The strength of EPR and ENDOR techniques in revealing structure–function relationships in metalloproteins

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Recent technological and methodological advances have strongly increased the potential of electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) techniques to characterize the structure and dynamics of metalloproteins. These developments include the introduction of powerful pulsed EPR/ENDOR methodologies and the development of spectrometers operating at very high microwave frequencies and high magnetic fields. This overview focuses on how valuable information about metalloprotein structure–function relations can be obtained using a combination of EPR and ENDOR techniques. After an overview of the historical development and a limited theoretical description of some of the key EPR and ENDOR techniques, their potential will be highlighted using selected examples of applications to iron-, nickel-, cobalt-, and copper-containing proteins. We will end with an outlook of future developments.

1. Introduction

Since the introduction of electron paramagnetic resonance (EPR) spectroscopy in 1944 by Zavoisky and of the related electron nuclear double resonance (ENDOR) spectroscopy in 1956 by Feher, both techniques have been applied extensively in the fields of biology, medicine, chemistry, physics and material sciences. The techniques can be used to determine the structure and dynamics of paramagnetic systems (i.e. systems containing one or more unpaired electrons), and can also be applied to diamagnetic systems using spin probes or spin labels. Until the end of the 1980s, the vast majority of the EPR/ENDOR studies were performed using continuous wave (CW) techniques at X-band microwave (mw) frequencies (~9.5 GHz). Although the use of these CW-EPR and ENDOR techniques have proven to be invaluable for many analyses, the amount of information that can be obtained is limited by factors inherent to the CW approach. In the 1980s, it was shown that the complementary use of pulsed EPR and ENDOR may overcome these problems, but it was only with the introduction of fast electronics and the commercialization of pulsed-EPR/ENDOR spectrometers in the late 1980s that these techniques started to be applied on a larger scale. Since then, the field of EPR has been revolutionized by new developments both in pulsed-EPR methodology and in types of applications, similar to the evolution observed in the field of nuclear magnetic resonance (NMR), EPR's (more famous) sister technique. Furthermore, the progresses in EPR have been paralleled by rapid instrumental developments, amongst which is the construction of so-called 'high-field EPR spectrometers'. These spectrometers operate at mw frequencies of 95 GHz and higher, and their development was initiated by the seminal work of Lebedev. Details on the new methodological and instrumental developments in EPR and on various applications are reported in the reference book on pulsed EPR by Schweiger and Jeschke and in recent review papers, including two recent Invited Articles in this journal on the applications of high-field EPR and high-field ENDOR. It should be noted that EPR and ENDOR are by no means routinely applicable techniques and the interpretation of EPR and ENDOR spectra is often very complex.

For decades, EPR has been one of the methods of choice to study paramagnetic metalloproteins. Indeed, many of the metal ions binding to proteins are paramagnetic: Fe(III), Cu(II), Ni(II), Co(II), Mn(II), VO(II), etc., and thus form ideal intrinsic probes for EPR. The recent instrumental and methodological developments in EPR and ENDOR have made these techniques unparalleled tools for unravelling the electronic and local geometric structure of the metal centre.

In this Invited Article, we will highlight some of the possibilities of advanced EPR and ENDOR techniques for the study of metalloproteins. The manuscript is structured as follows. After an introduction to EPR and ENDOR spectroscopy, we show how the recent developments in EPR and ENDOR techniques have given a new impetus to the elucidation of metalloprotein structure and function. We hereby focus on examples from our own research work, but extend the discussion with work of others for those applications we feel exemplify the field, even though they are not part of our current research. We will conclude with indicating some of the challenges for the future. Note that this overview is not meant to be exhaustive and we apologize to our colleagues whose work we may have inadvertently failed to quote.
2. Theoretical background of EPR spectroscopy

2.1. What information can be obtained using EPR spectroscopy?

The spin Hamiltonian, $H$, describing the unpaired electron(s) coupled to nuclear spins in an external magnetic field, forms the basis for understanding the outcome of EPR experiments. For an unpaired electron ($S = 1/2$), coupled to different nuclear spins ($I_k$), the spin Hamiltonian is given by:

$$H = \beta_0 B_0 g_S S / h + \sum_k (SA_k I_k - \beta_0 g_n B_0 I_k / h) + \sum_{k,l} I_k P_{kl} I_l$$

(1)

where $\beta_0$ is the Bohr magneton, $h$ is Planck’s constant, $\beta_n$ is the nuclear magneton and $B_0$ is the external magnetic field. We make use of the symbol $\sim$ to indicate the transpose of a vector.

The $g$ tensor in the field-dependent electron Zeeman term (first term of eqn (1)) contains information about the local symmetry of the paramagnetic site and its electronic state. The hyperfine interactions between the unpaired electron and the surrounding nuclear spins are described by the second Hamiltonian term in eqn (1). The hyperfine matrix $A$ can be written as the sum of the isotropic, Fermi contact interaction, $a_{iso}$, originating in the finite probability for the unpaired electron to be found at the nucleus, and of the dipole–dipole coupling, $T$. For some cases, in which the electron–nucleus distance, $r$, is larger than 0.26 nm and the spin delocalization over the ligand is negligible, the latter term can be approximated by a point-dipolar interaction that allows for a direct determination of $r$:

$$T_{ij} = \frac{\mu_0}{4\pi r^3} g_n g_i \beta_n \beta^2 (3r_ir_j - \delta_{ij})$$

(2)

with $r_x$, $r_y$, and $r_z$ the direction cosines defining the orientation of the magnetic field in the $g$ tensor frame.

For transition-metal centers, knowledge of the metal hyperfine tensor (for metal isotopes with nuclear spin) in combination with the $g$ tensor often provides a fingerprint to identify the metal ion, its oxidation state and the local symmetry of the metal center. Knowledge of the hyperfine interaction between the unpaired electron(s) and the transition-metal nucleus ($I \geq 1/2$) can corroborate this derivation. Furthermore, analysis of the hyperfine interaction of the surrounding nuclei allows one to unravel the spin distribution in the system (via the Fermi contact term) and to determine electron–nucleus distances and spatial information (dipole–dipole contribution). The nuclear quadrupole interaction reflects the electric-field gradient at the different quadrupolar nuclei. This gradient depends on the full electronic structure. Put together, these EPR parameters allow for the determination of both the local geometric and electronic structure of the metal site. Finally, since most of the EPR parameters are tensors (see eqn (1)), a detection of the time averaging of these tensors will give direct information about the dynamics of the system under study.

2.2. How to obtain this information

2.2.1. General. In order to determine the set of parameters described in the previous section, the EPR spectroscopist can use a multitude of CW and pulsed methods. The applicable techniques can differ in many ways. They can make use of a single mw frequency (e.g. X-band CW-EPR), multiple microwave frequencies (e.g. electron double resonance (ELDOR) spectroscopy), a combination of mw and radio frequencies (rf (ENDOR) or a combination of mw frequencies and optical frequencies (e.g. optically detected magnetic resonance (ODMR)). In order to enhance the obtainable information, the spectroscopist may choose to combine results of experiments performed at different mw frequencies. While the majority of experiments is still performed at X-band mw frequencies (~9.5 GHz), the impact of so-called high-field EPR and ENDOR experiments, performed at frequencies
from 95 GHz (W-band) upwards, has been growing extensively in recent years.\textsuperscript{10,16,18,19}

In practice, at all times the EPR spectroscopist will have to combine a number of techniques in order to obtain an as-complete-as-possible set of parameters. Although the selection of experiments in general not trivial, we outline a rough guideline for the study of transition metal complexes by means of the example of a histidine-bound Cu(ii). This scheme will help the reader to understand the examples given in section 3. Note that this guideline only includes the major techniques highlighted in this paper, but does not pretend to represent the full arsenal of techniques that can be used. In general, field-swept EPR techniques, such as CW-EPR, can reveal information about the $g$ tensor, the zero-field tensor, the strongest hyperfine interactions and possible electron spin–spin interactions. The type of information that can be retrieved is spin-system dependent (see section 2.1). In the case of the Cu(ii)–His example, the CW-EPR experiment will provide information about the $g$ and copper hyperfine tensor. Furthermore, ENDOR and ELDOR-detected NMR techniques can reveal the hyperfine, nuclear Zeeman and nuclear quadrupole couplings of nearby nuclei (Cu(ii)–His: information about the directly coordinating $^{14}$N of His and the nearby $^1$H). Finally, electron spin echo envelope modulation (ESEEM) techniques allow for an identification of these parameters for the nuclei that are even further away from the unpaired electron(s) (Cu(ii)–His: remote nitrogen of the His and protein backbone nitrogens). In short, as we move further away from the unpaired electron(s), we will shift from field-swept EPR techniques to ENDOR and ELDOR-detected NMR tools, and finally to ESEEM techniques. In practice, the application area of the different techniques will overlap partially and the choice of the appropriate techniques is also nucleus dependent (e.g. protons at a distance of 0.4 nm away from the unpaired electron are perfectly detectable with X-band ENDOR, whereas nitrogens at the same distance need to be probed with X-band ESEEM techniques).

In the following sections we outline in more detail the most important classes of techniques that are mentioned above and that will recur in section 3.

