Chapter 4

High Field ESR: Applications to Protein Structure and Dynamics

HF ESR Protein Structure and Dynamics

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Abstract: Electron Spin Resonance at high magnetic fields (HF ESR) is rapidly developing into a powerful biophysical tool which is uniquely positioned to address complex aspects of structure and dynamics of proteins, membranes, and macromolecular assemblies at molecular level. The source of the contemporary resurgence of interest in ESR as a biophysical tool is that there are, broadly speaking, three large groups of problems, which can be approached with ESR methods but cannot easily be studied by traditional structural techniques: (1) structure and dynamics of large molecular weight proteins in solution; (2) membrane and membrane-associated proteins: structure, location with respect to the membrane, side-chain dynamics, and interactions with other membrane components or DNA's and RNA's; (3) fast conformational transitions of proteins and RNA's in solution, protein folding and re-folding. The focus of this review is mainly on HF ESR spin-labeling techniques, primarily via nitroxide spin labels, as this method is the most flexible and is even applicable to proteins which are otherwise ESR-silent. We start with physical aspects of ESR of nitroxide spin labels at high magnetic fields in order to categorize the characteristic information that can be gained from such experiments. Then we describe practical applications of spin-labeling HF ESR to study structure and dynamics of complex biophysical systems. We also discuss several details of the ESR motional theory based on the stochastic Liouville equation (SLE) that are relevant to nitroxide line shape analysis in order to appreciate both the information available from and the limitations of the method. Particular emphasis is given to multifrequency HF ESR methods in studies of spin-labeled membranes and biopolymers. Finally, we review the use of HF ESR in applications to molecular structure including distance measurements, determination of molecular orientations from ESR of ordered samples, and structural studies based on g-factor measurements.

FROM:
Biological Magnetic Resonance
Vol. 22, Very High Frequency
(VHF) ESR/EPR
Edited by: Oleg Grinberg
and Lawrence J. Berliner
Kluwer Academic/Plenum Pub.
2004

Key words: Electron Spin Resonance, Electron Paramagnetic Resonance, High Field ESR, High Field EPR, spin labelling, nitroxide, protein structure, protein dynamics

1. INTRODUCTION

This review will cover applications of high field ESR to studies of proteins. It will focus mainly on spin-labeling techniques, primarily via nitroxide spin labels, as this method is the most flexible and is even applicable to proteins which are otherwise ESR-silent. We will also briefly describe other ESR approaches that may be used to study structure and dynamics, *e.g.*, in connection with photosynthetic systems. In addition, we will also discuss properties of membranes that are relevant for understanding protein structure and function, particularly in the case of transmembrane proteins. Finally, we will provide a brief overview of resources available to a researcher who decides to utilize these powerful and technologically advanced methods.

Spin labeling biomolecules with nitroxides was initially developed in the early to mid sixties to a large extent in the labs of McConnell (Stanford, USA) and Rozantzev (Institute of Chemical Physics, Russia), as well as others. Since then, spin-labeling ESR has matured into a valuable spectroscopic tool to study local structure and dynamics of complex macromolecules. Over the years, progress in spin labeling methodology and applications has been well documented in the literature.[1-5]

Recent innovations in ESR experimental methods and instrumentation resulted in a resurgence of interest in ESR, particularly high field ESR (HF ESR) and pulsed ESR. These advances, as well as the flexibility and applicability of spin labeling; position this method as one of several methodologies, such as single molecule fluorescence, atomic force microscopy, and multidimensional NMR, that can be used to study structure and dynamics in biological systems. The source of the contemporary resurgence of interest in ESR as a biophysical tool is that there are, broadly speaking, and three large groups of problems, which cannot easily be studied by traditional structural methods:

(1) structure and dynamics of large molecular weight proteins in solution;

(2) membrane and membrane-associated proteins: structure, location with respect to the membrane, side-chain dynamics, and interactions with other membrane components or DNA's and RNA's;

(3) fast conformational transitions of proteins and RNA's in solution, protein folding and re-folding.

HF ESR spin labeling can address these, and other complex problems, because it is now possible to routinely carry out new experiments that were not practical in the past. These new experimental capabilities emerged as a

synergetic effect of several disparate developments in biotechnology and ESR instrumentation. A clear drawback of spin labeling ESR of the sixties—a relative scarcity of unique protein residues suitable for labeling—was solved by the development and subsequent rapid exploitation of site-directed mutagenesis and solid-state peptide synthesis. The use of site-directed mutagenesis in spin labeling ESR was first introduced by Hubbell and coworkers.[6,7] Their pioneering efforts led to the development of the site-directed spin labeling (SDSL) method. Briefly, in its applications to proteins, SDSL involves cysteine-substitution mutagenesis at a position chosen by the experimenter. If a protein has some undesirable native cysteines, those are replaced by other suitable amino acids such as serine. Then, a spin label, such as the thiol-specific methanethiosulfonate (MTSSL, 1-oxyl-2,2,5,5-tetramethyl-3-pyrroline -3-methyl), is used to link the nitroxide moiety to the cysteine S-H group through a disulfide bond. That nitroxide group, which is now positioned at the desired location on the protein, or locations for doubly-labeled proteins, reports on local structure and dynamics[9] since its ESR spectrum is sensitive to local protein motion and spin-spin interactions. Rotational motion of the nitroxide is influenced by the side-chain protein dynamics while spin-spin interactions with paramagnetic relaxers (such as hydrophilic paramagnetic metal ion complexes or lipophilic molecular oxygen) provide the solvent accessibility parameter for the particular labeled site.[6-8] At conventional ESR frequencies, the nitroxide motion also reports on the overall tumbling rate of the labeled proteins in solution. At high frequencies, the slow overall tumbling of large biomolecules is frozen out on the ESR time scale.[10] Spin-spin interactions with another nitroxide label or protein metal ion site (native or engineered) can be used to study the distance between fragments of the protein chain. By studying the ESR spectra of spin labels as a function of their unique position(s) along the polypeptide chain (*i.e.*, by performing a so-called "nitroxide scan"), an exclusive set of local data on protein structure and dynamics can be obtained. The method is fully applicable to solutions of proteins obviating the need for the crystallizations that are required by several structural methods, is virtually independent of protein size, and might be applied to study membrane proteins under biologically relevant conditions. In this context it is important to note that structure determinations alone via, *e.g.*, X-ray crystallography, serve only as a guide to understanding the protein function. For the latter, careful consideration of dynamic parameters is crucial.[11] The same ESR spin-labeling methodology is also fully applicable to study the structure and dynamics of nucleic acids in solution.[12-16]

Although the field of SDSL and spin labeling ESR continues to grow through new techniques and applications, the great majority of these experiments is still carried out at X-band (9-10 GHz), and only a few are done at Q-band (35 GHz) and higher frequencies. However, high field/ high frequency HF ESR utilizing microwaves above *ca.* 34 GHz is uniquely positioned to offer quantitative and qualitative information on spectra derived from experimental spin labeling

methods. With an increase in magnetic field the ESR spectrum from a nitroxide spin label changes significantly. At high fields, for a typical nitroxide spin label the Zeeman anisotropy in the spin Hamiltonian starts to dominate over the nitrogen hyperfine interaction at magnetic fields above *ca.* 1.1 T, and at ever higher fields, the resolution of the Zeeman anisotropy continues to increase. Thus, while at the magnetic fields used at conventional X-band ESR (0.3 T, 9 GHz), the nitroxide spectrum is determined by an axial hyperfine term averaged by spin label motion, at magnetic fields above 3.4 T (95 GHz, W-band), the rhombic Zeeman term provides new information on protein structure and dynamics which are inaccessible by traditional means.

The technical problems in developing HF-ESR were first addressed successfully by Professor Yakov S. Lebedev and co-workers from the Institute of Chemical Physics (Moscow, Russia). Using a 140 GHz (5 Tesla) ESR spectrometer they were clearly the first who explored the enhanced sensitivity of HF ESR to molecular motion of spin labels, the effects of local polarity on the nitroxide g-factor, and pioneered many other useful applications.[17-19] However, in some sense, their pioneering studies of spin labels with HF ESR were a bit ahead of the supporting technology, as, *e.g.*, site-directed spin labeling had yet to be developed. Although the Moscow 140 GHz HF ESR spectrometer was clearly state-of-the-art at that time, the sensitivity for lossy liquid aqueous samples was often not sufficient for routine spin labeling experiments with fully hydrated proteins and membranes at ambient temperatures. Nevertheless, Lebedev and co-workers carried out several benchmark spin labeling HF ESR studies and were the true pioneers of this method.

Nowadays, HF ESR is undergoing rapid development powered by the efforts of many research groups to advance the field. In our view, HF ESR in general and its application to proteins and other complex biomolecules and assemblies in particular have tremendous potential and we confidently expect to see ever-increasing activity in the future. The intention of this Chapter is to review (*i*) what are the physical aspects of carrying out spin-labeling ESR experiments at high magnetic fields, (*ii*) what kind of new data on protein structure and dynamics could be obtained from such experiments and how these high field experiments are carried out, (*iii*) to outline areas of research for future exploration, and (*iv*) to detail how these experiments might be carried out and what resources in specialized HF ESR hardware and software are available. Special emphasis will be given to recent developments which are believed to be of general interest to researchers in the field and which also reflect the interests of the authors.

2. PHYSICAL ASPECTS OF ESR OF NITROXIDE SPIN LABELS AT HIGH MAGNETIC FIELDS

A general spin Hamiltonian for a nitroxide spin label is given by:

$$\mathrm{H} = \left(\beta\vec{B} \bullet g \bullet S + \beta_n \vec{B} \bullet g_n \bullet I\right) + hS \bullet A \bullet I + (other \quad terms) \qquad (1)$$

where the two terms in parentheses describe electronic and nuclear Zeeman interactions and the third term represents the hyperfine interaction of the electronic spin with the neighboring nitrogen nucleus (I=1 for ^{14}N or I=½ for ^{15}N-substituted labels). Typically, the nuclear Zeeman term is excluded unless the experiments are carried out at very high frequencies.[20,21]

Thus, in the absence of spin-spin interactions, the ESR spectrum of a nitroxide is mainly determined by an electronic *g*-matrix and a hyperfine tensor *A*. The latter two terms have different symmetry and different magnetic field dependences. In particular, for a typical nitroxide the hyperfine tensor *A* is almost completely axial with $A_z > \tilde{A_y} A_x$, while the *g*-matrix has a clear rhombic character: $g_z < g_y < g_x$. In the low-field limit, the powder pattern spectrum is determined by the anisotropy of the hyperfine term. Therefore the *x*- and *y*-orientations of a nitroxide in the magnetic field are essentially indistinguishable. In the high-field limit, the hyperfine tensor is small compared to the anisotropy of the Zeeman term and all principal axis orientations become resolved. For a typical nitroxide radical, the latter condition is satisfied at ESR frequencies of 94 GHz and higher. This is illustrated in *Figure 1*, which shows simulated rigid-limit ESR spectra from a typical nitroxide at conventional (9.0 GHz) and high (250 GHz) ESR frequencies. From this comparison, the advantages of HF ESR are becoming clear: all components of the *g*-matrix are well resolved and the lines corresponding to nitrogen hyperfine components may be resolved as well.

There are several conclusions that can be easily drawn from our discussion and from *Figure 1*. All of the conclusions are related to the dominance of the g-factor anisotropy over the hyperfine term at ESR frequencies of 95-250 GHz and higher:

1) High field ESR spectra of nitroxides have superior angular resolution compared to conventional X-band ESR. Thus, it is now becoming possible to study orientations of spin labeled fragments of complex molecules with greater accuracy and, in particular, to deduce the static and dynamic behavior along the so-called *x*- and *y*-orientations.

2) The sensitivity to molecular motion is also greatly enhanced. Indeed, a truncated spin Hamiltonian (1) can be broken into an isotropic H_0 and anisotropic parts H_1:

$$\mathrm{H} = \mathrm{H}_0 + \mathrm{H}_1(t) \qquad (2)$$

The anisotropic part is given by:

$$\mathrm{H}_1(t) = \beta\vec{B} \bullet \left[g(t) - g_{iso}\right] \bullet S + hS \bullet \left[A(t) - A_{iso}\right] \bullet I \qquad (3)$$

The isotropic parameters are defined as $g_{iso}=aTr\{g\}$ and $A_{iso}=aTr\{A\}$. With an increase in the magnetic field, the anisotropic part of the Hamiltonian proportionally increases due to the Zeeman term and this leads to an enhanced overall sensitivity of nitroxide spectra to molecular motion at high magnetic fields. Specifically, at high magnetic fields the fast motion condition:

$$\left|h^{-1}\mathrm{H}_1(t)\right|\tau_c \leq 1 \qquad (4)$$

breaks down for shorter correlation times τ_c than at X-band.

