley³⁹ to assess the possibility to control the unpaired spin localization by means of torsion angles of the three aryl rings of a TAM. They studied the influence of chemical functionalization and temperature on spin localization and found that generally, the average αC spin localization is determined effectively by $\langle \cos^2 \varphi_i \rangle$ [where $\langle \cos^2 \varphi_i \rangle = (\cos^2 \varphi_1 + \cos^2 \varphi_2 + \cos^2 \varphi_3)/3$ and φ_i are the dihedral angles of each aryl ring with respect to the central sp^2 carbon atom plane]. This result can be used for future chemical design of TAMs as well as for the synthesis of novel TAM-based materials.

Typical EPR spectra of TAM radicals are shown in Fig. 3 for the series of TAMs shown in the same figure.⁴² The spectra are characterized by narrow lines in the range of 0.01–0.05 mT. Due to deuteration of CH_3 groups, it was possible to resolve the hyperfine interaction (HFI) constants in the region of 0.01 mT and to obtain all ^{13}C HFI constants with high accuracy.⁴³ The position of an EPR line of satellites can be used for PELDOR experiments on TAM–TAM labeled biomolecules. Figure 4

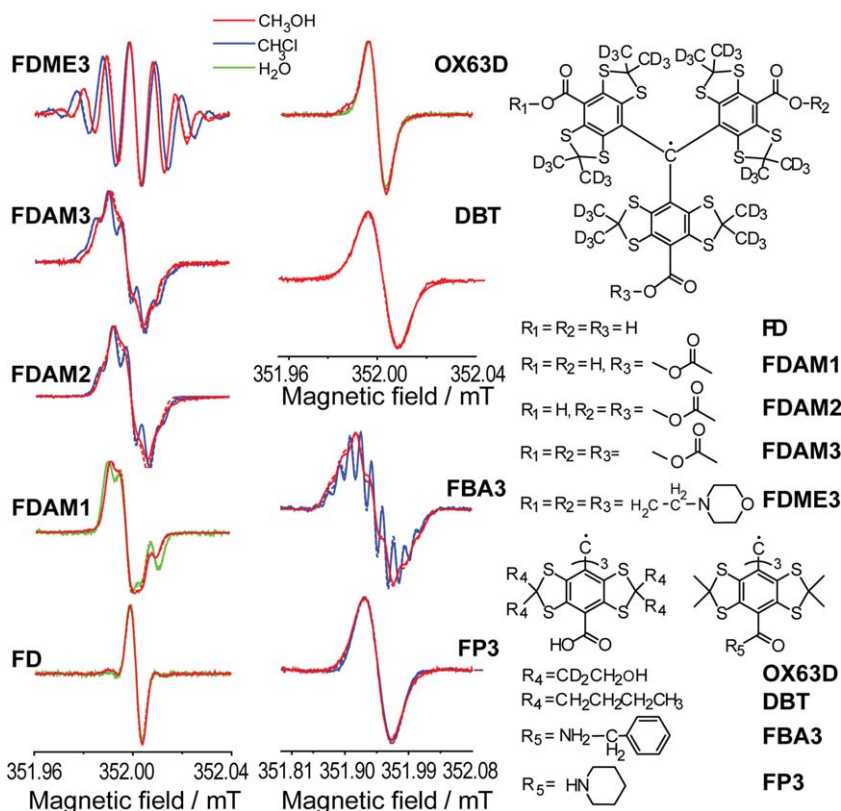


Fig. 3 Structures of TAM radicals and their X-band CW EPR spectra at 300 K in different solvents. Adapted with permission from ref. 42. Copyright © 2015 American Chemical Society.

shows EPR spectrum of Finland trityl at 293 K. $\Delta H_{pp} \sim 0.01$ mT including ¹³C satellites.⁴⁴

3 Synthesis of TAM radicals and TAM labeling of proteins and nucleic acids

TAM synthesis has been reviewed in numerous publications. Depending on the purpose, various chemical modifications of TAM radicals have been obtained, in particular amino derivatives allowing for dual pH/oxygen analysis,⁴⁵ aldehyde derivatives with high sensitivity to oxygen,⁴⁶ ester derivatives for intracellular oxygen quantification,^{47,48} a fluorinated TAMs,⁴⁹ dendritic trityl radicals with higher stability,⁵⁰ trityl-nitroxide biradicals for simultaneous determination of redox status and oxygenation,⁵¹ and dual-function oxygen- and pH-sensitive phosphonated trityl probes.^{52,53}

The synthesis of TAM radicals is substantially more complicated and time-consuming than that of nitroxide radicals. The synthesis of several widely used TAM radicals (OXO63, OXO31, and Finland) is described in patents,^{20–23} but the exact reproduction of the methods described in patents in most cases is not possible. Taking this into account, a simple

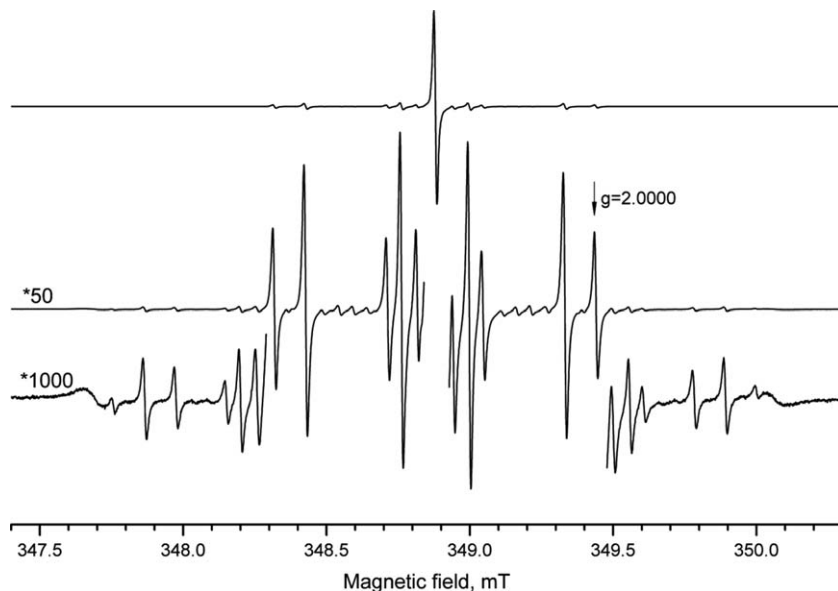
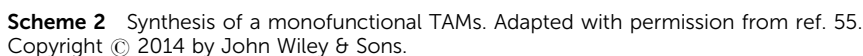
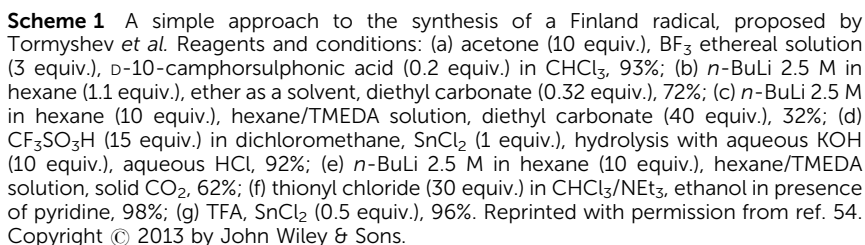


Fig. 4 EPR spectrum of 2.0 mM Finland trityl at 293 K. $\Delta H_{pp} \sim 0.01$ mT. The lines with least intensity are due to the hyperfine interactions in molecules containing two ^{13}C atoms. Reprinted from S. N. Truhan, V. F. Yudanov, V. M. Tormushev, O. Yu. Rogoshnikova, D. V. Trukhin, M. K. Bowman, M. D. Krzyaniak, H. Chen and O. N. Martyanov, Hyperfine interactions of narrow-line trityl radical with solvent molecules, *J. Magn. Reson.*, **233**, 29, Copyright © 2013, with permission from Elsevier.⁴⁴

approach to the synthesis of Finland TAMs, proposed recently by Tormyshev *et al.*,⁵⁴ seems to be helpful. This method is based on the original method of exhaustive carboxylation of a diamagnetic precursor (Scheme 1).

Suitable synthetical approaches to monofunctional TAM derivatives, a perspective on the TAM chemistry, and their use as spin labels were proposed recently.^{41,55,56} The approach⁵⁵ is based on a reaction of tris(2,3,5,6-tetratriaryl) cation with C-, N-, P-, or S-centered nucleophiles, yielding—as the main reaction products—asymmetrical monosubstituted triarylmethyl radicals (Scheme 2). The byproducts are unsubstituted TAMs formed due to one-electron reduction of the initial cations. The proposed approach allows for a variety of synthesis procedures for diverse TAM radicals and TAM-based spin labels. In particular, of a large series of TAM radicals including deuterium-substituted analogs was synthesized for future application as spin labels.⁴²

The approaches to spin labeling of proteins and nucleic acids are discussed in detail in several reviews.^{57–59} For protein labelling, in most cases, a highly selective reaction is carried out between a target cysteine residue in a biomolecule and a thiosulfonate group of a stable radical. In the absence of a cysteine group in the natural biomolecule and in the cases when a cysteine residue is located at a position not suitable for PD EPR analysis, it is necessary to use genetic engineering to prepare mutants that contain a special moiety (or moieties) at certain position(s) and can specifically react with a spin label. The latter is covalently



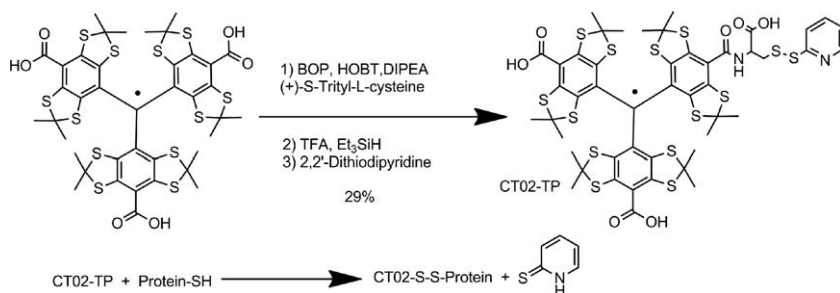
coupled with a cysteine moiety through a disulfide bond. Thiosulfonate coupling is highly selective but is not strong and can be broken easily in the presence of a reductant. Thus, in some cases, it is better to use less specific alkyl spin labels containing iodoacetamide or maleimide moieties. For higher accuracy of distance measurements, a rigid linker between a spin label and a biomolecule is preferred. A long and flexible linker leads to a wider distribution of distances between spin labels and complicates measurement of the dynamics and distances.

SDSL of nucleic acids includes different methods described in a recent review.⁸ A spin label can be introduced during oligonucleotide synthesis or postsynthetically. The first approach is usually limited to short sequences of 50–70 nucleotide residues (amenable to solid-phase synthesis). For longer sequences, incorporation of synthesized fragments into the structures of interest is required. The postsynthetic spin labeling requires site-directed attachment of the label to long and structured nucleic acids.

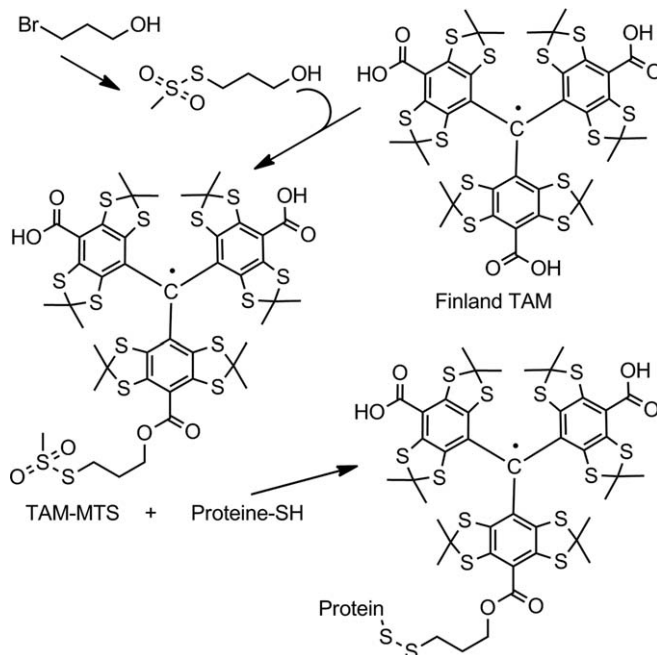
The first spin labeling of a protein by means of a TAM radical was performed by Hubbell and coworkers; they used a thiol-reactive trityl radical for SDSL of T4 lysozyme mutants.⁶⁰ The synthesis of a TAM-based spin-labeling reagent (CT02-TP) is shown in Scheme 3. The TAM radical CT-03 was conjugated with (+)-S-trityl-L-cysteine, followed by deprotection of the cysteine with trifluoroacetic acid and triethylsilane. The resulting thiol reacts with 2,2'-dithiodipyridine with formation of a TAM spin label. It should be noted that the proposed approach has limitations for general use because of low reactivity of the reagent.

Later, a TAM spin label for long-range distance measurement at physiological temperatures using T_1 RE was proposed by Yang *et al.*⁶¹ They synthesized a new TAM methanethiosulfonate reagent that undergoes a selective and high-yield reaction with cysteine (Scheme 4) generating a TAM-labeled side chain. The advantage of this reaction in comparison with a previous study, where a TAM radical was introduced at a cysteine *via* a reaction with an activated disulfide TAM reagent, is the highly reactive methanethiosulfonate function.

Recently Joseph *et al.*⁶² demonstrated the advantages of TAM labeling for selective and sensitive detection of protein–protein or protein–ligand interactions in complex native membranes. For TAM labeling of native



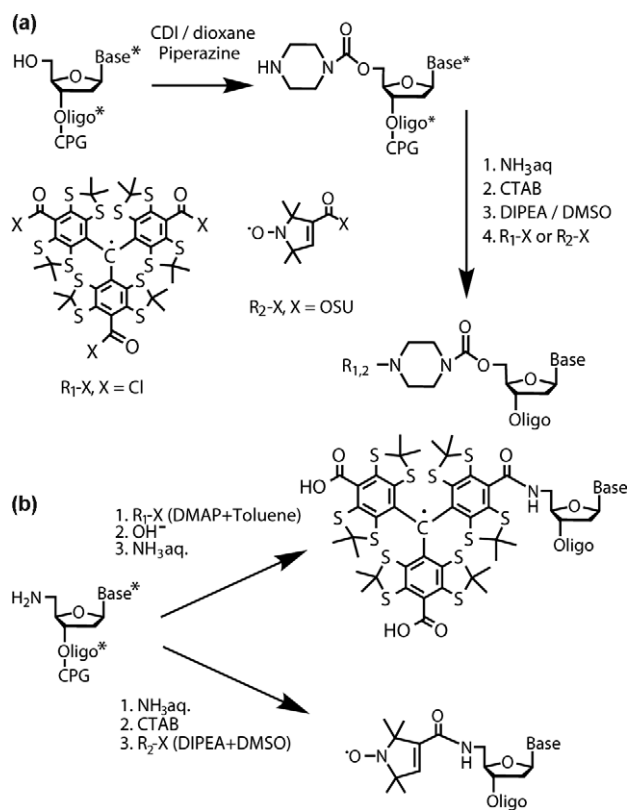
Scheme 3 Synthesis of CT02-TP and its reaction with a protein's SH group. Adapted with permission from ref. 60. Copyright © 2012 American Chemical Society.



Scheme 4 Preparation of the TAM methanethiosulfonate reagent and the TAM labeling of a protein. Adapted from Z. Yang, M. D. Bridges, C. J. López, O. Yu. Rogozhnikova, D. V. Trukhin, E. K. Brooks, V. Tormyshev, H. J. Halpern and W. L. Hubbell, A triarylmethyl spin label for long-range distance measurement at physiological temperatures using T1 relaxation enhancement, *J. Magn. Reson.*, **269**, 50, Copyright © 2016, with permission from Elsevier.⁶¹

outer membranes from *Escherichia coli*, those authors used a functionalized TAM radical containing a methanethiosulfonate group, which reacted with the SH group of a cysteine to form the side chain TAM1. Unexpectedly, room temperature continuous-wave (CW) EPR spectroscopy yielded an unusually broad spectrum for the TAM attached to the membranes containing BtuB 188C or BtuB WT. It was proved that TAM radicals formed an aggregate with the membrane, and a large proportion of the TAM binds nonspecifically. Thus, investigators should take the possibility of aggregation into account. Probably the best way to overcome this problem is the use of a hydrophilic TAM (OXO63 or OXO 31) as a spin label.

To date, only two examples of TAM labeling of a nucleic acid have been published.^{63,64} To demonstrate the possibility of dipolar EPR distance measurements at room temperature, model DNA duplexes labelled with a TAM were used. For formation of a DNA duplex, two 10-mer complementary oligonucleotides D1 and D2 labeled with TAM radicals were synthesized. A Finland TAM was chosen as a precursor for the synthesis of the two derivatives, *i.e.*, TAM-Cl and TAM-OSU (Scheme 5), used alternatively as spin-labeling reagents for oligonucleotides.⁶³ The latter were synthesized by means of phosphoramidite chemistry on a controlled pore glass support (see Scheme 1), and two linkers were used for spin labeling: rigid 1,4-piperazine and the short –NH– linker (Scheme 5).



Scheme 5 (a) Synthesis of TAM- and nitroxide-labeled oligonucleotides with a piperazine linker. Adapted with permission from ref. 63. Copyright © 2014 American Chemical Society. (b) The synthesis of TAM- and nitroxide-labeled oligonucleotides with a short (-NH-) linker. Adapted with permission from ref. 64. Copyright © 2015 American Chemical Society.

4 Electron spin relaxation of TAM radicals

The electron spin-lattice relaxation time values (T_1) and phase memory time ($T_m \approx T_2$) are crucial characteristics of spin labels and determine the range of distances amenable to measurement by EPR. The temperature dependence of T_1 and T_m for TAMs was studied by Halpern *et al.*⁶⁵ and Eaton *et al.*⁶⁶ Spin-lattice relaxation $1/T_1$ for TAMs in a frozen water: glycerol (1:1) solution at a temperature between 20 and 80 K is mainly determined by a Raman process (Fig. 5). For a Finland trityl, T_1 is ~ 2.5 ms in the X-band at 80 K. At higher temperatures in the X-band, including room temperature, $1/T_1$ is affected by a local mode process that modulates spin-orbit coupling. For a Finland trityl, T_1 is approximately 15–17 μs ^{42,67} in the X-band in an aqueous solution at ambient temperature and is sensitive to the substituent introduced into its carboxyl moiety.⁴² Two trends were observed: enhancement of $1/T_1$ after introduction of the ester moieties and a decrease in $1/T_1$ after introduction of amide moieties.

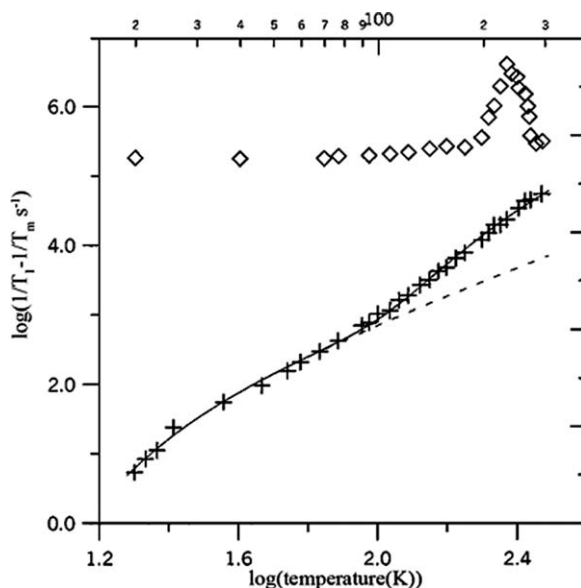


Fig. 5 X-band electron spin relaxation rates for *sym*-trityl-CD₃ in water : glycerol (1 : 1): (◇) $1/T_m$, (x) $1/T_1$, (—) a fit to the empirical data that were calculated, including Raman and local mode relaxation processes, and (---) a fit to empirical data including only the Raman process. Reprinted from L. Yong, J. Harbridge, R. W. Quine, G. A. Rinard, S. S. Eaton, G. R. Eaton, C. Mailer, E. Barth and H. J. Halpern, Electron spin relaxation of triarylmethyl radicals in fluid solution, *J. Magn. Reson.*, **152**, 156, Copyright © 2001, with permission from Elsevier.⁶⁵

The frequency dependence of T_1 for a Finland trityl and its derivative OXO63 was studied in the range of 250 MHz to 9.2 GHz.⁶⁷ It was shown that at 250 MHz and 300 K, T_1 is determined by inter- and intramolecular electron–proton dipolar couplings modulated by molecular tumbling at the rates comparable to the reciprocal of the resonance frequency. The contribution of this mechanism is reduced at higher frequencies and becomes negligible in the X-band.

T_m for TAMs at a low temperature in frozen water:glycerol (1:1) is determined by spin diffusion of solvent protons and is virtually independent of the microwave frequency or temperature up to 130 K.^{66,68} The T_m values are 3.9, 4.8, and 5.1 μ s for the Finland trityl, its deuterated analog, and OXO63, respectively, at 100 K in the X-band.⁶⁶ These T_m values are greater than those for nitroxyl radicals in the same solvent owing to greater radii of the trityl radicals and a correspondingly longer distance of the closest approach to solvent protons. Deuteration of the solvent makes T_m longer as compared with a proton-containing solvent at a temperature below 130 K and almost does not influence the T_m values at higher temperatures. Between approximately 130 and 170 K, the T_m value for the Finland trityl is reduced by rotation of the methyl groups. Between 200 and 300 K, where glassy solvent melts and softens, spin echo dephasing is enhanced by motional averaging of anisotropic interactions. The maximal value of $1/T_m$ in water:glycerol (1 : 1) is observed at $\sim$ 233 K

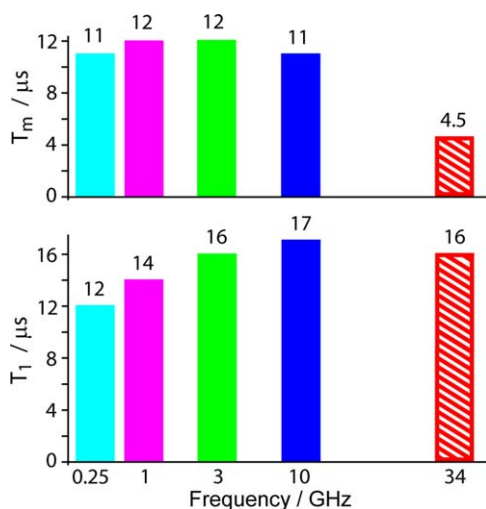


Fig. 6 Dependence of relaxation time values T_m and T_1 on microwave frequency for deuterated Finland trityl (FD) in H_2O at 300 K. Reprinted with permission from ref. 42. Copyright © 2015 American Chemical Society.

in the X-band, indicating that at this temperature, the tumbling rate is of the same order of magnitude as the anisotropy.

At room temperature in an aqueous solution in the X-band, a TAM is close to the fast tumbling limit, and T_m is mainly determined by T_1 .⁶⁷ For a Finland trityl, T_m is $\sim 9 \mu\text{s}$ in the X-band in an aqueous solution at 300 K and is slightly sensitive to deuteration of the radical and solvent.^{42,67} Note, however, that covalent attachment of the spin label to the biopolymer increases its rotational correlation time. This situation leads to enhancement of $1/T_m$ compared to that for a free radical in solution because of incomplete motional averaging of anisotropic interactions. There are two primary sources causing the decrease in T_m when the mobility of the spin label becomes restricted: anisotropy of an HFI and the g -factor. The multifrequency analyses of a Finland trityl in the X-, S-, and L-bands and at 250 MHz by Eaton *et al.*⁶⁷ revealed that HFI anisotropy plays the dominant role, whereas the contribution of g -anisotropy is negligible. Further research by Kuzhelev *et al.*⁴² showed that T_m values for a Finland trityl at 300 K are ~ 1.5 – 2.5 times smaller in the Q-band (34 GHz, 1.2 T) than in the X-band (9 GHz, 0.3 T; Fig. 6). This trend was ascribed to the contribution from g -anisotropy that is negligible in weaker magnetic fields but comes into play in the Q-band. Generally, nitroxide-based spin labels in PD EPR experiments at frequencies higher than the X-band allow a researcher to increase the signal-to-noise ratio.⁶⁹ Nevertheless, for a TAM, the relaxation data show that when the spin label is at least partly mobile, the X-band is most suitable for distance measurements by PD EPR.⁴²

5 Peculiarities of PD EPR approaches to distance measurement using TAM spin labels

Various methods of pulsed EPR dipolar spectroscopy (PDS) (DEER, DQC, or SIFTER) can be used for mapping objects spin-labeled by a trityl

radical on a commercial EPR spectrometer. The choice of a PDS method depends on the type of spin pair used: a spin pair with two trityl labels (TAM–TAM) or an orthogonal spin pair consisting of a trityl and nitroxyl label (TAM–NIT).

An EPR spectrum of a trityl is much narrower even in frozen solutions (~ 0.2 – 0.5 mT in the X-band) as compared to a widely used nitroxide (~ 7 mT in the X-band), resulting in a significant increase in the signal intensity in echo-detected EPR experiments for a TAM–TAM spin pair. The DQC^{14,70} and SIFTER¹⁵ methods require only one microwave frequency. The power of microwave pulses in these methods should be strong enough to excite the whole spectrum of the label. In contrast to nitroxides for a TAM–TAM spin pair, complete excitation of the single narrow spectrum is easily obtained on a commercial EPR spectrometer in the X-band (9.5 GHz),⁶³ Ku-band (17.2 GHz),⁶⁰ and Q-band (34 GHz).⁷¹ This state of affairs makes DQC and SIFTER seemingly ideal methods for a TAM–TAM spin pair.

Dipolar spectra and distance distributions from DQC⁶³ or SIFTER⁷¹ data can be obtained from the background-corrected time traces using DeerAnalysis.⁷² On the other hand, there is an obstacle for application of DQC or SIFTER: uncertainty in the definition of the background functions containing both an interspin and a relaxation-induced component. This ambiguity can affect widths of the distance distribution functions and the resulting mean distances. Today, there is no complete theoretical description of background functions of single-frequency PDS time domain traces, and in contrast to DEER, the background functions cannot be defined by studying a single-labeled sample. Nonetheless, application of the polynomial or exponential function to background correction allows an investigator to obtain a distance distribution function close to those that are obtained by DEER with the same samples.^{19,63} Comparison of the results obtained by DQC and SIFTER in the Q-band at 50 K revealed that the signal-to-noise ratio for SIFTER is fourfold higher than that for DQC.⁷¹

The most widely used two-frequency DEER^{12,73} method is also suitable for analysis of a TAM–TAM spin pair. In a DEER experiment in the X-band, the inversion pulse is placed at the center of the EPR spectrum, and the detection pulses are placed in the region of the ¹³C satellite, ~ 0.5 mT away from the inversion pulse (Fig. 7a).^{63,74} The time traces obtained by DEER in the X-band have a poor signal-to-noise ratio and low modulation amplitude as compared to those for DQC because of overlapping pulses. Nevertheless, DEER data can be useful in case of uncertainty regarding the baseline function in DQC or SIFTER traces.

The effects of orientation selection are not expected for TAM–TAM spin pair labeling in the X- and Q-band, owing to the relatively narrow line width of a TAM in a frozen solution and efficient excitation of the whole spectrum. At a higher microwave frequency, the EPR spectra of TAM in a frozen solution become wider and more asymmetric due to partially resolved *g*-tensor anisotropy.^{66,71} In the G-band, the spectral width is ~ 4.6 mT, which makes DEER preferable for distance measurements in a TAM–TAM spin pair. DEER measurements performed by Prisner *et al.*⁷¹

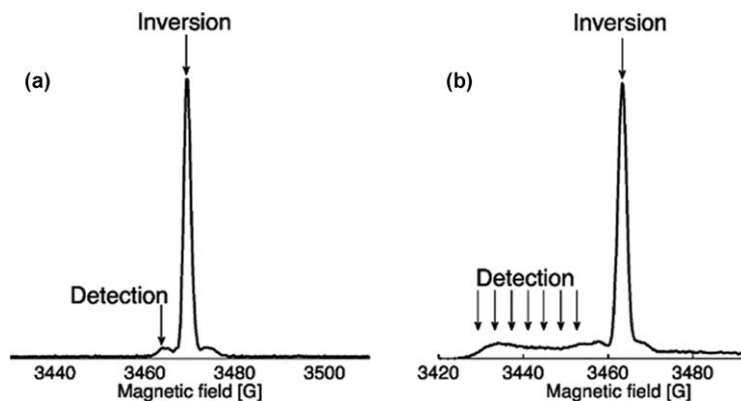


Fig. 7 Two-pulse echo-detected field sweeps of TAM–TAM and TAM–NIT spin pairs. The arrows show the positions of PELDOR inversion and detection pulses. Reproduced from ref. 74 with permission from the PCCP Owner Societies.

at various pump–probe positions across the G-band EPR spectrum for a TAM–TAM biradical revealed orientation selection. Nonetheless, the weak spectral anisotropy of the DEER time traces did not allow those researchers to obtain orientational information about the biradical in question.

For a TAM–NIT spin pair, the wide EPR spectrum of a nitroxide determines suitability of DEER for measurement of the interspin distance.^{63,74} The inversion pulse is placed on the maximum of the echo-detected EPR spectrum of a TAM. The detection pulse sequence is placed on the nitroxide EPR line at a frequency 30–90 MHz higher relative to the inversion pulse (Fig. 7b). Due to efficient pumping on the narrow EPR line of TAMs (~ 0.2 mT), the use of orthogonal labeling by TAM–NIT pairs allows investigators to obtain deeper dipolar modulation as compared with a NIT–NIT spin pair.

CW EPR measurements and DFT of TAMs showed that $\sim 70\%$ of the spin density is located on the central carbon atom and $\sim 30\%$ is delocalized across the rest of the molecule,³⁸ in contrast to widely used nitroxide radicals where spin density is mainly localized to the nitrogen and oxygen atoms.⁷⁵ Differences in the EPR spectral width and the spin density distribution for a TAM and nitroxide mean that the pseudo-secular part of the dipole–dipole coupling and spin delocalization for TAMs are substantial at longer distances as compared to nitroxides. To define the range of interspin distances where the pseudo-secular coupling should be included in the analysis, a series of TAM–TAM rigid biradicals with well-defined distances between the spin centers have been synthesized and studied using CW EPR, DQC, and DEER by Sigurdsson *et al.*⁷⁴ They found that TAM labels with interspin distances up to 2.5 nm fall into the strong-coupling regimen, and precise distances can be extracted only by taking into account both the spin density delocalization and the pseudo-secular part. Spin delocalization in a TAM was modeled by means of four-point spin center approximation where the central carbon atom is the major spin-bearing atom, and the spin-bearing carbon atoms in each phenyl group were combined into a single

point, located at the center of the phenyl group. The position and spin density of these points were determined by DFT calculations. In analysis of spin delocalization for a TAM–TAM biradical, taking into consideration the ~ 18.4 Å distance between the center carbon atoms leads to an increase in the mean interspin distance by 0.3 Å.

The range of distances that fall within the intermediate regimen (between a strong and weak coupling) was estimated to be 2.8–3.2 nm for a pair of TAMs, in contrast to 1.0–1.5 nm for a pair of nitroxides. In this case, the EPR data require careful analysis, and the resulting interspin distances are less reliable. CW EPR measurements enable assumptions about the dipole–dipole coupling for an unknown interspin distance. Special analysis of PDS time traces is needed if the width of the CW EPR spectrum of a dipole–dipole coupled trityl radical is broader than that for a single trityl radical.

Chemical properties differ significantly between trityl and nitroxyl spin labels. Generally, a trityl is certainly more bulky and hydrophobic than a nitroxide; this situation can cause discrepancies in the resulting distance distribution functions and mean distances. For TAM–TAM-labeled polymers with rigid structure, excellent agreement was observed with DEER data obtained for similar polymers labeled with a nitroxide.¹⁹ In contrast, in biological systems in the case of a flexible linker of spin labels, distance mapping using trityl and nitroxide labels can yield different results. For example, for the DNA duplex shown in Fig. 8, the width of the obtained distance distributions strongly depends on the type of radical: replacement of both nitroxides by TAMs in the same duplex spin-labeled with a piperazine linker caused narrowing of the distance distributions by the factor of three, thus resulting in improved accuracy of distance measurements.⁶⁴ Replacement of one nitroxide by a trityl also leads to a noticeable but less pronounced narrowing as compared with a TAM–TAM spin pair. The structure of the linker significantly influences the distance distributions in nitroxide–nitroxide pairs: the use of a short amine-based linker improves the accuracy by the factor of two as compared to a

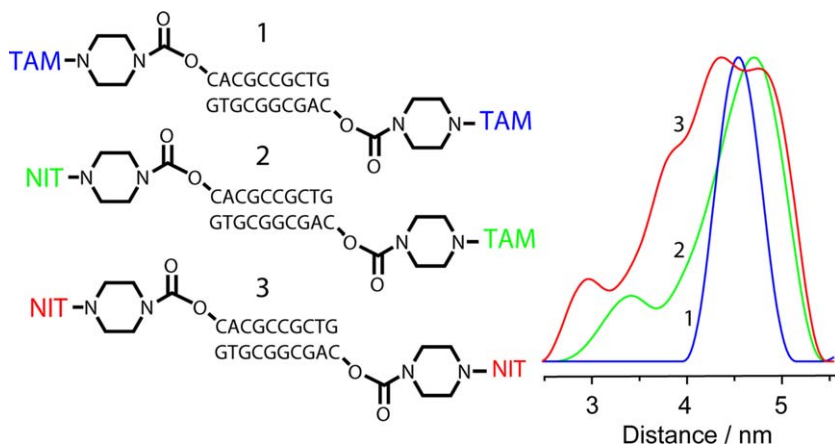


Fig. 8 The structure of spin-labeled DNA duplexes studied in ref. 64. Adapted with permission from ref. 64. Copyright © 2015 American Chemical Society.

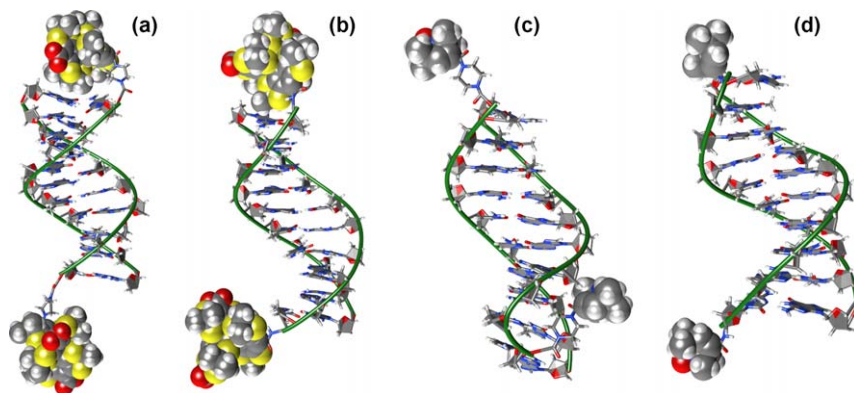


Fig. 9 Typical positions of spin labels relative to DNA duplexes: (a) I (D1-Pip-TAM/TAM); (b) VI (D1-NH-TAM/TAM); (c) IV (D1-Pip-NIT/NIT); (d) V (D1-NH-NIT/NIT). Adapted with permission from ref. 64. Copyright © 2015 American Chemical Society.

piperazine-based linker. Meanwhile, the distribution width in TAM–TAM pairs is mostly independent of the linker length.

Circular dichroism spectroscopy and melting temperature analysis⁶³ have shown that attachment of a TAM label does not strongly perturb the B-form conformation of a DNA duplex, indicating that the observed differences in the distance distributions are caused only by different conformational dynamics of trityl and nitroxyl spin labels. Molecular dynamics calculations (Fig. 9) were confirmed by the empirical results and revealed greater conformational disorder of nitroxide labels compared to TAM labels in the DNA duplexes being studied. It was concluded that there is a specific hydrophobic interaction of TAM radicals with terminal base pairs of the DNA duplex, and that this interaction causes TAM labels to occupy well-defined “capping” positions with respect to the termini of the duplex. In contrast to a trityl, the hydrophobic interaction of a nitroxide with terminal base pairs is weaker, and the labels can freely move relative to DNA.

Recently, this specific interaction was confirmed by orientation-selective high-frequency (94 GHz) DEER.⁷⁶ The double-frequency oscillations were observed in $\sim g_{\parallel}$ orientations of an echo-detected EPR spectrum, meaning that the (x, y) plane of TAMs is perpendicular to the DNA helix, and trityl radicals preferably occupy the “capping” positions. The specific interactions between a trityl and the system under study may complicate the translation of the obtained distance distributions into desired structural information. Nonetheless, when the interaction sites are known in advance, restriction of conformational flexibility can be used to improve the resolution of the distance measurements by narrowing the distance distributions.

6 Distance measurements at physiologically relevant temperatures

A PDS experiment is based on detection of the oscillation caused by a dipole–dipole interaction. For successful distance measurement, T_m of the spin label being used must be long enough to provide sufficient time

for recording at least one period of a dipolar oscillation. It is known that PD EPR is usually used for distance measurement at 80 K and lower because standard nitroxide-based spin labels have short T_m values at higher temperatures (T_m does not exceed 0.9 ns at a temperature >100 K).^{1–4} TAMs are characterized by long T_m (even at room temperature), which enables mapping the distance by PDS under conditions close to physiological.

To measure the distance by PDS, the object being studied must be immobilized in order to prevent averaging of the anisotropic dipolar interaction between the spins by rotational diffusion. In a frozen solution, this condition is automatically satisfied, but for measurements at the physiological temperature, it is necessary to use special approaches to immobilization. Recently, different ways to immobilize biopolymers were tested: covalent attachment to a solid support,⁶⁰ noncovalent interaction with silica gel,⁶³ and the use of an environmental medium such as disaccharides^{77,80} (Fig. 10a–c). It is important to find the conditions

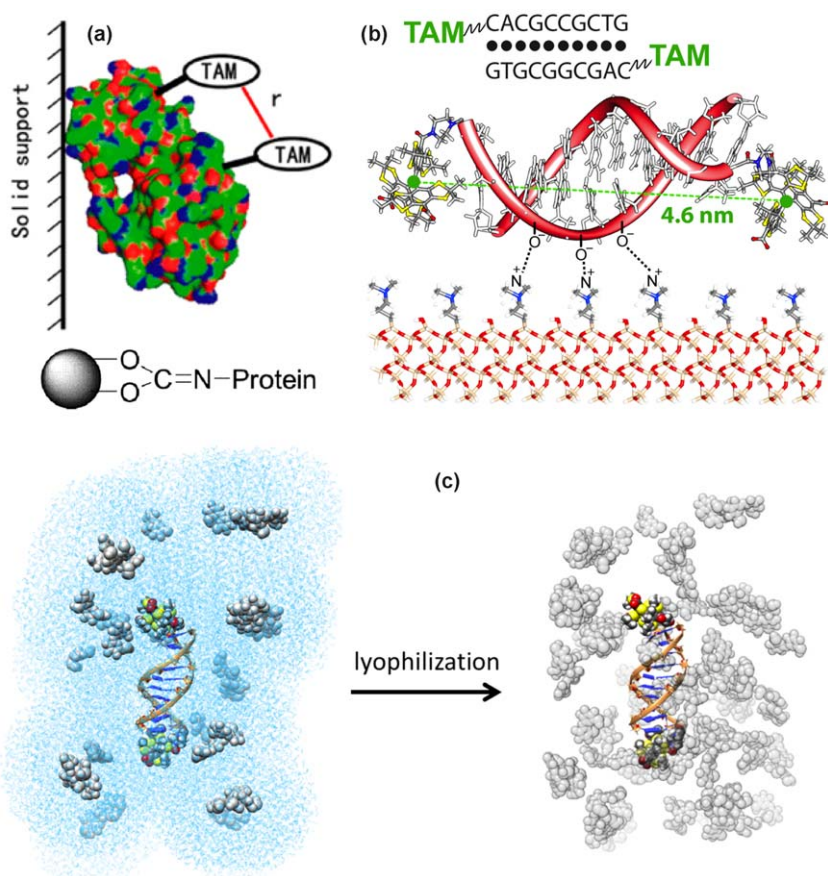


Fig. 10 Different types of immobilization of a biomolecule. (a) Covalent attachment to a solid support. Adapted with permission from ref. 60. Copyright © 2012 American Chemical Society. (b) Sorption on NucleosilDMA particles. Adapted with permission from ref. 63. Copyright © 2014 American Chemical Society. (c) Embedding in a dehydrated trehalose. Adapted with permission from ref. 80. Copyright © 2016 American Chemical Society.

where the immobilization does not disturb the native structure of the objects under study, and it is desirable to attain a long T_m value.

The first PDS distance measurements in a liquid solution (at 4 °C) were implemented using trityl spin labels and T4 lysozyme (T4L) as a model protein.⁶⁰ The immobilization was achieved *via* covalent attachment to a solid support (Fig. 10a). The T_m value at 4 °C for a trityl attached to an immobilized protein was 0.7 μ s, which allowed to measure a $\sim$ 2 nm distance. It should be noted that this range of distances may be measured by dipolar broadening of CW lineshapes of interacting nitroxides at room temperature.^{78,79} Furthermore, covalent attachment to a solid phase can induce disorder of the structure under study. Therefore, to minimize the impact of immobilization on the measured distances, the sites of attachment to the surface must be located at some distance from the moiety carrying the spin labels.

For nucleic acids, efficient immobilization can be achieved using the common ion exchange sorbent NucleosilDMA (Fig. 10b). DNA is held on the particle's surface by the electrostatic interactions between phosphate anionic groups of DNA and cationic groups of NucleosilDMA. This approach was applied to physiological-temperature distance measurement in a model 10-mer DNA duplex labeled with trityl radicals *via* a piperazine linker.⁶³ The T_m value for the electrostatically immobilized DNA duplex was 1.4 μ s at 37 °C in an aqueous solution; this situation allowed those authors to measure distances of $\sim$ 4.6 nm with high accuracy (Fig. 11). Comparison of the data obtained at 37 °C with the results of PDS experiments at 80 K in a water:glycerol solution and with results of a molecular dynamics simulation revealed that sorption of the DNA duplex on NucleosilDMA has no effect on the distances measured. This approach to immobilization is easy to implement: the NucleosilDMA sorbent is commercially available, and preparation of a sample requires only mixing an aqueous solution of DNA with dried NucleosilDMA particles.

Another method of immobilization involves embedding a biomolecule in a dehydrated disaccharide (Fig. 10c). Dehydrated trehalose provides effective immobilization in PDS room-temperature experiments and can be successfully applied to a wide variety of biological systems.^{77,80} During dehydration, trehalose can retain water molecules and create networks of hydrogen bonds.^{81,82} For proteins, slow drying of the water:trehalose solution at room temperature in most cases allows for preservation of the native structure in a dehydrated state and for restoration of their functional properties after rehydration.⁸³ Recently, trehalose was successfully applied to immobilization of a spin-labeled protein,⁷⁷ but the first attempt to perform PDS measurements on a spin-labeled DNA in dehydrated trehalose failed due to partial denaturation of double-stranded DNA.⁸⁴ The melting of DNA can be avoided if the sample is lyophilized from a frozen state in vacuum immediately after flash-freezing in liquid nitrogen.⁶⁴ Additionally, high vacuum (5×10^{-8} bar) enables deoxygenating the sample and obtaining longer T_m values. The distance distribution obtained for doubly TAM-labeled DNA in trehalose is similar to that obtained in a water:glycerol solution at 80 K (Fig. 11). The electron

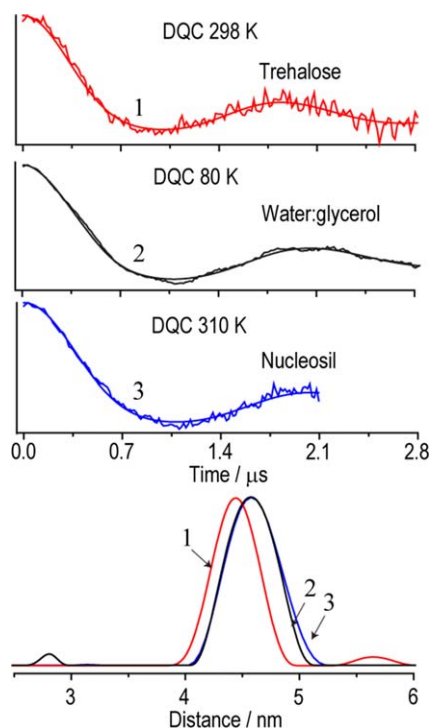


Fig. 11 (Top) Background-corrected X-band DQC time traces obtained for the doubly TAM-labeled DNA duplex: (1) in dehydrated trehalose at 298 K, (2) in water:glycerol at 80 K, (3) in the presence of Nucleosil/DNA particles at 310 K. (Bottom) Corresponding distance distributions obtained using DeerAnalysis.⁷² For (1) – adapted with permission from ref. 80. Copyright © 2016 American Chemical Society. For (2) and (3) – adapted with permission from ref. 63. Copyright © 2014 American Chemical Society.

spin dephasing time of a TAM-based spin label in dried trehalose is the longest in comparison with all other immobilizers studied previously ($T_m = 2.2 \mu\text{s}$ for TAM-labeled duplex DNA in trehalose). These findings suggest that the combination of a TAM-based spin label and trehalose can be considered a promising strategy for long-range distance measurements in nucleic acids and protein complexes at ambient temperature by EPR.

The first room temperature DEER using a ^{13}C satellite of a TAM for observation and the central line for pumping was reported recently,⁴² and the distances obtained match those observed previously by DQC for the same sample.⁶³ A two-pulse sequence was used for measurements of phase memory time T_m , and the latter was found to be slightly longer at the central (^{12}C) EPR line ($T_m = 2.2 \mu\text{s}$) than in the ^{13}C satellite ($T_m = 1.7 \mu\text{s}$). This difference is determined by the contribution from modulation of an anisotropic ^{13}C HFI. The value of T_m and the signal intensity in the ^{13}C satellite are sufficient for reliable DEER measurements at room temperature and the spin–spin distance obtained is in good agreement with the previously obtained data for this duplex.⁶³

Because of the relatively large size of a TAM radical, its possible influence on the structure of a biopolymer and validity of the data

obtained by PD EPR are subject to debate. Verification of interspin distances measured by PD EPR was performed recently by means of a combination of NMR and a molecular dynamics simulation.⁸⁵ It is known that NMR is widely used for structural analysis of biomolecules under natural conditions (room temperature and an aqueous solution). It was shown that the DNA duplex structures and conformation flexibility of a native duplex and a TAM-labeled duplex obtained by means of a molecular dynamics simulation are the same as the structure obtained by NMR refinement. To compare TAM-labeled, native, and NMR-refined structures, a cluster analysis of each molecular dynamics trajectory was performed. The average distance between structures in the most populated clusters was 0.5 Å in all cases. A comparison of the most representative structures in each type of molecular-dynamics trajectory is shown in Fig. 12.

It is necessary to emphasize that PDS measurements at ambient temperature can also be carried out using spirocyclohexane-substituted nitroxides (Fig. 13) in conjunction with immobilization in glassy trehalose.⁷⁷ The T_m value of commonly used nitroxide-based spin labels is too short to perform PDS measurements at a temperature >80 K because of rotation of the *gem*-methyl groups.⁸⁶ This major spin echo dephasing mechanism can be eliminated by replacement of the *gem*-methyl groups with spirocyclohexyl groups; this approach makes T_m long enough for PDS distance measurements at a temperature >80 K.^{87,88} Eaton *et al.* introduced spirocyclohexane-substituted nitroxide spin labels into some sites on T4 lysozyme.⁷⁷ Immobilization of doubly labeled T4 lysozyme in glassy trehalose allowed them to obtain long enough T_m (920 ns in the Q-band) to measure an interspin distance of 3.2 nm at ambient temperature.

On the other hand, T_m of nitroxide-based labels in dehydrated trehalose at 300 K is ~700 ns and does not strongly depend on the structure of substituents.⁸⁴ It was shown that the difference in substituents at a

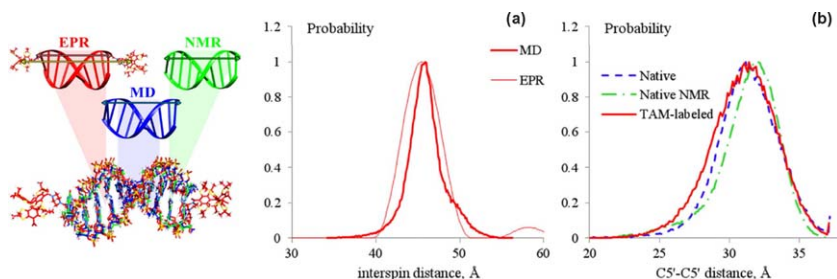


Fig. 12 Comparison of TAM-labeled (red), native (blue), and native NMR-restrained structures. Data on D1/D2 DNA duplexes (green) were obtained by molecular dynamics simulations. (a) The distribution of interspin distance probability in the TAM-labeled DNA duplex; this distribution was obtained by analysis of a 1.5 μ s trajectory (thick curve), molecular dynamics trajectory, and by the DQC method (thin curve, adapted from ref. 63. Copyright © 2014 American Chemical Society). (b) The C5'-C5' distance of the 5'-terminal nucleotides obtained in the MD simulation of the TAM-labeled DNA duplex (red line), native DNA duplex (blue dashed line), and native DNA duplex with NMR restraints (green dashed-dotted line). Adapted with permission from ref. 85. Copyright © 2016 American Chemical Society.

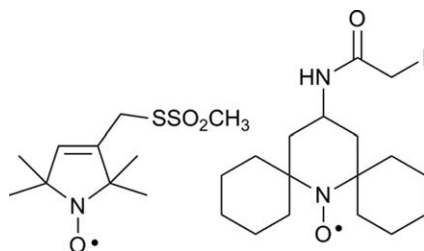


Fig. 13 The structure of spin labels: MTSL and spirocyclohexane-substituted piperidine nitroxides. Adapted from S. V. Meyer, M. A. Swanson, Clouston, P. J. Boratyński, R. A. Stein, H. S. Mchaourab, A. Rajca, S. S. Eaton and G. R. Eaton, Room-temperature distance measurements of immobilized spin-labeled protein by DEER/PELDOR, *Biophys. J.*, **108**, 1213, Copyright © 2015, with permission from Elsevier.⁷⁷

position close to the radical center and replacement of the pyrroline nitroxide ring with a piperidine nitroxide ring lead to only slight changes in electron spin phase relaxation time. A slight increase in T_m for a spirocyclohexane-substituted nitroxide covalently attached to a biomolecule in comparison with a tetrasubstituted analog is ~ 100 ns and is most likely due to the interaction of hydrophobic spirocyclohexyl groups with the biomolecule, thus leading to limited mobility and consequently longer relaxation time. If we take these data into account, then nitroxide-based labels at present cannot be used for long-range distance measurements at room temperature where application of trityl spin labels is preferable.

As mentioned above, trityl radicals have relatively long spin lattice (T_1) relaxation time, which makes them promising labels for studies on a distance-dependent spin lattice (T_1). Hubbel and coworkers used an alternative approach to measuring distances at room temperature; this method is based on the T_1 RE of trityl radicals by Cu^{2+} .⁶¹ The idea behind the RE approach is based on the changes in the electron spin relaxation of the observed spin label induced by Cu^{2+} or another transition metal with short electron spin relaxation. The changes in observed T_1 depend on the distance between the observed spin label and metal; thus, the analysis of electron spin relaxation in the presence and absence of the metal allows researchers to measure this distance. Jun *et al.* showed that RE of a nitroxide side chain in a rapidly tumbling peptide ($T_1 = 2$ μs) can help to measure distances up to 25 Å.⁸⁹ Due to substantially longer relaxation time T_1 for a trityl radical ($T_1 = 12$ μs), distances up to 50 Å can be measured at room temperature using RE (Fig. 14c). Yang *et al.* studied three Cu^{2+} -TAM1 pairs (shown in Fig. 14a) tethered using CNBr-activated Sepharose or coupled site-selectively using *p*-AcPhe at position 65. The values of spin lattice relaxation time were measured using long-pulse saturation recovery (SR) EPR. Figure 13 shows representative spin relaxation curves for T4L 23L/131TAM1 attached site-selectively to residue 65. Note that only an average distance except for distance distribution can be obtained by the RE method; this situation significantly limits applicability of this method in the case of several conformations of the spin label.

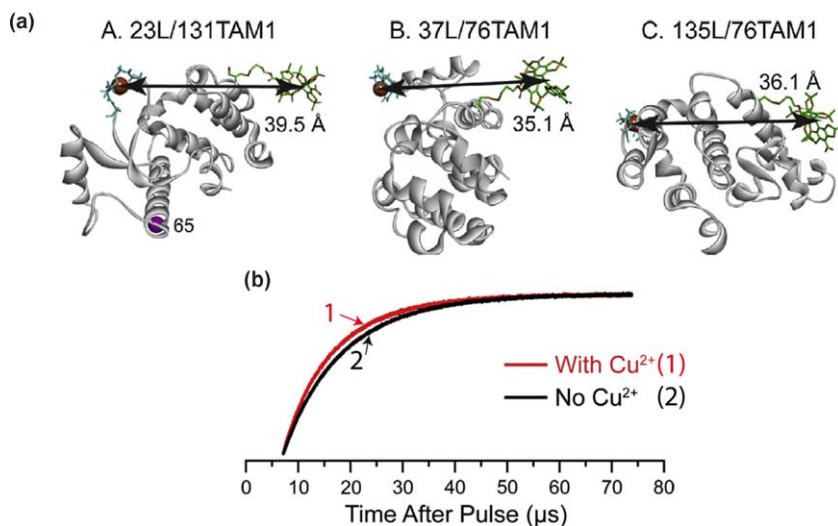


Fig. 14 (a) Models of the TAM1–Cu²⁺ pairs investigated in ref. 61. (b) Representative relaxation enhancement data. The saturation recovery traces obtained for the 23L/65p-AcPhe/131TAM1 sample in the absence (2) and presence (1) of Cu²⁺. Adapted from Z. Yang, M. D. Bridges, C. J. López, O. Yu. Rogozhnikova, D. V. Trukhin, E. K. Brooks, V. Tormyshev, H. J. Halpern and W. L. Hubbell, A triarylmethyl spin label for long-range distance measurement at physiological temperatures using T_1 relaxation enhancement, *J. Magn. Reson.*, **269**, 50, Copyright © 2016, with permission from Elsevier.⁶¹

7 Conclusion

Some investigators proposed to use TAM radicals as spin labels in SDSL PD EPR only four years ago. Since then, several advantages of these applications of TAMs were demonstrated, in particular (i) room temperature distance measurements in proteins and nucleic acids; (ii) higher accuracy of distance measurements in an oligonucleotide duplex in comparison with nitroxide spin labels; (iii) orientation dependence in the G-band providing information concerning specific interactions between a trityl and a biomolecule; (iv) spin labeling of a natural membrane allows for selective detection of the interaction between a TAM-labeled protein and its nitroxide-labeled ligand with high sensitivity as well as measurements at up to 175 K; (v) orthogonal spin labeling by means of a TAM allows for measurements of distances as long as 50 nm by RE. Many more achievements in TAM applications are expected in the coming years. Possible future improvements may include the development of novel hydrophilic spin labels based on OXO63 or OXO31, which will prevent aggregation of a TAM with a membrane or other biomolecules. Nowadays, the most appropriate pulse sequences for distance measurements using TAM radicals are DQC and SIFTER, and new pulse sequences are likely to be proposed.

Acknowledgements

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Molecules as electron spin qubits

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Electron spins confined to molecular compounds are promising candidates to base the elementary unit of a quantum computer: the qubit. The major advantage of molecular spin qubits stems from the ability to tailor their performance properties using synthetic chemistry and devise inexpensive new systems for experimental study. The article details the latest developments of the design and testing of new molecular spin qubits and how they are addressing the key challenges in the field of quantum computing.

1 Introduction

A quantum computer is a machine that will impact all areas of society. The potential lies in its operation; a device that utilises the principles of quantum physics to solve problems in a fraction of the time it would take any current computer.¹ The elementary building block of a quantum computer is a quantum bit, or “qubit”. In contrast to a classical bit, which takes one of two well-defined values, *e.g.* 0 or 1, a qubit can be either 0, 1, or any value in between. It is the latter property, coined superposition, where enormous increases in information processing are gained because calculations are performed simultaneously.

Electrons are seemingly the natural choice for a matter spin qubit, as the dipole moment of an electron is by definition a two-level system with spin “up” and spin “down” levels. Surprisingly, the physical realisation of an electron spin qubit was preceded by those composed of nuclear spins. The lack of enthusiasm for electron spins stems from the intractable obstacle of their short decoherence times – the lifetime of electron spin superposition states. The inherent isolation and confinement of nuclear spins from the influence of the surrounding environment endows them with enviable decoherence times, with a record of 39 minutes at room temperature.² Exploiting this characteristic of nuclear spins and the depth of expertise in spin manipulation by means of NMR, the execution of quantum algorithms have already been performed with nuclear spin qubits.^{3,4}

Despite the shorter coherence lifetime, electron spins offer a distinct advantage over nuclear spins in the ease with which they can be scaled. This feature has been central to the surge in activity over the last decade in the design and construction of molecular systems as matter spin qubits that utilise electron spins, occasionally in combination with nuclear spins. These constructs are different to materials-based systems such as nitrogen vacancies in diamond,^{5,6} doped silicon,^{2,7–9} or quantum dots.^{10,11} Confining electron spins to well-defined molecules provides unparalleled degree of control of their intrinsic properties, such as modifying the composition to optimise decoherence lifetime, as well as

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the ability to tune the interaction and therein the accessibility of superposition states that constitute quantum logic operations. Coupling these features with the tremendous advances made in the electron spin manipulation technology driven by pulsed EPR studies of protein systems,^{12,13} electron spins in the form of molecular qubits are poised to surpass their nuclear spin counterparts. The present review aims to provide a summary of the last four years of progress in the design and utilisation of molecular spin qubits, and their input towards the physical realisation of quantum computing.

2 Quantum hardware

In classical computing, a bit is either “on” or “off”, 0 or 1; in quantum computing, a qubit is in a superposition of 0 and 1 (defined as $|\psi\rangle = \alpha|0\rangle + \beta|1\rangle$). For a successful qubit the simplest requirement is that it is a two-level system. An electron spin ($S = 1/2$) achieves this with the spin-up regarded as one level and spin-down as the other, described as the $m_s = +1/2$ and $m_s = -1/2$, states, respectively. An $S = 1/2$ system is a qubit because it can be described as any superposition of these two states, as visualised by a position on the surface of a Bloch sphere (Fig. 1).

Among the various materials under consideration as the hardware of a quantum device, there are a number of features they all have in common. These characteristics were summarised by DiVincenzo into a set of criteria to which potential qubits must adhere.^{14,15} There are a total of five criteria for quantum computation with a further two for the implementation of quantum communication that will not be discussed here. As the field has developed and expanded, the criteria have been refined and elaborated,^{10,16,17} and are summarised as follows:

- I. a scalable physical system with well characterised qubits
- II. the ability to initialise the state of the qubits

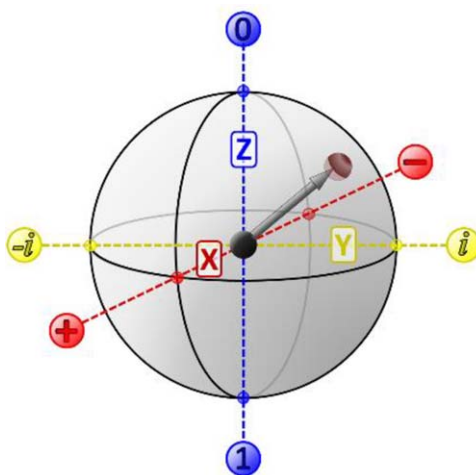


Fig. 1 Bloch sphere representation of a spin qubit.

- III. long coherence time much longer than the gate operation time
- IV. a “universal” set of quantum gates
- V. a qubit-specific measurement capability

The first two criteria are readily achieved by a single electron spin because an isolated $S = 1/2$ system can only adopt one of two levels that are well-defined and pure spin states. In molecules with more than one unpaired electron, like high-spin transition metal ions and molecular nanomagnets, access to a pure $S = 1/2$ ground state is achieved by lowering the temperature. It is important to exclude admixtures of higher spin or excited states that will pollute the ground state and lead to “leaking” of quantum information.^{16,18} It is therefore a requirement of a multispin system to have a sufficiently large energy gap between the ground state and its excited states, which can be engineered by synthetic chemistry.

The second criterion is equally straightforward, as simply cooling down any molecular system in the presence of an external magnetic field will lead to a Zeeman splitting of the m_s states, and preferential population of the lowest energy level. For $S = 1/2$ paramagnets, this will apply at room temperature; for multispin systems, the temperature is a function of the energy gap between the ground and excited states. An increase in the applied magnetic field will also lead to a population bias of the lowest energy level.

The idea of quantum coherence – and decoherence – is central to a qubit’s performance, and how effectively it functions. The lifetime of a cohered state – the formation of quantum phase information – is considered by many to be the most critical of the DiVincenzo criteria. This is largely because it is the only parameter over which qubit designers and engineers have the most control. The terms coherence and decoherence are used interchangeably throughout the literature, with the former being the goal of a qubit interaction – the formation of a cohered or “entangled” state – and the latter describing the decay or loss of this state. In this article, we shall stick with decoherence, and its lifetime is a direct measure of how rapidly quantum information is lost. Decoherence is essentially the interaction of the qubit with its surrounding environment. There are two general phenomena of how this occurs: interaction with phonons and vibrations within the material, and a through-space interaction with neighbouring spins – electronic and nuclear – within the qubit and between qubits. The latter exposes the irony that a quantum computer requires interaction between qubits which actually fuel the destruction of the sought after entangled state. The interaction with phonons is predominantly suppressed by operating at low temperature, which neatly augments the first two criteria. Eliminating through-space or dipolar interaction between spins is the more daunting challenge as the very atoms from which matter spin qubits are based constitute a “spin bath” that extends throughout space promoting decoherence. That said, chemists can be judicious in their choice of atomic ingredients, selecting elements with a low abundance of isotopes with non-zero nuclear spin or isotopes with a small gyromagnetic ratio. The target for

any potential qubit is to exhibit a decoherence lifetime that is a factor of 10^4 longer than the time required for a basic computation action, the gate time. Gate times are typically in the region of 10–20 ns, depending on the technology delivering the gating action. This places a lower limit on the decoherence lifetime of 100 μ s. This factor is known as the qubit figure of merit, Q_M , which is quantified as $2\Omega_R T_2$, where Ω_R is the Rabi frequency and T_2 the decoherence lifetime (*vide infra*).

Although addressing the second criterion nicely works in favour of eliminating decoherence from phonons, the downside is the need to scale qubits as described in the first criterion increases the number of dipolar decoherence pathways. In order for a quantum computer to operate there must be universal logic, and therein the fourth criterion. A quantum computer capable of implementing advanced algorithms such as those created by Shor or Grover,^{19,20} is an assemblage of quantum logic gates analogous to classical Boolean ones. This ranges from simple single qubit unitary rotations to selective operations on individual qubits within a larger construct. Many universal logic gates operate on coupled qubits which requires there be an interaction between them, known as entanglement, a consequence of operating within a quantum system.³ In principle, all classical logic gates can exist in a qubit form, as highlighted by the physical manifestation of NOT, CNOT and $\sqrt{\text{SWAP}}$ quantum logic operations.

Lastly there is the issue of correctability, where the requirement exists that it must be possible to extract the entropy of a system to maintain a quantum state. This issue is related to the aforementioned second and fifth criteria, that of a “qubit specific measurement capability”. Essentially, both the input and output from the system must be a simple fiducial state that is classical in nature. Therefore, qubits can never be completely isolated from the external environment as information needs to be retrieved from the system.

It is by these guidelines that the performance of a matter spin qubit is qualitatively and quantitatively evaluated. The current state-of-the-art in molecular electron spin qubits is primarily focused on the third criterion, the decoherence lifetime, as the measure by which different atomic architectures are compared. Only within the last few years has progress advanced to the implementation of quantum logic, which goes beyond qubit engineering towards constructing apparatus needed to perform and execute quantum logic in this truly multidisciplinary field.

3 Experimental methods

The five criteria detailed above are the starting point towards implementing quantum computing with matter spin qubits. Assessing the performance of molecular qubits primarily makes use of a variety of EPR spectroscopic methods. Typically the entry point for an examination of the electronic structure of a paramagnetic molecule is continuous-wave (cw) EPR spectroscopy, where the electron spin energy levels are described by the spin-Hamiltonian parameters g , A , D , E and J , which have their usual meanings. Because of the requirement of spin qubits to

exhibit very weak couplings, a standard commercial X-band spectrometer is well suited to probe these electronic interactions as they are typically of similar magnitude to the microwave quantum ($\sim 0.3 \text{ cm}^{-1}$). Electronic structure studies are routinely augmented with other physical characterisation techniques such as magnetometry, electronic and vibrational spectroscopy, and specific heat capacity that provide additional detail on the electron spin manifold, especially in the presence of stronger spin-spin interactions. Elucidation of the electronic and, in many cases the molecular structure by means of single-crystal X-ray diffractometry, means the first criterion is achieved.

To evaluate decoherence phenomena, pulsed EPR spectroscopy is sought. Although in many cases, cw EPR will identify and quantify dipolar spin-spin interactions such as hyperfine and zero-field splitting (ZFS), relaxation times are measured using electron spin echo envelope modulation (ESEEM) methods. Two experiments are employed to quantify the spin relaxation properties of the system; the pulse sequences are presented in Fig. 2. The spin-spin relaxation time, T_2 , also known as the transverse relaxation or phase memory time (T_2 , T_2^* and T_m are used to denote this parameter) is measured by fitting the decay profile of the intensity of a two-pulse Hahn echo sequence with increasing interpulse delay time τ (Fig. 2). The decay of the echo intensity plotted as a function of τ is modelled as either a monoexponential according to eqn (1), where 2τ is twice the interpulse delay time, $I(2\tau)$ is the integrated echo intensity for the pulse separation, $I(0)$ the intensity extrapolated to $\tau = 0$, and T_2 the decoherence time.²¹

$$I(2\tau) = I(0) \exp\left[-\frac{2\tau}{T_2}\right] \quad (1)$$

Often the decay profile is not adequately matched with a simple monoexponential which is the result when shallow proton modulation surface in the ESEEM measurement and the relaxation processes are

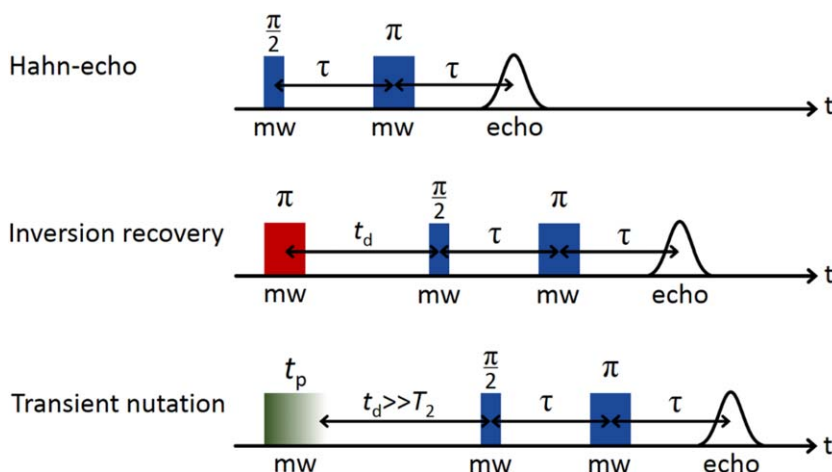


Fig. 2 EPR pulse sequences used in relaxation and coherence measurements.

altered. In this regime, a stretched exponential is employed, as described by eqn (2), where x is the stretch factor. When decoherence is dominated by spin diffusion – the flip-flop of nuclear spins – the stretch factor ranges between 2 and 3; when it is dominated by the physical motion of magnetic nuclei, the value is closer to 1.²¹ Occasionally two relaxation regimes, designated fast “ f ” and slow “ s ”, are operative, and these are accommodated by a biexponential function (eqn (3)), where A_f and A_s are the amplitudes of the fast and slow relaxation components with relaxation times $T_{2,f}$ and $T_{2,s}$, respectively.

$$I(2\tau) = I(0) \exp \left[- \left(\frac{2\tau}{T_2} \right)^x \right] \quad (2)$$

$$I(2\tau) = A_f \exp \left(- \frac{2\tau}{T_{2,f}} \right) + A_s \exp \left(- \frac{2\tau}{T_{2,s}} \right) \quad (3)$$

The spin-lattice or longitudinal relaxation, universally labelled T_1 , is measured by inversion recovery. The approach employs a three pulse ESEEM sequence where an inversion pulse, which flips the spin packets along the z -component of magnetisation, is followed by a standard Hahn-echo (Fig. 2). These magnetisation recovery curves are modelled using the same mono-, stretch or bi-exponential functions detailed for T_2 (eqn (1)–(3)).

Variable power nutation experiments are used to demonstrate coherent spin manipulation, an essential requirement of a spin qubit. The experiment utilises a nutation or tipping pulse (t_p) which manoeuvres the spins to a particular superposition, *i.e.* a spot on the surface of the Bloch sphere (Fig. 1), determined by the length of the pulse and its power. The existence of the superposition is established by the subsequent observation of an echo using the prescribed sequence which reveals damped oscillations at multiple powers. These so-called Rabi oscillations arise from coherent absorption and emission of microwave radiation within the resonant cavity,²² and represent oscillatory transitions between the ground and excited collective spin states within the system. Rabi oscillations that show a linear dependence of the frequency with the intensity of the oscillating field demonstrates the creation of an arbitrary superposition of states, and thus fulfilling part of the fourth criterion towards implementing universal quantum logic. The Rabi frequency (Ω_R) is a measure of gating time; in this case a simple unitary rotation, *i.e.* a spin flip.