2.2.2. Field-swept EPR techniques. Any EPR analysis will start with the measurement of the CW-EPR spectrum of the sample under study. In CW-EPR the absorption of continuously irradiated microwaves with a constant frequency is monitored as a function of the magnetic field, $B_0$. For technical reasons, the derivative of the absorption signal is measured. For a simple $S = 1/2$ system, the magnetic field will lift the degeneracy of the $m_S$ manifolds (see electron Zeeman term in eqn (1)), and the state of the electron spin can be changed by mw irradiation with an energy equal to the energy difference between the two spin states. In general, the allowed EPR transitions follow the selection rules $|\Delta m_S| = 1$, $|\Delta m_I| = 0$. The field at which the resonance occurs links to the $g$ value as described by eqn (1). The resonance depends on the orientation of the magnetic field versus the molecular frame, since the $g$ tensor in eqn (1) has, in its most general case, non-equivalent principal values. Since, in a powder or a frozen solution, the paramagnetic molecule will take on all possible orientations versus the static magnetic field, its CW-EPR spectrum is the sum of the resonances of all these orientations. Because of the magnetic-field dependence of the electron Zeeman interaction (eqn (1)), small differences in the principal values can be resolved at high mw frequencies, where they may remain unnoticed at the standard X-band frequency. The increased $g$ resolution is therefore one of the important advantages of going to higher mw frequencies.

The CW-EPR spectra become more complex if hyperfine interactions with the transition-metal nucleus or surrounding strongly coupled nuclei are (partially) resolved. Furthermore, the zero-field splitting will additionally complicate the spectrum for high-spin systems. If the zero-field interaction is considerably larger than the electron Zeeman term, it may become impossible to derive all parameters from the CW-EPR spectrum. Again, experiments at higher mw frequencies can help, since the field dependence of the electron Zeeman term allows for a fine-tuning of the ratio between the two terms.

CW-EPR can be used not only to determine the static structure of the paramagnetic molecule, but it is also a valuable tool for measuring dynamical aspects. Molecular motions that are very fast compared to the applied mw frequency will cause a total averaging of the orientation-dependent contributions and result in a so-called ‘isotropic’ spectrum (fast motion limit), whereas motions that are considerably slower than the mw frequency are observed as static (‘powder-like’) spectrum. All intermediate motions will give rise to complex spectral features that allow the determination of the nature and correlation time of the motion. Change of the mw frequency thus shifts the reference to which the motion is compared and a multi-frequency approach facilitates largely the interpretation of the complex spectra. Further details on CW-EPR can be found in the standard text books on this topic.\textsuperscript{20–23}

In principle, similar information can be obtained from electron spin echo (ESE)-detected EPR. In these experiments, a spin echo, usually a Hahn echo or a primary echo, is generated using a specific mw pulse sequence and the echo intensity is monitored as a function of the external magnetic field. For an $S = 1/2$ system, this approach generates a spectrum matching the integrated form of the corresponding CW-EPR spectrum. In cases where the unpaired electron(s) are interacting with surrounding nuclear spins, the ESE-detected EPR spectrum may be distorted due to echo modulations (see later) and special precautions should be taken when interpolating these data.\textsuperscript{9} ESE-detected EPR may be preferred over CW-EPR at high mw frequencies at which echo modulations are shallow and the poor phase stability of the CW-EPR experiments can cause severe spectral deformations. Further details about the advantages of ESE-detected EPR and alternative mw pulse sequences can be found in ref. 9.

2.2.3. ENDOR spectroscopy. Although in some cases the hyperfine interactions of the surrounding nuclei can partially be resolved by field-swept EPR experiments, other techniques need to be applied to map out the hyperfine and nuclear quadrupole couplings in detail. In particular, the spectroscopist will look for methods to directly probe the nuclear transitions ($|\Delta m_S| = 0$, $|\Delta m_I| \geq 1$). Fig. 1a shows the energy
levels for a $S = 1/2$, $I = 1/2$ system (e.g. an unpaired electron coupled to a proton). The nuclear frequencies are in this case given by

$$v_{m_S = 1/2} = v_2 = \sqrt{(A/2 + \nu_I)^2 + B^2/4}$$

$$v_{m_S = -1/2} = v_B = \sqrt{(-A/2 + \nu_I)^2 + B^2/4}$$

(5)

where $A$ and $B$ are related to the secular ($A_{zz}$) and pseudo-secular ($A_{xx}$ and $A_{yy}$) hyperfine couplings: $A = A_{zz}$ and $B = (A_{xx} + A_{yy})^{1/2}$. $\nu_I = -\beta g_s B_0 / h$ is the nuclear Lamor frequency. For isotropic hyperfine values and cases where $|\nu_I| \gg |A|$, $B$, the nuclear frequencies reduce to

$$v_{A,B} = |\nu_I \pm A/2|$$

(6)

In the case where $|\nu_I| > |A/2|$, one refers to the nuclei as being weakly coupled, whereas $|\nu_I| < |A/2|$ is typical for strongly coupled nuclei. The $|\nu_I| \approx |A/2|$ case is called the cancellation condition.

For nuclei with $I > 1/2$, the nuclear frequencies additionally depend on the nuclear quadrupole interactions.\(^{9,20,21,23}\)

In order to detect the nuclear frequencies, ENDOR experiments can be performed. In CW ENDOR, introduced by Feher\(^2\) in 1956, the CW-EPR signal at a given field is saturated by an increase in the mw power. The recovery of this signal is then monitored as a function of the frequency of continuously irradiated radiofrequency waves. Indeed, as the radio frequency matches a nuclear frequency, the populations of all energy levels will be changed and a net mw absorption can be detected. The typical ENDOR spectra for the cases described by eqn (6) are shown in Fig. 1b. Inspection of Fig. 1b reveals immediately the advantage of ENDOR at high mw frequencies. Indeed, higher mw frequencies imply the use of higher magnetic fields and lead, through the magnetic-field dependence of $\nu_I$, to a better peak separation in the ENDOR spectra. In many cases, a multi-frequency approach, combining ENDOR results obtained at different mw frequencies, proves to be favourable. Note that the ENDOR spectrum will only reflect the nuclear frequencies corresponding to the orientations selected by the magnetic-field setting at which the ENDOR experiment is performed. In order to reconstruct the full hyperfine and nuclear quadrupole tensors, experiments at different magnetic-field settings (‘observer positions’) should be performed.

One of the most often used pulsed-ENDOR techniques is Davies ENDOR spectroscopy\(^{25}\) (Fig. 2a). The first selective mw $\pi$ pulse inverts the population of a particular EPR transition. Each time the frequency of the subsequent frequency-swept rf pulse becomes equal to a nuclear frequency, the population of the EPR transitions will be affected. This change is monitored via a two-pulse-echo detection scheme (last two mw pulses). Mims ENDOR\(^6\) (Fig. 2b) offers an alternative scheme for recording the nuclear frequencies whereby the rf-induced changes in the gruted polarization created by the first two mw $\pi/2$ pulses are detected.

Pulsed ENDOR schemes offer the advantage over CW ENDOR that certain ENDOR signals can be selectively suppressed. An example of this is commonly used in Davies ENDOR in order to disentangle overlapping ENDOR signals of weakly coupled protons and strongly coupled nitrogens. The former signals can be largely suppressed by the use of short mw pulses (so-called hyperfine contrast selective ENDOR\(^9\)). Furthermore, more complex pulsed-ENDOR schemes allow for a correlation of the nuclear frequencies with other parameters in a second dimension. The HYENDOR technique, in which ENDOR frequencies are directly correlated to the hyperfine splitting, is a good example of this.\(^{26}\)

Since a recent Invited Article in this journal focused on pulsed ENDOR techniques,\(^{19}\) we refer to this work and other reviews and standard text books for further details on CW and pulsed ENDOR.\(^{9,11,19,27–29}\)

### 2.2.4. HYSCORE spectroscopy

The nuclear frequencies can also be determined using ESEEM techniques. In many cases these techniques give complementary information to the ENDOR experiments. Low-frequency signals ($<5$ MHz) are best detected using ESEEM. In this way, ESEEM will be the method of choice for the study of weakly coupled nitrogens, as are often found for protein-embedded transition-metal complexes.

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![Fig. 1](image)

(a) Energy level for an $S = 1/2$, $I = 1/2$ system, whereby $\nu_I < 0$, $A, B > 0$ and $|\nu_I| > |A/2|$. (b) The ENDOR spectrum described by eqn (6) for the weak-coupling case $|\nu_I| > |A/2|$ (top spectrum) and the strong-coupling case $|\nu_I| < |A/2|$ (bottom spectrum).

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In ESEEM experiments a sequence of mw pulses is applied that generates an electron spin echo. The modulation of the intensity of this echo is then monitored as a function of one of the pulse intervals.\(^9\) Fourier transformation of this time signal results in frequency spectra reflecting the nuclear frequencies similar to an ENDOR spectrum. The origin of this echo modulation is explained in detail in the standard text books\(^9,30\) and will not be treated here.

Here, we will only focus on the basic interpretation of hyperfine sublevel correlation (HYSCORE) experiments.\(^31\) HYSCORE is a four-pulse ESEEM technique (Fig. 2c) whereby the two interpulse distances \(t_1\) and \(t_2\) are varied independently. Two-dimensional Fourier transformation leads to frequency-domain spectra where the nuclear frequencies belonging to different \(m_f\) manifolds are correlated. The HYSCORE sequence is based on the three-pulse ESEEM experiment, that consists of three \(\pi/2\) mw pulses whereby the primary spin echo is detected as a function of the time between the last two pulses.