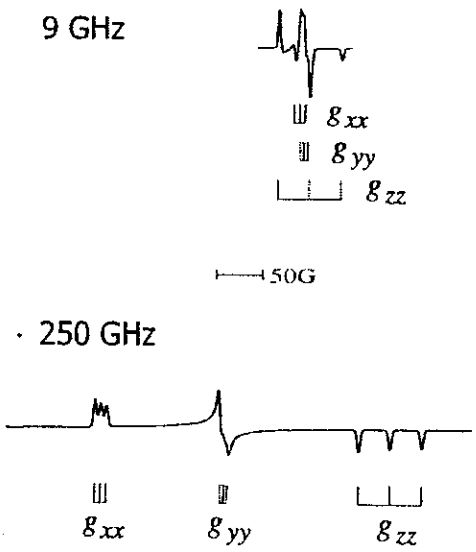

Figure 1. Simulations of the rigid-limit continuous wave ESR spectra of a typical nitroxide (*i.e.*, a spin label in a frozen amorphous matrix) illustrate the increased resolution and orientational sensitivity of HF ESR (250 GHz, bottom) compared with conventional ESR frequency of 9 GHz (top). The triplet hyperfine splittings (marked by sets of three vertical lines) are due to the interaction of the unpaired electron with the ^{14}N nucleus. Reproduced with permission from Ref. 27.

This causes an onset of slow-motional effects at shorter τ_c. Also, at these high magnetic fields, contributions from non-secular spectral densities to the

nitroxide ESR line shapes can be neglected even for very fast rotational motions.[21,23] This simplifies the data analysis for EPR nitroxide spectra in fast motional limit.

Analysis shows that in the fast motional limit the secular spectral density arising solely from rotational modulation of the electronic g-matrix grows quadratically with the resonance frequency ν, resulting in a large (by a factor of a hundred) enhancement at 95 GHz over conventional 9-10 GHz ESR. This trend continues at even higher frequencies. Furthermore, this spectral density contributes equally to all nitrogen hyperfine lines and is the dominant source of homogeneous broadening at 95 GHz and above.[22-23]

These rather substantial changes that the nitroxide ESR spectra undergo with an increase in the frequency of the experiment from 9 to 95 GHz and higher are especially useful for analysis of the complex dynamics of spin labeled biomolecules. One example, which we will consider later in this Chapter in more detail, involves the analysis of dynamics of spin-labeled protein chains. Indeed, these chains often undergo relatively fast reorientational motion with τ_c of the order of a few ns, while τ_c's for the global protein dynamics are typically longer. Thus, by increasing the magnetic field of the ESR experiment, one can increase the magnitude of the spectral anisotropy which is subjected to dynamic averaging and thus to obtain a faster "snapshot" of molecular dynamics than by conventional X-band ESR.[27] This feature of the multifrequency HF ESR approach is illustrated in *Figure 2* which shows the transformations of a nitroxide ESR spectrum upon increase in the magnetic field.

Another important feature is that for a typical nitroxide the rhombic component of the g-matrix $\Delta g=\frac{1}{2}(g_x-g_y)\approx 0.0016$ is comparable to the axial component $\delta g=\frac{1}{2}(g_x+g_y)-g_z\approx 0.0055$. Thus, at resonance frequencies of W-band and above, where superior orientational resolution is available, the full rotational diffusion tensor can be determined.[20,22-26]

The HF ESR studies of dynamics of spin-labeled molecules are especially informative when combined with analysis of ESR data obtained at conventional resonant frequencies. The combined data provides useful constraints on the overall tumbling rates involved. Overall, the above considerations outline one of the main features of spin labeling ESR at high magnetic fields is an enhanced sensitivity (and resolution) of HF ESR to molecular motion.

The dominance of the Zeeman term and its anisotropy in HF ESR spectra of nitroxides improves the spectral resolution as well. Indeed, the ability to resolve two spectra from a mixture of species with different g-factors ($\Delta g=g_1-g_2$) is defined by the difference in the field position of the spectra ΔB:

$$\Delta B \approx \frac{h\nu}{g_e^2\beta}\Delta g \qquad (5)$$

and the line width $\Delta B_{p\text{-}p}(\nu)$ at a given resonance frequency ν. Most spin-labels have similar g-factors ($\Delta g_{iso}<0.001$). These results in strongly overlapping X-

band EPR spectra from mixtures of different nitroxides or spin probes partitioned between the membrane aqueous and lipid phases.[28]

$R=10^8\ s^{-1}$

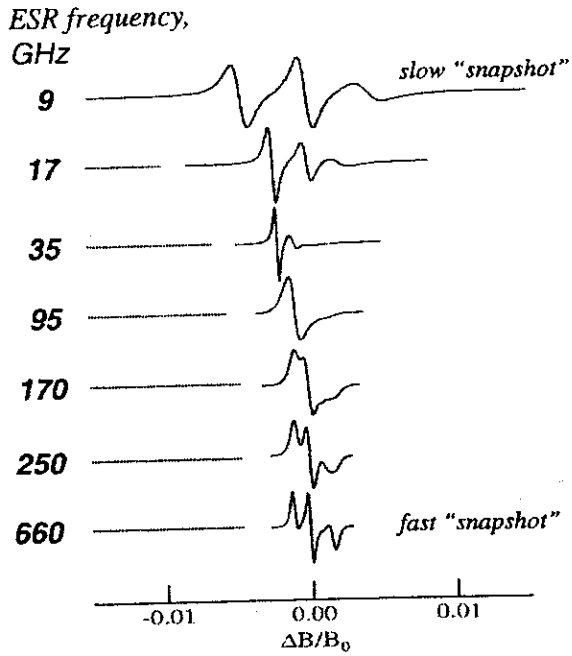

Figure 2. Simulations of ESR spectra of a typical nitroxide tumbling isotropically with the rotation diffusion rate $R=10^8\ s^{-1}$ as a function of the resonant frequency of the ESR experiment. The large variations that the spectrum undergoes as the frequency is changed are very sensitive to the character of the motion. While for the given rate of rotational diffusion the ESR spectrum at conventional 9.0 GHz (top) is in motionally narrowed regime, the spectra at high frequencies (bottom) display very slow motion, almost at the rigid limit. Reproduced with permission from Ref. 27.

A substantial spectral overlap might also occur for spin adducts formed by the same spin trap and different radical precursors. If the line width increase with the resonance field/frequency is less than the frequency-dependent line separation, then improved spectral resolution may be observed. For nitroxides, it is often the case that the spectral width is dominated by unresolved field-independent spin-spin interactions, including those with neighboring protons. Thus, in the limits of both fast and slow motion, information exploiting the improved spectral resolution and information from multiple species can be gained. We will illustrate that this feature of spin labeling HF ESR is especially

useful for the analysis of multi-component systems such as mixtures of different spin adducts[24] as well as for deducing local polarity and electric field effects from accurate measurements of *g*-factors.[29]

Besides the three major features of spin labeling experiments arising from the dominance of the *g*-anisotropy over that of *A* in the ESR spectra of nitroxides at high magnetic fields listed above, there are at least two other factors that could be exploited with great benefits in studies of nitroxides as well as other systems.

The first is the greatly improved absolute point sensitivity of HF ESR *vs.* conventional X-band. With the increase in the resonance field, both the population difference between the Zeeman levels and the magnitude of the microwave quantum increase while the dimensions of the resonators and the sample size scale down. In general, the frequency dependence of ESR spectrometer sensitivity is rather difficult to predict because of contributions from many factors, including but not limited to the performance of available millimeter-wave components and different design schemes (*e.g.*, wave-guide *vs.* quasioptical components, homodyne *vs.* heterodyne, *etc.*). Other practical factors to consider are the frequency dependence of line widths, relaxation times, and the maximum available amplitude of magnetic field modulation available for continuous wave spectrometers. Nevertheless, several groups in the field demonstrated absolute point sensitivity for non-lossy samples down to 10^7 spins/G when converted to standard conditions.[21,30-32] This is an improvement of about three orders of magnitude *vs.* the sensitivity of conventional X-band ESR (about 10^{10} spins/G). However, for HF ESR experiments the sample volume should be greatly decreased compared to conventional X-band. For example, the largest tubes which can be used for non-lossy samples in the University of Illinois W-band EPR spectrometer have i.d.=0.7 mm.[32] For a single-mode cylindrical W-band resonator the effective length of the sensitive region of the cavity is only about 2 mm, and thus the maximum sample volume in the resonator is only *ca.* 1.5 μl.

Another somewhat unexpected benefit of applying high magnetic fields for ESR experiments might be in the ability to utilize magnetic field alignment methods to improve the spectral resolution for membrane and membrane protein samples. Magnetic field alignment is a well-established technique in NMR of proteins.[36-38] The method is based on a spontaneous alignment of discoids formed from a mixture of long- and short-chain phospholipids at magnetic fields above 2.5 T and temperatures of 37-40 °C.[39] Typically, these discoids - also called bicelles - are made from *ca.* 3:1 mixture of a long-chain 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and a short-chain 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC). The magnetic alignment of bicelles is the consequence of anisotropy of the magnetic susceptibility tensor $\Delta\chi$. The latter can be manipulated by the addition of paramagnetic lanthanide ions. It has been discovered that certain lanthanides shift $\Delta\chi$ to a positive value causing the bicelles to flip 90° such that the membrane normal (*i.e.*, director vector) becomes parallel to the external magnetic field.[38,40] Although magnetic field alignment of

bicelles has been also demonstrated in X-band experiments,[41,42] the use of ESR at magnetic fields >3.3 T is clearly advantageous for at least two reasons: (i) the concentration of lanthanides, such as Yb^{3+}, can be dropped to 1 molar % (*vs.* 10 molar % at X-band[41,42]) and (ii) the bicelle alignment is spontaneous even at magnetic fields corresponding to W-band while field cycling is known to be a necessity for X-band studies.[43] Alignment effects are not always beneficial, however. In studies of vesicle dispersions carried out at 250 GHz, magnetic susceptibility-induced pinching effects may cause line shape distortions and complicate data analysis.[44] Nevertheless, the authors believe that this alignment technique should be further explored in HF ESR. It might be particularly useful for spin-labeling studies of transmembrane proteins.

The optimum sample size plays an important role in the design of HF ESR experiments and is determined by non-resonant dielectric losses. In some cases, adding glycerol and other non-polar solvents to the sample helps reduce these losses.[33] However, an exceptional concentration sensitivity has been demonstrated even for liquid samples which are almost purely aqueous. For example, the results of the recent tests of the Illinois W-band ESR spectrometer demonstrated concentration sensitivity for aqueous solution of perdeuterated Tempone (2,2',6,6'-tetramethyl-4-piperidone-1-nitroxide) at room temperature as low as 7 nM/G without extrapolation to maximum available power.[34] The optimum inside diameter of the capillary for an aqueous sample was found to be just 0.15 mm which corresponds to *ca.* 70 nl volume inside the cavity. This sensitivity is sufficient to carry out spin labeling ESR experiments with samples even at 20-100 μM concentrations and $<10^{-12}$ Mole sample quantity. The latter becomes a rather useful feature of the HF ESR method when only small amounts of spin-labeled proteins and/or nucleic acids are available. Because the size of the cavity-type resonators and optimum sample capillaries scales down with the wavelength of the mm-waves, the resonators of an open-type design, such as Fabry-Perot, are more common and convenient. The optimal volume for aqueous samples in such resonators is typically 0.1–1.0 μL and concentration sensitivity can be exceptionally high, reaching the μM range as demonstrated by the Cornell group.[21] Further discussion of sensitivity for a variety of sample conditions and resonant structures may be found in the contribution of Budil and Earle to this volume.[35]

3. APPLICATIONS TO LOCAL MOLECULAR DYNAMICS OF SPIN-LABELED PROTEINS AND MEMBRANES

Although spin labeling molecular dynamics studies carried out with HF ESR still represent a rather small fraction of recent spin-labeling literature, the advantages of the method have been demonstrated for a variety of systems

ranging from small nitroxides in simple fluids to complex multimode reorientations of spin-labeled protein side chains. Some of these studies and other recent technological developments in ESR were recently reviewed by Freed[45] and by Freed and coworkers.[27] A review of recent progress in spin labeling ESR in applications from materials science to molecular biophysics has been published.[46] Another review outlining the prospects of HF ESR of spin labels in membranes has also appeared.[33] In addition, a comparative catalogue of X- and W-band ESR spectra illustrating an enhanced sensitivity of HF ESR to molecular dynamics has been published.[23]

3.1 Spin-Labeling Studies of Complex Systems: Fast Motion Limit

Although at high magnetic fields the onset of slow-motional effects is seen at shorter rotational correlation times than at X-band, some spin-labeled systems still fall in such a regime. Clearly, the fast motion condition is satisfied for small nitroxides and spin adducts in simple fluids as was discussed in the section above. However, it was found that HF ESR spectra from spin-labeled peptides, DNA monomers, and flexible sections of proteins when studied at room temperature and above might also fall into fast motion regime. This is potentially advantageous for separating overlapping species since in the fast motion regime the ESR line widths are narrow. These narrow signals are also easier to detect than the broader slow-motion CW ESR spectra at the same spin label concentrations

One recent example is a HF ESR study of nitroxide side-chain dynamics in a helix-forming peptide which was labeled with MTSSL.[47] Experimental spectra recorded at 140 GHz over the temperature interval from 277 to 306 K was found to be amenable to analysis with simple fast-motion theory. The analysis revealed that the nitroxide motion is anisotropic with $\tau_{\perp}/\tau_{\parallel}$ approaching 2.6 at 306 K.