4 Organic molecular qubits

Main group elements with unpaired spins are, with a few exceptions, unstable. Of those that are easy to handle under standard laboratory conditions, most tend to be nitrogen-based. Nitroxyl radicals are the archetypal organic free radical and have been a cornerstone of EPR spectroscopy since its conception due to the combination of a stable electron spin and signature ^{14}N hyperfine coupling.²³ Given the ease with

which they can be modified and elaborated, Takui and co-workers have been world-leading in their use of nitroxyl radicals as spin hosts in single- and multi-qubit molecules.^{24–30} A selection of the chemical species they have synthesised and characterised is presented in Fig. 3.

From the selection shown, the biradical {2,2,6,6-tetramethylpiperidin-*N*-oxyl-4-yl}-3,5-dimethylbenzoate-4-yl}terephthalate is the only molecular system to demonstrate quantum gating.²⁴ The use of a 3,5-dimethylbenzoate-4-yl group in the covalent linkage of the two nitroxyl radicals is the crucial design feature. The steric bulk of its methyl substituents ensures this group is orthogonal to the adjacent terephthalate group, thus forcing the planes of the terminal piperidin-*N*-oxyl (TEMPO) radicals to be similarly orthogonal. This is the concept of *g*-engineering,²⁶ where enforced rotation of molecule ensures the local *g*-frame of each unpaired spin is different, which is accompanied by different resonant field positions. In this way the resonant field is used to selectively excite electron spins. This is exemplified by the cw EPR spectrum of this biradical with fully deuterated TEMPO groups and a ¹⁵N (*I* = 1/2) nucleus (Fig. 4). The bridge design also positions the two radicals ~2 nm apart, limiting the

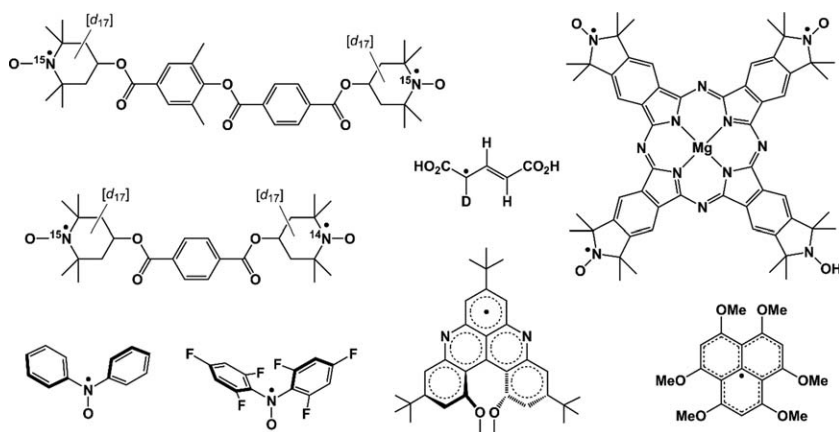


Fig. 3 Organic-based molecular spin qubit candidates.

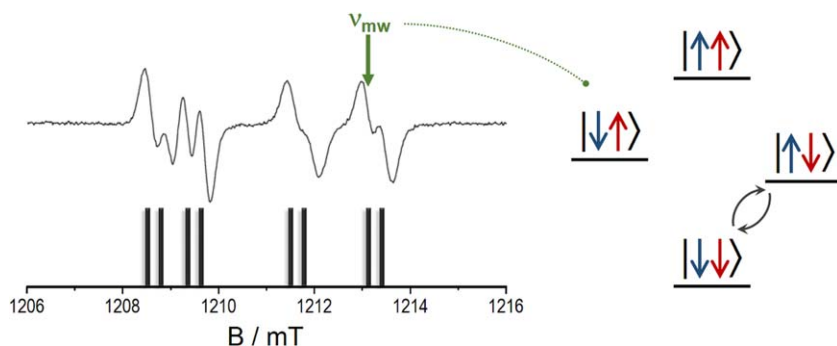


Fig. 4 Stick plot and Q-band EPR spectrum of a single-crystal of the TEMPO biradical diluted in the diamagnetic TEMPOL analogue. A mw pulse at the indicated resonant field flips the second electron (left arrow) spin relative to the first (right arrow), constituting a CNOT operation.

dipolar interaction to 0.0003 cm^{-1} ; the isotropic exchange interaction is an order of magnitude smaller.

The effects of intermolecular interactions and dipolar decoherence were minimised by diluting the biradical into a single crystal of the corresponding diamagnetic TEMPOL, where the nitroxyl group is replaced by a ketone. The CNOT operation was enacted by pumping at a specific field position that corresponds to a spin flip of the second unpaired electron relative to the first. The observation of Rabi oscillations by transient nutation spectroscopy confirms the CNOT operation described in the four-level energy diagram in Fig. 4.

More recent results from Takui and co-workers include designer organic radical spin qubits for adiabatic quantum computing.²⁸ Two three-qubit systems were tested: a magnesium phthalocyanine with three nitroxyl radicals on the periphery, and the 2-deuterated glutamic acid radical. The former is a three-electron qubit system while the latter is a one-electron, three nuclear spin qubit system (Fig. 3). The study focused on developing methodology for implementing Shor's factorisation algorithm using modern EPR instrumentation.⁴ The algorithm had been previously executed using NMR techniques.³¹ Using conquered pulse sequences, they discovered much faster gating times for these molecules on account of the stronger interaction strength between spins, in particular electron spins, when contrasted with their nuclear counterparts.³¹ This study ultimately demonstrated the importance of molecular spin qubits, not only as a viable platform on which to base a quantum computer, but moreover being well-characterised qubits that provide optimal conditions to test spin manipulation technologies which will be needed for the ultimate realisation of quantum computing irrespective of the qubit platform.

Takui and Morita and co-workers have also examined other stable organic radicals based on graphite-like fragments.^{32–34} Phenalenyl is one example – a neutral, carbon-centred π radical whose stability stems from the delocalisation of the unpaired electron over the planar D_{3h} -symmetric 13π -electron system. A quantum simulator was developed by attaching six methoxy groups to the α -carbons of the phenalenyl core (Fig. 3). Using pulsed EPR techniques applied to their nitroxyl radical systems, they investigated quantum spin simulators for studying a large number of interacting equivalent spins in molecular frames, in this instance the protons of the methoxy substituents. The quantum coherence afforded by this system arose from synchronised rotational motions of the interacting nuclear spins with the delocalised electron in the phenalenyl core.

There is no better example of an isolated spin qubit than an endohedral fullerene – a C_{60} with an atom or molecule trapped with its cage (Fig. 5). The space within the fullerene is sufficiently large to enclose a single nitrogen atom with half-filled p orbitals and a $^4S_0$ ground state. Even in the solid state, the EPR spectrum of $N@C_{60}$ is characterised by very sharp lines on account of the lack of interaction of the paramagnetic entity with its diamagnetic host.³⁵ Spin-Hamiltonian parameters of $g=2.0036$ and $A=17 \text{ MHz}$ describe a spherical spin system with isotropic hyperfine coupling of the ^{14}N ($I=1$) nucleus. The trapped atom can

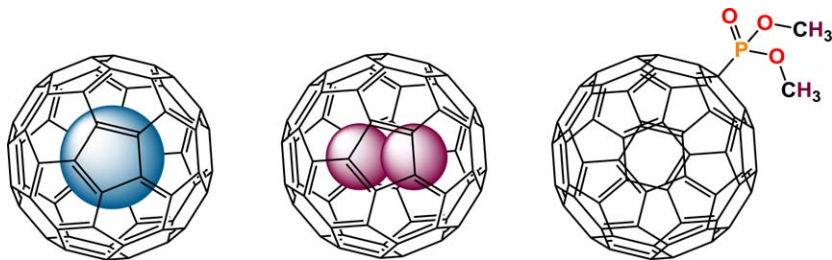


Fig. 5 Fullerene-based molecular spin qubits, endohedral N@C_{60} and $\text{H}_2\text{@C}_{60}$, and exohedral $\text{C}_{60}\text{-PO(OMe)}_2$.

be varied such as $^{15}\text{N@C}_{60}$ and $^{31}\text{P@C}_{60}$, both $I = 1/2$ nuclei, as well as using bigger fullerenes that accommodate larger spin centres such as metal ions.^{36,37} Interest in endohedral fullerenes was piqued by their remarkably long coherence times, 250 μs at a rather warm 170 K.^{35,38} This set the record for molecular spin qubits at the time, and over the last decade, nothing has approached this value at such an elevated temperature. The drawback of endohedral fullerenes, particular by those impregnated with group 5 elements, is the arduous and costly method of their production. As a result N@C_{60} is priced at £100m per gram and is the most expensive material on Earth. Despite this, fullerenes provide a unique testing ground for exploring quantum phenomena, and to this end new assemblies have been developed to capitalise on the incredibly long T_2 .^{39,40} Although data regarding the spin relaxation processes have not yet been measured, a novel means of implanting molecules using rudimentary organic synthetic techniques,⁴¹ researchers have examined the spin dynamics in $\text{H}_2\text{@C}_{60}$ (Fig. 5).³⁹ Electron spins are generated on the fullerene by photoexcitation to its triplet state ($S = 1$), and the effect on the proton spins of the trapped H_2 molecule investigated by ENDOR spectroscopy. The photoexcited triplet state is long-lived, exceeding T_2 by several orders of magnitude. A comparison is made with an exohedral fullerene – a C_{60} with an appended phosphate group with spin-active ^1H and ^{31}P nuclei (Fig. 5).

5 Inorganic molecular qubits

5.1 Monometallic complexes

In the design of molecules with unpaired electrons, coordination complexes bearing paramagnetic transition metal ions are the most obvious candidates to fulfill the DiVincenzo criteria. Only very recently has the search for viable molecular qubit candidates turned to a single metal ion with an $S = 1/2$ ground state. This is surprising as they have relatively long T_2 , in particularly at elevated temperatures because any excited spin state is energetically far removed from the ground state such that it cannot offer an alternative magnetic relaxation pathway. This is certainly the prized feature of organic radicals, though these are more arduous to synthesise than transition metal complexes. Coordination chemistry offers the convenience that the spin centre can be trapped within an organic housing composed of ligands, which can also exert significant

influence on the magnetic properties of metal ion to which they are bound. Furthermore, ligand design enables more than one metal ion to be linked, and the interaction between metal ions modulated by the architecture. The development of inorganic molecular spin qubits is arguably more about the organic ligand component as it is the choice of transition metal ion as spin host.

A recent trend in transition metal ion based qubits is the use of so-called nuclear spin free ligands to combat the deleterious effects of hyperfine or dipolar coupling on the decoherence lifetime. All the ligands presented in Fig. 6 are composed of carbon with either oxygen or sulfur atoms which are almost entirely composed of $I = 0$ isotopes. Even though maleonitrile dithiolate, $(mnt)^{2-}$, has nitrogen atoms that come with nuclear spin – ^{14}N $I = 1$, 99.7% abundant – this nucleus has a very weak gyromagnetic ratio, about 7% that of the proton. The overarching theme is the eradication of protons, as these are the greatest threat to achieving practical decoherence times. Two key reports that pushed the boundaries for coordination complexes as molecular spin qubits appeared almost simultaneously in 2014. They chose metals from either end of the first-row of the transition elements, V(IV) d^1 and Cu(II) d^9 . The impressively long relaxation times offered by these metal ions had already been investigated many years earlier by seminal cw and pulsed EPR studies by Eaton and Eaton,^{21,42–45} whose interest centred on optimising relaxation times for distance measurements in biological systems.⁴⁶ This included correlating the effects of spin–orbit coupling and spin state on T_1 and T_2 ,^{42,44,47,48} so it is not surprising to see many of these simple coordination complexes studied by Eaton and Eaton now retasked as electron spin qubits.

The Freedman group chose $[\text{V}^{\text{IV}}(\text{dbddto})_3]^{2-}$,⁵⁰ a complex ion possessing a trigonally distorted $\{\text{VS}_6\}$ polyhedron (Fig. 7).⁵⁶ Ignoring the tetrabutylammonium counteranions, the complex has no protons, and this feature alone is responsible for the impressive T_2 of 1.2 μs at 80 K, increasing to 1.5 μs at 20 K (Table 1).

Non-oxo vanadyl entities are characterised by isotropic g -values on account of their $(d_{z^2})^1$ ground state – $^2\text{A}_1$ in D_3 symmetry (Table 1).^{57,58}

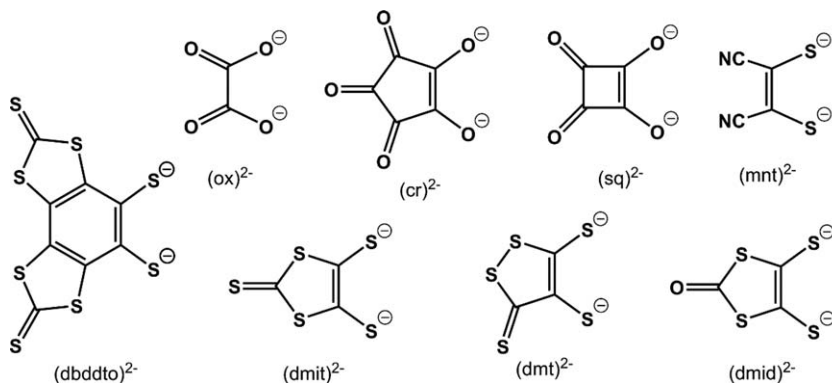


Fig. 6 Nuclear spin free ligands in coordination complexes as molecular spin qubits.

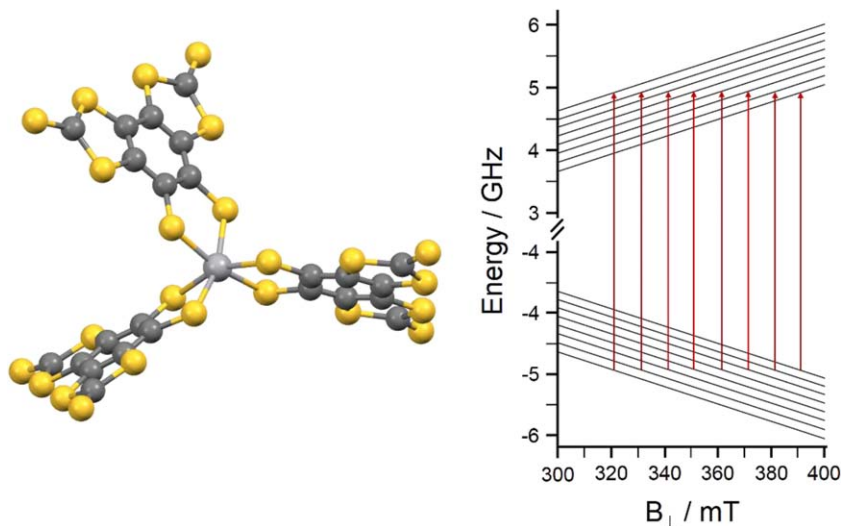


Fig. 7 Structure of the complex ion $[V(dbddto)_3]^{2-}$ (left) as viewed along the C_3 axis (colour palette: V, pewter; S, canary; C, charcoal), and the spin manifold of the V^{IV} ($S = 1/2$, $I = 7/2$) ion (right) determined from the simulation parameters listed in Table 1. Vertical arrows represent EPR transitions ($\Delta M_S = 1$; $\Delta M_I = 0$) at their corresponding resonant field positions. Adapted⁵⁰ from J. M. Zadrozny, J. Niklas, O. G. Poluektov and D. E. Freedman, *J. Am. Chem. Soc.*, 2014, **136**, 15841. Copyright 2014 the American Chemical Society.

This is juxtaposed by the highly anisotropic hyperfine coupling to the ^{51}V $I = 7/2$ (99.7% abundant) nucleus which gives the EPR spectrum its characteristic 8-line pattern. The hyperfine splitting is noticeably different to oxovanadium(IV), where the d_{xy} ground state delivers an $A_{zz} \gg A_{xx} \approx A_{yy}$ pattern. Although the strong ^{51}V hyperfine splitting is unavoidable, and a major contributor to decoherence in related molecular qubits,⁵⁹ Freedman and co-workers tapped into this spectral feature by performing transient nutation experiments at a variety of resonant field positions corresponding to peak maxima in the Hahn-echo. The relatively large spacing between these maxima from the ^{51}V hyperfine coupling enabled the observation of Rabi oscillations for different m_I levels of the hyperfine manifold ($\Delta M_I = 0$), establishing a large inventory of states that offer coherent spin control at X-band frequencies (Fig. 7).

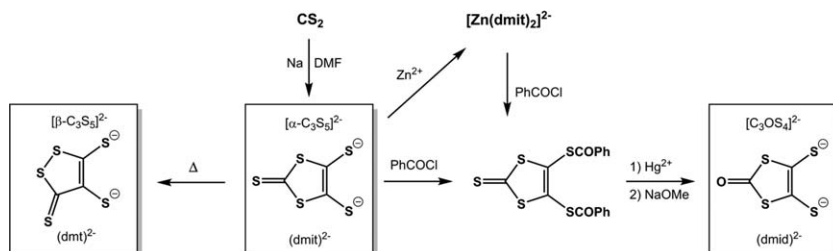
The Freedman group followed on from this communication to demonstrate how synthetic modifications can drastically enhance the decoherence lifetime by expanding the number of dianionic tris(dithiolene)vanadium compounds using three well-known sulfur-rich ligands (Fig. 5). The progenitor of this class of ligands is 1,3-dithiole-2-thione-4,5-dithiolate abbreviated (dmit) $^{2-}$ from its original name dimercaptioisotrithione.⁶⁰ The preparation is exceedingly simple: carbon disulfide is reacted with an alkali metal (Na or K) in DMF,^{61,62} an inexpensive procedure carried out on a multi-gram scale. Several nuclear spin free analogues are generated from (dmit) $^{2-}$, including 1,2-dithiole-3-thione-4,5-dithiolate, (dmt) $^{2-}$, and 2-oxo-1,3-dithiole-4,5-dithiolate, (dmid) $^{2-}$, as detailed in Scheme 1.⁵⁸

Firstly, replacing NBu_4^+ with PPh_4^+ in concert with a change of glassing solvent from butyronitrile to a mixture of deuterated

Table 1 Spin-Hamiltonian Parameters and Relaxation Times for $S = 1/2$ Monometallic Qubits.

Complex	Medium	$g_{x,y,z}$	$A_{x,y,z}/\text{MHz}$	T_1/ms	$T_2/\mu\text{s}$	T/K	Ref.
[PPh ₄ -d ₂₀] ₂ [V ^{IV} (dbddto) ₃]	0.01 mM CS ₂	1.968	−261	20.4	675	10	49
		1.970	−269				
		1.990	46				
[PPh ₄] ₂ [V ^{IV} (dbddto) ₃]	0.5 mM 1 : 1 DMF- <i>d</i> ₇ /toluene- <i>d</i> ₈	1.968	−261	10.8	6.5	10	49
		1.970	−269				
		1.990	46				
[NEt ₄] ₂ [V ^{IV} (dbddto) ₃]	1 mM BuCN	1.972	−258	1.5	1.5	20	50
		1.972	−258				
		1.992	6				
[PPh ₄] ₂ [V ^{IV} (dmit) ₃]	0.5 mM 1 : 1 DMF- <i>d</i> ₇ /toluene- <i>d</i> ₈	1.959	−348	2.4	6.0	10	49
		1.958	−310				
		1.980	46				
[PPh ₄] ₂ [V ^{IV} (dmt) ₃]	0.5 mM 1 : 1 DMF- <i>d</i> ₇ /toluene- <i>d</i> ₈	1.956	−342	1.2	6.1	10	49
		1.954	−338				
		1.986	65				
[PPh ₄] ₂ [V ^{IV} (dmid) ₃]	0.5 mM 1 : 1 DMF- <i>d</i> ₇ /toluene- <i>d</i> ₈	1.960	−319	1.1	6.3	10	49
		1.964	−341				
		1.981	57				
[V ^{IV} O(dpm) ₂]	1 mM 2 : 3 CH ₂ Cl ₂ /toluene	1.9980	168	50	2.7	4	51
		1.9815	190				
		1.9490	510				
[V ^{IV} O(Pc)]	0.1% [TiO(Pc)]	1.987	168	14	3.4	4.3	52
		1.987	168				
		1.966	477				
	0.5 mM D ₂ SO ₄	1.9760	480	2405	22	7	53
		1.9760	220				
		1.9975	85				

[PPh ₄] ₃ [Fe ^{III} (CN) ₆]	1 mM 1 : 1 H ₂ O/glycerol	2.35 2.10 0.915	–	–	2.4	4.7	54
[Co ^{II} (Pc)]	0.5 mM D ₂ SO ₄	2.2830 2.2830 2.0232	60 60 259	11.1	9.4	7	53
[PPh ₄] ₂ [Cu ^{II} (mnt) ₂]	0.001% [PPh ₄] ₂ [Ni ^{II} (mnt) ₂]	2.0227 2.0227 2.0925	118 118 500	87.4	9.2	7	55
[PPh ₄ -d ₂₀] ₂ [Cu ^{II} (mnt) ₂]	0.01% [PPh ₄ -d ₂₀] ₂ [Ni ^{II} (mnt) ₂]	2.0227 2.0227 2.0925	118 118 500	96.2	68	7	55
[Cu ^{II} (Pc)]	0.5 mM H ₂ SO ₄	2.0496 2.0496 2.1990	15 15 630	85	7.8	7	53
	0.5 mM D ₂ SO ₄	2.0502 2.0502 2.1965	30 30 610	103	41	7	
[Cu ^{II} (Pc ^F)]	0.5 mM D ₂ SO ₄	2.0470 2.0470 2.1925	15 15 630	60	40	7	53
[Cu ^{II} (Pc ^{Cl})]	0.5 mM D ₂ SO ₄	2.0525 2.0525 2.2050	30 30 620	140	43	7	53
K ₃ [Ru(ox) ₃]	1 mM 1 : 1 H ₂ O/glycerol	2.473	–	–	3.4	4.7	54
[PPh ₄] ₃ [Ru ^{III} (CN) ₆]	1 mM 1 : 1 H ₂ O/glycerol	1.976 1.991 2.003	–	–	2.6	4.7	54
[PPh ₄] ₃ [Os ^{III} (CN) ₆]	1 mM 1 : 1 H ₂ O/glycerol	1.82	–	–	4.1	4.6	54



Scheme 1 Synthetic route to (dmit)^{2−} and conversion to (dmt)^{2−} and (dmid)^{2−} ligands.

N,N-dimethylformamide (DMF) and toluene, increased T_2 by more than 400% (Table 1). Comparing the different nuclear spin free ligands revealed the largest, (dbddto)^{2−}, offered the longest decoherence time perhaps due to the increased isolation of the V(IV) ion from the surrounding environment. Variation among (dmit)^{2−}, (dmt)^{2−}, and (dmid)^{2−}, is negligible. The larger (dbddto)^{2−} also gave the longest T_1 by some margin; T_1 corresponds to the lifetime the qubit assumes the classical 0 and 1 states. The most profound impact on T_2 was produced by fully deuterating the counteranion, PPh₄⁺-d₂₀, of [V(dbddto)₃]^{2−} and then exploiting its modest solubility in carbon disulfide (0.01 mM), a distinctly apolar and nuclear spin free solvent. Under these conditions, T_2 reached its zenith of 675 μs at 10 K, and despite the boast of millisecond decoherence, the trajectory of T_2 suggests this threshold may be achieved below 5 K. This value eclipses many prominent solid state platforms, though is still well behind endohedral fullerenes (*vide supra*). It is interesting to note that the long decoherence times for N@C₆₀ were also measured in CS₂.^{35,38} The observation of coherent spin manipulations was confirmed by transient nutation experiments for each complex that yielded signature Rabi oscillations.

At about the same time, van Slageren and co-workers used dithiolene ligands bound to Cu(II) as their vehicle to achieve room temperature quantum coherence.⁵⁵ With a fully deuterated chemical system, [PPh₄-d₂₀]₂[Cu^{II}(mnt)₂] was doped into the diamagnetic matrix composed of [PPh₄-d₂₀]₂[Ni^{II}(mnt)₂] at 0.01% dilution. The use of dithiolene ligands makes this particularly advantageous, as the square planar geometry persists for all bis(dithiolene) complexes of late transition metals.⁵⁸ Dilution in a solid state matrix facilitated measurement of the relaxation times up to room temperature (Table 1). Rabi oscillations were observed at 293 K by recording transient nutation on the peak maximum of the Hahn-echo corresponding to the $| -1/2, +3/2 \rangle \rightarrow | +1/2, +3/2 \rangle$ transition of the $| m_s, m_I \rangle$ manifold. This molecular spin qubit yielded a T_1 of 96 ms and T_2 of 68 μs at 7 K using a Q-band spectrometer; T_2 approached 1 μs at room temperature.

The group of Sessoli and Sorace at the University of Florence capitalised on these results to push quantum coherence toward ambient temperature. Their first molecular spin qubit was [VO(dpm)₂],⁵¹ where (dpm)[−] is the anion of dipivaloylmethane, a member of the β-diketonato group of ligands (Fig. 8). The system offered impressive relaxation times

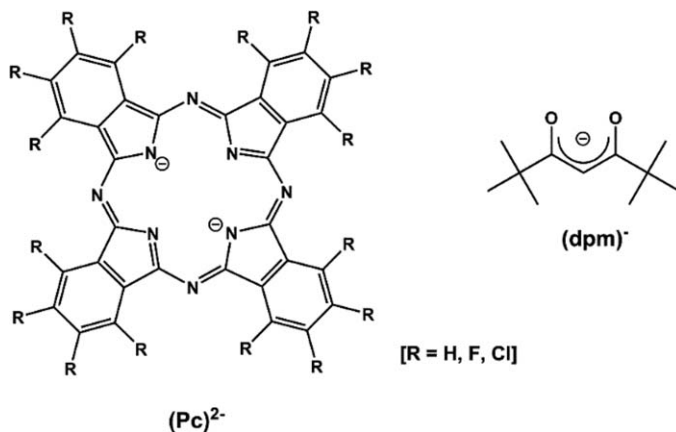


Fig. 8 Ligands used in transition metal based molecular spin qubits.

with $T_1 = 50$ ms and $T_2 = 2.7$ μ s at 4 K (Table 1). Rabi oscillations were also observed at this temperature from the interaction of the electron spin with the surrounding protons, where the ^tBu groups of the chelating ligand are presumably more influential than the non-deuterated solvent. The study introduced a novel method of evaluating T_1 by AC magnetic susceptibility, which tracks the field dependence of T_1 , in contrast to the resonance condition for EPR. A very slow decrease of T_1 was monitored with increasing temperature, which was not accelerated by an applied field up to several teslas. Furthermore they deposited the molecule on a Au(III) surface, where the ground state d_{xy} orbital is aligned parallel to the surface plane. This offers the promise of assembling many molecular spin qubits while keeping the magnetic and spin properties intact.

Following on was a study of vanadyl phthalocyanine, [VO(Pc)],⁵² where the V(IV) metal ion is the spin host locked into the N_4 coordination of a macrocycle that is easy to synthesise in large quantities and readily modified to tune the performance properties (Fig. 8). Measurements at room temperature were achieved by doping into the analogous titanium complex, [TiO(Pc)]. At 0.1% dilution and 4.3 K, T_1 reached 14 ms and T_2 a respectable 3.4 μ s at X-band frequency (Table 1). Bader *et al.* assessed the same compound, though in D₂SO₄ solution at Q-band frequency.⁵³ They recorded a fantastically long T_1 of 2.4 s and an equally improved T_2 of 22 μ s at 7 K (Table 1), highlighting the advantage of working at higher frequencies that offer inherently longer relaxation times.

The [VO(Pc)] qubit exhibited quantum coherence up to room temperature with $T_2 \sim 1$ μ s at 300 K, while T_1 decreased more substantially to 1.1 μ s. Not only is this the highest T_2 value to date at this temperature, but the first observation of Rabi oscillations at room temperature. In addition, AC susceptibility measurements revealed a linear increase of T_1 with temperature up to 20 K, characteristic of a direct mechanism. This reaffirmed the inextricable link between T_1 and T_2 on the performance of spin qubits. Because T_2 is limited by T_1 , strategies must be sought to extend the lifetime of both to ensure the DiVincenzo criteria are fulfilled.

The latest studies also focused on varying the metal ion while keeping the ligand constant. Freedman and co-workers looked at two series of compounds where the effect of spin state and spin-orbit coupling on T_2 could be assessed. The spin-orbit coupling series consisted of well-known hexacyano complexes of group 8 elements Fe, Ru and Os, with spin-orbit coupling constants of 464, 880, and 3100 cm^{-1} , respectively. With each metal in the +III oxidation state and an $S=1/2$ ground state on account of the strong ligand field, T_2 actually increased with increasing spin-orbit coupling, $\text{Fe} < \text{Ru} < \text{Os}$ (Table 1). This is surprising given spin-orbit coupling mediates spin-lattice relaxation which in effect limits T_2 . However, it would appear at the measurement temperature of 5 K, $T_1 \gg T_2$, and therefore removing the impact of spin-orbit coupling on the decoherence lifetime. Certainly the attenuation of T_2 between 13 and 22 K is more dramatic for Os than either Fe or Ru, though overall Ru is the least effected by temperature.

Variation in spin state was gauged by the series $[\text{M}(\text{ox})_3]^{3-}$, where $(\text{ox})^{2-}$ is the conjugate base of oxalic acid, and a nuclear spin free ligand (Fig. 6). Metals were chosen based on their spin ground state: Ru(III) $S=1/2$, Cr(III) $S=3/2$, and Fe(III) $S=5/2$, and the trend in T_2 followed expectation that decoherence is accelerated when the spin centre has several pathways to dissipate the energy, *i.e.* $2S$ energy levels. The strongly performing $[\text{Ru}(\text{ox})_3]^{3-}$, with $T_2 = 3.4\text{ }\mu\text{s}$ at 5 K afforded Rabi oscillations when subjected to variable power transient nutation experiments, and the first time this phenomenon has been observed with either a second or third row transition element. On the whole the impact of spin state on T_2 was rather insignificant and highlights the potential to make use of the many qubit operations that are available at a single metal site by tuning the zero-field splitting.

The Freedman group have demonstrated how this can be done with first row transition metals that prefer a high spin configuration. The first was $[\text{Fe}(\text{cr})_3]^{3-}$,⁶³ where the $S=5/2$ ferric ion showed a characteristic effective g -value of 4.3. Simulation of the X-band spectrum at 77 K gave spin-Hamiltonian parameters $g = (2.009, 2.010, 2.011)$, $D = -0.30\text{ cm}^{-1}$, and $|E/D| = 0.23$, underscoring the highly mixed m_s states for this rhombic system. Probing the maximum intensity of the Hahn-echo for a sample with 0.2% dilution in the corresponding gallium analogue, Rabi oscillations were observed that showed linear dependence on the mw power. The decoherence time of $0.72\text{ }\mu\text{s}$ is limited by the short T_1 of $11.3\text{ }\mu\text{s}$ measured at 5 K, highlighting the importance of T_1 on the performance of any molecular spin qubit.

A Cr(III) ion in an octahedral ligand field has a $(t_{2g})^3$ electron configuration. The strong exchange interaction within this ion isolates the ground state ensuring a small ZFS, typically the same magnitude as the X-band microwave quantum. This is the case for $[\text{Cr}(\text{dmit})_3]^{3-}$,⁶⁴ where the cw EPR spectrum is described by $g = (1.99, 2.02, 1.96)$ and ZFS parameters, $D = 0.326\text{ cm}^{-1}$ and $E/D = -0.328$. For this highly rhombic spin system, Freedman and co-workers were able to selectively access the allowed transition, $m_s = -3/2 \rightarrow -1/2$ as well as the first superposition on a forbidden transition, $m_s = -3/2 \rightarrow +3/2$, which is formally

" $\Delta M_S = 3$ ". The small D -value ensures the forbidden transition appears at a resonant field position accessible at X-band frequencies; the value for E/D at its maximum limit ensues mixing of m_s states intensifying this forbidden transition. Rabi oscillations were observed at field positions for the both allowed and forbidden transitions, characterised by T_2 values of 1.81 μs and 0.15 μs , respectively. This was the first example of quantum coherence of a forbidden transition, and the realisation of high spin molecules for quantum computing as forecast by Leuenberger and Loss in 2001.¹⁸

Most recently, Freedman and co-workers altered the composition of the counteranion in $[\text{Co}(\text{dmit})_2]^{2-}$ to convert it from a single-molecule magnet to a potential qubit.⁶⁵ Four counteranions were used: NBu_4^+ , PPh_4^+ , $[(\text{Ph}_3\text{P})_2\text{N}]^+$, and $[\text{K}(\text{18-crown-6})]^+$. The smaller, more flexible cation, NBu_4^+ cation enforced a greater deviation of the $[\text{Co}(\text{dmit})_2]^{2-}$ from D_{2d} symmetry. This fuelled an increase in the rhombicity of the zero-field interaction, as reported by the absence of hysteresis in coercive field susceptibility measurements. An EPR signal was recorded for this sample, as the mixing of m_s states driven by rhombicity brings the allowed transition of the effective $S = 1/2$ system into a resonant field, albeit accessible at L-band frequency. As with the $\text{Cr}(\text{III})$ system above, manipulating the ZFS parameters provides a new point of access to quantum transitions in high spin transition metal complexes.

5.2 Multimetallic clusters

The use of more than one metal centre is an alternative means to generate a large spin manifold. By careful orchestration of the coupling between individual spin centres, well-defined states can be accessed using current EPR techniques. One of the more simple examples of a polymetallic cluster is a hydroxo-bridge tricopper(II) complex with three $S = 1/2$ spin centres (Fig. 9). The remaining coordination sites are

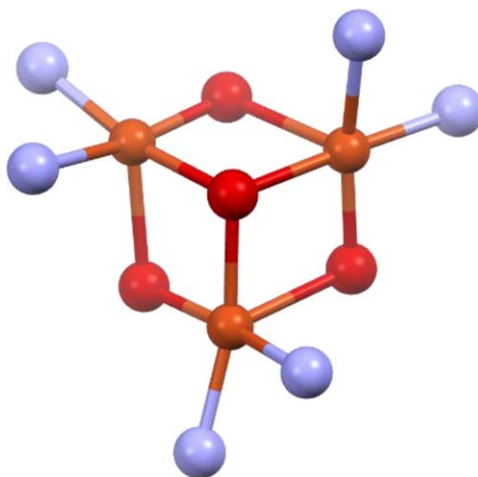


Fig. 9 Depiction of the cuboidal $\{\text{Cu}_3\text{O}_4\}$ cluster showing the first coordination sphere (colour palette: Cu, bronze; O, crimson; N, mauve).

occupied by a Schiff-base ligand,⁶⁶ synthesised from the condensation of *N*-methyl-1,3-propanediamine and salicylic acid in the presence of a templating Cu(II) ion. The cuboidal {Cu₃O₄} complex pivots on a tetrahedral bridging hydroxide ligand that occupies one vertex of the cube placing the Cu(II) ions at three other vertices (Fig. 9). The phenolate oxygen atom of each Schiff-base ligand occupies the other vertices and bridges the Cu(II) ions; these ions form an equilateral triangle. Exchange coupling is mediated by the bridging hydroxy and phenoxy groups, with the $S = 1/2$ total spin ground state generated by an antiferromagnetic coupling of about 22 cm^{-1} .⁶⁶ This gives a well isolated ground state. Examination of the relaxation properties of this effective spin-1/2 revealed $T_1 = 0.7\text{ ms}$ and $T_2 = 1.29\text{ }\mu\text{s}$ in methanolic solution at 5 K and X-band frequencies.⁶⁷ These values are comparable to monocopper spin qubits (Table 1). It was further shown that a powder sample of this compound also had a relatively long T_2 time of $0.39\text{ }\mu\text{s}$, which is unusual for an undiluted solid. This stems from the use of bulky Schiff-base ligands, the overall charge of the cluster balanced by perchlorate counterions, and the rather tame magnetic character of Cu(II) ions. T_1 decreased markedly with increasing temperature, shortening to 0.28 ms at 14 K, whereas T_2 remained robust, decreasing to $1\text{ }\mu\text{s}$ upon warming the system from 5 K to 20 K. At higher temperatures the relaxation lifetimes will be severely attenuated as the $S = 3/2$ excited state mixes with the ground state. As evidenced by ENDOR spectroscopy, the ligand protons are the main source of the quantum decoherence, though interestingly *via* a combination of through-space (dipolar) and through-bond (exchange) mechanisms.

Octanuclear clusters composed of Cr(III) ions are straightforward to make very stable. The series began with the homometallic octanuclear ring, [Cr₈F₈(O₂C^tBu)₁₆] (Fig. 10),⁶⁸ whose diamagnetic ground state stems from the cancellation of individual magnetic moments from an even number of $S = 3/2$ ions. It is an equally simple task to introduce a different spin centre into the parent structure, one that would have a different spin ground state as its neighbouring Cr(III) ions and therefore give a net non-zero spin to the octanuclear cluster. This is achieved by adding a stoichiometric amount of a divalent metal salt into the reaction mixture as well as a monocation to balance the charge. The inclusion of one divalent metal ion gives a monoanionic cluster, [Cr₇MF₈(O₂C^tBu)₁₆][−]. By selecting a secondary amine, not only is the charge balanced by protonation to an ammonium cation, but it acts as a template upon which the 8-member metal chain winds around. Following the general procedure summarised in eqn (4), an enormous family of heterometallic rings have been synthesised (Chart 1). The first were {Cr₇M} rings where M = Ni(II), Zn(II) and Mn(II), and rings have been made with as many as fourteen metal centres in a single domain structure with a periodic boundary condition. Their interior is lined with eight bridging fluorides while the exterior is decorated with twice as many bridging carboxylates that endow these inorganic clusters with high solubility in non-polar solvents. Inclusion of a sugar, *N*-ethyl-D-glucamine (H₃Etglu), displaces five fluorides from the final structure

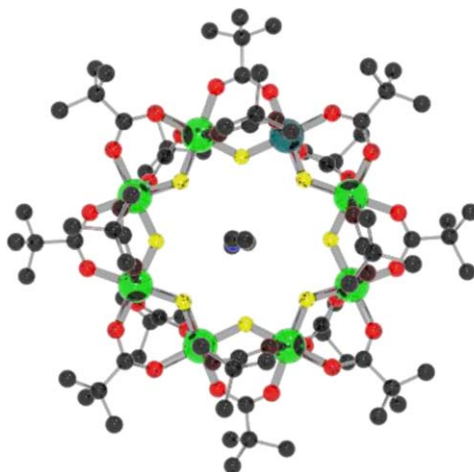


Fig. 10 Structure of the archetypal $[\text{R}_2\text{NH}_2][\text{Cr}_7\text{MF}_8(\text{O}_2\text{C}^t\text{Bu})_{16}]$ heterometallic ring (colour palette: Cr, lime; Ni, teal; F, lemon; N, zaffre; O, scarlet; C, onyx). Hydrogen atoms omitted for clarity. Reproduced from G. F. S. Whitehead, J. Fernando-Soria, L. Carthy, R. G. Pritchard, S. J. Teat, G. A. Timco and R. E. P. Winpenny, *Dalton Trans.*, 2016, **45**, 1638–1647 with permission from The Royal Society of Chemistry.

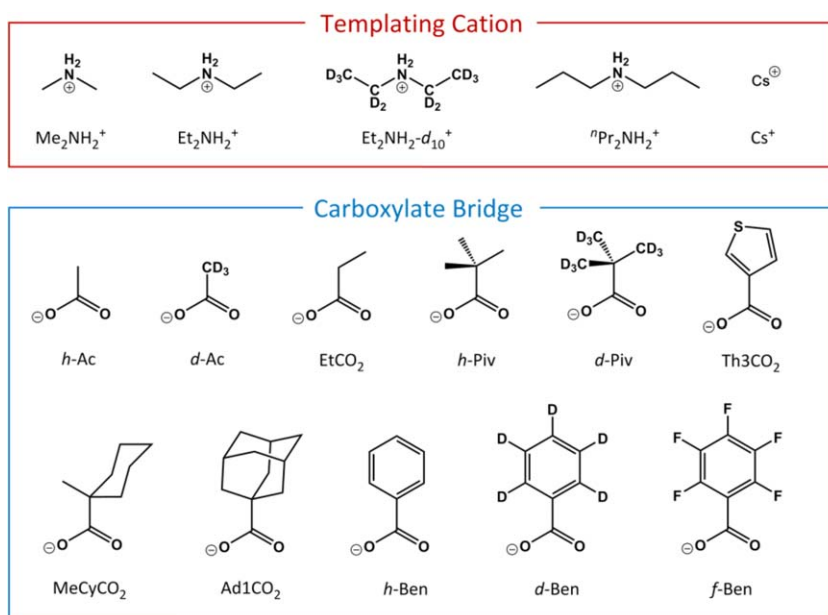
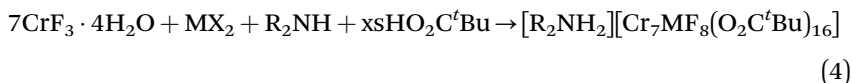


Chart 1 Chemical structures of central templating cation and bridging carboxylate variants with abbreviated names.

and gives the resultant heterometallic ring a purple hue.⁶⁸ These charge-neutral clusters with the general formula $[\text{Cr}_7\text{MF}_3(\text{Etglu})(\text{O}_2\text{C}^t\text{Bu})_{15}(\text{H}_2\text{O})]$ have a labile solvent ligand that is readily replaced with bifunctional ligands through which rings can be linked.^{69–71} The size of the rings is controlled through templates of which there are several options, including

the prototypical dialkylammonium cations, to imidazolium ions, alkali metals and coordination compounds.



The stability of these heterometallic rings is highlighted by the fact they are purified by simple column chromatography. This and other similarities to organic molecules has seen their proliferation by functionalising the ring to produce an elementary building block that has been incorporated into hybrid organic–inorganic rotaxanes and into molecules containing up to 200 metal centres.

The antiferromagnetic coupling between adjacent metal ions in $\{\text{Cr}_7\text{Ni}\}$ is sufficiently strong to give an effective spin- $S = 1/2$ ground state rings at cryogenic temperatures. The first excited state ($S = 3/2$) is populated above 8 K.^{72,73} At a measurement temperature of 5 K, simple EPR spectra with one resonance are observed. Although isotropic in its appearance, simulation is best achieved using axial pattern with $g_{\perp} = 1.78$ and $g_{\parallel} = 1.74$. These g -values are rather low and are a consequence of the net $S = 1/2$ ground state arising from antiferromagnetic coupling. The net g -value is given by: $g_{1/2} = 1.666g_{\text{Cr}} - 0.666g_{\text{Ni}}$.⁷⁴ The “single-ion” g -value for a Ni(II) ion exceeds 2.00; for the Cr(III) ion a value of 1.98 is typical. Applying the formula for the exchange-coupled $\{\text{Cr}_7\text{Ni}\}$ ring gives the observed experimental g -value around 1.80. This is remarkably useful as it allows quick diagnosis by EPR whether this slightly axial signal is shifted or distorted by the interaction of any covalently or non-covalently connected spin centres.

Coordination compounds such as the aforementioned metallic rings are known as nanomagnets due to their size. Although most coordination complexes tend to exceed 10 Å in at least one dimension, it is not customary to apply this label mono- and bi-metallic complexes. The application of molecular nanomagnets to the spin qubit cause effectively began in 2007 with a seminal study by Ardavan *et al.*⁷⁵ with the determination of decoherence times in 8-membered heterometallic rings $\{\text{Cr}_7\text{M}\}$ rings where $\text{M} = \text{Ni(II)}$ and Mn(II) .⁷⁶ The latter has an $S = 1$ ground state, and the broad EPR signal is described with spin-Hamiltonian parameters $g = 1.9$, $D = 0.7 \text{ cm}^{-1}$, $E/D = 0.09$. For the sought after $S = 1/2$ complex, $[\text{Et}_2\text{NH}_2][[\text{Cr}_7\text{NiF}_8(\text{O}_2\text{C}^t\text{Bu})_{16}]]$ a decoherence time of 0.38 μs was measured at 4.5 K; the Mn analogue registered the same performance. The temperature dependent profile of T_1 was also the same for both, exceeding 1 ms at 1.8 K, and dependent of the magnetic anisotropy of the heterometallic ring.

Significant enhancement of T_2 was achieved using a perdeuterated sample, with d -Piv ($\text{O}_2\text{C}(\text{CD}_3)_3$) bridging carboxylates and $\text{H}_2\text{NEt}_2\text{-}d_{10}^+$ templating cation. The decoherence time was increased by a factor of six, reaching a maximum of 3.8 μs at 1.5 K (Table 2). This indicated that electron-nuclear interactions, in particular proton hyperfine coupling,⁷⁷ is the major contributor to T_2 as this factor is exactly the same ratio as the gyromagnetic moments of the hydrogen and deuterium nuclei.

Table 2 Decoherence times for [Cat][Cr₇NiF₈(O₂CR)₁₆] variants.^a

Templating cation	Carboxylate bridge	Medium	$T_2/\mu\text{s}$	T/K	Ref.
Me ₂ NH ₂ ⁺	Piv	toluene	0.38	5	78
Et ₂ NH ₂ ⁺	Piv	toluene	1.01	3	78
		toluene- <i>d</i> ₈	0.73	3	78
ⁿ Pr ₂ NH ₂ ⁺	Piv	toluene- <i>d</i> ₈	0.62	5	78
Cs ⁺	Piv	toluene	1.04	2.75	78
		toluene- <i>d</i> ₈	1.20	2.75	78
Et ₂ NH ₂ ⁺	<i>d</i> -Piv	toluene- <i>d</i> ₈	0.93	5	78
Et ₂ NH ₂ ⁺ - <i>d</i> ₁₀	<i>d</i> -Piv	toluene	3.8	1.8	75
		toluene- <i>d</i> ₈	0.93	5	78
Cs ⁺	<i>d</i> -Piv	toluene	3.85	2.75	78
		toluene- <i>d</i> ₈	15.3	1.5	78
ⁿ Pr ₂ NH ₂ ⁺	Ad1CO ₂	toluene	3.10	3.25	78
		toluene- <i>d</i> ₈	0.55	5	78
Et ₂ NH ₂ ⁺	Ad1CO ₂	toluene- <i>d</i> ₈	0.66	5	78
Cs ⁺	Ad1CO ₂	toluene	3.12	2.75	78
		toluene- <i>d</i> ₈	5.1	1.5	78
ⁿ Pr ₂ NH ₂ ⁺	<i>h</i> -Ac	toluene- <i>d</i> ₈	0.44	5	78
ⁿ Pr ₂ NH ₂ ⁺	<i>d</i> -Ac	toluene- <i>d</i> ₈	0.54	5	78
ⁿ Pr ₂ NH ₂ ⁺	Th3CO ₂	toluene- <i>d</i> ₈	0.48	5	78
ⁿ Pr ₂ NH ₂ ⁺	MeCyCO ₂	toluene- <i>d</i> ₈	0.63	5	78
ⁿ Pr ₂ NH ₂ ⁺	EtCO ₂	toluene- <i>d</i> ₈	0.34	5	78
ⁿ Pr ₂ NH ₂ ⁺	<i>h</i> -Ben	toluene	0.59	3	79
		toluene- <i>d</i> ₈	2.68	3	79
ⁿ Pr ₂ NH ₂ ⁺	<i>d</i> -Ben	toluene	0.90	3	79
		toluene- <i>d</i> ₈	2.56	3	79
ⁿ Pr ₂ NH ₂ ⁺	<i>f</i> -Ben	toluene	2.28	3	79
		toluene- <i>d</i> ₈	2.42	3	79
Cs ⁺	<i>h</i> -Ben	toluene	1.71	3	79
		toluene- <i>d</i> ₈	3.06	3	79
Cs ⁺	<i>d</i> -Ben	toluene	2.49	3	79
		toluene- <i>d</i> ₈	2.23	3	79

^a Cat = templating cation; O₂CR = carboxylate bridge – see Chart 1.

The salient result from this study is that T_2 exceeds previous expectations by three orders of magnitude, thus marking molecular nanomagnets as viable hardware for spin qubits.⁷⁵

A systematic study was carried out on a number of heterometallic {Cr₇Ni} ring variants in order to quantify the input from the elemental constituents of the ligands and templating cations on the decoherence time (Chart 1).⁷⁸ For a fixed templating cation, ⁿPr₂NH₂⁺, the ^tBu group

of the archetypal pivolate bridging ligand was compared to other commercially available carboxylate ligands. The decoherence times measured in toluene- d_8 at 5 K followed the trend $\text{Et} < \text{Me} < \text{Th3} < \text{Ad1} < {}^t\text{Bu} < \text{MeCy}$, though only over a small range of 0.34–0.63 μs (Table 2). All Hahn-echo decay plots were modelled with a stretched exponential, with the stretch parameter, x , ranging between 0.97–1.53, revealing the physical motion of the spin active nuclei is dominant in these compounds. The trend is defined by the steric bulk of the specific substituent, with the larger groups unable to freely rotate as efficiently as more compact and linear Me and Et. Surprisingly, methyl group rotation, still operative at this measurement temperature (5 K) did not perturb the T_2 for methylcyclohexyl and ${}^t\text{Bu}$ substituents, suggesting their methyl groups are perturbed by the accompanying architecture.

Deuteration is the most effective synthetic strategy to improving decoherence lifetimes. Comparing deuterated and non-deuterated carboxylate ligands, Ac and Piv, the deuterated groups provided a 20% improvement in T_2 in analogous species with ${}^n\text{Pr}_2\text{NH}_2^+$ and Cs^+ counteranions, respectively (Table 2). The influence of protons in the solvent was tested by comparing analogous species in deuterated and non-deuterated toluene. Consistently T_2 is increased in toluene- d_8 by $\sim 20\%$ for $\text{Cs}[\text{Cr}_7\text{NiF}_8\{\text{O}_2\text{C}(\text{CH}_3)_3\}_{16}]$, $\text{Cs}[\text{Cr}_7\text{NiF}_8\{\text{O}_2\text{C}(\text{CD}_3)_3\}_{16}]$ and $\text{Cs}[\text{Cr}_7\text{NiF}_8\{\text{O}_2\text{CAd1}\}_{16}]$. Interestingly, deuteration of the templating cation gave no increase in T_2 , as shown by the identical time of 0.93 μs for Et_2NH_2^+ and $\text{Et}_2\text{NH}_2^+-d_{10}$. Evaluating the effect of the templating cation on T_2 , the $\{\text{Cr}_7\text{Ni}\}$ ring with Piv ligands was synthesised with Me_2NH_2^+ , Et_2NH_2^+ , ${}^n\text{Pr}_2\text{NH}_2^+$ and Cs^+ at its centre. The decoherence time followed the trend $\text{Me}_2\text{NH}_2^+ < {}^n\text{Pr}_2\text{NH}_2^+ < \text{Et}_2\text{NH}_2^+ \approx \text{Cs}^+$ (Table 2). By combining all the components that increased the decoherence lifetime into one $\{\text{Cr}_7\text{Ni}\}$ ring – d -Piv, Cs^+ in toluene- d_8 – a record T_2 of 15.3 μs was measured at 1.5 K.

Further chemical modification of the $\{\text{Cr}_7\text{Ni}\}$ rings was performed recently, specifically replacing protons with halogens.⁷⁹ Using halogenated derivatives of the bridging carboxylate ligands, acetate and benzoate (Chart 1), relaxation times in the presence of different decohering nuclei were contrasted: ${}^1\text{H}$, ${}^2\text{H}$, ${}^{19}\text{F}$, ${}^{35}\text{Cl}$, ${}^{37}\text{Cl}$, which span a range of gyromagnetic moments, abundances and spins. For the rings with bridging benzoate ligands templated about ${}^n\text{Pr}_2\text{NH}_2^+$, T_2 measured for the fluorinated ring was considerably longer than the protonated one. Although these nuclei, ${}^{19}\text{F}$ and ${}^1\text{H}$, are magnetically similar, it was found that the pentafluorophenyl ring is rotated away from the carboxylate CO_2 plane in contrast to the simple phenyl ring. This structural conformation has the effect of severely restricting rotation and libration of the methyl groups in the cation template, removing this decoherence pathway from the system. This trend is mapped in the T_2 times for the three benzoate ligands measured at 3 K in toluene: $h\text{-Ben} < d\text{-Ben} < f\text{-Ben}$, where the rather small difference between protonated and deuterated isotopologues stems from the fact that rotations and librations of aromatic C–H bonds are not operative at this temperature. Interestingly, the T_2 values are essentially the same in toluene- d_8 , where other forces are dominant in this medium (Table 2). This observation emerged when comparing T_2 and stretch

parameter values for *h*-Ben and *d*-Ben templated with Cs^+ . The absence of Cs ESEEM modulation in the Hahn-echo decay profile confirms this nucleus (^{133}Cs $I = 7/2$, 100%) does not contribute to decoherence, unlike the proton laden dialkylammonium cations. The magnitude of the stretch parameter ($0.8 < x < 1.2$) again indicates spectral diffusion to be the decoherence mechanism.

Having established that decoherence times are sufficient for spin manipulation, the next step was to link these units together to create constructs that could carry out computation. The chemical remedy to this problem is addressed by linking two or more rings together, in some instances using a chemically unique spin centre. There are three routes to linking these clusters: accessing the H-bonding interactions *via* the ammonium templating cation, using a bifunctional carboxylate, and generating a vacant coordination site on the ring in order to attach a monodentate ligand. The first examples of weakly coupled systems that could be envisaged to generate entanglement was achieved by reacting the parent $\{\text{Cr}_7\text{Ni}\}$ ring with isonicotinic acid in boiling *n*-propanol. The result was the replacement of a pivalate ligand with an isonicotinic one, effectively producing a very large, functionalised pyridine ligand. This was put to use with the addition of two equivalents of $\{^n\text{Pr}_2\text{NH}_2\}$ -py to copper nitrate and copper pivalate salts to form $\{^n\text{Pr}_2\text{NH}_2\}[\text{Cr}_7\text{MF}_8(\text{O}_2\text{C}^t\text{Bu})_{15}(\text{O}_2\text{CC}_5\text{H}_5\text{N})_2][\text{Cu}(\text{NO}_3)_2(\text{OH}_2)]$ and $\{^n\text{Pr}_2\text{NH}_2\}[\text{Cr}_7\text{MF}_8(\text{O}_2\text{C}^t\text{Bu})_{15}(\text{O}_2\text{CC}_5\text{H}_5\text{N})_2][\text{Cu}(\text{O}_2\text{C}^t\text{Bu})_4]$, respectively. These two compounds, abbreviated $\{\text{Cr}_7\text{Ni-Cu-Cr}_7\text{Ni}\}$ and $\{\text{Cr}_7\text{Ni-Cu}_2\text{-Cr}_7\text{Ni}\}$, are tripartite systems with three spin centres each. The difference between them is dependent on the temperature. At the measurement temperature of 5 K, the copper spin centre in $\{\text{Cr}_7\text{Ni-Cu}_2\text{-Cr}_7\text{Ni}\}$ is diamagnetic owing to the strong exchange coupling between adjacent Cu(II) ions. Thus this compound is described as the “switched off” tripartite system on account that the long-range coupling between terminal $\{\text{Cr}_7\text{Ni}\}$ rings is non-existent. This is in contrast to the permanent $S = 1/2$ spin of the mononuclear Cu(II) ion in $\{\text{Cr}_7\text{Ni-Cu-Cr}_7\text{Ni}\}$, designated “switched on”. The effect is seen in their EPR spectra recorded on powder samples at Q-band frequencies. The $\{\text{Cr}_7\text{Ni-Cu}_2\text{-Cr}_7\text{Ni}\}$ gives the simple $g = 1.80$ signal of an unperturbed $\{\text{Cr}_7\text{Ni}\}$ ring; the $\{\text{Cr}_7\text{Ni-Cu-Cr}_7\text{Ni}\}$ gives a rich spectral profile of three weakly coupled $S = 1/2$ centres. Using a three spin Hamiltonian, simulation gave $g = (1.83, 1.83, 1.77)$, $A^{\text{Cu}} = (30, 30, 594)$ MHz, $D = -0.028 \text{ cm}^{-1}$, $J = 0.306 \text{ cm}^{-1}$. From these values, the authors constructed an energy level diagram and identified the transitions within this manifold that would constitute two fundamental forms of entanglement, the Greenberger-Horne-Zeilinger, $|\text{GHZ}\rangle$, and Werner, $|\text{W}\rangle$, states. Given the magnetic properties of $\{\text{Cr}_7\text{Ni-Cu}_2\text{-Cr}_7\text{Ni}\}$, these could be executed using pulsed EPR technology in a 0.5 T magnetic field.

Two- and four-spin systems were generated by linking rings with Cu(II) ions.⁸⁰ This was achieved by using a functionalised secondary amine during the ring forming reaction. Appended to the alkyl substituents was placed a phenyl (Ph) ring and a pyridyl (Py) ring. Both of these groups are large enough to stop the $\{\text{Cr}_7\text{Ni}\}$ from slipping off the ammonium “thread”; these are coined [2]-rotaxanes because the two components,

ring and thread, are non-covalently interlocked.^{81,82} A bipartite system was produced by coordinating this [2]-rotaxane to the copper ion in $[\text{Cu}(\text{hfac})_2]$, where $(\text{hfac})^-$ is the chelating 1,1,1,6,6,6-hexafluoroacetylacetonato ligand, giving $[\text{Cu}(\text{hfac})_2][\text{PyCH}_2\text{NH}_2\text{CH}_2\text{CH}_2\text{Ph}][\text{Cr}_7\text{MF}_8(\text{O}_2\text{C}^t\text{Bu})_{16}]$ (Fig. 11a). Single-crystal X-ray diffraction revealed the centroid of the $\{\text{Cr}_7\text{Ni}\}$ ring is 7.34 Å away from the $\text{Cu}(\text{II})$ ion. In a similar reaction with a diammonium dication spaced by a 10-carbon chain, a [3]-rotaxane $[\text{Cu}(\text{hfac})_2(\text{H}_2\text{O})_2][\{\text{PyCH}_2\text{NH}_2(\text{CH}_2)_5\}_2][\{\text{Cr}_7\text{MF}_8(\text{O}_2\text{C}^t\text{Bu})_{16}\}_2]$ was synthesised, where the capping Py groups of the organic thread are bound to a $[\text{Cu}(\text{hfac})_2]$ unit that retains a H_2O ligand (Fig. 11b). The length of this thread is the minimum distance necessary to have two adjacent $\{\text{Cr}_7\text{Ni}\}$ rings.

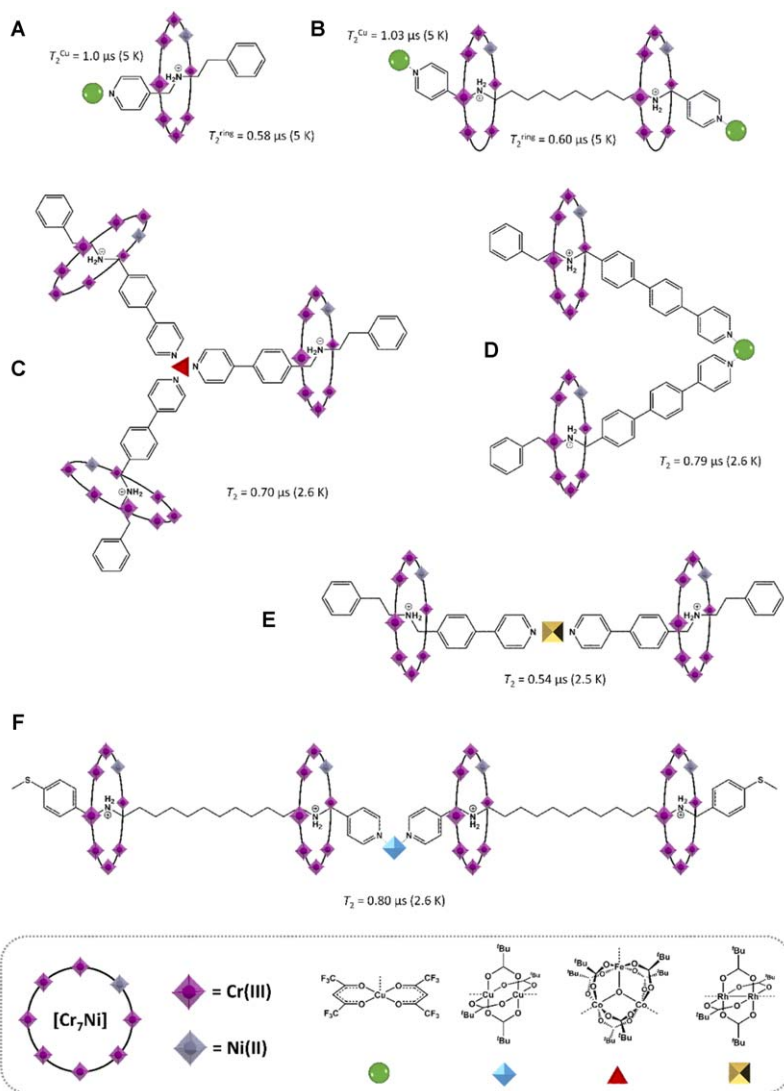


Fig. 11 Structures of $[n]$ -rotaxane assemblies linked through mono-, bi- and tri-metallic coordination complexes. T_2 times and measurement temperatures are noted for each.

The effect of using a non-covalent linkage to connect the spin centres was the dramatically weaker exchange interaction. This manifested in their EPR spectra with a miniscule splitting of the individual spin centres.⁸⁰ The two-spin compound, $[\text{Cu}(\text{hfac})_2][\text{PyCH}_2\text{NH}_2\text{CH}_2\text{CH}_2\text{Ph}][\text{Cr}_7\text{MF}_8(\text{O}_2\text{C}^t\text{Bu})_{16}]$, was described with spin-Hamiltonian parameters $g_{\text{Cr7Ni}} = (1.780, 1.765, 1.710)$, $g_{\text{Cu}} = (2.065, 2.045, 2.325)$, $A^{\text{Cu}} = (0, 0, 450)$ MHz, $J = -0.032 \text{ cm}^{-1}$; the larger [3]-rotaxane was characterised with $g_{\text{Cr7Ni}} = (1.780, 1.775, 1.705)$, $g_{\text{Cu}} = (2.060, 2.045, 2.322)$, $A^{\text{Cu}} = (0, 0, 450)$ MHz, $J = -0.020 \text{ cm}^{-1}$. The exchange coupling constants are an order of magnitude smaller than for the covalently linked $\{\text{Cr}_7\text{Ni}-\text{Cu}-\text{Cr}_7\text{Ni}\}$ species (*vide supra*), and therefore these molecules are described as AB and AB₂ spin systems, respectively, drawing on NMR nomenclature. The effect of the weak coupling was gauged by studying the relaxation times, where each spin centre was selectively targeted by shifting the resonant field. The data revealed the Cu(II) and $\{\text{Cr}_7\text{Ni}\}$ T_2 values are essentially the same as those measured for the individual components.

Using the principles of supramolecular chemistry and the robustness of the hybrid inorganic-organic system, large, multi-centred molecules were produced.⁸³ The architectures studied by EPR spectroscopy are rotaxanes C, D, and F in Fig. 11 and [3]-rotaxane C in Fig. 12. Using long-chain diammonium cations with pendant pyridine groups as “stoppers” to prevent the detachment of the H-bonded heterometallic cluster, several $\{\text{Cr}_7\text{Ni}\}$ rings are brought together in a single molecule that addresses the challenge of scaling these spin qubits.¹⁵

Simple coordination complexes are used to tie the [2]- and [3]-rotaxanes together. Depending on the transition metal ion or cluster, which in itself is a spin centre, two or three of these rotaxanes are connected giving multi-qubit assemblies of up to seven qubits in one molecule. The $\{\text{Cr}_7\text{Ni}\}$ rings are sufficiently well-spaced that their mutual presence (intramolecular coupling) does not influence the decoherence of the system, as noted by T_2 times of 0.54–0.80 μs recorded at 2.6 K which match the single ring values (Table 2). The dipolar interaction between $\{\text{Cr}_7\text{Ni}\}$ rings was assessed by Pulsed Electron Double Resonance (PELDOR), a four-pulse experiment that involves flipping the spin on one centre and examining the change in the effective local magnetic field of a second spin in the system – essentially a CNOT operation. For the copper pivalate linked [3]-rotaxanes (Fig. 11f), a two-qubit gate time of 260 ns was recorded. This establishes that the dipolar coupling between qubits is sufficiently weak to implement quantum logic before the entangled state has decohered.⁸³

This two-qubit gate time is somewhat longer than that measured on a suite of [3]-rotaxanes.⁸⁴ By altering the composition of the $\{\text{Cr}_7\text{Ni}\}$ rings and threads to adjust the interspin distance, gate times of 77–225 ns were measured using the four pulse PELDOR sequence. The shorter times were recorded for the simple [3]-rotaxanes without a connecting inorganic node (Fig. 12a and 12b), where the $S = 1/2$ rings are separated by 1.64–1.94 nm. The longer gate time of 225 ns was determined for a dirhodium bridged rotaxane (Fig. 11e), with an inter-ring distance of 2.64 nm. The ability to manipulate one spin relative to another is purely a

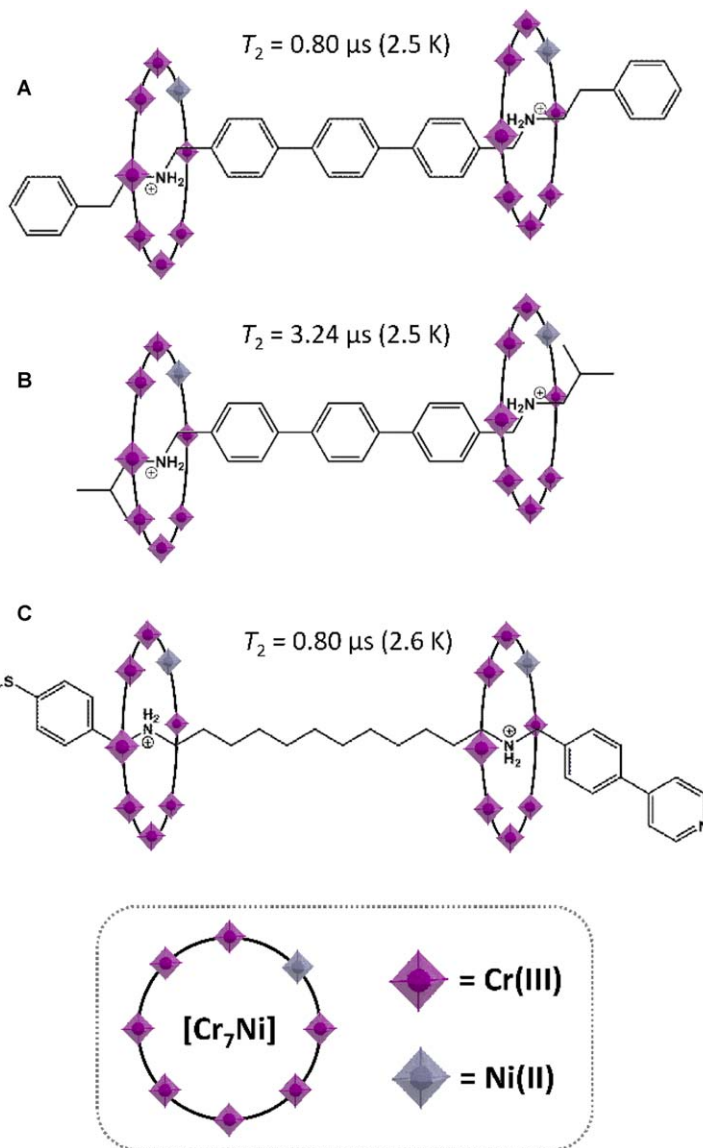


Fig. 12 Structures of the [3]-rotaxanes with two-qubit gate times measured by PELDOR spectroscopy. T_2 times and measurement temperatures are noted for each.

function of the dipolar coupling strength. Overall the gate time is an order or magnitude shorter than the decoherence time, which is the salient requirement for the realisation of quantum logic with these multi-qubit systems.⁸⁴

The alternative to supramolecular approaches exploiting non-covalently linked spin centres is a covalent bonding pathway accessed by adding a bifunctional ligand to the $\{\text{Cr}_7\text{Ni}\}$ ring.^{85,86} Following on from earlier work with isonicotinate, where the carboxylate binds to the ring metal ions rendering the whole unit as a substituted pyridine ligand, a 2,2':6'2''-terpyridyl (tpy) appended carboxylate was synthesised and

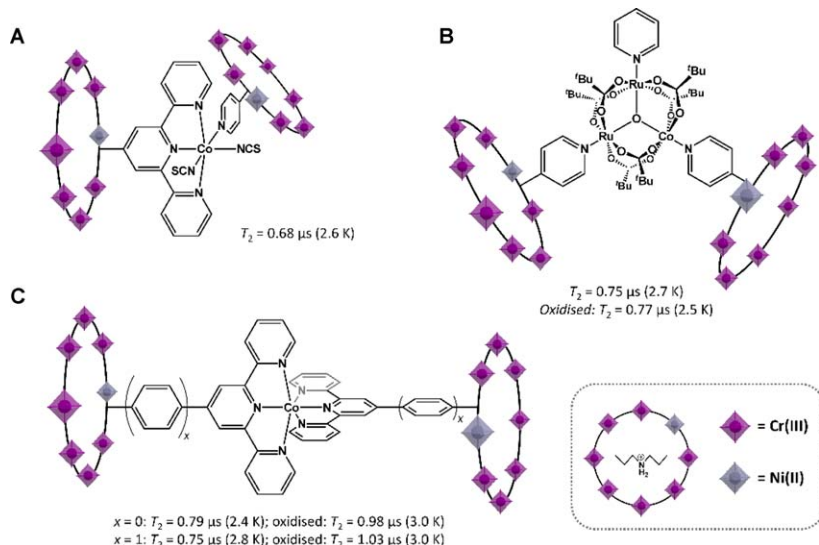


Fig. 13 Multi-qubit assemblies derived from $\{\text{Cr}_7\text{Ni}\}$ -py and $\{\text{Cr}_7\text{Ni}\}$ -tpy functionalised ligands. T_2 times and measurement temperatures are noted for each.

connected to $\{\text{Cr}_7\text{Ni}\}$ rings. The polydentate ligand provides greater stability than the monodentate pyridine ligand; two of these groups readily coordinated to Co(II) giving an octahedral, low-spin ($S = 1/2$) complex sandwiched between two $\{\text{Cr}_7\text{Ni}\}$ rings (Fig. 13). These $\{\text{Cr}_7\text{Ni}\}$ -py and $\{\text{Cr}_7\text{Ni}\}$ -tpy ligands are modular units for the assembly of multi-qubit molecules.