For the \(S = 1/2, I = 1/2\) system described earlier, the HYSCORE spectrum is characterized by a correlation pattern as shown in Fig. 3a. For the weak-coupling case, the cross peaks are found in the \((++)\) quadrant, whereas they appear in the \((-+\) quadrant for the strong-coupling case. As can be seen from Fig. 3a, the hyperfine value can easily be estimated from the peak positions. Furthermore, the correlation pattern considerably facilitates spectral assignment for complex systems where the electron is interacting with many nuclei. For disordered systems, \(e.g.\) frozen solutions of proteins, each magnetic-field setting corresponds to a selection of molecular orientations (so-called orientation selection) and the HYSCORE signals will no longer be sharp cross peaks but extended ridges representing the sum of the cross peaks corresponding to each of these orientations. This is shown in Fig. 3b, where a proton HYSCORE spectrum of a frozen solution of a Cu(II)-bound prion protein (PrP(23-231)) is depicted. The intersection of the diagonal and the anti-diagonal (dashed line) represents the proton Larmor frequency for this field. This is a typical example of the weak-coupling case (signals in \((++)\) quadrant). Analysis of the HYSCORE spectra of disordered systems requires spectral simulation and is non-trivial.

For an \(S = 1/2, I = 1\) system (\(e.g.\) an unpaired electron interacting with a \(^{14}\)N nucleus), the correlation pattern becomes more complicated. In cases where only one molecular orientation is selected by the magnetic field setting (single-crystal case), six nuclear frequencies can be detected: four single-quantum frequencies (\(\Delta m_I = 1\)) and two double-quantum (DQ) frequencies (\(\Delta m_I = 2\)) (Fig. 3c). The corresponding HYSCORE spectrum will then reveal information about the nine correlations linking the nuclear frequencies of the two \(m_f\) manifolds. For cases with a considerable nuclear quadrupole contribution (which is generally the case for \(^{14}\)N nuclei), the HYSCORE spectra are dominated by the double-quantum (DQ) cross peaks, linking the frequencies\(^32\)

\[
\nu_{\text{DQ}}^{m_f} = 2 \sqrt{\left( \nu_I \pm \frac{\eta}{2} \right)^2 + \left( \frac{e^2 q Q}{4\hbar} \right)^2 \left( 3 + \eta^2 \right)}
\]

where \(a\) is the hyperfine coupling at the particular observer position. These DQ cross peaks will appear in the \((++)\) quadrant for a weak-coupling case and in the \((-+\) quadrant for a strong-coupling case. The other cross peaks can be found spread over both quadrants. This is exemplified in Fig. 3d, showing the \(^{14}\)N HYSCORE spectrum of a VO(II) salen-type molecule taken at an observer position known to excite all molecular orientations in the ligand plane. This example was chosen because it nicely represents some of the essential characteristics of nitrogen HYSCORE spectra of disordered systems. Similar spectra have been reported for VO(II) model systems of the enzyme bromoperoxidase.\(^33\) Similar to the case depicted in Fig. 3b, the HYSCORE features in Fig. 3d are broad, because several molecular orientations are excited. The SQ ridges are clearly broader than the DQ cross peaks. This results from the fact that the SQ nuclear frequencies depend in \textit{first order} on the nuclear quadrupole interaction, whereby the DQ nuclear frequencies only depend in \textit{second order} on this coupling (eqn (7)), resulting in less extended and hence more intense DQ ridges. Using eqn (7) and the position and width of the DQ cross peaks in Fig. 3d, we can immediately estimate that the in-plane \(^{14}\)N principal hyperfine values will lie
In the HYSCORE spectra, sharp cross-peaks will be observed linking the above frequencies with the DQ nuclear frequency from the other m_S manifold.

The nuclear quadrupole couplings of 2H nuclei are in general very small, so that in this case, the HYSCORE spectra are dominated by cross peaks between the basic frequencies, rather than by the DQ frequencies. The typical forms of these HYSCORE spectra are described in ref. 35.

Significant sensitivity enhancement can be obtained in ESEEM experiments by the use of matched mw pulses. Matched pulses are high turning angle (HTA) pulses that enhance the efficiency of forbidden transfers and thus drastically increase the intensity of basic- and combination-frequency transitions. In the case of three-pulse ESEEM and HYSCORE spectroscopy, the second and third \( \pi/2 \) pulse will be matched to increase the efficiency of the forbidden transfers between allowed electron coherence and nuclear coherence. If one wants to enhance the ESEEM signals of a weakly coupled nucleus, the field strength of the matching mw pulses, \( \nu_{\text{match}} \), should be taken to be equal to the nuclear Zeeman frequency of this nucleus. For a strongly coupled nucleus, the field is matched on the hyperfine coupling, which, in practice, usually means that maximal mw power needs to be applied. The optimal length of the HTA pulse then has to be determined experimentally using a 2D three-pulse ESEEM experiment, where the pulse length of the second and third \( \pi/2 \) pulse is varied simultaneously in one dimension, and the time between these two pulses is varied in the second dimension (normal three-pulse ESEEM dimension). Fig. 4 shows a nice example of the effect of mw pulse matching on the three-pulse ESEEM spectra of an organic radical in polymethylmethacrylate. The matching is done on weakly coupled protons (\( \nu_{\text{match}} = 15.125 \) MHz). Fig. 4a shows the echo modulation for a standard three-pulse ESEEM (all mw pulse lengths equal to 16 ns) showing shallow proton modulations. Fig. 4b depicts the huge change in the modulation pattern when optimal matching pulses are used (\( t_{\pi/2, \text{match}} = 48 \) ns). This difference is also reflected in the corresponding frequency-domain spectrum (figure inset), showing now intense proton signals. Not only is a clear signal centered around \( \nu_{1} \) observed, but also the low frequency component of a proton hyperfine coupling of \( \sim 28 \) MHz is now clearly visible (indicated by an asterisk in the inset). Similar effects can be observed for the corresponding HYSCORE spectra (not shown).

It is evident that the use of matched mw pulses has largely increased the potential of the different ESEEM techniques.

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**Fig. 3** (a) Schematic representation of a HYSCORE spectrum of an \( S = 1/2, I = 1/2 \) system, with \( \nu_1 < 0, A, B > 0 \) and \( B < < A, \nu_1 \). For the weak-coupling case, the cross-peaks between the basic frequencies, \( \nu_\alpha \) and \( \nu_\beta \), will appear in the (–+) quadrant (squares), in the strong-coupling case, the cross peaks are found in the (–+) quadrant (circles). (b) Experimental proton HYSCORE spectrum of a frozen solution of Cu(II)-bound PrP(23–231) taken at an observer position corresponding to \( g = 2.37 \). \( \tau = 96 \) ns, and the pulse lengths, \( t_{\pi/2} = 16 \) ns and, \( t_\tau = 16 \) ns, were taken. (c) Schematic representation of the energy levels for an \( S = 1/2, I = 1 \) system indicating the double-quantum (DQ) and single-quantum (SQ) nuclear frequencies. The example represents a strong-coupling case. (d) Experimental \({}^{14}\)N HYSCORE spectrum of a frozen toluene solution of \((R,R)\) N,N’-bis(3,5-di-tert-butylsalicylidene)-1,2-cyclohexane-diamino vanadyl (II) at \( g = 2.056 \) (strong-coupling case). The DQ and SQ cross peaks are indicated. \( \tau = 176 \) ns, and the pulse lengths, \( t_{\pi/2} = 16 \) ns and, \( t_\tau = 16 \) ns, were taken.
Where ESEEM analyses would have been abandoned before because of lack of significant echo modulations, the relevant nuclear information can now be obtained.

Matched pulses can also be used to overcome one of the most annoying problems of HYSCORE experiments, namely the fact that the intensity of the HYSCORE signals depends strongly on the time \( t \) (so-called \( t \)-dependent blind spots). Because of this, HYSCORE spectra with different interpulse distances, \( t \), should be recorded and added for each observer position. This considerably increases the measurement time. The blind spots can be avoided by the use of an alternative SMART HYSCORE pulse sequence\(^3\) (Fig. 2d). In this scheme, the original \( \pi/2-t-\pi/2 \) nuclear-coherence generator is replaced by a one-pulse nuclear-coherence generator (matched pulse), avoiding the \( t \)-dependent blind spots. The second \( \pi \) pulse in the sequence is needed to refocus the free induction decay (FID) signal and allow echo detection.

Finally, since the modulation depth is proportional to \( I(I + 1) \), the echo modulation tends to be dominated by the modulations of the nuclei with the highest nuclear spin. In complex situations where the unpaired electron is interacting with several nuclei, this may lead to cross-suppression of the weaker modulations.\(^3\) This can be circumvented by the use of matching pulses\(^3\) or more extended ESEEM schemes.\(^3\)

Further information on HYSCORE, ESEEM and other pulsed EPR sequences can be found in ref. 9.