A second example is a 220 GHz ESR study of an oligonucleotide with a newly synthesized cytosine spin-label.[48] The spectrum from a monomer in an aqueous solution exhibited a three-line pattern characteristic of a nitroxide in the fast motion regime. The anisotropic diffusion tensor estimated from this fast motion spectrum was consistent with the expected rapid motion of the probe molecule around its tether.

The W-band ESR spectra of a recombinant human growth hormone, labeled at NH_2-terminus with a succinimidyl 2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-caboxylate,[23] show a three-line pattern of broad lines similar to that observed by Millhauser and coworkers[47] and is illustrative of a mobile protein fragment.

Although fast-motion HF ESR spectra of spin-labeled proteins and peptides are relatively uncommon because they are indicative of rather flexible and/or disordered parts, they have the advantage of being well resolved from otherwise

broad HF ESR spectra characteristic of slow or intermediate motion. This could be useful for resolving the fractions of proteins which are disordered or partially unfolded. *Figure 3* shows comparative W-band and X-band ESR spectra from iso-1-cythochrome c from *Saccharomyces Cerevisiae* labeled at the native cysteine 102 with MTSSL. Recently, the local folding/unfolding kinetics of this spin-labeled protein has been studied with X-band stop-flow ESR based on measurements of the peak height.[49,50] From comparisons of the double-integral normalized ESR spectra at X- and W-band (*Figure 3*), it is clear that even for this rather mobile site, HF ESR is more sensitive to the changes in local dynamics upon protein unfolding.

On the other hand, if the focus of the study is the complex modes of the molecular dynamics of complex systems, then the fast-motion ESR spectra, generally speaking, are of limited value. Typically, only the effective diffusion tensor can be determined from such spectra. In order to learn more about the details of local dynamics of spin-labeled molecules and the relaxation mechanisms involved, one should analyze slow motion spectra, which, when properly analyzed, provide a wealth of information about molecular dynamics, and allow one to critically test detailed models of motion in protein and membranes. The most important aspect of spin-labeling HF ESR is that because of the enhanced contribution of the Zeeman term anisotropy to the spectra at high magnetic fields, many nitroxide-labeled systems fall into slow-motion and/or intermediate motion regime. This calls for more elaborate models and computationally efficient methods of spectral analysis.

3.2 Spin-Labeling Studies of Complex Systems: Intermediate and Slow Motion

HF ESR spectra in the slow motion limit are especially informative because of the superior angular resolution resulting from the dominance of the g-matrix anisotropy. Another feature of the slow motion HF ESR spectra is that some faster modes of rotation that would appear as "pre-averaged" in conventional X-band ESR spectra fall into an intermediate motional regime at high magnetic fields. Thus, more realistic and complicated models that include rearrangements of molecules in a local solvent cage are required to adequately describe the multifrequency and HF ESR spectra. This is the basis of the so-called slowly relaxing structure or cage model, developed by Freed and co-workers.[51,10] We review this model and some of the applications below.

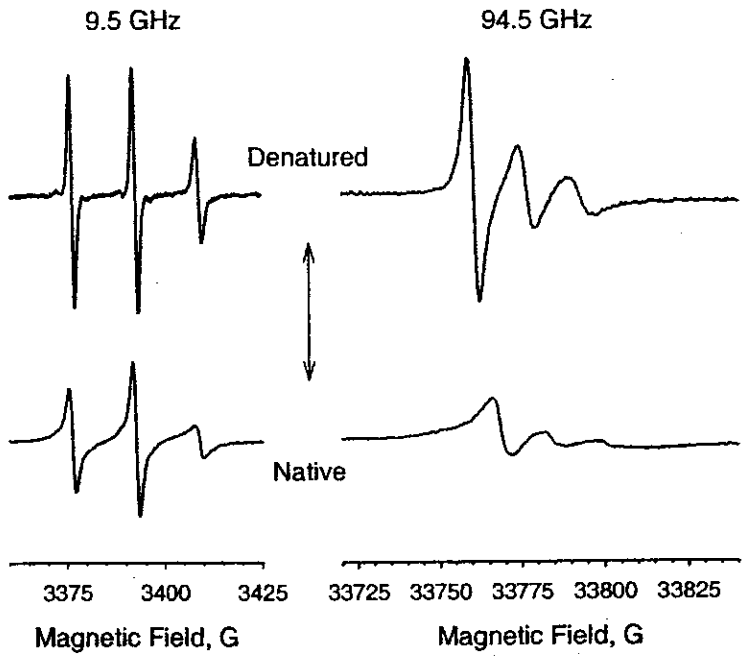

Figure 3. Comparison of 9.5 and 94.4 GHz EPR spectra of iso-1-cythochrome *c* from *Saccharomyces Cerevisiae* labeled at native cysteine 102 with MTSSL illustrates an enhanced sensitivity of W-band ESR line shape to changes in local protein dynamics upon protein denaturing. The spectra at each of the frequencies were taken at room temperature and are normalized by the value of the double integral. The protein was denatured by addition of 2 M of guanidine hydrocholoride (Pierce, Illinois). Protein concentration was 0.2 mM in a HEPES buffer at pH 6.5 (Smirnov *et al.*, in preparation).

3.2.1 Modeling of slow motional ESR spectra of nitroxides: stochastic Liouville equation and MOMD model

Over the years several approaches to quantify slow-motional ESR spectra of nitroxides have been developed. Here we wish to concentrate on the slow motion theory that is based on solving the stochastic Liouville equation (SLE). This approach has been the focus of the group of Prof. Freed (Cornell) and has proven to be an important means of analyzing the sometimes rather complex spectra that can be observed both in biological and model systems.

It is not the purpose of this review to provide a detailed introduction to the SLE theory and its use in magnetic resonance. There are a number of excellent

texts, which deal with these topics,[52,53] and those works should be consulted for the relevant theoretical background. Nevertheless, we believe it is useful to discuss here several details of the SLE-based motional theory that are relevant to nitroxide line shape analysis in order to appreciate both the information available from and the limitations of the method.

The SLE is an equation of motion for the density matrix describing the coupled spin and spatial degrees of freedom. The density matrix description is an inherently statistical one and as such is well suited to describe the response of the macroscopic sample probed in an ESR experiment. The equation of motion for the density matrix is governed by the Liouville-von Neumann equation:

$$\frac{d}{dt}\rho = \frac{1}{i\hbar}[H_0 + H_1(t), \rho] \tag{6}$$

Here H_0 defines the energy levels in the system and includes such terms as the electron and nuclear Zeeman interactions, hyperfine interactions, and zero field splittings if present. The quantity $H_1(t)$ is considered to be time-dependent due to, for example, rotational and/or translational diffusion. This term may be said to modulate the energy levels and cause the system to relax to equilibrium via life-time broadening effects. The term $H_1(t)$ is also orientation dependent and may be described by a traceless second rank tensor, which is small compared to H_0. If the correlation time τ_c describing the relaxation-inducing random fluctuations of the system is sufficiently short, then the effects of $H_1(t)$ may be treated using perturbation theory, as is common, for example, in motional narrowing theory. When the τ_c is not so short, one must use more general methods of analysis to incorporate the effects of $H_1(t)$ into a description of the dynamics. By performing an ensemble average over the density matrix, it is possible to show that the resulting equation of motion for the density matrix is:

$$\frac{d}{dt}\langle\rho\rangle = \frac{1}{i\hbar}[H, \langle\rho\rangle] + \Gamma\langle\rho\rangle \tag{7}$$

where Γ is a stochastic operator which describes the random processes giving rise to relaxation in the system. As such, it models in a consistent way the details of the ensemble averaging. The operator H is the full quantum mechanical Hamiltonian of the system. While the previous equation (6), when solved using the perturbation theory, is valid only in the motional-narrowing regime, the ensemble averaged SLE (7) is valid in a broader interval of τ_c that spreads from the motional narrowing regime to the rigid limit. Once the model for stochastic averaging is chosen, one may solve the eq. (7) and determine the ESR line shape.

There are several useful models for the operator Γ. The simplest possible stochastic process responsible for spin label relaxation would be the isotropic rotational diffusion occurring through small, uncorrelated, angular steps (simple Brownian motion). This model is appropriate for small spherical spin labels in

simple fluids, such as toluene. Then the stochastic operator has the form $\Gamma=RL^2$ where L^2 is the square of the quantum mechanical angular momentum operator, and R is the isotropic rotational diffusion constant. The physical interpretation of the chosen form for the stochastic operator Γ is rather straightforward: because L is the generator of infinitesimal rotations, this forms of Γ models the small angular steps appropriate to Brownian rotational diffusion. The Γ operator has the same analytical form as the Hamiltonian for a symmetrical quantum mechanical rotor if one makes the identification $R=(2I)^{-1}$, where I is the moment of inertia of the rotating object. For non-spherical spin-labeled objects with a symmetry axis, but still diffusing in simple fluids, the operator Γ takes the following form:

$$\Gamma = R_{\perp}L^2 + \left(R_{\parallel} - R_{\perp}\right)L_z^2 \qquad (8)$$

where $R_{\parallel}$ is the rotational diffusion rate around the symmetry axis, and $R_{\perp}$ is the rate about the axes which are orthogonal to the symmetry axis. This choice for the operator Γ is appropriate for elongated spin labels such as, *e.g.*, cholestane (3-doxylcholestane or CSL), diffusing in simple fluids. For such molecules the two diffusion rates $R_{\parallel}$ and $R_{\perp}$ are related to the corresponding moments of inertia $I_{\parallel}$ and $I_{\perp}$ as $R_i=(2I_i)^{-1}$ and the operator Γ is the Hamiltonian operator for the axially symmetric quantum-mechanical rotor. Analogously, the operator Γ for the completely asymmetric rotor should be used to model rotational motion for the general case of an asymmetric spin label.[20] The eigenfunctions for such an operator are specified by three quantum numbers: L, K, and M.

One of the most commonly used methods for solving the SLE relies on the fact that the eigenfunctions of Γ are given by the eigenfunctions of the Hamiltonian of the rigid rotor. When the tumbling of the label falls into motional narrowing regime, *i.e.* when $|h^{-1}H_1(t)|\tau_c \leq 1$, the relaxation rates are essentially insensitive to the details of the averaging and, thus, it is only necessary to consider a very small number of L, K, and M modes in order to model the spectra.[52,53]

When the tumbling rates are slow, *i.e.* when $|h^{-1}H_1(t)|\tau_c > 1$, then the time dependent part of the spin Hamiltonian will couple rotational modes with different L, K, and M values making the ESR spectrum sensitive to the details of the stochastic mechanism responsible for partial averaging of spectral anisotropies. The increase in the spectral anisotropy achieved at high magnetic fields spreads the ESR spectrum of nitroxides over a larger spectral window and makes the spectral shapes exquisitely sensitive to the details of the rotational dynamics, and provides the means for testing more sophisticated models of probe dynamics. These advanced models do require more parameters, and therefore larger basis sets spanned by the L, K, and M quantum numbers, to specify the problem satisfactorily and to fit the spectrum.

Turning now from the stochastic operator Γ to the spin Hamiltonian, we wish to note that the relevant Hamiltonian for the system may be expressed in terms of irreducible spherical tensors (IST's). Each magnetic interaction contributing

to the Hamiltonian has a simple expression but only in a particular coordinate system. In order to proceed with solving the SLE, it is then necessary to refer the IST's to a common reference frame. This is one of the most tedious tasks in calculating the slow-motional spectra. One has to consider that the elements of the magnetic tensors are time independent but only in a body-fixed frame of the nitroxide moiety. In contrast, the polarization magnetic field is static in the laboratory frame. For definition of the coordinate systems involved, construction of the matrix elements of the Liouville superoperator, and computationally efficient ways to solve the SLE the readers referred to the literature.[52,53] We will discuss some of the coordinate frames and the transformations involved in the next section of this Chapter.