Homoleptic and heteroleptic complexes were synthesised and characterised by X-ray crystallography, mass spectrometry and EPR. The strength of the interspin coupling is similar to that measured for the $\{\text{Cr}_7\text{Ni}\}\text{-Cu}\text{-}\{\text{Cr}_7\text{Ni}\}$ compound,⁸⁷ albeit the exchange interaction is anisotropic on account of the relative orientation of the $\{\text{Cr}_7\text{Ni}\}$ rings to the inorganic node and each other. The decoherence times of these assemblies are the same as recorded for the free ligands (Table 2). Moreover, the Co(II) and $\{\text{Ru}_2\text{Co}\}$ inorganic nodes are redox-active, with reversible one-electron oxidation waves evident in their cyclic voltammograms. By selecting an appropriate oxidant, in this case ferrocenium hexafluorophosphate $[\text{FcP}_2]\text{PF}_6$, the central Co ion is oxidised to the diamagnetic Co(III) species, and the exchange coupling interaction is switched off.⁸⁶ In the case of the oxo-centred $\{\text{Ru}_2\text{Co}\}$ triangle (Fig. 13b), oxidation generated an unpaired electron on this unit, switching on the exchange coupling between it and its two $\{\text{Cr}_7\text{Ni}\}$ rings. The process is reversed by reducing the complexes with cobaltocene. Although it was performed in separate stages using chemical reagents, this is nonetheless a successful demonstration of electrochemical switching of the spin-spin interaction, whereby entanglement can be instigated by modulating the potential. Similar switching of a two-qubit system was performed previously on a polyoxometalate controlled by the applied potential from an STM tip.⁸⁸

5.3 Lanthanoids

Recent progress in the chemistry of f-block elements stems largely from the profound impact on the field of molecular magnetism, specifically single-molecule magnets where the spin-orbit coupling dwarfs crystal field splitting giving large magnetic anisotropy.⁸⁹ It is the case that what makes a good single-molecule magnet – a large ZFS – unfortunately makes it unsuitable as a qubit, where the goal is to eliminate all spin-spin coupling, aside from the necessary qubit-qubit interaction in order to create entanglement. So it would seem counterproductive to employ lanthanoid ions as spin hosts in molecular qubits. As investigations into the dependency of relaxation times on spin state and spin-orbit coupling have revealed that spin qubits are not disadvantaged by high spin and large spin-orbit coupling,^{42,44,47,48,54} it seems prudent not to dismiss lanthanoids from the field.

Gadolinium, holding a position in the middle of the f-block, has a $[\text{Xe}]6s^25d^14f^7$ electron configuration. As lanthanoids have a penchant for the +III oxidation state, this gives a $[\text{Xe}]4f^7$ configuration for the Gd(III) ion. The half-filled shell with $S = 7/2$ ground state exhibits similar features to d-block elements with half-filled levels, such as Mn(II) and Fe(III) , both $S = 5/2$, and octahedral Cr(III) $S = 3/2$. These spin ground states are characterised by vanishingly small ZFS parameters – D -values – as the main contributor to large D comes from spin-conserving transitions that are not operative in half-shell configurations. The small D sought for molecular qubits also makes these spin centres useful probes for distance measurements in biological systems.⁹⁰ Coronado and co-workers had produced several promising molecular spin qubits based on lanthanoid spin hosts.^{91–96} Specifically they targeted isotropic Gd(III) with their signature polyoxometalate ligands that are metal oxide clusters typically composed of Mo and W, prized for the high water solubility and lack of decoherence damaging protons. The weak gyromagnetic moments and low natural abundances of the spin-active isotopes of Mo and W renders this inorganic ligand nuclear spin free.

A powder sample of the polyoxometalate molecular magnet $[\text{GdW}_{30}\text{P}_5\text{O}_{110}(\text{H}_2\text{O})]^{14-}$ (Fig. 14),⁹² doped at 1% and 0.1% in the diamagnetic yttrium analogue, exhibited Rabi oscillations demonstrating coherent spin control. By matching the Rabi frequency to the proton Larmour frequency (15 MHz at X-band; $B_0 = 349.6$ mT), it was shown that the long-term oscillations were dependent on the static field (B_0) rather than the microwave power, indicating coherence between the predominantly $m_s \pm 5/2 \rightarrow \pm 3/2$ transition and the proton nuclear spin from the coordinated water molecule. The achievement marked a potential for quantum algorithms realised with single-molecule magnets.

More recently, Coronado and co-workers used this same feature of the field dependency of the Rabi oscillation frequency to suppress dipolar contributions from protons that constitute the spin bath leading to short decoherence times.⁹⁴ They utilised a series of complexes where the lanthanoid ion is encapsulated within a polyoxopalladate, $[\text{LnPd}_{12}(\text{AsPh})_8\text{O}_{32}]^{5-}$; the chosen lanthanoids – Tb, Dy, Ho, Er, Tm – represent a cross-section of Kramers and non-Kramers ions with a rich

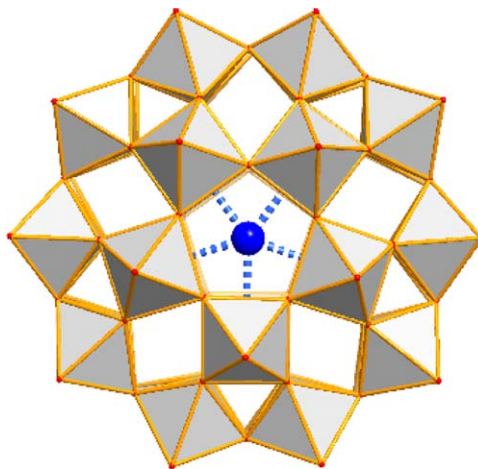


Fig. 14 Molecular structure of $[\text{GdW}_{30}\text{P}_5\text{O}_{110}(\text{H}_2\text{O})]^{14-}$ viewed down the C_5 symmetry axis. The Gd ion is depicted by the azure coloured sphere. The coordinated water molecule is not shown.

energy level structure in this highly symmetric, near-cubic ligand field. In the Ho complex, a moderate axial compression of 1% instigated by a rather small applied magnetic field of 410 mT produced a scenario where the electron spins became invisible to the nuclear spins that constitute the surrounding environment. This neat observation obviates the need to chemically combat the decohering nuclear spin bath and stems from the presence of sizeable tunnelling splittings inherent to lanthanoid molecular magnets.

The Coronado group's entry into this field began in 2010 with the assessment of the relaxation profile of lanthanoid single-ion magnets with polyoxometalate ligands.⁹⁷ The molecular magnets under examination have an 8-coordinate lanthanoid ion sandwiched between two tungsten polyoxometalate ligands, with the general formula $[\text{Ln}(\text{W}_5\text{O}_{18})_2]^{9-}$ (Fig. 10). Rabi oscillations detected from the holmium complex, $[\text{Ho}(\text{W}_5\text{O}_{18})_2]^{9-}$,⁹⁸ even at high concentration (25% diluted in the Y analogue), indicated a long decoherence time sustained by the aforementioned tunnelling splitting of the ground state. In collaboration with Hill at the National High Field Magnetic Laboratory in Tallahassee, the $m_J = \pm 4$ ground state of this square antiprismatic Ho single-ion magnet with D_{4d} point symmetry is split by the ^{165}Ho $I = 7/2$ (100% abundant) nuclear spin (Fig. 15).⁹⁶

The large tunnelling splitting gave rise to atomic clock transitions, so named because atomic clocks are composed of similarly isolated ions that are immune to magnetic field variations and other environmental perturbations and are therefore excellent timekeepers. The clock transitions are those within the m_I levels of Ho, where at specific field positions the electron spin flipping between spin-up and spin-down manifolds ($\Delta M_I = 0$) is shielded from the surrounding environment comprising a myriad of magnetic field effects principally those of nuclear spins. The decoherence time for a quantum operation at one of these optimal

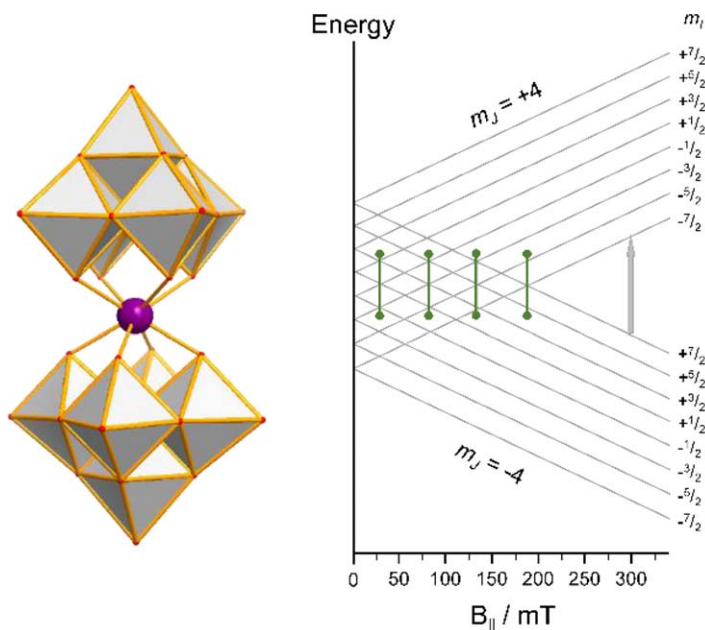


Fig. 15 Molecular structure of the square antiprismatic $[\text{Ln}(\text{W}_5\text{O}_{18})_2]^{9-}$ single-ion magnets (left; colour palette: Ln, rhubarb), and the Zeeman splitting of the $m_J = \pm 4$ ground state of the Ho complex, where the vertical lines identify the field position of atomic clock transitions between the hyperfine energy levels (m_I).

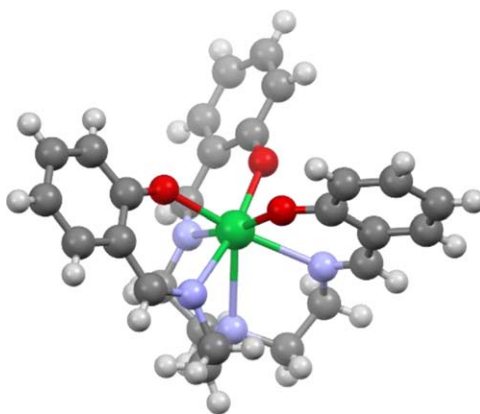


Fig. 16 Molecular structure of the $[\text{Yb}(\text{trensall})]$ spin qubit (colour palette: Yb, jade; O, garnet; N, lavender; C, lead; H, chiffon).

positons was $8.4 \mu\text{s}$ at 5 K with a comparatively high sample concentration when comparing with the dilution ratio of other spin qubits with similarly long T_2 .

An organic tripodal ligand presents a designer pocket to trap a single lanthanoid ion to give a charge neutral complex. Pedersen *et al.* recently characterised such a molecular spin qubit with a $\text{Yb}(\text{III})$ spin host encased in a trensal – a Schiff-base ligand formed from the condensation of salicylic acid and tris(2-aminoethyl)amine (Fig. 16).⁹⁹ The benefit of the

neutral charge, compared with the polyoxometalate systems, is that the complex is sublimable providing a means to scale up the number of qubits by using vapour deposition techniques on an appropriate substrate. The EPR spectrum of single crystals of [Lu(trensal)] doped with 7% [Yb(trensal)] is simulated as an effective $S = 1/2$ system with $g_{\parallel} = 4.3$ and $g_{\perp} = 2.9$.¹⁰⁰ A long T_1 of 10 ms at 3.5 K also afforded a reasonable T_2 of 0.5 μs at a field position corresponding to the $I = 0$ EPR transition in the Hahn-echo for $B_{\perp}$ to the C_3 axis of the complex. Transient nutation experiments at different slices of the $B_{\parallel}$ and $B_{\perp}$ spectra resulted in Rabi oscillations extending to 4 μs , attributable to coherent spin manipulation with protons in the system.

A unique multidentate ligand devised by Aromí and co-workers has led to the development of two qubit system.^{101–103} A combination of three of these asymmetric ligands, composed of different donor types, ketone, alcohol, amine (Fig. 17), leads to the formation of different sized coordination pockets that discriminate lanthanoids based on their ionic radius.¹⁰⁴ In this way, a family of dilanthanoid complexes with varying degrees of ligand protonation were synthesised and structurally characterised, with the general formula $[\text{LnLn}'(\text{HL})_2(\text{H}_2\text{L})]^+$, for $\text{Ln} = \text{La}, \text{Ce}$, $\text{Ln}' = \text{Er}$; $\text{Ln} = \text{Ce}$, $\text{Ln}' = \text{Y}$; $\text{Ln} = \text{Ln}' = \text{Y}$ (overlooking the fact that Y is strictly not a lanthanoid). The coordination sphere is topped up by solvents (pyridine, water) and NO_3^- counterions.

The ability of the ligand to direct the positioning of different lanthanoids based on their size gave the two-qubit system [CeEr] (Fig. 18). Both ions are in the +III oxidation state. The $\text{Ce(III)} 4f^1$ ion is characterised by

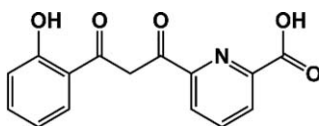


Fig. 17 Structure of the multidentate pro-ligand, H_2L .

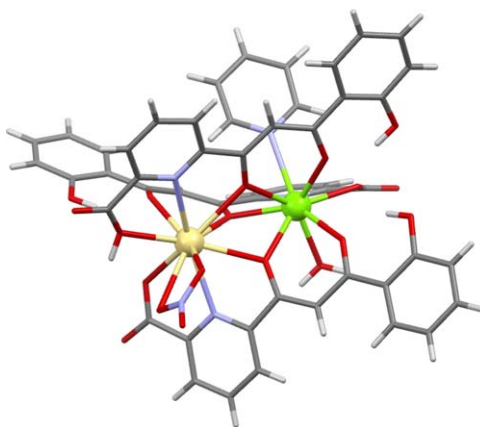


Fig. 18 Molecular structure of $[\text{CeEr}(\text{HL})_2(\text{H}_2\text{L})(\text{NO}_3)(\text{py})(\text{H}_2\text{O})]$ (colour palette: Ce, buff; Er, chartreuse).

$J = 15/2$ and $g_J = 6/5$, while the smaller $\text{Er(III)} 4f^{11}$ has $J = 5/2$ and $g_J = 6/7$. At low temperatures these Kramers ions are treated as effective spin- $S = 1/2$ species, validated by magnetic susceptibility measurements, and so described by a simple spin-doublet Hamiltonian. Details regarding the electronic structure of the individual two spin hosts, Ce(III) and Er(III) , was obtained by pairing each with a diamagnetic ion in the adjacent site: Er with La, and Ce with Y. These values were then summed to describe the magnetic properties of the $[\text{CeEr}]$ complex. The combination of these two effective $S = 1/2$ ions constitutes a viable two-qubit logic gate. In a similar manner as was experimentally demonstrated with a nitroxyl biradical,²⁴ Aguilà *et al.* mapped out the theoretical implementation of a CNOT gate. Each spin site can be addressed individually on account of their different g -values and a miniscule exchange coupling of $J = 0.01 \text{ cm}^{-1}$. A promising decoherence time of $0.4 \text{ }\mu\text{s}$ was recorded at 5 K , though on a non-optimised system.

6 Outlook

There has been significant activity in the area of molecular spin qubits over the last four years with many of the key criteria for a viable qubit being addressed simultaneously. Significant ground has been covered improving decoherence lifetimes, with monometallic complexes, particular those with nuclear spin free ligands eclipsing their organic rivals when the system is fully optimised. The benefit of this, and extending the phenomenon to room temperature by solid dilution, has fostered new insight into the synergistic relationship between decoherence and spin-lattice relaxation mechanisms. At the other end of the spectrum, high spin systems either housed within a single metal ion or through the combination of several metal ions, exhibit much shorter decoherence lifetimes than their simple $S = 1/2$ counterparts. Here the focus is on combining single qubit molecular building blocks into multi-qubit arrays that are needed to test the implementation of quantum coherence and logic operations. This is an area which molecular spin qubits excel, as they are well-characterised and easy to deploy. This will be most beneficial in overcoming the bottleneck that is designing and constructing the apparatus needed to execute quantum computing.

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Status and perspectives of a virtual multifrequency spectrometer for ESR

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ESR spectroscopy is still one of the most useful experimental techniques to analyze the structure, dynamics, and magnetic properties of open-shell species. However, disentanglement of the different factors tuning the overall spectra requires effective yet reliable theoretical calculations. Recent progresses in this field are very encouraging, and it is becoming often possible to compute reliable magnetic parameters as well as to simulate the whole spectra from first principles without adjustable parameters. This goal requires the proper account of different aspects concerning stereo-electronic, environmental and dynamical properties of the molecular system under investigation. Approaches rooted into the stochastic Liouville equation then provide, at present, the most effective theoretical background for integrating magnetic and dynamic ingredients to directly give the spectrum. The main topic of the present contribution is the description of a recent implementation of one of these approaches in a virtual molecular spectrometer including also a powerful graphical user interface. Nitroxide radicals will be used throughout to provide specific examples of the different topics.

1 Introduction

The role of molecular interactions and thermal effects in determining the macroscopic properties of matter cannot be overemphasized, as these generate both internal and global dynamics which set the chemical and physical properties of the latter. Said dynamics – complex as they are – thus constitute the link between interactions (be it molecular or thermal) on one hand, and the chemical as well as physical properties on the other. This explains the central spot they occupy in many branches of molecular sciences, including spectroscopy. In this review we shall focus on biological macromolecules – which can be considered as references in what concerns paradigms of complexity for the spectroscopic study and the modelization of several relaxation processes. Here, dynamics play an important role in the stability, functionality, recognition and catalysis of such macromolecules like proteins. In a protein for instance, it is possible to recognize various time scales that range from those originating from dynamics due to entire protein movements or part of its domains (milli-, microseconds) to the internal ones that involve groups of atoms (nano-, pico-, femtoseconds). The synergistic consequences of the various dynamics collaboratively define the chemical and physical characteristics of biological macromolecules – an all important well-known concept widely exploited in the literature,^{1–3} for example, to further our understanding of vital biological processes through the study of the several

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kinds of motions that occur during reactive events in which biological macromolecules play an active role.

In addition, the huge influence played by dynamics on the macromolecule's shape and stability consequently implies the capability of the same dynamics to modify the structure of the latter, making the transition between diverse conformations possible. Take for instance the flexibility of a protein around a bond; this can be very crucial when regarding the selectivity over a substrate as during the recognition step, the time scales and/or the degrees of freedom of motion of the protein are modified around the bond site. Thus, the study and comprehension of the complex global and internal dynamics of macromolecules by means of theoretical and experimental tools lead to the understanding of the mechanisms with which such molecules explicate their functions.

The experimental methods widely employed today in this effort rely on spectroscopic techniques, both magnetic and optical ones. Several comparisons of different spectroscopic techniques (NMR, ESR, optical spectroscopies, thermal factors analyses with X rays) potentially useful for the study of dynamical properties of proteic systems are available in the literature.⁴ Just to mention, amongst the optical spectroscopies, Fluorescence Polarization Anisotropy (FPA),⁵ Dynamic Light Scattering (DLS)⁶ and Time Resolved Stokes Shift (TRSS)⁷ are the most common techniques used in the biological field and they are all time resolved.

Among the magnetic spectroscopies, we mention Nuclear Magnetic Resonance (NMR) which plays a pivotal role. With this technique, the molecular dynamics are followed through the observation of the nuclear spin relaxation, which is in turn caused by the variations of the effective magnetic field acting on the examined nucleus, resulting therefore in the mixing of the nuclear spin states – thus ensuing transitions.⁸ NMR gives information on the motions at various time scales, from microseconds to picoseconds. A common view widely held is that the fast motions of atoms express themselves over the “slow” times of spin relaxations.

Another important and frequently used technique, central theme of this review, is the Electron Spin Resonance (ESR) – also known as Electron Paramagnetic Resonance (EPR). The principles of this technique are analogous to the nuclear ones. ESR is in principle one of the most powerful methods of investigating the electronic distribution in molecules and the characteristics of their environments. In fact, the relaxation of the excited electronic spin levels occurs due to their coupling to the motions of the chemical environment. The main investigative strategy used in ESR is that of having a paramagnetic site on the interested molecule upon which measurements are to be performed. Compared to NMR spectra in which it is often difficult to distinguish among the resonant atoms (especially when working at low magnetic fields), the convenience of ESR spectra lies in the fact that the vast majority of the information retrieved are pertinent to the paramagnetic center and its chemical surrounding. An experimental technique commonly adopted for example is the so called Site Directed Spin Labeling (SDSL) in which a particular paramagnetic compound is bonded to a particular site of the macromolecule in order to study its ESR spectrum in

that given chemical environment. Different kinds of nitroxides linked to conveniently substituted cysteines are typically used in this scope.⁹ Since the ESR spectra of nitroxides depend on both the polarity of the medium and the motional dynamics of these radicals, they can provide valuable information on the microenvironment of the latter. Given their importance in ESR studies, several of nitroxide radicals have been synthesized to be either used as spin probes (when dispersed in an environment) or as spin labels (when chemically attached to a biological molecule, *e.g.* a protein). In addition, a large part of ESR studies in biology is based on nitroxide radicals since these organic radicals are remarkably persistent because the unpaired electron is nested between the four methyl groups; they can therefore be involved in various reactions without the unpaired electron being affected. In particular, because of their favorable time scale, ESR experiments can be highly sensitive to the details of the rotational and internal dynamics of the macromolecule. Indeed, from an ESR spectrum, it is possible to recover three kinds of information¹⁰ relative to the nitroxide group: namely, information regarding (a) the accessibility to the side chain (which allows us to understand the shape of the secondary structure); (b) the dynamics of the side chain, obtainable from the spectrum line amplitude and from the inverse of the second moment of the spectrum; (c) the inter residue distance, which is measurable in the presence of two magnetic probes.¹¹ Furthermore, there is also the possibility to perform a time resolved measurement that allows the unraveling of the conformational variations of the examined protein. Secondary structure movements, tertiary structure folding and domain motions can be highlighted with this very technique.^{10–13} In Fig. 1 we report some common nitroxide radicals (also called nitroxyl radicals) used in biological ESR experiments.¹⁴ We also mention that in

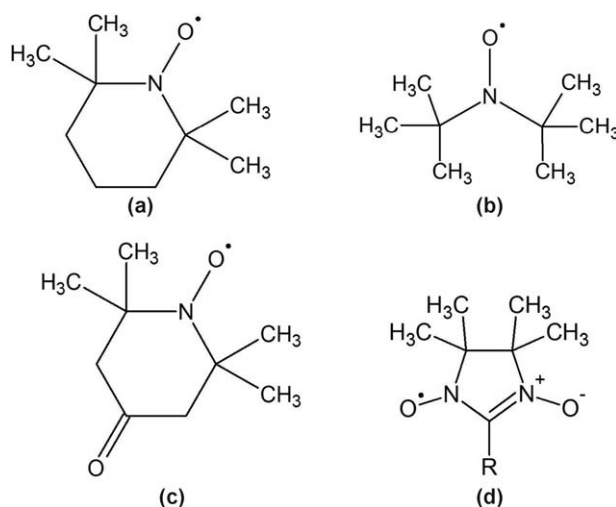


Fig. 1 Most common nitroxide radicals used in SDSL. (a) 2,2,6,6-tetramethyl-piperidine-1-oxyl also known as TEMPO, (b) di-*tert*-butylamine-*N*-oxyl also known as di-*tert*-butyl-nitroxide, (c) 2,2,6,6-tetramethyl-4-piperidone-1-oxyl also known as TEMPONE, (d) type of persistent radicals similar to nitroxides commonly called nitronyl nitroxides.

the field of material science persistent nitronyl nitroxides are frequently used. The ability of these radicals to act as bidentate ligands for various transition and rare earth metal ions often results in composite materials with new magnetic properties.

Concerning the interpretation of ESR spectra, it must be said that the task is quite involved since there are both experimental (a huge amount of data at different frequencies must be collected) and theoretical (interpretative advanced models are necessary to properly describe the molecular dynamics, and they must be computationally feasible) problems to face. Data interpretation often makes use of stochastic models and/or molecular dynamics simulations. At the same time ESR spectroscopy is intrinsically amenable to an advanced theoretical interpretation in terms of quantum chemistry and statistical thermodynamics. In recent years, these tools have reached a degree of accuracy, accessibility, and a power given by efficient computer implementation – a fine combination which promises a leap from fitting simulation to prediction.

The systematic application of modeling and numerical simulations to interpret experimental observables afford the relationship between ESR spectroscopic measurements and molecular properties. The main information needed in order to simulate a spectrum are the magnetic interactions present in the examined system and how the same undergoes relaxation processes, *i.e.* a model for the molecular dynamics. This goal is traditionally achieved by defining a phenomenological model including a number of molecular, mesoscopic, and macroscopic parameters to be fitted in order to reach the best agreement between experimental and simulated spectra. The main problem of this fitting procedure is encountered when several minima are present and is related to the use of various parameters and best-fit parameters. Consequently, the fitting approach is useful only when few fitting parameters are used. The usage of a Stochastic *Ab initio* Spectra Simulation (SASS) can solve some of these problems. The SASS as sketched in Fig. 2 combines

- Quantum mechanical (QM) calculations of structural parameters and magnetic tensors, possibly including discrete-continuum solvent models (that take into account average interactions with the environment);^{15,16}
- Inclusion of stochastic effects into dynamic models provided by calculated molecular parameters.

As we write, the SASS guarantees the best parameter free approach to the calculation of ESR spectra to be compared with the measured ones. The Stochastic Liouville Equation (SLE), which serves as the powerhouse of SASS,^{17,18} accounts for all the different aspects regarding the structural, dynamical, and magnetic properties of the molecular system under investigation at the cost of basic input parameters such as the magnetic tensors of the examined molecule and solvent macroscopic parameters. This integrated procedure for Continuous-Wave Electron Spin Resonance (CW-ESR) is well suited, and part of it has been implemented in the framework of the Virtual Multi-frequency Spectrometer (VMS) project – a project recently embarked on by our group and still under active development.^{19–21} A Graphical User Interface (GUI) composed of both

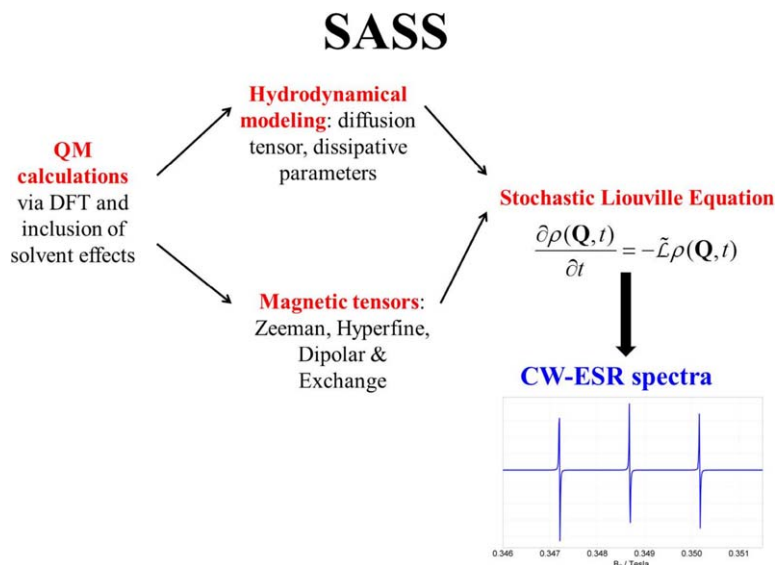


Fig. 2 Scheme of the Stochastic *Ab initio* Spectra Simulation Approach (SASS).

interactive tools and direct graphical visualization of data gives to both the novice and the expert user the possibility to easily treat almost all the spectroscopies. Until now, the “fast” spectroscopies have been implemented, while we are still working on the slow spectroscopies such as NMR and in particular ESR. The E-SpiReS code²² has also been successfully implemented for the calculation of CW-ESR spectra. We are currently working on new developments for what concerns both the magnetic (see subsection 6.1) and the diffusive part (subsection 6.2).^{23,24} Future developments will include also the advanced ESR spectroscopies.

In the following, we shall curtail our attention only on CW-ESR spectroscopy in solution of organic monoradicals and biradicals, leaving out, for the sake of simplicity, advanced ESR spectroscopies (such as time domain multiple pulse spin echo) and double resonance methods (like Electron Nuclear Double Resonance (ENDOR)), not to mention the ESR of metal ions in solution or solid state CW-ESR. In particular, as usual, CW-ESR spectra lines will be presented as first derivatives of the absorption spectrum of the spins with respect to the field.

The paper is organized as follows: in the next section, “Interpretative models for magnetic spectroscopies”, we shall discuss the main information required in order to simulate a CW-ESR spectrum and the advanced method of the SLE. Then, in sections “Spin Hamiltonian” and “Stochastic diffusion operator, relaxation” we provide in detail the description of the two main ingredients that enter in the SLE (*viz.* the spin Hamiltonian and the stochastic diffusion operator), and consider how to calculate QM magnetic tensors, besides discussion on how to tackle the diffusive problem. Technical details about the SASS from the perspective of VMS are given in section “Stochastic *Ab Initio* Spectra Simulation of CW-ESR” together with some recent results about the application of this integrated methodology to some biological

macromolecules. Current workings on our recent improvements of the two main SLE ingredients will be given in section “Towards a multi-body ESR: novel computational techniques”. Finally, in section “Conclusions” some space is reserved for discussion and future perspectives.

2 Interpretative models for magnetic spectroscopies

CW-ESR spectra are very important since they bear the fingerprint of many microscopic processes which are highly informative regarding, primarily, the structural properties (which in essence determine the number of peaks) and the dynamical behavior (responsible for the broadening of peaks) of the probed molecule in solution. The need to breakdown and pinpoint this wealth of information to specific microscopic underlying processes has been, both, encouraged and in harness with the advancement in computing power achieved over recent decades, bringing to light many more complex models and algorithms for the simulation and interpretation of ESR spectra. In order to elucidate such microscopic dynamic processes – which occur on time scales corresponding to frequencies above 100 GHz, on average – it is imperative to have theoretical models capable of treating explicitly the system's degrees of freedom involved in said processes. The extent to which these degrees of freedom (for example, rotational and internal degrees of freedom) are explicitly taken into account specifies (1) the amount and quality of information retrievable from the experimental CW-ESR spectra, and (2) the computational cost one has to pay. It is often the case that the more refined the quality of information, the higher the computational cost.

In order to simulate a CW-ESR spectrum, the first step is to gather information regarding the structure and motional dynamics of the paramagnetic species. But where and how do we fetch this information? On one hand, the knowledge of both may come from experiments. For example, estimations of the structural and magnetic parameters can be gathered directly from the recorded CW-ESR spectra, while the dynamical behavior may be understood from spectroscopic fluorescence anisotropy experiments, where it is possible to collect dissipative information regarding the system. Meanwhile, on the other hand, direct computations can also provide good estimations of the above mentioned properties. QM calculations can be performed in order to retrieve structural and magnetic properties, while dynamical behavior may come from Molecular Dynamics (MD) simulations. MD simulations can provide very valuable information on the dynamics, both the global and internal ones. In addition to allowing detailed study of the faster motions that occur on time scales of the order of ns and ps, they also make it possible to study coupled relaxations of more spin systems.²⁵ Nonetheless, the simulation time frame of ns and ps constitutes a serious drawback on their application in spin relaxations given that the latter evolve on time scales that range from ps to ms. The only way to get around the problem in order to have realistic results from a MD trajectory is to impose steps of 1–2 fs on the simulation, but this would mean running a single simulation for an unreasonable number of months if not years. To illustrate the point, for a

1 ms trajectory, for example, with 1 fs step, 10^{15} steps are required, which will translate into a prohibitive computation time.

Once information concerning structural and dynamical properties are assembled, numerical simulations of CW-ESR spectra can be ran based on a careful theoretical model. Fitting procedures are then pursued in the scope of minimizing deviations between experimental and calculated spectra in order to refine both the structural and the dynamical information. As mentioned earlier, this stage can suffer from the burden of choice of the best-fit parameters due to the presence of several minima when the number of fitting parameters in the model grows. Needless to say, one can avoid this problem by resorting to a model that does not depend heavily on fitting parameters. Undoubtedly, this is one of the advantages of CW-ESR spectra modeling based on the Stochastic Liouville Equation (SLE) methodology. And our SASS approach – which performs both the direct calculation of CW-ESR spectra and fitting of all the aforementioned parameters in an integrated fashion – is certainly a good example. Within the SLE approach, structural and motional dynamics of the paramagnetic probe are treated consistently. In particular, the former is described *via* an effective spin Hamiltonian that takes into account all the magnetic interactions, while the latter is taken care of by adopting a stochastic diffusive model. Just to mention, a MD trajectory can also be used²⁶ to modulate the spin Hamiltonian by making use of an orientation dependent component of the paramagnetic probe. Then inserting the obtained spin Hamiltonian in the SLE gives the time evolution for the spin density matrix, from which it is possible to calculate the ESR spectrum. Stochastic and inertial many-body models that diffuse in a coupled manner render the interpretation of the motional dynamics starting from magnetic resonance measurements more efficient. Consequently, it can be said that MD simulations can be a great asset to stochastic models in the final goal of understanding the probe's motional dynamics in the presence of, for example, complex fluid systems.

Over the years, there have been many approaches to theoretical and computational ESR. These approaches seem to focus on or (1) the appropriate spin Hamiltonian to use under a given set of conditions, or (2) the evaluation of magnetic tensors, or (3) relaxation processes responsible for line broadening. The approaches which fall under (1) are mainly relativistic quantum mechanical, while those approaches which fall under (2) are primarily relativistic but can also be based on Density Functional Theory (DFT), or a hybrid of the two. Approaches (3) are mainly application of the classical theory of stochastic processes. All three approaches are unquestionably vital for the analysis and interpretation of ESR spectra but it appears the majority of ESR research groups are specialized in only one of the above mentioned approaches, adopting diverse conventions and nomenclature. As a result, the communication between the three communities has been difficult in these years. With VMS, through our SASS approach, we are trying to build a computational tool which will serve as a bridge between the various communities and harmoniously integrate all three approaches efficiently

as possible. In order to fully let the reader appreciate the SASS approach, in Section 3.1 we analyze the spin Hamiltonian and how we get to SLE model from the former. In Section 3.2 we shall see how the magnetic tensors are calculated and how the effect of the solvent are accounted for in VMS. Section 3 is structured in such a way that all three communities see where their research fit in the general setting of SASS. However, we give a very brief introduction to the SLE in Section 2.1.

2.1 Advanced model: stochastic Liouville equation

The motion of the probe in the solvent is more or less random. The degree of randomness depends on factors like the temperature and viscosity of the solvent. A realistic model for the evolution of reduced density matrix of the probe cannot ignore this fact. Besides, several researches have shown how significant the influence of this random motion can be on the line shapes of ESR spectra.²⁷ For example, if the viscosity of the solvent is very low, the temperature relatively high and the probe relatively small so as to have very rapid fluctuations in its motion, the line shapes observed are simple Lorentzian (*fast motional regime*). In this case, one can assume a complete loss of coherence between the probe's motion and its spin states, thus on the ESR spectra. The Bloch-Wagness-Redfield theory can be used here to compute the ESR spectra. On the other hand, when the experimental conditions (for example, high viscosity of solvent and low temperature) are such that coherence between the probe's motion and its spin states (*slow motional regime*) cannot be ignored, one needs a formalism which explicitly takes into consideration the random motion of the probe and the correlation between motion and spin states. The Stochastic Liouville (SL) approach is devised for this task. Moreover, the same formalism can be used in the fast motional regime.¹⁷

In the SL approach, one specifies a collection $\mathbf{q}$ of the probe's coordinates to consider as evolving stochastically. Say, $\mathbf{Q} = (\mathbf{q}, \sigma)$, where σ denotes spin operators of the probe, one then defines an operator $\rho(\mathbf{Q}, t)$ which in essence is the *conditional* spin density matrix given specific values of the stochastic variables $\mathbf{q}$. Its equation of motion – referred to as the Stochastic Liouville Equation (SLE) – is

$$\frac{\partial \rho(\mathbf{Q}, t)}{\partial t} = -i[H(\mathbf{Q}), \rho(\mathbf{Q}, t)] - \Gamma \rho(\mathbf{Q}, t) \quad (1a)$$

$$= -\mathcal{L} \rho(\mathbf{Q}, t) \quad (1b)$$

where we have introduced the Liouvillian operator $\mathcal{L} \equiv iH^\times + \Gamma$, where $H^\times \equiv [H, \bullet]$. As we see later on, the Hamiltonian $H(\mathbf{Q})$ (which has been scaled by the factor $\hbar^{-1}$) is an effective Hamiltonian which depends on the stochastic variables $\mathbf{q}$ as well as on the spin operators. The first term of eqn (1a) gives the time evolution of the spin states at constant coordinate $\mathbf{q}$ whilst the second term accounts for the time evolution of the stochastic variables $\mathbf{q}$ according to the stochastic operator Γ when the spin states are held constant.

The CW-ESR spectrum of the probe $I(\omega - \omega_0)$ (which is in function of the sweep frequency ω and the Larmor frequency $\omega_0 \equiv \gamma_e B_0 = g_0 \beta_e B_0 / \hbar$, where g_0 is related to the $\mathbf{g}$ - tensor through the relation $g_0 \equiv \text{Tr}(\mathbf{g})/3$) is calculated as the real part of the auto-correlation function of the vector $|\nu\rangle$ (*starting vector*) – which is nothing but the x -component of the magnetization vector of the electrons. For example, in the case of a single unpaired electron and a single nucleus of spin I_N (e.g. a nitroxide, NO), $|\nu\rangle \equiv [I_N]^{-1/2}(\mathbf{s}_x \otimes \mathbb{I}_N)$ (where $[I_N] \equiv (2I_N + 1)$ and $\mathbb{I}_N$ represent the dimension and unit operator of the Hilbert space of the isolated nucleus, respectively). More explicitly, if we use the vector $|\nu p_{\text{eq}}^{1/2}(\mathbf{q})\rangle$, where $p_{\text{eq}}(\mathbf{q})$ is the equilibrium Boltzmann-type distribution of the variables $\mathbf{q}$, then

$$\begin{aligned} I(\omega - \omega_0) &= \frac{1}{\pi} \text{Re} \langle \nu p_{\text{eq}}^{1/2}(\mathbf{q}) | [i(\omega - \omega_0) + \tilde{\mathcal{L}}]^{-1} | \nu p_{\text{eq}}^{1/2}(\mathbf{q}) \rangle \\ &= \frac{1}{\pi} \text{Re} \langle \nu p_{\text{eq}}^{1/2}(\mathbf{q}) | [i(\omega - \omega_0) + iH^\times + \tilde{\Gamma}]^{-1} | \nu p_{\text{eq}}^{1/2}(\mathbf{q}) \rangle \end{aligned} \quad (2)$$

where we have introduced the symmetrized stochastic operator $\tilde{\Gamma} \equiv p_{\text{eq}}^{-1/2}(\mathbf{q}) \Gamma p_{\text{eq}}^{1/2}(\mathbf{q})$. Iterative numerical algorithms are employed to compute $I(\omega - \omega_0)$ from eqn (2); among these, the widely used are the Lanczos and the conjugate gradient algorithms. Then the first derivative of the adsorption line I with respect to the applied external magnetic field B_0 must be taken.

In the following, we shall discuss how the SLE emerges from a series of well-thought approximations beginning with the full molecular Hamiltonian.

3 Spin Hamiltonian

3.1 From pin Hamiltonian to stochastic Liouville Equation

The effective spin Hamiltonians employed in magnetic resonances depend on the spin angular magnetic momenta of electrons and nuclei, which have no analogues in classical mechanics. Thus the usual way of determining a classical Hamiltonian and then promoting appropriate classical variables to corresponding quantum operators fails. We shall not give here a systematic derivation of the spin Hamiltonian since it is far beyond the scope of this review and will require at least an entire review on its own. What we would like to rather do is to highlight some important features of the derivation to keep in mind when trying to solve the SLE.

The Dirac equation

The appropriate derivation of the spin Hamiltonian can be done within quantum electrodynamics (QED), whose root traces back to Dirac's equation²⁸

$$i\hbar \frac{\partial \psi}{\partial t} = H_D \psi \quad (3)$$

which is a relativistically covariant Schrödinger-like equation for massive particles with $\frac{1}{2}$ -spin (or Dirac particles) like the electron interacting with

an electromagnetic field. The operator H_D in eqn (3) is termed as the Dirac Hamiltonian and it is given by the expression

$$H_D = (c\boldsymbol{\alpha} \cdot \boldsymbol{\pi} + q\phi + \beta mc^2) \quad (4a)$$

$$\boldsymbol{\pi} = \mathbf{p} - q\mathbf{A} \quad (4b)$$

where c is the speed of light; ϕ and $\mathbf{A}$ are the scalar and vector potentials of the electromagnetic field, respectively; q is the charge of the particle (for an electron, $q = -e$); m and $\mathbf{p}$ are its mass and momentum, respectively; $\boldsymbol{\alpha} \equiv (\alpha_x, \alpha_y, \alpha_z)$ and β are in general $n \times n$ matrices (also called Dirac matrices) which components satisfy a set of conditions.^{28–33} As Dirac proved, n must be an even number not less than 4, which implies that the wave function ψ in eqn (3) is a column vector of dimension $n(\geq 4) \times 1$ (with n even as stated earlier) called an n -component spinor. When $n = 4$, a possible choice of the Dirac operators are (these are also the original matrices Dirac used²⁸)

$$\alpha_i = \begin{pmatrix} \mathbf{0} & \boldsymbol{\sigma}_i \\ \boldsymbol{\sigma}_i & \mathbf{0} \end{pmatrix} \quad \beta = \begin{pmatrix} \mathbf{1} & \mathbf{0} \\ \mathbf{0} & -\mathbf{1} \end{pmatrix} \quad (i = x, y, z) \quad (5)$$

where the matrix elements are all 2×2 matrices. In particular, $\mathbf{0}$ and $\mathbf{1}$ correspond to null and unit 2×2 matrices, respectively; the $\{\boldsymbol{\sigma}_i\}$ are the Pauli matrices

$$\boldsymbol{\sigma}_x = \begin{pmatrix} 0 & 1 \\ 1 & 0 \end{pmatrix} \quad \boldsymbol{\sigma}_y = \begin{pmatrix} 0 & i \\ -i & 0 \end{pmatrix} \quad \boldsymbol{\sigma}_z = \begin{pmatrix} 1 & 0 \\ 0 & -1 \end{pmatrix}. \quad (6)$$

The Pauli matrices are related to the components of the spin operator $\mathbf{s}$ of a Dirac particle through the relation $\mathbf{s}_i = \frac{\hbar}{2} \boldsymbol{\sigma}_i$, $i = \{x, y, z\}$. The simple case $n = 4$ was shown by Dirac to yield a wave function ψ whose two components correspond to positive energies and the other two are associated with negative energies. The states corresponding to positive energies can be decoupled from those of negative energies in the non-relativistic limit ($v \ll c$, where v is the particle's velocity) using for example the Foldy–Wouthuysen transformation;^{34,35} this yields two set of independent wave equations each involving a 2 component spinor (ψ_+ and ψ_- , wave functions of the positive and energy states, respectively). For example, in the case electron, ψ_+ is a 2 component spinor whose components are nothing but the well-known spin-up ($\uparrow$) and spin-down ($\downarrow$) states of the electron while the ψ_- counterpart is said to describe the positron.

The multi-electron Hamiltonian

The original Dirac equation though, applies only to a single Dirac particle interacting with an external electromagnetic field. Its extension to the case of two or more interacting Dirac particles is not trivial. Shortly after Dirac published his electron theory, Breit³⁶ made an important step in deriving a relativistic wave equation for two Dirac particles interacting

with each other by defining a relativistic Hamiltonian H_B (called the Breit or Dirac–Coulomb–Breit Hamiltonian)

$$H_B = H_0 + H_{\text{int}} \quad (7a)$$

$$H_0 \equiv H_{D,1} + H_{D,2} \quad (7b)$$

$$H_{\text{int}} \equiv H_{\text{int,Coulomb}} + H_{\text{int,Breit}} + \mathcal{O}(mc^2\alpha^5) \quad (7c)$$

$$H_{\text{int,Coulomb}} \equiv \frac{q_1 q_2}{4\pi\epsilon_0 r_{12}} \quad (7d)$$

$$H_{\text{int,Breit}} \equiv -\frac{q_1 q_2}{8\pi\epsilon_0 r_{12}} \left[\boldsymbol{\alpha}_1 \cdot \boldsymbol{\alpha}_2 + \frac{(\boldsymbol{\alpha}_1 \cdot \mathbf{r}_{12})(\boldsymbol{\alpha}_2 \cdot \mathbf{r}_{12})}{r_{12}^2} \right] \quad (7e)$$

which is simply the sum of (1) two Dirac Hamiltonians $H_{D,1}$ and $H_{D,2}$ (corresponding to the isolated Dirac particles, for example electrons) and (2) an interaction term H_{int} which is the sum of the Coulomb interaction Hamiltonian $H_{\text{int,Coulomb}}$ between the two Dirac particles and a third term $H_{\text{int,Breit}}$, called the Breit operator, which is a quantum electrodynamic second-order perturbation correction. The latter accounts for the spin–spin and retarded interactions. Basically, higher order correction terms can be obtained by resorting to quantum electrodynamics. Significant as Breit's equation is, it is not Lorentz invariant even though it is gauge invariant. The complete relativistic covariant wave equation for two interacting Dirac particles is given by the Bethe–Salpeter (BS) equation^{31,37} – which is an integro-differential equation – proposed two decades after Breit's seminal work in 1951 by Hans Bethe and Edwin Salpeter. As one would expect, the leading terms of the expansion of the BS equation coincides with the Breit equation. Despite the fact that the Bethe–Salpeter equation contains all possible QED effects, it demands separate times for the two electrons, a requirement which is essential for its Lorentz invariance but in sharp contrast to conventional non-relativistic quantum mechanics where the evolution of a system involves a unique time. Moreover, the BS equation is very difficult to solve and may also lead to abnormal solutions as discussed by some authors.^{38–40} In the vast majority of problems dealt with in theoretical chemistry, the refinement of results by adding QED correction terms beyond the second-order leads to no significant improvement. These observations, together with the fact that the Breit operator is valid for electrons moving with relatively very low velocities compared to the speed of light c (for example, valence electrons in an atom or the HOMO electrons of a molecule), make the Breit equation suitable for our purposes. The important thing we would like to emphasize here is that the Breit operator – from which the spin–spin interaction term, which we shall discuss later and very important in ESR when there are more than one spin, stems from is a perturbation term which, together with the Coulomb term, makes the Breit equation valid to the order $mc^2\alpha^4$; therefore, it is only appropriate to seek first order solutions of the said operator.³⁵

As one could imagine, the Hilbert space on which H_B operates is one of dimension 16 (which can be seen as the tensor product of the two Hilbert spaces – each of dimension 4 – of the individual electrons), thus the wave function ψ in Breit's equation for two electrons is 16 component spinor. Given that the Breit equation is a fair approximation of the BS equation for interacting Dirac particles of velocities $v \ll c$ (which is mostly the case in molecular science), we may reduce it to a non-relativistic form by employing for example a Foldy-Wouthuysen-like transformation to separate the subspace in which both electrons have positive energy from the original 16-dimensional Hilbert space. This subspace is of dimension 4 (which can be viewed as the tensor product between the positive energy subspace of the two electrons).

It is worth noting that the associated effective two-particle Hamiltonian (which is similar to the non-relativistic Schrödinger equation with some relativistic terms) may be employed to define the Hamiltonian of a collection of interacting electrons if we assume that the only admissible interactions between the particles are pairwise. In fact, the approximate nature of the Breit equation allows only for such type of interactions. Following McWeeny,⁴¹ we shall term this multi-electron Hamiltonian as the pure electronic term H_{el} ,

$$H_{el} = H_{el,0} + H_{el-el} \quad (8)$$

where $H_{el,0}$ is the sum of the non-relativistic form of the Dirac Hamiltonian of the separate electrons; H_{el-el} is the electron–electron interacting Hamiltonian (see pages 176 and 177 of ref. 35 for explicit equations).

Molecular Hamiltonian

The extension of these results to molecular systems present significant theoretical hurdles which are still not satisfactorily overcome today. These and other theoretical problems related to the molecular Hamiltonian⁴² (and so to the spin Hamiltonian, as we shall shortly see) seem to be major concerns of the relativistic quantum chemistry and associated ESR communities only even though they are of fundamental importance. The transition from the Breit Hamiltonian to a molecular one is simply an *ad hoc* one and still lacks a solid theoretical justification, despite the fact that the obtained theoretical framework agrees satisfactorily with experiments. Indeed the most common way to extend the non-relativistic limit of the multi-electron Breit Hamiltonian to a molecular Hamiltonian is to assume that: (1) every isolated nucleus obey a Dirac-type equation, (2) there exists Breit-type Hamiltonian for two interacting nuclei (or between a nucleus and an electron), (3) by means of a Foldy-Wouthuysen-like transformation, the above mentioned Breit-type Hamiltonian can be reduced to non-relativistic form, and that (4) only pairwise interactions (electron–electron, nucleus–nucleus, electron–nucleus) are possible. With these assumptions, one may readily write down the non-relativistic approximated Hamiltonian H_{probe} for a molecular system by simply adding to the above defined pure electronic term H_{el} , a pure nuclear term

H_{nuc} (which includes all nucleus–nucleus interactions) and a third term, the electron–nuclear term $H_{\text{el–nuc}}$ (which accounts for all electron–nuclear interactions). Similar to the electron magnetic moment operator $\hat{\mu} \equiv g\mu_{\text{B}}\mathbf{s}$ (where g is the electron g -factor, μ_{B} is the Bohr magneton and $\mathbf{s}$ is the Pauli spin operator of the electron), one defines based on phenomenological arguments a magnetic moment $\hat{\mu}_K \equiv g_K\mu_N\mathbf{I}_K$ for the K -th nucleus, where $\mu_N (\equiv e\hbar/m_p)$, where m_p is the mass of the proton) is the nuclear magneton, and the value of g_K and the spectrum of $\mathbf{I}_K$ – the nuclear g -factor and spin operator of the K -th nucleus – are to be determined experimentally. The H_{nuc} term is derived from H_{el} by simply replacing the mass, the position vector and the magnetic moment of the electron with those of the nucleus, whilst the $H_{\text{el–nuc}}$ term is obtained from $H_{\text{el–el}}$ by making the same substitutions for only one electron in each term present in the latter. We report here the explicit equations for H_{probe} from Moss³⁵ (we have omitted some terms which are not of interest as far as this review is concerned. The interested reader should consult the cited literature):

$$H_{\text{probe}} = H_{\text{el}} + H_{\text{nuc}} + H_{\text{el–nuc}} \quad (9)$$

$$H_{\text{el}} = H_{\text{el},0} + H_{\text{el–el}} \quad (10a)$$

$$H_{\text{el},0} \equiv \sum_i \left\{ m_e c^2 + \frac{\pi_i^2}{2m_e} - e\phi(\mathbf{r}_i) + g\mu_B \mathbf{s}_i \cdot \mathbf{B}(\mathbf{r}_i) + \cdots + \mathcal{O}(m_e c^2 \alpha^5) \right\} \quad (10b)$$

$$\begin{aligned} H_{\text{el–el}} \equiv & \sum_{j>i} \frac{1}{4\pi\epsilon_0} \frac{e^2}{r_{ij}} \\ & + \sum_{j>i} \frac{1}{4\pi\epsilon_0} \left\{ \left(\frac{g\mu_B}{c} \right)^2 \left[\frac{\mathbf{s}_i \cdot \mathbf{s}_j}{r_{ij}^3} - 3 \frac{(\mathbf{s}_i \cdot \mathbf{r}_{ij})(\mathbf{r}_{ij} \cdot \mathbf{s}_j)}{r_{ij}^5} - \delta(\mathbf{r}_{ij}) \left(\frac{8\pi}{3} \right) (\mathbf{s}_i \cdot \mathbf{s}_j) \right] \right\} \\ & + \cdots + \mathcal{O}(m_e c^2 \alpha^5) \end{aligned} \quad (10c)$$

where the first two terms in eqn (10b) are the rest energy and the kinetic energy of the electron, respectively; the other two terms account for the interaction between the electron and the external electromagnetic field, *viz.* the term $(-e\phi(\mathbf{r}_i))$ is the potential energy of the interaction between the i -th electron and the scalar potential ϕ of the external field, whilst $g\mu_B \mathbf{s}_i \cdot \mathbf{B}(\mathbf{r}_i)$ is the so-called Zeeman term which plays a vital role in magnetic resonance studies and accounts for the interaction between the electron and the external magnetic field. The first term in eqn (10c) is the Coulomb interaction between a pair of electrons and the second term is called the spin–spin term; whilst the first two terms of the spin–spin term relates to the dipole–dipole interaction between the magnetic moments of the pair, the last term describes a Fermi–contact interaction.

Similar equations to 10 may be written for H_{nuc} :

$$H_{\text{nuc}} = H_{\text{nuc},0} + H_{\text{nuc-nuc}} \quad (11a)$$

$$H_{\text{nuc},0} \equiv \sum_K \left\{ m_K c^2 + \frac{\pi_K^2}{2m_K} + Z_K e \phi(\mathbf{r}_K) - g_K \mu_N \mathbf{I}_K \cdot \mathbf{B}(\mathbf{r}_K) + \cdots + \mathcal{O}(m_r c^2 \alpha^4) \right\} \quad (11b)$$

$$H_{\text{nuc-nuc}} = \sum_{K' > K} \frac{1}{4\pi\epsilon_0} \left\{ \frac{Z_K Z_{K'} e^2}{r_{KK'}} + g_K g_{K'} \left(\frac{\mu_N}{c} \right)^2 \left[\frac{\mathbf{I}_K \cdot \mathbf{I}_{K'}}{r_{KK'}^3} - 3 \frac{(\mathbf{I}_K \cdot \mathbf{r}_{KK'})(\mathbf{r}_{KK'} \cdot \mathbf{I}_{K'})}{r_{KK'}^5} \right] \right\} \\ + \cdots + \mathcal{O}(m_r c^2 \alpha^4) \quad (11c)$$

where $m_r \equiv (m/m_p)^2$; $\pi_K \equiv \mathbf{p}_K - Z_K e \mathbf{A}(\mathbf{r}_K)$ and Z_K is the atomic number of the K -th nucleus. For the electron–nuclear interaction Hamiltonian $H_{\text{el-nuc}}$ we report only the following terms

$$H_{\text{el-nuc}} = - \sum_{i,K} \frac{1}{4\pi\epsilon_0} \frac{Z_K e^2}{r_{iK}} \\ - \sum_{i,K} \frac{1}{4\pi\epsilon_0} \frac{g_K g_K \mu_B \mu_N}{c^2} \left[\frac{\mathbf{s}_i \cdot \mathbf{I}_K}{r_{iK}^3} - 3 \frac{(\mathbf{s}_i \cdot \mathbf{r}_{iK})(\mathbf{r}_{iK} \cdot \mathbf{I}_K)}{r_{iK}^5} - \delta(\mathbf{r}_{iK}) \left(\frac{8\pi}{3} \right) (\mathbf{s}_i \cdot \mathbf{I}_K) \right] \\ + \cdots + \mathcal{O}(m_r c^2 \alpha^4) \quad (12)$$

We remind the reader that in writing eqn (10)–(12) we have omitted certain terms like the spin–orbit coupling terms which may prove to be important in certain magnetic resonance studies, in particular ESR of heavy metals.

Shifting the spectrum of H_{probe} with respect to the rest energy of the electrons and nuclei (which are constants), we may rewrite eqn (9) as

$$H_{\text{probe}} = H_{\text{classical}} + H_{\text{spin}} \quad (13)$$

where

$$H_{\text{classical}} \equiv \sum_i \left[\frac{\pi_i^2}{2m_e} - e \phi(\mathbf{r}_i) + \sum_{j>i} \frac{e^2}{4\pi\epsilon_0 r_{ij}} + \cdots + \mathcal{O}(m_e c^2 \alpha^5) \right] \\ + \sum_K \left[\frac{\pi_K^2}{2m_K} + Z_K e \phi(\mathbf{r}_K) + \sum_{K'>K} \frac{Z_K Z_{K'} e^2}{4\pi\epsilon_0 r_{KK'}} + \cdots + \mathcal{O}(m_r c^2 \alpha^4) \right] \\ + \sum_{i,K} \left[- \frac{Z_K e^2}{4\pi\epsilon_0 r_{iK}} + \cdots + \mathcal{O}(m_r c^2 \alpha^4) \right] \quad (14)$$

$$\begin{aligned}
H_{\text{spin}} \equiv & \sum_i \left[\mu_B \mathbf{s}_i \cdot \mathbf{g} \cdot \mathbf{B}_i + \sum_{j>i} \mathbf{s}_i \cdot \mathbf{D}_{ij} \cdot \mathbf{s}_j + \cdots + \mathcal{O}(mc^2\alpha^5) \right] \\
& + \sum_K \left[-g_K \mu_N \mathbf{I}_K \cdot \mathbf{B}_K + \sum_{K'>K} \mathbf{I}_K \cdot \mathbf{Q}_{KK'} \cdot \mathbf{I}_{K'} + \cdots + \mathcal{O}(m_r c^2 \alpha^4) \right] \quad (15) \\
& + \sum_{i,K} [\mathbf{s}_i \cdot \mathbf{A}_{iK} \cdot \mathbf{I}_K + \cdots + \mathcal{O}(m_r c^2 \alpha^4)]
\end{aligned}$$

where $\mathbf{D}$ is the Zero-field splitting tensor, while $\mathbf{g}$, $\mathbf{Q}$ and $\mathbf{A}$ are the Zeeman, nuclear quadrupole and hyperfine interaction tensors, respectively. The Zeeman tensor has many contributions as we shall see in subsection 3.2. It may be expressed as

$$\mathbf{g} = g \mathbf{1}_3 + \Delta \mathbf{g} \quad (16)$$

where $\mathbf{1}_3$ is the 3×3 unit matrix and $\Delta \mathbf{g}$ is the correction term. $H_{\text{classical}}$ is the part of the Hamiltonian one could derive based on classical mechanics with some relativistic corrections, and it contains no spin operators. Meanwhile, the H_{spin} (the spin Hamiltonian) originates directly from the combination of quantum mechanics and special relativity and dates back to Dirac's equation. For ESR, the contribution of the second square parenthesis in eqn (15), *i.e.* the nuclear Zeeman and quadrupole terms, is usually very small and may be neglected, as we shall do here.

The paramagnetic probe Hamiltonian in the presence of solvent

The probe Hamiltonian derived so far is valid only for molecules in vacuum. Since we are interested in ESR spectra of solvent embedded probes, our theoretical model needs to take into consideration the effect of the solvent.

An easy way to account for solvent effects is to do a system-bath partition whereby the paramagnetic probe is considered as the focus system with isolated Hamiltonian H_{probe} , and the solvent as the bath – whose isolated Hamiltonian will be denoted as H_{solvent} . The total Hamiltonian H_{tot} of the bipartite system is

$$\begin{aligned}
H_{\text{tot}}(\{\chi_i\}, \{\chi_K\}, \{q_x\}) = & H_{\text{probe}}(\{\chi_i\}, \{\chi_K\}) + H_{\text{solvent}}(\{q_x\}) \\
& + H_{\text{probe-solvent}}(\{\chi_i\}, \{\chi_K\}, \{q_x\}) \quad (17)
\end{aligned}$$

where $H_{\text{probe-solvent}}$ is the Hamiltonian which describes the system-bath interaction; $\chi_l \equiv (\mathbf{r}_l, \mathbf{p}_l, \mathbf{s}_l)$ denotes the spatial ($\mathbf{r}_l$), momentum $\mathbf{p}_l$ and spin ($\mathbf{s}_l$) coordinates of the l -th electron (indexed i) or nucleus (indexed K) of the probe; $\{q_x\}$ is the set of position-spin coordinates of the electrons and nuclei of the solvent molecules. All position-spin vectors are expressed in reference to the laboratory frame (LF). It goes without saying that a theoretical model based on a total Hamiltonian H_{tot} as given in eqn (17) is computationally intractable. Again, a series of approximations is inevitable at this point.

Notice that the solvent appears explicitly in eqn (17) as a collection of molecules (*explicit model*). A very reasonable approximation is to (1) assume a continuum model for the solvent (*implicit model*), and (2) treat it at a classical mechanical level (while we continue to treat the probe system quantum mechanically). The model becomes now a semi-classical one. Established implicit solvation models like the polarizable continuum model (PCM) are mainly devised for this purpose as we shall see in Section 3.2. Using these models, an effective Hamiltonian $H_{\text{probe}}^{\text{eff}}(\{\chi_i\}, \{\chi_{Ii}\})$ for the probe can be derived by averaging H_{tot} over solvent degrees of freedom. Limiting ourselves to ESR studies, and supposing we subject the probe to a uniform static external magnetic field $\mathbf{B}_0$, we may write

$$H_{\text{probe}}^{\text{eff}} = H_{\text{classical}}^{\text{eff}} + H_{\text{spin}}^{\text{eff}} \quad (18)$$

In this case, the effect of the solvent in the PCM model primarily translates into a fluctuating electromagnetic field with scalar and vector potentials ϕ_{solvent} and $\mathbf{A}_{\text{solvent}}$, respectively, which shall be in general time-dependent. Thus, from eqn (14)–(15), we may write (retaining only terms significant in the ESR studies of systems of interest to the present review and keeping in mind the big $\mathcal{O}$ notations for the various terms),

$$\begin{aligned} H_{\text{classical}}^{\text{eff}} \equiv & \sum_i \left[\frac{\tilde{\pi}_i^2}{2m_e} - e\langle\phi_{\text{solvent}}\rangle(\mathbf{r}_i) + \sum_{j>i} \frac{e^2}{4\pi\epsilon r_{ij}} \right] \\ & + \sum_K \left[\frac{\tilde{\pi}_K^2}{2m_K} + Z_K e\langle\phi_{\text{solvent}}\rangle(\mathbf{r}_K) + \sum_{K'>K} \frac{Z_K Z_{K'} e^2}{4\pi\epsilon r_{KK'}} \right] + \sum_{i,K} \left[-\frac{Z_K e^2}{4\pi\epsilon r_{iK}} \right] \end{aligned} \quad (19)$$

$$H_{\text{spin}}^{\text{eff}} \equiv \sum_i \left[\mu_B \mathbf{s}_i \cdot \mathbf{g}'_i \cdot \mathbf{B}_0 + \sum_{j>i} \mathbf{s}_i \cdot \mathbf{D}'_{ij} \cdot \mathbf{s}_j + \sum_K \mathbf{s}_i \cdot \mathbf{A}'_{iK} \cdot \mathbf{I}_K \right] \quad (20)$$

where $\langle\phi_{\text{solvent}}\rangle$ and $\langle\mathbf{A}_{\text{solvent}}\rangle$ are the mean scalar and vector potentials of the electromagnetic field inside the probe's cavity due to the solvent; $\tilde{\pi}_i \equiv \mathbf{p}_i + e(\mathbf{A}_0 + \langle\mathbf{A}_{\text{solvent}}\rangle_i)$, and $\tilde{\pi}_K \equiv \mathbf{p}_K - Z_K e(\mathbf{A}_0 + \langle\mathbf{A}_{\text{solvent}}\rangle_K)$. The tensor $\mathbf{g}'$ now accounts also for the magnetic field originating from the interaction between the probe and the solvent.

The tensors $\mathbf{g}'$, $\mathbf{D}'$ and $\mathbf{A}'$ are averaged over the interaction between the probe and the solvent. The reader should also note that we have substituted ϵ_0 with ϵ , the latter being the dielectric constant inside the probe's cavity.

Tracing out very fast motional dynamics

The typical time scale of ESR experiments being in the same range as that of molecular rotations and internal dynamics, the effective probe Hamiltonian (eqn (18)) may be further averaged over fast relaxing motional degrees of freedom like modes of vibration and libration.

In order to achieve this, $H_{\text{probe}}^{\text{eff}}$ must be expressed as function of the various motional degrees of freedom (for example, translation, rotation, vibration) of the probe. This require performing a set of transformations (which we simply indicate as Λ) which yields

$$\Lambda(\{\chi_i\}, \{\chi_K\}) = (\mathbf{R}_{\text{cm}}, \mathbf{P}_{\text{cm}}, \{\chi_i\}, \{\eta_v\}, \{\mathbf{I}_K\}) \quad (21)$$

$$\Lambda[H_{\text{probe}}^{\text{eff}}(\{\chi_i\}, \{\chi_K\})] = H_{\text{probe}}^{\text{eff}}(\mathbf{R}_{\text{cm}}, \mathbf{P}_{\text{cm}}, \{\chi_i\}, \{\eta_v\}, \{\mathbf{I}_K\})$$

where $\mathbf{R}_{\text{cm}}, \mathbf{P}_{\text{cm}}$ are the position and momentum of the probe's center of mass; $\{\eta_v\}$ is the set of generalized coordinates which characterizes the collective modes of motion (e.g. vibration, libration, rotation) of the probe's nuclear network, and $\{\mathbf{I}_K\}$ indicate nuclear spins. Implicit in the transformation Λ is the Born–Oppenheimer approximation. We shall not go through the explicit transformations here since they are widely treated in the literature.^{43–46} $H_{\text{probe}}^{\text{eff}}$ now becomes the sum of various operators defining the Hamiltonian of the various degrees of freedom and their couplings. Assuming a significant coupling between (1) the electronic and vibrational modes, and (2) between the roto-translational modes and the spin states, but (3) a very weak coupling between the two sets, then the density matrix of the probe ρ_{probe} may be written as direct product between the density matrix ρ_{Σ} of the vibronic degrees of freedom (denoted as Σ) and ρ_{s+R}

$$\rho_{\text{probe}} \approx \rho_{\Sigma} \otimes \rho_{s+R} \quad (22)$$

where ρ_{s+R} is the density matrix for the roto-translational (indicated collectively as $\mathbf{q}$) and spin degrees of freedom. Accordingly, the equation of motion for ρ_{probe} follows directly from the Liouville–von Neumann equation, viz.

$$i\hbar \frac{\partial \rho_{\text{probe}}}{\partial t} = [H_{\text{probe}}^{\text{eff}}, \rho_{\text{probe}}]. \quad (23)$$

The vibronic transitions being very fast compared to the experimental probing in ESR experiments, the corresponding degrees of freedom may be traced out in eqn (23) and we obtain an effective Liouville–von Neumann equation for ρ_{s+R}

$$i\hbar \frac{\partial \rho_{s+R}}{\partial t} = [H_{s+R}^{\text{eff}}, \rho_{s+R}] - i\hbar \rho_{s+R} \omega_{\Sigma} \quad (24)$$

where,

$$H_{s+R}^{\text{eff}} \equiv \text{Tr}_{\Sigma} [\rho_{\Sigma} H_{\text{probe}}^{\text{eff}}] \quad \omega_{\Sigma} \equiv \text{Tr}_{\Sigma} [\partial_t \rho_{\Sigma}]. \quad (25)$$

where H_{s+R}^{eff} is the effective Hamiltonian one obtains after tracing out vibronic degrees of freedom; on the other hand, ω_{Σ} may be interpreted as the vibronic dissipation factor. In the normal conditions of ESR experiments, the vibronic state of the probe remain, to a fair degree of

approximation, in an equilibrium state. Thus, we may take the limit $\omega_{\Sigma} \rightarrow 0$.