### 2.2.5. ELDOR-detected NMR.

Although ELDOR-detected NMR (Fig. 2e) is by far not as commonly applied as the different pulsed-ENDOR or ESEEM sequences described above, its use can sometimes be very advantageous.\(^3\) The basics of the method are quite simple. The selective \( \pi \) pulse with mw frequency 1 inverts the population of an EPR transition. Whenever the variable microwave frequency of the mw2 pulse matches one of the allowed or forbidden EPR transitions, the population in the observed transition will change. Observation of the FID signal as a function of the difference between the two mw frequencies again results in a spectrum reflecting the nuclear frequencies. Alternatively, the mw1 \( \pi \) pulse can be replaced by a \( \pi/2-t-\pi \) scheme whereby the spin echo is then monitored as a function of the varying mw frequency 2. A drawback of the ELDOR-detected NMR method is the fact that the central hole (around \( v_{mw1} = v_{mw2} \)) will cover the signals at low frequencies. Fig. 5a shows an extreme example of a broad central hole, but also in more favourable cases, the central hole can still be significant (Fig. 5b). The ELDOR-detected NMR approach is therefore
best suited to the detection of strongly coupled nuclei. Alternatively, the experiments can be performed at higher mw frequencies (making use of the increase in the nuclear Zeeman frequencies). The experiment depicted in Fig. 5a was performed at Q-band mw frequencies. The proton Zeeman frequency lies here at \( \sim 50 \) MHz and the proton frequencies are clearly separated from the central hole. At X-band mw frequencies, \( \nu_H \) lies around 15 MHz and a similar experiment would then not allow an analysis of the proton interactions.

Note that the width of the central hole can be reduced by pulse shaping and/or going to lower temperatures. It depends on the local concentration of the paramagnetic centres (links to the phase-memory time). The example in Fig. 5a can thus be further optimized, but was chosen here to illustrate the worst-case scenario.

At W-band mw frequencies, the ELDOR-detected NMR approach can lead to better signal-to-noise ratios than pulsed ENDOR, especially in the case of strongly coupled nuclei.\(^{40}\) Detection of these interactions implies the use of Davies ENDOR, and the detection of the ENDOR spectrum of strongly coupled nitrogen nuclei is notoriously difficult (and sometimes impossible) at W-band, requiring thousands of scans. The corresponding W-band ELDOR-detected NMR spectrum can usually be detected in a few scans. The explanation for this is probably manifold and factors such as sample heating due to the rf pulse in the Davies scheme, the different relaxation paths involved in the two experiments and the smaller amount of pulses in the ELDOR-detected scheme should be considered.

Fig. 5b shows an example of a single-scan W-band ELDOR-detected NMR spectrum of a Cu(II)-bound small His-containing peptide. The spectral contributions of the directly binding nitrogen of the histidine ligand can be readily identified after only one scan. The full hyperfine and nuclear quadrupole tensor can in this way be determined by recording the W-band ELDOR-detected NMR spectra at different magnetic-field settings (Maria Fittipaldi, work in progress).

2.3. From spectrum to model

In order to extract the EPR parameters from the different experimental results, adequate simulation and fitting programs are imperative. Due to the complexity and large diversity of the EPR experiments and the spin systems that can be addressed by them, an accurate simulation of the spectra is not trivial. Although simulation programs targeted at the simulation of specific EPR problems have been developed over the years,\(^{41,47}\) it is only recently that attempts to develop software packages that can tackle CW and pulsed EPR/ENDOR simulations on a general level have been started.\(^{48}\) The matlab-based EasySpin program\(^ {49}\) is at present the most extended and flexible simulation package for both CW and pulsed EPR spectra, but it still does not cover everything. It is evident that the further development and generalization of the simulation programs will remain one of the most important aims in the field.

Once the EPR parameters have been determined experimentally, the key question is how to interpret the different spin Hamiltonian parameters in terms of useful electronic and geometric structural information. In section 2.1, we already outlined how the individual EPR parameters can in principle be linked to different structural and electronic parameters. In practice, this step proves to be very difficult. Let us take the example of the hyperfine coupling. The \( A \) matrix can be split in an isotropic and an anisotropic part. While the isotropic part (Fermi contact part) can still be easily linked to the spin density on the nucleus, the interpretation of the anisotropic part is often less straightforward. This part contains orbital contributions and an electron–nuclear-distance dependence that are not easily separated.

Although several useful approaches have been explored and used for many decades\(^ {9,20-24}\) such as the point–dipolar approximation (see eqn (2)), there are many cases reported in which the full information hidden in the \( A \) matrix could not be satisfactorily decoded. This has, in combination with the fast increase in EPR methodology and its linked increase in experimental data, triggered the development of different quantum chemical approaches to compute the different spin Hamiltonian parameters.\(^ {49,50}\) In this, a symbiotic relation has now been formed between the theoretical and experimental approaches, as will also become clear from some of the examples in section 3. Experimental EPR data provide the ultimate control for the quality of the quantum chemical computation. Although for some cases density functional theory (DFT) and other \( \text{ab initio} \) methods have proven to generate theoretical spin Hamiltonian parameters that agree quantitatively with the experiment, a qualitative agreement is, for the majority of cases, still the best one can currently achieve, especially for transition metal complexes.\(^ {49,50}\) Even with these limitations, the quantum chemical computations are starting to play an essential role in EPR analysis. As mentioned in the previous section, the spectral simulation of EPR/ENDOR spectra can become very complex and at some points only a fraction of the hyperfine and nuclear quadrupole information can be extracted in a first analysis. If these parameters are then used to evaluate different theoretical models, valuable hints about the principal values and directions of the \( A \) and \( P \) tensors of the remaining magnetic nuclei can be derived from the quantum chemical computations. This information can then be used again as starting values to optimize the spectral simulations in order to derive the full set of spin Hamiltonian parameters for the system under study.\(^ {51,52}\)

Furthermore, once a satisfying agreement is found between the experimental data and the theoretical model, not only important structural information can be obtained from the best-matching model, but also the computed ground-state wavefunction can be linked to the inner working of the transition-metal site. Indeed, important characteristics, such as redox functions or ligand binding, are not only governed by the geometric structure of the site, the electronic state plays an equally important factor. Knowledge of the electronic structure is therefore essential if one wants to understand, for instance, why apparently small changes can induce function loss in one protein, and why seemingly more drastic changes still lead to (partial) preservation of the metalloprotein’s function in another case. Once a good theoretical model is found, the effect of changes in the structure (\( e.g. \) mutagenesis) can be probed theoretically. Thus, the amount of further experiments can be reduced to a selective and well-targeted set. In this way, the final outcome of the study exceeds what could possibly be reached by a separate approach. One of the
essential goals for the future is therefore a flawless matching of the two approaches, whereby the earlier-mentioned experimental and theoretical problems have to be overcome.

2.4. Advantages and drawbacks of using EPR to study metalloproteins

One of the important advantages of EPR is the large variety of experiments that can be performed. For many paramagnetic transition metal complexes, CW-EPR spectra can be obtained in a large temperature range up to room temperature. For these complexes, EPR can reveal important information about the molecular dynamics on timescales between 10 ps and a few μs.53,54 Information on the static structure of the transition-metal site can be obtained from the different advanced pulsed-EPR/ENDOR methods described in the previous sections. However, in order to be able to use the latter methods, the samples need to be cooled to very low temperatures (<25 K). The predominant reason for the latter is the need to slow down different relaxation pathways.9 Cooling is generally done using liquid helium and together with the high cost of the EPR equipment, this makes running an EPR facility expensive. However, other advanced techniques, such as XRD, neutron diffraction or NMR, are also very costly. A second drawback is the fact that the spectral complexity requires specialist’s input and a full structural analysis using EPR cannot be done on a high through-put basis.

The amount of (protein) material needed for the analysis depends on many factors, including the specific spin Hamiltonian parameters, the sensitivity of the specific spectrometer at hand, and even the time (and money) the particular spectroscopist is willing to invest (measurement time and liquid-helium consumption). In the most favourable cases, protein concentrations of 50–100 μM are sufficient. This is the case when the paramagnetic centre is characterised by a $g$ tensor exhibiting little anisotropy and when the (metal) hyperfine coupling is small. An example of this is the NO-ligated ferrous form of a heme protein. In extreme cases, protein concentrations in the mM range are needed. This is the case for systems exhibiting large $g$ anisotropy and/or large hyperfine couplings. The EPR study of low-spin ferric heme proteins requires concentrations of 1–3 mM (see section 3.1.1.). Note that the spin–spin relaxation will become faster as the concentration of paramagnetic molecules increases. At a certain concentration, the relaxation will be so fast that no spin-echo signal will be detectable. Hence, an increase in the concentration of a protein does not necessarily lead to a higher echo intensity and the optimum concentration needs to be established for each system separately. The volume needed for the experiment depends on the mw frequency. While X-band EPR measurements require about 100 μl of the sample, only 1 μl is needed at W-band mw frequencies. In practice, the available material will thus partially determine the type of experiments that can be performed.

In many cases, EPR provides valuable (and sometimes essential) information complementary to other structure characterization methods. In NMR, the presence of the unpaired electron(s) will usually cause large shifts and line broadenings, making it difficult if not impossible to gain structural information in the neighbourhood of the unpaired electron. EPR can provide this essential information and thus complete the experimental data. Single-crystal X-ray diffraction structure analyses and neutron-diffraction analyses have proven their extreme value in the determination of (metal)protein structures. However, EPR studies can also provide unique complementary information that may put XRD results in another perspective, as will be exemplified in sections 3.1.1 and 3.2.1. Other spectroscopic methods, such as extended X-ray absorption fine structure (EXAFS), optical and laser spectroscopies are also often used complementarily to EPR spectroscopy.

3. Applications

In this section, we will describe a few examples of EPR and ENDOR studies of iron-, nickel-, cobalt-, and copper-containing proteins. The examples are chosen so as to illustrate the potential of state-of-the-art EPR and are not meant to be exhaustive.