While solutions of the SLE in the slow motion regime for a probe in simple fluids have proven to be very sensitive to the microscopic details of molecular dynamics, making quantitative assessments of the elements of the rotational diffusion tensor feasible,[20] one has to extend the definition of the Γ operator in order to describe more complicated systems of biophysical relevance. In particular, it is important to consider (and to study!) the effects of a local orienting potential such as one might find in liquid crystals or lipid bilayers. One can modify the Γ operator to account for the existence of environmental constraints on spin label motion by noting that in such a system the spin label experiences reorienting torques, which may be described by specifying a potential well that favors certain orientations of the spin probe. The reorienting torques may, in turn, be quantified by specifying angular momentum operators with coefficients determined by the particular form of the local orienting potential.[52,53] An explicit form for the Γ operator accounting for these effects may be written as follows:

$$\hat{\Gamma} = \hat{\Gamma}_{iso} + \hat{\Gamma}_{poten} = J \cdot R \cdot J + J \cdot \left\{ \left[R \cdot J \left[\frac{U(\Omega)}{k_B T} \right] \right] \right\} \qquad (9)$$

where J is the generator of infinitesimal rotations (angular momentum) in the diffusion frame, R is the diffusion tensor of the spin label, k_B is Boltzmann's constant, T is the temperature, and $U(\Omega)$ is the potential incorporating specific environmental constraints.[53] A general form for the potential may be written as:

$$U(\Omega) = -k_B T \sum_{L,K} c_K^L D_{0K}^L(\Omega) \qquad (10)$$

where Ω is the set of (time-dependent) Euler angles which specify the orientation of the diffusing spin label with respect to the local director (symmetry axis of the potential responsible for ordering), $D_{0K}^L(\Omega)$ is a Wigner rotation matrix element (quantum mechanical rigid rotor eigenfunction), which, for the specified choice of indices, is simply related to the associated Laguerre polynomials. In practice, one typically specifies even L and K values less than or equal to 4 such that $K \leq L$. The c_K^L coefficients specify the relative

magnitudes of the terms contributing to the orienting potential.[54] We will mention in passing that specifying a potential with $L=4$ was found to be useful for describing restricted rotational diffusion within a cone. While addition of the Γ_{poten} to the Γ operator allows one to model more complicated rotational dynamics of the probe, the presence of the Γ_{poten} term couples more diffusion modes via the SLE and this leads to an increases of the required basis set size.

It is worthwhile to mention here that the form of the operator Γ given by eq. (10) is particularly useful for studying spin-labels diffusing in lipid bilayers, which are good models of cellular membranes. In such systems, translation and reorientation of the lipids is constrained by the surrounding lipids and other molecules, such as, for example, membrane proteins and peptides, cholesterol, and, if present, polysaccharides. Detailed line-shape analysis of ESR spectra of spin labels in such an environment allows one to determine the elements of the rotational diffusion tensor, which is an indication of the local membrane fluidity. In addition, details of the ordering of spin-labeled molecules in the membrane and the resulting order parameter can be determined.

When the global tumbling modes are frozen out on the ESR time scale, which is often the case for HF ESR spectra of spin-labeled proteins, one can model the dynamics by assuming that the system has microscopic order and macroscopic disorder—the so-called MOMD model. In this model, environ-mental constraints define a local orienting potential well in which the spin label diffuses and also the local director direction. This is the microscopic order part. Then, one has to account for various orientations of the local directors in the laboratory coordinate frame. Typically, this distribution of orientations is more or less isotropic and this is the macroscopic disorder alluded to in the MOMD acronym. To calculate spin label ESR spectra under this model one has to compute a set of spectra for a discrete set of director orientations and then sum the results with a weight function corresponding to the chosen director distribution. The practical details of carrying out MOMD calculations are discussed elsewhere.[54]

One of the important biophysical objects that are well descried by the MOMD model is a membrane vesicle. While in a particular vesicle region the spin labels may undergo a quite restricted motion, generally, the local directors are randomly oriented with respect to the laboratory frame. The local motion of spin labels leads to orientation-specific homogeneous broadening (hb) of the local spectra that in turn produces inhomogeneous broadening (ib) due to a weighted summation over all the director orientations, just as in a powder-spectrum average. The degree of ib depends on how well the system is ordered locally. Each MOMD orientation may be subjected to a different, orientation dependent ib. If there is a substantial ib, it can mask the hb and reduce the spectral resolution and the sensitivity of the spectral shapes dynamical and ordering parameters. However, modeling of the ESR spectra using the MOMD methods reviewed here can compensate for such reduction in resolution, and provide important insights into the relevant dynamics and structural characteristics of the system. We will illustrate these points below.

For dynamics that occur on a particular time scale, *e.g.*, slow global tumbling of a macromolecule, or rapid internal motions of protein side chains, one can "tune" the spectral response of the ESR line shapes by choosing the appropriate magnetic field/resonant frequency for the experiment. For example, X-band ESR spectra of spin labeled proteins are generally sensitive to the overall protein tumbling modes, whereas these modes are often "frozen out" in HF ESR spectra. On the other hand, the remaining diffusion modes corresponding to rapid internal motions may be studied in great detail *via* a thorough analysis of the HF ESR line shape. To summarize, the fast tumbling modes are much easier to analyze from HF ESR spectra while the slower modes have the greatest effect on the line shape at lower frequencies. This is a manifestation of the so-called "snapshot" effect.[27]

3.2.2 Slow relaxing local structure (SRLS) model

In more complicated systems, where the motion of the nitroxide spin label is constrained by a local orienting potential, but the potential-defining environment also changes on the ESR time scale, as one might find in a slowly tumbling spin-labeled protein, one must extend the Γ operator. Taking a spin labeled protein as an example, the motions that influence the appearance of the observed spectra are the constrained diffusional modes of the tether which links the nitroxide to the protein and the overall tumbling modes of the protein. In such a system, the constrained environment of the tethered spin probe defines the local orienting potential. This constrained environment slowly reorients with respect to the laboratory frame as the protein tumbles in solution, providing the coupling to the over-all, or global diffusion.

The slowly relaxing local structure (SRLS) model recognizes that a useful approach to describe the composite dynamics is to account only for a few modes of diffusion. The main motivation behind this simplification is the limited resolution of the observed ESR line shapes. In the SRLS model, the spin probe is assumed to be reorienting in a local environment which itself is relaxing on a longer time scale. In applications to macromolecular systems, the faster motion describes the internal dynamics, while the slower motions account for the global rotation of the macromolecule. It is important to note that the SRLS model contains the MOMD model as a limiting case. Another important limiting case is provided by the fast internal motion (FIM) model. In the FIM model, the internal dynamics lead to a partial averaging of the magnetic tensors, which is quantified by an effective order parameter. The global motion is then described by the rotational diffusion tensor.[10]

In order to understand the parameters utilized in the SRLS model, it is necessary to consider coordinate transformations among several reference frames as we discussed in the previous section if this Chapter. A chain of coordinate transformations has to be carried out that refers all of these IST's and ISTO's (irreducible spherical tensor operator) to a common reference frame in

order to compute numerical solutions of the SLE that describes the dynamics.[55,51] Understanding these coordinate transformations and the roles that the various ISTs and ISTO's play in describing the dynamics is crucial for constructing a reasonable model for the system under study as well as for understanding the output of the fitting programs. *Figure 4* illustrates how the various coordinate transformations are related.

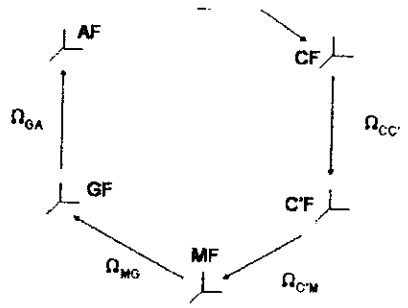

Figure 4. Reference frames which define the structural and dynamic properties of the combined system of spin-labeled moiety and macromolecule. LF=Lab Frame, CF=Cage Frame, C'F=Internal Director Frame, MF=Internal Diffusion Frame, GF=g-matrix frame, AF=A-matrix frame.

The laboratory frame (LF) is taken with the z-axis parallel to the polarizing magnetic field B_0. In the cage frame (CF), which describes the overall tumbling of the macromolecule, the z-axis is typically chosen to lie along one of the principal axes of, *e.g.*, the protein under study. The Euler angles, which set the coordinate transformation from LF to CF, are called collectively Ω_{LC}. These angles are modulated by the global tumbling of the macromolecule. Thus, the set of Euler angles Ω_{LC} parameterizes the instantaneous orientation of the macromolecule with respect to the LF and is time-dependent.

To model constraints on the internal motions of the system, it is useful to define an internal ordering frame C'F, which is fixed with respect to the macromolecule. In accordance with conventional usage in parallel constructions, this frame is also known as the tilt cage frame. The set of Euler angles, which specify the transformation from CF to C'F are $\Omega_{LC} = (0, \beta_{CC'}, 0)$, are considered to be time-independent. The internal modes of diffusion are modeled in the MF frame, which spans the symmetry axes of internal modes of diffusion. The Euler angles relating MF and LF, Ω_{LM}, are modulated by both the internal dynamics and the overall tumbling.

The set of Euler angles relating C'F and MF is $\Omega_{C'M}$. In order to simplify the problem somewhat, note that MF defines both the principal axes for diffusion and for orientational restriction of the internal modes of motion. Note further that MF may be tilted from the g-matrix symmetry axes, defined by the frame GF, by a static set of Euler angles $\Omega_{MG} = (\alpha_{MG}, \beta_{MG}, \gamma_{MG})$. Finally, one may also allow for a tilt between the hyperfine matrix frame AF and GF, the g-matrix frame, via Ω_{AG}, although this effect is not considered further in the problems we have analyzed to date.

The time-dependent Hamiltonian that enters into the solution of the SLE relevant for this two-body system, where the overall, or global, diffusion constitutes one body, and the internal diffusion modes constitute the second body, is given by:

$$\hat{H} = \sum_{\mu=g,A} \sum_{l=0,2} \sum_{m=-l}^{l} \sum_{m'=-l}^{l} \sum_{m''=-l}^{l} \hat{A}^{(l,m)}_{\mu,L} D^{l}_{mm'}(\Omega_{LM}) D^{l}_{m'm''}(\Omega_{MG}) F^{(l,m'')*}_{\mu,G} \quad (11)$$

where $X^{(l,m)}_{\mu,N}$ stands for the m^{th} component of the l^{th} rank IST or ISTO X defined in the N frame, with μ specifying the kind of magnetic interaction - g for Zeeman or A for hyperfine. The $D^{l}_{nn'}(\Omega_{NN'})$'s are the Wigner rotation matrix elements which affect the coordinate transformation from the N to the N' frames. The set of Euler angles Ω_{LM} relates the laboratory frame to the internal diffusion frame as outlined above, and, thus, depends on the overall tumbling diffusion modes described by Ω_{LC} and the internal modes described by $\Omega_{C'M}$. The detailed form of $\hat{A}^{(l,m)}_{\mu,N}$, which is an ISTO dependent on spin operators, and $F^{(l,m)}_{\mu,N}$, which is an IST component containing contributions from spin label magnetic parameters (g-matrix and A-tensor), may be found elsewhere.[52]

The diffusion operator describing the global and internal tumbling modes may be written as:

$$\hat{\Gamma} = \hat{\Gamma}^{global}(\Omega_{LC}) + \hat{\Gamma}^{\text{int}}(\Omega_{LM}) + \hat{\Gamma}^{global}(-\Omega_{C'M}) + \hat{\Gamma}^{\text{int}}(-\Omega_{C'M}) \quad (12)$$

where the first two terms describe the isotropic contributions to the global and internal diffusion modes and the last two terms refer to the internal orienting potential which couples the internal and global diffusion modes. This construction of the Γ operator is analogues to the one we wrote down in the presence of an ordering potential and is discussed above.

The principal difference between the local ordering case discussed above and the SRLS model under consideration here, is that one must specify additional parameters to describe the global diffusion modes. In the original implementation of the SRLS model, only interaction potentials corresponding to a cylindrically symmetric cage mean field were considered:

$$V^{\text{int}}(\Omega) = -\sum_{l=2,4} c_0^2 D^{l}_{0,0} + c_2^{l}\left[D^{l}_{02}(\Omega) + D^{l}_{0-2}(\Omega)\right] \quad (13)$$

where $D^{l}_{ml}(\Omega)$ are Wigner rotation matrix elements. That restricted SRLS model requires only one additional dynamical parameter $R^{C}_{\perp}$, which describes the cage diffusion, and two cage quantum numbers L^{C} and M^{C} on account of the cylindrical symmetry of the mean field cage potential.[51] Even restricting attention to the l=2 case in the original SRLS model allows one to derive axial and rhombic cage order parameters S^{C}_{0} and S^{C}_{2} from the specific form of the interaction potential. Since that time, the SRLS model has been generalized to more complex cases.[10] Upon relaxing the restriction to cylindrically symmetric cage mean field potentials, an additional dynamical parameter $R^{C}_{//}$ and an additional cage quantum number K^{C} are required to completely specify the extended SRLS model. Explicit forms for $\hat{\Gamma}^{global}$ and $\hat{\Gamma}^{int}$ as well as the potential terms may be found elsewhere.[10] The original SRLS formulation,[51] although not as general as the models available now, does have the advantage of (relative) simplicity as well as a lower computational overhead due to the smaller set of required basis set quantum numbers and thus smaller basis set size.

