**Towards Increased Concentration Sensitivity for Continuous Wave EPR**

**Investigations of Spin-Labeled Biological Macromolecules at High Fields**

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**Introduction**

 High-field, high-frequency electron paramagnetic resonance (EPR) spectroscopy at W- (~95 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 1 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 spin-labeled biological macromolecules.

**Experimental**

 High-field EPR experiments were carried out on HiPER and a Bruker E680 W-band spectrometer (Billerica, MA) at the National High Magnetic Field Laboratory (NHMFL). The thin-layer sample holders made by Rexolite® (cross-linked polystyrene) were fabricated in-house by the NHMFL machine shop. A cysteine mutant of IA3 protein (E10C) was expressed in E.coli and subsequently purified and labeled with IAP spin label (E10P1).

**Results and Discussion**

 Several newly developed thin-layer sample holders were designed and tested for signal sensitivity (**Fig. 1A**). We first assessed the sensitivities of these holders using the radical TEMPO as a standard. We then evaluated the performance of an optimized sample holder, with a ~50L sample volume, by collecting W-band EPR spectra reflective of the unstructured to helical transition of an intrinsically disordered protein IA3. We also demonstrated a second application involving characterization of the base dynamics in large RNAs. A concentration sensitivity of 2 µM was achieved for a TEMPO standard and 20-30 µM for the biological samples (**Fig. 1B**). A ~10-fold enhancement in concentration sensitivity was achieved using these thin-layer sample holders with HiPER compared to a TE011 cylindrical cavity on a commercial Bruker W-band spectrometer (**Fig. 1C**).

**Conclusions**

**Fig. 1.** **(A)** Schematic diagram of the sample position of a thin-layer holder in the electromagnetic field. The sample is placed at half wavelength (λ) from the mirror, where the B1-field component of the reflected microwave radiation from the mirror is at a local maximum while the E-field is at a minimum. **(B)** Spectra of 2 µM and 5 µM TEMPO using an optimized sample holder H2. **(C)** Comparison of concentration sensitivity of the H2 holder in HiPER and a TE011 cylindrical cavity in a Bruker E680 spectrometer.

 These results 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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