3.1. Iron-containing proteins

A glance in one of the many standard books on bioinorganic chemistry reveals that various proteins contain iron-binding sites or have an iron co-factor, many of them being paramagnetic. We will focus in this section on examples of ferric forms of heme proteins from our own research and on iron–sulfur proteins. The latter proteins are not studied by us, but their importance in biological processes merits some attention in this overview.

3.1.1. Ferric form of heme proteins. The most well-known iron-containing co-factor is probably the heme group (Fig. 6a), which is for instance found in globins. The iron ion of the heme group is reported to occur in the ferrous, ferric and ferryl form. The (paramagnetic) ferric (Fe(III)) forms of cytochromes, peroxidases and catalases are biologically active. The ferric or so-called ‘met-form’ of the globins was long thought to be biological inactive, because oxygen can only reversibly bind at the ferrous heme iron to realize the globin’s most documented functions of oxygen storage and transport. However, recently discovered new functions of vertebrate myoglobin55 and the discovery of new globin-type proteins in all kingdoms of nature with unknown functions56 have renewed the interest in the ferric form of globins.

Ferric heme proteins can have different spin states: a low-spin (LS) state ($S = 1/2$), or a high-spin (HS) state ($S = 3/2$). The spin states are governed by the strength of the axial ligands. All globins have a conserved histidine at position F8 that binds to the heme iron (proximal side), whereby the distal ligand can vary. A weak axial ligand, such as a distal water, will lead to a HS state, which is typically observed for the met form of vertebrate myo- and hemoglobin. Coordination of a strong axial ligand, such as an endogenous histidine, then leads to a LS iron(III) state, as found for the recently discovered vertebrate neuroglobin and cytoglobin.57 The X-band CW-EPR spectra of frozen solutions of LS and HS ferric heme proteins differ considerably. The LS ferric heme proteins are characterized by a highly rhombic $g$ tensor, as exemplified by the principal $g$ values of ferric mouse neuroglobin: $g_x = 1.29$, $g_y = 2.15$, $g_z = 3.12$.58 The X-band
CW-EPR of HS globins can be described by the effective $g$ values ($g_x,\text{eff} \approx g_y,\text{eff} \approx 6$ and $g_z,\text{eff} \approx 2$) (see later). X-Band CW-EPR thus offers a fast and easy method to determine the spin state of the iron.

Peisach and Blumberg systemized the analysis of the CW-EPR spectra of several biological molecules and related model systems. They constructed the so-called “truth tables”, which provide a means to relate the EPR parameters of a paramagnetic site to structural information. This approach was applied successfully to LS ferric heme proteins whereby the $g$ values give indications on the type and orientation of the axial ligands binding to the heme iron. Although these tables are based on crude assumptions, they can nevertheless give important initial information on the system. Indeed, using this simple approach, we could show for the case of ferric neuroglobin that the iron center is bis-histidine coordinated, and the histidine planes are not eclipsing the Fe–pyrrole nitrogen bonds. Furthermore, the EPR parameters indicate that a conformation in between parallel and near to perpendicular alignment of the histidine planes is present. This was later confirmed by the X-ray data.

In 1986, Scholes and co-workers used CW-ENDOR to study frozen solutions of bis(imidazole)-ligated LS ferric heme complexes. Although they could derive interesting information on the electronic and geometric structure of the heme environment from this study, there were several method–inherent limitations to the work, mainly due to the high degree of spectral overlap of the contributions of the individual nuclei. The same problems were observed for X-band Davies ENDOR and three-pulse ESEEM studies of ferric heme proteins, since in all cases the spectra are one-dimensional and the spectral contributions of different nuclei will show a similar degree of overlap.

Hoffman and co-workers showed that some of the above-mentioned problems can be solved by using a combination of CW, Davies- and Mims-ENDOR techniques at Q-band (35 GHz) mw frequencies. Due to the magnetic-field dependence of the Larmor frequency, $\nu_L$, the spectral contributions of the different nuclei can then be better separated at higher fields. In this way, the authors established that the allylbenzene-bound heme of inactivated chloroperoxidase is in fact an N-alkylhemin metallocycle with a carbon of allylbenzene bonded to the pyrrole nitrogen.

Raitsimring, Walker and Astashkin showed in a series of papers that it is advantageous to perform two- or four-pulse ESEEM experiments at different settings of the magnetic field and different mw frequencies. From the magnetic field dependence of the proton combination peaks, detailed information about the orientation of the proton hyperfine tensor in the $g$ tensor frame can then be obtained. For the nearby protons of the axial imidazole ligands, this information can be directly linked to the orientation of imidazole ligands in the heme pocket.

The first two-dimensional ESEEM experiments on ferric LS heme proteins were performed by García-Rubio et al. They compared the HYSCORE spectra of a bis(imidazole) ligated heme compound, with selective isotopic substitution of the nitrogens, to those of cytochrome $b_{559}$. In this way the HYSCORE peaks of cytochrome $b_{559}$ could be assigned. The large spectral similarity between cytochrome $b_{559}$ and the model system led to a detailed identification of the heme–ligand orientations.

In our recent work on a ferric porphyrin model system, we combined X-band CW-EPR, HYSCORE, and four-pulse ESEEM experiments to maximize the information that can be obtained from an EPR study of ferric heme complexes. The strategy consists of three steps. In a first step, the
orientation of the hyperfine and nuclear quadrupole tensors of the pyrrole nitrogens is determined in the $g$ tensor frame by the simulation of the nitrogen HYSCORE spectra. For all metalloporphyrin complexes the highest nuclear quadrupole value of the pyrrole nitrogen is found to lie in the porphyrin plane perpendicular to the metal–nitrogen bond. This provides a means to determine the orientation of the $g$ tensor frame in the molecular frame. The analysis of the proton HYSCORE and combination-peak experiments then provides the hyperfine tensors of the imidazole protons. These parameters can be decoded into the proton–iron distance and orientation of the Fe–H vector in the $g$ tensor frame. Using the results of the first step, this information is then linked to the orientation of the imidazole planes versus the porphyrin. In a final control step, the imidazole nitrogen hyperfine and nuclear quadrupole tensors are determined from the nitrogen HYSCORE spectra. The in-plane rotation of the principal axes of these tensors is linked to the orientation of the imidazole plane and should match the earlier proton data. In a later work, this methodology was extended with pulsed ENDOR experiments and applied to ferric mouse neuroglobin.\textsuperscript{74} Fig. 7a shows a typical nitrogen HYSCORE spectrum of ferric mouse neuroglobin. The DQ cross peaks of the heme and histidine nitrogens are well separated. As outlined in section 2.2.4., initial spin Hamiltonian parameters can be derived from the positions of these cross-peaks using eqn (7), and this can then be refined by spectral simulation. The structural parameters obtained by this approach for the F8His and E7His of ferric mouse neuroglobin matched the earlier X-ray data. A similar procedure was also successfully applied to unravel the direct heme environment of tomato hemoglobin, a globin for which no prior structural information was available.\textsuperscript{75} In our recent study of ferric cytoglobin, the strategy could also be used to solve an ambiguity between two conflicting X-ray studies (I. Ioanitescu, to be published). Although the error margins on the structural parameters are larger than for those obtained from X-ray diffraction studies, the method does not require single crystals and can thus be used to study every ferric LS heme protein without prior crystallization. The methodology now also opens the possibility of identifying disulfide-bridge-induced changes in the heme pocket of human neuroglobin. It was earlier found that most neuroglobins, with the exception of Rodentia and zebrafish neuroglobins, can form a disulfide bridge between the cysteines on positions CD7 and D5.\textsuperscript{76} Up until now, all efforts to isolate and crystalize the latter form of the protein have failed. The combined pulsed EPR/ENDOR approach may well provide the only way to identify the structural change occurring in the heme pocket upon disulfide bridge formation. A large part of the ferric forms of heme proteins are in a HS state. The mw quantum energy at X-band ($\sim 9.5$ GHz) is much smaller than the zero field splitting ($5\text{–}10$ cm$^{-1}$), so that the observed EPR spectrum only arises from the transitions of the lower Kramers doublet and can be described as an $S_{\text{eff}} = 1/2$ system whereby

\begin{align*}
H_{\text{eff}} &= \frac{\beta_e B_0 g_{\text{eff}} S_{\text{eff}}}{\hbar} + \sum_k \left( S_{\text{eff}} A_{k,\text{eff}} \mathbf{I}_k - \frac{g_\alpha g_{\text{eff}} B_0}{\hbar} \mathbf{I}_k \right) + \sum_{k, l > 1/2} \tilde{I}_k \bar{P}_{k,\text{eff}} \mathbf{I}_k \\
&\text{(9)}
\end{align*}

whereby the effective spin Hamiltonian parameters link to the general spin Hamiltonian values of eqn (1) and (4) by

\begin{align*}
g_{k, x, \text{eff}} &= \frac{g_k}{3} \left( 1 - \frac{12E}{D} \right), \\
g_{k, y, \text{eff}} &= \frac{g_k}{3} \left( 1 + \frac{12E}{D} \right), \\
g_{k, z, \text{eff}} &= \frac{g_k}{3} \\
A_{k, x, \text{eff}} &= \frac{A_k}{3} \left( 1 - \frac{12E}{D} \right), \\
A_{k, y, \text{eff}} &= \frac{A_k}{3} \left( 1 + \frac{12E}{D} \right), \\
A_{k, z, \text{eff}} &= \frac{2A_{k, z} A_{k, y}}{D}, \text{(11)}
\end{align*}
work now focuses on testing the performance of different splitting. The effective nuclear quadrupole tensor, $D_{\text{eff}}$, usually shows only minor deviations from the $P$ tensor in eqn (1).\textsuperscript{77}