3.2.3 Applications of the SRLS model in multifrequency and HF ESR

In this section, we will review several examples of increasing complexity that will illustrate the utility and the flexibility of the SRLS model for analyzing HF ESR and multifrequency ESR spectra. The first system we would like to consider is *o*-terphenyl (OTP), an isotropic, fragile glass-former. One might assume that for such a simple solvent the stochastic operator Γ could be chosen to have a rather simple form, perhaps, accounting for anisotropic diffusion but not including local ordering. However, Freed and co-workers have found that the rotational diffusion of spin labels dissolved in OTP can be strongly influenced by the dynamic solvent cage formed. Although the lifetime of the dynamic cage formed in OTP above the glass temperature T_G is expected to be short on physical grounds, the local ordering potential can be quantified by the SRLS model.[56] For the cholestane spin label, Freed and co-workers found that a transient solvent cage comprising approximately ten OTP molecules was adequate to describe the dynamics of the system from the melting temperature T_M to the so-called critical temperature T_C. Interestingly, it was observed that above T_M there were no significant cage effects. This served as a strong indication that in this range the OTP is a true isotropic solvent. The model parameters derived from non-linear least-squares fitting of the spectra were also consistent with rapid rotational diffusion of the cholestane around its long axis, as it should be for a molecule of such geometry. A fit to a simple Brownian

diffusion model failed to reproduce all of the spectral features observed in the experiment, *Figure 5*.

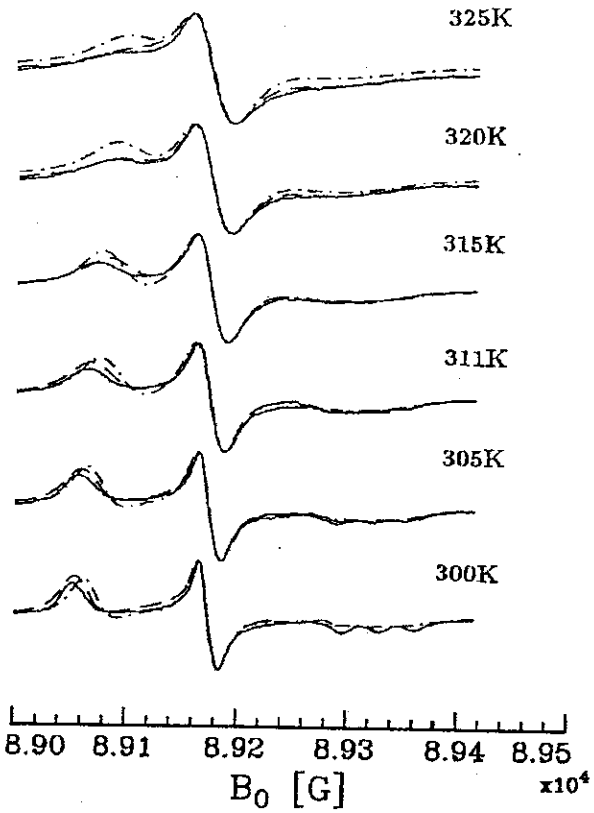

Figure 5. 250 GHz ESR spectra of CSL solution in *o*-terphenyl (OTP) at several temperatures. The solid line is the experimental spectrum. The dash-dot line is a non-linear least-squares fit of a Brownian diffusion model. The dashed line is a non-linear least-squares fit of the SRLS model. Reproduced with permission from Ref. 45

The SRLS mechanism has also been invoked to explain the segmental rotational dynamics of spin-labeled polystyrene in toluene solution.[57] This study provides an example of a system in which the dynamic cage is defined by constrained diffusion of the spin label attached to a polymer. The SRLS model is appropriate to analyze these spectra since the local ordering environment is coupled to the global tumbling modes of the polymer. However, the HF ESR spectra of this system are not sensitive to the global tumbling modes of the polymer as they are too slow on the 250 GHz ESR time scale. As we have

argued above, one may analyze such HF ESR spectra by taking the MOMD limit of the SRLS model. Then the 250 GHz best-fit dynamical parameters may be used as the fixed values for the internal dynamics in a constrained SRLS model for fitting the 9 GHz spectra. It was found that a simultaneous MOMD fit of the 250 and 9 GHz ESR spectra produced systematic discrepancies in the fitting parameters. The work of Liang and Freed, who derived the MOMD limit from the more general SRLS model,[10] validated the procedure by which the dynamical parameters determined from the MOMD analysis of the HF ESR spectra were used as inputs to the SRLS analysis of ESR spectra at X-band.

Another illustrative example of the utility of the SRLS model is provided by a multifrequency ESR study of spin labeled T4 lysozyme.[58] Two spin-labeled cysteine mutants at positions 44 and 69 were studied. Similar to the dynamics of spin-labeled polystyrene we discussed above, the overall tumbling modes of spin-labeled T4 lysozyme were too slow to affect the 250 GHz ESR spectra in any significant manner. Thus, for HF EPR spectra the MOMD analysis, as we have argued above, is fully appropriate. At 9 GHz, the spectra are still sensitive to the overall tumbling. A combined multifrequency analysis, in which the contributions of the internal modes to the system dynamics are fixed from the analysis of HF ESR spectra and the overall tumbling rates are extracted from a suitable analysis of the coupled internal and overall tumbling observed at X-band, can then be used to gain important insights into complex dynamic modes of the system. In this system, the spin label reorientation, side chain fluctuation, and global protein tumbling all were found to contribute to the observed multifrequency ESR spectra, but their relative contributions vary depending on the frequency of the experiments.

Detailed analysis of the T4 lysozyme ESR data demonstrated the existence of two distinct motional modes with different ordering parameters. The origin of these modes is thought to be due to two different preferred rotomeric sites of the disulfide-linked nitroxide side chain about the C_α-C_β bond. Depending on the rotomer occupied, the nitroxide ring experiences different local environments with different constraints on the diffusive modes. It was also found that the nitroxide reorients more rapidly when it is *normal* to its tether instead of being *co-linear* as one may expect based on the physics of the tether. One intriguing possible explanation of the observed effect is that $R_\perp$ actually reports on fast fluctuations of the protein backbone at the site to which the nitroxide label is attached.

As a final example, we would like to illustrate how the SRLS model was successfully utilized to study the dynamics of spin labeled lipids in pure DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine) and cholesterol-rich DPPC membrane vesicles.[59] *Figure 6* reproduced from that work demonstrates two noteworthy features: the excellent signal-to-noise ratio of the HF ESR spectra and the barely observable deviations of the MOMD fits from the experimental HF ESR spectra. Such high quality experimental data was crucial to obtain reliable least squares spectral simulations. It is expected that multifrequency

ESR spectra from site-specifically labeled mutant proteins in oriented systems such as bilayers should have similar signal-to-noise ratios in order to be studied using the SRLS approach to extract all of the structural and dynamical data available from the spectra.

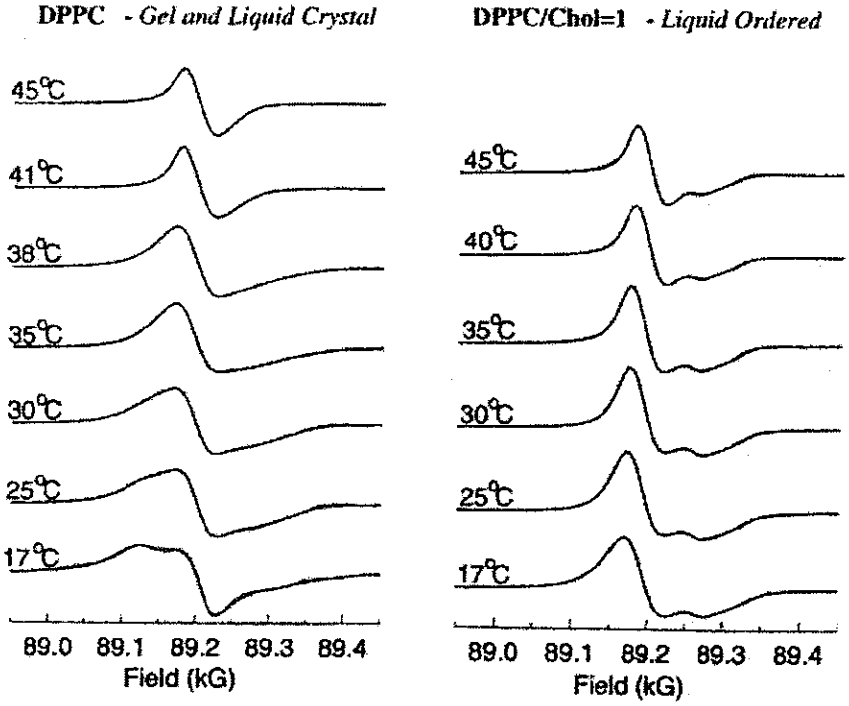

Figure 6. Variable temperature experimental (solid lines) and the MOMD least-square simulations (dotted lines) of 250 GHz ESR spectra of 16-PC in membrane vesicles. Spectra from pure DPPC vesicles are shown in the left-hand column. Spectra from DPPC/cholesterol at 50:50 molar ratio are shown in the right-hand column. Reproduced with permission from ref. 59.

The principal result of this study by Freed and co-workers was that the addition of cholesterol to the lipid membrane vesicle has a pronounced effect on the end chain dynamics of the spin labeled lipid. Analysis of the ESR spectra using the MOMD model provided further evidence for the accepted view that in lipid bilayers cholesterol promotes diffusional modes of acyl chains that are characterized by high mobility and high ordering. Another feature that emerged from this multifrequency ESR fits is that these acyl chain modes are clearly distinguished from the overall or global ordering and rotational mobility of the lipid molecules. The acyl chain ordering of the pure DPPC membranes is

significantly reduced upon the phase transition from the gel to liquid crystal phase occurring at approximately 40 °C. In the cholesterol-rich bilayers, high acyl chain order is maintained above the phase transition. As we have emphasized, the addition of cholesterol has only a limited effect on the global diffusion modes.

3.2.4 Recent progress in multifrequency and HF ESR of spin-labeled membranes and biopolymers

Rapid improvements in HF ESR instrumentation and progress in spin-labeling methodology and applications drive the efforts of many research groups today. Through these continuing efforts the method of spin-labeling HF ESR emerges as a powerful and versatile spectroscopic tool to uncover the complex dynamics of biopolymers from slow-motional nitroxides spectra.

One example is provided by Marsh and co-workers who studied anisotropic motion and, in particular, nonaxial lipid ordering in cholesterol-rich phospholipid membranes by W-band ESR. Using fully hydrated bilayer membranes of dimyristoyl phosphatidylcholine containing 40 mol % of cholesterol, Gaffney and Marsh obtained an indication of the way in which lateral ordering of lipid chains in membranes is induced by cholesterol.[60] More recently, Livshits and Marsh have undertaken a theoretical study to identify the dynamic origin of the residual $(g_{xx}\text{-}g_{yy})$ anisotropy observed in nonaxial HF ESR spectra of cholesterol-containing membranes.[61] It was concluded that this anisotropy arises from in-plane ordering of the lipid chains by cholesterol. The theory was further tested by Marsh and co-workers in their W-band ESR study of cholesterol-rich membranes.[62] This result could be a significant step towards understanding domain formation in cellular membranes. A timely review of HF ESR of spin labels in membranes by Marsh and co-workers has now appeared.[33] It should be emphasized, however, that while Marsh and co-workers' model is useful for obtaining qualitative insights into the data trends, quantitative analysis by rigorous slow-motional theory would be necessary to extract all of the available information from the spectrum. We believe that these studies of model bilayer systems will also be very useful in providing a solid background for understanding the dynamics of trans-membrane proteins in the future.

Understanding global and local dynamics of DNA continues to be a fruitful field of application for spin labeling ESR. Clearly, the faster time scale of HF ESR, where the overall tumbling modes of diffusion are frozen out, and the unique sensitivity to nonaxial (*i.e., x- y-*) modes of motion, would be very beneficial in providing new insights on position-dependent DNA dynamics. Recently, Robinson and co-workers reported a W-band rigid-limit ESR spectrum of a DNA to which a perdeuterated and ^{15}N-substituted nitroxide was attached in a rigid manner.[63] The spectrum demonstrated excellent resolution and negligible strain effects thus indicating the great potential of such labels in DNA research. In separate efforts, Budil, Strauss, and co-workers reported a different approach to the labeling of oligonucleotides using a newly synthesized

cytosine label which has a flexible attachment.[48,64] Variable temperature 220 GHz ESR studies of spin-labeled mono- and pentamers in aqueous buffers with 75 and 35 volume % of glycerol respectively showed good agreement of the slow-motional line shapes with the MOMD model.[48] The viscosity of the DNA solution was adjusted by adding glycerol in order to bring the motion of an otherwise rather mobile label, due to its flexible attachment, into the slow motion regime. Least-squares simulations of 220 GHz ESR spectra demonstrated that for the monomer species the rotational rates around all three molecular axes of the label could be measured independently because the principal axis orientations are well resolved in 220 GHz ESR spectra.[48]

HF ESR studies on spin-labeled T4-lysozyme using ficoll as the viscosity increasing agent are currently being undertaken at Cornell in collaboration with Hubbell and co-workers. At this time, there is some experimental evidence that ficoll puts less strain on the protein structure than other common solutes.[11] The enhanced sensitivity of HF ESR to internal modes of motion will be a useful test of this hypothesis.