We may take a closer look at H_{s+R}^{eff} before moving on to consider how to simplify and solve eqn (24) using the Stochastic Liouville approach. First of all, according to eqn (18), H_{s+R}^{eff} in eqn (25) can be rewritten as

$$H_{s+R}^{\text{eff}} = H_{s+R,\text{classical}}^{\text{eff}} + H_{s+R,\text{spin}}^{\text{eff}} \quad (26)$$

where,

$$H_{s+R,\text{classical}}^{\text{eff}} \equiv \text{Tr}_{\Sigma} [\rho_{\Sigma} H_{\text{classical}}^{\text{eff}}] \quad H_{s+R,\text{spin}}^{\text{eff}} \equiv \text{Tr}_{\Sigma} [\rho_{\Sigma} H_{\text{spin}}^{\text{eff}}] \quad (27)$$

where $H_{s+R,\text{classical}}^{\text{eff}}$ and $H_{s+R,\text{spin}}^{\text{eff}}$ are the effective classical and spin Hamiltonians after tracing out vibronic degrees of freedom, respectively. In particular, it follows from eqn (20) that $H_{s+R,\text{spin}}^{\text{eff}}$ may be explicitly written as,

$$H_{s+R,\text{spin}}^{\text{eff}} \equiv \sum_i \left[\mu_B \mathbf{s}_i \cdot \bar{\mathbf{g}}_i \cdot \mathbf{B}_0 + \sum_{j>i} \mathbf{s}_i \cdot \bar{\mathbf{D}}_{ij} \cdot \mathbf{s}_j + \sum_K \mathbf{s}_i \cdot \bar{\mathbf{A}}_{iK} \cdot \mathbf{I}_K \right] \quad (28)$$

where the tensors $\bar{\mathbf{g}}$, $\bar{\mathbf{D}}$, $\bar{\mathbf{A}}$, defined as

$$\bar{\mathbf{g}} \equiv \text{Tr}_{\Sigma} [\mathbf{g}' \rho_{\Sigma}] \quad (29a)$$

$$\bar{\mathbf{D}} \equiv \text{Tr}_{\Sigma} [\mathbf{D}' \rho_{\Sigma}] \quad (29b)$$

$$\bar{\mathbf{A}} \equiv \text{Tr}_{\Sigma} [\mathbf{A}' \rho_{\Sigma}] \quad (29c)$$

are the vibronic-averaged g -tensor, zero-field splitting and hyperfine tensors, respectively. These tensors are formally in function of the roto-translational degrees of freedom ($\mathbf{q}$). Thus, $H_{s+R,\text{spin}}^{\text{eff}}$ now depends solely on the spin operators $\{\mathbf{s}_i, \mathbf{I}_K\}$ and $\mathbf{q}$, whilst $H_{s+R,\text{classical}}^{\text{eff}}$ depends on just $\mathbf{q}$.

3.1.1 Stochastic Liouville approach. The roto-translational and the slow internal degrees of freedom of the probe – which we collectively indicate as $\mathbf{q}$ – are subject to fluctuations due to interaction with the solvent. As explained earlier, the stochastic Liouville approach to the computation of ESR spectra is one which explicitly takes into consideration the randomness characterizing $\mathbf{q}$. We show here how this randomness can be introduced into the equation of motion for ρ_{s+R} (eqn (24)) to obtain the stochastic Liouville equation (SLE). There have been many derivations and discussions on the SLE.⁴⁷ The heuristic derivation given here is simply aimed at making the reader unfamiliar with this formalism grasp some of its significant features. For a more precise derivation we invite the reader to see.^{47,48}

We begin by considering the components of $\mathbf{q}$ as stochastic variables. Say $\rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t)$ the density matrix of probe at the instant t given a

specific set of values $\mathbf{q}_0$ for the stochastic variables $\mathbf{q}$, then it is easy to see that

$$\rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t) = \text{Tr}_R[\delta(\mathbf{q} - \mathbf{q}_0)\rho_{s+R}(\mathbf{q}, \boldsymbol{\sigma}, t)]. \quad (30)$$

where $\boldsymbol{\sigma} \equiv \{\mathbf{s}_i, \mathbf{I}_K\}$. In other words, $\rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t)$ determines an effective spin density matrix for the probe assuming that the random variables are of value $\mathbf{q}_0$ at the instant t . To find a master equation for $\rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t)$, we take the partial derivative of eqn (30) with respect to t , which we may write as

$$\frac{\partial}{\partial t}\rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t) = \left[\frac{\partial}{\partial t}\rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t) \right]_{\mathbf{q}_0} + \left[\frac{\partial}{\partial t}\rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t) \right]_{\boldsymbol{\sigma}}, \quad (31)$$

where the first term on the right is the time evolution of ρ_s when $\mathbf{q}_0$ is left unchanged, whilst the second term is the converse. Thus, the latter represents the time evolution of the spin density matrix and, as such, must have the canonical Liouville–von Neumann form

$$\left[\frac{\partial}{\partial t}\rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t) \right]_{\mathbf{q}_0} = \frac{1}{i\hbar} [H_{\text{spin}}^{\text{eff}}(\mathbf{q}_0, \boldsymbol{\sigma}, t), \rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t)] \quad (32)$$

where $H_{\text{spin}}^{\text{eff}}$ is the effective spin Hamiltonian which depends parametrically on $\mathbf{q}_0$. From eqn (28), it follows that

$$H_{\text{spin}}^{\text{eff}}(\mathbf{q}_0, \boldsymbol{\sigma}, t) \equiv \sum_i \left[\mu_B \mathbf{s}_i \cdot \mathbf{g}_i(\mathbf{q}_0) \cdot \mathbf{B}_0 + \sum_{j>i} \mathbf{s}_i \cdot \mathbf{D}_{ij}(\mathbf{q}_0) \cdot \mathbf{s}_j + \sum_K \mathbf{s}_i \cdot \mathbf{A}_{iK}(\mathbf{q}_0) \cdot \mathbf{I}_K \right] \quad (33)$$

where $\boldsymbol{\sigma} \equiv \{\mathbf{s}_i, \mathbf{I}_K\}$, and where the magnetic tensors $\mathbf{g}$, $\mathbf{D}$, $\mathbf{A}$ now depend only on the stochastic variables $\mathbf{q}_0$.

On the other hand, the second term in eqn (31) is basically the time evolution of the probability density of $\mathbf{q}_0$ with the spin states held constant. As such, we may write,

$$\begin{aligned} \left[\frac{\partial}{\partial t}\rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t) \right]_{\boldsymbol{\sigma}} &= -\nabla_{\mathbf{q}_0} \cdot \dot{\mathbf{q}}_0 \rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t) \\ &= -\Gamma \rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t) \end{aligned} \quad (34)$$

where $\Gamma \equiv \nabla_{\mathbf{q}_0} \cdot \dot{\mathbf{q}}_0$. The stochastic nature of $\mathbf{q}_0$ is introduced at this level through the vector $\dot{\mathbf{q}}_0$. The operator Γ is called the stochastic operator and it operates only on $\mathbf{q}_0$ space.

Making use of eqn (31)–(34), we get

$$\frac{\partial}{\partial t}\rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t) = \frac{1}{i\hbar} [H_{\text{spin}}^{\text{eff}}(\mathbf{q}_0, \boldsymbol{\sigma}, t), \rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t)] - \Gamma \rho_s(\mathbf{q}_0, \boldsymbol{\sigma}, t). \quad (35)$$

The form of the stochastic operator Γ depends on a number of factors such as the nature of the variables $\mathbf{q}$ one finds convenient and/or

appropriate to consider and the assumptions made on the stochastic process (usually assumed to be Markovian). Furthermore, in the scope of rendering the model more realistic, Γ can be defined so as to reach thermal equilibrium at finite temperature. We shall elaborate on these points in depth in Section 4 but for now we consider the simple case in which we assume a very weak correlation between spin states and the degrees of freedom $\mathbf{q}$. Then,

$$\rho_{s+R}(\mathbf{q}, \sigma, t) \approx \rho_R(\mathbf{q}, t) \otimes \rho_s(\sigma, t) \quad (36)$$

where $\rho_R(\mathbf{q}, t)$ and $\rho_s(\sigma, t)$ are the density matrices for the $\mathbf{q}$ and spin degrees of freedom, respectively. Then, from eqn (30) it follows that

$$\rho_s(\mathbf{q}_0, \sigma, t) = p(\mathbf{q}_0, t) \rho_s(\sigma, t). \quad (37)$$

where $p(\mathbf{q}_0, t)$, the probability function

$$p(\mathbf{q}_0, t) \equiv \text{Tr}_R[\delta(\mathbf{q} - \mathbf{q}_0) \rho_R(\mathbf{q}, t)] \quad (38)$$

gives the probability that $\mathbf{q} = \mathbf{q}_0$ at t . It then follows from eqn (34) that

$$\frac{\partial}{\partial t} p(\mathbf{q}_0, t) = -\Gamma p(\mathbf{q}_0, t) \quad (39)$$

since the operator Γ operates only on the $\mathbf{q}$ variables.

3.2 QM evaluation of interaction tensors and solvent effects

The evaluation of magnetic tensors by quantum mechanical methods possibly including vibrational averaging and environmental effects has been reviewed several times in recent years.^{49,50} As a consequence we provide in the following just a short summary of the most relevant aspects making explicit reference only to organic free radicals involving only one unpaired electron and negligible relativistic effects.

g-Tensor

One obtains the following expressions for the four contributions:

$$g_{kl} = g_c \delta_{kl} + \Delta g_{kl}^{\text{RMC}} \delta_{kl} + \Delta g_{kl}^{\text{GC}} + \Delta g_{kl}^{\text{OZ/SOC}} \quad (40)$$

$$\Delta g_{kl}^{\text{RMC}} = -\frac{\alpha^2}{S} \sum_{\mu, \nu} P_{\mu\nu}^{\alpha-\beta} \langle \varphi_\mu | \hat{T} | \varphi_\nu \rangle \quad (41)$$

$$\Delta g_{kl}^{\text{GC}} = \frac{1}{2S} \sum_{\mu, \nu} P_{\mu\nu}^{\alpha-\beta} \left\langle \varphi_\mu \left| \sum_A \zeta(r_A) (\mathbf{r}_A \mathbf{r} - \mathbf{r}_{A,k} \mathbf{r}_l) \right| \varphi_\nu \right\rangle \quad (42)$$

$$\Delta g_{kl}^{\text{(OZ/SOC)}} = \frac{1}{2S} \sum_{\mu, \nu} \frac{\partial P_{\mu\nu}^{\alpha-\beta}}{\partial B_k} \langle \varphi_\mu | \hat{z}_l^{\text{SOMF}} | \varphi_\nu \rangle \quad (43)$$

Here $\hat{T}$ is the kinetic energy operator. It is noted that the g -tensor expressions make reference to the global origin of the coordinate system. This would seem to imply the unphysical and unfortunate situation that the results of the computations depend on the choice of origin except at the basis set limit. The way around this artefact is to employ magnetic field dependent basis functions ("gauge including atomic orbitals", GIAO's).

Hyperfine coupling

One finds for the three parts of the hyperfine coupling the following expressions:

$$A_{kl}^{(A;c)} = \delta_{kl} \frac{8\pi P_A}{3} \frac{1}{2S} \rho^{\alpha-\beta}(\mathbf{R}_A) \quad (44)$$

$$A_{kl}^{(A;d)} = \delta_{kl} \frac{P_A}{2S} \sum_{\mu,\nu} P_{\mu\nu}^{\alpha-\beta} \langle \varphi_\kappa | r_A^{-5} (r_A^2 \delta_{\mu\nu} - 3r_{A;\mu} r_{A;\nu}) | \varphi_\tau \rangle \quad (45)$$

$$A_{kl}^{(A;SO)} = -\frac{P_A}{S} \sum_{\mu,\nu} \frac{\partial P_{\mu\nu}^{\alpha-\beta}}{\partial \hat{I}_k^{(A)}} \langle \varphi_\mu | \hat{z}_l^{\text{SOMF}} | \varphi_\nu \rangle \quad (46)$$

with $P_A = g_e g_N \beta_e \beta_N$. Thus, the first two terms are straightforward expectation values while the spin-orbit (SOC) contribution is a response property. In this case, one has to solve a set of coupled-perturbed equations with the nucleus-orbit interaction taken as the perturbing operator. However, for the light atoms considered in the present contribution the SOC term is negligible and it will not be considered in the following.

Zero-field splitting

The Zero-field splitting is the least well developed Spin Hamiltonian (SH) parameter in ESR spectroscopy. It is also the most complicated one since the spin-spin contribution is a genuine two electron property. For this contribution, it can be shown that

$$D_{kl}^{\text{SS}} = \frac{g_e}{16S(2S+1)} \frac{\alpha^2}{\sum_{\mu\nu} \sum_{\kappa\tau}} (P_{\mu\nu}^{\alpha-\beta} P_{\kappa\tau}^{\alpha-\beta} + P_{\mu\kappa}^{\alpha-\beta} P_{\nu\tau}^{\alpha-\beta}) \langle \mu\nu | r_{12}^{-5} (3r_{12,k} r_{12,l} - \delta_{kl} r_{12}^2) | \kappa\tau \rangle \quad (47)$$

The introduction of methods rooted into the Density Functional Theory (DFT) represents a turning point for the calculations of spin-dependent properties.⁴⁹⁻⁵⁴ Before DFT, QM calculations of magnetic tensors were either prohibitively expensive already for medium size radicals⁵⁵⁻⁵⁸ or not sufficiently reliable for predictive and interpretative purposes. Today, last generation functionals coupled to purposely tailored basis sets allow to compute magnetic tensors in remarkable agreement with their experimental counterparts.^{49,50,59,60} On the basis of recent studies, the PBE0 or B3LYP global hybrid functionals in conjunction with the EPR-II or SNSD basis sets represent remarkable compromises between reliability and computer time.^{49,50,61,62}

A good geometry is a mandatory prerequisite to evaluate in the right way different effects, like the direct and spin polarization contributions to the hyperfine coupling constant, for example. Next, in the framework of the Born–Oppenheimer approximation, we can speak of a potential energy surface (PES) and of a “property surface” (PS), which can be obtained from quantum mechanical computations at different nuclear configurations. In this scheme, expectation values of observables are obtained by averaging the different properties on the nuclear wave functions. Semirigid molecules are quite well described in terms of a harmonic model, but a second-order perturbative inclusion of principal anharmonicities provides much improved results at a reasonable cost.^{63–65} As a first approximation, the vibrationally averaged value of a property can be written:

$$\langle \Omega \rangle = \Omega_{\text{ref}} + \left(\frac{\partial \Omega}{\partial s} \right)_{\text{ref}} \langle s \rangle + \frac{1}{2} \left(\frac{\partial^2 \Omega}{\partial s^2} \right)_{\text{ref}} \langle s^2 \rangle \quad (48)$$

Even when the linear term is absent for symmetry reasons and the key role is played by mean square amplitudes, which, however, can be quite large: thus quantitative (and even semiquantitative) agreement with experiment cannot be obtained by static models, irrespective of the quality of the electronic model. Large amplitude motions cannot be described by the perturbative approach sketched above, but a classical treatment is usually sufficient. Then, the computational strategy involves two independent steps: first, MD simulations are run for sampling with one or more trajectories the general features of the configuration space; then, observables are computed as averages over a suitable number of snapshots. The *a posteriori* calculation of spectroscopic properties, compared to other on-the-fly approaches, allows us to exploit different electronic structure methods for the MD simulations and the calculation of physical–chemical properties. In this way, a more accurate treatment for the more demanding molecular parameters, of both first (*e.g.* hyperfine coupling constants) and second (*e.g.* electronic *g*-tensor shifts) order, could be achieved independently of structural sampling methods: first-principles, semi-empirical force fields, as well as combined quantum-mechanics/molecular-mechanics approaches could be all exploited to the same extent, once the accuracy in reproducing reliable structures and statistics is proven.

The accuracy with which the magnetic tensors are now calculated with Gaussian09⁶⁶ package is sufficient to use them directly in the SASS approach, probably with some minor corrections. As a matter of fact, the Mean Absolute Deviation (MAD),

$$\text{MAD} = \frac{\sum_{i=1}^N [\mu_{\text{iso,cal}}(i) - \mu_{\text{iso,exp}}]}{N}, \quad \mu = (g, A) \quad (49)$$

of the isotropic magnetic tensors $g_{\text{iso}} (=1/3 \text{ Tr}(\mathbf{g}))$ and $A_{\text{iso}} (=1/3 \text{ Tr}(\mathbf{A}))$ was calculated for elements of the second and third rows in a wide range of molecular species using Gaussian03.⁶⁷ The results – obtained using different basis sets for Zeeman and hyperfine interaction tensors reported

Table 1 Data analysis for isotropic g_{iso} of 92 molecular species containing second and third row atoms. MAD is expressed in Gauss.

	exp	B3LYP/N07D	PBE0/N07D
92 Molecules			
MAD		0.0006	0.0006
Max absolute error		0.0048	0.0050
Slope		0.9704	0.9317
Intercept		0.0595	0.1369
R2		0.8525	0.8159
Min	2.0000	1.9994	1.9995
Max	2.0130	2.0121	2.0120

in Table 1 and Fig. 3, respectively for the said tensors – demonstrate how quite accurate these calculations are.

In general terms, different strategies have been proposed to model environmental effects on energetics and spectroscopic properties of molecular systems. One common approach relies on the same MD simulations followed by QM evaluations of average values over selected snapshots employed for large amplitude motions of isolated molecules. However, proper treatment of boundaries can play a significant role in determining the reliability of the results. Although periodic boundary conditions (PBC) are usually employed in this connection, non-periodic boundary conditions (NPBC) show a number of significant advantages and are a more natural choice for the localized basis functions (normally Gaussians) used in computational chemistry approaches. On these grounds, a general multi-layer approach can be introduced for describing localized phenomena, in which the central part of the system is described by a QM approach, intermediate region by an atomistic description, and the boundary in terms of an effective “steric” potential together with the reaction field introduced by the (dielectric or conductor) continuum describing bulk solvent. Such a scheme was proposed some years ago with encouraging results.^{68–70} With the recent implementation of a QM/MM polarizable embedding scheme based on the fluctuating charge (FQ) approach, a new feature can be included in such a model, since the solvent molecules in the nearby of the solute can be polarized by the QM charge density.^{71–74}

Complete neglect of the atomistic part leads naturally to the last-generation continuum models, in which the solute is embedded in a molecularly-shaped cavity (with proper boundary conditions), which follows the motion of the solute. This approach allows a significant decrease in the computational cost, leading in state-of-the-art implementations to negligible overheads with respect to a corresponding computation *in vacuo*. Implicit models are particularly suitable for the treatment of bulk effects, where the effect of solvent molecules is included in an averaged way by the definition of a cavity. Among the implicit solvent models, the Polarizable Continuum Model (PCM) in all its different variants (“Conductor-like” C-PCM, “Dielectric” D-PCM, and “Integral Equation Formalism” IEF-PCM)^{15,16,75} is one of the most reliable methods,

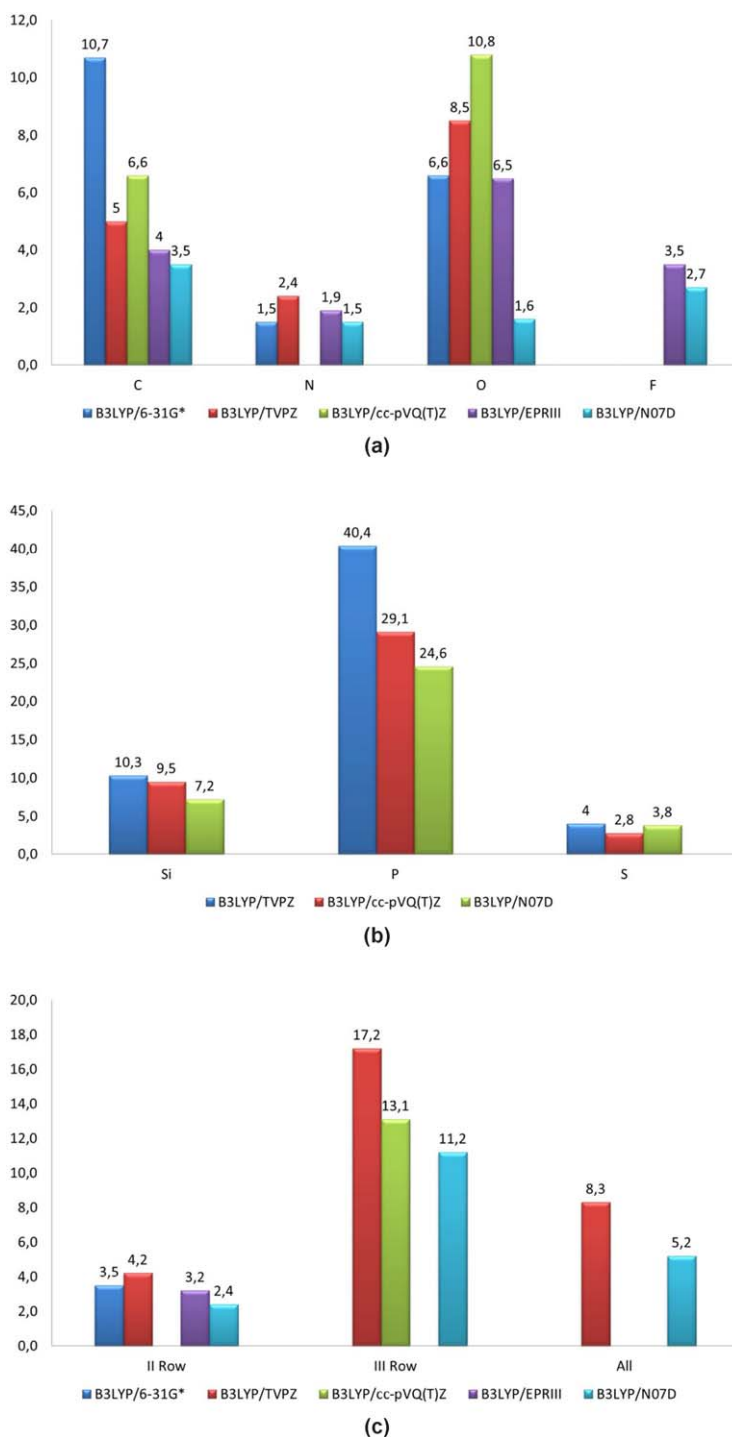


Fig. 3 MADs of isotropic hyperfine coupling for 199 molecular species containing nuclei of second and third row: (a) C, N, O, F nuclei; (b) Si, P, S nuclei; (c) others molecules containing II and III row atoms, and other elements. MADs are expressed in Gauss.

and it has been successfully applied for the simulation of many different properties of molecules in condensed phase.

4 Stochastic diffusion operator, relaxation

Having discussed the spin Hamiltonian in Section 3, we now turn our attention to the other main ingredient that enters into the SLE, namely the stochastic diffusion operator Γ . This operator describes the diffusive motion of some degrees of freedom and it has the general form of the so-called Fokker–Planck (FP) or Smoluchowski operator.^{76–78} The use of stochastic operators approach constitutes a reasonable compromise between the necessity to gather principal aspects of molecular dynamics and to extract significant dynamics parameters, and the demand to have at hand a relatively simple methodology and of practical implementation. In the SLE context, it models the dependence of the reduced density matrix ρ_s (from now on, simply ρ) on the relaxation process induced by the often slow stochastic variables $\mathbf{q}_0$ (henceforth $\mathbf{q}$). The molecular motions, which are described by $\mathbf{q}$, are assumed to be Markovian and diffusive. The Markovian assumption made is the same as admitting that for every molecule the probability $p(\mathbf{q}, t_i)$ to have a certain configuration $\mathbf{q}_i$ at time t_i depends exclusively on the configuration $\mathbf{q}_{i-1}$ at time t_{i-1} ; in other words, the motional dynamics of the system is considered to be memory-less. However, the diffusive assumption made implies the molecular motions are in a high friction limit (also known as the overdamped regime), which is the most common situation encountered experimentally when in the presence of an isotropic liquid phase, for example. These assumptions made on the dynamics are simply equivalent to assuming the stochastic variables $\mathbf{q}$ to be Markovian and diffusive. If we further assume a very weak correlation between the spin σ and spatial degrees of freedom $\mathbf{q}$ such that eqn (36) holds, then, disregarding the inertial effects of motion description, eqn (39) also holds, which we rewrite below for the sake of convenience

$$\frac{\partial}{\partial t} p(\mathbf{q}, t) = -\Gamma p(\mathbf{q}, t). \quad (50)$$

Eqn (50) is termed the Smoluchowski equation for a generic stochastic process $\mathbf{q}$. The form of the stochastic operator Γ is defined according to the physical interpretation of the system under examination. To make the model more realistic, Γ is defined in such way that the probability density $p(\mathbf{q}, t)$ is evolved to a stationary equilibrium state at some finite absolute temperature T . The probability distribution $p_{\text{eq}}(\mathbf{q})$ corresponding to this stationary state is assumed to be a Boltzmann distribution,

$$p_{\text{eq}}(\mathbf{q}) = \frac{e^{-V(\mathbf{q})/k_B T}}{\langle e^{-V(\mathbf{q})/k_B T} \rangle} \quad (51)$$

where V is the potential to which the stochastic coordinates are exposed, and k_B is the Boltzmann constant. With $\langle \dots \rangle$ the integral over the stochastic coordinates $\mathbf{q}$ in $\mathbf{q}$ -space is meant.

In the course of years of research in this field, many procedures have been proposed for a proper identification and selection of which variables to treat as slow stochastic variables. One of these is based on “time coarse-graining” procedures. In the overdamped regime the graining is simple: the “solvent” coordinates are the fast ones, while the three Euler angles Ω that give the orientation of the molecule with respect to the laboratory inertial frame are the slow ones.

However, in the presence of internal degrees of freedom, time-coarse graining methods can become in most cases unmanageable. Even more, the definition of relevant dynamics with these methods in the presence of complex macromolecules is still an open problem. The strategy usually adopted by many in this case is to consider the surrounding solvent molecules as constituting a cage with a slow relaxation rotational dynamics coupled to the rotational motion of the probe. This is true, for example, in ordered media and/or glasses where it is necessary to preserve some information about the dynamics of the solvent.⁷⁹ Sometimes, the motion of the system is conveniently approximated as a two-body diffusion motion coupled through a potential. The “Slowly Relaxing Local Structure” (SRLS) model is an example of a model based on this approximation and it has been successfully used for the interpretation of magnetic resonance measures (both NMR and ESR) of more complex systems as liquid crystals,^{27,79,80} glasses and others.^{81–85} SRLS is a more powerful theoretical tool since it takes rigorously into account the global and internal motion coupling of the system under study.

It is often possible to resort to chemical intuition in order to guess relevant coordinates. At present there are no satisfactory methodologies to clearly detect relevant coordinates in large molecules. In Section 6.2 we shall discuss the identification and usage of a generalized coordinate of the system, which in this context can be interpreted as the slow stochastic variable.

In the next subsections we shall discuss in detail the form of the diffusive operator for two important diffusive models, namely the one-body and the two-body (SRLS) models. These models have been applied successfully in the past to systems such as liquid crystals, glasses and biological macromolecules. We make the assumption that the potential has two separated contributions: an “external” term acting on the global orientation (*e.g.* ordering effect in liquid crystals) and an internal term acting on the torsional angle, if present. Thus,

$$V(\mathbf{q}) = V_{\text{ext}}(\Omega) + V_{\text{int}}(\theta) + V_{\text{coupling}}(\mathbf{q}) \approx V_{\text{ext}}(\Omega) + V_{\text{int}}(\theta). \quad (52)$$

Once the form of the stochastic operator has been decided based on the relevant physics of the system, the full diffusion tensor of the system enters as a new parameter in the SLE and proper evaluation of diffusive properties is required.

4.1 One-body model

The one-body model considers the molecule as a single rigid body that diffuse in a medium. This model is suitable for small molecules or

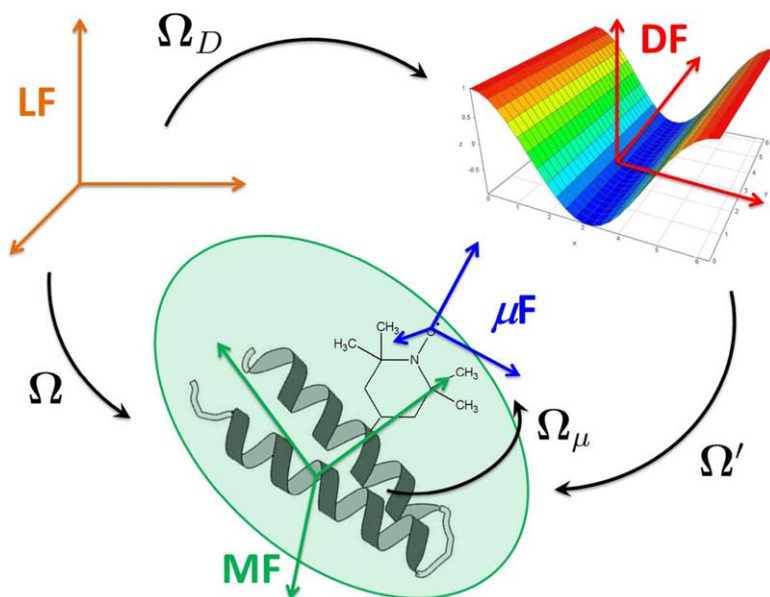


Fig. 4 Scheme of the reference frames involved in the definition of the one-body model. See text for the symbols meaning.

extremely rigid structures, and it is relatively simple. Though the internal and global motions of molecules can occur at different characteristic times, this model somehow neglects the former by just keeping track of the global reorientation. Before going on to describe the model, a clear understanding of the various reference frames involved and how they are related to each other is highly imperative. These are sketched in Fig. 4, where LF refers to the inertial laboratory frame where the spin and magnetic field operators are defined. The set of Euler angles Ω_D transform LF to the potential frame, DF. The latter, which is also an inertial frame, is that frame in which the orienting potential V is defined. With Ω' we denote the set of Euler angles that take the frame DF to the molecular frame, MF, which is conventionally chosen to be aligned with the principal axis of the diffusion tensor (*i.e.* MF is that reference frame in which the diffusion tensor has a diagonal form). Ω is the stochastic variable of the diffusive part of the model and it is composed of three Euler angles which take LF to MF; the relation $\Omega' = \Omega - \Omega_D$ holds. We still need two more reference frames (collectively labeled as μF , $\mu \in \{g, A\}$), one for each magnetic (Zeeman, hyperfine) interaction tensor. In particular, gF indicates the frame in which the Zeeman tensor for the electron has a diagonal form, while with AF we indicate the analogous for the hyperfine interaction tensor. The μF s are fixed on the molecule and the set of Euler angles that transform MF to μF are indicated as Ω_μ .

In the one-body model, the molecule is considered as a single rigid body that diffuse in a medium according to a purely rotational motion without inertial effects. In the overdamped regime, this stochastic

process is described by just the set of Euler angles $\mathbf{\Omega}$ that take LF to MF, *i.e.* we are assuming $\mathbf{q} \equiv \mathbf{\Omega}$. Following eqn (50), we have that the Smoluschowski equation for the body's orientation is

$$\frac{\partial}{\partial t} p(\mathbf{\Omega}, t) = -\Gamma p(\mathbf{\Omega}, t). \quad (53)$$

Bearing in mind that the motion is rotationally diffusive – thus the only spatial coordinates are the rotational coordinates $\mathbf{\Omega}$ – the diffusive operator Γ can be obtained starting from the generalized Langevin equation of the system. With these considerations, we can write

$$\Gamma = \mathbf{J}^\dagger \mathbf{D} p_{\text{eq}} \mathbf{J} p_{\text{eq}}^{-1} \quad (54)$$

where $\mathbf{J}$ is the angular momentum operator that acts on $\mathbf{\Omega}$, $\mathbf{D}$ is the diffusion tensor.

For what concerns symmetry, it is common place to assume a cylindrical symmetry – upon which the diffusion tensor defined in MF becomes characterized by the two principal values $D_\perp (= D_{xx} = D_{yy})$ and $D_\parallel (= D_{zz})$.

Given that $\mathbf{q} \equiv \mathbf{\Omega}$, it follows straightforwardly from eqn (52) that $V(\mathbf{q}) \equiv V(\mathbf{\Omega}) = V_{\text{ext}}(\mathbf{\Omega})$. As a result, from eqn (52) the equilibrium probability distribution simply becomes

$$p_{\text{eq}}(\mathbf{\Omega}) = \frac{e^{-V(\mathbf{\Omega})/k_B T}}{\langle e^{-V(\mathbf{\Omega})/k_B T} \rangle}. \quad (55)$$

It is readily inferred from eqn (55) that the equilibrium distribution p_{eq} reduces to a constant ($p_{\text{eq}} = 1/8\pi^2$) in the absence of an external orienting potential $V(\mathbf{q})$. We may emphasize here that in general the origin of the external potential depends greatly on the system under study and may not be necessarily due to the interaction of the system with an external field in the conventional sense. In the case of probes linked to proteins for example, the orienting potential can be thought of as that which originates from the structural constrictions imposed by the protein itself towards the binding site magnetic probe is located. The choice of orienting potential that acts on the body is very crucial since, in combination with the diffusion tensor, it is highly responsible for the spin relaxation. A typical model for the potential that has proved quite successful in the one-body dynamics is the axial potential model, in which one assumes that the body's potential depends exclusively on the orientation (quantified by the angle β' , since in this case $\alpha' = \gamma' = 0$ in $\mathbf{\Omega}' = (\alpha', \beta', \gamma')$) of MF's principal axis with respect to that of DF, where the potential is defined and diagonal. The potential can be expressed in terms of Wigner matrices $\mathcal{D}_{00}^n(\mathbf{\Omega}')$; in the reference frame DF we have

$$V(\mathbf{\Omega}') = - \sum_{n=1}^N \epsilon_n \mathcal{D}_{00}^n(\mathbf{\Omega}') \quad (56)$$

where ϵ_n are constants. Making use of the properties of Wigner matrices^{86,87} V in eqn (56) can be rewritten explicitly in function of the stochastic coordinate Ω :

$$V(\Omega) = - \sum_{n=1}^N \epsilon_n \sum_{m=-n}^n \mathcal{D}_{0m}^n(\Omega_D) \mathcal{D}_{m0}^n(\Omega). \quad (57)$$

Given that Ω_D is the orientation between the two inertial frames LF and DF, it is evident from eqn (57) that $V(\Omega)$ depends parametrically on the former. Thus, “powder spectra”, for instance, can be calculated through a parametric variation of Ω_D .

As we discussed in Section 3.1, in the SLE (see eqn (35)), the effective spin Hamiltonian $H_{\text{spin}}^{\text{eff}}$ depends parametrically on the stochastic variable, which in the present case is Ω . If we were to write the spin Hamiltonian for an unpaired electron interacting with diverse nuclei assuming a one-body model (together with all the assumptions made above) for the orientation of the paramagnetic probe, then eqn (33) reduces to

$$H(\Omega, \sigma) = \mu_B \mathbf{s} \cdot \mathbf{g}(\Omega) \cdot \mathbf{B}_0 + \sum_K \mathbf{s} \cdot \mathbf{A}_K(\Omega) \cdot \mathbf{I}_K \quad (58)$$

where we have simply written $H_{\text{spin}}^{\text{eff}}$ as H (this notation will be used in the following). The spin Hamiltonian H in eqn (58) is expressed in Cartesian basis. Transforming it into spherical basis, and making use of spherical irreducible tensors, releases us of those asymmetric components deeply buried in the Cartesian representation. If this transformation is carried out, followed by a transition from the Hilbert space to the Liouville space, we obtain¹⁸

$$H^\times = \sum_{\mu=g,A} \sum_{l=0,2} \sum_{m,m'=-l}^l \mathcal{D}_{mm'}^l(\Omega) F_{\mu,MF}^{(l,m')*} A_{\mu,LF}^{(l,m)\times} \quad (59)$$

where $H^\times$ is the superhamiltonian. Employing once again the properties of the Wigner matrices, the components $F_{\mu,MF}^{(l,m)}$ may be further expressed in their respective magnetic tensor frames μF , and we obtain

$$F_{\mu,MF}^{(l,m)*} = \sum_{m'=-l}^l \mathcal{D}_{mm'}^l(\Omega_\mu) F_{\mu,\mu F}^{(l,m')*}. \quad (60)$$

Since we are now in the Liouville space, the eigenvalue problem to solve is

$$\frac{\partial |\rho\rangle\rangle}{\partial t} = -\mathcal{L} |\rho\rangle\rangle \quad (61)$$

where $\mathcal{L}$, the stochastic Liouville operator, is defined as

$$\mathcal{L} = \mathcal{L}_\sigma + \mathcal{L}_q, \quad (62)$$

where $\mathcal{L}_\sigma \equiv \frac{i}{\hbar} H^\times$, $\mathcal{L}_\mathbf{q} \equiv \Gamma$, and $|\rho\rangle\rangle$ is the vector representation of the density matrix ρ in Liouville space. The first term in eqn (62) is the spin part of the stochastic Liouville operator and it depends parametrically on the stochastic variables $\mathbf{q}$; the second term $\mathcal{L}_\mathbf{q}$ is the equivalent of the stochastic operator Γ in Liouville space. Now that we have all the elements needed in order to set up and solve the SLE, we are left with a basis set $\{|\Sigma\rangle\rangle\}$ in which to expand $|\rho\rangle\rangle$.

In view of eqn (62), the natural choice is to opt for a basis set which is simply the direct product of a basis set of the two operators $\mathcal{L}_\sigma$ and $\mathcal{L}_\mathbf{q}$. To this end, the normalized Wigner matrices $\{|L M K\rangle\rangle\}$ constitute a good choice for $\mathcal{L}_\mathbf{q}$; specifically,

$$|L M K\rangle\rangle \equiv \sqrt{\frac{[L]}{8\pi^2}} \mathcal{D}_{MK}^L(\boldsymbol{\Omega}) \quad (63)$$

where $\boldsymbol{\Omega}$ is as usual the set of Euler angles that take LF to MF and is stochastic. When computing CW-ESR spectra, a maximum value for L (*i.e.* $L_{\max}$) is specified; this leads to a truncation of the basis $\{|L M K\rangle\rangle\}$. The higher $L_{\max}$, the more precise the calculations related to the diffusion part are. If the diffusive operator Γ , and so $\mathcal{L}_\mathbf{q}$ in Liouville space, describes the evolution of n stochastic variables, the basis set is just built from the direct product of the basis set for each single coordinate.

We can draw on the last remark we have just made to build a basis set for the spin part. As a matter of fact, this basis set can be constructed by taking the direct product of the Liouville space basis set of each spin present (uncoupled representation). In the elementary case a single electron and a nucleus, the spin part basis set $\{|\sigma\rangle\rangle\}$ is constructed as

$$\{|\sigma\rangle\rangle\} = \{|\sigma\rangle\rangle_{\text{el}}\} \otimes \{|\sigma\rangle\rangle_{\text{nu}}\} = \{|\sigma\rangle\rangle_{\text{el}} \otimes |\sigma\rangle\rangle_{\text{nu}}\} \quad (64)$$

where $\{|\sigma\rangle\rangle_{\text{el}}\}$ and $\{|\sigma\rangle\rangle_{\text{nu}}\}$ are the superbasis sets of the electron and nucleus, respectively. But $\{|\sigma\rangle\rangle_{\text{el}}\} \equiv |S m'_s\rangle\langle S m''_s|$, and $\{|\sigma\rangle\rangle_{\text{nu}}\} \equiv |I m'_I\rangle\langle I m''_I|$ and so the basis set $\{|\sigma\rangle\rangle\}$ can be written more explicitly as

$$\{|\sigma\rangle\rangle\} = \{|s m'_s\rangle\langle s m''_s| \otimes |I m'_I\rangle\langle I m''_I|\} = \{|m'_s m''_s, m'_I m''_I\rangle\rangle\} \quad (65)$$

where s and $\{m\}$ are the electron spin and its projection along the quantization axis, respectively. Likewise can be said of I and $\{m_I\}$ for the nucleus. Nevertheless, it has been shown⁸⁸ that it is more convenient to consider the following combination of m' and m'' in order to have a more sparse matrix representation of the Liouvillian $\mathcal{L}$:

$$p = m' - m''$$

$$q = m' + m''.$$

Finally, we can write the basis $\{|\Sigma\rangle\rangle\}$ as

$$\{|\Sigma\rangle\rangle\} = \{|\sigma\rangle\rangle \otimes |L M K\rangle\} \quad (66a)$$

$$= \{|m'_S m''_S, m'_I m''_I\rangle\rangle \otimes |L M K\rangle\} \equiv \{|m'_S m''_S, m'_I m''_I, L M K\rangle\rangle\} \text{ or,} \quad (66b)$$

$$= \{|p^S q^S, p^I q^I, L M K\rangle\rangle\} \quad (66c)$$

While eqn (66a) is generally valid, eqn (66b)–(66c) only apply to the case of two spins. From eqn (66b)–(66c) we observe that the stochastic Liouville operator $\mathcal{L}$ in the case of two spins is a tensor with seven indexes, and this is a forewarning of how large the total dimension of the basis set $\{|\Sigma\rangle\rangle\}$ can grow. To emphasize on this very important point, we note that if we assume for example $L = 2$, $I = 1$ and $s = 1/2$ we end up with a basis set $\{|\Sigma\rangle\rangle\}$ of dimension 1260 – a concrete reminder of how rapidly large $\dim\{|\Sigma\rangle\rangle\}$ can become as the number of spins and parameters like L are increased. In fact, this very unwanted characteristic of the SLE approach has thwarted the application of this approach to more complicated scenarios whereby one has, for example, an unpaired electron interacting with a significant number of nuclei. Symmetry considerations may be introduced so as to make computations more manageable but these usually do not amount to a substantial improvement, computationally speaking. We shall take a closer look at this problem in Section 6.1 when we discuss about multi-spin ESR. There, we shall also discuss very recent approaches in condensed matter physics and quantum information theory which we think may possess the right solutions to this problem.

In conclusion, once the basis set $\{|\Sigma\rangle\rangle\}$ is defined, the matrix elements $\langle\langle\Sigma_1|\mathcal{L}|\Sigma_2\rangle\rangle$ can be calculated (see ref. 18, 88 for some of these). In the same references, the choice of the starting vector $|v\rangle\rangle$ and its projection on the base $\{|\Sigma\rangle\rangle\}$, $\langle\langle\Sigma|v\rangle\rangle$, are discussed.

4.2 Two-body model

Restricting motional dynamics to only global ones as it is the case with the one-body model may not give reliable results, especially in the case of macromolecules whereby diverse domains mostly happen to have characteristic motional properties. A more reliable alternative to the one-body model is the so-called two-body model, also referred to as the slowly relaxing local structure (SRLS) model,^{80,89–92} in which the motion of the system is likened to that of two rigid bodies coupled to each through an interacting potential and undergoing rotational diffusion, all in the presence of a medium. With this model, both the global and relevant internal motions can be explicitly taken care of. The two bodies here are the macromolecule and the magnetic probe, bonded at some site but free to reorient relative to each other. This model allows for even more generalizations which can be applicable to many diverse situations.

As we did for the one-body model, we begin by distinguishing all the reference frames useful in the model description. In Fig. 5 we illustrate

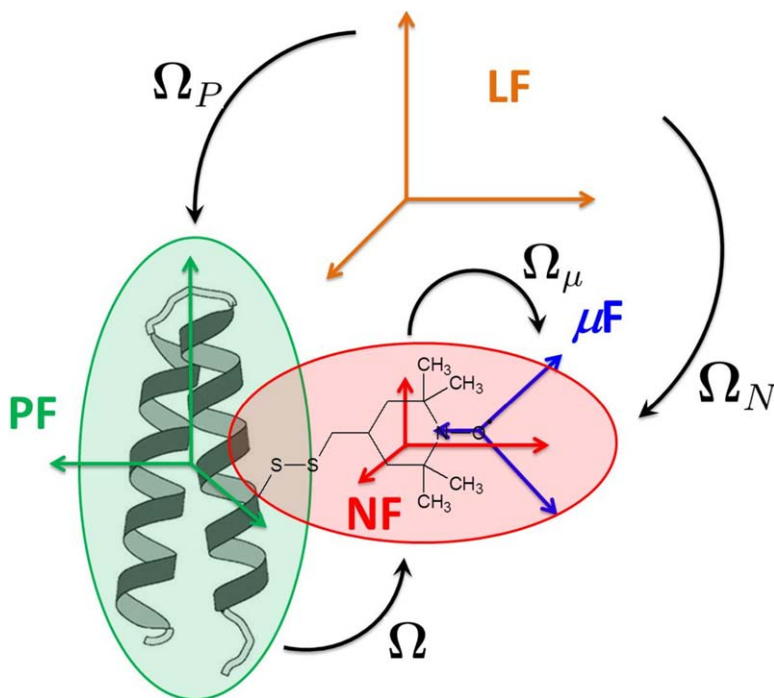


Fig. 5 Scheme of the reference frames involved in the definition of the two-body model. See text for the symbols meaning.

these reference frames. Again, it is convenient to start by defining an inertial LF, then a reference frame NF on the magnetic probe (which in Fig. 5 is represented by the nitroxide group) and another one on the protein, PF. The NF and PF frames are such that the relative diffusion tensors are diagonal with respect to them. Finally, the reference frames – Zeeman (gF) and hyperfine interaction (AF) – for the magnetic tensors are defined; both magnetic frames are fixed on the nitroxide and their respective tensors have a diagonal form with respect to them. The Euler angles involved in the transformation between the frames are also reported in Fig. 5.

The interaction potential V may be chosen to be of rhombic symmetry with principal component along the $\hat{z}$ -axis of PF. The analytical expression for the potential, in function of the angle between NF and PF, thus happens to be,

$$V(\Omega) = -\epsilon_0 \mathcal{D}_{00}^2(\Omega) - \epsilon_2 [\mathcal{D}_{02}^2 + \mathcal{D}_{0-2}^2]. \quad (67)$$

This potential is bistable, *i.e.* it has two minima corresponding to the angles $\beta = (0^\circ, 180^\circ)$. In setting up the SLE, we may ask what the stochastic coordinates $\mathbf{q}$ are in this case. Though there are multiple choices one can make in this regard, the natural choice seems to be that of taking $\mathbf{q} \equiv (\Omega_N, \Omega_P)$, where Ω_N, Ω_P are the Euler angles that take LF to NF and LF to PF, respectively. We now need to derive expressions for the diffusive Γ and spin superhamiltonian $H^\times$ operators, which will in turn

be inserted into the SLE (eqn (61)). It can be shown that these operators are given by the expressions^{80,89–92}

$$\Gamma = [{}^N\mathbf{J}(\mathbf{\Omega}_N)^N \mathbf{D}p_{\text{eq}} {}^N\mathbf{J}(\mathbf{\Omega}_N) + {}^P\mathbf{J}^\dagger(\mathbf{\Omega}_P)^P \mathbf{D}p_{\text{eq}} {}^P\mathbf{J}(\mathbf{\Omega}_P)] p_{\text{eq}}^{-1} \quad (68)$$

$$H^\times = \sum_{\mu=g,A} \sum_{l=0,2} \sum_{m,m'=-l}^l \mathcal{D}_{mm'}^l(\mathbf{\Omega}_N) F_{\mu,NF}^{(l,m')*} A_{\mu,LF}^{(l,m)\times}. \quad (69)$$

It is worth observing here that: (1) even though the spin superhamiltonian $H^\times$ does not depend explicitly on the other stochastic variable – namely, $\mathbf{\Omega}_P$ – the influence of the latter is manifested on $H^\times$ through its coupling with $\mathbf{\Omega}_N$ *via* the interaction potential V , (2) the stochastic part $\mathcal{L}_{\mathbf{q}}$ of $\mathcal{L}$ is given by the sum of two operators, each depending on only one of the two stochastic variables:

$$\mathcal{L}_{\mathbf{q}} = \mathcal{L}_{\mathbf{\Omega}_N} + \mathcal{L}_{\mathbf{\Omega}_P} \quad (70)$$

where,

$$\mathcal{L}_{\mathbf{\Omega}_N} \equiv {}^N\mathbf{J}^\dagger(\mathbf{\Omega}_N)^N \mathbf{D}p_{\text{eq}} {}^N\mathbf{J}(\mathbf{\Omega}_N) p_{\text{eq}}^{-1} \quad (71a)$$

$$\mathcal{L}_{\mathbf{\Omega}_P} \equiv {}^P\mathbf{J}^\dagger(\mathbf{\Omega}_P)^P \mathbf{D}p_{\text{eq}} {}^P\mathbf{J}(\mathbf{\Omega}_P) p_{\text{eq}}^{-1} \quad (71b)$$

The physical significance of eqn (70) plays into the notion that we are considering two stochastic processes, which though are not independent of each other given their coupling through the potential V .

As it happens to be, choosing the stochastic variables to be $\mathbf{\Omega}_N, \mathbf{\Omega}_P$ is not that convenient since the expression of the potential V in these variables can be very complicated. Choosing rather $\mathbf{q} \equiv (\mathbf{\Omega}, \mathbf{\Omega}_P)$, where $\mathbf{\Omega}$ is the set of Euler angles that take PF to NF, proves to yield simple expressions for V , and eqn (67) hold in the case of rhombic symmetry.

In light of this choice of stochastic variables, the diffusive operator Γ and the spin superhamiltonian become

$$\Gamma = [{}^N\mathbf{J}^\dagger(\mathbf{\Omega})^N \mathbf{D}p_{\text{eq}} {}^N\mathbf{J}(\mathbf{\Omega}_N) + [{}^P\mathbf{J}(\mathbf{\Omega}) - {}^P\mathbf{J}(\mathbf{\Omega}_P)]^\dagger {}^P \mathbf{D}p_{\text{eq}} [{}^P\mathbf{J}(\mathbf{\Omega}) - {}^P\mathbf{J}(\mathbf{\Omega}_P)]] p_{\text{eq}}^{-1} \quad (72)$$

$$H^\times = \sum_{\mu=g,A} \sum_{l=0,2} \sum_{m,m',m''=-l}^l \mathcal{D}_{mm'}^l(\mathbf{\Omega}_P) \mathcal{D}_{m'm''}^l(\mathbf{\Omega}) F_{\mu,NF}^{(l,m'')*} A_{\mu,LF}^{(l,m)\times} \quad (73)$$

Similar to eqn (70), it is clear from eqn (72) that we can write $\mathcal{L}_{\mathbf{q}} = \mathcal{L}_{\mathbf{\Omega}} + \mathcal{L}_{\mathbf{\Omega}_P}$, where the adding terms can be easily inferred from eqn (72).

In all generality, we see that the stochastic Liouville operator $\mathcal{L}$ in the two-body model is the sum of three operators: $\mathcal{L} = \mathcal{L}_{\sigma} + \mathcal{L}_{\mathbf{\Omega}} + \mathcal{L}_{\mathbf{\Omega}_P}$. The Liouville space basis (or superbasis) set $\{|\Sigma\rangle\rangle\}$ to use here may be chosen as the direct product of basis sets of the three superoperators,

$$\{|\Sigma\rangle\rangle\} = \{|\sigma\rangle\rangle\} \otimes \{|L M K\rangle\} \otimes \{|L^P M^P K^P\rangle\} \quad (74)$$

where as usual, $\{|\sigma\rangle\}$ is a superbasis set for $\mathcal{L}_{\mathbf{q}}$, and $\{|L M K\rangle\}$, $\{|L^P M^P K^P\rangle\}$ the basis sets (normalized Wigner matrices, eqn (63)) for $\mathcal{L}_{\Omega}$ and $\mathcal{L}_{\Omega^P}$, respectively. In the case of a single electron and nucleus, analogous to eqn (66), we have

$$\{|\Sigma\rangle\} = \{|p^S q^S, p^I q^I, L M K, L^P M^P K^P\rangle\} \quad (75)$$

The linear combinations of the K^P and M indexes

$$m^{\pm} = K^P \pm M \quad (76)$$

are shown to bring some simplifications if combined with the system's symmetry.

4.3 Diffusion tensor

The dependence of the stochastic operator Γ on the diffusion tensor $\mathbf{D}$ is writ large both in the case of the one-body (eqn (54)) and two-body models (eqn (72)). In this section, we discuss how it is evaluated. In order to evaluate the diffusion properties of a molecular system we adopt a hydrodynamical model that describes the system as an ensemble of beads, linking the overall molecular shape directly to roto-translational (and) internal friction properties. The model, originally pioneered by Moro,^{93,94} has been computationally implemented in the DiTe code of Barone and coworkers.⁹⁵ Without going into much details, we shall present here the focal ideas behind this hydrodynamical model. In contrast to the original model which deals with only dihedral angles, we show in Section 6.2 our recent extension of the model to a generalized coordinate.²⁴

It is worth remembering here that the coordinates $\mathbf{q}$ are in general the remaining degrees of freedom of the paramagnetic probe after all the fast motional degrees of freedom has been traced out (or coarse-grained), as we saw in subsection 3.1.1 when deriving the effective Hamiltonian to be used in the SLE. The set $\mathbf{q}$ is composed of three types of degrees of freedom: (1) the probe's translation embodied in the position vector $\mathbf{R}_{cm}$, (2) its orientation given by the set of Euler angles Ω , and (3) the remaining internal coordinates $\mathbf{x}$ after averaging out the fast ones. That is, $\mathbf{q} = [\mathbf{R}_{cm}, \Omega, \mathbf{x}]$. The velocity of these coordinates may be indicated as $\mathcal{V} = [\mathbf{v}, \omega, \dot{\mathbf{x}}]$, where $\dot{\mathbf{q}} \equiv \mathcal{V}$, $\dot{\mathbf{R}}_{cm} \equiv \mathbf{v}$, $\dot{\Omega} \equiv \omega$. In the same manner, we can define a force vector $\mathcal{F} = [\mathbf{f}, \boldsymbol{\tau}, \boldsymbol{\tau}_x]$, whose components are the translational force, the torque and the internal forces. The vectors $\mathcal{F}$ and $\mathcal{V}$ are related through the following equation,

$$\begin{pmatrix} \mathbf{f} \\ \boldsymbol{\tau} \\ \boldsymbol{\tau}_x \end{pmatrix} = -\xi \begin{pmatrix} \mathbf{v} \\ \omega \\ \dot{\mathbf{x}} \end{pmatrix} \quad (77)$$

where the proportionality matrix ξ is termed as the constrained friction matrix and it is in turn related to the diffusion tensor through Einstein's relation

$$\mathbf{D} = k_B T \xi^{-1} = \begin{pmatrix} \mathbf{D}_{TT} & \mathbf{D}_{TR} & \mathbf{D}_{TI} \\ \mathbf{D}_{RT} & \mathbf{D}_{RR} & \mathbf{D}_{RI} \\ \mathbf{D}_{IT} & \mathbf{D}_{IR} & \mathbf{D}_{II} \end{pmatrix} \quad (78)$$

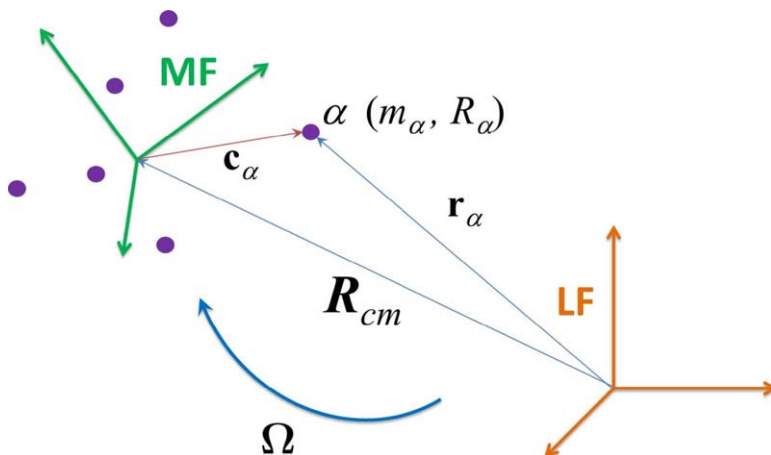


Fig. 6 Sketch of a molecular system and physical quantities involved in the diffusion tensor model construction.

where with the subscripts T, R, I we imply translational, rotational, and internal contributions, respectively. We infer from eqn (77) that if the set $\mathbf{x}$ has a total of n components, *i.e.* $\mathbf{x} = \{x_1, \dots, x_n\}$, then ξ happens to be a $(6+n) \times (6+n)$ matrix. But how do we evaluate the constrained friction matrix ξ ?

For the sake of illustrating how ξ is computed, imagine the N atoms of the molecule (viewed as an ensemble of beads) immersed in a continuous medium with viscosity η and with no constraints restraining them to each other whatsoever (see Fig. 6). What one will end up seeing is simply a collection of beads, each with a position vector $\mathbf{r}_\alpha$ and velocity $\mathbf{v}_\alpha \equiv \dot{\mathbf{r}}_\alpha$, $\alpha = 1, \dots, N$, and subject to a force $\mathbf{F}_\alpha$. We may likewise define here a collective velocity vector $\mathbf{v} = [\mathbf{v}_1, \dots, \mathbf{v}_N]$ and force vector $\mathbf{F} = [\mathbf{F}_1, \dots, \mathbf{F}_N]$. In analogy to eqn (77), we can write

$$\begin{pmatrix} \mathbf{F}_1 \\ \vdots \\ \mathbf{F}_\alpha \\ \vdots \\ \mathbf{F}_N \end{pmatrix} = -\Xi \begin{pmatrix} \mathbf{v}_1 \\ \vdots \\ \mathbf{v}_\alpha \\ \vdots \\ \mathbf{v}_N \end{pmatrix} \quad (79)$$

where Ξ is the $3N \times 3N$ (unconstrained) friction matrix.

It can be proved that the collective force vectors, $\mathcal{F}$ and $\mathbf{F}$, are related through a matrix. The same can be said of the collective velocity vectors $\mathcal{V}$ and $\mathbf{v}$.^{94,96} It thus goes on that

$$\mathcal{F} = \mathbf{A}\mathbf{F} \quad \mathbf{v} = \mathbf{B}\mathcal{V}, \quad (80)$$

where $\mathbf{A}$ and $\mathbf{B}$ are matrices of dimension $(6+n) \times 3N$ and $3N \times (6+n)$, respectively, and are the transpose of each other: $\mathbf{B} = \mathbf{A}^{\text{tr}}$. In addition,

these matrices solely depend on the instantaneous geometry of the molecule. Accordingly, we conclude from eqn (77)–(79) and (80) that

$$\xi = \mathbf{B}^{\text{tr}} \Xi \mathbf{B} = \begin{pmatrix} \xi_{TT} & \xi_{TR} & \xi_{TI} \\ \xi_{RT} & \xi_{RR} & \xi_{RI} \\ \xi_{IT} & \xi_{IR} & \xi_{II} \end{pmatrix} \quad (81)$$

which is an equation that relates the two friction matrices ξ , Ξ through the purely geometrical matrix $\mathbf{B}$.

We may now consider finding an expression for the $\mathbf{B}$ matrix. In order to do so, we first of all express the Cartesian velocities $\mathbf{v}_\alpha$ with respect to the external coordinates,⁹⁷

$$\mathbf{v}_\alpha = \mathbf{v} + \mathbf{E}^{\text{tr}}(\Omega) \cdot \left(\omega \times \mathbf{c}_\alpha + \sum_{\mu=1}^n \frac{\partial \mathbf{c}_\alpha}{\partial x_\mu} \dot{x}_\mu \right) \quad (82)$$

with $\mathbf{E}(\Omega)$ being the Euler matrix which transforms the LF into MF. Rewriting eqn (82) more explicitly, we have

$$\begin{pmatrix} \mathbf{v}_1 \\ \vdots \\ \mathbf{v}_\alpha \\ \vdots \\ \mathbf{v}_N \end{pmatrix} = \underbrace{\begin{pmatrix} \mathbf{1}_3 & -\mathbf{E}^{\text{tr}}(\Omega) \mathbf{c}_1^\times & \mathbf{E}^{\text{tr}}(\Omega) \frac{\partial \mathbf{c}_1}{\partial x_1} & \cdots & \mathbf{E}^{\text{tr}}(\Omega) \frac{\partial \mathbf{c}_1}{\partial x_n} \\ \vdots & \vdots & \vdots & & \vdots \\ \mathbf{1}_3 & -\mathbf{E}^{\text{tr}}(\Omega) \mathbf{c}_\alpha^\times & \mathbf{E}^{\text{tr}}(\Omega) \frac{\partial \mathbf{c}_\alpha}{\partial x_1} & \cdots & \mathbf{E}^{\text{tr}}(\Omega) \frac{\partial \mathbf{c}_\alpha}{\partial x_n} \\ \vdots & \vdots & \vdots & & \vdots \\ \mathbf{1}_3 & -\mathbf{E}^{\text{tr}}(\Omega) \mathbf{c}_N^\times & \mathbf{E}^{\text{tr}}(\Omega) \frac{\partial \mathbf{c}_N}{\partial x_1} & \cdots & \mathbf{E}^{\text{tr}}(\Omega) \frac{\partial \mathbf{c}_N}{\partial x_n} \end{pmatrix}}_{\mathbf{B}} \begin{pmatrix} \mathbf{v} \\ \omega \\ \dot{\mathbf{x}} \end{pmatrix} \quad (83)$$

where $\mathbf{c}_\alpha^\times$ is the 3×3 matrix with elements $(\mathbf{c}_\alpha^\times)_{mn} = \sum \varepsilon_{mnl} (\mathbf{c}_\alpha)_l$, where ε_{mnl} is the Levi-Civita symbol.

We also note from eqn (83) that the derivative $\frac{\partial \mathbf{c}}{\partial x}$ is required for the computation of the vector $\mathbf{v}$. For natural internal coordinates such as dihedrals,⁹⁵ bond angles and/or bond lengths, analytical expressions⁹⁸ for these derivatives are well-known.

Having finally computed the matrix $\mathbf{B}$, the only impediment which stands in our way as we want to calculate the matrix ξ is the unconstrained friction tensor Ξ , which for now is totally obscure to us. The simplest model one can resort to in evaluating Ξ is that of independent beads, *i.e.* one assume all the beads experience the same friction, and so Ξ becomes simply proportional to the $3N \times 3N$ identity matrix $\mathbf{1}_N$,

$$\Xi = \Xi_0 \mathbf{1}_N \quad (84)$$

with $\Xi_0 = CR_e \eta \pi$, where R_e is the hydrodynamical radius of a bead and $C = \{4, 6\}$ according to whether the slip or stick friction models are

assumed, respectively. With these assumptions, the constrained friction tensor, eqn (81), turns out to be of the form

$$\xi = \Xi_0 \mathbf{B}^{\text{tr}} \mathbf{B}, \quad (85)$$

which in combination with Einstein's relation, eqn (78), returns the diffusion tensor $\mathbf{D}$.

Of course one can turn to more elaborate models like the Oseen^{99,100} or the Rotne–Prager¹⁰¹ models if the effects of hydrodynamic interactions between the beads are to be accounted for. With these models, a perturbative correction to the friction felt by a generic bead in a medium due to the motion of other particles is done. The Rotne–Prager model is an good choice given that it always yield positive-definite diffusion tensors. Considering for example two generic atoms α, β , elements of the $3\text{N} \times 3\text{N}$ the unconstrained diffusion tensor Ξ are

$$\left\{ \begin{array}{l} \Xi_{\alpha\alpha} = \frac{k_B T}{\Xi_0} \mathbf{1}_3 \\ \Xi_{\alpha\beta} = \frac{k_B T}{8\pi\eta r_{\alpha\beta}} \left[\left(1 + \frac{2R_e^2}{3r_{\alpha\beta}^2} \right) \mathbf{1}_3 + \left(1 - \frac{2R_e^2}{r_{\alpha\beta}^2} \right) \frac{\mathbf{r}_{\alpha\beta} \otimes \mathbf{r}_{\alpha\beta}}{r_{\alpha\beta}^2} \right] \quad \text{if } r_{\alpha\beta} \geq 2R_e \\ \Xi_{\alpha\beta} = \frac{k_B T}{8\pi\eta r_{\alpha\beta}} \left[\frac{r_{\alpha\beta}}{2R_e} \left(\frac{8}{3} - \frac{3r_{\alpha\beta}}{4R_e} \right) \mathbf{1}_3 + \frac{\mathbf{r}_{\alpha\beta} \otimes \mathbf{r}_{\alpha\beta}}{8R_e^2} \right] \quad \text{if } r_{\alpha\beta} \leq 2R_e \end{array} \right. \quad (86)$$

according to the Rotne–Prager model, where $\mathbf{r}_{\alpha\beta} = \mathbf{r}_\alpha - \mathbf{r}_\beta$, $r_{\alpha\beta} = |\mathbf{r}_{\alpha\beta}|$. The diffusion tensor $\mathbf{D}$ then is easily found to be

$$\mathbf{D} = (\mathbf{B}^{\text{tr}} \Xi^{-1} \mathbf{B})^{-1} \quad (87)$$

following eqn (81) and Einstein's relation, eqn (78).

5 Stochastic *ab initio* spectra simulation of CW-ESR

5.1 Computational implementation

In Sections 3 and 4 our discussions dealt with the two main ingredients of the SLE: the spin Hamiltonian (or superhamiltonian $H^\times$) and the stochastic diffusion Γ operator, respectively. Once the spin Hamiltonian and a model for Γ are defined, together with a basis set, a software for the calculation of CW-ESR spectra can be built. Since the '90s, there has been a surge in the search for new sophisticated models for the simulation, analysis and/or interpretation of experimental ESR results, leading to the development of quite a number of new softwares – mostly independent of each other. When it comes to the SLE approach, we have at our disposal today several software packages implementing numerical solutions to the SLE for CW-ESR. We mention here for example the open-source ACERT package by Freed and co-workers for the simulation and analysis of ESR spectra, which includes the basic simulation program EPRLL⁴⁷ and the nonlinear least-squares fitting code NLSL.¹⁰² Some of these programs have been used in conjunction with the SRLS model as described in

ref. 103 Stoll's EasySpin¹⁰⁴ – a free simulation toolbox for the MatLab package for mainly solid-state ESR and which implements a partial solution to the SLE – is another example. Many other softwares are available for advanced ESR spectroscopies, such as ENDOR and particular kinds of pulsed ESR.¹⁴

The E-SpiReS code²² developed more recently seems to fill up all the lacking features past codes for the calculation of CW-ESR spectra had; it follows the SASS scheme sketched in Fig. 2. With the aid of a simple Graphical User Interface (GUI), it makes the setting of parameters to be used in the spectrum calculation easier for a generic user. This code has been implemented in the VMS tool^{19–21} preserving all the main features of E-SpiReS, which are (1) the high modularity of the code that gathers both the structural and dynamical information, (2) the GUI, (3) the possibility to call independent softwares (Gaussian, in this specific case) for QM calculation of magnetic tensors, (4) the parallelization of the code apt to speed up the spectrum calculation, especially when the base dimension is huge.

VMS has the great advantage to be an all-in-one application which provides several tools. Among the various advantages, VMS facilitates the creation of complicated input files for spectroscopy computations and the understanding of complex output files, and allows the visualization of optical electronic, IR, Raman spectra and CW-ESR spectra. It also provides an integrated environment for direct comparison between collected experimental spectra and theoretically calculated spectra in order to correctly interpret complex results. It also features an integrated scientific data visualizer for the analysis and processing of data along with different graphical tools. Finally, an interactive 3D molecule builder is embedded in VMS to allow the user to build and manipulate complex structures loaded from quantum chemical software packages.

In what follows we shall present a simple tutorial case in the scope of illustrating how by using VMS the user can setup the various parameters needed and run a CW-ESR spectrum calculation (based on the SLE approach). The basic example of phenyl nitronyl nitroxide (R = Ph in Fig. 1, panel d) in aqueous solution will be considered. Then in the next subsection we shall present some novel applications.

Tutorial: phenyl nitronyl nitroxide

To calculate a new CW-ESR spectrum with VMS, the user need to click on the “EPR” button (see Fig. 7). He/She is then required to provide the molecular structure of the system under study, typically as xyz, protein data bank (pdb) or Z-matrix formats (zmt). At this point, the user can also load a Gaussian output file (in general a log file) containing the molecular geometry and the computed magnetic tensors. Once loaded, a 3D representation of the molecule appears in a window where the user can use the typical features of molecule viewer softwares to rotate or enlarge the structure, to name the least. Together with that –following the program work flow – a parameter selector window appears, where all the physical properties of the system under study can be set. To begin with, it is necessary to select the dynamical model for the molecule, namely the one-body or the two-body model. In this last case, the user is also

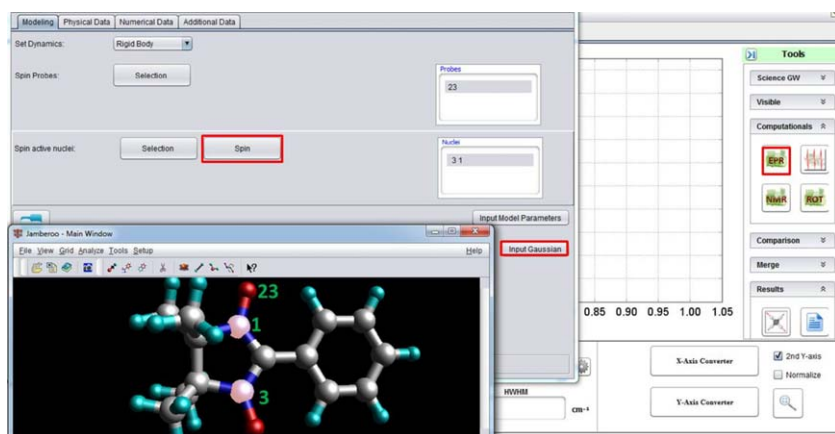


Fig. 7 VMS interactive 3D molecule builder, main panels. The buttons recalled in the text ("EPR", "Spin", "Input Gaussian") are highlighted with a rectangular box.

required to specify the two atoms bonded by the torsional angle and this information can be provided directly by clicking on the related atoms on the 3D representation of the molecule. In essence, by selecting either model (one- or two-body), the user is indirectly defining the form of the diffusive operator Γ to be used; considering the small phenyl nitronyl nitroxide molecule, the one-body model can be adopted.