As mentioned before, the X-band CW-EPR spectrum of HS ferric heme compounds can be simulated with an effective electron spin of 1/2, and effective $g$ values. Unfortunately, the X-band CW-EPR spectra of different HS ferric heme proteins tend to be alike and reveal little information on the system other than the identification of the spin state. A direct determination of the zero-field splitting using CW-EPR is only possible at high mw frequencies.\textsuperscript{78}

In 1982, Scholes \textit{et al.} published a seminal paper on the CW-ENDOR analysis of a single crystal of aquometmyoglobin, unravelling the hyperfine and nuclear quadrupole tensors of the heme and histidine nitrogens.\textsuperscript{79} Since then, only a few ESEEM or pulsed ENDOR studies of HS heme compounds have been reported,\textsuperscript{80} contrasting with the recent evolution in the field of EPR. There are two reasons for this. First of all, the effective hyperfine values of the heme and imidazole nitrogens reach values of 30–45 MHz for orientations in the heme plane.\textsuperscript{79} These interactions cannot be observed using standard ESEEM techniques. Furthermore, the pseudo-nuclear contributions to the nuclear Zeeman term given in eqn (12) considerably complicate the analysis of ENDOR and ESEEM spectra, especially when no single crystals are available. In a recent study, we showed how some of these problems can be circumvented by using matched HYSCORE spectroscopy.\textsuperscript{77} Fig. 7b shows a matched X-band nitrogen HYSCORE spectrum of the ferric form of the E7Q mutant of neuroglobin taken at an observer position $g = 3.48$. Because of the sensitivity enhancement by the matched mw pulses mentioned earlier, the nitrogen DQ cross peaks can readily be detected in this spectrum. These signals are not revealed in the standard HYSCORE experiment. Furthermore, clear ridges stemming from nearby protons can be recognized in the $(+ +)$ quadrant. The ridges in the $(- +)$ quadrant at frequencies lower than 15 MHz are complex to interpret and agree with the SQ nitrogen frequencies and different combination frequencies (nitrogen–nitrogen and nitrogen–proton).

The HYSCORE study of the ferric form of the E7Q-mutant of human neuroglobin also revealed that this protein lacks the characteristic distal water at neutral pH and that the lysine at position E10 coordinates to the iron at high pH.\textsuperscript{77} Our current work now focuses on testing the performance of different advanced pulsed-ENDOR and ENDOR techniques, e.g. HYSCORE, ELDOR-detected NMR, and HYEND, at different mw frequencies for the analysis of HS ferric heme proteins (work in progress). The ELDOR-detected NMR technique at W-band (95 GHz) mw frequencies in particular promises to become a valuable tool in the analysis of ferric HS systems.

$$g_{n,k,x,\text{eff}} = g_{n,k} + \frac{2g_A \beta_A A_{n,k}}{\beta_B D},$$
$$g_{n,k,y,\text{eff}} = g_{n,k} + \frac{2g_B \beta_A A_{n,k}}{\beta_B D},$$
$$g_{n,k,z,\text{eff}} = g_{n,k}.$$

where $D$ and $E$ are the tetragonal and rhombic zero-field splitting. The effective nuclear quadrupole tensor, $P_{\text{eff}}$, usually shows only minor deviations from the $P$ tensor in eqn (1).\textsuperscript{77}

3.1.2. Iron–sulfur proteins. Another important class of iron proteins comprises the so-called iron–sulfur proteins. These proteins have different functions, but are most frequently involved in electron transport. Different iron–sulfur clusters ([2Fe–2S], [3Fe–4S], and [4Fe–4S]) are observed. Characteristics of iron–sulfur proteins is the coordination of the iron ion by cysteines, or occasionally by other amino acids of the protein side chains, and, for polymeric Fe–S centers, the remarkable ligation with bridging ‘inorganic’ sulfide ions. It is safe to say that EPR has played a crucial role in iron–sulfur protein research. Indeed, based on their CW-EPR analysis, Bertrand \textit{et al.}, could propose the existence of a new class of [2Fe–2S] proteins, the so-called “Rieske-type” proteins (Fig. 6b), which are characterized by a larger $g$ tensor anisotropy compared to the classical ferredoxin type ($g_{av} \sim 1.91$ instead of $g_{av} \sim 1.96$).\textsuperscript{81} They pointed out that the unusual $g$ values of this new type of [2Fe–2S] protein are consistent with the presence of ligands at the ferrous site that are more electronegative than sulfide, which is in contrast to the classical ferredoxin-type clusters where all ligation is provided by sulfide. In recent years, more advanced pulsed EPR techniques have been used successfully to identify the direct environment of the iron–sulfur clusters as exemplified in ref. 82–84. A detailed overview of the EPR and ENDOR work done on iron–sulfur proteins is presented in ref. 84 and 85.

3.2. Nickel-containing proteins

Ni(i) (d$^9$) and Ni(ii) (d$^8$) are paramagnetic ions. In many cases, identification of the oxidation state of the nickel ion is not straightforward and the nickel centre of many nickel-containing enzymes is poorly characterized, as will become apparent from the following examples. We focus here on the EPR analyses of different forms of methyl-coenzyme M reductase (MCR) to which one of us (S.V.D) also contributed. In the second part, we also summarize some of the EPR work done in field of [FeNi] hydrogenases. This is not our own research field, but we feel it illustrates the potential and also future challenges of EPR in the analysis of metal-containing proteins nicely and it can therefore not been left out of this overview.

3.2.1. Methyl-coenzyme M reductase (MCR). MCR plays a crucial role in the methane-forming step of the energy metabolism of archaea, since it catalyzes the reduction of methyl-coenzyme M (2-(methylthio)ethane-sulfonate, CH$_3$SCoM) with coenzyme B (7-thioheptanoyl-threonine-phosphate, HSCoB) to methane and CoMS-SCoB.\textsuperscript{86} The enzyme contains the nickel-centred porphinoid co-factor, F430 (Fig. 6c). Different forms of the enzyme and their interconver-

The CW-EPR and ENDOR spectra of the isolated F430 co-factor of and of the active form of the enzyme, MCR$_{red}$, are very alike and typical for a d$^8$ Ni(i) complex with the unpaired electron residing in the d$_{x^2-y^2}$ orbital.\textsuperscript{88} The principal $g$ values are given in Table 1. Although the hyperfine couplings of the four pyrrole nitrogens show small differences, they are
of the same order and typical for strongly coupled nitrogens ($A_{iso} \approx 28.5$ MHz for MCRred1 and $A_{iso} \approx 27.8$ MHz$^{89}$ for Ni(I) F430$^{88}$). Fig. 8a shows the X-band Davies ENDOR spectra of the Ni(i) form of F430 pentamethyl ester. The top spectrum was recorded using selective, soft mw pulses. It is quasi-symmetrical around the proton Larmor frequency, $v_L$, and is dominated by the spectral contributions of the nearby protons of the F430 macrocycle. When using hard mw pulses (bottom spectrum in Fig. 8a), the spectrum changes completely. The proton signals are now suppressed and the underlying signals of the strongly coupled nitrogens become apparent. Since at this observer position all in-plane orientations are excited, the nitrogen ENDOR spectrum is complex, stemming from many orientations. The two groups of four lines in Fig. 8a indicate the basic nuclear frequencies corresponding to the two in-plane principal directions of the hyperfine and nuclear quadrupole tensor ($P_x \approx -1.85$ MHz, $P_y \approx 1.1$ MHz$^{28}$). The extra features stem from the other in-plane orientations. Fig. 8a illustrates the hyperfine contrast selective ENDOR effect mentioned in section 2.2.3.

In the presence of coenzyme M (2-mercaptoehanesulfonate, HSCoM) and HSCoB, MCRred1 is converted into MCRred2 (maximum conversion is 50%). The CW-EPR spectrum of this form is characterized by an unusual, highly rhombic $g$ tensor, whereby the broadening of the EPR line widths observed for $^{61}$Ni-labeled MCRred2 reveals that the paramagnetic centre is nickel based.$^{87}$ Combination of X-band and Q-band ENDOR and HYSCORE spectroscopy allowed us to determine the hyperfine and nuclear quadrupole parameters of the pyrrole nitrogens. Contrasting the case of MCRred1, two sets of nitrogens with hyperfine values differing by about a factor of two could be identified for MCRred2$^{90}$. The smaller hyperfine coupling could be detected using Q-band HYSCORE (Fig. 8b). It was assigned to the nitrogen of pyrrole A. The pyrrole ring A is not $\pi$-conjugated and therefore more flexible than the other pyrrole rings (Fig. 6c). Reduction of the nitrogen hyperfine values therefore indicates that pyrrole A is slightly bent out of the macrocycle plane. The Q-band HYSCORE spectrum of $^{33}$S-labeled coenzyme M revealed additional features (signals marked by the arrows in Fig. 8b) that stem from the $^{33}$S interactions. The two cross-peaks in the ($+ +$) quadrant at $(10.8, 31.8)$ MHz and $(-31.8, 10.8)$ MHz are assigned to the triple-quantum transitions. The appearance of the $^{33}$S-related cross-peaks proved unambiguously the coordination of CoM to the nickel center in MCRred2$^{91}$.