A recent series of works by Jeschke, Spiess, and co-workers demonstrated a potentially wide field of applications of spin labeling ESR[65-67] and HF ESR in polymer studies.[68-71] Although the systems studied are not biopolymers, we would like to briefly review this very interesting body of work because the same ideas are fully applicable to the problem self-assembly of biopolymers and the emerging field of biofilms.

One series of papers by Jeschke, Spiess, and co-workers was devoted to ESR studies of polymer film formation from colloidal dispersions using a set of different spin probes.[67,68,71] This is a very important area of research because of the wide applicability of polymer films for coating, painting, printing, and binding. The main conclusions of these ESR studies were that the use of W-band ESR led to a more reliable determination of rotational anisotropies, and that the line shapes are more sensitive to slow motion than conventional X-band ESR specrta.[68,71] HF ESR data indicated that the surfactant aggregates do exhibit a low molecular order, whereas the ionic additives are strongly attached to immobilized ionic clusters. Another recent report of this group describes the use of HF ESR and spin labels to study the structure of highly cross-linked porous polymer resins which are based on a styrene-divinylbenzene matrix.[70] It was found that sufficiently large spin probes exhibit a distribution of mobilities. This distribution was clearly seen in W-band ESR spectra because of enhanced sensitivity to molecular motion observed at high magnetic fields. A preference of the surfactant head group for the surface of pores created by imprinting with reverse micelles was also observed. The difference in the site selectivity of probe molecules or additives was subtle but very much detectable by HF ESR. These examples illustrate a new and growing interest in utilizing spin labeling ESR in studies of heterogeneous polymeric materials to uncover the principles that govern self-assembly in these systems. Applications of the spin-labeling

technique to studies of the self-assembly of biopolymers is a natural extension of the method and should be explored further.

3.2.5 Time Domain ESR Studies of Slow Molecular Motion

Although the line shapes of HF ESR are more sensitive to details of molecular order and reorientations at faster time scales than those of traditional ESR, there would be several advantages to extending high field ESR time domain methods beyond the rigid limit. With a decrease in the rate of molecular motion, the spin label spectrum may become insensitive to the changes in rotational correlation time once the spectrum approaches the rigid limit. In other words, spectral changes due to homogeneous mechanisms may become too small and too difficult to measure because of the inhomogeneous broadening (ib) of continuous wave (CW) ESR spectra. We have already mentioned this problem in the context of the MOMD model. Clearly, if ib dominates the CW ESR spectra, one has to turn to modern time-domain ESR techniques to measure hb effects in order to be able to study the mechanisms of nitroxide dynamics. Nowadays, these studies have become possible with the growing availability of time-domain HF ESR instrumentation.

As an example of the utility of high field, field-swept electron spin echo methods to study torsional modes in biologically relevant systems, we note the contributions of Möbius and co-workers who took advantage of the superior resolution of HF ESR to study dynamical modes in the photosystem I complex.[72,73]

Another example is a study of the molecular dynamics of an elongated nitroxide in frozen toluene matrices by two-dimensional electron spin echo spectroscopy (ESE) carried out at 95 GHz.[115] This study was carried out at temperatures of 130 K and below at which the rigid limit for CW ESR spectra is already achieved. Two-pulse field-swept ESE spectra recorded with variable pulse separation time indicated that while the electronic relaxation remains essentially isotropic at 130 K, it becomes strongly anisotropic at 90 K. To explain the observed relaxation anisotropy the authors suggested a new relaxation mechanism associated with stochastic modulation of the g-matrix caused by residual motion of the solvent molecules.[115] Clearly, this hypothesis should be further tested in time-domain HF ESR studies.

In an application to polymers, time domain W-band ESR was recently utilized to study confinement effects in ionomers.[69] Electronic T_1 and T_2 relaxation times were measured for spin probes localized at the interface between the ionic clusters and the polymer as a function of temperature. The excellent angular selection of W-band ESR resolved x-, y-, and z- orientations of the nitroxides in the magnetic field and allowed Leporini and co-workers to probe electronic relaxation along those orientations for samples which remained macroscopically disordered. Based on these measurements of the electronic relaxation as a function of the nitroxide orientation, it was concluded that

reorientation of these spin probes has clear uniaxial features. Moreover, evidence was presented that the dynamic constraints on the poly(isoprene) chains in the diblock copolymer propagate over the whole chain consisting of approximately 170 monomer units.

Clearly, HF ESR and especially time-domain techniques have enormous potential in studies of slow molecular motion in complex and heterogeneous systems. It is our belief that the ongoing development of more sophisticated time-domain HF ESR instrumentation as well as experimental and theoretical methods will lead to new understanding of molecular motion in condensed matter and open new areas of applications for the method. So far, these HF ESR studies have been limited to the rigid limit, or near the rigid limit, where T_2's are relatively long and selective pulses can be used without complicating the spectral analysis unduly. As the tumbling rate goes just above the slow motion regime and T_2's become of the order of nanoseconds, it will be necessary to develop pulse spectrometers that can deliver non-selective pulses with large spectral bandwidths in order to excite the relevant coherences. Such large bandwidths are required in order to perform time-domain ESR experiments based on coherence transfer techniques, as well as to exploit the multiplex advantage of Fourier transform ESR. Steps towards these goals are currently being undertaken at Cornell University, where a high-power W-band pulsed spectrometer is under development. Non-selective $\pi/2$ pulses as short as 4 ns have been already demonstrated. The Cornell W-band spectrometer is capable of phase-cycling sequences suitable for 2D-FT-COSY, 2D-FT-SECSY and 2D-FT-ELDOR experiments. Preliminary experiments demonstrating 2D-FT-COSY for solution of Tempo (2,2',6,6'-tetramethyl-4-piperidone-1-nitroxide) in *n*-decane have been performed in the motional-narrowing limit. Currently, the Cornell group is focused on extending the capabilities of the pulse spectrometer to 2D-FT-ELDOR on complex systems in aqueous solution.

4. APPLICATIONS TO MOLECULAR STRUCTURE

Spin-labeling and site-directed spin labeling ESR are powerful structural methods of molecular biophysics. While hitherto in this review we have concentrated mainly on nitroxide spin labels, HF ESR studies of non-nitroxide radicals are becoming increasingly important for studies of structure and dynamics. Recent examples include a comparative study of neutral tryptophan and tyrosine radicals in ribonucleotide reductase (RNR) that was able to distinguish between mutated and wild type proteins.[74] Lubitz and co-workers have also recently demonstrated the utility of single-crystal rotation studies of dark-stable tyrosine radicals in photosystem II.[75] Time-domain ESR techniques at high magnetic fields have also been utilized to study the structure of the

tyrosil radical of yeast RNR.[76] As these examples show, HF ESR studies of non-nitroxide radicals is an important and growing field. We will discuss more examples of this technique below. Time domain HF ESR studies can also take advantage of differing relaxation rates in order to discriminate between spectral components.[77]

Another emerging direction of research is to explore molecular-scale sensitivity of HF ESR spectra of paramagnetic *S*-state ions, such as Mn^{2+} or Gd^{3+}, to the environment. Although the changes in the CW ESR line shape of such ions in biological environment are well documented in literature,[78] the low spectral resolution and broad line shapes observed in conventional 9.5 GHz ESR complicated efforts to fully exploit these effects for biophysical applications. With increasing magnetic field, the relative contribution of the zero-field splitting (ZFS) terms to the spin Hamiltonian of these paramagnetic metal ions decreases, and this leads to substantial simplification of the ESR spectra as well as significant line width narrowing. Smirnova, Clarkson and co-workers made use of this improved spectral resolution to study interactions of various Gd^{3+}-based MRI contrast agents with proteins and membranes.[79-81] For example, these authors have shown that at W-band the ESR signals arising from Gd^{3+} complexes partitioned between aqueous and lipid phases of phospholipid bilayers, as well as from free and protein bound complexes become partially resolved due to differences in the ZFS.[79-81] Recent experiments carried out at the North Carolina State University Department of Chemistry have shown that solution Mn^{2+} W-band ESR spectra are also uniquely sensitive to the coordination of this ion in biomolecules. It appears that Mn^{2+} may serve as a useful and a very informative probe to study the interactions of similar divalent ions which are more biologically relevant, such as Mg^{2+}, with proteins in RNA's in solutions at physiological temperatures.

HF ESR can also be also utilized to directly measure distances and angles in spin labeled systems. A detailed review of various ESR methodologies which are now used for structural studies of biological systems has recently been published.[82] This review shows that although many of the current ESR methods rely on MTSSL as the spin label of choice for SDSL, the use of unnatural ESR-active amino acids, such as TOAC (2,2,6,6-tetramethyl-piperidine-1-oxyl-4-amino-4-carboxylic acid), might offer several important advantages since in this label the nitroxide moiety is fused into the peptide backbone producing. Therefore this nitroxide directly reports on the dynamics and the position/orientation of the peptide backbone. Furthermore, perturbation of the system due to spin labeling is clearly minimal.[83] Although many examples of spin-labeling structural studies can be found in the literature,[82] only a few of those, especially using TOAC, have been carried out at high magnetic field. We have no doubts that several of these studies would benefit from being carried out at high magnetic fields. Among them are distance measurements which are based on dipolar coupling of nitroxide pairs, determination of angular orientations of spin-labeled molecules with respect to the magnetic field, and

structural studies which are based on g-factor measurements. These methods are reviewed below in more detail.

4.1 Distance Measurements

Distance determination by spin labeling ESR is based on measurements of the magnetic interaction of a spin label with another nitroxide, paramagnetic metal ion, or a nuclear spin. Although electronic spin-spin interactions, such as spin exchange, dipolar, or a combination thereof, are field- independent, application of high magnetic fields both simplifies and improves the accuracy of the data analysis.

Perhaps some of the first studies of nitroxide pairs with HF ESR were carried out by Lebedev and co-workers in Moscow.[19,84] The authors noted that although the number of spectral components of the rigid-limit 140 GHz ESR spectra of a nitroxide biradical is greater than at conventional X-band, the spectrum is still easier to interpret because of the excellent angular resolution which allows one to sort out the spectral components and the corresponding dipolar coupling.[84] Basically, for well-resolved spectra, the initial estimates for the components of the dipolar tensor can be deduced from measurements of dipolar splittings directly from the spectrum.

More recently, these advantages of dipolar spin labeling HF ESR were further explored by Hustedt and co-workers in a study of ^{15}N-spin-labeled coenzyme NAD^+ (SL-NAD^+) bound to a microcrytalline, tetrameric glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[86] The structure of the tetramer was such that for the purpose of dipolar ESR the four spin can be considered as two independent pairs. The work was carried out with an ammonium sulfate-precipitated form of the SL-NAD^+-GAPDH which was resuspended in a small volume of saturated ammonium sulfate as a slurry at a concentration of *ca.* 200 μM. For such a complex the spin labels populate unique conformations and remain virtually immobilized with respect to the protein. ESR spectra were recorded at 4 °C at X-, Q, and W-bands and fit simultaneously with a least-squares software package developed at Vanderbilt University.[85-87]

The results, shown in *Figure 7*, illustrate excellent agreement between the experimental spectra and the fits. Based on the results of this study, several conclusions regarding the prospects of HF ESR in such experiments can be drawn:

(1) The spectral features corresponding to all principal axis components of the nuclear hyperfine and dipolar coupling interactions are clearly resolved and at the conditions of the experiment no strain broadening of the lines was observed. The line assignment is significantly simplified at W-band *vs.* lower ESR frequencies.

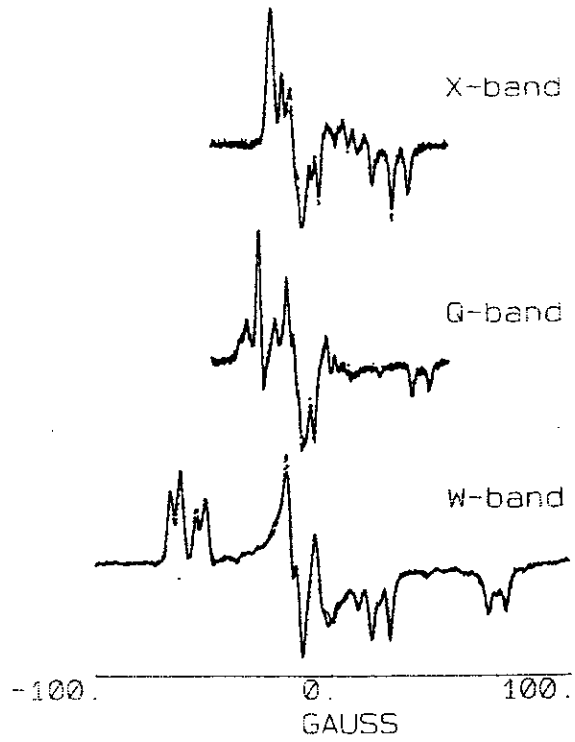

Figure 7. Experimental CW ESR spectra (dots) at X-, Q-, and W-band of spin-labeled NAD^+ bound to GAPDH at a ratio of 3.4 labels per GAPDH tetramer. Dipolar coupling between the nitroxides results in well resolved splitting of the spectral components corresponding to principal axis orientations. Note a progressive improvement in spectral resolution and angular selectivity from X- to W-band. The simultaneous multifrequency least-squares simulations are shown as solid lines superimposed with the spectra. Details of this study are given by Hustedt *et al.*[86] Reproduced with permission from Ref. 86.