After selecting what dynamical model to use, some information regarding the magnetic tensors must be given. This step is equivalent to defining the effective spin Hamiltonian. The user can again directly click on (a) the atoms bearing the unpaired electrons and (b) the nuclei coupled to these electrons, setting also the nuclear spin momentum, with the "spin" button. In the specific case of the molecule considered here, the O–N bond is involved, and the electron is coupled to both N nuclei ($I=1$). In the other panels (see Fig. 8), the user is required to specify important physical data such as the magnetic external field B_0 applied, the field range of acquisition of the signal, the temperature T and the viscosity of the solution. The numerical data panel collects the truncation limit $L_{\max}$ (see subsection 4.1) of the basis set for the global tumbling and the number of steps for the Lanczos algorithm (if not specified the number of steps is taken to be 10% of the basis' dimension). Finally, additional data can be provided. These include, for example, information such as the form of the potential V (if present) and its coefficients (see subsection 4.1), the linewidth, specific hydrodynamical information like the sphere radius R_e , the boundary condition coefficient C (see subsection 4.3), *etc.* In order to set the magnetic tensors' eigenvalues, the user can either feed them directly through the proper window or click the "Input Gaussian" button. In this last case, a QM calculation of the magnetic tensors with Gaussian will be launched and the results will be automatically fed to the main program through an input file. In the specific case of phenyl nitronyl nitroxide being considered here, we chose the second route when providing the Zeeman tensor and the two hyperfine coupling interaction tensors to the program.



Fig. 8 VMS panels showing the various input data to be set. The calculated spectrum is shown in the lower right panel.

The program then proceeds to the Diffusion environment where the diffusion tensor calculation is launched according to the model presented in Section 4.3. Once the results are in, the single eigenvalues and/or the trace of the diffusion tensor can be edited, on the user's discretion. Here, VMS creates a parameters file that can be modified by the user. Everything is now in place and the calculation of the CW-ESR spectra can be ran. The spectrum is calculated by numerically solving the SLE. When the calculation is finished, the spectrum is directly plotted by VMS (see lower right panel in Fig. 8). At this stage, the user can make use of a refine mode which makes it possible to conduct refinement operations. Here, a fitting of the various physical parameters (the user can specify which ones) of the calculated spectra are conducted in comparison with a loaded experimental one, taken as reference. The fitting algorithm is based on the Levenberg–Marquardt method of nonlinear least squares minimization.^{105,106} Since we don't have the digitalized experimental spectra of phenyl nitronyl nitroxide, here we just show the theoretical one.

5.2 Novel applications

With the purpose of making some practical examples on how the theoretical aspects discussed till now are applicable to molecular systems, we now discuss here two cases of novel applications in the context of CW-ESR spectroscopy.

We begin with the case of [60]fulleropyrrolidine bisadducts with nitroxide probes.¹⁰⁷ Two TOAC spin probes (Fig. 1, panel a) are bonded to the fullerene molecule in different sites of the icosahedron. The dynamics of the system was described adopting the one-body model, and so a unique stochastic process – consisting of the three Euler angles that

take LF to MF – was involved. For simplicity, the absence of an external potential V (*i.e.* free diffusion assumption) was assumed.

Let us now consider the spin Hamiltonian. Given that the number of unpaired electrons involved here are two, the spin Hamiltonian is the sum of the individual Zeeman and hyperfine interaction terms for the two electrons, plus new terms accounting for the interaction between the two electrons. These new additional terms are the dipolar interaction and the (isotropic) Heisenberg exchange coupling terms:

$$H = \frac{\beta_e}{\hbar} \mathbf{B}_0 \mathbf{g}_i \mathbf{s}_i + \frac{\beta_e}{\hbar} \mathbf{B}_0 \mathbf{g}_j \mathbf{s}_j + \gamma_e \mathbf{I}_i \mathbf{A}_i \mathbf{s}_i + \gamma_e \mathbf{I}_j \mathbf{A}_j \mathbf{s}_j - 2\gamma_e J \mathbf{s}_i \mathbf{s}_j + \frac{\mu_0 g_e^2 \beta_e^2}{4\pi \hbar r^3} \left[\mathbf{s}_i \mathbf{s}_j - \frac{3}{r^2} (\mathbf{s}_i \mathbf{r})(\mathbf{r} \mathbf{s}_j) \right]. \quad (88)$$

The Heisenberg exchange term ($-2\gamma_e J \mathbf{s}_i \mathbf{s}_j$) is added to account for the difference in energy between the singlet and triplet states; J is called the exchange energy. The position of the two nitroxides must be known in order to do calculations with the spin Hamiltonian. The vector $\mathbf{r}$ was expressed in spherical coordinates with respect to MF and the N–O bond was taken as reference point for the calculation of the contribution due to dipolar interaction. The super spinhamiltonian was then built in the same way as in eqn (59).

The value of the exchange energy may succumb to possible modulations due to the flexibility of the molecules. This phenomenon can be taken into consideration, for example, introducing in the SLE a new operator, the so called restructuring operator⁷⁸ which operates on a discrete space of possible values $\{J_n\}$ of the exchange energy J through a master equation.

The Liouvillian operator $\mathcal{L}$ was then constructed as we discussed in the previous sections. The basis set for $\mathcal{L}$ was then constructed taking the direct product of the spin transitions spaces $\{|p_i^s q_i^s, p_i^t q_i^t\rangle\}$ (see subsection 4.1, the normalized Wigner functions and the J_n values' space

$$\{|\Sigma\rangle\rangle\} = \{|p_i^s q_i^s, p_i^t q_i^t\rangle\rangle \otimes |p_j^s q_j^s, p_j^t q_j^t\rangle\rangle \otimes |L M K\rangle \otimes |n\rangle\} \quad (89)$$

where n is an integer number that runs upon the possible conformations. The starting vector $|v\rangle\rangle$ of the system, also related to the magnetization operator which gives the spectral line, is given by the sum of the starting vector of the two electrons multiplied by some factor

$$|v\rangle\rangle = N[I_i, I_j]^{-\frac{1}{2}} (|\mathbf{s}_{x,i}\rangle\rangle + |\mathbf{s}_{x,j}\rangle\rangle). \quad (90)$$

The dimension of the basis set was quite huge and a *pruning* procedure was necessary. This method considers the weight of each basis function over the spectrum and eliminates those ones that are less important, with a specific criterion.^{18,108}

Five spectra of different fullerene bisadducts with the two TOAC molecules was simulated; in all the cases a fitting procedure was used in order to reach the best fit with the experimental spectra. In all the cases,

quite an excellent agreement was shown between the calculated and experimental spectra.

Another interesting biradical system that has been studied is the Fmoc-(Aib-Aib-TOAC)₂-Aib-OMe for which the CW-ESR spectra has been computed in different solvents.^{109,110} The spin labeling of biological macromolecules provides remarkable information such as the distance between the two nitroxides; also at short distances, the CW-ESR spectrum in liquid solution can be very informative because its line shapes depend on several structural and dynamic parameters which characterize the double labeled peptide. This heptapeptide assumes a 3₁₀-helix conformation and the motional dynamics is again described adopting the one-body model. The spin Hamiltonian was again described by eqn (88), neglecting also this time the explicit hyperfine coupling with nuclei other than ¹⁴N. Moreover, the basis and the starting vector used are the same as those given in eqn (88)–(90).

QM calculations, in different solvents using PCM, have been done in order to locate the energy minima, respect to which a CW-ESR spectra computation was started. The calculated spectra have been compared with the experimental ones and they are in quite a good agreement, confirming the same trend in the change of line shape as a change of solvent was made.

A recent study on which we are currently working on is the CW-ESR spectra simulation of phospholamban (PLB) in a lipid bilayer probed by single TOAC spin labels. PLB is a 52-aminoacid amphipathic integral membrane protein that regulates the active transport of calcium in the heart. Different CW-ESR spectra has been recorded^{111,112} by attaching the magnetic probe to different sites of the peptide backbone and different structural dynamics have been shown. In Fig. 9 we sketch the peptide immersed in the lipid bilayer membrane (represented by the shadowed rectangle). The four positions to which the TOAC was attached are reported in the figure.

The CW-ESR spectrum recorded in the transmembrane domain site (position 46) has shown that the probe is strongly immobilized, and this is consistent with the presence of a stable and highly ordered transmembrane helix. In going out towards the cytoplasmic domain (positions 24, 11, 0), the isotropic nanosecond dynamic disorder emerges. In conjunction with NMR measurements it has also been shown that the outer part of PLB (the one that is directly exposed in the cytoplasmic domain) is in dynamic equilibrium between an ordered conformation – which is in direct contact with the membrane surface – and a more dynamic conformer, leading to the hypothesis of a two-state model. A recent MD simulation made in our group with a modified AMBER Force Field for TOAC residue has verified at position 11 this conformational jump by measuring the angle ϕ formed by the three positions 0, 24, 46. In addition, by measuring the distance between the C_α atoms of positions 0 and 11 it can be shown that the helix out of membrane has a “breathing” motion (see Fig. 10).

We are currently trying to reproduce the experimental CW-ESR spectrum of this system when the TOAC probe is attached in position 11

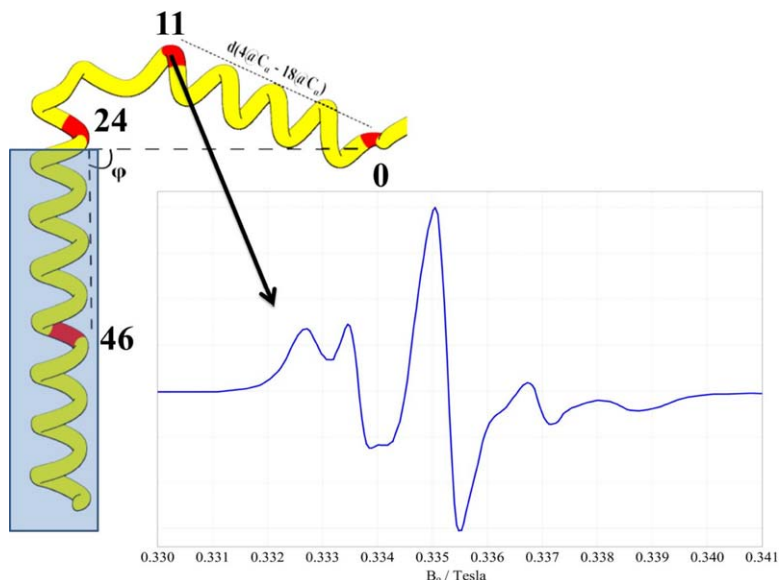


Fig. 9 Sketch of the PLB system immersed in the lipid bilayer membrane (shaded rectangle). The experimental CW-ESR spectrum for the TOAC probe linked at position 11 is reported.

using the SASS implemented in VMS. As first stage of this effort, we are modeling PLB considering only the outer part of the peptide as a single rigid body that is strongly oriented; to this end, the MD trajectory is useful to parametrize the external orienting potential (eqn (67)). The main problem is due to the strong orientation of the body. This implies a huge value of $L_{\max}$ for the Wigner basis functions and consequently a very long computation time. For what concerns the spin Hamiltonian we are taking into account only the Zeeman and the hyperfine coupling interactions since the system is monoradical and therefore the exchange and dipolar interaction terms are absent. The large size of this molecular system, together with all its peculiar features, makes this novel study quite challenging as regards the CW-ESR spectra simulation.

6 Towards a multi-body ESR: novel computational techniques

6.1 Multi-spin Hamiltonian

The SL approach described in the previous sections, like many other approaches, is mostly suitable (computationally speaking) in cases in which a relatively low number of spins are allowed to interact. This has to do with the exponential growth of the Liouville space which accompanies an increase in the number of spins N . For example, for N 1/2-spins, the dimension of the Liouville space generated from the static spin Hamiltonian is 2^{2N} . The dimension further increases if spatial degrees of freedom (such as orientation) are taken into account. Although the purely spin part of the Liouvillian scales exponentially with the number of interacting spins, the spatial part scales polynomially with parameters

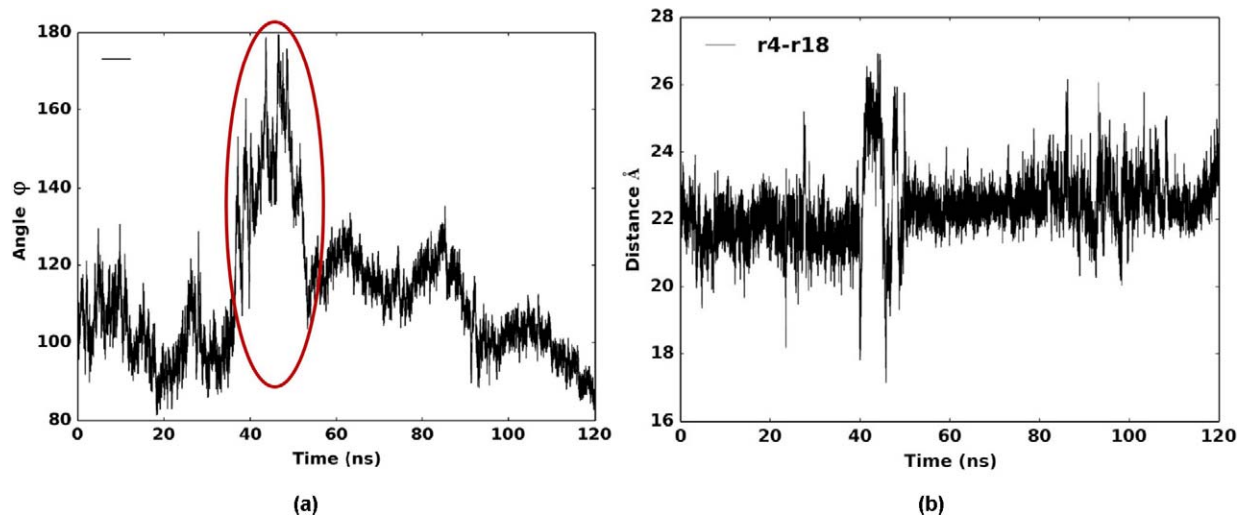


Fig. 10 (a) Variation of angle φ along the MD trajectory. (b) Variation of the distance between C_α at positions 0 and 11 along the MD trajectory.

like L (see Section 4). The exceedingly fast pace at which the spin space increases has botched many efforts aimed at applying the SL approach to large spin systems. Among the many important aspects of this extension is the fact that it will enable detailed analysis of the dynamics of biological macromolecules.¹¹³

A very promising approach developed in recent years by Hore, Kuprov and co-workers is the state-space restriction (SSR)^{114,115} algorithm which – as its name suggests – consists of restricting numerical evaluations to those states which are essential to the dynamics of the system. Originally applied to solid-state nuclear magnetic resonance, the SSR method may be applied also in the simulation of solid-state ESR spectra. Interacting spins as many as 10^4 can be handled to give reliable results with just a few basis set. The success of the SSR algorithm rests on the fact that it partitions the spin network into small collections called “spin clusters” and then construct a local Liouvillian for each cluster, which are subsequently evaluated exactly. The global Liouvillian is then obtained from the cluster Liouvillians by a sort of recoupling under specific rules. As mentioned by the authors,¹¹⁴ the SSR method also allows for a rapid convergence of the system’s propagator. The method relies heavily on the topology of the spin network, and the cluster Liouvillians are observed to be significantly sparse. The ZTE (zero track elimination) method developed by Kuprov augments the efficiency of the method by further pruning the cluster Liouvillians yielding a minimal basis set which captures remarkably the dynamics of the system.

In our view, an even more promising approach to tackle the exponential growth problem lies in the new vibrant research field of tensor network (TN) theory.^{116,117} With great contributions coming from the quantum information community, and their inherent versatility in adaptation to complex computations, TN methods are now setting new standards in the scientific computation paradigm as they continue to excel as promising alternatives to conventional numerical methods. Research in this field has seen a steep growth in recent years and this is confirmed by the plethora of new TN algorithms which are being published by various research groups year after year. Though the first TN method may be said to be Kenneth Wilson’s numerical renormalization group (NRG)¹¹⁸ proposed in 1975 and which led to the solution of the Kondo problem,¹¹⁹ the most famous TN algorithm today is the density matrix renormalization group (DMRG)¹²⁰ – first proposed by Steven White in 1992^{121,122} – which belongs to the class of TN states called the matrix product states (MPS).^{116,123,124} Recently proposed TN states like the projected entangled pair states (PEPS)¹²⁵ and the multi-scale entanglement renormalization ansatz (MERA)¹²⁶ are also gaining grounds. Indeed, the SSR algorithm may somehow be seen as a TN-like method but which makes an unconscious minimal use of the core ideas characteristic of the latter – which is very interesting because it signals the feasibility of simulating the ESR spectra of, for example, a macromolecule spin-labeled at various sites to extract information about how these labeled sites interact with each other in great details. Moreover, TN methods are known to be incredibly effective when simulating

strongly correlated systems; this property makes them even more appealing because it can be exploited in the simulation of multi-spin ESR where correlations between some groups of spin are likely to be very strong.

Our group is currently working to devise efficient computational ways of simulating multi-spin CW-ESR spectra. For the time being, our focus is on multi-spin monoradicals, *i.e.* a single unpaired electron interacting with an arbitrary number of nuclei. We believe TN methods provide the necessary elements for solving the many computational problems that plague conventional methods of simulation. To illustrate the convenience of TNs, consider a time independent spin Hamiltonian of N_{el} unpaired electrons and N_{nu} nuclei. Before writing down the spin Hamiltonian, we introduce the *substitution operator* $\mathfrak{G}$ of order n defined as

$$\mathfrak{G}(x_1, x'_1; \dots; x_n, x'_n) f(x_1, \dots, x_n) = f(x'_1, \dots, x'_n). \quad (91)$$

Then, following eqn (28), the matrix representation of the spin Hamiltonian of the system in the uncoupled representation⁸⁶ can be written as

$$\begin{aligned} H = & \sum_i \sum_{\beta} \mu_B B_0 \mathfrak{G}(\mathbb{I}^{[i]}, g_{z\beta}^{[i]} \mathbf{s}_{\beta}) \bigotimes_{n=1}^N \mathbb{I}^{[n]} \\ & + \sum_{i, i' (> i)} \sum_{\beta, \beta'} D_{\beta\beta'}^{[i, i']} \mathfrak{G}(\mathbb{I}^{[i]}, \mathbf{s}_{\beta}; \mathbb{I}^{[i']}, \mathbf{s}_{\beta'}) \bigotimes_{n=1}^N \mathbb{I}^{[n]} \\ & + \sum_{i, K} \sum_{\beta, \beta'} A_{\beta\beta'}^{[i, K]} \mathfrak{G}(\mathbb{I}^{[i]}, \mathbf{s}_{\beta}; \mathbb{I}^{[K]}, \mathbf{I}_{\beta}^{[K]}) \bigotimes_{n=1}^N \mathbb{I}^{[n]} \end{aligned} \quad (92)$$

or more succinctly,

$$H = \mathfrak{G}_{\text{spin}} \bigotimes_{n=1}^N \mathbb{I}^{[n]} \equiv \mathfrak{G}_{\text{spin}} \mathbb{I}^{\otimes N} \quad (93)$$

where $N = N_{\text{el}} + N_{\text{nu}}$ and where,

$$\begin{aligned} \mathfrak{G}_{\text{spin}} \equiv & \sum_i \sum_{\beta} \mu_B B_0 \mathfrak{G}(\mathbb{I}^{[i]}, g_{z\beta}^{[i]} \mathbf{s}_{\beta}) + \sum_{i, i' (> i)} \sum_{\beta, \beta'} D_{\beta\beta'}^{[i, i']} \mathfrak{G}(\mathbb{I}^{[i]}, \mathbf{s}_{\beta}; \mathbb{I}^{[i']}, \mathbf{s}_{\beta'}) \\ & + \sum_{i, K} \sum_{\beta, \beta'} A_{\beta\beta'}^{[i, K]} \mathfrak{G}(\mathbb{I}^{[i]}, \mathbf{s}_{\beta}; \mathbb{I}^{[K]}, \mathbf{I}_{\beta}^{[K]}). \end{aligned} \quad (94)$$

In eqn (91)–(94): (a) we have assumed the presence of an homogeneous static magnetic field of modulus B_0 along the z -axis; (b) the index $i(K)$ runs over all unpaired electrons(nuclei); (c) the index β runs over the axes x, y, z ; (c) $g_{z\beta}^{[i]}$ is the z, β component of the g -tensor of the i -th electron (see eqn (96)); (d) the scalar $D_{\beta\beta'}^{[i, i']}$ denotes the β, β' element of the electron spin–spin interaction tensor between the electrons i and i' (eqn (96)); (e) $A_{\beta\beta'}^{[i, K]}$ is element β, β' of the hyperfine interaction tensor

between i -th unpaired electron and the K -th nucleus (eqn (96)); (f) $\mathbf{s}_\beta$ and $\mathbf{I}_\beta^{[K]}$ represent the matrix representation of the component β of the electron and K -th nucleus' spin angular momentum operator, respectively; (g) the index n runs over both electrons and nuclei; (h) $\mathbb{I}^{[n]}$ is the matrix representation of the identity operator of the Hilbert space of the isolated n -th particle and is a square matrix of order 2 for electrons and $(2I_K + 1)$ for the K -th nucleus; (i) $\mathbb{I}^{\otimes N}$ is the *ordered* Kronecker product of the N identity matrices and it is also the unit operator on the global Hilbert space of the system. For example, we may choose an order in which we list first the electrons and then the nuclei, obtaining

$$\mathbb{I}^{\otimes N} \equiv \bigotimes_{n=1}^N \mathbb{I}^{[n]} = \mathbb{I}^{[i=1]} \otimes \mathbb{I}^{[i=2]} \otimes \dots \otimes \mathbb{I}^{[i=N_{\text{el}}]} \otimes \mathbb{I}^{[K=1]} \otimes \mathbb{I}^{[K=2]} \otimes \mathbb{I}^{[K=N_{\text{nu}}]}. \quad (95)$$

$$\mathbf{g}^{[i]} = \begin{pmatrix} g_{xx}^{[i]} & g_{xy}^{[i]} & g_{xz}^{[i]} \\ g_{yx}^{[i]} & g_{yy}^{[i]} & g_{yz}^{[i]} \\ g_{zx}^{[i]} & g_{zy}^{[i]} & g_{zz}^{[i]} \end{pmatrix} \quad \mathbf{D}^{[i,i']} = \begin{pmatrix} D_{xx}^{[i,i']} & D_{xy}^{[i,i']} & D_{xz}^{[i,i']} \\ D_{yx}^{[i,i']} & D_{yy}^{[i,i']} & D_{yz}^{[i,i']} \\ D_{zx}^{[i,i']} & D_{zy}^{[i,i']} & D_{zz}^{[i,i']} \end{pmatrix} \quad (96)$$

$$\mathbf{A}^{[i,K]} = \begin{pmatrix} A_{xx}^{[i,K]} & A_{xy}^{[i,K]} & A_{xz}^{[i,K]} \\ A_{yx}^{[i,K]} & A_{yy}^{[i,K]} & A_{yz}^{[i,K]} \\ A_{zx}^{[i,K]} & A_{zy}^{[i,K]} & A_{zz}^{[i,K]} \end{pmatrix}$$

Note that the tensors $\mathbf{g}$, $\mathbf{D}$ and $\mathbf{A}$ are all Cartesian. We could have equally made the choice of working in spherical basis (which automatically removes non-contributing terms due to symmetry) as we saw in Section 4 but we shall stick to the use of Cartesian tensors just to make the discussion more intelligible.

What becomes pretty clear from the use of the substitution operator (see for example eqn (93)) is the fact that the total spin Hamiltonian is obtained from the identity operator $\mathbb{I}^{\otimes N}$ through a series of operations in which at most 2 local identity operators (matrices) are substituted with an equal number of spin matrices, multiplied by some constants and summed over. The bound on the number of substitution – which stems from the fact that the spin Hamiltonian allows for only pairwise interactions as assumed when we discussed its derivation in Section 3 – gives rise to the sparsity which characterizes the spin Hamiltonian matrix. Furthermore, since this bound is independent of the number of spins, we observe that as N increases, the more sparse the spin Hamiltonian becomes. Furthermore, it is evident from eqn (93)–(95) that the order of the square matrix H , $\wp(H)$, is

$$\wp(H) = 2^{N_{\text{el}}} \prod_{K=1}^{N_{\text{nu}}} (2I^{[K]} + 1). \quad (97)$$

where $I^{[K]}$ is the spin of the K -th nucleus. Thus, the dimension of H grows exponentially with the number of the different spins. Moreover, for a generic wave function $|\psi\rangle$ of the system in the uncoupled representation, we have

$$|\psi\rangle = \sum_{m_s^{[1]}, \dots, m_s^{[N_{\text{el}}]}, M_s^{[1]}, \dots, M_s^{[N_{\text{nu}}]}} C_{m_s^{[1]}, \dots, m_s^{[N_{\text{el}}]}, M_s^{[1]}, \dots, M_s^{[N_{\text{nu}}]}} \times |s^{[1]}m_s^{[1]}, \dots, s^{[N_{\text{el}}]}m_s^{[N_{\text{el}}]}, I^{[1]}M_s^{[1]}, \dots, I^{[N_{\text{nu}}]}M_s^{[N_{\text{nu}}]}\rangle \quad (98)$$

where the set $\left\{ |s^{[1]}m_s^{[1]}, \dots, s^{[N_{\text{el}}]}m_s^{[N_{\text{el}}]}, I^{[1]}M_s^{[1]}, \dots, I^{[N_{\text{nu}}]}M_s^{[N_{\text{nu}}]}\rangle \right\}$ is the basis and $m_s(M_s)$ indicate the eigenvalues of the z-component of the spin operator of the corresponding electron(nucleus). The coefficients $C_{m_s^{[1]}, \dots, m_s^{[N_{\text{el}}]}, M_s^{[1]}, \dots, M_s^{[N_{\text{nu}}]}}$ are elements of a complex tensor with N indexes, each capable of assuming a number of values corresponding to the possible $m_s(M_s)$ values of the associated electron(nuclear) spin. In other words, we need a number of coefficients in the order of $\mathcal{O}(\wp(H))$ in order to specify a quantum state. This way of indicating quantum states is impractical due to the exponential growth of these parameters with the number of spins. Besides, the increase in sparsity of H as N increases proves the inefficiency of this approach. This poses a significant computational problem because although we would like to find an efficient way of specifying a quantum state, we would also like not to lose relevant information on the system.

Let us now see how the same problem is approached using a TN method. Rather than dealing directly with the tensor C , a TN method decomposes the latter into a collection of small tensors with a certain interconnection which maintain the salient entanglement features of the system. From another point of view, the decomposition is equivalent to the creation of maximally entangled states projected locally on smaller Hilbert spaces,^{117,125} yielding a construction which depends polynomially on the number of distinct spins. This is the very gist of the efficiency and success of TN approaches.

The decomposition though comes at a cost, and the prize to pay is the emergence of extra degrees of freedom – called *bond indexes* – in the system. In fact, the factor tensors are linked together by these degrees of freedom, which are actually indexes that can take up to a certain number of different values. For this reason, the bond indexes may be interpreted as virtual spins. The rank D of an index is defined as the number of different values it can assume. Index ranks are very important because they provide a quantitative measure of the entanglement present in the quantum state. Moreover, the higher the rank of D , the larger portion of the total Hilbert space explored. We shall not delve further into ranks and the closely related concept of area laws. (See^{116,123} for further information.) What we are interested in at the moment is the quantum states representation according to TNs and why this can be crucial in the simulation of multi-spin ESR.

Before moving on, it must be mentioned that important ingredients in the TN approach are the concept of *site* (*i.e.* the location of a spin) and the topology of the TN (*i.e.* the spatial arrangement of the sites). The site is not necessarily a spatial one and orbitals may be used as sites as it is normally done in QC-DMRG.¹²⁷ For the sake of clarity, we shall limit our discussion to spatial sites and consider a stable paramagnetic probe with a certain number of single unpaired electrons which may be assumed to be located on some nuclei. From the TN approach perspective, we may for example define each atom of the probe as a site hosting a nucleus and a single unpaired electron. To make the arguments in the following clearer, assume that we divide the whole system into two parts: the electronic and nuclear parts. Then we shall have a TN for each of the two subsystems. If we choose a matrix product state (MPS) to represent the quantum state of each subsystem, then that of the nuclear part $|\psi\rangle_{\text{nu}}$ can be written as

$$|\psi\rangle_{\text{nu}} = \sum_{M^{[1]}, M^{[2]}, \dots, M^{[N_{\text{nu}}]}} \text{Tr} \left[\Gamma_{M^{[1]}}^{[1]} \Gamma_{M^{[2]}}^{[2]} \cdots \Gamma_{M^{[N_{\text{nu}}]}}^{[N_{\text{nu}}]} \right] |M^{[1]}, M^{[2]}, \dots, M^{[N_{\text{nu}}]}\rangle \quad (99)$$

where the $\Gamma_{M^{[K]}}^{[K]}$ are matrices of dimension $D_K \times D_{K+1}$, where D_{K+1} is the bond index between the K -th and $(K+1)$ -th sites. The bond dimension D of the MPS is defined as the maximum bond index, *i.e.* $D = \max_K D_K$. If $D_1 = D_{N_{\text{nu}}+1}$, *i.e.* if $\Gamma_{M^{[1]}}^{[1]}$ and $\Gamma_{M^{[N_{\text{nu}}]}}^{[N_{\text{nu}}]}$ are row and column vectors, respectively, then eqn (99) simplifies to

$$|\psi\rangle_{\text{nu}} = \sum_{M^{[1]}, M^{[2]}, \dots, M^{[N_{\text{nu}}]}} \Gamma_{M^{[1]}}^{[1]} \Gamma_{M^{[2]}}^{[2]} \cdots \Gamma_{M^{[N_{\text{nu}}]}}^{[N_{\text{nu}}]} |M^{[1]}, M^{[2]}, \dots, M^{[N_{\text{nu}}]}\rangle. \quad (100)$$

The use of eqn (99) or eqn (100) depends on the geometry of the system. The former is suitable for closed structures (periodic boundary conditions), while the latter is excellent for linear structures (open boundary conditions).

More importantly, we see from eqn (99) (and also from eqn (100)) that the number of parameters P needed to specify $|\psi\rangle_{\text{nu}}$ is simply

$$P(|\psi\rangle_{\text{nu}}) = \sum_{K=1}^{N_{\text{nu}}} \wp(H^{[K]}) D_K D_{K+1} = \sum_{K=1}^{N_{\text{nu}}} (2I^{[K]} + 1) D_K D_{K+1} \quad (101)$$

where $\wp(H^{[K]})$ is the dimension of the Hilbert space of the local site K . The remarkable thing here is that the number of parameters now grow polynomially in the number of distinct spins rather than exponentially as discussed earlier. In fact, if the nuclei are all the same and $D_K = D_{K+1} = D$, then $P(|\psi\rangle_{\text{nu}}) = N_{\text{nu}} D^2 (2I + 1)$, where I is the spin of a generic nucleus.

Let us now bring the unpaired electrons into the picture. Each of the N_{nu} sites is now occupied by a nucleus and a number of unpaired electrons. We begin by indexing the sites as $\nu (=1, \dots, N_{\text{nu}})$ instead of K , for the sake of clarity. We can extend the argument we have just made in the last two paragraphs to the present case as well by defining at each site ν a *new*

spin $I^{[v]}$ which depends on the spin number of the nucleus $I^{[v]}$ occupying that site and its unpaired electrons. It can be easily proved that

$$\tilde{I}^{[v]} = (2^{\nu_e} - 1) \left(\frac{1}{2} + I^{[v]} \right) + I^{[v]} \quad (102)$$

where ν_e is the number of unpaired electrons in site v . Then, following eqn (99), we can express a generic quantum state of the system as

$$|\psi\rangle = \sum_{\tilde{M}^{[1]}, \tilde{M}^{[2]}, \dots, \tilde{M}^{[N_{\text{nu}}]}} \text{Tr}[\tilde{\Gamma}_{\tilde{M}^{[1]}}^{[1]} \tilde{\Gamma}_{\tilde{M}^{[2]}}^{[2]} \cdots \tilde{\Gamma}_{\tilde{M}^{[N_{\text{nu}}]}}^{[N_{\text{nu}}]}] |\tilde{M}^{[1]}, \tilde{M}^{[2]}, \dots, \tilde{M}^{[N_{\text{nu}}]}\rangle \quad (103)$$

Again, $\tilde{\Gamma}_{\tilde{M}^{[v]}}^{[v]}$ are $\tilde{D}_v \times \tilde{D}_{v+1}$ matrices, where $\tilde{D}_{v+1}$ is the bond index between the v -th and $(v+1)$ -th sites. The number of parameters $P(|\psi\rangle)$ needed in this MPS representation is thus

$$P(|\psi\rangle) = \sum_{v=1}^{N_{\text{nu}}} \wp(\tilde{H}^{[v]}) \tilde{D}_v \tilde{D}_{v+1} = \sum_{v=1}^{N_{\text{nu}}} (2\tilde{I}^{[v]} + 1) \tilde{D}_v \tilde{D}_{v+1} \quad (104)$$

where $\tilde{H}^{[v]}$ is the Hilbert space of site v . We see also here that the number of parameters does not grow exponentially with the number of spins. We shall not discuss here how the factor tensors $\tilde{\Gamma}$ (or Γ) are constructed because it goes beyond the scope of this review (the interested reader should see the already cited literature or Perez-Garcia *et al.*¹²⁸ The spin Hamiltonian may also be written in the MPS-like form, called matrix product operator (MPO)¹²⁹ representation.

So far, we have been talking about wave functions and Hilbert spaces, but not density matrices and Liouville spaces, which are actually of primary importance in theoretical and computational magnetic resonances. All that has been discussed above about Hilbert spaces can be generalized to Liouville spaces if we exploit the homomorphism¹³⁰ between the two. In particular, we know that just as the wave function is a vector in the Hilbert space (HS), so is the density matrix operator in the Liouville space. And just as we have Schrödinger's equation in HS, in Liouville space (LS) we have (eqn (61))

$$\frac{\partial |\rho\rangle\rangle}{\partial t} = -\mathcal{L}_\sigma |\rho\rangle\rangle \quad (105)$$

where $|\rho\rangle\rangle$ is the (column) vector in LS representing the density matrix operator ρ as we saw in Section 4, and

$$\mathcal{L}_\sigma \equiv \frac{i}{\hbar} (H \otimes \mathbb{I}^{\otimes N} - \mathbb{I}^{\otimes N} \otimes H^{\text{tr}}). \quad (106)$$

The important thing to note here is that the vector $|\rho\rangle\rangle$ can also be expressed as an MPS state, and so the number of parameters needed will scale as $\mathcal{O}([P(|\psi\rangle\rangle)]^2)$ – *i.e.* still polynomial in the number of distinct

spins – instead of $\mathcal{O}[\phi(H)]^2$). And this clearly shows how efficient the MPS and TN protocols are, in general.

The use of TN methods to simulate the time evolution of multi-spin ESR is practically feasible. The advances made in TN theory has made it possible to simulate the time evolution of quantum states, even in the presence of dissipation.¹³¹ Recent developments have also led to the formulation of a linear response theory in DMRG,¹³² which is good news for researchers who want to simulate CW-ESR using DMRG approaches. Making use of the homomorphism between HSs and LSs, one can treat the spin part of the stochastic Liouville operator $\mathcal{L}$ (eqn (1)) by an MPS protocol, for example, rather than using conventional diagonalization schemes based on basis sets which explode exponentially with the number of spins. Meanwhile, one can retain the conventional treatment of the stochastic spatial degrees of freedom as discussed for example in Section 4. The computational gain is that one ends up with an efficient algorithm which takes good care of entanglement and scales polynomially with the number of spins (as discussed above, the CW-ESR simulation of a number of spins beyond 20 is unattainable with conventional methods due to exponential growth of the basis). It is also interesting to note that magnetic tensors of nitrosils and other radicals are already being calculated using TN approaches like DMRG.¹³³ This means that it is possible to one day simulate CW-ESR using only TN protocols given that the stochastic diffusion part may be treated using modern developments in renormalization theory or recent developments in continuous-MPS.¹³⁴ The nature of the type of interactions considered is very crucial as certain TN methods are good for only short range interactions. For a basic understanding of DMRG and other TN methods as applied to quantum chemistry we invite the reader to see Szalay *et. al.*'s excellent review.¹³⁵

6.2 Diffusive problem along a generalized coordinate

As the reader might have appreciated by now, a reliable model for the diffusion of the stochastic variables $\mathbf{q}$ is all-important if the spin-relaxation is to be described genuinely with great care. Molecular systems are certainly subject to diffusive processes which involve their translational, rotational and internal degrees of freedom. Working out and implementing hydrodynamical models capable of yielding realistic diffusion tensors is by no means plain sailing.

In Section 4.3 we discussed to some length a reliable diffusion tensor model that enters the FP equation and which, together with the Spin Hamiltonian in the SLE, describes the relaxation process of the spin due to its coupling to external degrees of freedom $\mathbf{q}$. However, it must be added that the primary handicap of this model and that of the DiTe code⁹⁵ consists of the very fact that the only internal degrees of freedom it can handle are the torsional angles. Such a disturbing limitation clearly calls for an extension of the model to other internal coordinates in a generalized fashion – a call two of us (AP and VB) have adhered to in a recent work where they generalize the previous model.²⁴ The generalization proceeds from the consideration that the vast majority of diffusive

processes encountered in chemistry, in particular, can be properly parametrized by a generalized coordinate (GC) – in general a nonlinear combination of the internal ones – along a path in some generalized space.

The construction of GCs can be done following a three-step procedure. First of all, one needs an ordered series of geometrical structures of the system under study. Secondly, the variation of angular momenta between two consecutive geometries is minimized, *i.e.* the coupling between the GC and the global rotation of the system at the inertia tensor level is minimized; once this is achieved, the results become manifest in the diffusion tensor as well. Finally, the GC is then calculated as the distance in mass-weighted Cartesian coordinates between two consecutive structures. An important advantage of using mass-weighted coordinates is that reduced-mass can be set to unity when performing kinematic calculations.

The path along which the diffusive process occurs through the GC can be computed for example as follows: (1) one performs a rigid scan (also known as non-relaxed scan) along a chosen internal coordinate, keeping the rest of the system rigid at any scan step; (2) one then goes on to perform a relaxed-scan along an internal coordinate, in which all the internal coordinates of the system (except the scanned one) are optimized; (3) finally, an Intrinsic Reaction Path (IRC)^{136,137} is then computed, *i.e.* a minimum energy path in mass-weighted Cartesian coordinates joining a first order saddle point with two minima.

Once the GC has been computed, it can be used, for example, in the quantum mechanical context, as reported in many studies.^{49,52,138,139} The one-dimensional GC can also be treated as a diffusive process, with the possibility of taking into account the relaxation of the system. This method avoids many problems like the unnatural approaching of atoms of which DiTe was fond of. Moreover, taking the GC as a classical coordinate, it is possible to set up and solve the one-dimensional Smoluchowski equation using the so called Discrete Variable Representation (DVR).²³ We also believe that this generalized diffusion tensor model applied in the context of the SLE could be of great benefit. If we consider a biological macromolecule such as a protein, we see that given a GC (for example calculated from the sequence of structures generated by a MD simulation), it is possible to make use of this integrated machinery to compute both the interacting potential and the diffusion tensor, and also the magnetic tensors that enter in the SLE. In this very case, the GC would be considered as the stochastic variable of the diffusive problem. In comparison to the previous diffusion tensor model discussed in Section 4.3, with this new model the variables **a** are reduced to a single GC, called *s*, which can be determined by following the steps briefly outlined above. Eqn (83) still holds; the derivatives will be now $\frac{\partial \mathbf{c}}{\partial s}$, and the diffusion tensor:

$$\mathbf{D} = k_{\text{B}} T \xi^{-1} = \begin{pmatrix} \mathbf{D}_{TT} & \mathbf{D}_{TR} & \mathbf{D}_{TG} \\ \mathbf{D}_{RT} & \mathbf{D}_{RR} & \mathbf{D}_{RG} \\ \mathbf{D}_{GT} & \mathbf{D}_{GR} & \mathbf{D}_{GG} \end{pmatrix} \quad (107)$$

where G now stands for generalized. Since we are dealing with just one GC, $\mathbf{D}_{GG}$ is a scalar function of s , $\mathbf{D}_{GG}(s)$. From the scan geometries and the knowledge of s , the quantity $\frac{\partial \mathbf{c}}{\partial s}$ is directly computed inside the code.

On the basis of this new model, we have also been able to compute kinetic constants from various diffusive problems, showing the validity of the method. The other route is the classical one and in this view we extended the theory to a diffusion tensor model function of a GC calculated with both a non-relaxed scan (1) and a relaxed one (2). The details, here omitted, on how this GC is computed, can be found for instance in ref. 139.

A code which computes the energy and other properties such as the diffusion tensor along the GC has been successfully integrated in a development version of the Gaussian code.¹⁴⁰

7 Conclusions

The interpretation of the dynamical behavior of biological macromolecules is of fundamental importance for the comprehension of their stability, reactivity and lastly of their biological functionality. Relaxation processes present on proteic systems are distributed among a vast range of time scales that includes the global dynamics of the macromolecule (ms – μ s), fluctuations that involve localized chemical sites (ns – ps), rapid dynamics of charge or energy transfer (ps – fs). The physico-chemical properties of molecules depend essentially on the synergical action of these components that act on different time scales.

Direct or indirect information about multiscale dynamics of proteins can be obtained from a variety of spectroscopic techniques both magnetic (NMR, CW-ESR, ENDOR) and optical (FPA, DLS, TRSS). In particular, ESR measurements performed on proteic systems with SDSL can represent a reliable investigation technique. The study of their magnetically probed derivatives allows us in principle to collect a wealth of information about secondary structure, the inter-residuals distances and both the global and local motions of the protein. This richness of information is by now limited by two factors (1) the experimental difficulty to collect extensive data at various frequencies and (2) the necessity to employ advanced interpretative models that describe explicitly the molecular dynamics and that are computationally manageable.

The theoretical approach sketched in this review relies on the SLE, by means of the direct inclusion of molecular dynamics – or at least of some relevant degrees of freedom – under the form of stochastic operators (Fokker–Planck, diffusive) on the super spinhamiltonian that governs the temporal evolution of density matrix/conditional probability ρ of the system. In general it is possible to describe multiple relaxation processes and to take into account the presence of interacting magnetic probes inside the model, by a numerical solution of the SLE. The choice of the Γ operator allows to model the various relaxation processes of the system (*bodies*), each one characterized by an appropriate set of dissipative parameters.

In the last two decades, single and multi-body models have been developed. These models have initially been applied to simple liquids

and anisotropic phases; however, they have found recent and fruitful applications also in the interpretation of ESR spectroscopies in proteic systems. On the other hand, together with the potentiality of the general methodology, several limitations have emerged, that are ascribable to factors such as (1) the limited efficiency of the computational implementation, due to the disordered growth of the softwares developed during the years around an original core of Fortran codes and (2) the necessity to extend the available models in order to include the presence of additional degrees of freedom.

With this review we have presented, without the claim of exhausting the subject, a first systematic analysis of these problems, with the final goal to facilitate the application of developed methodologies and SDSL experiments preformed for different proteic systems.

Future works will surely foresee the extensions of the models to multi-body through the generalization of the modeling and of the software in order to include the presence of more interacting magnetic probes. Finally the creation of new formal tools for both the exact and approximated treatment of multi-bodies models will be central. Also, new directions will be surely taken into account in the dynamical description, adopting generalized coordinates and in the formalization of multi-spin hamiltonians, searching for the best computational route.

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Application of site-directed spin labelling for studying conformational changes in the catalytic cycle of G proteins activated by dimerization

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Site-directed spin labelling (SDSL) combined with electron paramagnetic resonance (EPR) spectroscopy is an efficient method to study the structure and the conformational dynamics of proteins. In particular, long range distance measurements (up to ~ 100 Å) between pairs of spin labels by pulse EPR methods enable quantitative analysis of conformational equilibrium dynamics and allow identification and characterization of conformational changes in the course of a proteins function. This review summarizes contributions SDSL EPR made to understanding of how the structure and dynamics of a specific group of GTP-hydrolyzing proteins, G proteins activated by nucleotide-dependent dimerization (GADs), change during their functional cycle.

1 Introduction

Proteins exhibit their function – acting as catalysts, sensors, signalling molecules, transporters, regulated channels or as mechanical effectors – by exhibiting a variety of conformational changes, *i.e.* they are dynamic entities. The structural alterations during the functional cycle of the molecules range from subtle side chain rearrangements, bringing, for example, specific charges into the right position to assemble a catalytic centre, to large-scale domain motions that, *e.g.*, govern intermolecular interactions or makes them act as “molecular motors”. In some cases crystal structures, obtained for enzymes in the presence of ligands/substrates, that carry mutations of *e.g.* catalytically active residues or that have been crystallized under specific conditions, provide snapshots of putative intermediate steps of a protein’s function. Nevertheless, a full mechanistic description of biological function requires knowledge not only about structure but also about dynamics.

In the last decade remarkable progress has been made in understanding the dynamic properties of proteins and how they are related to function, and a significant fraction of our current knowledge is based on investigations on nucleotide-hydrolysing enzymes (NTPases). This ubiquitous and diverse class of enzymes, mainly comprising ATP- and GTP hydrolysing proteins, is an ongoing subject of intense and widespread research due to its utmost medical relevance. ATP hydrolysing enzymes (ATPases) are the “motors” driving almost all kinds of cellular functions, including the translocation of various substrates across membranes by ATP binding cassette (ABC) transporters.¹ Proteins hydrolysing GTP play

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a major role in cell signalling and a vast number of other processes including regulatory functions, and have been implicated in human diseases like for example cancer, neurodegenerative disorders like Parkinson's and Alzheimer's disease, retinitis pigmentosa and certain cardiovascular diseases.

Significant contributions to understanding the structure and function of ABC transporters and of numerous other systems, and how dynamics play a significant role in the functional cycle of proteins come from the studies applying site-directed spin labelling (SDSL) in combination with (pulse) electron paramagnetic resonance (EPR) spectroscopy.² This review summarizes SDSL EPR studies that contributed to understanding of a specific group of GTPases, namely the so-called G proteins activated by dimerization (GADs),³ which have been carried out in the authors lab. It provides an introduction into the basic properties of GTPases and especially GADs, followed by a brief overview of the method of SDSL EPR, and in particular the inter spin distance measurements. In the main part the data obtained for three members of the GAD protein family is presented, namely the tRNA-modifying enzyme MnmE from *E. coli*, human GBP1 and the RocCOR tandem domain from the *C. tepidum* Roco protein.

1.1 GTP hydrolyzing proteins (GTPases, G proteins)

Proteins that bind guanosine-triphosphate (GTP) and catalyse the exothermic reaction to form the diphosphate form (GDP) by cleaving the terminal (γ)-phosphate group (and sometimes also forming the monophosphate by additional cleavage of the β -phosphate) are a large and ubiquitous class of enzymes. They usually act as signalling molecules but can also execute other enzymatic functions, using the energy released during GTP hydrolysis to, for example, modify a second substrate. Generally speaking, GTPases are molecular switches that cycle between “off” and “on” states, thereby controlling processes like cell growth and differentiation as well as vesicular and nuclear transport.⁴

Activation of the G proteins (switching “on”), *i.e.* the exchange of GDP for GTP, requires dissociation of bound GDP, which is a very slow process that is accelerated by accessory proteins called guanine nucleotide-exchange factors (GEFs). Switching “off” takes place by hydrolysis of the GTP to GDP. This process is also slow in most GTPases and therefore has also to be accelerated by another class of proteins named GTPase-activating proteins (GAPs).⁴ The resulting “classical” G protein cycle is depicted in Fig. 1A.

The best-well known representatives of “classical” signalling GTPases are the Ras (Rat sarcoma) proteins. Ras proteins are small (~21 kDa) G proteins, which are active when bound to GTP and inactive in the GDP bound state. The whole family of Ras-like small GTPases contains >100 members in 5 sub-families (Ras, Rho, Rab, Arf, and Ran) that function as molecular switches, thereby regulating multiple cellular functions like cell proliferation and differentiation, organization of the cytoskeleton, and intracellular membrane trafficking.⁴ The specificity of the different subfamilies relates to different intracellular localizations and interaction

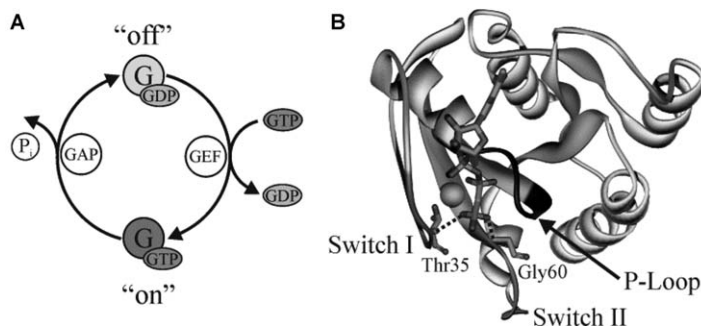


Fig. 1 (A) Scheme showing the classical G protein cycle involving accessory proteins for nucleotide exchange (GEFs) and GTP hydrolysis (GAPs). (B) Crystal structure of H-Ras p21 (pdb 5P21) in ribbon representation. The P-loop and the switch I and switch II regions are indicated. The GppNHP moiety is shown as sticks. The magnesium ion is shown as a sphere.

with distinct sets of effectors. Their common structure, the so-called G domain fold is depicted in Fig. 1B showing the structure of H-Ras p21⁵ with the structural motifs characteristic for this type of GTPases: (i) the conserved P-loop (phosphate binding loop), interacting with the β - and γ -phosphates of the nucleotide, and (ii) two segments called “switch” regions, which form two hydrogen bonds to the γ -phosphate oxygens by means of the main chain amino groups of conserved Thr and Gly residues in switches I (T35 in Ras) and II (G60), respectively, and thus exhibit structural transitions upon nucleotide hydrolysis (cleavage of the γ -phosphate).⁶ The conformational change taking place was described as a “loaded spring” mechanism where release of the γ -phosphate after GTP hydrolysis allows the two switch regions to relax into the GDP specific conformation.⁷

Ras GTPases have extremely high affinity for GDP and GTP with dissociation constants in the pico- to nano-molar range. Thus, exchange of GDP for GTP to “recharge” the GTPase after hydrolysis is a rare event and hence the intrinsic activity of the proteins is low. GEFs accelerate GDP dissociation and thereby facilitate reloading of GTP.⁸ For the GTP hydrolysis reaction a fully assembled catalytic site requires groups that (i) can stabilize the negative charge build-up at the β - or γ -phosphate, depending on whether the mechanism is dissociative or associative, and (ii) correctly position the nucleophilic water molecule in the hydrolysis transition state. In Ras GTPases a conserved switch II Gln residue is suggested to orient the nucleophilic water molecule for attack of the γ -phosphate group. The aforementioned group needed to stabilize the negative charge building up at the phosphate groups during the hydrolysis reaction is missing in Ras GTPases, but it can be provided in *trans* by their respective GAPs. Upon Ras-Ras·GAP complex formation a positively charged Arg residue (R789, named “arginine finger”) in Ras·GAP takes a catalytic position at the Ras nucleotide binding site. Remarkably, mutations that reduce the Ras GTP hydrolysis rate, thereby prolonging the active GTP-bound conformation and thus also the activation time of cell proliferation, are frequently found in a variety of cancers.⁹

1.2 G proteins activated by dimerization (GADs)

As previously discussed, Ras superfamily proteins need to form complexes with their cognate GAPs to complement and/or stabilize the active site, thereby increasing the rate of GTP hydrolysis by several orders of magnitude.^{7,10} Furthermore, nucleotide exchange needs to be accelerated by GEFs that strongly reduce nucleotide affinity. Thus, Ras GTPases are regulated by interaction with these two accessory proteins.

Members of a second group of GTPases, named G proteins activated by nucleotide-dependent dimerization (GADs),³ exhibit low nucleotide affinity, thereby rendering the need for GEFs to exchange GDP for GTP obsolete. Furthermore, these proteins “utilize” dimerization for reciprocal complementation of their active sites. GADs dimerize in a nucleotide-dependent manner where GTP binding appears to moderate the interaction between the two G domains. The mode of dimer formation varies between different GADs – as will be shown in this review – but the unifying principle is that each protomer provides residues for the active site of the other protomer, which either stabilize flexible parts of the partner or directly participate in catalysis, like an arginine finger. GADs are thus not regulated by either GEFs or GAPs as they seem to carry all elements needed for a nucleotide-regulated GTPase switching cycle. Figure 2A depicts the resulting scheme of the G protein cycle for GADs. Figure 2B shows active site complementation observed in the crystal structure¹⁸ of the dimer of the isolated G domains of MnME obtained in the presence of the transition state mimick GDP-AlF_x. MnME and two other representatives of the group of GAD proteins, hGBP1 and the RocCOR tandem domain found in Roco proteins, will be introduced in more detail in the following paragraphs.

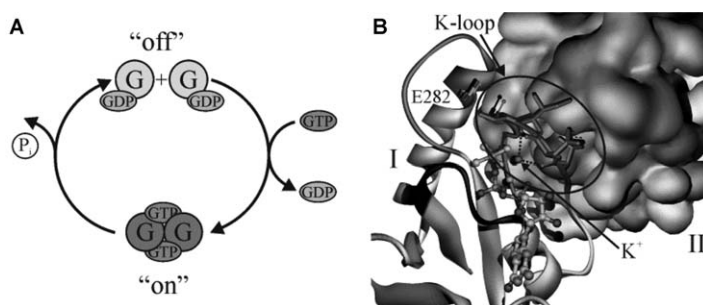


Fig. 2 (A) Scheme of the catalytic cycle of G proteins activated by nucleotide-dependent dimerization (GADs). They do not require GEFs due to their low (μM) affinity for nucleotides, and also do not need GAPs for activation of the hydrolysis reaction. Their GTPase reaction is triggered by dimerization across the nucleotide-binding site where the active site of one protomer is complemented by the other protomer. (B) Active site complementation in GADs. Depicted is the crystal structure of the isolated G domains of EcMnME in complex with GDP-AlF_x (shown in ball-and-stick representation) (pdb 2GJ8). Interaction with monomer II (shown as the molecular surface) orients the K-loop (backbone and side chains shown as sticks) in monomer I (ribbons) to position the K⁺ ion which takes the position of the positive charge of arginine fingers in Ras/Ras-GAP complexes. Furthermore dimerization positions a conserved Glu residue (E282) also implicated in catalysis by stabilizing or polarizing the attacking water molecule in the GTP hydrolysis step. For better comparability with Fig. 1B the P-loop of monomer I is also marked in black.

MnmE. MnmE is an evolutionary conserved GTPase found in bacteria, fungi and humans that catalyzes (in complex with another protein named MnmG/GidA) the formation of a carboxymethylaminomethyl-group (cmnm) at the 5-position of the so-called wobble uridine (U34) of certain tRNAs.¹² This modification assists to enhance the interaction with A and G, but suppresses base-pairing with C and U,¹³ and furthermore plays a regulatory role in gene expression.¹⁴ The human homologues of MnmE and MnmG, termed hGTPBP3 and Mto1,¹⁵ respectively, have been implicated in the development of severe mitochondrial myopathies such as MERRF (myoclenic epilepsy ragged red fibres), MELAS (mitochondrial encephalomyopathy lactic acidosis stroke) and nonsyndromic deafness.¹⁶

MnmE is a three-domain protein consisting of a N-terminal tetrahydrofolate-binding domain, a central helical domain, and a canonical Ras-like G domain inserted into the helical domain. As shown by several crystal structures of MnmE from different organisms, the protein forms a constitutive dimer *via* the N-terminal domains. The two G domains face each other with a distance of about 50 Å between the two catalytic sites (Fig. 3A).¹⁷

It has been postulated that nucleotide-dependent G domain dimerization across the nucleotide binding site activates the GTPase and the distinct biological functions of MnmE, based on biochemical data and the crystal structure of the isolated MnmE G domains in complex with the transition state mimic GDP-aluminium tri- or tetrafluoride (AlF_x).¹⁸ Nevertheless, despite significant efforts no crystal structure of the protein dimer with the two G domains being in a “closed” state could be obtained.¹⁹

hGBP1. Human guanylate binding protein 1 (hGBP1) belongs to the dynamin superfamily of large GTPases, that exhibit nucleotide-dependent oligomerisation, leading to stimulation of the GTPase activity.²⁰ Expression of hGBP1 is mainly activated by interferon γ .²¹ The protein has been shown to be involved in immune responses against viral and bacterial targets, *e.g.* hepatitis C virus, influenza A virus, and bacterial meningitis, and furthermore exhibits antiangiogenic and antitumor activities.²²

Interestingly, hGBP1 can bind all three guanine nucleotides and hydrolyzes GTP to GDP and GMP in two successive steps utilizing the same catalytic center.^{12,23} The elongated protein consists of three domains (Fig. 3B). The LG domain also resembles the canonical GTPase domain of Ras, but with insertions and extensions, and is followed by the purely α -helical middle and α 12/13 domains. The latter domain



Fig. 3 Structural models of (A) the MnmE dimer (pdb 1XZP), (B) hGBP1 (pdb 1F5N), and (C) the RocCOR tandem (model, based on pdb 3DPU) from *C. tepidum* Roco. The G domains are highlighted by gray shading.

comprises a long helix (α_{12}) which flanks the full length of the protein. At the C-terminal end the $\alpha_{12/13}$ domain interacts with the LG domain, stabilizing its position.²⁴ It was shown by other methods that binding of the non-hydrolyzable GTP analogue GppNHp causes the formation of hGBP1 dimers, and that binding of GDP $\cdot$ AlF_x leads to the formation of tetramers.²⁴ Oligomerization of hGBP1 leads to a self-stimulation of its GTPase reaction.²⁵

A crystal structure of the isolated LG domains with GppNHp¹¹ suggests that dimerization of hGBP1 takes place *via* the LG domains, and a putative head-to-head dimer model can be constructed by superimposing the LG domains of the full-length crystal structure onto the LG domains in the dimer structure. Furthermore, structural rearrangements observed within the LG domain upon dimerization are believed to be transmitted to $\alpha_{12/13}$, thereby controlling the oligomerization state of the protein and the second step of nucleotide hydrolysis.^{26,27} However, no detailed structural information on the full length protein in the dimeric or tetrameric state could be obtained so far.

The RocCOR tandem. Roco proteins are large multi-domain proteins that are characterized by the presence of a Ras-like G domain called Ras of complex proteins (Roc) that always occurs in tandem with a C-terminal of Roc (COR) domain.²⁸ Roco family proteins are found in bacteria, plants and animals, and four of them are identified in vertebrates, namely leucine-rich repeat (LRR) kinase 1 (LRRK1), LRRK2, death-associated protein kinase 1 (DAPK1) and malignant fibrous histiocytoma amplified sequences with leucine-rich tandem repeats (MASL). In these proteins, the RocCOR tandem is always preceded by an LRR domain. The human proteins LRRK1 and LRRK2 comprise, in addition to the RocCOR tandem and the N-terminal LRR domain, a C-terminal kinase domain. DAPK1, only found in metazoans, exhibits a tumour-suppressor DAPKs domain. Although there is wide variation in the architecture of the Roco proteins, previous studies suggest that the function and structure of the catalytic core is conserved.²⁹ The most prominent member of the Roco protein family is LRRK2 that has been found to be mutated and activated in cases of familial Parkinson's disease.³⁰

Bacterial Roco proteins as well as presumably also LRRK2 form an active dimer *via* the COR domains.³¹ Crystal structures of, for example, the *Chlorobium tepidum* RocCOR (*CtRocCOR*) tandem domain³² (Fig. 3C) reveal a COR domain dimer in which the N-termini interact with the – between vertebrates and bacteria highly conserved – Roc domain of the same protomer. The less conserved C-termini function as the dimerization device. Consistent with other GADs, dimerization is essential for GTPase activity, and some bacterial Roco proteins use an arginine finger of one monomer to complete the catalytic machinery of the other monomer.

However, the present crystal structures were only solved for nucleotide-free and GDP-bound states of the proteins, and the exact mechanism of the Roc G-protein cycle is still not well understood.

2 Site-directed spin labelling and EPR spectroscopy

Introduced by Wayne L. Hubbell in the late 1980's,³³ site-directed spin labelling (SDSL) combined with electron paramagnetic resonance (EPR) spectroscopy became a very powerful technique to elucidate the structure and conformational dynamics of biomolecules. The technique can be applied to soluble as well as membrane proteins and nucleic acids, and the size and complexity of the system under investigation is almost arbitrary.^{34,35} Furthermore, sample conditions (*e.g.* temperature, buffer composition and sample concentration) usually can be chosen much closer to the physiological state of the system compared to other structural techniques like X-ray crystallography, NMR or cryo-EM, or the experiments can even be carried out under *in vivo* conditions.³⁶ These advantages render SDSL EPR the most flexible and widely applicable tool for investigation of protein structure and conformational dynamics.

Spin labels can be introduced at specific sites within proteins using various coupling schemes,³⁵ but by far the most common method works by cysteine substitution mutagenesis followed by modification of the sulfhydryl group with a nitroxide reagent. The most widely used spin label is the methanethiosulfonate spin label ((1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate, MTSSL (Fig. 4A). In the case of nucleic acids, the spin label can be attached either *via*

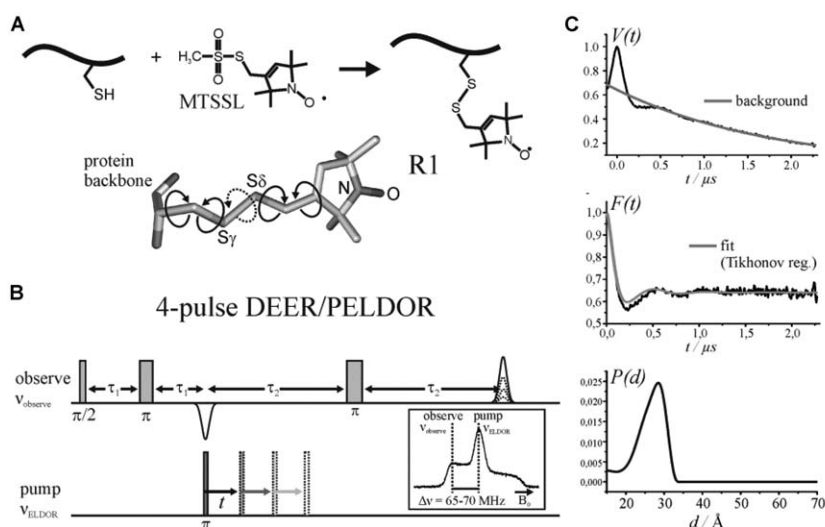


Fig. 4 Site-directed spin labelling (A) Reaction of the methanethiosulfonate spin label (MTSSL) with the sulfhydryl group of a cysteine side chain, generating the spin label side chain R1. The flexible bonds within the R1 side chain (stick representation) are indicated by curved arrows. (B) The DEER experiment. 4-Pulse sequence and excitation scheme (inset, shown is an echo-detected field swept EPR absorption spectrum, positions in the spectrum where pump and observer pulses are set are indicated). (C) DEER data analysis. Primary data is the echo amplitude as a function of the pump pulse position, $V(t)$, that is modulated with the dipolar frequency(ies). Background correction removes contributions from surrounding molecules in the sample, resulting in the DEER form factor, $F(t)$. Tikhonov regularization yields the inter spin distance distribution, $P(d)$.

nucleobases modified with a reactive group, or can be attached at the sugar moieties during oligonucleotide synthesis.

EPR spectroscopy of spin labelled molecules yields information about the spin label side chain mobility, its solvent accessibility, the polarity of its immediate environment, and intra- or intermolecular distances between two spin labels.^{34,35} Inter spin distance measurements for a series of labelled protein variants allows for example modelling of the protein structures with a spatial resolution at the level of the backbone fold, but can also be used for detecting and characterizing conformational changes occurring upon protein activation *e.g.*, by trapping the molecules in activated or intermediate states.³⁷ The use of continuous wave (cw) and foremost pulse EPR spectroscopy for distance measurements is described in the following.

Inter spin distance measurements

In EPR spectroscopy, two basic approaches are used to determine the dipolar spin–spin interaction. First, if the interspin distance is less than about 20–25 Å, the static dipolar interaction in an unordered immobilized sample, *i.e.* in frozen or highly viscous solution, considerably broadens the cw EPR spectrum and can be quantified by a detailed line shape analysis. Distances in the range from 15 Å up to about ~80 Å can be measured using pulse EPR methods. The range of accessible distances in these experiments can be extended >100 Å, if fully deuterated samples are used. Two major protocols exist, namely the most commonly used four-pulse double electron–electron resonance (DEER),³⁸ also called pulse electron double resonance (PELDOR), and Double Quantum Coherence (DQC).³⁹

In DEER/PELDOR spectroscopy the signal from which the distance information can be derived is a modulation of the echo amplitude of one spin population (observer spins) caused by dipolar interaction with another spin population (pumped spins), which has been excited by a strong microwave pulse of different microwave frequency (Fig. 4B). The frequency of oscillation of this modulation is given by the dipolar frequency ω_{DD} , which is proportional to the inverse cube of the inter spin distance r . DEER/PELDOR experiments are routinely performed at X- (~9 GHz) or Q-band (~35 GHz) frequencies. Evaluation of the data – usually by deconvolution methods, which represent model free approaches, like the most commonly used Tikhonov regularization – yields an inter spin distance distribution.⁴⁰ In DQC all pulses are applied at the same frequency but with high intensity in order to excite the whole spectral distribution of spins. Data analysis is performed using the same approaches used for DEER/PELDOR as the resulting data contains the same information.

Thus, the combination of cw and pulse EPR techniques provides means to determine inter spin distances in the range from ~10 to >100 Å, thereby covering the most important distance regime necessary for structural investigations and for detecting conformational changes in proteins and protein complexes.

Interpretation of inter spin distances in terms of molecular structure

The use of distance constraints from EPR spectroscopy is complicated due to the length and flexibility of the spin label side chain (see Fig. 4A). The distance between the C α atom and the NO group of the spin label can vary by up to ~ 8 Å, depending on the conformational freedom of the label side chain at the attachment point. Therefore the distances obtained from the experiment can differ significantly – in both directions – from the corresponding C α –C α distances, that could directly provide information about the protein's secondary and tertiary structure. To take the length and flexibility of the spin label side chain into account different approaches can be used. If a structural model for the labelled molecule is available, molecular dynamics (MD) simulations with the structure with spin labels attached to it can be performed to sample the conformational space of the label and (partly) of the protein. Monte Carlo methods vary the torsional angles of the spin label stochastically and are often combined with MD simulations to account also for dynamics of the protein. Both approaches are computationally demanding. Alternatively, and computationally much less “expensive”, rotamer libraries can be used to represent the conformational space of the spin label side chain. In this approach, a discrete set of possible conformations, provided by a library containing the energetically most favourable spin label conformers that have been derived from MD and/or Monte Carlo simulations, is used.⁴¹ In the examples presented in the next section the rotamer library implemented in the software package MMM⁴¹ was used. It consists of 210 rotamers of MTSSL bound to cysteine. These rotamers are used to replace the native residues at the positions of interest in a structural model of the protein/system. Energies and resulting populations for the individual rotamers are calculated using a Lennard–Jones potential, and are considered as weights in the simulation of the distance distributions. For more details about the rotamer library analysis (RLA) approach see Polyhach *et al.*⁴¹ Rotamer libraries represent the fastest method to account for the spin label conformational flexibility and it has been shown that the accuracy of this method is comparable to that of the more sophisticated sampling techniques mentioned before.⁴²

With a structural model at hand, these approaches provide the computed distance distributions between the spin labels that can be compared to the experimental distance distributions to validate or disprove the structural model and to identify conformational changes. Discrepancies between calculated and experimental distances can be used for refinement of the model. Alternatively, distance distributions between the respective backbone atoms can be computed from inter spin distances, providing means not only for validating structural models but also for *de novo* structure prediction.⁴³

3 Conformational changes in the catalytic cycle of GADs

In this section the results of DEER/PELDOR distance measurements on spin labelled variants of the three members of the GAD protein family

introduced above, MnME, hGBP1 and the RocCOR tandem domain from a bacterial Roco protein, are presented. For MnME and RocCOR, conformational changes in the course of the GTPase cycle are investigated by trapping the GTP-bound form (with GppNp), the hydrolysis transition state (with GDP- AlF_x), the GDP-bound state (after hydrolysis and release of the cleaved phosphate group), and the apo state (appearing during nucleotide exchange). The study on hGBP1 is focused on GTP-induced dimerization of the protein and the conformational changes taking place during this process.

3.1 Large-scale G domain motions in MnME

To test whether an “open” G domain arrangement found in GDP- and GppCp-bound structures¹⁹ of MnME represents the conformation found in solution and furthermore to structurally characterize the putative transition state with closed G domains, Meyer *et al.*¹⁹ carried out DEER distances measurements between nitroxide spin labels in the G domains of full-length *Ec*MnME in the different steps of the GTPase cycle.

Single labelling positions (MTSSL) in this study are E287, close to the top of the G domain ($\text{G}\alpha 2$), S278 in switch II, and D366 at the bottom of the G domain ($\text{G}\alpha 5$), as well as I105 in the N-terminal dimerization domain, resulting in the introduction of two symmetry-related spin labels in the MnME dimer (Fig. 5A). All positions chosen for cysteine substitutions are non-conserved, surface-exposed residues. Mutant proteins assayed for K^+ -stimulated GTPase activity with and without attached MTSSL show no impairment of GTPase activity in comparison to wild type.