MCRox1 is an enzymatically inactive but ‘ready’ form of MCR, which can be readily reduced to the active MCRred1. The oxidation state of MCRox1 is highly debated, Ni(i) ($d^9$)$^{89,92}$ Ni(ii) ($d^7$),$^{93}$ a Ni(iii)-thiolate and a high-spin Ni(ii) ion ($S = 1$) antiferromagnetically coupled to a thyl radical$^{94}$ have all been proposed. The fact that MCRox1 is converted to MCRred after addition of Ti(III) citrate at pH 9 and 60 °C indicates that MCRox1 is a Ni(II) complex.$^{95}$ On the other hand, MCRox1 can also be generated by cryoreduction of MCRox1-silent, an enzymatically inactive EPR-silent Ni(II) form, which makes a Ni(i) oxidation state more probable. The g values (Table 1) and pyrrole nitrogen hyperfine values ($A_{iso} \approx 25$ MHz) are typical of an $S = 1/2$ species with an unpaired electron predominantly residing in the nickel $d_{z^2}-p_z$ orbital. Since both Ni(i) and Ni(III) complexes with the unpaired electron in the $d_{z^2}-p_z$ orbital have been reported, this does not shed light on the oxidation state of nickel in MCRox1. Furthermore, the X-ray absorption (XAS) spectroscopy,$^{96}$ UV-Vis$^{97}$ and magnetic circular dichroism$^{98}$ data indicate a close resemblance of MCRox1 to the EPR-silent Ni(II) states.

### Table 1 Principal $g$ values of coenzyme F430 and different MCR forms

<table>
<thead>
<tr>
<th>Complex</th>
<th>$g_x$</th>
<th>$g_y$</th>
<th>$g_z$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni(i)-F430</td>
<td>2.063</td>
<td>2.063</td>
<td>2.244</td>
<td>88</td>
</tr>
<tr>
<td>MCRred1</td>
<td>2.060</td>
<td>2.070</td>
<td>2.2485</td>
<td>89</td>
</tr>
<tr>
<td>MCRred2</td>
<td>2.1753</td>
<td>2.2313</td>
<td>2.2869</td>
<td>90</td>
</tr>
<tr>
<td>MCRox1</td>
<td>2.1527</td>
<td>2.1678</td>
<td>2.2312</td>
<td>94</td>
</tr>
</tbody>
</table>

![Fig. 8](https://example.com/fig8.png)

Fig. 8 (a) X-Band Davies ENDOR spectra of the Ni(i) form of F430 pentamethyl ester taken at an observer position corresponding to $g \approx g_z$. The top spectrum was taken using selective mw pulses ($\tau_{m2} = 200$ ns, $\tau_p = 400$ ns) and a long rf $\pi$ pulse (10 $\mu$s), the bottom spectrum was taken using hard mw pulses ($\tau_{m2} = 26$ ns, $\tau_p = 52$ ns) and a shorter rf pulse (5.4 $\mu$s). The meaning of the two groups of four lines is explained in the text. (b) Q-Band HYSCORE spectrum recorded at 25 K of MCR in the MCRred2 state with H$^{33}$S-CoM, observer position $g = 2.28$. The arrows identify the peaks that originate from $^{33}$S interactions. The remaining cross-peaks stem from an interaction with the nitrogen of pyrrole A. The spectrum was reproduced with permission from C. Finazzo, J. Harmer, C. Bauer, B. Jaun, E. C. Duin, F. Mahlert, M. Goenrich, R. K. Thauer, S. Van Doorslaer and A. Schweiger, J. Am. Chem. Soc., 2003, 125, 4988-4989. Copyright 2003 American Chemical Society.
Only recently, our multi-frequency EPR, ENDOR and HYSCORE approach in combination with $^{61}$Ni, $^{33}$S and $^2$H isotope labeling led to an identification of MCR$_{ox1}$ as a Ni(III) thiolate in resonance with a thyl radical/high-spin Ni(II) complex. Analysis of the $^{61}$Ni and $^{33}$S hyperfine values proved that Ni–S coordination was present in which 7 ± 3% of the spin density is on the sulfur. The spin-density distribution and Ni–S bonding show the non-innocent electron donating character of the sulfur ligand on the oxidation state and can explain the apparent Ni(II)-like spectra observed with XAS, UV-Vis and MCD. Deuteration of the CoM ligand revealed that the distance between the nickel centre and β-protons of CoM is very similar to those of Ni(II)-MCR$_{ox1}$-silent for which an X-ray study exists. Exchange experiments in D$_2$O also suggest that the binding of the SO$_3^-$ group of CoM may be stabilized by hydrogen bonding with two nearby tyrosines (Tyr$^{233}$ and Tyr$^{567}$)$_{94}$. In the context of the previous descriptions it is worthwhile to mention that all known X-ray structures of MCR describe inactive, oxidized (Ni(II)) forms. At present, no crystals of the important paramagnetic forms have been grown, so the EPR technique provides the main characterization method for the active centre of MCR.

### 3.2.2. [NiFe] hydrogenases

Hydrogenases catalyze the heterolytic splitting of molecular hydrogen. The metal-containing hydrogenases are usually classified by the type of metal in their active site: [NiFe] and [Fe]-only hydrogenases, of which the former class is the largest. The active site of the [NiFe] hydrogenases consists of a heterobimetallic cluster, in which the nickel and iron atoms are bridged by the sulfur atoms of two cysteines. Furthermore, two additional cysteines bind terminally to the Ni atom. The iron atom is ligated by three non-protein di-atomic molecules (two CN$^-$ and one CO). At least seven oxidation states have been reported for [NiFe] hydrogenases. Advanced EPR techniques have played and are still performing a crucial role in the elucidation of the structure and activation mechanisms of hydrogenases.

In the oxidized form, two EPR-active forms co-exist: the ‘unready’ Ni–A and the ‘ready’ Ni–B form, which can be activated by reduction under a H$_2$ atmosphere in a few minutes (Ni–B) or after incubation for hours (Ni–A). In the aerobically ‘as-isolated’ enzyme (Ni–A) an additional bridging of the Ni and Fe atom is achieved by (presumably) an oxygen species as could be inferred from $^{17}$O ENDOR at Q-band mw frequencies. Combination of the EPR and ENDOR data with density functional theory (DFT) calculations lead to two possible models: (i) OH$^-$ as bridging ligand and (ii) the bridging ligand is a diatomic-type ligand. A similar approach for Ni–B allowed for the identification of an OH$^-$ bridging ligand.

Reduction of the enzyme by two electrons leads to the catalytically active Ni-C state. Illumination at low temperature causes the conversion of Ni–C into another paramagnetic state Ni–L. Using $^2$H$_2$O exchange experiments in combination with ENDOR and HYSCORE spectroscopy the presence of a hydride as a bridging ligand could be proven for a regulatory hydrogenase and for a catalytic hydrogenase. After illumination at 10 K, the strong hyperfine coupling assigned to the bridging hydride is lost, indicating a cleavage of the metal–hydride bond.

The EPR story of [NiFe] hydrogenases can, in our view, be considered a textbook example of the potential of modern-day EPR. Not only are the experimental data gathered using a variety of multi-frequency EPR and ENDOR techniques revealing information unattainable by other spectroscopic methods, the interpretation of the EPR data is also corroborated by DFT computations. As stated earlier, one of the great challenges is the theoretical modelling of the magnetic parameters extracted from the experimental data in order to obtain direct structural information about the system hand. The example of the study of [NiFe] hydrogenases shows the potential of an integrated EPR-DFT approach. Note that we focused in this section on the Ni-related centres in [NiFe] hydrogenases. However, these proteins contain 11 more iron atoms besides the one in the Ni–Fe subunit. These additional Fe atoms are part of three iron–sulfur clusters and can also be studied by EPR (see section 3.1.2).

### 3.3. Cobalt-containing proteins

Cobalt(II) is found in natural proteins, such as some active forms of B$_{12}$ enzymes, but is also a good functional mimic of other metal ions. Furthermore, Co(II) is an excellent spectroscopic probe since it is both optically and EPR active. For these reasons, the unique oxygen binding properties of Co(II) porphyrins have obtained considerable attention in biochemical studies, because these complexes can be used as model systems for the ligand-binding characteristics of globins. Because of the fast autoxidation rates, the analysis of the oxygenated native heme proteins is often very difficult. Cobaltous-substituted porphyrins can then offer a way to study both the oxy and deoxy species using CW and pulsed EPR and ENDOR. Similarly, the EPR analysis of Co(II)-substituted zinc-dependent aminopeptidases revealed essential structural information that was not obtained by other techniques.

We here focus on the study of the EPR-active forms of B$_{12}$ proteins.

#### 3.3.1. Cobaltous forms of B$_{12}$ proteins

B$_{12}$ derivatives are involved in unique organometallic biological reactions. The B$_{12}$ co-factors are essentially cobalt-containing corrin complexes. CoB$_{12}$ derivatives in solution at neutral pH and in the solid state, the base-on form is the most stable constitution. In contrast, cobaltous B$_{12}$ derivatives, but they are only transiently formed and therefore difficult to observe. In some cases, the dimethylbenzimidazole (DBI) group is coordinated to the cobalt atom to form a base-on form, whereas, if such a coordination is absent or replaced by an exogeneous ligand, these complexes are referred to as base-off complexes.