(2) Not only the *z*- but also the *x*- and *y*-turning points are well resolved in the W-band ESR spectrum. This makes the least-squares simulations much more sensitive to the mutual orientations of the spin labels on the proteins and allows one to determine mutual orientations of spin labeled protein residues with better accuracy.

(3) The time scale of motional averaging at W-band is moved toward faster motions bringing the whole spectrum essentially into the rigid limit at the conditions of the experiment. We have already discussed this 'snapshot' effect previously with examples of 250 GHz spin label ESR spectra.

Another example of the use of CW dipolar EPR for distance determination was given recently by Bottle and coworkers.[88] In their initial ESR studies of a carboxy-derivative of an anionic isoindoline aminoxyl, CTMIO, carried out at X-band, they observed an extra broadening of the ESR spectrum recorded in frozen toluene-d_8 at 123 K.[89] The broadening was interpreted as an indication of dimmer formation although no resolved dipolar splittings were observed. Consequent re-examination of this system with W-band ESR demonstrated a well resolved dipolar splitting of the g_z-components essentially at the same temperature of the experiment (130 K).[88] The direct observation of dipolar interaction led to a more accurate estimate of the interspin distance of 19.3 Å. This study is a good illustration of the resolution gain achieved with HF ESR.

It is very likely that with the growing availability of HF ESR and specialized least-squares simulation programs more double-label experiments will be carried out at high magnetic fields. Clearly, the excellent angular and g-factor resolution achieved at high magnetic fields would facilitate accurate determination of distances and angles between the interacting spins from CW and time-domain ESR experiments. Some of the advantages of high magnetic fields also hold for double resonance experiments such as ENDOR and ELDOR. While ELDOR at high magnetic fields remains to be demonstrated, HF ENDOR and ESEEM have proven to be powerful methods in structural studies of free radicals and paramagnetic metal ion centers in enzymes.[90] These methods are awaiting detailed exploration in applications to spin-labeled molecules. A review of recent applications of spin labels and ENDOR at conventional magnetic fields to various structural problems – ranging from small molecules to enzyme reaction intermediates – has been published.[91] Researchers from the Freie Universität in Berlin have discussed the advantages and disadvantages of high field ESEEM and, in a study of a dianisyl nitroxide, have shown that W-band ESEEM measurements of nitrogen couplings are feasible.[92] A recent application of time-domain HF ESR combined with photoexcitation is provided by a D-band study of the primary electron donor triplet state of in photosynthetic reaction centers of *Rhodabacter sphaeroides* R26 by Hoff and co-workers.[93]

4.2 Measurements of Molecular Orientations from Ordered Samples

As was discussed in section 2 of this review, with an increase in resonant frequency ESR spectra of spin labels become extremely sensitive to molecular orientations with respect to the external magnetic field because of the dominance of the g-factor anisotropy with its rhombic character. This

sensitivity can be exploited in studies of microscopic ordering and mutual spin orientations for disordered samples as was discussed above. When carried out at high magnetic fields, spin labeling HF ESR provides even more information on studies of samples which are macroscopically aligned even if this alignment is only partial. If the macroscopic order is relatively high, then the orientation of the spin-labeled fragment, at least in the first approximation, can be read directly from the spectrum. This puts spin labeling HF ESR in a unique position among other spectroscopic techniques because only a few of these techniques are capable of allowing the determination of molecular orientations in (partially) aligned samples. Currently, there is a particular interest in the study of orientations of biological molecules such as peptides and proteins with respect to membrane surfaces. Additionally, there is a growing interest in inferring the orientations of proteins and peptides imprinted on the surface of hybrid nanoscale devices.

A striking demonstration of the excellent resolution of HF ESR to the orientations of spin labeled molecules was provided by a 250 GHz ESR study of Barnes and Freed.[94] The study was carried out on macroscopically aligned model membranes composed of mixtures of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylserine (DMPS) and utilized a cholestane spin probe which served as a cholesterol analog. Using a specially designed sample holder for a Fabry-Perot resonator these authors examined two different orientations of membrane samples mechanically aligned on a quartz support.[95] *Figure 8* shows an example of 250 GHz ESR spectra from membrane samples of various DMPC:DMPS compositions.

The membranes were fully hydrated and aligned with the director perpendicular to the external magnetic field. The excellent angular resolution at 250 GHz ESR allowed the authors to draw provisional, qualitative conclusions directly from the spectra and then to enhance the analysis by least-squares modeling.

Indeed, the spectra shown in *Figure 8* are very illustrative. For example, the top spectrum, which corresponds to the DMPC:DMPS mixture in an 8:2 molar ratio, shows well resolved spectral features corresponding to g_x and g_z components, while the intensity of the g_y components is almost completely suppressed (compare with *Figure 1* which shows a typical spin label HF ESR spectrum from a randomly dispersed sample). The reason why the intensity of the g_y component is greatly diminished is that for this sample at this particular orientation of the quartz plate in the magnetic field almost none of the cholestane molecules are aligned with their nitroxide y-axis along the external magnetic field. As we have already discussed, clolestane is an elongated molecule in which the nitroxide *y*-axis is approximately aligned with its long axis. Thus, the spectrum in *Figure 8* is illustrative of cholestane molecules aligned along the bilayer director's vector. With an increase of the fraction of DMPS in the phospholipid mixture a progressive change in the position and the amplitude of spectral components was observed (*Figure 8*). Analysis of these

spectral changes in this particular data set together with a series of spectra at the perpendicular bilayer orientations led the authors to conclude that the cholestane probe in the DMPS-rich membrane exhibits a biaxial behavior which is markedly different from the axial orientation adopted by this probe in the DMPC membranes. The exquisite sensitivity of HF ESR to these ordering effects is of great significance, particularly as a preparation for spin-labeling HF ESR studies of transmembrane proteins.

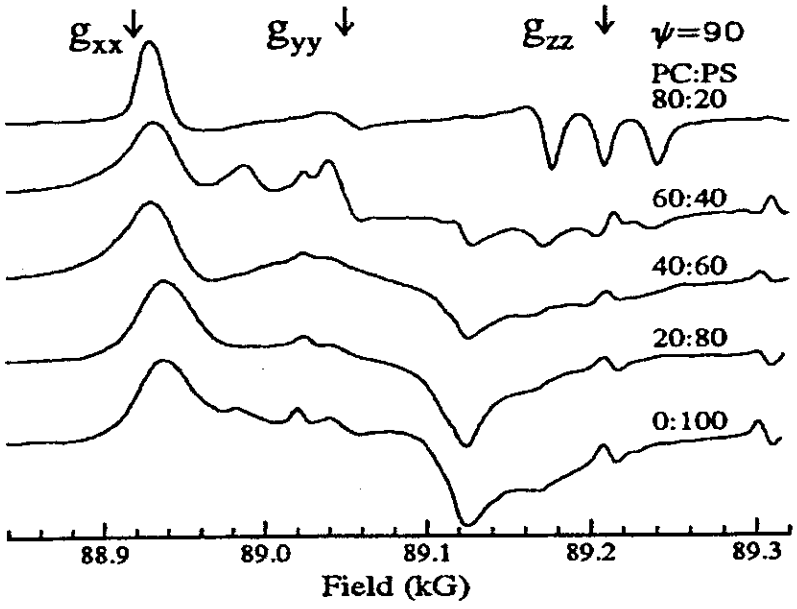

Figure 8. Experimental 250 GHz ESR spectra from membrane samples of various DMPC: DMPS compositions. The membranes were fully hydrated and mechanically aligned with the director vector perpendicular to the external magnetic field. All spectra were recorded at 10 °C in the gel phase. Reproduced with permission from Barnes and Freed.[94]

Another example of the use of HF ESR to study orientations of spin-labeled molecules is given in Refs. 43 and 46. These studies utilized a different alignment method, which is based on spontaneous ordering of lipid bicelles in external magnetic field. We have already discussed these results in Section 2 of this review.

HF ESR spin-labeling appears to be the method of choice for studying details of structural organization of nanoscale lipid assemblies. Recently, Smirnov and Poluektov described self-assembly of lipid nanotubes inside nanoporous anodic aluminum oxide (AAO) substrates.[116] These authors utilized high-resolution spin labeling EPR at 95 GHz to verify orientations of lipid molecules in such lipid nanoscale structures. Because the local order parameter S of the lipid fatty acid chains is known to decrease progressively towards the bilayer center, 1-palmitoyl-2-stearoyl-(5-doxyl)-sn-glycero-3-phosphocholine (5PC) lipids containing nitroxide moiety at position 5 were chosen for the studies. Dynamic lipid disorder and partial averaging of spectral anisotropies were further reduced by taking EPR spectra at a low temperature (150 K) at which dynamics of the phospholipids is approaching the rigid limit. It was observed that the largest changes in 5PC spectra upon reorientation of the AAO substrate in the magnetic field were observed at 95 GHz (W-band) because of an enhanced angular resolution of HF EPR over conventional X-band. The relative intensities of characteristic peaks of these spectra, Fig. 2 (left), are clearly different for two orientations of the substrate in the magnetic field. Particularly

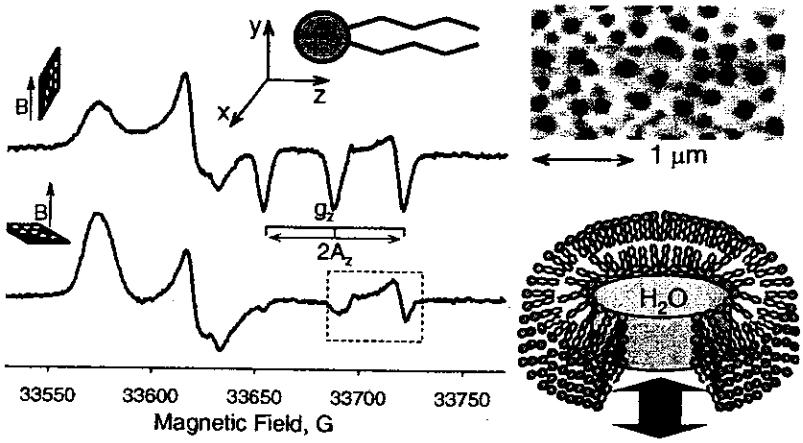

Figure 9. Left: Experimental rigid limit (T=150 K) high resolution 94.4 GHz (W-band) EPR spectra of AAO substrate with deposited DMPC:5PC (100:1) at two orientations of the substrate surface in the magnetic field. A cartoon on the top shows orientations of the magnetic axes with respect to the phospholipid. Note that the bottom EPR spectrum has a low intensity in the g_z-region (the feature in the dashed box is due to a paramagnetic AAO impurity) indicating that at this substrate orientation the lipid chains are perpendicular to the magnetic field. Right: SEM of the substrate after heat treatment and a cartoon of a lipid nanotube formed inside the substrate nanopores. Reproduced with permission from Smirnov and Poluektov.[116]

noticeable are the changes in the g_z-region (*i.e.*, high-field component which spreads from *ca.* 33850 to 33940 G): when the surface of the AAO substrate is perpendicular to the magnetic field (bottom spectrum), the *z*-component almost completely disappears (the signals inside the dashed box are mainly due to paramagnetic impurities in the AAO substrate). This means that at this substrate orientation only a very small fraction of molecules has the *z*-axis of the N-O frame aligned with the external magnetic field. The orientation of the nitroxide magnetic axes of 5PC is such that the z-axis is approximately aligned along the phospholipid chain (Fig.9, cartoon in the right top corner). Thus, it must be concluded that a majority of the phospholipids inside the nanopores (since the surface phospholipids were mechanically removed during sample preparation) are positioned with their long axis perpendicular to the magnetic field and therefore perpendicular to the direction of pores. The AAO surface is known to be hydrophilic[117] and therefore in fully hydrated samples the lipids are organized in bilayers rather than in monolayers. The bilayer lipid organization was further confirmed by experiments with the lipids labeled at the end of the acyl chain (16PC; not shown): these samples produced EPR spectra essentially identical to those from unsupported liposomes (not shown). This is consistent with a lipid nanotube geometry shown in Fig.9 (bottom right). The static order parameter of lipids in such a nanotube was determined by a "center of gravity" method[118] and was found to be exceptionally high $S_{static} \approx 0.9$.

