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Toward increased concentration sensitivity for continuous wave EPR investigations of spin-labeled biological macromolecules at high fields

Likai Song [a,1], Zhanglong Liu [c,1], Pavanjeet Kaur [a,b,1], Jackie M. Esquiaqui [c], Robert I. Hunter [d], Stephen Hill [a,b], Graham M. Smith [d], Gail E. Fanucci [c,*]

[a] National High Magnetic Field Laboratory, Florida State University, Tallahassee, FL 32310, USA
[b] Department of Physics, Florida State University, Tallahassee, FL 32306, USA
[c] Department of Chemistry, University of Florida, PO Box 117200, Gainesville, FL 32611, USA
[d] School of Physics and Astronomy, University of St. Andrews, North Haugh, St. Andrews KY16 9SS, United Kingdom

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ABSTRACT

High-field, high-frequency electron paramagnetic resonance (EPR) spectroscopy at W-(~94 GHz) and D-band (~140 GHz) is important for investigating the conformational dynamics of flexible biological macromolecules because this frequency range has increased spectral sensitivity to nitroxide motion over the 100 ps to 2 ns regime. However, low concentration sensitivity remains a roadblock for studying aqueous samples at high magnetic fields. Here, we examine the sensitivity of a non-resonant thin-layer cylindrical sample holder, coupled to a quasi-optical induction-mode W-band EPR spectrometer (HiPER), for continuous wave (CW) EPR analyses of: (i) the aqueous nitroxide standard, TEMPO; (ii) the unstructured to α-helical transition of a model IDP protein; and (iii) the base-stacking transition in a kink-turn motif of a large 232 nt RNA. For sample volumes of ~50 μL, concentration sensitivities of 2–20 μM were achieved, representing a ~10-fold enhancement compared to a cylindrical TE_{011} resonator on a commercial Bruker W-band spectrometer. These results therefore highlight the sensitivity of the thin-layer sample holders employed in HiPER for spin-labeling studies of biological macromolecules at high fields, where applications can extend to other systems that are facilitated by the modest sample volumes and ease of sample loading and geometry.

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1. Introduction

Structure and structure–function relationships of macromolecules are areas of intense EPR effort [1–16]. Coupled with site-directed spin-labeling (SDSL), EPR is oftentimes used to characterize protein and nucleic acid structures and dynamics, conformational changes, molecule folding, macromolecule complexes, and oligomeric structures [1–15,17]. The majority of biomolecules do not contain unpaired electrons from which one can obtain an EPR signal; therefore, spin-labeling approaches have been developed [15,18–25] where site-specific persistent radicals or paramagnetic metal-probes are incorporated at specific locations within a biomolecule. Properties of the EPR spectra that originate from these probes, positioned at well-defined vantage points, provide structural and dynamic constraints. High-field EPR approaches offer the possibility of increased signal sensitivity and information content, thus potentially extending the scope of research aimed at biological macromolecules. High fields increase EPR sensitivity and broaden its practice through improved *g*-factor resolution for extracting biophysical constraints, which include topics such as dynamics of molecular motion, local polarity and hydrogen bonding, relative orientation information, and membrane ordering [26–33]. Moreover, multi-frequency EPR is especially powerful for describing complex local biophysical dynamics [34–36].

With regards to applications in studying macromolecular conformational dynamics and flexibility, the increased Larmor frequency of high-field EPR allows for a "shift" in the motional averaging timescale toward fast motion. Traditionally, X-band (9.5 GHz) EPR spectroscopy has been utilized for SDSL investigations of biological macromolecules. At W-band (~94 GHz), the sensitivity to intermediate motion shifts to a time regime of faster motion with smaller correlation times, which affords increased spectral sensitivity to motions that appear in the "fast-motion" limit at X-band. Examples of nitroxide spin-labeled molecules falling into a fast motion regime (i.e. correlation times < 2 ns) include small peptides/proteins, small nucleic acids, unstructured

* Corresponding author.
E-mail address: fanucci@chem.ufl.edu (G.E. Fanucci).
[1] These authors contributed equally to this work.

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proteins/protein segments, and backbone/nucleobase labeling of RNAs [37–42]. For these biological systems, it is oftentimes challenging to extract dynamic parameters from the subtle differences of the narrow line shapes of CW EPR spectra at X-band. In this time regime, molecular motion nearly completely averages the *g*-tensor and hyperfine-tensor anisotropy. The resulting narrow line shapes show only slight changes in empirical mobility parameters, such as the central line width (ΔH_o) and the inverse of the second moment ($\langle H^2 \rangle^{-1}$). Alternatively, high-field EPR spectra obtained at W-band provide "sweet-spots" with enhanced line shape sensitivity for motions with correlation times ranging over 0.1–2 ns [43].

A prime example of X-band EPR studies that fall within this "fast-motion" regime is the characterization of dynamics of intrinsically disordered proteins (IDPs) [37]. IDPs are described as proteins or protein segments that lack a well-defined secondary or tertiary structure [44–48]. IDPs often exist in different structural states *in vivo*, from disordered to fully structured; and carry out their function either through a structural transition between different states or through their disordered flexible structure. IDPs oftentimes play key roles in cellular activity, such as transcription and translation regulation, signal transduction, post-translation modification, cellular transport, and protein assembly [45,49]. Structural and dynamic investigation of IDPs is of particular interest to elucidate their structure–function relationships.