Figure 5B shows the results of the DEER measurements, where the left panel shows inter spin distance distributions obtained by Tikhonov regularization of the background-corrected dipolar evolution data shown in the right panel. The results for S278R1 in switch II reveal broad distance distributions ranging from 25 to 50 Å for apo- and GDP-bound MnME, with increasing probabilities for larger distances. Possible explanations for such a broad distance distribution could be that the labelled position is located in a loop region, which is flexible in the free and GDP bound states, and/or that the spin label side chain itself is hardly restricted in its conformational space. Nevertheless, the most pronounced distances between 40–50 Å as well as the minor fractions between 30 and 40 Å observed by DEER are in strong agreement with an open state of the G domains as observed in the apo and GDP-bound crystal structures.

E287R1 close to the top of the G domain in $\text{G}\alpha 2$ reveals one major peak centred at a distance of 55 Å for the apo- and 53 Å for the GDP bound state, corresponding well to the $\text{C}\beta$ – $\text{C}\beta$ distance of 53 Å in the *T. maritima* MnME crystal structure. For D366R1 well defined inter spin distance distributions centred at 67 Å in the apo state and 65 Å in the GDP bound state are observed. Also here, the agreement with the inter-atom distances obtained from different crystal structures (62 Å for *Tm*MnME, 63 Å in *No*MnME-GDP) is good, suggesting an open conformation of the G domains in the “off” states of MnME. From the well defined distance distributions for E287R1 and D366R1 it can be further concluded that the

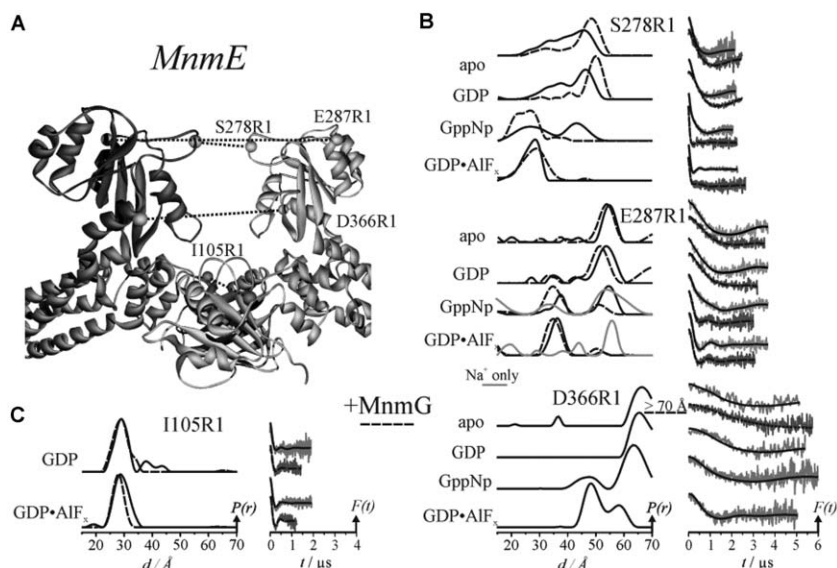


Fig. 5 Nucleotide-dependent G domain motions in MnME and MnME/MnmG. (A) View onto the N-terminal and G domains of MnME with label positions indicated by spheres at the C α positions of the respective residues. (B) DEER data recorded at X band for spin labels located in the G domain of MnME without nucleotide or in the presence of either GDP, GppNp or GDP·AlF $_x$. Left: distance distributions, $P(d)$, obtained by Tikhonov regularization (solid lines). Right: Corresponding background-corrected dipolar evolution data, $F(t)$. Data obtained in the presence of MnMG are indicated as dashed lines. Distance distributions shown in gray have been obtained for samples with sodium instead of potassium. The form factor for the two experiments performed with sodium-only buffer are not shown but largely resemble those shown for the apo and GDP-bound state of this mutant. (C) DEER data obtained for MnME spin labelled in the N-terminal domain (I105R1). Distance distributions shown in dashed lines have been obtained in the presence of MnMG and correspond to the form factors shown in dark gray.

MnME G domains adopt defined conformational states rather than a large continuum of conformations with different G domain orientations.

In contrast, the distance distributions for the GppNHP bound state reveal two fractions with different inter spin distances for all three label positions. The larger distance (E287R1: 55 Å, D366R1: 63 Å and S278R1: 43 Å) characterizes the open state of the G domains as observed for the apo and GDP-bound forms. The shorter distance (E287R1: 37 Å, D366R1: 47 Å and S278R1: 27 Å), contributing about 30% to the distance distribution, indicates the presence of a second conformation, where the two G domains are in close proximity. In the crystal structure of the AlF $_x$ -complexed G-domain dimer, the C β –C β -distances of the S278- and E287-pairs are shorter (18 Å and 28 Å, respectively) as compared to the GppNp DEER data, however, the spin label side chain itself has an average length of 7 Å between the nitroxyl-radical and the C β -atom, what can easily account for the observed differences for a pair of R1 side chains. Thus, the results obtained for the GppNp bound form indicate the presence two conformations, an open state and a closed conformation, in thermal equilibrium.

In the presence of the transition state mimic GDP·AlF $_x$, S278R1 and E287R1 again show single population maxima, with inter spin distances

of 28 and 36 Å, respectively. Again this is close to the C β –C β distances in the crystal structure of the GDP- AlF_x -bound G domain dimer structure (see above) and therefore in line with a closed conformation of the G domains. For position D366R1 the distance distribution exhibits two major fractions with distances of 58 and 48 Å, presumably due to two different rotamer populations of the spin label side chain. It is obvious from differences in the distance distributions for the GppNp and the GDP- AlF_x -state that the structure of the closed state in the presence of GDP- AlF_x slightly differs from that in the presence of GppNp. This suggests that on the reaction pathway from the triphosphate bound state to the hydrolysis competent conformation further rearrangements in the active site of the G domains take place.

For a spin label at position I105 in the N-terminal domain a comparison of the distance distributions obtained for the GDP state (open conformation, “off”) and GDP- AlF_x state (closed conformation, “on”) does not reveal any significant differences, indicating that closing of the G domains does not significantly influence the constitutive dimer formed *via* the N terminal domains. Deviations from the corresponding C β –C β distance in the available dimer crystal structures (36–37 Å) are likely due to spin label rotamer conformations.

MnmE/MnmG. To obtain insights into the influence of complexation with MnmG, distance measurements on the same set of spin label mutants of MnmE in the presence of *Ec*MnmG/GidA in the different steps of the GTPase cycle have been carried out.⁴⁴

Figure 5C shows that for position 105 in the N-terminal dimerization domain in the GDP bound state no significant changes can be detected. Thus, the overall arrangement of the MnmE dimer is not significantly influenced by MnmG. Nevertheless, the observed narrowing of the distance distribution in the presence of GDP- AlF_x indicates reduced conformational dynamics of the constitutive dimerization interface in the assembled and active catalytic MnmE/MnmG machinery.

In contrast, the G domains of MnmE are in part strongly affected by the presence of MnmG (Fig. 5B). A significantly narrower and more defined distance distribution is observed for a spin label at position S278 in the apo and GDP-bound states. In addition, the mean distance is shifted towards longer distances. This observations suggest that switch II becomes significantly more ordered and presumably somehow “attached” to other secondary structure elements of the G domain. With GppNp bound the addition of MnmG clearly shifts the equilibrium between “open” and “closed” conformations towards the closed state. In the GDP- AlF_x bound state the distance distribution becomes broader and slightly shifted to longer distances in the presence of MnmG, indicating a likewise but different arrangement of switch II. The distance distributions for position 287 appear to be hardly affected by the presence of MnmG – with the exception of the GppNp bound state, where the shift of the equilibrium towards the closed conformation is also clearly observed. For position 366 the inter spin distances in the presence of MnmG appear to be shifted to significantly longer distances, being beyond the detection

limit of the given experiment ($>70 \text{ \AA}$) although deuterated buffer was used for sample preparation.

Interestingly, the effects of MnmG on the MnmE G domains are even more pronounced in the absence of potassium that has been shown to significantly increase GTPase activity. DEER data collected in the absence of potassium, but with other monovalent cations of different size reveal that the formation of closed G domain conformations depends on the presence of ions of a specific size, in line with biochemical data and with identification of a potassium ion bound to the K-loop within switch I in the G domain dimer crystal structure. The K-loop is a sequence (*Ec*MnmE: T245-A248) that has been identified to be responsible for binding of a potassium ion *via* the main chain carbonyl oxygen atoms of T245 and I247.¹⁸ In the presence of sodium instead of potassium (Fig. 5B, gray lines), the DEER distance distributions reveal hardly any indications for the presence of the closed state. However, complexation with MnmG appears to abolish this requirement of K^+ for dimerization of the G domains and the hydrolysis reaction, although K^+ is much more potent in stimulating GTP-hydrolysis than MnmG.⁴⁴

3.2 GppNp-induced dimerization of hGBP1 involves α -13 association

To investigate how dimerization of hGBP1 upon binding of GppNp takes place, Vöpel *et al.*⁴⁵ spin labelled, separately, two positions in the LG domain, C225 on helix $\alpha 4'$ and N18 on the opposite side of the domain (Fig. 6A). Furthermore, based on the proposed involvement of $\alpha 12/13$ in dimerization, two positions in $\alpha 13$, K567 and Q577, where subjected to SDSL. DEER experiments performed with the singly labelled hGBP1-C225R1 without any nucleotide or in the presence of GDP (Fig. 6B) reveal, as expected, the complete absence of dipolar modulations. The raw DEER trace, $V(t)$, can be completely fitted with a background function. This is in accordance with the protein being monomeric as suggested by the gel filtration experiments.²⁴

Contrarily, in the presence of GppNp (Fig. 6C) well-defined inter spin distance distributions are obtained that indicate that the protein oligomerizes under these conditions. The distance distribution for position 225 exhibits a single peak at $40 \text{ \AA}$, whereas labels at position 18 on the opposite side of the LG domain exhibit a mean inter spin distance of about $54 \text{ \AA}$. Small peaks at shorter distances in the distance distribution for N18R1 are noise artefacts that are not reproducible. These experimental distances differ by $+5 \text{ \AA}$ (C225R1) and $+9 \text{ \AA}$ (N18R1) from the $C\alpha$ - $C\alpha$ distances calculated from the crystal structure of the isolated LG domains with GppNp,²⁶ but already indicate that dimerization of hGBP1 might indeed take place *via* the LG domains as suggested from the crystal structure. Naturally, when comparing inter spin distances with structural models the length and flexibility of the spin label side chain (see Fig. 4A and inset in Fig. 6) has to be taken into account as described in the previous section. A rotamer library analysis (RLA)⁴¹ with the crystal structure of the isolated LG domain dimer reveals good agreement with the experimental data, indicating that the

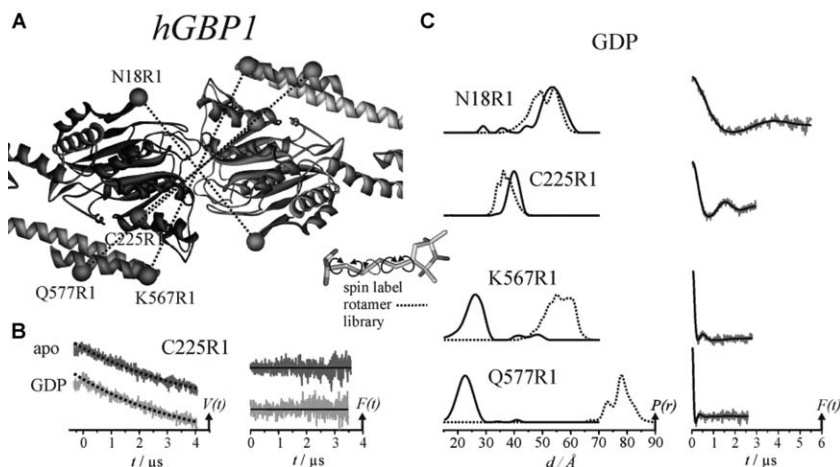


Fig. 6 GppNp-induced dimerization of hGBP1. (A) Putative hGBP1 dimer model obtained by superimposing the LG domains of the full-length crystal structure in the GppNp bound state (pdb 1F5N) onto the isolated LG domain dimer structure also obtained in the presence of GppNp (pdb 2BC9). Label positions are indicated by spheres at the α positions of the respective residues. (B) DEER data for the apo and GDP state for hGBP1-C225R1. Left: raw dipolar evolution data, $V(t)$, and respective background fits (broken line). In both cases, $V(t)$ can be entirely fitted with a background function, as can also be seen from the residuals shown in the right hand panel. (C) DEER data for label positions in the G domain (N18R1, C225R1) and in helix α 13 (K567R1, Q577R1). Left: distance distributions, $P(d)$, obtained by Tikhonov regularization (solid lines). Right: Corresponding background-corrected dipolar evolution data, $F(t)$. The results of the rotamer library analysis on the dimer model shown in panel A are shown as dotted lines.

LG domains exhibit an orientation in the hGBP1 dimer resembling the crystal structure dimer.

Using the thereby supported arrangement of the LG domain dimer as a template for constructing a model of the full-length dimer and performing the RLA on this model for positions 567 and 577 reveals calculated distance distributions centred at ~ 57 Å and ~ 78 Å, respectively (Fig. 6C, dotted lines). Strikingly, the DEER distance distributions (Fig. 6C, solid lines) exhibit significantly shorter distances – 26 Å for K567R1 and 22 Å for Q577R1 – indicating a substantially different arrangement of the N-terminal end of α 12 and of helix α 13. The short distances observed can only be explained assuming that α 12 and α 13 detach from the LG domain, thereby bringing the two α 13 helices into close vicinity, what would allow for an association to take place. This notion appears to be in line with the observations that in the presence of GDP- AlF_x helix α 4' in the LG domain undergoes a conformational change leading to a steric clash with α 12/13, and that point mutations distort the contact site between the LG domain and α 12/13.^{12,27} The results of this study⁴⁵ indicate that an uncoupling between α 4' and α 12/13 occurs already upon dimer formation in the presence of GppNp.

Based on the two DEER distance constraints a model of two interacting α 13 helices was constructed and the stability of such helix dimer evaluated and confirmed by molecular dynamics (MD) simulations. An RLA performed on such α 13 dimer yields for K567R1 a mean

distance ~ 7 Å shorter compared to the experimentally determined one, but a more distant location of the N-terminal ends of the helices is expected from their connection to $\alpha 12$, creating additional constraints on the helices. A remarkably good match of the experimental data with the RLA is observed for Q577R1, corroborating the notion that the two $\alpha 13$ helices in the hGBP1-GppNp dimer associate, forming a short parallel coiled-coil structure.

Notably, DEER experiments are carried out in frozen solution (50 K), always raising the question whether the observed state of the system under investigation is of physiological relevance (*i.e.* the state is populated also at physiological temperatures $\approx$ room temperature). In this study, quantitative FRET distance measurements by eTCSPC of hGBP1 labelled with fluorescent dyes at two of the spin label positions, N18 or Q577, carried out at room temperature, corroborated the observations made by DEER.⁴⁵ Moreover, the combination of DEER and fluorescence spectroscopy based inter label distance measurements enabled to identify two conformers of the hGBP1 dimer. A major state ($\sim 90\%$ populated) characterized by formation of the helix $\alpha 13$ dimer, and a low populated state where positions 567 and 577 exhibit significantly larger inter label distances (FRET: 67 Å for position 577; DEER: ~ 40 –50 Å for K567R1, deduced from reproducible minor contributions in this distance range, see Fig. 6C) that appear to be in line with the modelled dimer structure (Fig. 6A).

3.3 Conformational heterogeneity of the G domains in RocCOR

A study by Rudi *et al.*⁴⁶ follows structural changes in CtRocCOR (in the following: RocCOR) that occur upon binding of different nucleotides using three positions within the G domain (Roc) for spin labeling (Fig. 7A): T476 (close to the P-loop), S542 (close to the switch II region), and the native cysteine at position 600. Furthermore, to verify the assumption that the COR dimer serves as a rigid scaffold for the two moving Roc domains, a spin-label side chain was introduced at position S928 at the 'top' of the COR domains. None of these variants displayed significant impairment of its GTPase activity in comparison to the wild-type.

The results of the DEER distance measurements for these four variants are shown in Figure 7B and 7C. For position 928 in the COR domain only minor changes are observed in the distance distributions recorded in the presence of different nucleotides and in the apo state, indicating that no significant conformational changes take place in the COR dimer. Calculated inter-spin distance distributions for comparison with the experimental results obtained from a structural model for the RocCOR dimer⁴⁶ are obtained by applying an RLA approach⁴¹ to account for the dynamics of the spin-label side chain, combined with a short (16 ns) molecular dynamics simulation to consider also small-scale protein backbone dynamics and especially side chain motions around the spin label. The results of RLA analyses on snapshots equally distributed over the MD trajectory (MD-RLA) are averaged to obtain the final (calculated) distance distributions shown as dashed lines in the distance distributions in Fig. 7B and 7C. The MD-RLA distance distribution for S928R1 exhibits one broad peak that clearly differs from the experimental

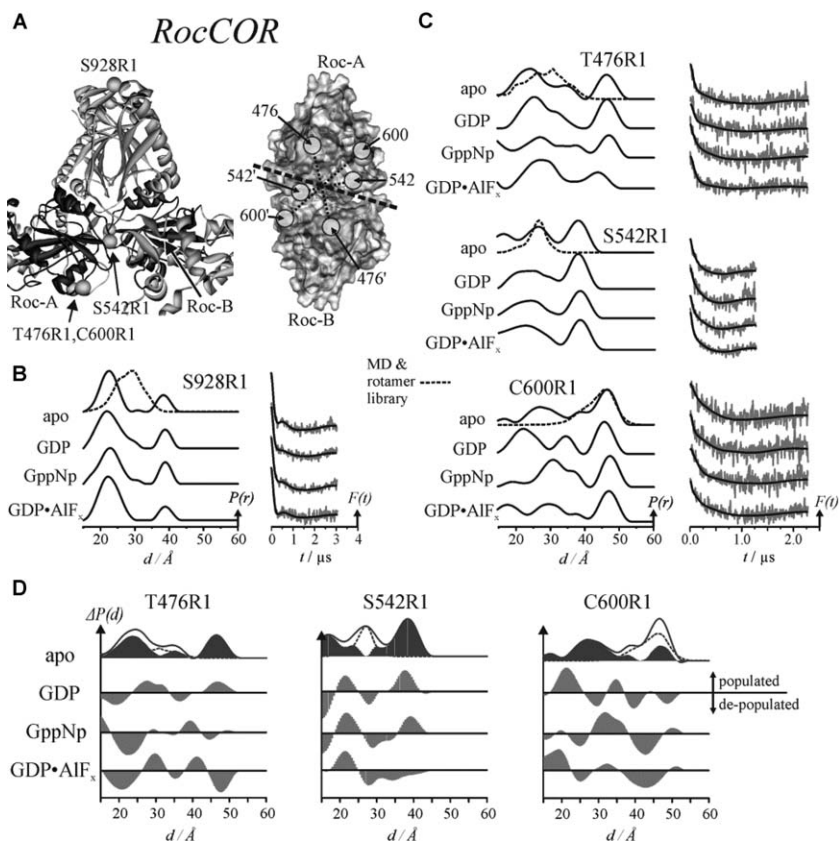


Fig. 7 Inter spin distances in CtRocCOR. (A) Model of the RocCOR dimer (side view and bottom view) indicating residues replaced by cysteine (except for Cys600) and subsequently labelled with MTSSL by spheres at the positions of their $C\alpha$ atoms. In the bottom view the interface between the two Roc domains is marked by a dashed line. (B) DEER data recorded at X band for CtRocCOR-S928R1, located in the COR domain. Left: distance distributions $P(d)$ obtained by Tikhonov regularization (solid lines) and by MD-RLA (see text) of the dimer model shown in panel A. Right: Background-corrected dipolar evolution data $F(t)$. (C) DEER data for CtRocCOR spin labelled at positions in the Roc domain: T476R1, S542R1 and C600R1. (D) Difference distance distributions $\Delta P(d)$. From left to right: $P(\text{apo}) - P(\text{MD-RLA})$, $P(\text{GppNp}) - P(\text{apo})$, $P(\text{GDP-AlF}_x) - P(\text{apo})$ and $P(\text{GDP}) - P(\text{apo})$. The $P(\text{apo}) - P(\text{MD-RLA})$ plots are shown together with both experimental $P(d)$ s. The difference amplitudes have been scaled for better visualization.

distance although approximately the same distance range is covered. From this observation a more tight arrangement of the COR dimer in solution was concluded, which could be explained by the observed crystal contacts that one COR domain is involved in.²¹ Nevertheless the invariability of the distance distributions in all cases confirms that the COR domains serve as a stable dimerization device independent of binding of the different nucleotides.

The DEER distance distributions for the three spin-label mutations in the Roc domain (Fig. 7C) obtained for the apo state are all broad and characterized by multiple peaks. The MD-RLA also predicts broad and multimodal distance distributions from the structural model, but the exceptional widths of experimental distributions indicate additional

conformational heterogeneity of the Roc domain dimer. Thus, the Roc domains in the model represent only one “snapshot” of a conformational ensemble for the G domains in the RocCOR tandem, explaining the absence of electron density for the one of the Roc domains in the crystal structure and further supporting the suggestion that the Roc domains are highly mobile entities and that the other Roc domain is only visible due to stabilizing crystal contacts.³²

To identify additional conformational states of the RocCOR dimer, difference distance distributions $\Delta P(d)$ (Fig. 7D, upper row) are calculated by subtracting the $P(d)$ of the MD-RLA from that of the (experimental) apo state. Dark areas indicate the remaining contributions that can be attributed to conformational states different from the crystal structure but present in the sample. The shape of the “remaining” distance distribution indicates the presence of at least two additional conformations, most obvious for position 542 where $\Delta P(d)$ exhibits two clearly distinct populations. This implies that the Roc domains in the RocCOR dimer in solution are characterized by at least three conformational states in equilibrium.

The distance distributions observed in the presence of GppNp, GDP- AlF_x or GDP exhibit clear differences to the apo state and in between them, but the conformational heterogeneity appears to prevail in all steps of the GTPase cycle. Difference distance distributions (Fig. 7D) obtained by subtracting $P(d)$ for the apo state from $P(d)$ obtained in the presence of the respective ligand indicate possible shifts in the occupancy of these states that are also reflected in changes of the means of the experimental distance distributions. For S542R1 in the dimer interface of the two Roc domains slightly increased mean distances are observed upon binding of GppNp (+0.5 Å) and GDP (+1.2 Å), whereas GDP- AlF_x binding leads to a slight decrease (−1.3 Å). Similar observations are made for the other two label positions in the Roc domain. Closer inspection of the distance distributions and $\Delta P(d)$ plots reveals that this can be explained by depopulation of the conformational state characterized by the crystal structure and increased population of other conformational states, e.g. characterized by distance distributions with mean distances of ~20 and 40 Å, respectively, for S542R1. Remarkably, in the GppNp- and GDP-bound states the population of these two state states increases, whereas in the presence of GDP- AlF_x only the state with short inter spin distances displays an increased population.

Further conclusions are drawn from the width of the short-distance peak observed in the distance distribution for S542R1 that appears to be significantly decreased compared with the distribution width in the apo state. A significantly reduced distance distribution width indicates reduced backbone and/or spin label dynamics, which could be characteristic for a fully assembled active site for GTP hydrolysis, bearing in mind that a putative arginine finger, Arg543,³² is located right next to the spin label position. The width in the GppNp- and GDP-bound states is further decreased compared to that for GDP- AlF_x , but the mean distances are about 2 Å larger. Taken together, these observations imply that nucleotide binding in general shifts the conformational equilibrium towards stabilization of the Roc dimer in the RocCOR tandem.

Rudi *et al.* further investigated spin labelled RocCOR constructs carrying mutations located at the interface between Roc and COR analogous to mutations in the most prominent member of the Roco family, LRRK2, that were shown to be implicated in late-onset Parkinson's disease. Remarkably, these mutations had significant influence on all inter spin distance distributions, thus influencing the structure of the Roc dimer and the nucleotide induced conformational changes taking place in the course of the GTPase cycle. This influence on the conformational equilibrium the Roc dimer is involved in appears to be the cause for the decreased GTPase activity observed for such mutants.⁴⁶

4 Discussion

The tRNA-modifying GTPase MnmE forms a constitutive dimer where the two G domains face each other (Fig. 8A). DEER inter spin distance measurements with *Ec*MnmE spin labelled at positions in the G domain and in the N-terminal dimerization domain,¹⁹ reveal that the MnmE GTPase cycle is characterized by an “open” conformation in the inactive states (apo and GDP-bound) and a “closed” conformation additionally formed in the presence of GTP. GTP hydrolysis appears to shift the equilibrium between these two conformations almost completely to the “closed” state. Strikingly, in the MnmE/MnmG complex the propensity of the MnmE G domains to form an active dimer increases, suggesting that MnmE is to be considered to be a so-called GTPase co-stimulator, as defined by Gasper *et al.*¹¹ The stimulation mechanism of MnmG differs from that of GAPs for the Ras like GTPases: (i) MnmG does not only interact with the GTP-bound form of the G proteins like classical effectors. This is shown by the DEER experiments that reveal that MnmG also

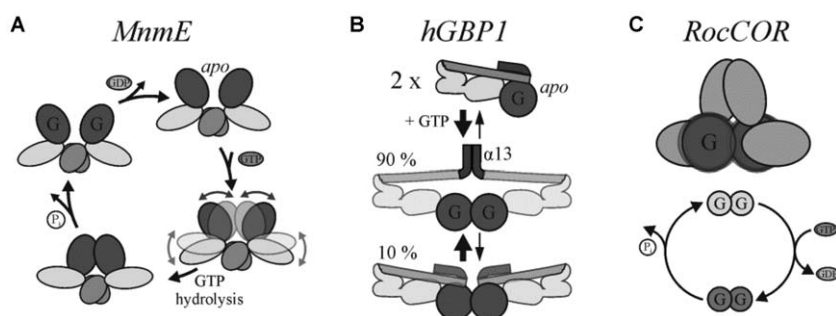


Fig. 8 Overview of nucleotide-induced conformational changes in GADs based on SDSL EPR. (A) The constitutive dimer MnmE exhibits large scale G domain motions with an “open” conformation in the apo and GDP-bound form, and a “closed” state starting to be populated upon GTP binding. GTP hydrolysis stabilizes the closed conformation. G domain movements appear to be coupled to motions of the helical domains according to SAXS data (see text). (B) GTP induces dimerization of hGBP1 via the G domains. The hGBP1 dimer is involved in a conformational equilibrium, where in ~90% of the dimers $\alpha 12/13$ detaches upon GTP binding. GTP hydrolysis stabilizes the closed conformation. (C) The Roc domains in the constitutive RocCOR tandem dimer remain associated throughout the GTPase cycle. GTP hydrolysis and nucleotide exchange are facilitated by small-scale alterations in a complex conformational equilibrium the constitutive G domain dimer is involved in.

influences the apo and GDP-bound conformations of MnmE, and (ii) it does not supply a catalytic residue into the G protein's active site. Otherwise, it would have been able to increase GTPase activity to a comparable extent as a potassium ion bound to the K-loop does.

The G domain motions observed for MnmE in the course of the GTPase cycle (Fig. 8A) suggest that this large-scale conformational change induces further rearrangements in the MnmE/MnmG complex to facilitate modification of the bound tRNA. Indeed, recently published SAXS-based models of MnmE and MnmE/MnmG in different nucleotide-states suggest that dimerization of the MnmE G domains is coupled to an upward movement of the wing-like helical domains (see Figs. 3 and 8A).⁴⁷ How these conformational changes could “drive” the modification reaction in the assembled MnmE/GidA/tRNA complex still remains to be elucidated.

hGBP1 is, contrary to MnmE, a monomer in the apo and GDP-bound states, but dimerization also takes place upon GTP binding and *via* the G domains, where active site complementation takes place (see Fig. 2B) *via* appropriate positioning of the arginine finger R48 by the second protomer in the complex. Combined results from DEER and FRET inter label distance measurements identify two conformers of the hGBP1 dimer (see Fig. 8B). One (90%) appears to be the “fully assembled” dimer characterized by the formation of an additional binding interface by helix $\alpha 13$. The minor fraction lacks this additional interaction. Remarkably, hGBP1 is farnesylated at the end of helix $\alpha 13$ at a “CaaX” motif *in vivo*.⁴⁸ Thus, dimerization of helices $\alpha 13$ (Fig. 8B) in the major fraction of the GTP bound proteins brings two lipid modifications as well as polybasic sequences directly adjacent to the CaaX box into close vicinity, thereby significantly increasing membrane affinity.⁴⁹ This strongly suggests that membrane association of hGBP1, that has already been proposed to be regulated by dimerization,^{27,49} could be permitted by association of the $\alpha 13$ helices. Noteworthy in this context, two other isoforms, hGBP2 and hGBP5, which are isoprenylated *in vivo* form not only homo- but also heterodimers,⁴⁹ suggesting dimerization of the C-terminal “membrane-association” domains to be a general and maybe regulatory feature for membrane localization and physiological function of this subclass of G proteins. For hGBP1 no effectors have been identified yet.

In the RocCOR tandem of the *C. tepidum* Roco protein the G domain (Roc) dimer does not show “open” and “closed” states in the course of the GTPase cycle (Fig. 8C) like they are observed for MnmE and, “in a sense”, for hGBP1 as well. The G domains rather remain associated, and more subtle molecular motions “embedded” in a complex conformational equilibrium facilitate the structural requirements for GTP hydrolysis. They sample multiple conformations, one of which seems to be represented by the *CtRocCOR* crystal structure except for the absence of defined electron density for the second Roc domain in the RocCOR dimer. Here, the DEER/PELDOR data provide an explanation for this observation and further support the notion that the Roc G-domains are highly mobile entities. Furthermore, mutations that are located in the Roc/COR interface and that also have strong effects on GTPase activity³²

significantly alter the conformational equilibrium of the G-domains, supporting the notion that the interaction between the N-terminal half of the COR domain and Roc controls this conformational equilibrium, and that mutations disfavour population of the catalytically active conformational state(s).⁴⁶

For MnmE and also for hGBP1 crystal structures obtained for the isolated G domains revealed how active site complementation by dimerization positions a positive charge required for efficient GTP hydrolysis. In both cases dimerization creates a “scaffold” to orient a loop region – the K-loop coordinating a potassium ion in MnmE and the P-loop carrying Arg48 in hGBP1 – so that a positive charge can coordinate the phosphate groups of the nucleotide. Also in *CtRocCOR* an arginine residue, Arg543, is suggested to act as an arginine finger.³² Indeed, removal of this positive charge in *CtRocCOR*-R543A completely abolishes GTPase activity, whereas its ability to bind nucleotides remains unchanged.³² But, contrary to MnmE and hGBP1, where active site complementation takes place *via* dimerization-induced conformational changes that arrange elements *within* each protomer to form the catalytically active state, R543 in the Roc domains appears to protrude into the active site of the *other* protomer to promote GTP hydrolysis,³² what is supported by the DEER data for the neighbouring position, S542.

Thus, although the RocCOR tandem at first sight appears to be a typical G protein activated by dimerization, the DEER/PELDOR results show that the underlying mechanisms differ significantly from those of most other proteins of the GAD family. First, only minor structural alterations upon binding and hydrolysis of GTP are observed, contrary to the large-scale domain motions in the constitutive MnmE dimer or dimerization of hGBP1 protomers. In the two latter cases, and in numerous other GADs – the septin family, signal recognition particle and its receptor (SRP-SR), the Toc family and the dynamins – first dimerization of the G domains has to take place, before nucleotide hydrolysis can proceed, although another SDSL EPR study revealed a slightly different mechanism for the Toc34 GTPase homodimer involved in chloroplast pre-protein translocation. In this case, the GTP bound state was found to exhibit an “open” and very dynamic conformation, whereas the G-domains in the GDP- and GDP- AlF_x -bound states form a quite rigid “closed” dimer.⁵⁰

In most cases, nucleotide-induced G domain dimerization is directly related to the protein's physiological function, for example in the dynamins that oligomerize to act in membrane tubulation and fission, in SRP-SR to couple the ribosome-bound nascent polypeptide chain with the translocation channel on the ER-membrane, or in the Toc proteins, where dimerization is assumed to be necessary for assembly of the translocon complex.³ Also in the constitutive MnmE dimer the observed large scale G domain motions seem to induce further conformational changes that are related to the function in tRNA modification, as described above.⁴⁷ Thus, in these enzymes GTP is hydrolysed to drive another process. In the RocCOR tandem the G domains remain associated throughout the whole GTPase cycle and the conformational changes

observed by inter spin distance measurements appear to facilitate GTP hydrolysis rather than to “execute a task”, indicating significant mechanistic variations within this protein class that need to be further elucidated – with SDSL EPR spectroscopy clearly being one of the methods of choice for such investigations.

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Nitroxides in host–guest chemistry: 2010–2016

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This report covers mainly the Electron Paramagnetic Resonance aspect of the last seven year of progress in host–guest chemistry involving nitroxides with cyclodextrins, cucurbiturils, calixarenes, capsules, nanoporous materials, metal organic framework (MOF), and nanotubes. Applications as probes for materials, as agents for Dynamic Nuclear Polarization, and as pH-probes in biology are also reported.

1 Introduction

Host–guest chemistry is a very broad field covering many types of hosts and guests.^{1–4} This chapter is focused on electron paramagnetic resonance (EPR) analysis of nitroxides as a guest, with several hosts such as cyclodextrins (CDs),^{5,6} calixarenes,⁷ and cucurbiturils (CBs).⁸ Other hosts such as micelles,⁹ Metal Organic Frameworks (MOFs),¹⁰ and porous based materials¹¹ are briefly mentioned in this review although they provide interesting and amazing results.

Despite several reviews on specific hosts where the use of a nitroxide as a guest is mentioned, and in spite of several available reviews on nitroxides^{12–15} in host–guest chemistry, this field has not been carefully reviewed since the reports of Lucarini and colleagues in 2010.¹⁶ Thus, the aim of this review is to cover the period 2010–2016 and to report the hosting of nitroxides by cyclodextrins, calixarenes, cucurbiturils, capsules, and resorcinarenes. We would like to mention that if some relevant articles are not cited in this review, this does not low quality of these studies, but rather they are either outside the scope or were simply overlooked by us. Moreover, articles about nitroxide host–guest chemistry with spin-trap systems are not covered by this review.

Hereafter, a few fundamental data on both nitroxides and hosts are mentioned and then, the chapter is subdivided into 3 main sections devoted to cyclodextrins, to cucurbiturils, and to other hosts such as calixarene, capsules, resorcinarene, nanotubes and MOFs.

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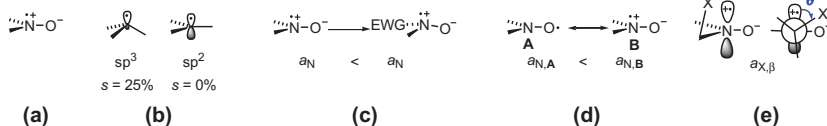


Fig. 1 (a) a_N in nitroxides. (b) Hybridisation of the SOMO. (c) The effect of the pyramidalisation on a_N . (d) Mesomeric forms of the nitroxyl moiety. (e) The effect of dihedral angle on hcc .

1.1 Fundamentals of EPR of nitroxides

The nitrogen hyperfine coupling constant (hcc) a_N corresponds to an α -coupling between the nitrogen atom and the unpaired electron (Fig. 1a). This hcc depends both on hybridisation of the SOMO (Single Occupied Molecular Orbital), *i.e.*, the Fermi contact term, and on the spin density on the atom. The Fermi contact term is the largest for a SOMO of s -type, and depends on the amount of character s used to describe the SOMO, *i.e.*, 25% for a SOMO of σ -type (sp^3 hybridisation, Fig. 1b) to $\approx 0\%$ in p -type (sp^2 hybridisation, Fig. 1b). The hybridisation, and then, the character s , is modified both by the steric strain and the presence of electron-withdrawing groups (EWGs) attached to the nitroxyl moiety. The latter, in general, increase the pyramidalisation at the N-atom, *i.e.* shifts sp^2 hybridisation to sp^3 hybridisation, and, hence, a_N increases (Fig. 1c). The nitroxyl moiety is described by two mesomeric forms (Fig. 1d): the neutral form **A** and the zwitterionic form **B**. Consequently, large values of a_N are ascribed to the larger weighting of form **B** because it exhibits greater spin density on the nitrogen atom. The weighting of each form depends both on the presence of EWGs attached to the nitroxyl moiety which favour form **A** over for **B** and on polarity of the solvent, *i.e.*, highly polar and/or hydrogen bonding solvent stabilizes zwitterionic species, and hence favours form **B** over form **A**.¹⁷ The sensitivity of a_N and of the Landé factor g to the environment makes nitroxides highly valuable probes used in many fields.^{12–14} Moreover, the changes in line shapes of the EPR spectra reflect the probe's motional dynamics (given by the correlation time τ) and therefore provides information on the freedom of motions and on the spin–spin interactions which depend on the distance between the spin labels.¹⁸

In the case of nitroxides carrying at least one non-zero spin atom X at the position β (Fig. 1e), inclusion into the cavity of the cyclodextrin may change the conformation of the nitroxide, resulting in a change in the dihedral angle θ between the SOMO on the nitrogen atom and the C–X bond implying a change in the corresponding hcc $a_{X,\beta}$ as given in the Heller–McConnell relationship, eqn (1).¹⁹

$$a_{X,\beta} = \rho_N^\pi (B_0 + B_1 \cdot \cos^2 \theta) \quad (1)$$

2 Cyclodextrins as hosts

2.1 Generality and fundamentals

Commonly, cyclodextrins (CDs) are described as a truncated cone made up of 6 (α), 7 (β) or 8 (γ) $\alpha(+)$ -glucopyranose units (Fig. 2).²⁰ The smaller

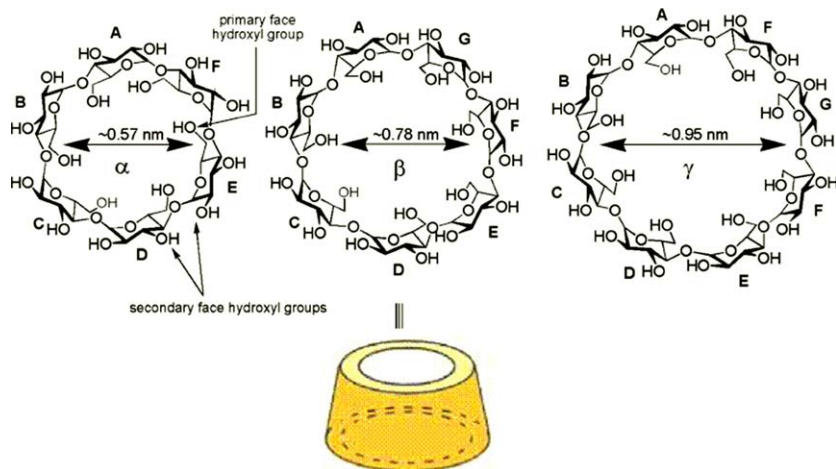


Fig. 2 Structures of the CDs commonly used for complexation investigations with nitroxides. Adapted with permission from E. Engeldinger, D. Armspach and D. Matt, Capped Cyclodextrins, *Chem. Rev.*, 2003, **103**, 4147. Copyright 2003 American Chemical Society.



Scheme 1 Basic scheme of the interaction between a nitroxide (guest) and CD (host).

rim is bordered by 6–8 hydroxymethyl groups and the larger rim by 12–16 hydroxy groups. The breadth of the cavity varies from 5.7 Å to 9.5 Å depending on the size of the CD. Because the inside wall is covered by protons, the cavity exhibits interesting lipophilic properties.²¹ Moreover, the presence of hydroxyl groups on both rims affords an easy and efficient way to prepare mono- and multi-functionalised CDs.²⁰ It offers an opportunity to develop CDs mono- and multi-substituted with a nitroxide group.

The simplest host–guest interaction between a nitroxide and a CD is the bimolecular system displayed in Scheme 1, where the nitroxide is outside the cavity (mode *out* on the left) or inside the cavity of the CD (mode *in* on the right). The inclusion of the nitroxide into the cavity of a CD is reflected in a decrease in a_N due to low polarity of the hydrophobic cavity, in line broadening caused by restricted motions, and in a change in $a_{X,\beta}$ due to a change in the conformation of the nitroxide to fit the cavity.¹⁶ Because nitroxides can carry two different alkyl groups attached to the nitroxyl moiety, 1:1 and 1:2 complexes nitroxides:CD can be formed depending on the CD and the nitroxides as displayed in Fig. 3. However, Spulber and Schlick²² using di-*tert*-butyl nitroxide and β -CD showed that, depending on the experimental conditions, *e.g.* acetic acid or difluoroacetic acid, the inclusion event is not as simple as depicted in Scheme 1. Indeed, in the presence of acetic acid and β -CD, the process of

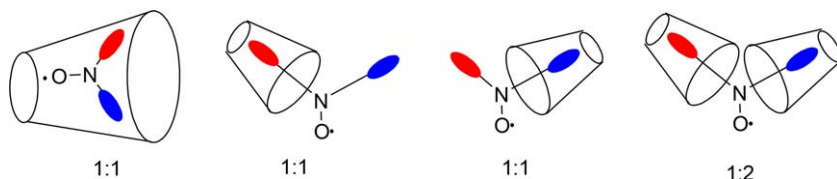


Fig. 3 Main types of complexes formed between a CD and a nitroxyl moiety carrying two different alkyl groups.

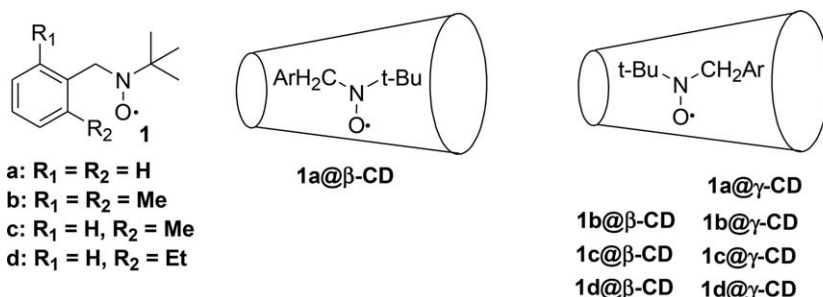


Fig. 4 Nitroxides **1a–d** investigated by Lucarini *et al.*²³ and their corresponding inclusion complexes.

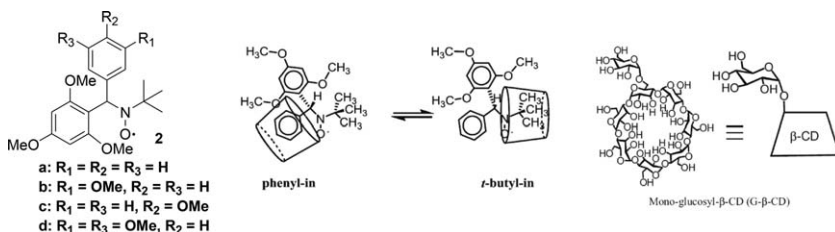


Fig. 5 Nitroxides **2a–d** investigated by Sueishi *et al.*²⁴ Equilibrium phenyl-in-*trans*-butyl-in is reproduced from ref. 24. K. Miyazono, T. Hanaya and Y. Sueishi, Characteristically Steric Effects of Substituent on Bidirectional Inclusion Complexation of α -Substituted Phenyl-2,4,6-Trimethoxybenzyl(*trans*-Butyl)Nitroxides with β -Cyclodextrins, *J. Inclusion Phenom. Macrocyclic Chem.*, 2015, **83**, 193, © Springer Science+Business Media Dordrecht 2015, with permission of Springer.

inclusion of the di-*tert*-butyl nitroxide is better described as a nitroxide inside the cavity (mode *in*) and a nitroxide on the *rim*.

These different modes of inclusions have been investigated by Lucarini *et al.*²³ and Sueishi *et al.*²⁴ with nitroxides **1a–d**²³ and **2a–d**,²⁴ respectively, using β - and γ -CD as well as the 6-O- α -D-glucosyl- β -cyclodextrin G- β -CD (Figs. 4 and 5, respectively) combining EPR and nuclear magnetic resonance (NMR) studies. Lucarini *et al.*²³ showed that **1** is completely included in β - and γ -CD to form 1:1 complexes (left in Fig. 3), this observation is supported by a decrease in a_N by more than 0.5 G. Nonetheless, orientation of the included nitroxide is a result of a subtle interplay of its bulkiness, the hydrophobicity of its substituents and the size of the CD cavity as highlighted by **1a** and **1b–d**. That is, the *t*-Bu group is bulkier than the PhCH₂ group but more hydrophobic than the ArCH₂; furthermore, the ArCH₂ group is deep inside **1a@β-CD**

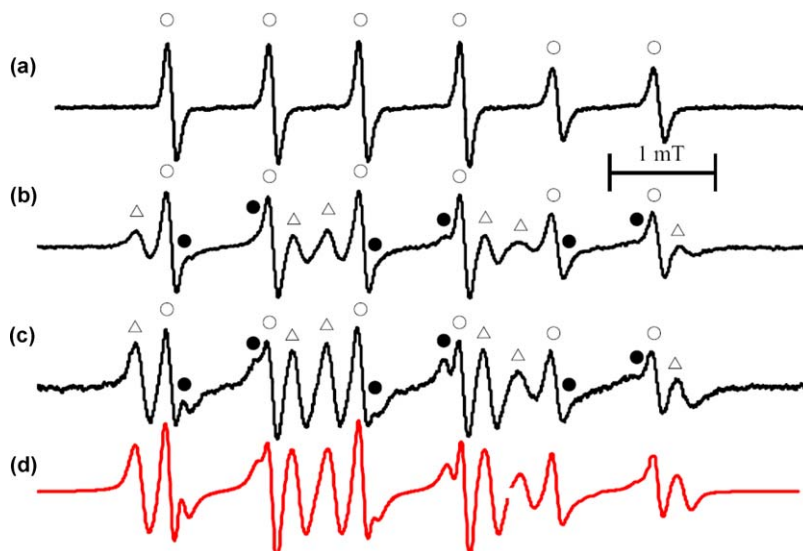


Fig. 6 EPR spectra of probe **2a** ($[2a] = 2 \times 10^{-4} \text{ mol dm}^{-3}$) in the presence of β -CD at 298 K: $[\beta\text{-CD}] = 0$ (a), 3.74×10^{-3} (b), and $1.47 \times 10^{-2} \text{ mol dm}^{-3}$ (c). (d) Simulated spectrum of (c). Assignment: (open circles) free probe ($a_N = 16.40 \text{ G}$ and $a_{H,\beta} = 10.50 \text{ G}$), (filled circle) phenyl-in complex ($a_N = 16.00 \text{ G}$ and $a_{H,\beta} = 6.50 \text{ G}$), and (open triangles) *t*-butyl-in complex ($a_N = 16.10 \text{ G}$ and $a_{H,\beta} = 12.4 \text{ G}$) of **2a**. K. Miyazono, T. Hanaya and Y. Sueishi, Characteristically Steric Effects of Substituent on Bidirectional Inclusion Complexation of α -Substituted Phenyl-2,4,6-Trimethoxybenzyl(*t*-Butyl)Nitroxides with β -Cyclodextrins, *J. Inclusion Phenom. Macrocyclic Chem.*, 2015, **83**, 193, © Springer Science+Business Media Dordrecht 2015, with permission of Springer.

whereas *t*-Bu group is deep inside **1a**@ γ -CD for which the CD cavity is larger. On the other hand, when ArCH_2 is bulkier than the *t*-Bu group as in **1b–d**, hydrophobicity and bulkiness combine to favour deep inclusion of the *t*-Bu group whatever the cavity. It has to be mentioned that the depth of the inclusion as well as the strength of the complex (express by the equilibrium constant) depends strongly on the bulkiness of the groups attached to the nitroxyl moiety.²³ It was also shown that the inclusion strikingly decreases the values of $a_{H,\beta}$ by several G.

The small difference (less than 0.5 G in a_N) reported by Sueishi and coll.²⁴ suggest a nitroxyl moiety not completely included in the CD cavity. The presence of 3 signals in the EPR spectrum of **2a** (Fig. 6) indicates the presence of two different 1 : 1 complexes (Fig. 3) in equilibrium with free **2a**. They were ascribed to the inclusion of either the phenyl group or the *t*-Bu group (Fig. 5). The strength of each complex depends on the bulkiness of the aryl fragment and on the pressure. That is, the *t*-Bu-in inclusion is favoured by an increase in the bulkiness of the aryl group and in the pressure. The use of G- β -CD reduces the influence of pressure on the group inclusion.

The effect of the hydrophobicity of the nitroxide on the inclusion process was simultaneously evaluated by the group of Chechik²⁵ and Freed.²⁶ For their work, Chechik *et al.*²⁵ investigated the use of **TEMPO**, **CAT16**, **16DSA**, **C12NO**, **5DD**, **5DSA** and different types of CDs such as α - and β -CD, methylated β -CD (MCD), and alkylated β -CD (HPB), and

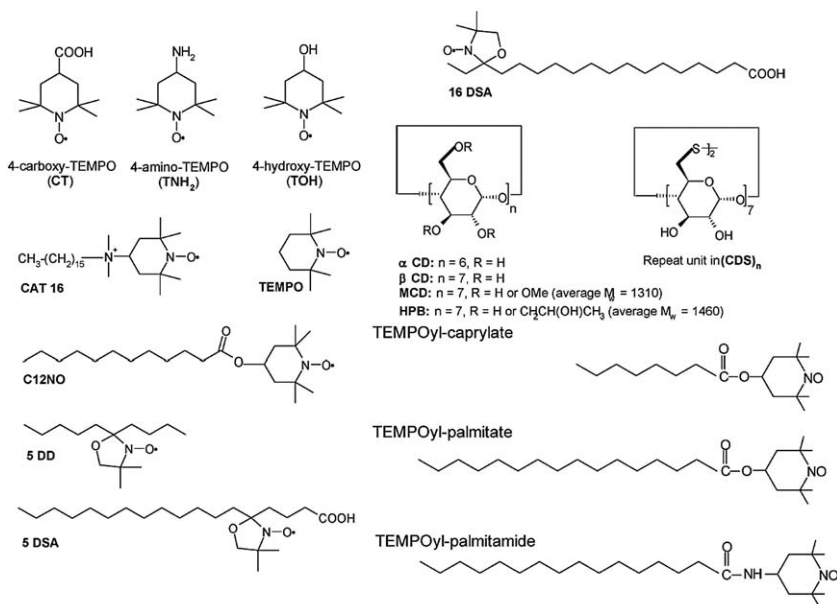


Fig. 7 Nitroxides investigated by Chechik *et al.*^{25,27} and Freed *et al.*²⁶ Adapted from ref. 25 with permission from The Royal Society of Chemistry. Adapted from ref. 26 and 27 with permission from the PCCP Owner Societies.

polymer-based nanocapsules of β -CD (CDS)_n (Fig. 7). It was noted that the inclusion of TEMPO in α -CD did not occur in sharp contrast to β -CD (a decrease in a_N by more than 0.5 G and an increase in correlation time τ). For other nitroxides, the small decrease in a_N despite a significant increase in τ meant that the inclusion of the hydrophobic thread was preferred over the more polar nitroxide group. For the type-5 series of nitroxide, 1 : 2 complexes are also observed. Due to the short alkyl chain in 5DD, inclusion of a nitroxide group is observed. On the other hand, Freed *et al.*²⁶ investigated 16DSA, 5DSA, TEMPOyl-caprylate, TEMPOyl-palmitate, and TEMPOyl-palmitamide (Fig. 7) in the presence of β - and γ -CD in solid state using X-band EPR and high-field high-frequency EPR at 170 and 240 GHz. They found that complexes of high order are formed due to the inclusion of the lipophilic thread in several CDs. Ionita and Chechik²⁷ investigated the effects of the inclusion into α - and β -CDs on the mobility of TEMPO, CT, TNH₂ and TOH at different temperatures in viscous solutions (water : glycerol 8 : 2 v/v). They observed 1 : 2 complexes rather than 1 : 1 complexes. They showed that tumbling of the probe inside the cavity is faster than in a free solution; this result indicates a less restricted motion in a viscous solvent and at a low temperature. Consequently, such an approach makes it possible to differentiate between a probe included and a probe non-included in the CD cavity. Both in solution and in a solid state, complexes of TEMPO or of its analogues with α -CD were not detected. In the solid state, it was found that the presence of a co-crystallised solvent has no influence on a_N . It was also noted that the tumbling rates of encapsulated probes are almost identical to those reported in the solid state.

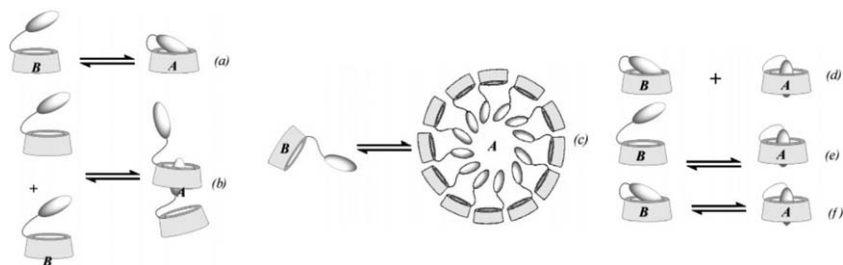
As mentioned above, the presence of free hydroxyl groups on the rim of the CD offers a nice opportunity to functionalise it with different groups and, especially with nitroxides. Because of the difference in the simple model of inclusion between free nitroxides and CDs, a nitroxide attached to a CD provides more possibilities for complexation:²⁸ (a) equilibrium between the probe out and a capping probe, (b) an intermolecular complex, (c) formation of aggregates or micelles, (d) the presence of a nitroxide in and a capping nitroxide as two independent species, (e) equilibrium between a probe out and a probe in, (f) equilibrium between a capping nitroxide and nitroxide in, and (g) a nitroxide out which is not depicted in Scheme 2. The occurrence of events (a), (e)–(g) (and the non-occurrence of events (b), (c), and (d)) has been evidenced combining X-^{29–31} and W-band EPR,²⁹ Electron Spin Echo Envelop Modulation (ESEEM), Double Electron–Electron Resonance (DEER),³¹ and competitive experiments with EPR,^{28,29,31} NMR,²⁹ ESEEM³¹ and DEER.³¹

Some other nitroxide-capped CDs (NCDs) based on permethylated- β -CD (TRIMEB) and on various linker and nitroxides were investigated using X-band EPR and ESEEM to unravel the events involved ((a), (e) and (g) in Scheme 2).³² As expected, occurrence of different events depends strikingly on the type of probes and on the type of linker, as shown in Scheme 3. In sharp contrast to previous results, addition of a competitor (adamantylmethanol) favors the inclusion of the nitroxide group for **NCD1–NCD4** (Fig. 8). However, it does not increase the resistance to bioreductants and it has no effect on the pK_a of **NCD5**.

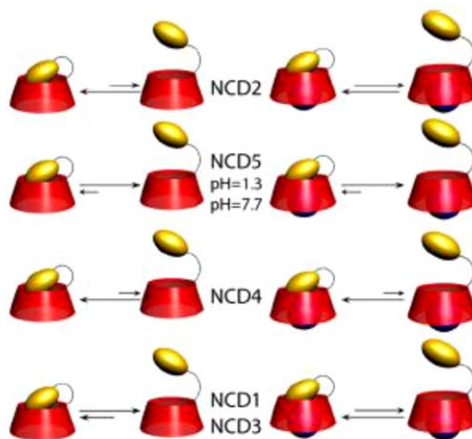
Due to the importance of the detection of NO,³³ nitroxyl nitroxide attached to TRIMEB **3** was prepared (Fig. 9).³⁴ EPR and ESEEM investigations showed that **3** is nicely described as a capping nitroxide (model (a) in Scheme 2). The EPR signal of the reduction of **3** into the corresponding imino nitroxide was detected as efficiently as for free nitronyl nitroxide and no extra resistance to the bioreductant was observed.

2.2 Application as a spin probe

Ionita *et al.*^{35–38} took advantage of these enhanced probing properties of encapsulated nitroxides in CD to investigate the structure of polymer gel. They used both free probes (**DT**,^{36,38} **L62NO**,³⁸ **F127NO**,³⁸ **AT**,^{35,37} **P3T2**,³⁵ and **TEMPO**³⁷) and β -CD as well as nitroxide-capped CDs (**D- β -CD**,^{36,38}



Scheme 2 Various possibilities for complexes to be formed with CD-capped nitroxides. Reprinted with permission from D. Bardelang, A. Rockenbauer, L. Jicsinszky, J.-P. Finet, H. Karoui, S. Lambert, S. R. A. Marque and P. Tordo, Nitroxide Bound β -Cyclodextrin: Is There an Inclusion Complex? *J. Org. Chem.*, 2006, **71**, 7657. Copyright 2006 American Chemical Society.



Scheme 3 Structural equilibrium in the studied NCDs before (left) and after (right) complex formation with adamantlylmethanol (AM). Note that the lengths of arrows are not proportional to the corresponding rate constants and reflect the structural equilibria only qualitatively. The nitroxide moiety is yellow; TRIMEB is red; AM is blue. Reprinted with permission from O. A. Krumkacheva, M. V. Fedin, D. N. Polovyanenko, L. Jicsinszky, S. R. A. Marque and E. G. Bagryanskaya, Structural Equilibrium in New Nitroxide-Capped Cyclodextrins: CW and Pulse EPR Study, *J. Phys. Chem. B*, 2013, **117**, 8223. Copyright 2013 American Chemical Society.

MTCYC,³⁸ **TCYC**^{35,37} and **MCT**³⁵) with X-band EPR and ESEEM³⁸ methods (Fig. 10). When **TCYC** or β -CD and isocyanate end-capped polyethylene glycol were used, the gel containing CD at the terminal position of the chain and in network nodes (Fig. 11a) was investigated. For example, adsorption of **AT** in the gel affords a two-components EPR signal corresponding to the signal (Fig. 11b) of an immobilised probe as expected for **AT** included in the CD at the nodes (*a* in Fig. 11a) and to the signal of a mobile probe trapped in the solvent pool in the gel (*c* in Fig. 11a). The changes in the gel network due to the temperature, solvent, polymer chain length and the initial ratio of reactants were also investigated using **TCYC**, **AT**, **TEMPO** and β -CD as probes.³⁷ It was shown that the mobility of the nitroxide-CD at the nodes was higher in aprotic solvents than in protic solvent, where H-bonding plays an important role in immobilising the gel network. It was also shown that short polymer strands results in a less mobile gel network.^{35,37} These probes were also applied to investigate the micellar-to-gel transition in F127 (*co-block*-poly(ethylene oxide PEO)-poly(propylene oxide PPO)-poly(ethylene oxide)) solutions.³⁸ Nitroxides **L62NO** and **F127NO** are located (Fig. 11c), respectively, at the interface PPG/PEO in the micelle (core–corona interface) and at the frontier of the micelle, with the solvent affording different information on the micelle-to-gel transition. Indeed, **L62NO** is more sensitive to the micelle-sol-gel transition because it implies the dehydration of the polyethylene oxide (PEO) after a rise in the temperature, affording a more hydrophobic environment. The use of **MTCYC**, **L62NO** and **F127NO** with hydroxypropyl- β -CD (HPB) combined with ESEEM studies showed that HPB does not significantly alter the organisation of the micelle because the formation of rotaxane is not detected according to Fig. 11c.³⁸

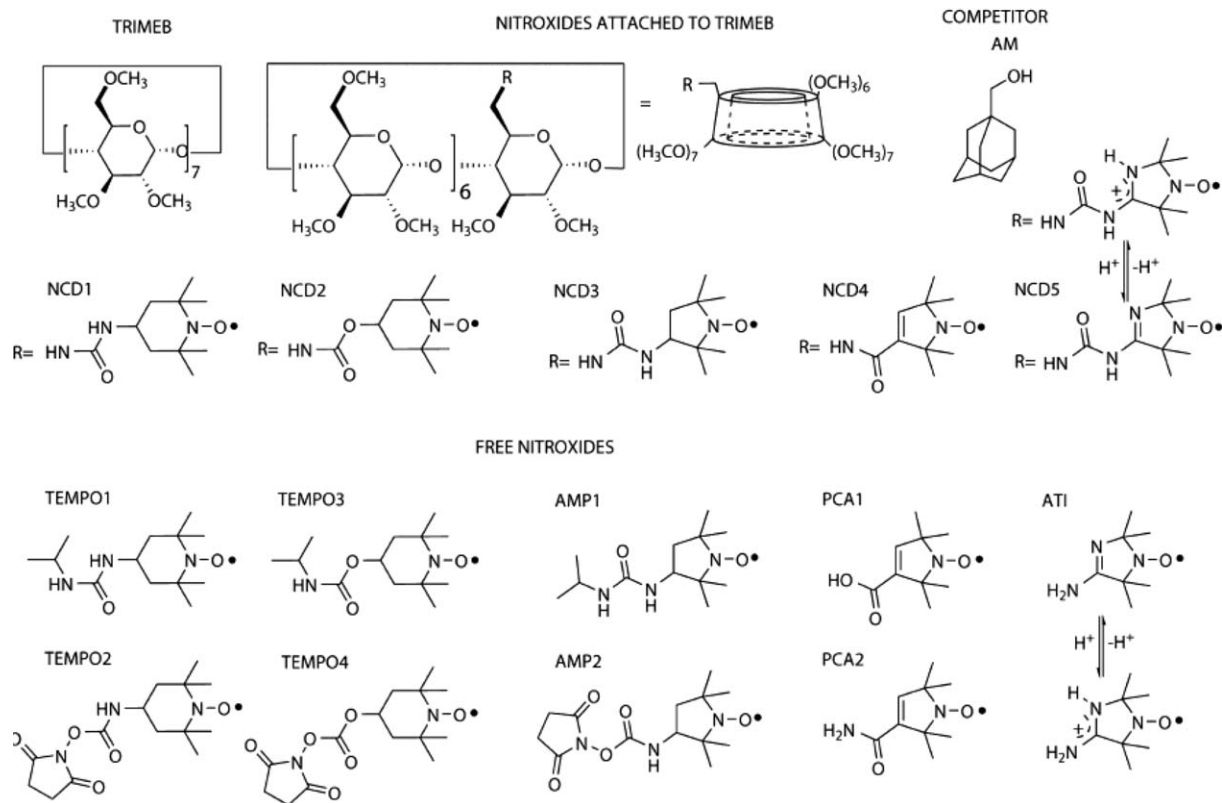


Fig. 8 Nitroxides investigated by Krumkacheva *et al.*³² Reprinted with permission from O. A. Krumkacheva, M. V. Fedin, D. N. Polovyanenko, L. Jicsinszky, S. R. A. Marque and E. G. Bagryanskaya, Structural Equilibrium in New Nitroxide-Capped Cyclodextrins: CW and Pulse EPR Study. *J. Phys. Chem. B*, 2013, **117**, 8223. Copyright 2013 American Chemical Society.

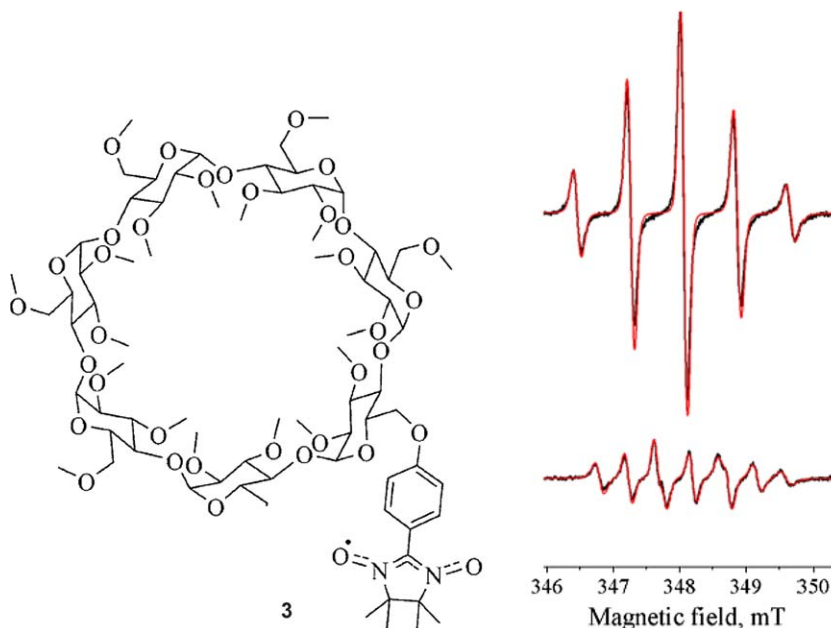


Fig. 9 Signal of **3** (0.5 mM) during the photolysis at $t=0$ s (upper signal) and $t=94$ s (lower signal) in the presence of sodium nitroprusside (50 mM, used to generate NO) in water at 300 K. Red lines show simulations obtained using the parameters listed for **3** ($a_{\text{N1}}=a_{\text{N2}}=8.2$ G, $\tau=7.9 \cdot 10^{10}$ s) and its corresponding imino-nitroxide ($a_{\text{N1}}=9.8$ G, $a_{\text{N2}}=4.4$ G, $\tau=7.9 \cdot 10^{10}$ s). R. K. Strizhakov, E. V. Tretyakov, A. S. Medvedeva, V. V. Novokshonov, V. G. Vasiliev, V. I. Ovcharenko, O. A. Krumkacheva, M. V. Fedin and E. G. Bagryanskaya, Permethyl- β -Cyclodextrin Spin-Labeled with Nitronyl Nitroxide: Synthesis and EPR Study, *Appl. Magn. Reson.*, 2014, **45**, 1087. © Springer-Verlag Wien 2014, with permission of Springer.

NCD5 (Fig. 8) has been applied as a quencher of luminescence of the excited (singlet or triplet) state of diphenyl ketone or benzylphenylketone immersed in the cavity of **NCD5**.³⁹ Zweier *et al.*⁴⁰ prepared trityl-nitroxide biradical **4** (Fig. 12) and observed increased lifetime of the nitroxide group as a result of encapsulation in the β -CD cavity serving as a shield against the bioreductants.^{16,41} Ionita *et al.*⁴² used the ability of a CD to include a nitroxide in their cavity to investigate the denaturation of bovine serum albumin under various conditions. Ionita *et al.*⁴³ applied nitroxide-capped β -CD to investigate the adsorption of a CD on functionalised gold@silica and magnetite@silica particles. Gotti *et al.*⁴⁴ used the exchange properties of **1a** to investigate the effect of various CD and sodium dodecyl sulphate (SDS) micelles. Grammenos *et al.*^{45,46} showed that the inclusion properties of CD can be used to investigate and quantify the action of drugs on cell membranes.

Casano *et al.*⁴⁷ showed that the inclusion of **bPTO** in α -CD (50 mM), β -CD (10 mM), γ -CD, and 2,6-di-*O*-methyl- β -CD (**DM- β -CD**) and (100 mM) occurred as shown in Fig. 13 for **DM- β -CD**. In sharp contrast to the observation done with **CB7** an **CB8** (*vide infra*), no protection due to the inclusion process is observed because all EPR signals decayed in less than 45 seconds after addition of ascorbic acid.

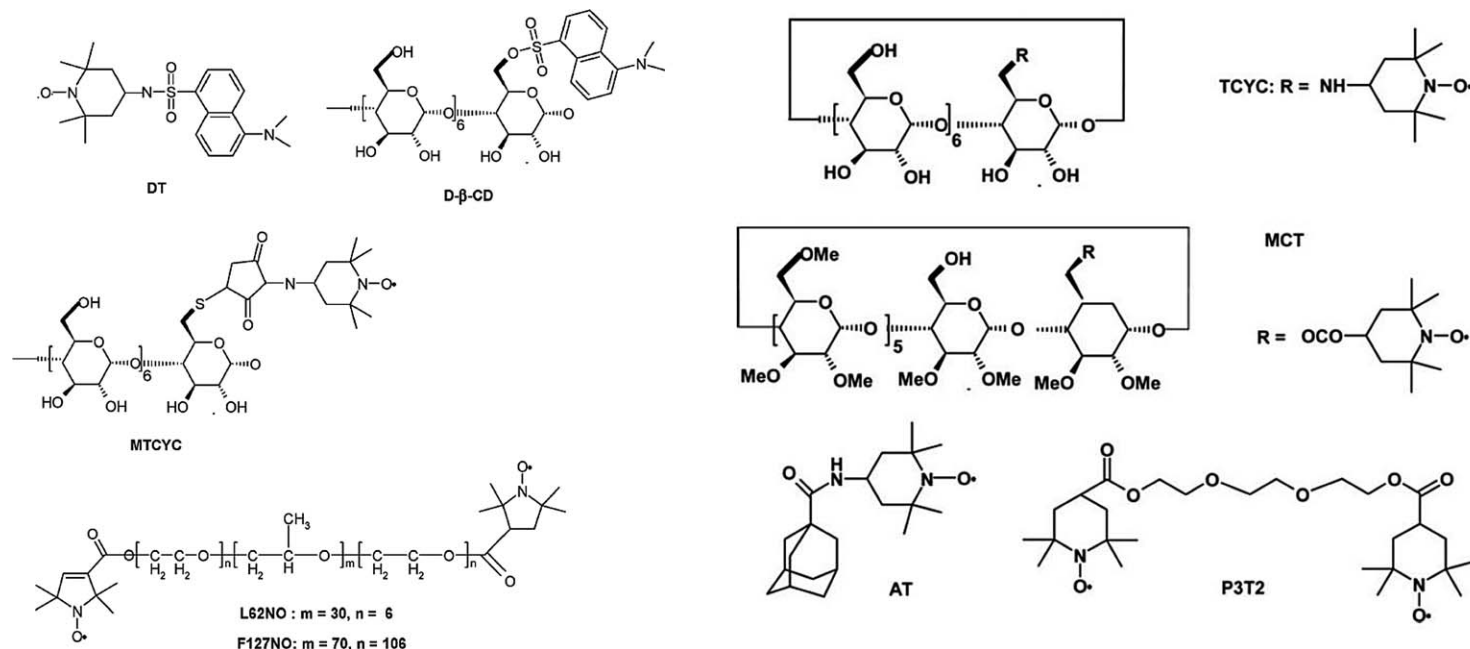


Fig. 10 Nitroxides investigated by Ionita *et al.*^{35–38} Adapted from ref. 35 and 37 with permission from The Royal Society of Chemistry. Adapted from ref. 36 and 38 with permission from the Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry.