4.3 Structural Studies Based on *g*-factor Measurements

Accurate measurements of magnetic parameters provide important data about the structure of free radicals. These data also represent required parameters for the analysis of the molecular dynamics of spin probes. The superior resolution of HF ESR makes it possible to accurately determine the principal axis components of the *g*-matrix and *A*-tensor from powder pattern spectra, in many cases eliminating the need for preparing and studying single crystals.

The magnetic parameters of nitroxides are also known to be sensitive to intermolecular interactions and, in particular, to hydrogen bonding and local solvent polarity.[96-98] In general, when a spin-labeled molecule is transferred from an aqueous (polar) to a hydrocarbon (non-polar) environment, the isotropic nitrogen hyperfine constant A_{iso} decreases by up to 1-2 G while the isotropic *g*-factor, g_{iso}, increases only slightly (by ≈ 0.0004). At X-band this change in g_{iso} corresponds to ≈ 0.68 G shift of the ESR line, which is smaller than the typical nitroxide line width (*ca.* 1 G). Therefore X-band EPR spectra from spin labels residing in different environments are only partially resolved for non-perdeuterated nitroxides with line widths of a Gauss or more. At W-band frequencies the shift in the resonance line positions due to the *g*-factor differences is magnified tenfold and is greater than the corresponding changes in

A_{iso}. At higher fields, the line shift is correspondingly greater. In conjunction with SDSL techniques, the sensitivity of the rigid-limit HF ESR line shape to the hydrophobic or hydrophilic nature of the spin label's immediate environment can provide useful structural information.[99,100]

In the past, the changes in magnetic parameters (both g-matrices and A-tensors) determined with high accuracy from the rigid limit EPR spectra at high magnetic fields were utilized to characterize nitroxide-solvent interactions and the local solvent polarity.[98] More recent applications involved 250 GHz studies of a polarity profile in phospholipid membranes with a series of doxyl-labeled stearic acids[101] and the differentiation of the microenvironment of spin-labeled side chains of a membrane protein bacteriorhodopsin.[99,100] Both studies utilized correlation plots between two magnetic parameters, g_{xx} and A_{zz} principal axis components, because those parameters are the most sensitive to local polarity changes. Both parameters are expected to be proportional to the π spin density ρ_0 at the oxygen atom of the nitroxide moiety.[98] More detailed interpretation of the polarity and electric field effect on g_x can be obtained using the theory of Stone for the g-matrix of π-radicals.[102] Because electrostatic interactions and hydrogen bonding play a critical role in the structure and function of biomolecules, several new theoretical studies devoted to calculations of the A-tensor and g-matrix have been published in the last few years.

Bales and co-workers reported a calibration between the shift in the isotropic nitrogen hyperfine coupling constant A_{iso} and the local charge on the nitroxide.[103] In their study the aqueous solvent was approximated with a continuum model and the effect on A_{iso} was calculated within the framework of the standard Hückel theory. Gullá and Budil extended the calibration of electric field effects to g-matrix calculations which were compared with the anisotropic g-factors of nitroxides containing internal groups which can be ionized.[104] Using the Stone theory they have shown that the electric fields of the magnitude typically found in proteins may lead to g-factor shifts measurable by HF ESR. They also suggested that the g-matrix might be sensitive not only to the magnitude of the electric field but also to its orientation. Recently, Budil and co-workers also presented the results of *ab initio* calculations of electric field effect on the g-matrix of nitroxide radicals.[105] Their approach utilized an intermediate level Rayleigh-Schrödinger perturbation theory based on unrestricted Hartree-Fock ab initio calculations. It was found that the calculated g-matrices agree well with the experimentally measured data.

Hydrogen bonding is another factor, which might affect the magnetic parameters of nitroxide radicals. Results from previous studies suggest that nitroxides may form hydrogen-bonded complexes when immobilized.[97,106-108] The complex formation and its life time are modulated by the chemical environment and temperature, thereby complicating polarity assignment. Recently, hydrogen bonding effects on nitroxide ESR spectra were revisited. Engström and co-workers applied the restricted open-shell Hartree-Fock (ROHF) linear response method with the atomic mean field approximation

(AMFI) to calculate the effect of hydrogen bond formation on the g-matrix of MTSSL.[109] Their results shows that the g_x component is reduced the most. The decrease in g_x is traced to a higher excitation energy and lower spin-orbit coupling and angular momentum matrix elements for the n-π^* excitation. Subsequently, a density functional theory (DFT) calculation of g and A for MTSSL and MTSSL covalently attached to β-mercaptoethanol were performed under conditions of varying dielectric constant of the medium and the number of hydrogen bonds formed with the nitroxide oxygen.[110] This work should be continued to further assist the interpretation of the rigid-limit data from spin-labeled peptides and proteins in terms of environmental properties such as polarity, proticity and local electric field. The W-band spin labeling ESR study of bacteriorhodopsin is a good illustration of the power of HF ESR in characterizing structural details of protein organization in the membrane.[99,100] Extending this technique to studies of the accommodation of transmembane proteins and their effects on dynamics, ordering and water penetration in lipid bilayers would be a useful generalization of this direction of research.

Accurate measurements of magnetic parameters in the fast motional regime are also useful for structural assignments and for the characterization of self-assemblies with multiple compartments. One example of this was given by Smirnov and Smirnova in W-band studies of lipid bilayer interdigitation using a small nitroxide, Tempo.[29] The polarity assignment for multiple components of Tempo W-band ESR spectra was carried out using an empirical relationship between g_{iso} and A_{iso} established for this nitroxide for a series of protic and aprotic solvents and their mixtures. In addition to an aqueous Tempo signal, at least two components attributed to the membrane environment were observed for the bilayer which is known to be interdigitated upon addition of 2 M ethanol. It was determined that the first membrane Tempo compartment had effective polarity close to the pure lipid phase, while the second compartment was close in polarity to the 7:3, v/v, ethanol-water mixture.[29]

In addition to the nitroxide spin labeled systems that we have discussed previously, one can make use of magnetic parameters of organic free radicals naturally occurring in proteins for structural assignments. It is timely to mention here some of the accomplishments in this field. The enhanced g-resolution achieved by HF ESR is well demonstrated by a recent determination of all three components of the g-matrix of perdeuterated chlorophyll radicals in a frozen solution.[113] In another recent study, Möbius and co-workers report a fully resolved g-matrix for a neutral flavin cofactor of *Escherichia Coli* photolyase.[114] That work, carried out at 360 GHz, yielded the following principal values for the rhombic g-matrix: $g_X = 2.00431(5)$, $g_Y = 2.00360(5)$, and $g_Z = 2.00217(7)$. This high accuracy provides an example of the excellent g-factor resolution achievable by state-of-the art HF ESR. From these HF ESR measurements, the relative orientation of the observed proton hyperfine tensor with respect to the principal axes of the g-matrix could also be assigned.

5. COMPUTATIONAL RESOURCES FOR SPIN LABELING AND HF ESR

Since the publication of the "User's Guide" for simulations of slow motional ESR spectra by solving the stochastic Liouville equation,[52] the Freed group has made serious efforts to aid users who wish to take advantage of the powerful and versatile simulation packages that were made available at that time.

Since then, the group has made significant progress in developing new models and computationally efficient software packages. By combining the spectral simulation capability with non-linear least-squares optimization, automatic fitting of experimental spectra became possible.[54] In addition, the simulation and fitting capabilities of these programs were extended to pulsed ESR experiments. More sophisticated and realistic models of spin label relaxation in complex biological systems have also been developed. Those codes have been available from the Cornell group via its FTP site since 1997.

Since the establishment of the ACERT NIH NCRR center, the Freed group has been in the process of porting the codes for their non-linear least-squares CW ESR fitting programs to a variety of operating systems such as AIX, Linux, and Windows (in collaboration with Prof. Budil, Northeastern, and also as a result of independent work within the Freed group). While at this moment the capabilities of the packages for different computational environments vary somewhat, efforts are under way to provide a suite of programs that would have an equally powerful functionality independent of the operating environment. However, at this time there are no plans to port the codes to the Mac platform.

The source codes continue to be available from the ACERT website http://www.acert.cornell.edu together with instructions for compiling the codes under the relevant operating system.

6. HARDWARE RESOURCES FOR HF ESR

Currently, the only turnkey HF ESR system is made by Bruker Biospin (Billerica, MA). This spectrometer operates at 95 GHz, is equipped by a versatile split-pair superconducting magnet, and is available in continuous wave and pulse configurations. The design and performance of this spectrometer has been described by Schmalbein and coauthors.[30]

Complete HF ESR microwave bridges (both pulse and CW), single-mode resonators, complete probe heads, and various high-performance components for mm-wave frequencies of 95 and 130 GHz can be purchased from HF EPR Instrumentation (Brooklyn, NY). This company was founded from nucleus of engineers who built the fist 140 GHz ESR spectrometer for the Lebedev group

in Moscow. Nowadays this company has multiple installations of mm-wave bridges throughout the world.

Although efforts have been made to launch commercial HF ESR spectrometers with resonant frequencies above 140 GHz (*e.g.*, Kyospin, Thomas Keating, Ltd., United Kingdom), at the time of this writing, the authors are not aware of any multiple installations of a turnkey spectrometer of such a kind. However, a variety of components and complete mm-wave bridges with excellent characteristics are currently available from the abovementioned suppliers.

Nevertheless, the rapid development of reliable millimeter and submillimeter wave technologies leads us to suggest that complete turnkey commercial systems will eventually operate above the current 95 GHz limit. As the part of the dissemination, service and training charge of the ACERT NIH NCRR center, one of the authors (KAE) would be happy to discuss HF ESR instrumentation and to consult on issues related to construction of HF ESR spectrometers with interested researchers. As the commercial marketplace changes and develops, the ACERT Center can also assist in selecting the proper options for turnkey systems depending on particular experimental needs.

7. CONCLUSIONS

The field of HF ESR studies of proteins is rapidly expanding as more research groups are exploiting SDSL techniques and studying other ESR-active moieties. The method has already produced valuable and path-breaking results for complex systems ranging from spin-labeled membranes and DNA's to membrane proteins. The latter are particularly difficult to study by other spectroscopic means. The new developments in time domain and double resonance techniques remain to be fully explored in studies of structure and dynamics of biopolymers and other molecules. The work on syntheses of new nitroxide labels should continue and labels tailored for high field applications should be developed. Development of improved computer hardware, software, and dynamical models will lead us to better understanding of complex dynamics and structural organization of labeled systems. A very important effort in this area is electrostatic mapping of membrane proteins using spin labeling ESR at high magnetic fields. The handling of miniature HF ESR samples and optimizing signals from lossy aqueous samples at high magnetic fields remains an active area of research, and much progress has already been made. It is the authors' expectation that generally applicable, readily available techniques for handling such samples will be developed in a timely and effective matter. We also expect that many accessories already applied in conventional ESR will be adapted for use at high magnetic field. For example, studies of miniature single crystals have been mentioned in this review. Light excitation is another

common method that has also been used. We also expect that the studies of linear electric field effect will become more common.

We also foresee many advantages to extending spin labeling protein folding studies initiated by rapid mixing to high magnetic field. Other alternatives to initiating protein conformational changes, such as T-jump, should also be explored. Studies involving the determination of orientations of spin-labeled molecules and distance-angle measurements should become increasingly important and popular. The excellent absolute point sensitivity of HF ESR should be fully exploited. Extending time domain techniques such as DEER or PELDOR and DQC to HF ESR would also be useful for exploiting the information available from distance studies. Another promising direction would be the development of microimaging techniques which one of the authors (AIS) initially demonstrated a decade ago with 140 GHz ESR.[111] Field-step methods for performing ELDOR have been introduced at high fields,[112] and it would be useful to extend these techniques to even higher fields.

ACKNOWLEDGMENTS. Both authors would like to thank the NSF and NIH for support of much of the work reported here. AIS thanks the US Department of Energy (DOE Contract W-31-109-Eng-38), National Science Foundation (NSF DMR-0114200), North Carolina Biotechnology Center (2001-ARG-0033), NATO (LST. CLG. 977528), and the North Carolina State University for the grant support. Special thanks are due to Prof. Tatyana I. Smirnova (NCSU) who contributed enormously to some of the studies reported here as well as to Profs. Belford, Clarkson, and Nilges from the Illinois EPR Research Center ((NIH RR01811 and R01-01811). KAE is supported by grants from NIH NCRR P41RR016292-02 and NSF Chemistry CHE-0098022. The resources of the Cornell Theory Center for the computation of some of the spectral fits reported here is gratefully acknowledged. KAE thanks AIS for stimulating scientific discussions over a period of many years. KAE also thanks the Cornell HF ESR group, which has consisted over the years of Profs. W. Bryan Lynch and David Budil, Drs. J. P. Barnes, D. S. Tipikin, W. T. Hofbauer, S. Pachtchenko, and Ms. Yan Lou for their contributions to the work reported on in part here. KAE also thanks Prof. Jack Freed for providing the environment that led to the establishment of ACERT and the opportunities ACERT provides to disseminate the know-how necessary for performing HF ESR.

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