For isolated B$_{12}$ co-factors in solution at neutral pH and in the solid state, the base-on form is the most stable constitution. In contrast, at low pH, a base-off form is found. This transition between the two forms is clearly reflected in the X-band CW-EPR spectra. In the EPR spectrum of the base-on form, a clear three-fold splitting of the high-field lines can be observed, due to the hyperfine interaction with the $^{14}$N nucleus ($I = 1$) of the coordinating DBI ligand. This disappears in the EPR spectrum of the base-off form, since DBI is no longer coordinating. In a recent work, we studied the solvent effects...
on base-off Co(ii) corrinates. Previous literature addressed Co(ii) corrinates in solution often as four-coordinated Co(ii) corrins. Using pulsed EPR and ENDOR in combination with $^3$H$_2$O exchange experiments we could clearly prove axial solvent ligation for Co(b)(ii)ester (heptamethyl cobyrinate perchlorate) and the base-off form of cob(n)alamin (B$_{12}$r) in polar solvents. Fig. 9 shows the difference HYSCORE spectrum of Co(b)(ii)ester in CD$_3$OD and CH$_3$OH. The $^2$H cross-peaks reveal direct information on the hyperfine coupling to the methanol deuterons proving axial coordination of the solvent. The additional cross-peaks stem from combination between the $^2$H basic frequency and one of the DQ frequencies of the ligand nitrogens as indicated in Fig. 9. Since only combinations between nuclear frequencies within the same $m_S$ manifold can occur, the appearance of these cross-peaks give direct information on the relative sign of the $^2$H and $^{14}$N hyperfine couplings.

In addition, the axial ligation of Co(b)(ii)ester in methanol was shown to be influenced by the phase transition of the solvent. Axial ligation of one methanol molecule is observed in the $\alpha$-crystalline phase, whereas six-coordination becomes possible in the glassy state. In a methanol : H$_2$O mixture, only five-coordination of Co(b)(ii)ester is observed. Addition of water to methanol changes its phase-transition properties and leads to frozen solutions with a large degree of crystalline structure. Differences in the solvent ligation could be related to changes in the network formed by the solvent molecules and the ring substituents. Furthermore, we could show that in apolar solvents Co(b)(ii)ester forms a contact-ion pair with its counter ion, perchlorate. No evidence of pure four-coordinated Co$^{\text{III}}$ corrins in solution is found. These observations were corroborated by DFT computations, whereby again the power of a combined EPR-DFT approach could be proven.

In addition, the hyperfine and nuclear quadrupole parameters of the magnetic nuclei in the vicinity of the unpaired electron could be determined. Combined with our earlier study on the base-on form of cob(n)alamin, these results can be used as reference data to study in detail the electronic structure of the Co(ii) forms of B$_{12}$ proteins.

The initial EPR studies on B$_{12}$ proteins focused on the analysis of the cobalamin form and of possible intermediately generated radicals using X-band CW-EPR. The base-on binding mode has been found in diol dehydratase (DD) and in B$_{12}$-dependent ribonucleotide reductase (RNR). For other B$_{12}$ proteins, the intramolecular dimethylbenzimidazole base is replaced by the imidazole side chain of a protein histidine residue. One such example is glutamate mutase from Clostridium cochlearium. The EPR spectrum of this protein shows a typical base-on pattern with the three-fold splitting of the high-field line. Upon binding of cobalamin to the $^{15}$N-labeled protein, the three-fold splitting changed into a two-fold splitting ($^{13}$N, $I = 1/2$), indicating that the cobalt is coordinating to a nitrogen of the enzyme. Furthermore, the EPR spectrum of a sample of the $^{15}$N-labelled protein in which only the histidines were $^{15}$N labeled, showed again the characteristic three-fold splitting. This EPR study was the first unambiguous proof that in the carbon-skeleton rearranging mutase a histidine residue of the enzyme rather than DBI coordinates to the cobalt center. Finally, the base-off form of cob(n)alamin is found to occur as an intermediate state in a number of proteins. As an example, methanol:5-hydroxybenzimidazolylcobamide methyltransferase (MT1) is the first of two enzymes playing a role in the transmethylation of methanol to 2-mercaptoethanesulfonic acid in Methanosarcina barkeri. Binding of MT1 to the methyl group of CH$_3$OH only occurs when the cobalt corrin is reduced to the Co(i) state. This reduction requires H$_2$, hydrogenase, methyltransferase activation protein and ATP. Using CW-EPR, it could be shown that the as-isolated oxidized Co(iii) state is reduced to a base-on cob(n)alamin (DBI internal coordination) by addition of H$_2$ and hydrogenase. Subsequent addition of methyltransferase activation protein and ATP leads to base-off cob(n)alamin.

At present, advanced pulsed EPR studies on B$_{12}$ proteins are still very scarce. The Co(n)-product radical pair state of ethanolamine deaminase is the B$_{12}$ protein that is by far the most studied by pulsed-EPR methods, but even for this system only X-band three-pulse ESEEM techniques and no advanced multi-dimensional pulsed EPR methods were applied. In our opinion, a large potential to understand the inner workings of these proteins is in this way left aside and we share the surprise of Bennett that relatively few EPR studies of Co(n)-containing or Co(n)-substituted enzymes are reported.

### 3.4. Copper-containing proteins

Undoubtedly, the majority of the EPR work on metalloproteins is performed on copper-containing proteins. Cu(n) (d$^9$, $S = 1/2$) is an ideal EPR probe. The ion is detectable by CW-EPR in a broad range of temperatures, making it a perfect tool to determine the dynamics and static structure of the protein via the study of the temperature dependence of the $g$ and copper hyperfine values ($^{63}$Cu, $I = 3/2$, abundance 69.15%, $^{65}$Cu, $I = 3/2$, natural abundance 30.85%). Although
the examples of EPR studies of copper-containing proteins are legion, including our own work on the Cu(II)-binding of prions and prion analogues,122,125 we will here focus only on the study of azurin using multi-frequency EPR techniques,126–136 because we think it offers the reader a clear insight into the possibilities of what can be obtained by these advanced EPR techniques.

3.4.1. Unravelling the structure of azurin. Although a quick scan through the EPR/ENDOR literature shows that the majority of the studies on copper proteins are performed at X-band mw frequencies, the use of high-field EPR techniques has great potential. This is exemplified by the study of wild-type azurin from *Pseudomonas aeruginosa* and some of its mutants. The Cu(II)-binding site in azurin is a typical type-I copper centre characterized by the strong ligation of two histidines and a cysteine and a weak ligation of commonly a methionine residue (Fig. 6e). In order to understand the electron-accepting and electron-donating processes occurring in this blue-copper protein, the electronic structure of the copper sites should be mapped out. EPR is one of the techniques of choice. However, at X-band frequencies, it is impossible to accurately determine the principal $g$ values. This high resolution can be obtained when using 95 GHz EPR spectroscopy. The EPR lines in the W-band ESE-detected EPR spectra of single crystals of wild-type azurin could be related to the 16 protein molecules in the unit cell.126 Similar analyses for the M121Q and M121H mutants of azurin revealed clear spectral changes that could be assigned to admixture of $d_{yz}$ (M121H) or $d_{xy}$ (M121Q) character into the wave function.127,128 W-Band nitrogen ENDOR and X-band ESEEM studies allowed for a complete determination of the hyperfine and nuclear quadrupole tensors of the remote nitrogens $N_5$ of the histidine ligands.129–133 In a seminal work, Groenen and co-workers detected for the first time single-crystal W-band ESEEM spectra arising from the directly coordinated nitrogens of the histidines that ligate copper in azurin.134 The set of experimental data was completed by a recent W-band deuterium ENDOR study of single crystals of azurin selectively deuterated at the $CP$ position of the copper-binding cysteine-112.135 The magnitude of the isotropic hyperfine coupling of the $\beta$ deuterons can directly be related to the spin density on the cysteine sulfur. All these EPR studies have not only provided a direct insight into aspects of the electronic structure of the copper site in azurin, they also form a critical and necessary test for the different quantum-chemical studies performed on azurin.136–138 Only a good qualitative agreement between theory and experiment gives confidence in the quantum-chemical approach and in the deduced conclusions on the inner working of the protein.

3.5. Other metal-containing proteins

The above paragraphs highlighted the type of metal-containing proteins we are familiar with from our own research. However, the list of paramagnetic ions reported to bind in *vivo* to proteins is much larger (Mn(II), Mo(v), ...) and so are the type of EPR studies performed on them. For a detailed overview of these systems, we refer to a number of recent reviews.15,84,89,139,140

4. Conclusions and challenges for the future

Recent methodological advances in EPR, amongst which are the development of different microwave pulse sequences and the construction of high-performance CW and pulsed EPR and ENDOR spectrometers operating at high mw frequencies, have largely increased the potential of applying these techniques to different problems. However, EPR remains a specialist’s tool and because of this, the technique is still relatively unknown to other scientific communities despite the recent booming of the field. In this overview we have tried to give the non-specialist reader an insight into the advantages and disadvantages of using EPR to study metalloproteins. It should be noted, however, that the application domains of EPR reach much further than the field of metalloproteins, comprising applications in biology, chemistry, material sciences, physics, medicine and pharmacology.

In our opinion, the most important challenges for the future include instrumental aspects, such as improvement of sensitivity and spectral resolution through new spectrometer design at high mw frequencies, the further development and automation of adequate simulation programs, and the improvement of the quantum chemical strategies for computation of EPR parameters (see section 2.3). The ultimate goal is to create an integrated toolbox that includes the spectral derivation of the different spin Hamiltonian parameters up to the interpretation of these magnetic interaction parameters in terms of (geometric and electronic) structure and dynamics of the complex under study.

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