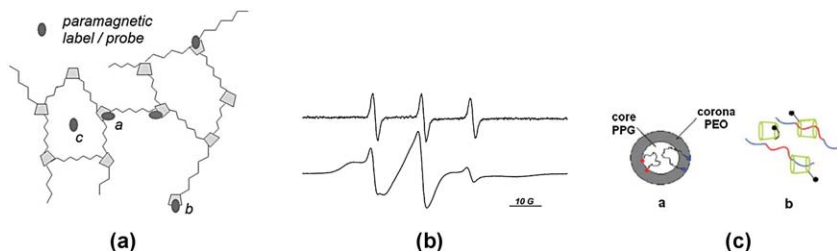


Fig. 11 (a) Schematic representation of the positions occupied by a paramagnetic species in the hydrogel network: (a) network nodes, (b) terminal position of the chain, (c) entrapped in the gel voids. (b) EPR spectra of AT in water, saturated solution (top spectrum), and adsorbed in gel network from a solution 10^{-3} M of AT in 5% aqueous ethanol (bottom spectrum). (c) (a) Schematic representation of the location of **F127NO** (blue) and **L62NO** (red) in the F127 micelle; (b) possible location of **MTCYC** relative to the F127 chain (green – CD cavity, black – paramagnetic moiety, blue – PEO block, dark red – PPG block). For (a) and (b), reproduced from ref. 35 with permission from The Royal Society of Chemistry. For (c), reproduced from ref. 38 with permission from the Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry.

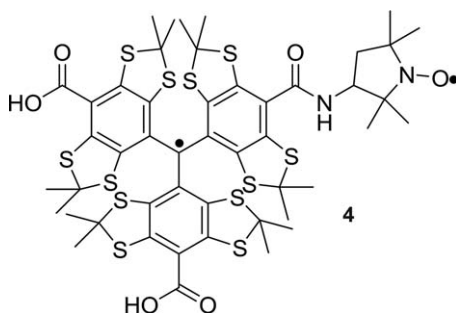


Fig. 12 Nitroxide-trityl prepared by Zweier *et al.*⁴⁰

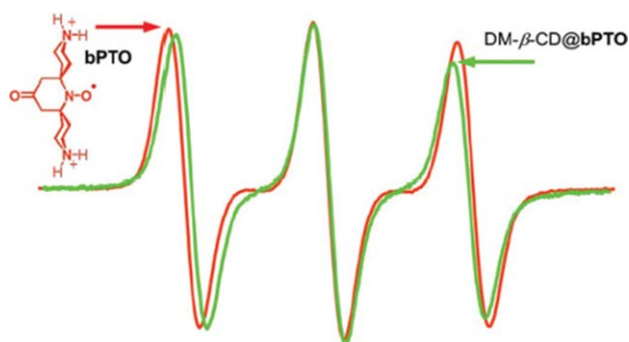


Fig. 13 EPR spectra of **bPTO** (0.2 mM) alone (red line) and in the presence of **DM-β-CD** (200 mM, green line). Reproduced from ref. 47 with permission from The Royal Society of Chemistry.

2.3 Spin-spin interaction applications

In the last decade,^{48,49} the spin-spin interaction in bisnitroxides was revisited in the context of the Dynamic Nuclear Polarisation (DNP) field; consequently the control of this interaction *via* structural changes is a major challenge. Mao *et al.*⁵⁰ nicely highlighted the use of CD to enhance

the DNP signal. Porel *et al.*⁵¹ showed that the communication (spin–spin exchange interaction) between spins in **T1** (Fig. 14) is controlled by the presence of β -CD and γ -CD. Indeed, in water **T1** yields a 5-line pattern of EPR signals with 88% of exchange interaction whereas in the presence of a CD, this interaction decreases by half but is not suppressed (Fig. 15). Clearly one equivalent of a CD is not sufficient to suppress the spin–spin interaction between the two nitroxide groups in sharp contrast to the efficiency of cucurbituril as a host (*vide infra*).

Lucarini *et al.*⁵² successfully prepared the bisnitroxide [2]rotaxane **7** using a nitroxide-capped thread and the spin-labelled α -CD **6** (Fig. 14). Model **5** and **6** afforded the expected 3 lines EPR signals exhibiting linewidth broadening due to a slower tumbling rate of large molecules whereas **7** showed a 5-line EPR signal with the 2nd and 4th lines broader and smaller than the 3 others (Fig. 16). Combining molecular dynamic calculation, NMR and PELDOR experiments, the EPR signal (Fig. 16) is unambiguously ascribed to **7**. Nevertheless, this unusual signal for the spin–spin⁵³ coupling in bisnitroxide is ascribed to the equilibrium between 3 species, with the Van't Hoff parameters $\Delta H = 6.1 \text{ kJ mol}^{-1}$ and $\Delta S = 16.1 \text{ J mol}^{-1} \text{ K}^{-1}$ for the slow reaction (Scheme 4): species **A**, in which the two nitroxide groups are too far apart to interact (exchange coupling constant $J = 0$) and afford the conventional intense and sharp 3-line signal ($a_N \approx 17 \text{ G}$) in slow chemical exchange with species **B** and **C** in which the two nitroxide groups are closer favouring the spin–spin interaction ($J \gg a_N$). On the other hand, the small broad lines in Fig. 16 point to a fast chemical exchange between **B** and **C** (Scheme 4).

With γ -CD and the polynitroxides **T2** and **T3** (Fig. 14), Ottaviani *et al.*⁵⁴ used the host–guest properties of CD to highlight their usefulness for the control of a spin–spin interaction. Polynitroxides **T2** (not shown) and **T3** (Fig. 17a) showed, respectively, the expected two-component 5- and 7-line patterns due to *close* conformations (1:2:3:2:1 for **T2** and 1:3:6:7:6:3:1 for **T3**) and to *far* conformations (conventional 1:1:1 triplet signal for free nitroxides), whereas in the presence of γ -CD, both exhibited the conventional triplet meaning that the encapsulation of nitroxide groups in the cavity of γ -CD shields both from spin–spin interaction (Fig. 17d). The effect of the ratio $[\gamma\text{-CD}]/[\text{T2 or T3}]$ on the EPR signal of **T2** (not shown) and **T3** (Fig. 17c) was monitored from 0 (57% of the *close* conformer showing a 7-line pattern signal) to 40 ($\approx 2\%$ of the *close* conformer showing a 5-line pattern signal), and 98% of *far* conformer with a 3-line pattern signal) through a ratio of 5 exhibiting both 7- ($\approx 2\%$) and 5-line (18%) signal patterns for the *close* conformers and the expected 3-line pattern signal (80%) for the *far* conformers.⁵⁵ These results are nicely described by sequential simplified Scheme 5 in which nitroxides are encapsulated step by step. It was then possible to estimate the van't Hoff parameters for the transformations from **T2** to **T2**@ γ -CD ($\Delta H = 45 \text{ kJ mol}^{-1}$ and $\Delta S = 48 \text{ J mol}^{-1} \text{ K}^{-1}$) and from **T3** to **T3**@(γ -CD)₂ ($\Delta H 14 \text{ kJ mol}^{-1}$ and $\Delta S = 42 \text{ J mol}^{-1} \text{ K}^{-1}$).

Liu *et al.*⁵⁶ showed that the spin–spin interaction between the trityl and the nitroxide moieties in **CT02-GT**, **CT02-AT**, and **CT02-VT** (Fig. 14) depends on both the length of the spacer and the concentration and the

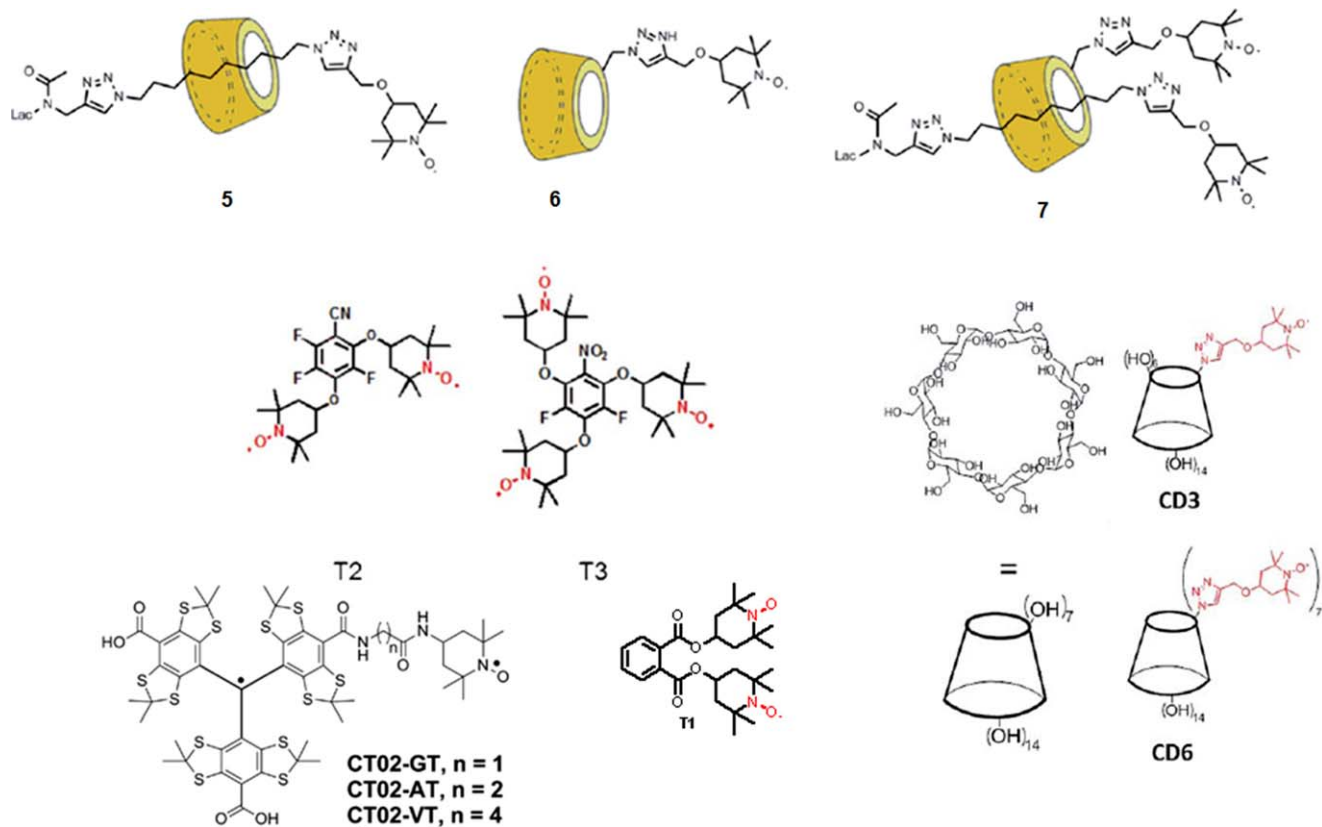


Fig. 14 Nitroxides investigated by Porel *et al.* (T1–T3),^{51,54} Lucarini *et al.* (5–7, CD3 and CD6),⁵² and Liu *et al.* (CT02).⁵⁶ Adapted from ref. 51, 54, 56 and 57 with permission from The Royal Society of Chemistry. In part with permission from C. Casati, P. Franchi, R. Pievo, E. Mezzina and M. Lucarini, Unraveling Unidirectional Threading of α -Cyclodextrin in a [2]Rotaxane Through Spin Labeling Approach, *J. Am. Chem. Soc.*, 2012, **134**, 19108. Copyright 2012 American Chemical Society.

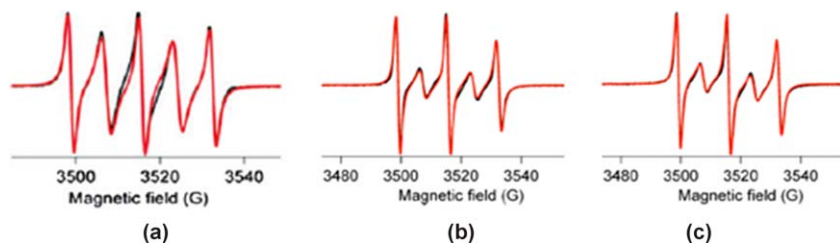
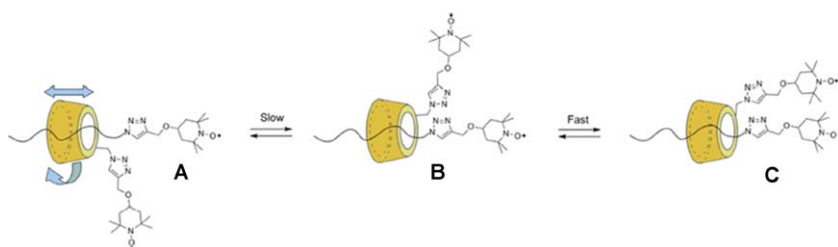


Fig. 15 EPR spectra (black lines) and their simulations (red lines) for **T1** (1 mM) in water (a) three-line component: $a_N = 16.9$ G, $\tau_c = 0.06$ ns, five-line component: 88% (b) **T1**/ β -CD (1 : 2), $a_N = 16.7$ G, $\tau_c = 0.15$ ns, five-line component: 40% (c) **T1**/ γ -CD (1 : 2), $a_N = 16.8$ G, $\tau_c = 0.12$ ns, five-line component: 40%. Reproduced from ref. 51 with permission from The Royal Society of Chemistry.



Fig. 16 EPR signal of **7** (0.05 mM) recorded in water at 328 K. Reprinted with permission from C. Casati, P. Franchi, R. Pievo, E. Mezzina and M. Lucarini, Unraveling Unidirectional Threading of A-Cyclodextrin in a [2]Rotaxane Through Spin Labeling Approach, *J. Am. Chem. Soc.*, 2012, **134**, 19108. Copyright 2012 American Chemical Society.



Scheme 4 Possible conformations of the bis-labelled rotaxane **7**. Reprinted with permission from C. Casati, P. Franchi, R. Pievo, E. Mezzina and M. Lucarini, Unraveling Unidirectional Threading of A-Cyclodextrin in a [2]Rotaxane Through Spin Labeling Approach, *J. Am. Chem. Soc.*, 2012, **134**, 19108. Copyright 2012 American Chemical Society.

type of CD (γ -CD, TRIMEB, and HPB) as shown in Fig. 18. Furthermore, as already reported for the simple CD-nitroxide inclusion,¹⁶ the presence of a CD strikingly increased the lifetime of the nitroxide part in the biradical.

Melone *et al.*⁵⁷ observed a conventional 3-line signal for **CD3** whereas **CD6** (Fig. 14) showed a broad (peak-to-peak line width of 11.5 G) ill-resolved peak with the conventional Landé factor of a nitroxide, *i.e.*, $g = 2.0058$ (Fig. 14). Combining EPR, DC-SQUID magnetometry, and relaxivity measurements, they showed that the spin-spin interaction in **CD6** cannot be described by the highly symmetrical arrangement of the nitroxide on the CD rim as displayed in Fig. 19a but by one of the 3 others (Fig. 19b–d); which requires one central nitroxide.

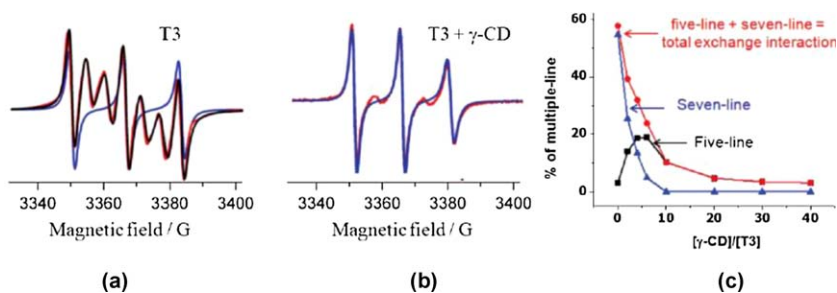
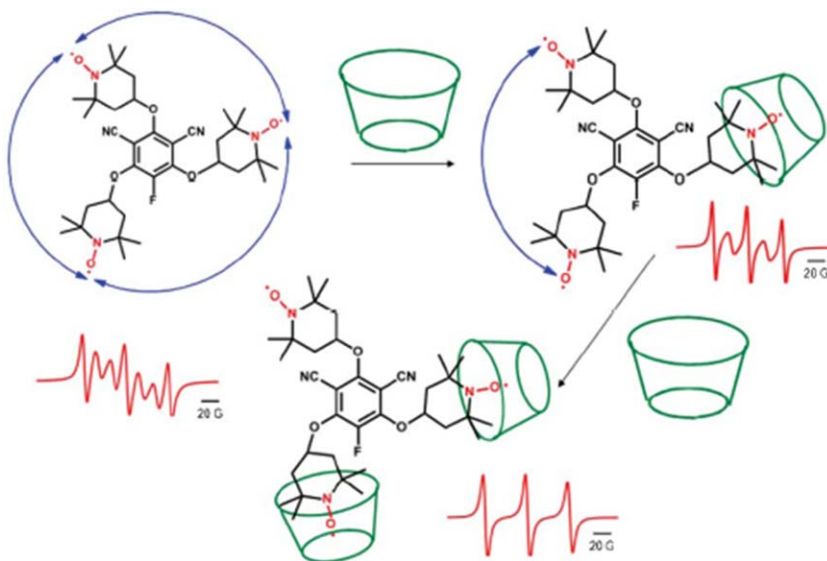


Fig. 17 Experimental EPR spectra (black line) and their simulations (red line: addition of Lorentzian lines; blue line: computation of the three-line component) for (a) T3 in 1:1 MeOH–water mixture, $a_N = 16.5$ G, $t = 0.08$ ns, seven-line component = 57%; (b) T3: γ -CD = 1:20, $a_N = 16.6$ G, $t = 0.25$ ns, five-line component = 5%, $T = 293$ K. (c) Plot for the percentage of multiple-line component of T3 vs. molar ratio of γ -CD:T3. $[\text{guest}] = 0.1$ mM. Reproduced from ref. 54 with permission from The Royal Society of Chemistry.



Scheme 5 Schematic representation of the stepwise encapsulation of T3 with γ -CD (green bucket) and observed EPR spectra (red). Blue arrows highlight the spin–spin interactions. Reproduced from ref. 54 with permission from The Royal Society of Chemistry.

3 Cucurbituril as a host

3.1 Generality and fundamentals

The cucurbituril (CB) host,^{58,59} a name due to its pumpkin-like shape, is composed of 5 to 10 glycoluril units bound together by 10 to 20 methylene bridges (Fig. 20). Consequently, the size of the portal (entrance of the cavity), the cavity diameter, and the cavity volume increase with the number of glycoluril units whereas the height is the same and corresponds to the size of one glycoluril unit (Fig. 20). It ensures a highly hydrophobic cavity closed by two highly polar portals (carbonyl groups)

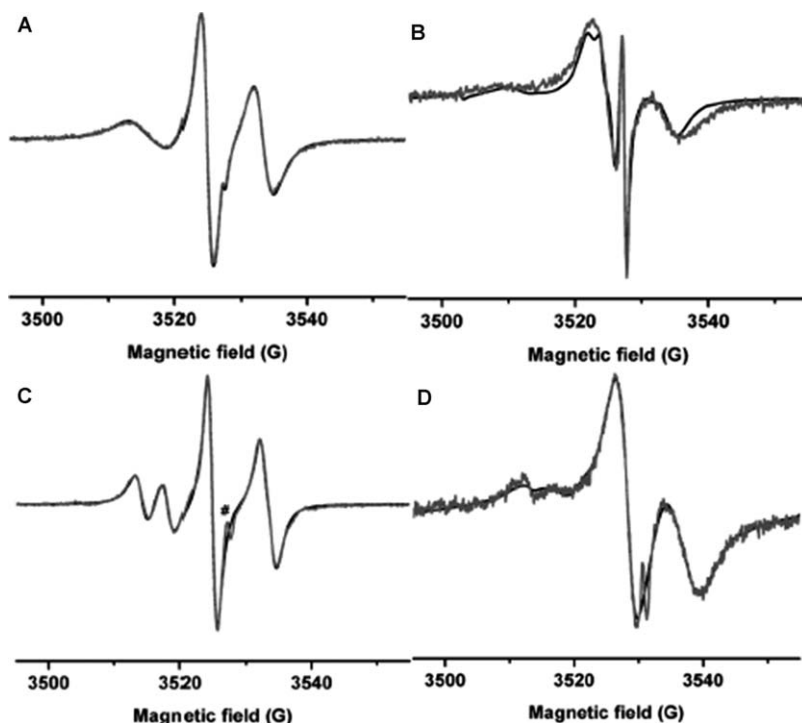


Fig. 18 Experimental (grey) and simulated (black) EPR spectra of free CT02-AT (A) and its complex with TRIMEB (B) and free CT02-GT (C) and its complex with TRIMEB (D). Reproduced from ref. 56 with permission from The Royal Society of Chemistry.

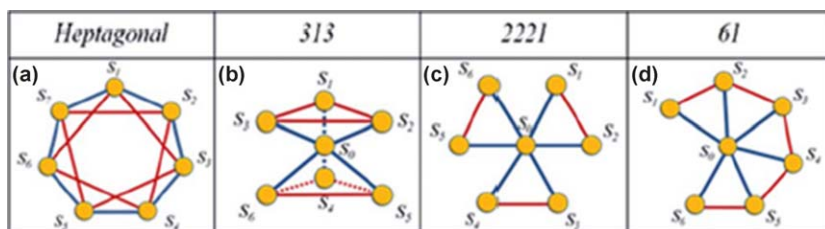


Fig. 19 Four different configurations for the seven radicals: (a) highly symmetric heptagonal configuration: the blue and red lines represent the J_1 and the J_2 coupling constants respectively. (b–d) In this case J_1 represent the exchange interaction between the central spin and the others ones whereas J_2 takes into account all other possible interactions excluding the central spin (see ref. 57 for details). Reproduced from ref. 57 with permission from The Royal Society of Chemistry.

leading to the unique guest properties of this family: the ability to dissolve a highly lipophilic molecule in water and the ability to efficiently host a molecule composed of hydrophobic and hydrophilic parts. More details on both the preparation and binding properties are provided in various reviews.^{58,60–64} Unlike CDs, CB were not easily functionalised until recently.^{65,66}

As for a CD, several host–guest interaction are possible for CB affording different types of host:guest complexes: 1:1 complexes exhibiting either an empty cavity (exclusion complex) and the host maintained outside of

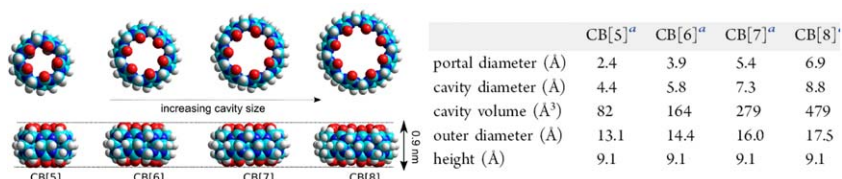


Fig. 20 Structure and geometrical parameters of the main CBs used to investigate complexation with nitroxides. Adapted with permission from ref. 59. Copyright 2000 American Chemical Society. Adapted with permission from S. J. Barrow, S. Kasera, M. J. Rowland, J. del Barrio and O. A. Scherman, Cucurbituril-Based Molecular Recognition, *Chem. Rev.*, 2015, **115**, 12320. Copyright 2015 American Chemical Society.

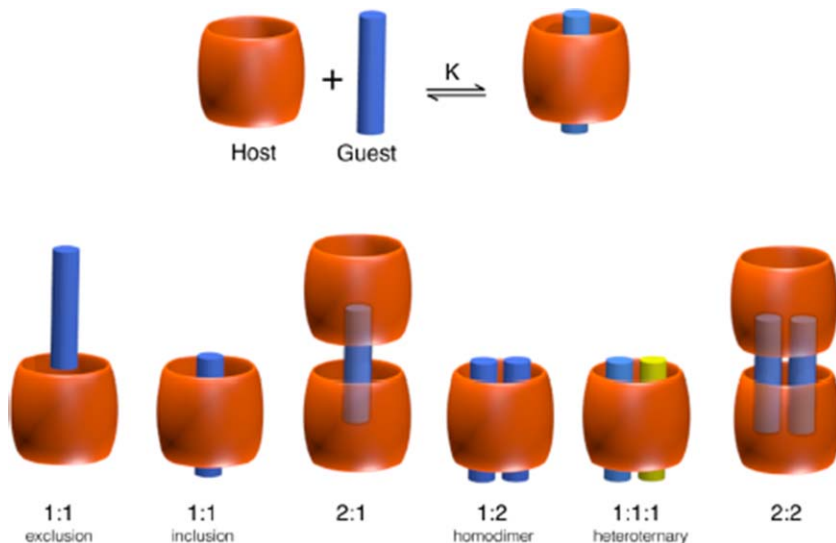


Fig. 21 The most frequently observed complexes with CBs. Adapted with permission from S. J. Barrow, S. Kasera, M. J. Rowland, J. del Barrio and O. A. Scherman, Cucurbituril-Based Molecular Recognition, *Chem. Rev.*, 2015, **115**, 12320. Copyright 2015 American Chemical Society.

the cavity by the H-bonding network with the carbonyl's portals or an inclusion complex with the guest immersed into the cavity of CD; a 2 : 1 complex where the guest is immersed in the cavities of two CBs; a 1 : 2 complex where the CB cavity hosts two guests; a 1 : 1 : 1 complex where two different guests are immersed in the same cavity; and a 2 : 2 complex where two guest are paired and immersed in two CB cavities. These are the most obvious cases for which several examples are reported (see also Fig. 21). Besides the EPR investigations, the process of inclusion as well as the position of the spin probes, *i.e.*, deeply immersed into the cavity or localised in the portal, have also been investigated using calculations.^{67,68}

As it is described below, CBs are, in general, applied to control the spin-spin exchange interaction, *i.e.*, to suppress or to regenerate it on demand. Nevertheless, Vostrikova *et al.*⁶⁹ reported one case where **CB8** is used to favour the spin-spin exchange interaction. Indeed, they observed (**8** · HCl)@**CB8** and **8**@**CB8** 1 : 1 complexes (Fig. 22) showing the expected

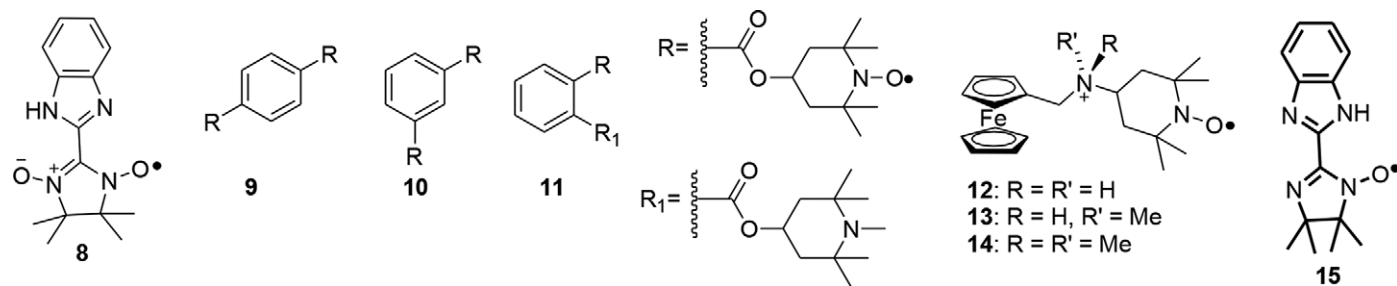
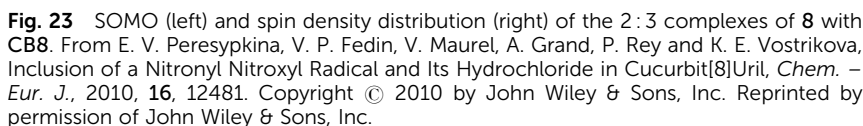


Fig. 22 Nitroxides investigated by Vostrikova *et al.* (8 and 15),^{69–71} Porel *et al.* (9–11),⁵¹ Kaifer *et al.* (12–14).⁷² Adapted from ref. 51 and 72 with permission from The Royal Society of Chemistry. Reprinted from K. E. Vostrikova, E. V. Peresypkina, V. A. Drebuschak and V. A. Nadolinny, Tuning of the Nitronyl Nitroxide Radical Magnetic and Electronic Properties by Inclusion in Cucurbit[N]Urils, *Polyhedron*, 2011, 30, 3083–3087. Copyright (2011), with permission from Elsevier.



Vostrikova *et al.*^{70,71} applied the host-guest properties of CB towards nitroxides to develop their use as nanoreactors. They reported on the association of **8** and **15** (Fig. 22) with CB7 and CB8 affording **8**·HCl@CB7, **8**·HCl@CB8, **15**·HCl@CB7 and **15**·HCl@CB8 as 1:1 complexes. The expected 1:2:3:2:1 5-line pattern for **8**·HCl@CB7 and **8**·HCl@CB8, and the 1:1:2:1:2:1:1 7-line pattern for **15**·HCl@CB7 and **15**·HCl@CB8 were observed. After heating 150 °C in the crystal state, and then dissolution in water, the EPR signal of **8**·HCl@CB7 or **8**·HCl@CB8 was replaced by the EPR signal of **15**·HCl@CB7 or **15**·HCl@CB8 (Fig. 24).

Porel *et al.*⁵¹ showed that, as for **11**, **9** and **10** (Fig. 22) exhibit a 3-line EPR signal meaning that the two nitroxide groups are far apart so that the spin-spin interaction is suppressed. Addition of **CB8** did not significantly change the values of a_N for **9** and **10** whereas t_C was significantly increased meaning for **9** and **10** that a 1:2 or 1:1 guest:host exclusion (Fig. 21) is likely formed whereas for **11**, NMR analysis supported the formation of an inclusion complex with the R₁ group in **11**.

Kaifer *et al.*⁷² investigated the inclusion of nitroxide-ferrocenes **12–14** because the binding constants between **CB7** or **CB8** and ferrocene are strong.⁷³ Combining, EPR, NMR, and cyclic voltammetry techniques, they showed that the increase in a_N of **12–14** in the presence of **CB7** is accounted for by a favoured deep immersion of the ferrocene moiety into the cavity ensuring a nitroxide moiety outside with a better solvation cage

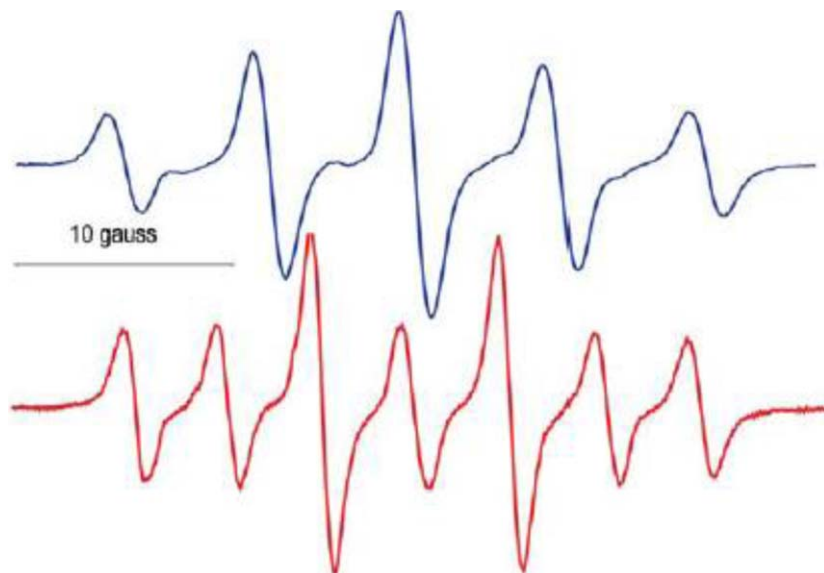


Fig. 24 EPR spectra in aqueous solutions for: $8 \cdot \text{HCl}@\text{CB8}$ (top), $15 \cdot \text{HCl}@\text{CB8}$ (obtained by thermal treatment of $8 \cdot \text{HCl}@\text{CB8}$) (bottom). In the case of CB7 the respective spectra are identical (see ref. 70). Reproduced from ref. 71 with permission from The Royal Society of Chemistry.

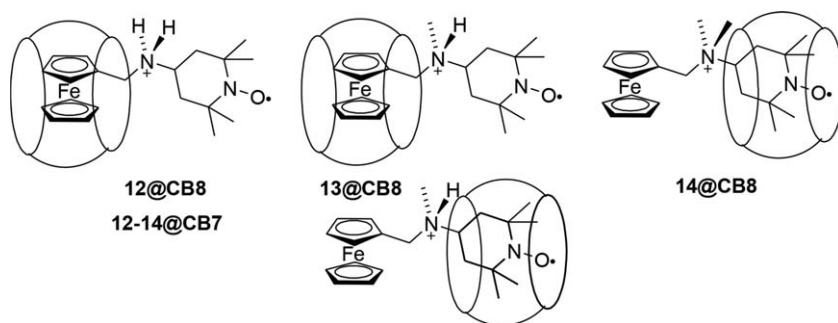


Fig. 25 Different 1:1 complexes depending on the type of CB and on the bulkiness of the linker.

which in turn favoured form **B** over form **A** (Fig. 1d). As observed for CB7 , a_{N} increases slightly when **12** is in the presence of CB8 . On the other hand, in the presence of CB8 , a_{N} values of **13** and **14** decrease meaning that the nitroxyl moiety is immersed into the cavity. Nonetheless, the different changes in a_{N} for **13** and **14** mean that the ferrocene and the nitroxyl moiety in **13** are in competition to be hosted by CB8 whereas for **14**, the association of CB8 with the nitroxyl moiety is strongly favoured (Fig. 25).

Schlick *et al.*⁷⁴ reported either the non-inclusion or the formation of a 1:1 exclusion complex (Fig. 21) for the association of di-*tert*-butyl nitroxide (**DTBN**) and CB6 as highlighted by the absence of significant changes in a_{N} , *i.e.*, $a_{\text{N}} = 17.1 \text{ G}$ both in water and in the presence of CB6 . On the other hand, in the presence of CB7 , **DTBN** showed a

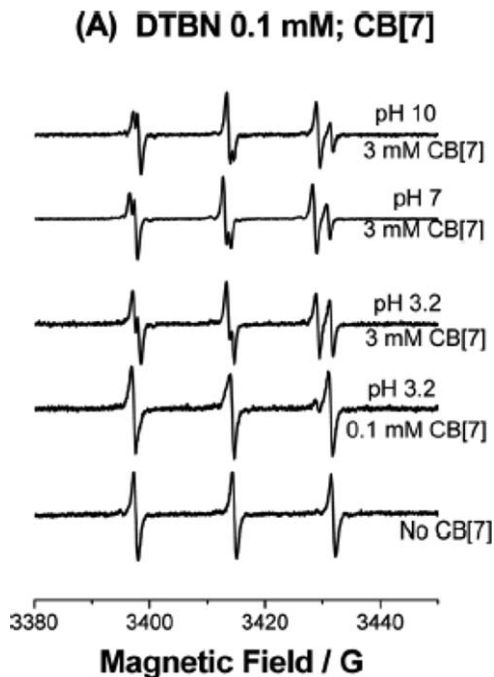


Fig. 26 EPR spectra of DTBN at 300 K in neat water, at the indicated pH and CB7 concentrations. Reprinted with permission from M. Spulber, S. Schlick and F. A. Villamena, Guest Inclusion in Cucurbiturils Studied by EPR and DFT: the Case of Nitroxide Radicals and Spin Adducts of DMPO and MNP, *J. Phys. Chem. A*, 2012, **116**, 8475. Copyright 2012 American Chemical Society.

two-component signal, *i.e.*, $a_N = 17.1$ G assigned to **DTBN** in water and $a_N = 15.5$ G for **DTBN** immersed in the cavity of **CB7** as a 1 : 1 inclusion complex **DTBN@CB7** (Fig. 26). In the presence of **CB8**, only one signal exhibiting a low $a_N = 16.4$ G ascribed to **DTBN** deeply immersed into the cavity is observed. The larger a_N value in **CB8** than in **CB7** is likely due to a higher degree of pyramidalisation of the nitrogen to adapt the nitroxide to the smaller cavity in **CB7** (7.3 Å) in comparison with **CB8** (8.8 Å). The signal of **DTBN** in **CB7** is modified only at a high concentration of NaCl because the sodium cation may be complexed in the portal, causing a redistribution of the electron on the nitroxyl moiety thus favouring form **B** over form **A** providing a larger a_N as observed. The equilibrium constant K for the “in” and “out” process was determined at several pH for **DTBN** (0.1 mM) and spanned from 700–1200 M^{-1} for **CB7** concentrations spanning from 0.5 mM to 3 mM and pH ranging from 3.2 to 10.

Ionita *et al.*⁷⁵ investigated the association properties of **CB6** with TEMPO-derivatives **16–20** (Fig. 27) variously substituted. The association of nitroxides **16–20** with **CB6** is rather moderate as expected for **CB6** (*vide infra*). Equilibrium constants K turned out to be very low ($K < 200 M^{-1}$ for **19**). For **17**, van't Hoff parameters for the association with **CB6** are given as $\Delta H = -21.2$ kJ mol⁻¹ and $\Delta S = -32.9$ J mol⁻¹ K⁻¹.

Bardelang *et al.*⁴⁷ investigate the dynamics of **21–24@CB7–8** 1 : 1 complexes (Fig. 27) and their stability in the presence of reductants.

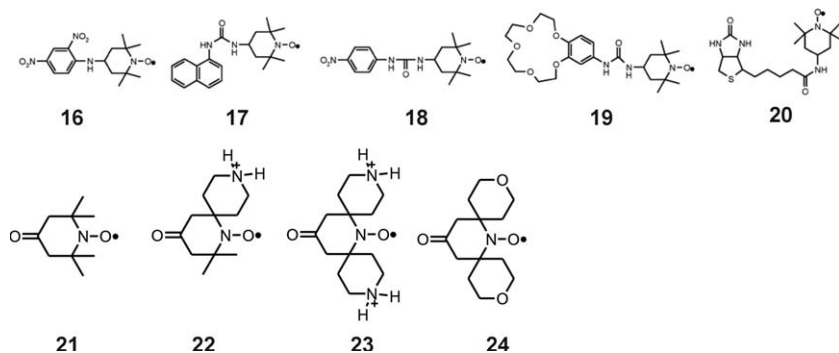


Fig. 27 Nitroxides investigated by Ionita *et al.*⁷⁵ and Bardelang *et al.*⁴⁷ Adapted from ref. 47 with permission from The Royal Society of Chemistry. Adapted from ref. 75 with permission from the Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry.

As expected, a shift of 0.8 G in a_N is observed for **21** in the presence of **CB7** or **CB8**, resulting in K_a values of 10^{-3} M^{-1} and 40 M^{-1} , respectively. For **22**, a_N shifted from 15.7 G in water to 15.0 and 14.5 G in **CB7** and **CB8**, respectively, K_a values are in the range 10^4 to 10^5 M^{-1} . The low values of *ca.* 14.0 G in a_N for **23** ($a_N = 15.3 \text{ G}$ in water) for both **CD7** and **CD8** pointed to the deeper immersion into the cavity (Fig. 28a) and to a strong binding constant as highlighted by the large K_a values, *i.e.*, $1.8 \times 10^5 \text{ M}^{-1}$ for **CB7** and more than 10^6 M^{-1} for **CB8**. These values are likely among the largest ones observed for the association of nitroxides with **CB**. Moreover, **23@CB7** and **23@CB8** do show the line width broadening expected for strongly associated and immersed nitroxides (Fig. 28a). Indeed, **23** carries two piperidinium salt groups as substituents that have 3 main conformations for the 6-membered rings: *trans-trans*, *cis-trans*, and *cis-cis* (Fig. 28b). DFT calculations on **23@CB7** and **23@CB8** showed that **23** is deeply immersed in the cavity and is maintained inside by an important hydrogen bonding network between the ammonium groups and the carbonyl functions of the portals (Fig. 28c). It showed that the *trans-trans* conformation of **23** is preferred in **CB7** whereas the *cis-cis* conformation is preferred in **CB8**. Both the H-bond network and the size of the cavities arrange the nitroxide so that the nitroxyl moiety is perpendicular to the axis of CB affording a free motion inside. Consequently, the motion of the nitroxide immersed in the cavity was de-correlated from the motion of the cavity affording, hence, *unexpected* narrow EPR lines. Although **23** (0.1 mM) is instantly reduced in the presence of ascorbic acid (2 mM), signals of **23@CB7** and **23@CB8** do not decay for almost 90 minutes (Fig. 28d), pointing again to the protective effect of the CB due to both a strong association with the nitroxide and its deep immersion into the cavity.

3.2 Spin-spin interaction as applications

Lucarini *et al.*⁷⁶ investigated the control of spin-spin exchange interactions in **25** and **26** (Fig. 29) using **CB6**, **CB7** and **CB8** through either the encapsulation of the nitroxide moiety or the formation of a

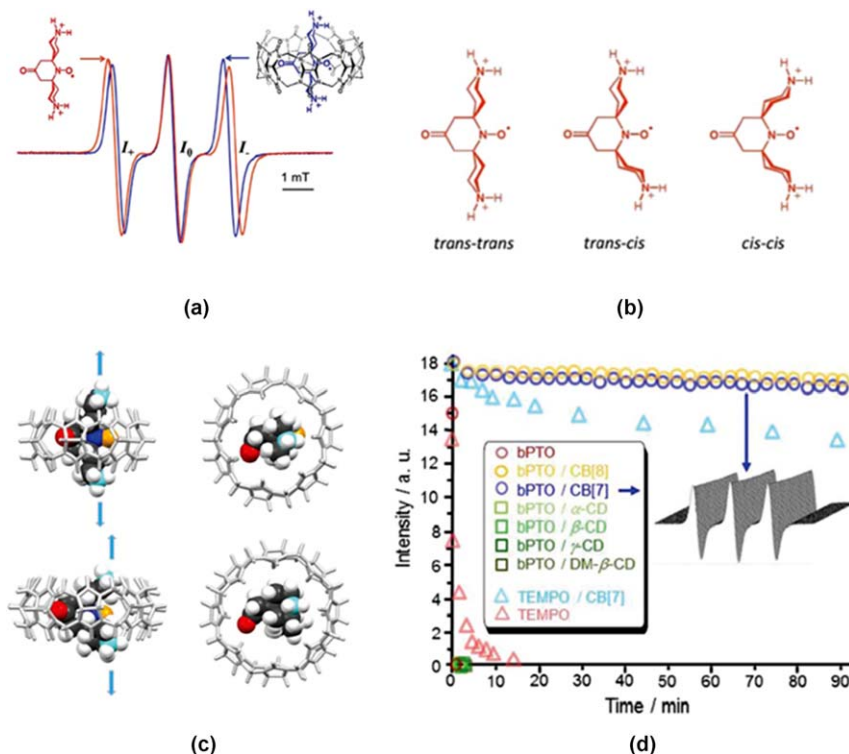


Fig. 28 (a) EPR spectra in water of **23** alone (0.2 mM, red line) and in the presence of **CB7** (1.4 equiv., blue line). (b) The three most stabilised conformers of **23** (within 0.3 kcal mol⁻¹). (c) Side (left) and top (right) views of the inclusion complex of **23** in **CB7** (top, *trans-trans* conformer) and in **CB8** (bottom, *cis-cis* conformer) as found after DFT minimisation (colours of the N and O atoms of the N–O• group are dark blue and yellow respectively). (d) Reduction experiments (decay of the EPR signal) of **23** (○, 0.2 mM) and **TEMPO** (△, 0.1 mM) and in the presence of **CB7** (12.75 mM for **TEMPO** (△) and 0.35 mM for **23** (○)), **CB8** (0.35 mM, ○), α-CD (50 mM □), β-CD (10 mM □), γ-CD (100 mM □) and DM-β-CD (200 mM □) by ascorbic acid (2 mM), and sodium ascorbate: 2 mM for **TEMPO** and **TEMPO/CB7**. Reproduced from ref. 47 with permission from The Royal Society of Chemistry.

pseudorotaxane (Fig. 30a). Whatever the CB, the typical 5-line pattern of **25** due to the spin–spin exchange interaction is suppressed after addition of an excess of CB (Fig. 30b). The changes in ΔB (Fig. 30c) reveal that **25@CB6** is better described by a 1:1 exclusion complex (Figs. 21 and 30a). The larger change in ΔB for **25@CB7** supports formation of a pseudorotaxane 1:1 complex (Fig. 30c) which collapses with increasing basicity. On the other hand, the large ΔB observed for **25@CB8** is better ascribed to the immersion of the TEMPO-end of the thread into **CB8** cavities (Fig. 30c). Thus, **TEMPO** is too large for a **CB6** cavity and can interact only with the portal, and **CB7** is large enough to be crossed by **TEMPO** but not large enough to accommodate it efficiently, and **CB8** has a cavity large enough to provide the best stabilizing interactions in the hydrophobic cavity. Thread **26** showed the expected 5-line pattern for a biradical with a spin–spin exchange interaction whereas for [3]rotaxane **27**

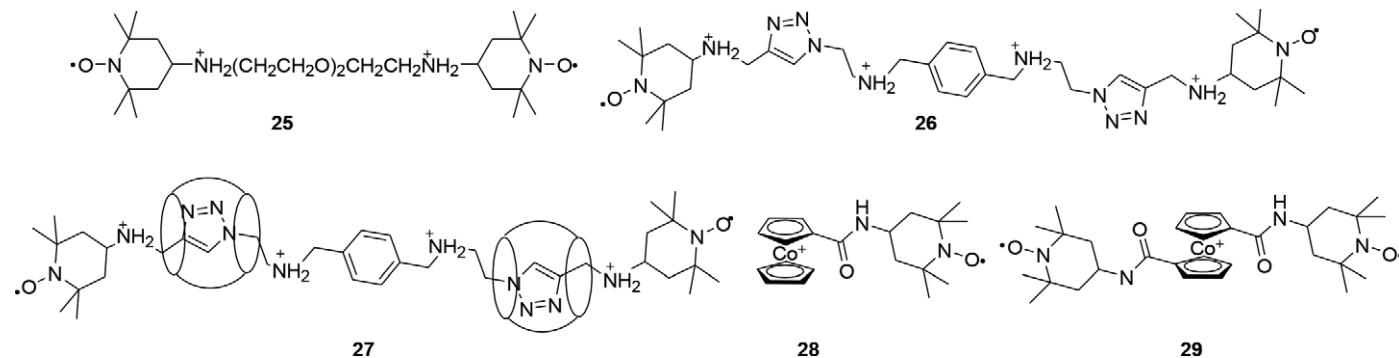


Fig. 29 Nitroxides investigated by Lucarini *et al.* (25–27),⁷⁶ and Kaifer *et al.* (28–29).⁷⁸ Adapted from ref. 76 with permission from The Royal Society of Chemistry. Reprinted with permission from S. Yi, B. Captain, M. F. Ottaviani and A. E. Kaifer, Controlling the Extent of Spin Exchange Coupling in 2,2,6,6-Tetramethylpiperidine-1-Oxyl (TEMPO) Biradicals via Molecular Recognition with Cucurbit[N]Urils Hosts. *Langmuir*, 2011, 27, 5624. Copyright 2011 American Chemical Society.

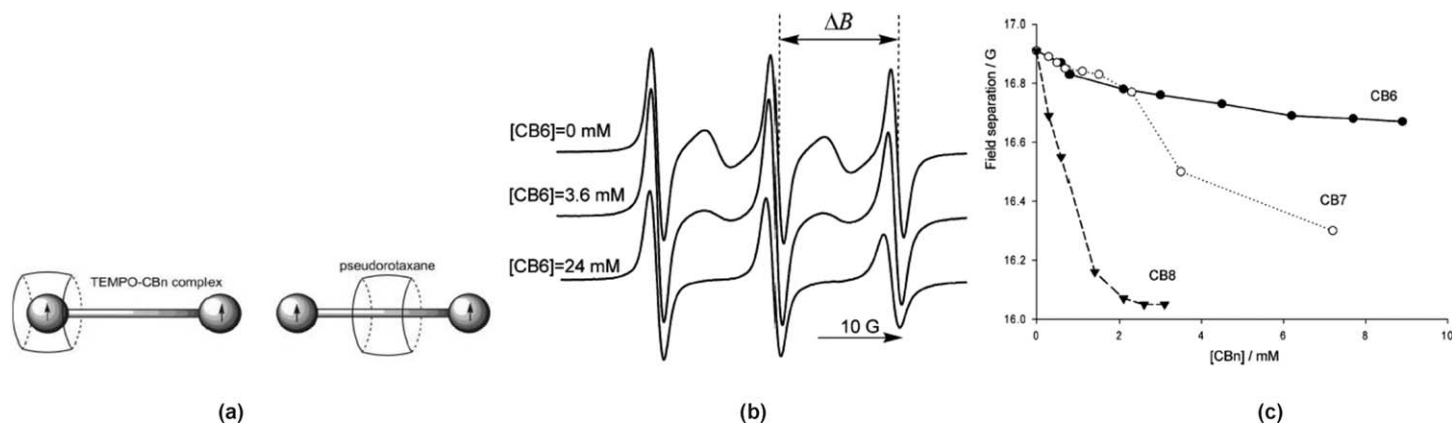


Fig. 30 (a) 1 : 1 exclusion complex (left) and pseudorotaxane. (b) EPR spectra of 25 (0.26 mM) in the presence of different amount of CB6 in water at 298 K as example. (c) Field separation between the ESR central and high field lines (ΔB) for biradical 25 in the presence of different amount of CB6 (●), CB7 (○) and CB8 (▼) in water at 298 K. With CB6 NaCl 0.1 M was added to the solution. Adapted from ref. 76 with permission from The Royal Society of Chemistry.

(two **CB6** and thread **26**, Fig. 29) this interaction is suppressed regardless of the conditions as the **CB6** cannot be removed due to the bulky **TEMPO**-end groups. Their purpose⁷⁷ was nicely highlighted by PELDOR experiments which showed a narrow distribution of the end-end distance between the two **TEMPO** groups in **27** as expected for threaded **26** because of the presence of two **CB6**. In contrast, the distribution for free **26** was broader, which has been assessed on several conformations showing a spin-spin interaction between the two **TEMPO**-ends of the thread **26**.

Turro *et al.*⁵¹ investigated the control of the spin-spin exchange interaction in **T1** (Fig. 14) using **CB7** and **CB8**. They showed that one equivalent of **CB8** was sufficient to suppress the spin-spin exchange interaction in **T1** according to the shift from a 5-line pattern to the conventional 3-line pattern after the addition of **CB8** (Fig. 31a). This observation is ascribed either to a barrier due to the cage wall suppressing the spin-spin interaction or to conformational changes due to the complexation that keeps the two nitroxide groups apart as displayed in Fig. 31b (or to both events).⁵¹ In comparison with other hosts such as **CB7**, **CA8**, β - and γ -**CD**, and an SDS micelle, **CB8** showed a unique behaviour because it suppressed completely the spin-spin communication in **T1** (Fig. 31c).

In agreement with their previous report on hosting ferrocene attached **TEMPO** by **CB7**,⁷² Kaifer *et al.*⁷⁸ observed for cobaltocenium-nitroxide **28** (Fig. 29) a strong selectivity of hosting the cobaltocenium moiety by **CB7** and the nitroxide moiety by **CB8** (Fig. 32) as supported by EPR and NMR data. The slight increase in a_N observed in the presence of **CB7** is likely due to better solvation of the whole molecule by water after immersion of the cobaltocenium part, which favoured form **B** over form **A** (Fig. 1d). The large decrease of a_N in the presence of **CB8** is ascribed to the deep immersion of the **TEMPO** group of **28** in the hydrophobic cavity of **CB8**. As expected the typical 5-line pattern for the binitroxide **29** decayed after addition to **CB8** (Fig. 33a–e), which associated specifically with the **TEMPO** group, to be almost removed for one equivalent of **CB8** (Fig. 33g). The 5-line pattern is completely cancelled for two equivalents of **CB8** and a_N did not decrease anymore, thus supporting deep immersion of two **TEMPO** groups in two **CD8**. The reversibility of the process has been checked by adding adamantyltrimethylammonium salt as a competitor which restored the 5-line pattern (Fig. 33f). The **29**@(**CB8**)₂ 1 : 2 host complex has been confirmed by X-ray analysis and the host-guest process can be described as displayed in Fig. 33h. The same behaviour is observed in the presence of **CB7** except that the decrease in a_N is smaller meaning that the **TEMPO** groups are partially immersed into the cavity, probably lying in the portal; 20 equivalents of **CB7** are necessary to suppress the spin-spin interaction. These data point to the weak association between **TEMPO** and **CB7**, despite the formation of the 1 : 2 host complex **29**@(**CB7**)₂.

Lucarini *et al.*⁵² investigated the control of the spin-spin exchange interaction in [2]rotaxane **7** (Fig. 14) with **CB7** as a host. The typical 5-line pattern (Fig. 16) for the spin-spin exchange interaction in **7** is cancelled out (Fig. 34a) upon the addition of an excess of **CB7** (even though it is not the best host, *vide supra*) and regenerated upon the addition of aniline at

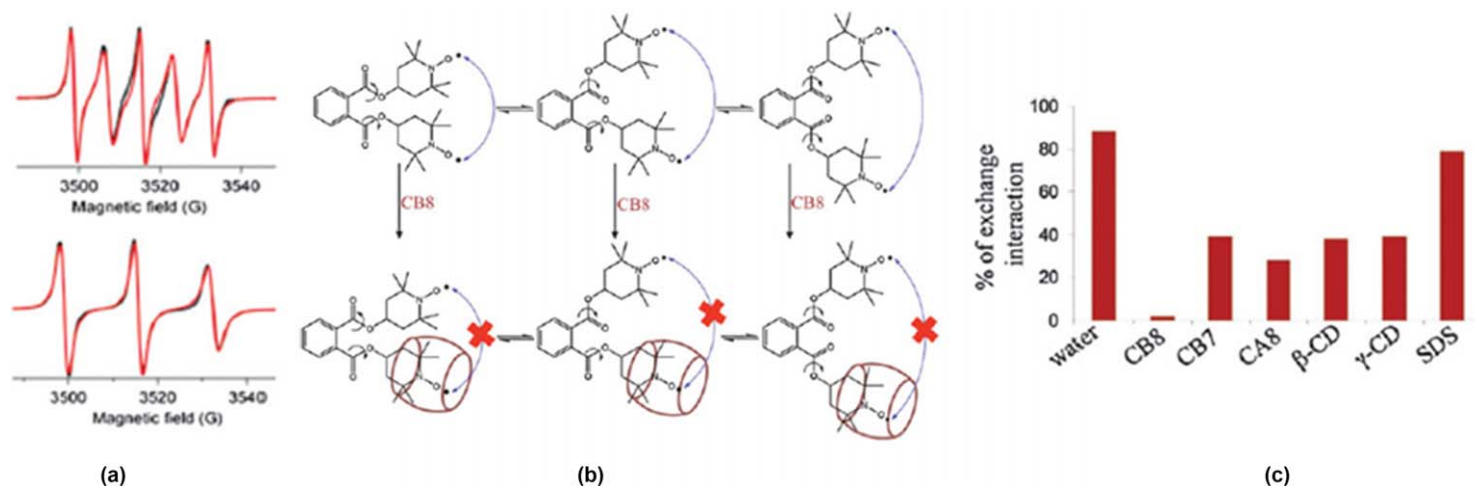


Fig. 31 (a) EPR spectra (black lines) and their simulations (red lines) for T1 (1 mM) in water (top three-line component: $a_N = 16.9$ G, $\tau_c = 0.06$ ns, five-line component: 88%) and in the presence of CB8 (bottom, 1 mM, $a_N = 16.7$, $\tau_c = 0.21$ ns, T1@CB8 (1:1)). (b) Representation of the possible conformers of T1 and T1@CB8. (c) Comparison of percentage of spin–spin exchange interaction in T1; note the exchange interaction in CB8 was 0%. For γ -CD and SDS micelles the broad Heisenberg component was subtracted before calculation of % exchange interaction. T1:host = 1:2 and in SDS (1:1200), CA8 is for calix[8]arene. Reproduced from ref. 51 with permission from The Royal Society of Chemistry.

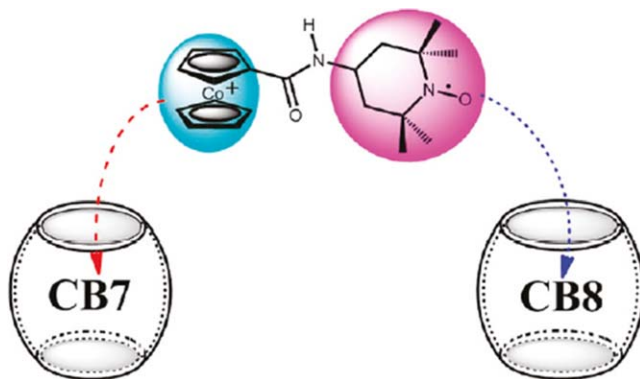


Fig. 32 Self-sorting of CB7 and CB8 by guest **28**. Reprinted with permission from S. Yi, B. Captain, M. F. Ottaviani and A. E. Kaifer, Controlling the Extent of Spin Exchange Coupling in 2,2,6,6-Tetramethylpiperidine-1-Oxyl (TEMPO) Biradicals via Molecular Recognition with Cucurbit[N]uril Hosts. *Langmuir*, 2011, 27, 5624. Copyright 2011 American Chemical Society.

pH = 3 (anilinium chloride as a competitor, Fig. 34b) and again cancelled when pH is risen to 8 (aniline is a poor competitor, Fig. 34c).

Bardelang *et al.*⁷⁹ investigated the modulation of the spin-spin exchange interactions in tetranitroxide **4T** (Fig. 35a) in the presence of **CB8**. At 90 °C, a 9-line pattern signal with hcc of 1/4 of a_N is observed for **4T** as for 4 nitroxides in interaction but not in the expected ratio 1 : 4 : 10 : 16 : 19 : 16 : 10 : 4 : 1 because the very rapid chemical exchange is not yet achieved (Fig. 36a). At room temperature, the signal of **4T** looks like the superimposition of a sharp 3-line signal and a broad 6-line signal (Fig. 36b) because of a major contribution of the J modulation to the transverse electron relaxation time. This signal changes dramatically after the addition of **CB8** up to two equivalents of **CB8** (Fig. 36c). The reversibility of the probes has been verified using the benzylamine as a competitor to restore the signal observed in Fig. 36b. Assuming that the EPR signal is due to 5 species, namely **4T**, **4T** with a triplet component, **4T@CB8**, **4T@CB8**₂ and **4T@CB8**₃, and due to a step-by-step immersion process, all spectra were nicely simulated. The equilibrium constants $K_{n,m}$ were determined, as was the evolution of each species (Fig. 36d). It appears that the first complexation of a **TEMPO** group by **CB8** has an equilibrium constant $K_1 = 4027 \text{ M}^{-1}$, and the immersion of a second **TEMPO** group in another **CB8** exhibits a 50-fold larger K_2 , *i.e.*, $K_2 = 202\,800 \text{ M}^{-1}$, whereas the inclusion of a third **TEMPO** group has a much lower $K_3 = 157 \text{ M}^{-1}$. These values are indicative of a strong cooperative effect from **4T@CB8** 1 : 1 complex to **4T@CB8**₂ 1 : 2 complex whereas no cooperative effect is observed for the formation of **4T@CB8**₃. These results are more consistent with the folded model of **4T@CB8**₂ (right complex in Fig. 35b) than with the highly symmetrical sandwich complex (left complex in Fig. 35b). Several H-bonding interactions between host-guest and guest-guest stabilised the folded complex. This assumption is supported by HRMS and NMR analysis as well as DFT calculations.

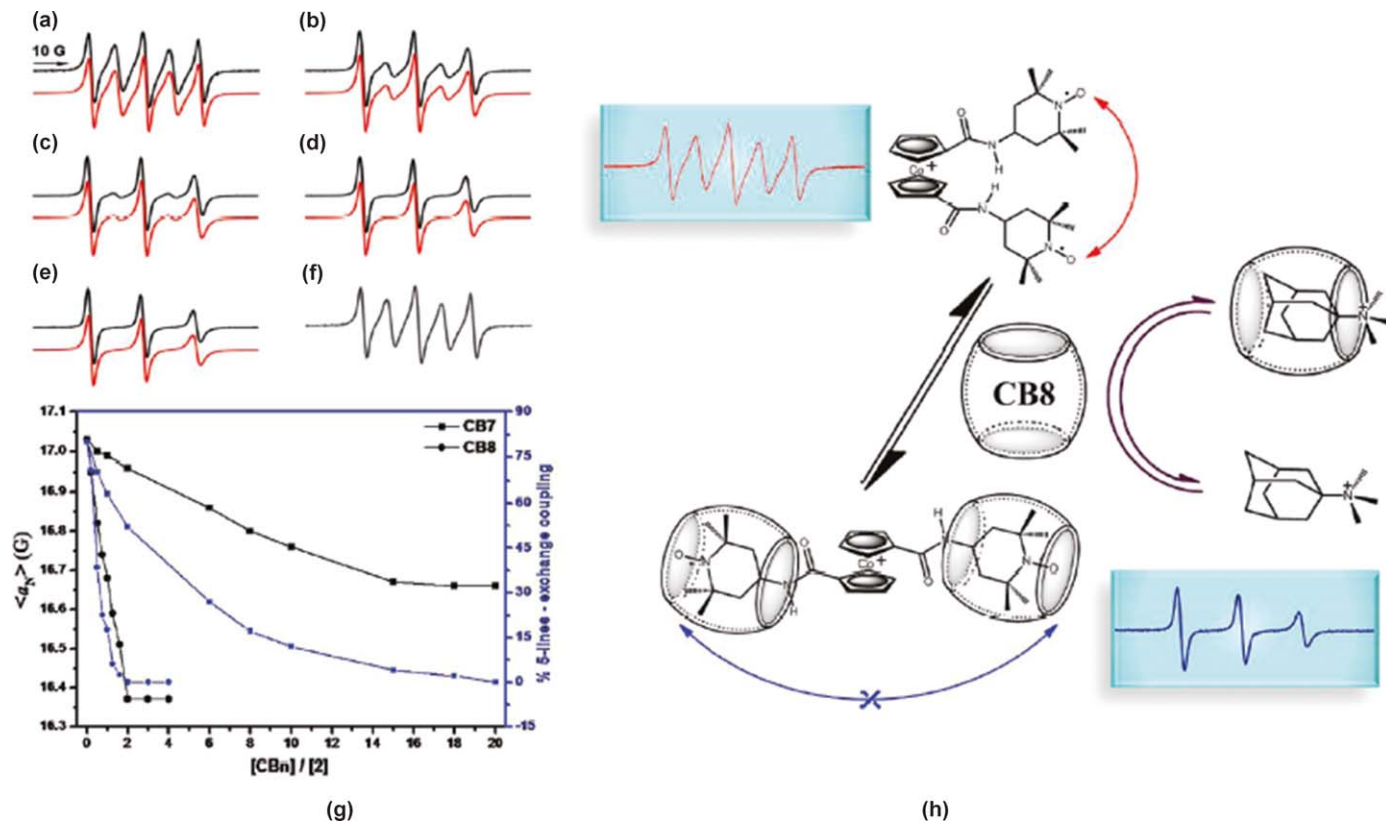


Fig. 33 EPR spectra of 0.05 mM **29** (H_2O , 295 K) (a) in the absence and in the presence of (b) 0.5, (c) 1.0, (d) 1.6, (e) 2.0 equiv. **CB8** and (f) 2.0 equiv. **CB8** plus excess adamantyltrimethylammonium salt as a competitor. (g) Variation of the $\langle a_N \rangle$ value (black line) and the computed extent of exchange coupling (blue line) as a function of added host equivalents **CB7** (■) and **CB8** (●). (h) Host-guest equilibria modulating the degree of spin exchange coupling in biradical **29**. Reprinted with permission from S. Yi, B. Captain, M. F. Ottaviani and A. E. Kaifer, Controlling the Extent of Spin Exchange Coupling in 2,2,6,6-Tetramethylpiperidine-1-Oxyl (TEMPO) Biradicals via Molecular Recognition with Cucurbit[N]uril Hosts. *Langmuir*, 2011, 27, 5624. Copyright 2011 American Chemical Society.

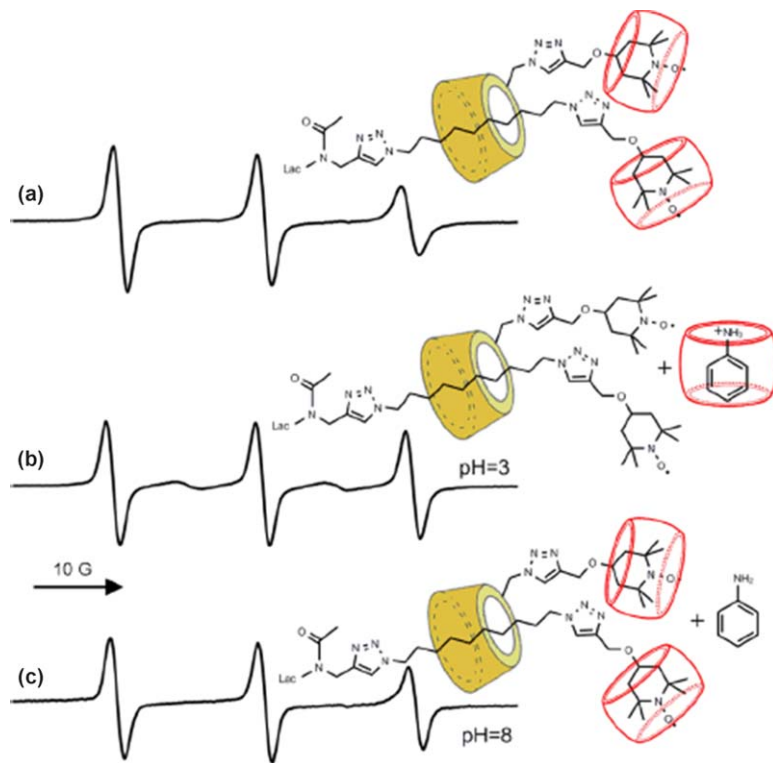


Fig. 34 EPR spectra of the bis-labelled rotaxane **7** (0.05 mM) in the presence of CB7 (10 mM): (a) initial solution and in the presence anilinium chloride (25 mM) at (b) pH 3 and (c) pH 8. Reprinted with permission from C. Casati, P. Franchi, R. Pievo, E. Mezzina and M. Lucarini, Unraveling Unidirectional Threading of α -Cyclodextrin in a [2]Rotaxane Through Spin Labeling Approach, *J. Am. Chem. Soc.*, 2012, **134**, 19108. Copyright 2012 American Chemical Society.

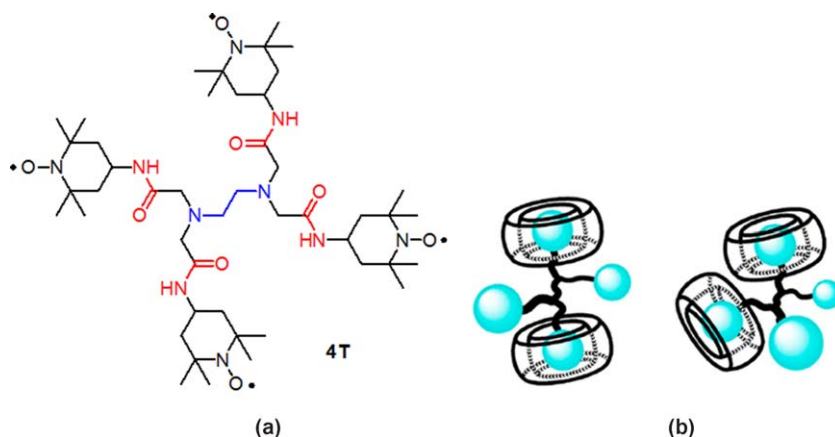


Fig. 35 (a) Structure of **4T**. (b) Two possible modes of inclusion for the complex **4T@CB8**₂. Reprinted with permission from D. Bardelang, G. Casano, F. Poulhès, H. Karoui, J. Filippini, A. Rockenbauer, R. Rosas, V. Monnier, D. Siri, A. Gaudel-Siri, O. Ouari and P. Tordo, Spin Exchange Monitoring of the Strong Positive Homotropic Allosteric Binding of a Tetra-radical by a Synthetic Receptor in Water. *J. Am. Chem. Soc.*, 2014, **136**, 17570. Copyright 2014 American Chemical Society.