Concentration sensitivity is a challenge for high-field EPR studies of biologically relevant samples in non-frozen aqueous solutions. This stems primarily from the shorter wavelengths associated with the higher microwave frequencies. First and foremost, if using a resonator, both the dimensions of the resonant structure and the corresponding active volume associated with the microwave B_1-field maximum (*E*-field minimum) shrink appreciably at high fields. This is particularly important for studies of aqueous samples because of the need to minimize dielectric losses in the solution due to interaction with the electric (*E*-field) component of the microwaves. For a typical enclosed cavity, this usually requires shrinking the sample holder in at least two dimensions. At X-band (wavelength = 3.3 cm), capillaries with ~1 mm inner diameter (I.D.) have an active volume in the 3–10 μL range, whereas capillaries of 0.1–0.2 mm I.D. present a volume of only ~0.1 μL in a typical Bruker W-band (wavelength = 3.3 mm) cylindrical cavity. This reduction in sample volume oftentimes results in decreased concentration sensitivity at W-band, relative to X-band, even though the absolute spin sensitivity is enhanced due to increased magnetization at high field. Even when using a quasi-optical approach with no resonator, as in the present study, or even with a Fabry-Pérot resonator [50], the reduction in penetration depth with increasing frequency also results in an unavoidable decrease in active sample volume. Moreover, broader spectral lines typically observed for nitroxides at high fields lead to further reductions in overall sensitivity. Therefore, concentration sensitivity remains a major problem for CW EPR studies at high fields, with technological challenges limiting widespread applications.

A recently developed quasi-optical 94 GHz EPR spectrometer (HiPER) provides exceptional sensitivity and time resolution [51,52]. For instance, it has demonstrated ~1 μM concentration sensitivity for pulsed dipolar EPR, or DEER (Double Electron-Electron Resonance), owing to its unique quasi-optical inductive-mode design that gives exceptional cross-polar isolation, its high power (kilowatt), high instantaneous bandwidth pulses, and the possibility to use non-resonant sample holders of large volume [52].

The present study examines the concentration sensitivity of several newly developed non-resonant thin-layer cylindrical sample holders, designed specifically for low-power CW EPR studies of aqueous samples using HiPER. We first assess the sensitivities of these holders using the radical TEMPO as a standard. We then evaluate the performance of an optimized sample holder, with a ~50 μL sample volume, by collecting W-band EPR spectra reflective of the unstructured to helical transition of an intrinsically disordered protein IA_3. We also demonstrate a second application involving characterization of the base dynamics in large RNAs. A ~10-fold enhancement in concentration sensitivity is achieved using these thin-layer sample holders with HiPER compared to a TE_{011} cylindrical cavity on a commercial Bruker W-band spectrometer. This study therefore highlights the advantages of using non-resonant induction mode sample holders for EPR studies of low concentration aqueous solutions at high magnetic fields, and their application to fast-motion biological samples.

2. Materials and methods

2.1. Materials

3-(2-Iodoacetamido)-PROXYL (IAP) spin labels and 2,2,6,6-Tetra methyl-1-piperidinyloxy (TEMPO) were purchased from Sigma Aldrich (St. Louis, MO). TEMPO solutions for EPR measurements were prepared in autoclaved deionized water (Barnstead NANOpure, CA). The primers for site-directed mutagenesis were purchased from Integrated DNA Technologies (Coralville, IA). *Escherichia coli* codon-optimized DNA fragment for encoding the inhibitor (IA_3) of yeast proteinase A (YprA) was purchased from DNA2.0 (Menlo Park, CA) and the corresponding protein sequence is shown in supplementary materials. Cysteine was introduced to Glu-10 via site-directed mutagenesis by using polymerase chain reaction (PCR) with the assistance of the custom designed primers. The IA_3 gene contained 8 additional codons which could be translated into two linking amino acids leucine and glutamic acid (LE) and a 6xHis-tag. The gene was then cloned into the pET-22b vector by two restriction enzyme digestion sites. The DNA sequence of IA_3 in this article was confirmed with Sanger sequencing in the Interdisciplinary Center for Biotechnology Research in the University of Florida. BL21 (DE3) PlysS cells were purchased from Invitrogen (Carlsbad, CA). Synthetic RNA fragments containing 4-thiouridine modified nucleobases were purchased from Dharmacon (Pittsburgh, PA). 0.15 mm inner diameter quartz capillary tubes for W-band Bruker EPR measurements were purchased from VitroCom (Mountain Lakes, NJ). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise indicated.

2.2. IA_3 protein expression and purification

Protein expression, purification and spin-labeling of the E10C IA_3 construct proceeded as described previously [37,43]. Briefly, protein expression was induced by Isopropyl-β-D-thiogalactopyranoside (IPTG); which was optimized by pilot expression and monitoring the intensity of the target protein band intensity in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Afterwards, the cell lysate was boiled in water and centrifuged to remove precipitants. The soluble supernatant was loaded into a nickel affinity column (GE Healthcare Life Sciences, Pittsburgh, PA) for further purifications. The residual nickel ion from the column was removed by buffer exchange with the addition of EDTA (1 mM final concentration). Finally, cysteine reduction was ensured by addition of dithiothreitol (DTT, 10 mM final concentration) for overnight reaction at 4 °C.

2.3. Spin-labeled IA_3 sample preparation

DTT was removed from the E10C IA_3 sample by buffer exchange into phosphate buffer (50 mM sodium phosphate, 300 mM sodium chloride and pH 7.4) by using a HiPrep 26/10 desalting column (GE

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