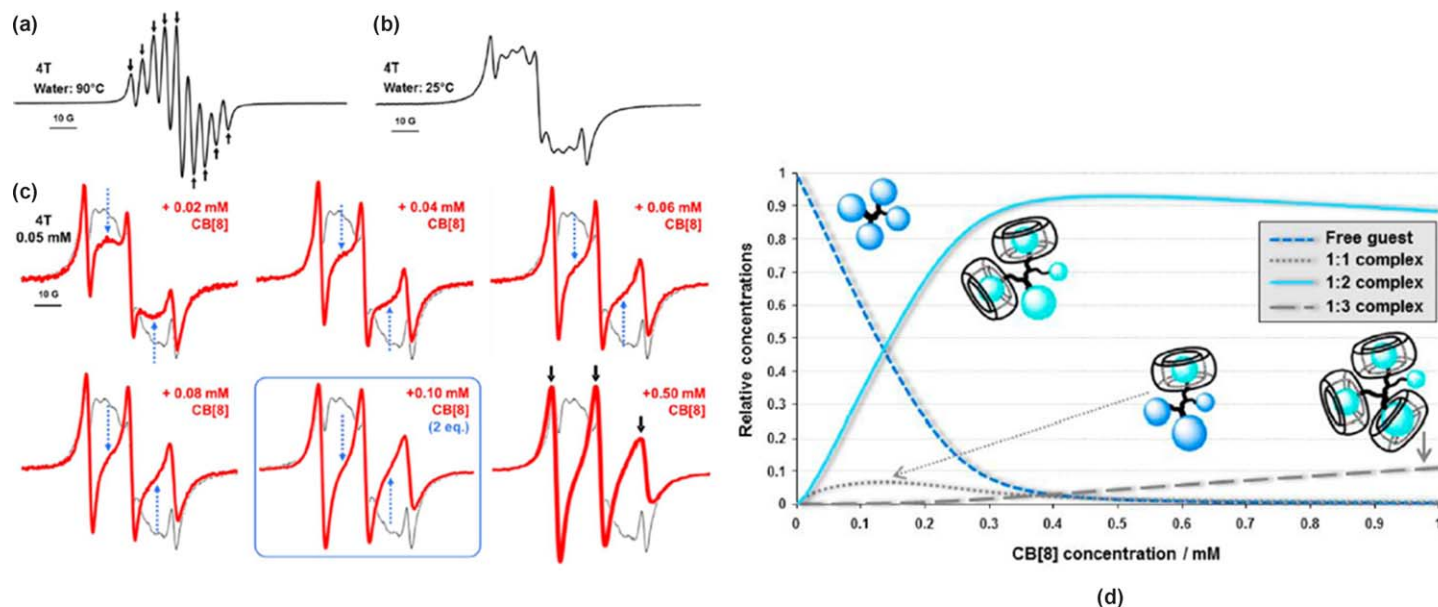


Fig. 36 EPR spectra in water solution of 4T (a) at 363 K, (b) at 298 K, and (c) in the presence of increasing amounts of CB8 highlighting the absence of noticeable changes above two equivalents of CB8. (d) Product distribution for the binding of tetranitroxide 4T (0.05 mM) with CB8 in water. Reprinted with permission from D. Bardelang, G. Casano, F. Poulhès, H. Karoui, J. Filippini, A. Rockenbauer, R. Rosas, V. Monnier, D. Siri, A. Gaudel-Siri, O. Ouari and P. Tordo, Spin Exchange Monitoring of the Strong Positive Homotropic Allosteric Binding of a Tetradical by a Synthetic Receptor in Water, *J. Am. Chem. Soc.*, 2014, **136**, 17570. Copyright 2014 American Chemical Society.

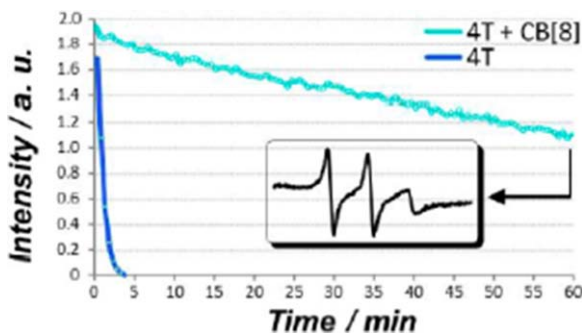


Fig. 37 Kinetics of the signal decay before (top cyan line) and after (bottom blue line) addition of CB[8] (1 mM) with ascorbic acid (5 mM) to a solution of tetradical 4T (0.1 mM) in water. Adapted with permission from D. Bardelang, G. Casano, F. Poulhès, H. Karoui, J. Filippini, A. Rockenbauer, R. Rosas, V. Monnier, D. Siri, A. Gaudel-Siri, O. Ouari and P. Tordo, Spin Exchange Monitoring of the Strong Positive Homotropic Allosteric Binding of a Tetradical by a Synthetic Receptor in Water, *J. Am. Chem. Soc.*, 2014, **136**, 17570. Copyright 2014 American Chemical Society.

Bardelang *et al.*⁷⁹ observed a striking protective effect of CB8 against ascorbic acid used as a reductant: 4T decayed in 180 s and 4T@CB₈ in more than 75 minutes (at this time, 75% of 4T@CB₈ was reduced) (Fig. 37). This result highlights a very slow exchange process of CB8 and that TEMPO groups are deeply immersed into the cavity.

Using CB6, Ionita *et al.*⁷⁵ investigated the control of the spin-spin communication in bis- and trisnitroxides (10^{-4} M) attached to a rigid linker as in bisnitroxides 30 and 33, to a mobile linker as in bisnitroxides 31 and 32, and the trisnitroxide 34 (Fig. 38). More than 3-line patterns are observed for 30, 31, and 33 pointing to spin-spin exchange interactions of various intensities. The high mobility of the linker in 32 reduced the probability that the 2 nitroxide groups would end up in the vicinity of each other and, thus, no spin-spin exchange interaction are observed by EPR. More surprising is the absence of a spin-spin exchange interaction for the trinitroxides 34 in sharp contrast to the tetranitroxide 4T reported by Bardelang *et al.*⁷⁹ In the presence of CB6 (10^{-2} M), no association is observed for 30 and 34 whereas weak associations are reported for 31–33 as highlighted by the low values of equilibrium constants K , *i.e.*, $K < 200 \text{ M}^{-1}$ for 33. Nevertheless, a complete suppression of the spin-spin exchange interaction is observed for 31 and 33. For 32, van't Hoff parameters for the association with CB6 are given as $\Delta H = -31.7 \text{ kJ mol}^{-1}$ and $\Delta S = -65.2 \text{ J mol}^{-1} \text{ K}^{-1}$.

4 Miscellaneous

4.1 Nanocapsules as host

Octa acid (OA) is a water-soluble host molecule and the peculiarity of OA in comparison with other host molecules is that in most cases two host molecules encapsulate a guest with formation of a closed capsule (Fig. 39a–c). Ramamurthy and co-workers⁸⁰ employed ¹H NMR and EPR techniques to study the dynamics of a guest molecule of different shapes

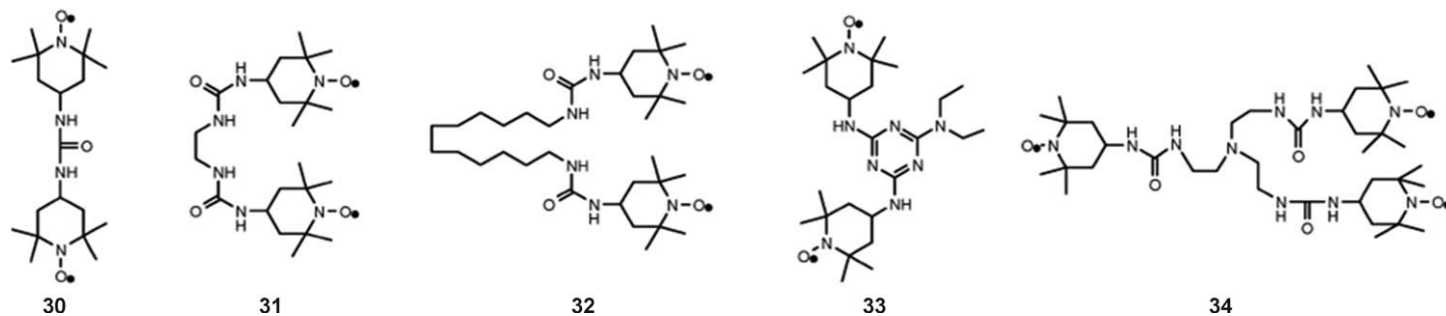


Fig. 38 Nitroxides investigated by Ionita *et al.*⁷⁵ Adapted from ref. 75 with permission from the Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry.

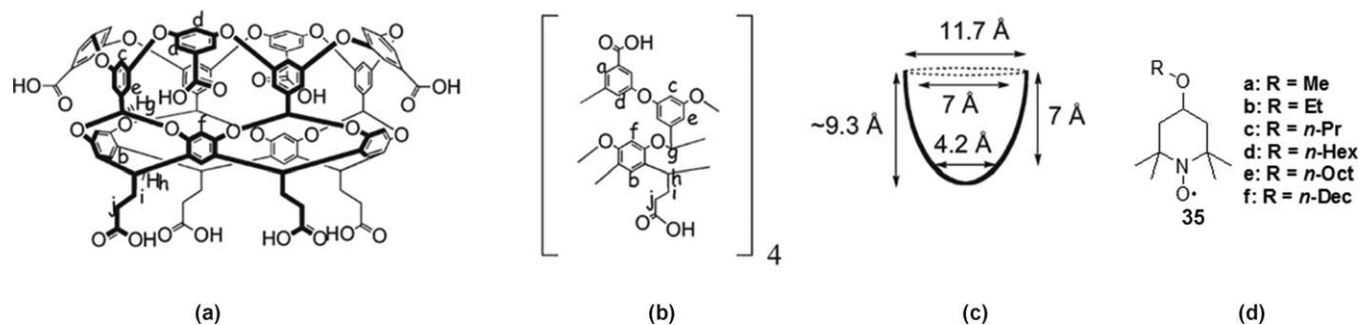


Fig. 39 Chemical structure (a), structure of a single panel (b), and (c) cartoon representation with dimensions of the host (octa acid) and (d) various nitroxides investigated. Adapted with permission from R. Kulasekharan, N. Jayaraj, M. Porel, R. Choudhury, A. K. Sundaresan, A. Parthasarathy, M. F. Ottaviani, S. Jockusch, N. J. Turro and V. Ramamurthy, Guest Rotations Within a Capsuleplex Probed by NMR and EPR Techniques, *Langmuir*, 2010, **26**, 6943. Copyright 2010 American Chemical Society.

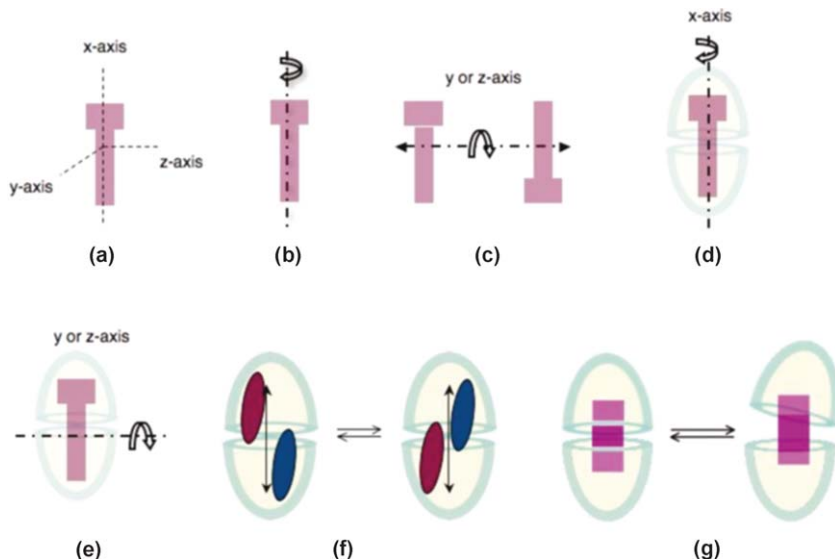


Fig. 40 Cartoon representations of various motions of host–guest complexes being investigated (b–f), host opening and closing (g), and the three axes of the guest (a). Reprinted with permission from R. Kulasekharan, N. Jayaraj, M. Porel, R. Choudhury, A. K. Sundaresan, A. Parthasarathy, M. F. Ottaviani, S. Jockusch, N. J. Turro and V. Ramamurthy, Guest Rotations Within a Capsuleplex Probed by NMR and EPR Techniques, *Langmuir*, 2010, **26**, 6943. Copyright 2010 American Chemical Society.

and sizes within an **OA** capsuleplex (Fig. 39a–c). Addition of nitroxides allowed them to understand the dynamics of the host, the guest and the host–guest complex on the timescale of a photochemically triplet excited guest molecule. Taking into account the difference in time scales among an excited singlet, triplet states, NMR, and EPR, the system may be static on the NMR timescale but dynamic on the EPR timescale. The authors used nitroxides **35a–f** (Fig. 39d) with substituted alkyl chains of different size. Motions in the capsuleplex may occur on different axes (Fig. 40a–d). The EPR data analysis leads to the conclusion that paramagnetic guest molecules undergo rotation along the molecular *x*-axis within the capsule and that their rotational motion is dependent on the length of the alkyl chain (Fig. 40b–d). Rotational correlation time of nitroxides attached to an alkyl chain of different length changes from 2 to 10 ns.

Later in a series of papers by Ramamurthy, Turro and co-workers,^{81–84} octaacid nanocapsules that formed guest–host complexes were used for investigation of the occurrence of electron–electron spin communication. Different types of systems were studied, in particular:

(1) a free nitroxide and photoexcited triplet chromophore inside **OA** (Fig. 41a) for studies on the communication between a confined excited singlet donor or a triplet donor and a free acceptor (singlet–singlet and triplet–triplet energy transfers respectively);⁸³

(2) a free nitroxide and a chromophore molecule covalently attached to nitroxides in **OA** (Fig. 41b) for studies on the communication between a confined radical and a ‘free’ radical (spin–spin exchange);⁸²

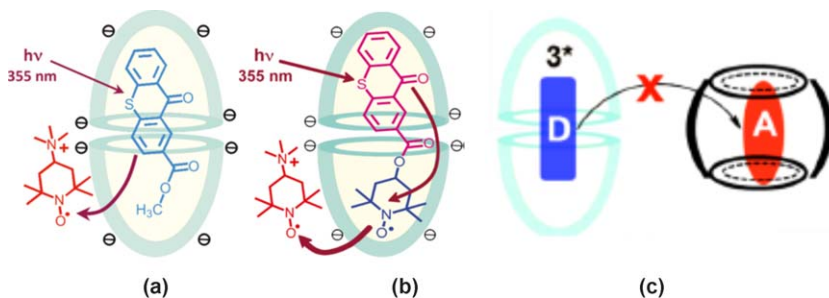


Fig. 41 The scheme of experiments to investigate the occurrence of electron–electron spin communication. (a) Polarisation transfer from an encapsulated triplet excited molecule to a positively charged nitroxide in the water solution. (b) Polarization transfer from encapsulated nitroxide covalently attached to triplet excited molecule, to a positively charged nitroxide in the water solution. (c) No electron transfer or polarisation transfer from an encapsulated nitroxide covalently attached to a triplet excited molecule, to nitroxide encapsulated in cucurbituril in water solution. For (a), reprinted with permission from S. Jockusch, M. Porel, V. Ramamurthy and N. J. Turro, From a Polarized Ketone Triplet State Incarcerated Within a Nanocapsule to a Nitroxide in the Bulk Aqueous Solution, *J. Phys. Chem. Lett.*, 2011, **2**, 2877. Copyright 2011 American Chemical Society. For (b), reprinted with permission from S. Jockusch, O. Zeika, N. Jayaraj, V. Ramamurthy and N. J. Turro, Electron Spin Polarization Transfer From a Nitroxide Incarcerated Within a Nanocapsule to a Nitroxide in the Bulk Aqueous Solution, *J. Phys. Chem. Lett.*, 2010, **1**, 2628. Copyright 2010 American Chemical Society. For (c), reprinted with permission from V. Ramamurthy, S. Jockusch and M. Porel, Supramolecular Photochemistry in Solution and on Surfaces: Encapsulation and Dynamics of Guest Molecules and Communication Between Encapsulated and Free Molecules, *Langmuir*, 2015, **31**, 5554. Copyright 2015 American Chemical Society.

(3) nitroxide in complex with another container (cucurbituril, calixarene, or cyclodextrin) and a chromophore molecule covalently attached to nitroxides or without nitroxide in OA (Fig. 41c) for studies on the communication between an confined excited molecule and confined nitroxide.⁸⁴

Steady-state EPR and the relaxation time of ^1H NMR signals of the host and the guest in water was used to prove the location of the nitroxide in relation to the capsule. The basic idea behind these experiments was to use chemically induced dynamic electron polarisation (CIDEP) detected by time-resolved EPR. Spin-polarised nitroxides were generated by quenching of triplet excited states of chromophore molecule (Fig. 42) covalently attached to ^{15}N -labelled nitroxides and were detected using time-resolved EPR experiments on ^{14}N -labeled nitroxides localised outside the capsule.⁸⁴ It was found that spin polarisation of the ^{15}N -labelled nitroxide was transferred through the walls of the nanocapsule to a ^{14}N -labelled nitroxide in the external bulk solvent (Fig. 42a). Three paramagnetic molecules, cationic (Tx), anionic (TQ), and neutral (T) nitroxides and eight guest molecules were used. The authors showed that the efficiency of the communication between the encapsulated guest and molecules in the bulk solvent is controlled by supramolecular factors such as Coulombic attraction and repulsion between the guest@host complex and charged molecules in the bulk solvent phase. In particular

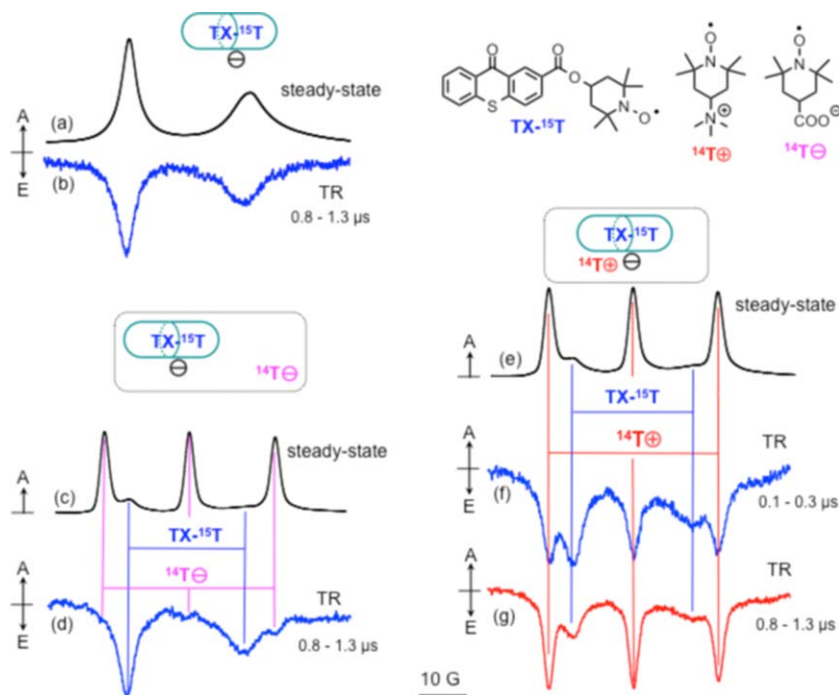


Fig. 42 Steady-state EPR (a, c, e; integrated form) and TR-EPR (b, d, f, g) spectra of OA-encapsulated TX- ^{15}T in the absence (a, b) and presence of $^{14}\text{T}^\ominus$ (c, d) or $^{14}\text{T}^\oplus$ (e, f, g) recorded in different time windows after pulsed laser excitation at 355 nm (5 ns pulse width) in deoxygenated aqueous buffer solutions at room temperature. $[\text{TX-}^{15}\text{T}] = 0.5 \text{ mM}$; $[\text{OA}] = 1 \text{ mM}$, $[^{14}\text{T}^\oplus] = 1 \text{ mM}$, $[^{14}\text{T}^\ominus] = 1 \text{ mM}$, 10 mM borate buffer, pH 9. Reprinted with permission from V. Ramamurthy, S. Jockusch and M. Porel, Supramolecular Photochemistry in Solution and on Surfaces: Encapsulation and Dynamics of Guest Molecules and Communication Between Encapsulated and Free Molecules, *Langmuir*, 2015, **31**, 5554. Copyright 2015 American Chemical Society.

molecules localised inside negatively charged (COO^-) organic container could be deactivated by a cationic nitroxide at a nearly diffusion controlled rate. Thus it was concluded that the presence of a wall between the excited molecule and a nitroxide affects neither exchange nor charge interaction between them and that spin-spin communication between an excited molecule and a free radical can occur even without direct overlap of their orbitals.⁸⁴

In the third type of experiments control of the close interaction between species was used with the help of the second container (cucurbituril, calixarene, or cyclodextrin). The results revealed that the presence of a double wall between the excited molecule and the nitroxide completely prevents the deactivation process; the best effect is achieved with cucurbit[8]uril.⁸⁴

It can be seen that addition of negatively charged nitroxide $^{14}\text{T}^\ominus$ affects spin polarisation negligibly, whereas for positively charged nitroxide $^{14}\text{T}^\oplus$ strong polarisation is observed on $^{14}\text{T}^\oplus$ due to polarization transfer.

4.2 Calixarenes (hexameric resorcinarene capsule)

4.2.1 Calixarene–nitroxide complexes in solution. The first paper concerning nitroxide encapsulation in a hexameric resorcinarene capsule in water-saturated nonpolar media (chloroform and dichloromethane) was performed by Kaifer and co-workers.⁸⁵ It was found that the a_N value measured for encapsulated tempamine is slightly greater than that of the free nitroxide probe indicating a small increase in the polarity of the microenvironment around the spin probe after encapsulation. A similar conclusion was drawn from the small decrease observed in the g factor. The data obtained in this paper revealed that encapsulation of guests with hexameric resorcinarene capsules **37** and **38** (Fig. 43) is affected by the electrostatic nature of the guests involved (Fig. 44a). It was found that a guest molecule such as **21** (Fig. 27) and **TOH** (Fig. 7) whose surface is predominantly covered by negative charge density, fails to undergo encapsulation, whereas the nitroxide probes with positive charge density, **TNH2** (Fig. 7), **36** (Fig. 43), and **28** (Fig. 29), show good encapsulation in the hexameric resorcinarene capsule (Fig. 44b).

Recently,⁸⁶ several nitroxide spin probes, **TEMPO**, **21** (Fig. 27), **DTBN**, and banana shaped nitroxide **39** (Fig. 43), were used to investigate the formation and inner space of a water-soluble capsular assembly. The use of the EPR/spin-probing technique to monitor the binding process enabled (1) to evaluate the inner cylindrical space metrics providing the maximum guest width and height, (2) to quantitatively determine the strength of molecular recognition, and (3) to show the ability of the capsule to widen in the central part *via* bending of one of the aromatic panels. The banana shape of **39** was used to redefine the limits of guests that can be included.

4.2.2 Calixarene–nitroxide complexes in solid state. In series of papers,^{87–89} guest–host complexes based on calixarene–nitroxides in a solid state were synthesised and their structure and dynamics of the nitroxide inside the capsule at different temperatures were studied using single-crystal X-ray diffraction and wide-frequency-range EPR at X, W-band and 360 GHz. The structures of the calixarene guest–host systems are shown in Fig. 46. It should be noted that the first efforts to

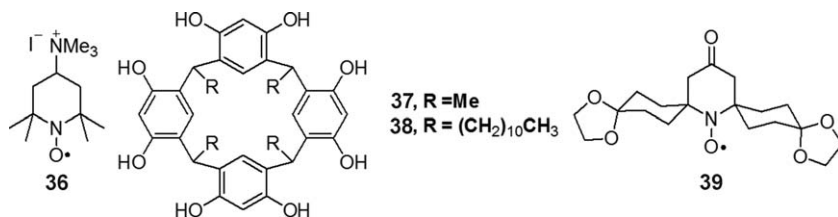


Fig. 43 Structures of nitroxides **36** and **39**, and of the resorcinarene hosts **37** and **38**. From E. Mileo, S. Yi, P. Bhattacharya and A. E. Kaifer, Probing the Inner Space of Resorcinarene Capsules with Nitroxide Guests, *Angew. Chem.*, 2009, **48**, 53373. Copyright © 2009 by John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.

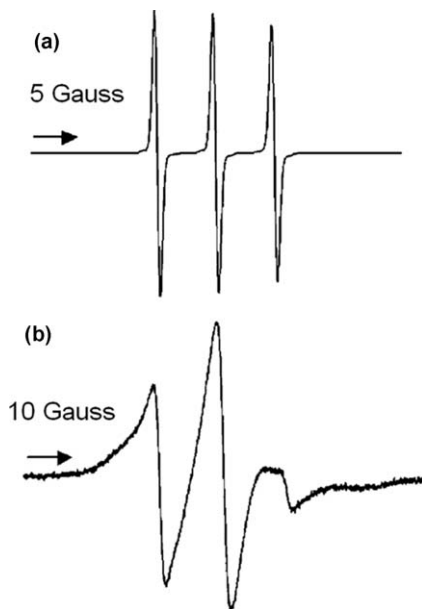


Fig. 44 Experimental EPR spectra in water-saturated CH_2Cl_2 at 293 K. (a) 0.1 mM **28**, (b) 0.1 mM **28** + 4.0 mM of **38**. From E. Mileo, S. Yi, P. Bhattacharya and A. E. Kaifer, Probing the Inner Space of Resorcinarene Molecular Capsules with Nitroxide Guests, *Angew. Chem.*, 2009, **48**, 53373. Copyright © 2009 by John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.

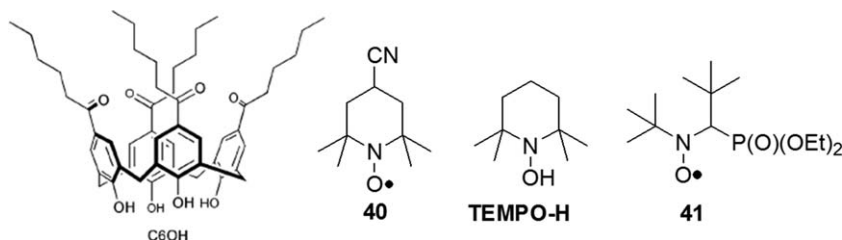


Fig. 45 Nitroxides encapsulated in **C6OH**. Reproduced from ref. 88 with permission from the PCCP Owner Societies.

encapsulate **TEMPO** in *para*-hexanoyl calix[4]arene **C6OH** (Fig. 45) were not successful.⁹⁰ The result of synthesis was the crystal structure of a diamagnetic inclusion complex of calix[4]arene with **TEMPO-H**. It is known that complexes of acyl calixarenes are stabilised by host–guest and host–host van der Waals interactions. Thus, the presence of alkyl groups in the nitroxide is an important factor allowing for the isolation of such complexes. The use of nitroxides **35a** and **41** (Fig. 45) enabled to obtain the paramagnetic inclusion complexes consisting with of one nitroxide confined in a capsule of two calixarene molecules (Fig. 46a and b, respectively). The EPR spectra of complexes in the crystal lattice are broad due to the dipole–dipole interaction between nitroxides

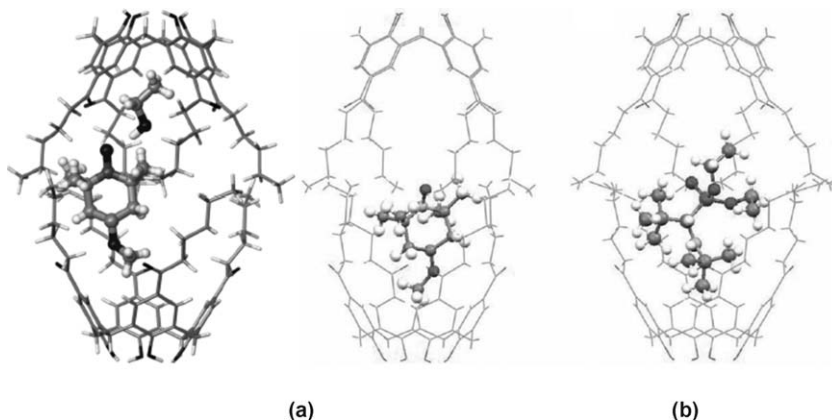


Fig. 46 The capsule of complexes (a) $35 + \text{MeOH} @ (\text{C6OH})_2$ (left) and $35 @ (\text{C6OH})_2$ (right) and (b) $41 @ (\text{C6OH})_2$. Reproduced from ref. 88 and 89 with permission from the PCCP Owner Societies.

occupying neighbouring capsules in the crystal lattice, and are not informative. In order to solve this problem the nanocapsules containing nitroxides were separated from each other by nanocapsules containing diamagnetic molecules (dibenzylketone **DBK**). Application of high-field EPR at 360 GHz, helped to find two different capsule occupations (i) a nitroxide alone and (ii) an ethanol molecule occupying the same capsule as the nitroxide. These two cases were distinguished by shifts of their g_{xx} component, between encapsulated nitroxide molecules forming a hydrogen bond between their O–(N) group and the OH group of an ethanol molecule.

Application of multifrequency EPR methods is very important for retrieval of detailed information on characteristics of the motional process of guests in the nanocapsules. EPR at different microwave frequencies correspond to different motional regimes (fast, intermediate or slow) depending on the Larmor frequency and rotational correlation time τ_c and thus the analysis of wide-range multifrequency EPR spectra (Fig. 47) can be used for estimation of τ_c . Thus, due to different ratios between field-dependent Zeeman and field-independent hyperfine interactions, additional information can be obtained on the anisotropy of the spin probe motion.

Temperature dependent CW-EPR measurements as well as Electron Spin Echo Detected EPR (Fig. 48) revealed an orientational anisotropy in the motion of **35a** encapsulated in **C6OH**. Simulation of EPR spectra while taking into account different model of anisotropic motion allowed to obtained map of motion shown in Fig. 49.

The possibility to use the solid-lipid nanoparticles of calixarene with nitroxides as a new magnetic materials, MRI contrast agents, *in situ* pH probes, or models for oxidation stress studies were considered.⁹¹ The nanoparticles were re-dispersed in water by applying ultrasound. It was shown that the calixarene nanocapsules protect nitroxides against reduction by ascorbic acid.⁸⁸

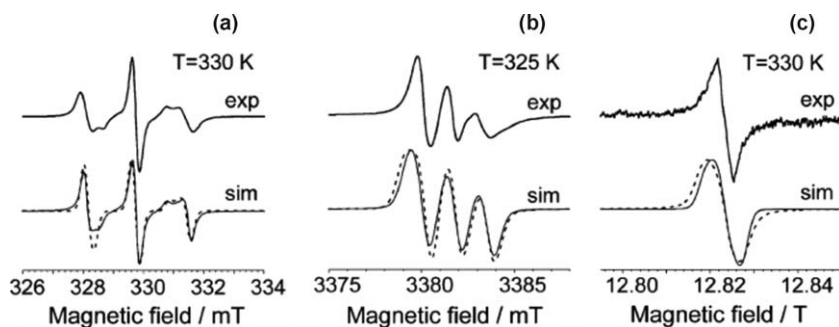


Fig. 47 Experimental (solid lines) and simulated (dashed lines and thin solid lines) X-band (a); W-band (b) and 360 GHz EPR spectra of $35a@C6OH_2$ at $T = 330$ K. Simulations were performed using the fast-restricted-motion model $\beta = 36^\circ$ and $\theta \in [75^\circ, 105^\circ]$ (solid lines) and the MOMD model with the orientation potential Y20, the motion-anisotropy parameter $\lambda_{20} = -1.0$, and the rotational correlation time. Reproduced from ref. 89 with permission from the PCCP Owner Societies.

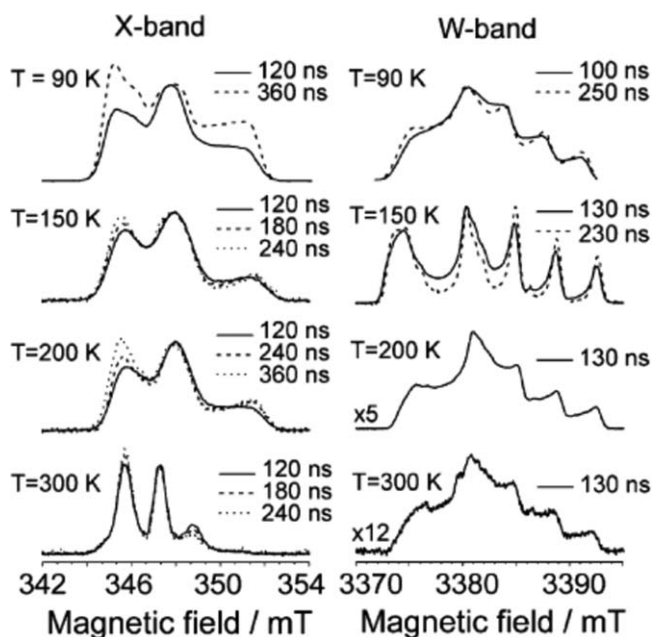


Fig. 48 ED-EPR spectra of $35a@C6OH_2$ ($35/DBK = 1/30$) at various temperatures and different t -delays. The spectra are normalised to the maxima of the central components. Reproduced from ref. 89 with permission from the PCCP Owner Societies.

4.2.3 Calixarene covalently attached to nitroxides. A series of paper that started from the articles by Rajca *et al.*,⁹¹ Takui *et al.*,⁹² Tretyakov *et al.*⁹³ deals with the synthesis of calixarene-based mono-, bi- and tetra-radicals and with the investigation of their magnetic properties. The main interest in these systems is related to slow electron spin relaxation rates, which are relevant for magnetic resonance imaging (MRI) or dynamic nuclear polarisation (DNP) applications. In addition, the model studies revealed an antiradical activity, a superoxide

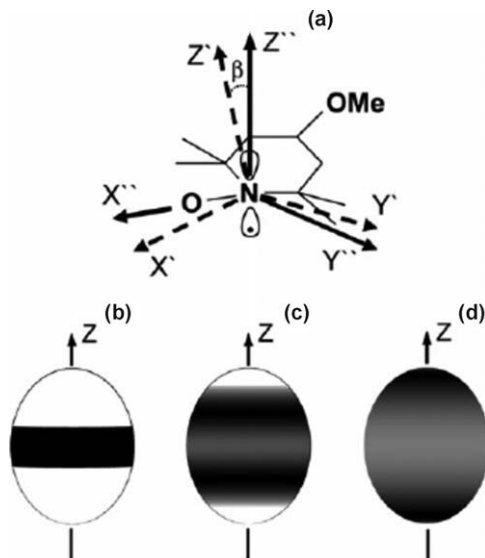


Fig. 49 Orientations of the coordinate systems $X''Y''Z''$ and $X'Y'Z'$ with respect to the nitroxide moiety. Allowed motion regions in the model of fast restricted motion: (b) for the Z' axis, (c) the Z'' axis and (d) the X'' axis coinciding with the NO bond direction. Colour density corresponds to the probability of orientation. Parameters are as follows: restriction for the θ angle $\theta \in [75^\circ, 105^\circ]$ and angle $\beta = 36^\circ$. Reproduced from ref. 89 with permission from the PCCP Owner Societies.

dismutase-like activity and efficient inhibition of ABAP-induced peroxidation of linoleic acid by these compounds.⁹⁴

Tretyakov *et al.*⁹³ synthesised a new calix[4]arene (1,3-*alternate*) functionalised with four nitronyl nitroxyl groups. EPR study of these compounds revealed weak intra- and inter-molecular exchange interactions of the antiferromagnetic character between the unpaired electrons of the para-magnetic centres.

CW-EPR and HSCORE spectroscopy (Fig. 50) in combination with DFT calculations were used by Takui *et al.*⁹² to study magnetic properties of calix[4]arene-based biradical **42** with two *tert*-butyl nitroxides in the toluene glass and monoradical **43**. The authors investigated the structural environmental information on the local spin sites relevant to the hydrogen bonds. The temperature dependence of the fine-structure EPR signal intensity has shown that the triplet state for the biradical is thermally accessible with the energy gap of 4 cm^{-1} above the singlet ground state. The analysis of EPR spectra determined by anisotropic hyperfine structures due to nitrogens in non-oriented media allowed Takui *et al.* to obtain information on the relative orientation of the nitroxide groups and determine the electronic and molecular structures of nitroxide-based biradicals.⁹²

4.3 Nitroxides in nanotubes, MOF and nanoporous materials

Applications of nitroxides as guests and mesoporous materials as hosts are aimed at solving various issues, including efficient solid-phase DNP

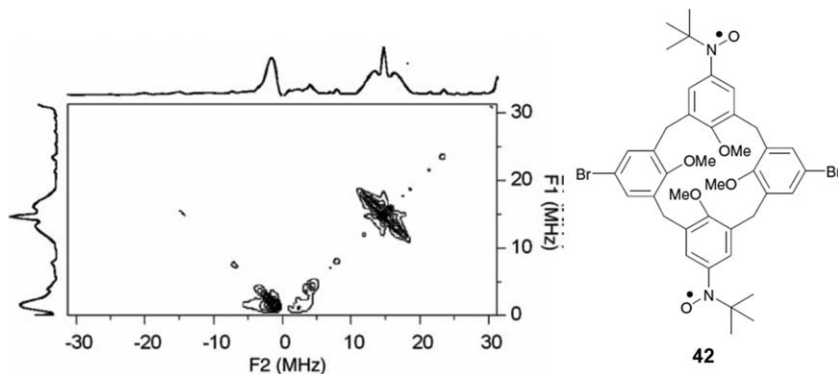


Fig. 50 2-D HSCORE pattern observed in the toluene glass at 10 K for the monoradical **43** of **42**. The magnetic field was set to the central ESR signal of the monoradical **43** ($B_0 = 0.3451$ T, mMW = 9.6827 GHz). T. Sawai, K. Sato, D. Shiomi, K. Toyota, Q. Wang, J.-S. Wang, Y. Li, G.-S. Wu and T. Takui, Intramolecular Hydrogen Bonding in Calix[4]Arene-Based Nitroxide Monoradical and Biradical as Studied by CW-ESR and Pulse-ESR HSCORE Spectroscopy, *Appl. Magn. Reson.*, 2011, **41**, 337. © Springer-Verlag Wien 2011, with permission of Springer.

matrixes to improve cross-effect DNP,⁹⁵ a contrast agent for MRI,⁹⁶ or magnetic materials.⁹⁷

Efficient solid-phase DNP matrixes based on using hybrid silica meso-structured materials (Fig. 51) with attachment of mono- or bisnitroxide functionalised materials (**Mat-TEMPO** and **Mat-bTurea**) were prepared using a versatile procedure which enables attachment of various polarizing agents (PAs) to the material.⁹⁵ The authors⁹⁵ found that the most important factor for obtaining a high DNP coefficient is the homogeneous distribution of radical moieties within the porous network of the materials which prevent aggregation of radicals. It was shown that the use of this solid matrix with different small molecules ($[2,3-^{13}\text{C}]$ -L-alanine and $[1-^{13}\text{C}]$ pyruvic acid) allows the solvent to be polarised (Fig. 52). The advantages of this approach are (i) easy separation of the matrix from the solution allowing to obtain a pure polarised solution suitable for *in vivo* studies without any need for chemical treatment and without undesired by-products, (ii) a possibility to extend it to biradicals for improved cross-effect DNP, *via* incorporation of insoluble radical **bTurea**, which is then used to polarise aqueous solutions or to narrow line radicals such as OX063 (trityl type-radical) for DNP with dissolution at the liquid helium temperature.⁹⁵

The advantages of EPR of spin probes for characterisation of porosity and photostability of polymers – that hold promise for inherent microporosity design for applications of pressure-sensitive paints – were shown by Sheveleva *et al.*⁹⁸ The estimation of pore sizes and comparative estimation of free volume of the porous polymers were performed *via* analysis of EPR data. In addition the nitroxide guest molecules were used to investigate the UV degradation of polymers and even to obtain information on spatial (surface *vs.* volume) localisation of decomposition processes by means of EPR and time-resolved EPR.^{98,99}

In a series of article by Kovaleva *et al.*,^{100–103} the guest imidazolidine and imidazoline nitroxides in different mesoporous media **AAO** were

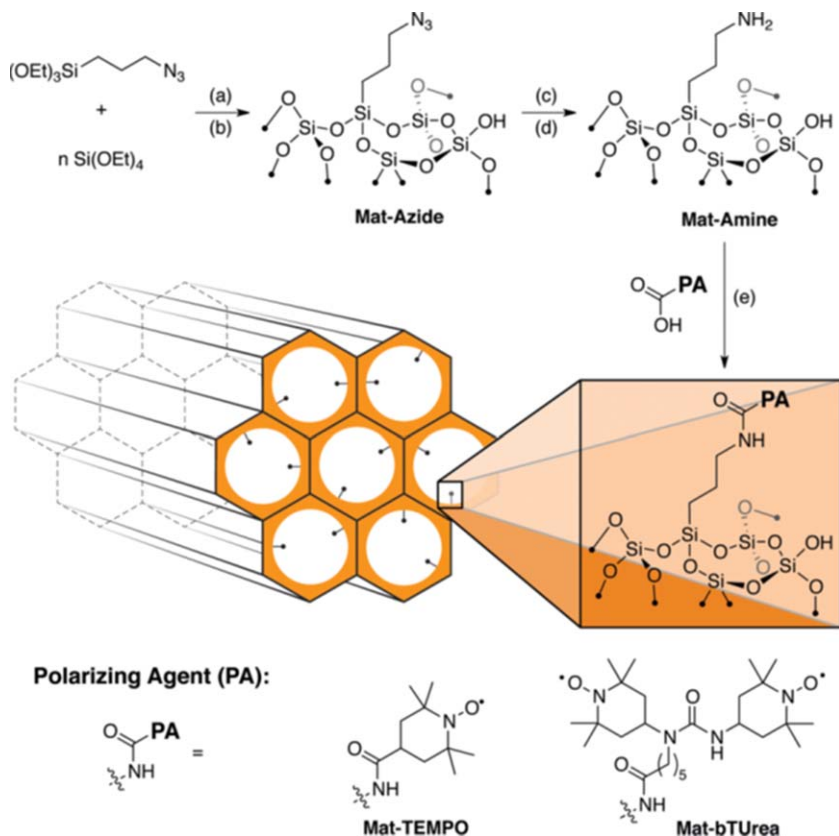


Fig. 51 Synthesis of Mat-TEMPO and Mat-bTUrea. Reagents: (a) Pluronic P123, NaF, HCl(aq); (b) HCl(aq), pyridine; (c) PPhMe₂, THF; (d) H₂O; (e) HBTU, HOBT, DIPEA, THF, and DMF. Reprinted with permission from D. Gajan, M. Schwarzwälder, M. P. Conley, W. R. Grüning, A. J. Rossini, A. Zagdoun, M. Lelli, M. Yulikov, G. Jeschke, C. Sauvé, O. Ouari, P. Tordo, L. Veyre, A. Lesage, C. Thieuleux, L. Emsley and C. Coperet, Solid-Phase Polarization Matrixes for Dynamic Nuclear Polarization From Homogeneously Distributed Radicals in Mesoporous Hybrid Silica Materials, *J. Am. Chem. Soc.*, 2013, **135**, 15459. Copyright 2013 American Chemical Society.

used to determine local pH. These nitroxides are known to reveal reversible protonation of the nitrogen group leading to EPR spectrum dependent on pH (Fig. 53).

CW X-band (9 GHz) EPR of two pH-sensitive nitroxide radicals was used to study acid-base properties of anodic aluminium oxide (AAO) ceramic membranes with macroscopically aligned and hexagonally packed nanopore architecture (inset in Fig. 53). For AAO membranes with pore diameters $\geq 58 \pm 8$ nm the value of pH was the same inside the pores, as in the bulk external solution while for the nanopores of smaller diameters (*ca.* 29 and 18 nm), it was 0.5–0.8 pH units lower than in the bulk. Thus, the authors showed that an EPR titration method could be used for characterisation of meso- and nanoporous membranes for catalysis and sorption.¹⁰³

pH-sensitive nitroxides as a guest in a host system of the channels of the mesoporous molecular sieves **MCM-41** and **SBA-15** with diameters

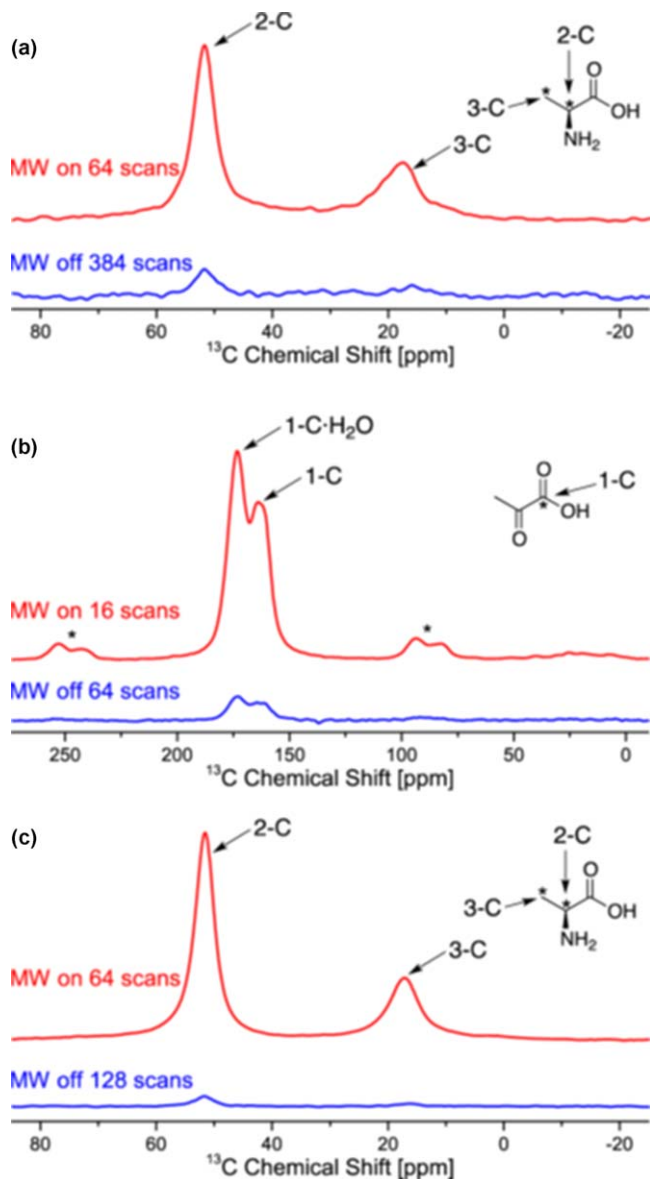


Fig. 52 ^{13}C CP MAS NMR spectra of (a) [2,3- ^{13}C]-L-alanine and (b) [1- ^{13}C]pyruvic acid both enhanced by 1/34 Mat-TEMPO and (c) [2,3- ^{13}C]-L-alanine enhanced by 1/100 Mat-bTUrea. In all three cases, the probe molecule was dissolved in pure H_2O . The microwave on in the spectrum is highlighted in red, and the microwave off spectrum is in blue. In (a) εC CP was 39, in (b) εC CP was 34, and in (c) εC CP was 45. The unlabelled carbons in these samples were not detected. In (b), the two 1-C resonance values correspond to the keto acid and the 2-hydrated pyruvic acid ($\text{MeC}(\text{OH})_2\text{COOH}$). Reprinted with permission from D. Gajan, M. Schwarzwälder, M. P. Conley, W. R. Grüning, A. J. Rossini, A. Zagdoun, M. Lelli, M. Yulikov, G. Jeschke, C. Sauvé, O. Ouari, P. Tordo, L. Veyre, A. Lesage, C. Thieuleux, L. Emsley and C. Coperet, Solid-Phase Polarization Matrixes for Dynamic Nuclear Polarization From Homogeneously Distributed Radicals in Mesostuctured Hybrid Silica Materials, *J. Am. Chem. Soc.*, 2013, **135**, 15459. Copyright 2013 American Chemical Society.

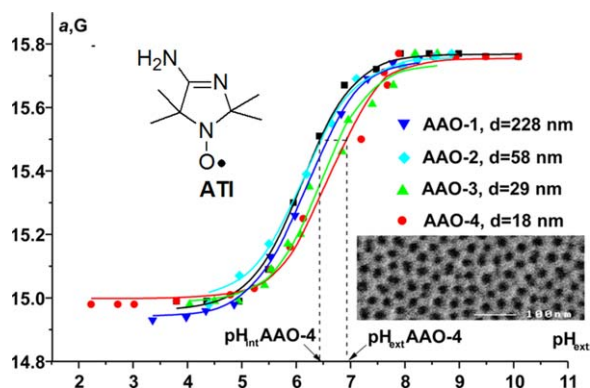


Fig. 53 Experimental room temperature EPR titration $a = f(\text{pH}_{\text{ext}})$ curves for aqueous solutions of ATI (0.1 M) in a bulk aqueous phase (black squares) and inside the nanopores of AAO-1 (blue triangles), AAO-2 (magenta diamonds), AAO-3 (green triangles), and AAO-4 (red circles). The best fits to the modified Henderson–Hasselbalch equations are shown as solid lines of the same colour as the corresponding symbols. d for diameter of pores in nm highlighted by the inset for AAO-4. Reprinted with permission from E. G. Kovaleva, L. S. Molochnikov, U. Venkatesan, A. Marek, D. P. Stepanova, K. V. Kozhikhova, M. A. Mironov and A. I. Smirnov, Acid–Base Properties of Nanoconfined Volumes of Anodic Aluminum Oxide Pores by EPR of pH-Sensitive Spin Probes, *J. Phys. Chem. C*, 2016, **120**, 2703. Copyright 2016 American Chemical Society.

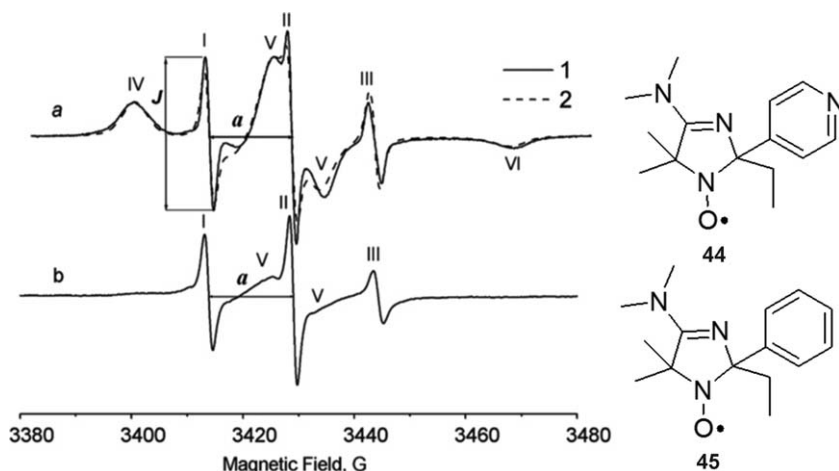


Fig. 54 Representative EPR spectra of nitroxides **44**, **45** (a) and ATI (b) in the channels of SBA-15 at 293 K and pH = 6.22: (1) experimental spectrum, (2) spectrum simulated using Freed's program; I, II, and III – signals of isotropic spectrum; IV, V, and VI – signals of anisotropic spectrum. Reprinted from E. G. Kovaleva, L. S. Molochnikov, E. L. Golovkina, M. Hartmann, I. A. Kirilyuk and I. A. Grigor'ev, Dynamics of pH-Sensitive Nitroxide Radicals in Water Adsorbed in Ordered Mesoporous Molecular Sieves by EPR Spectroscopy, *Microporous Mesoporous Mater.*, 2013, **179**, 258–264. Copyright (2013), with permission from Elsevier.

ranging from 2.3 to 8.1 nm were used for estimation of the solution acidity inside the channels and of the near-surface electrical potential.¹⁰⁰

The EPR spectra of the nitroxide located in the channels of nanoporous materials consist of a superposition of isotropic and anisotropic signals (Fig. 54), corresponding to the coexistence of nitroxides in the process of fast and slow motion. The authors studied the NR retention by the

mesoporous molecular sieves after washing with an aqueous KCl solution. The analysis of EPR spectra afforded the ratio of the radical molecules in the fast and slow motional regimes in the channels of the mesoporous molecular sieves and its dependence on pH. At high pH, nitroxides **44** and **45** exhibit the EPR spectra characteristic only of the fast-motional nitroxides regardless of their size (Fig. 54). According to the results, it was suggested that the pyridine and benzene fragments of the radicals interact with the silica surface both with the hydrophilic and hydrophobic patches.

4.4 Application of guest–host systems as contrast agent for MRI

In recent years, many attempts were made to use guest–host systems as a contrast agent for MRI.¹⁰⁴ Among them are the encapsulation or functionalisation of nitroxide radicals into calix[4]arenes,^{88,91} polymers,^{105–107} anticancer drugs,¹⁰⁸ dendrimers,^{109–111} or biomolecules such as DNA oligomers,¹¹² liposomes,¹¹³ or human serum albumin.¹¹⁴ In most cases, enhancement of proton relaxivity and the tissue targeting capability were not very good. A nice demonstration of an application of a guest–host system comprising nitroxide encapsulated within US-tubes (Fig. 55) as a

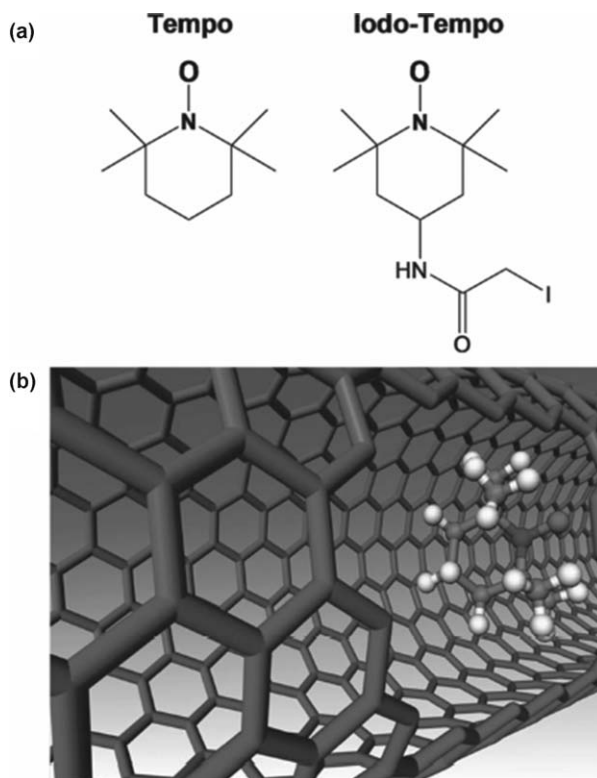


Fig. 55 (a) Structures of TEMPO and iodo-TEMPO nitroxides. (b) Depiction of a US-tube loaded with a single Tempo molecule. From E. J. Rivera, R. Sethi, F. Qu, R. Krishnamurthy, R. Muthupillai, M. Alford, M. A. Swanson, S. S. Eaton, G. R. Eaton and L. J. Wilson, Nitroxide Radicals@US-Tubes: New Spin Labels for Biomedical Applications, *Adv. Funct. Mater.*, 2012, **22**, 3691. Copyright © 2012 by John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.

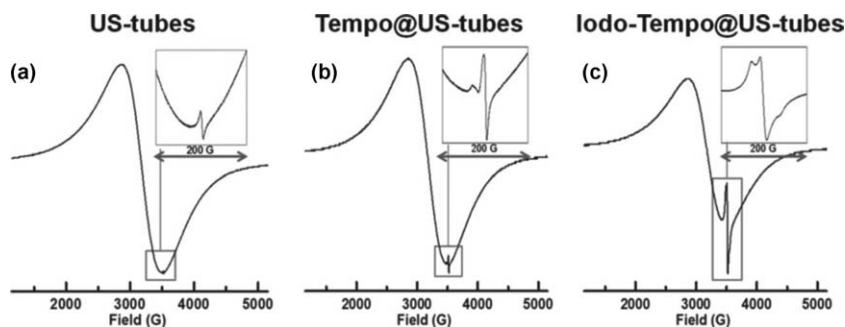


Fig. 56 EPR spectra from powders of the empty US-tube and the nitroxide-labelled US-tube. From E. J. Rivera, R. Sethi, F. Qu, R. Krishnamurthy, R. Muthupillai, M. Alford, M. A. Swanson, S. S. Eaton, G. R. Eaton and L. J. Wilson, Nitroxide Radicals@US-Tubes: New Spin Labels for Biomedical Applications, *Adv. Funct. Mater.*, 2012, 22, 3691. Copyright © 2012 by John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.

Sample	T_1 [ms]	T_2 [ms]	r_1 [mM ⁻¹ s ⁻¹]	r_2 [mM ⁻¹ s ⁻¹]	r_1/r_2
Tempo@US-tubes	3159	232	2 ^{a)}	132 ^{a)}	66
Iodo-Tempo@US-tubes	3278	366	2 ^{a)}	110 ^{a)}	55
US-tubes	2333	188	0.1 ^{b)}	4 ^{b)}	40
Tempo	-	-	0.15 ^{c)}	0.10 ^{c)}	0.7
Iodo-Tempo	-	-	0.27 ^{c)}	0.43 ^{c)}	1.6

Fig. 57 Water proton relaxation properties of TEMPO- and iodo-TEMPO@US-tubes and empty US-tubes in 0.17% pluronic F-108 surfactant as obtained with a Bruker Minispec (mq60) NMR spectrometer at 1.41 T (60 MHz) and 37 °C. Relaxivity values determined from: (a) the nitroxide concentration by N% from elemental analysis; (b) Ni concentration determined by ICP-OES. The relaxivity data from the US-tubes represent the lowest relaxation values achieved assuming a total contribution of all the nickel paramagnetic species in the tubes; and (c) the inversion recovery method and multi-echo sequence for different concentrations of the nitroxides in an aqueous solution. From E. J. Rivera, R. Sethi, F. Qu, R. Krishnamurthy, R. Muthupillai, M. Alford, M. A. Swanson, S. S. Eaton, G. R. Eaton and L. J. Wilson, Nitroxide Radicals@US-Tubes: New Spin Labels for Biomedical Applications, *Adv. Funct. Mater.*, 2012, 22, 3691. Copyright © 2012 by John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.

contrast agent for MRI was recently presented by Rivera *et al.*⁹⁷ The nitroxides were encapsulated within ultrashort (*ca.* 50 nm) single-walled carbon nanotubes. The complexes formation was characterised by thermogravimetric analysis (TGA), X-ray photoelectron spectroscopy (XPS), Raman spectroscopy and EPR spectroscopy. The EPR spectra show that the US-tubes affect the tumbling rates of the encapsulated nitroxides and therefore their ability to induce water proton relaxation (Fig. 56). The encapsulation leads to excellent relaxation rates and efficiency in MRI because complexes showed good contrast enhancement due to their high relaxivity shown in Fig. 57. Thus nitroxide radicals within US-tubes hold promise for application to MRI, EPRI, and the measurement of oxygen concentration *in vivo*.

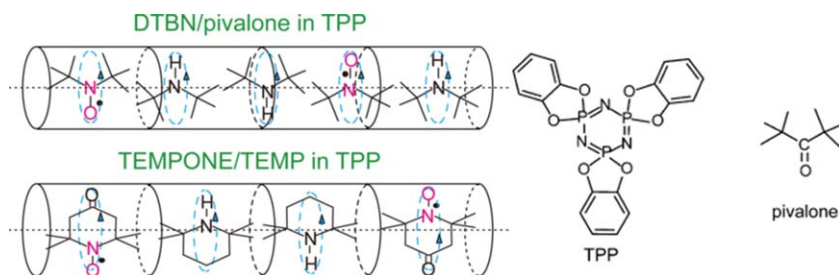


Fig. 58 Distribution of **21** and **DBN** in **TPP** nanochannels and structures of **TPP** and pivalone. Reprinted with permission from H. Kobayashi, K. Takeuchi and T. Asaji, *Molecular Orientation and Dynamics of Different Sized Organic Radicals Included in Organic 1D Nanochannels*. *J. Phys. Chem. A*, 2013, **117**, 2093. Copyright 2013 American Chemical Society.

In papers by Kobayashi *et al.*,^{115–117} molecular orientation and dynamics of nitroxides in the organic 1D nanochannels of tris(*o*-phenylenedioxy)-cyclotriphosphazene (**TPP**) (Fig. 58) and organic nanochannels of 2,4,6-tris-4-(chlorophenoxy)-1,3,5-triazine (**CLPOT**) were investigated using EPR. The inclusion nitroxides (**DTBN**, **21** and **TEMPO**) were diluted by co-inclusion of diamagnetic molecules. A different character of motion was observed for these two nanochannels. For **TPP**, a comparison of experimental spectra with the simulated ones (Fig. 59) showed that the axial rotation of **DTBN**, **TEMPO** and **21** (**TEMPONE**) molecules occur in nanochannels with rotation axes almost parallel to the principal *y*-axis of the *g* tensor with activation energies of 3, 5 and 10 kJ mol^{−1}, respectively. In **CLPOT** nanochannels, the axial rotation of **TEMPO** or **TOH** molecules occurs with the rotation axis tilted towards the principal *x* direction in the axis system of the *g*-tensor and activation energies of rotation were estimated at 8 and 7 kJ mol^{−1}, respectively. These results indicate that the molecular orientations of guest nitroxides are almost independent of the molecular sizes of the guest radicals in **TPP** nanochannels, although the molecular dynamics are dependent on the molecular sizes. The differences in the character of motion for **TPP** and **CLPOT** nanocapsules are attributed to the larger nanospace in the **CLPOT** nanochannels.

For nitroxides of a larger size in particular **PhNN** (X = H) and ***p*-NPNN** (X = NO₂ in Fig. 60) included in **CLPOT** nanochannels the anisotropic rotational diffusion around the rotation axis tilted to the principal axis system of the *g*-tensor by (θ , ϕ) = (68°, 78°) for **PhNN** (X = H in Fig. 60) and (74°, 90°) for ***p*-NPNN** (X = NO₂ in Fig. 60) was observed. The parameters of rotational correlation time and activation energy were relatively longer and much larger than those for **TEMPO** or **TOH** in the **CLPOT** nanochannels. These results are explained by the larger molecular size of the nitronyl nitroxide radicals and stronger interaction between nitronyl nitroxides and the surrounding host or guest molecules (Fig. 60).

4.5 Nitroxides in MOFs

Successful application of EPR to studies of the structure, functions and corresponding guest–host interactions in diamagnetic nano- or

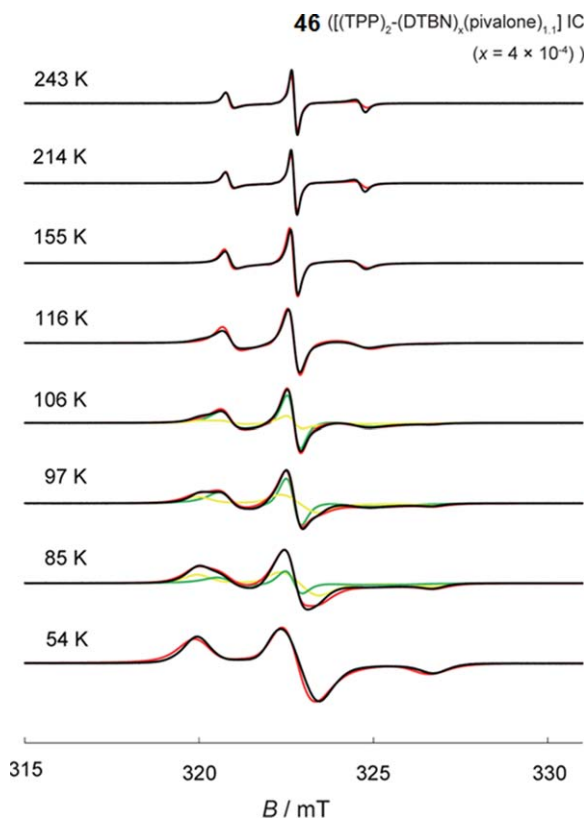


Fig. 59 Measured and simulated (red lines; obtained using the EasySpin software package²²) EPR spectra of **46** ($[(\text{TPP})_2-(\text{DTBN})_x(\text{pivalone})_{1.1}] \text{IC}$ ($x = 4 \times 10^{-4}$)). Each spectrum was well reproduced by a rigid-limit powder pattern of a typical nitroxide at 54 K and axially rotating components originating from the anisotropic slow-rotation of the DTBN radical above 116 K. The spectra were temperature-independent above 243 K. At an intermediate temperature, the spectra were reproduced by a superposition (red) of the rigid-limit powder pattern (yellow) and axially rotating components (green). Reprinted with permission from H. Kobayashi, K. Takeuchi and T. Asaji, Molecular Orientation and Dynamics of Different Sized Organic Radicals Included in Organic 1D Nanochannels, *J. Phys. Chem. A*, 2013, **117**, 2093. Copyright 2013 American Chemical Society.

meso-porous metal-organic frameworks (MOFs) was performed recently by Fedin and co-workers.^{118,119} Nitroxides (**TEMPO** and **4-oxo-TEMPO**, **4-hydroxo-TEMPO**, and **4-acetamido-TEMPO**) were incorporated into MOF in particular **MIL-53(Al)** revealing so called “breathing” behaviour using two ways of post-synthetic adsorption: (i) sorption of radicals from the gas phase, and (ii) the impregnation method. **MIL-53(Al)** is known to reversibly change the size of pores under a temperature change. The authors reported that in addition to changes in mobility upon large pore $\leftrightarrow$ narrow pore conversion, unexpected changes in the magnetic susceptibility of the sample were observed: the effective amount of spins decreased by a factor of ~ 3 upon transition. This observation was ascribed to the reversible reaction of **TEMPO** with the μ_2 -hydroxo group of **MIL-53(Al)** and formation of diamagnetic hydroxylamine (**TEMPO-H**).

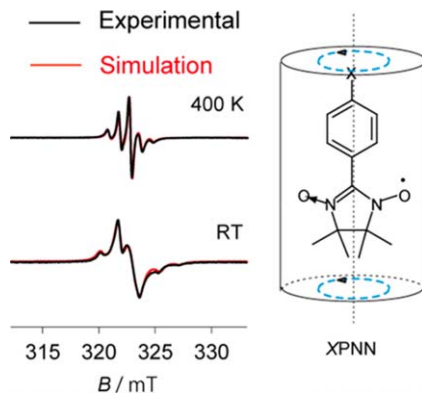


Fig. 60 Cartoon showing the orientation of nitronyl nitroxide in nanochannel (right) and its experimental (black line) and simulated (red line) EPR signal at room temperature (bottom) and 400 K (top). Reprinted with permission from H. Kobayashi, Y. Morinaga, E. Fujimori, T. Asaji, ESR Study of Molecular Orientation and Dynamics of Nitronyl Nitroxide Radicals in CLPOT 1D Nanochannels. *J. Phys. Chem. A*, 2014, **118**, 4907–4917. Copyright 2014 American Chemical Society.

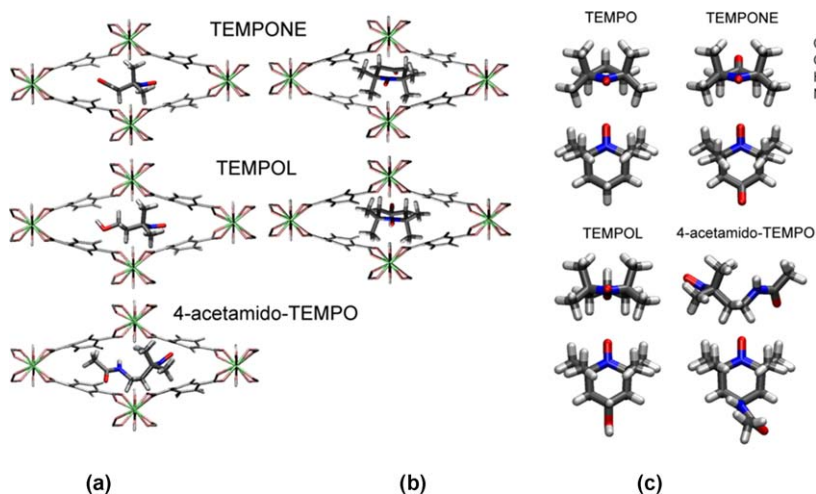


Fig. 61 Two representative locations of TEMPO derivatives in narrow pores of MIL-53(Al) with long (a) and short (b) distances from the NO group to the μ_2 -hydroxo group of MIL-53(Al). (c) Structure of TEMPO, TEMPONE, TEMPOL, and 4-acetamido-TEMPO.

The EPR data and molecular dynamics simulations revealed that in large pores (LP state) nitroxide undergoes molecular motion at a large solid angle, whereas in narrow pore (NP state), it can be “trapped” in a set of orientations, some of which enable the weak hydrogen bonding between nitroxyl moiety of the nitroxide and a proton of the μ_2 -hydroxo group of MIL-53(Al) (the $\text{OH} \cdots \text{ON}$ distance is $\sim 1.1 \text{ \AA}$) (Fig. 61).

Depending on substituents in the piperidine ring, different mobility and reactivity of the corresponding nitroxides were observed. Based on a comparison of molecular dynamics calculations with EPR data, practical suggestions on selecting spin probes for in-MOF EPR studies were formulated.

5 Conclusion

Since the last report by Lucarini *et al.*,¹⁶ the applications of nitroxides@CD and nitroxides@CB have expanded substantially. Now, systems suitable for imaging techniques have been exemplified. At this time, it is not possible to foresee one or several key research directions because many of the results discussed above are quite promising. Nevertheless, it is likely that systems based on nanotubes and systems investigating the control of the spin–spin communications between unpaired electrons will be one of the hot topics in this field of research. Looking at the first reports in this field dating back 10 to 15 years ago, applications of host–guest chemistry as an agent for DNP are likely to be implemented and will gain popularity in the scientific community in the near future.

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