Sample Preparation for Service at the National Biomedical EPR Center

Brian Bennett
Marquette University, brian.bennett@marquette.edu
Sample Preparation for Service at the National Biomedical EPR Center

The National Biomedical EPR Center at the Medical College of Wisconsin is an NIH-funded research resource with a three decade history of technology and methodology development, technological and scientific collaboration, home-grown scientific and biomedical research, and training and service to the EPR community [1]. Service is an important mission of the Center, and service activities include rapid-freeze-quench sample preparation, multiple EPR spectroscopies, assistance with interpretation of spectra and computer simulations, assistance with resonator and spectrometer design and maintenance, and provision of novel EPR-specific chemicals such as spin traps. Potential users are encouraged to explore our service web site [2].

Resources for EPR spectroscopy are available in a number of ways, depending on the number and types of samples and the level of experience of the user. Experienced users may visit the Center and use spectrometers with little assistance from the Center staff. Inexperienced users are encouraged to apply for a training grant and visit the Center, typically for 3–5 days, to either learn how to use a technique or a spectrometer, or else to at least get an appreciation of how the EPR data are collected, processed and interpreted in the context of their research project. Users with samples that are straightforward to prepare and interrogate may simply send samples to the Center to be analyzed by Center personnel. Whichever route is taken, it should be appreciated that the Center does not have all of the sample preparation facilities of a comprehensively equipped chemistry or biochemistry department at a research university, and users should aim to make samples as “EPR-ready” as possible at their home institution. This article is intended to provide some guidance to users with regard to sample preparation. Sample requirements vary depending on the samples, the experiment and the spectrometer characteristics, and criteria for some of the more common scenarios are described.

Frozen solutions. For frozen solutions, we offer X-band cwEPR (parallel and perpendicular B1), cwENDOR and pulsed EPR methods including ESEEM, HYSCORE, ENDOR, ELDOR, and DEER, each over a temperature range of 4–120 K. Multifrequency options include L- and S-band cwEPR and L-band NARS [4] at T > 77 K, and W-band cwEPR, Q-band cwEPR and Q-band ENDOR at T = 4–120 K. Q-band pulsed EPR is coming soon! Table 1 summarizes the EPR tube and filling requirements for each of the currently available systems; tubes are generally available as stock from Wilmad [5] or special order from VitroCom [6]. For very low background signals synthetic quartz tubes are recommended but clear fused quartz tubes suffice for most applications.

For X-band cwEPR/ENDOR at low temperatures (<70 K) the sample height is important. Samples much less than 3.5 cm in height may have the surface of the sample in an active part of the resonator and exhibit signals due to condensed molecular oxygen; this can often be dealt with by annealing, but is time (and helium) consuming. Samples >4 cm in height will not be completely surrounded by the cooling helium stream and will experience a temperature gradient along the sample. Samples much greater than 4 cm in height will partially thaw in the cryostat and experience a temperature gradient along the sample. Samples that are frozen generally do not need to be sealed, and we recommend cutting the sample tube to 16.0 ± 0.2 cm in length (Figure 1A), to allow for annealing in the cryostat to remove O2 signals if necessary. Shorter tubes can be accommodated if the experiment requires it (e.g. rapid-freeze-quench). For tubes that must be sealed under vacuum or inert atmosphere, tubes should be sealed as close to the original top of the tube (usually 25 cm) as possible, with at least 20 cm of undistorted tube beneath the seal. For L- & S-band EPR, the same applies as for X-band, except that we do not recommend cutting the 25 cm tubes.

For X-band pulsed EPR, tubes must fit into a holder and are ideally ~10 cm in length (Figure 1B). The sample height should be greater than the resonator active length. As the entire tube is cooled, there is no rigid upper limit on sample height. Sealed sample tubes must be sealed very carefully because the seal itself should not protrude outside the walls of the cylinder projected by the EPR tube, otherwise the sample may not fit into the holding rod. The “4 mm” pulsed resonator is designed for 3.8 mm tubes, but samples in 4 mm tubes can be loaded manually through the bottom of the resonator and held in place with a PTFE tape harness (Figure 1C). The tubes will be cut to ~7 cm in length and users should be generous with the sample height because the sample moves during the coupling procedure and precise sample location is not assured (Table 1).

Two common questions relate to sample concentration and the use of cryoprotectants. For X-band DEER of spin labels, a concentration of 0.2 mM is generally recommended. Though spectra have been obtained on as low as 70 μM, an eight-fold longer acquisition time is needed to collect data of the same nominal quality as from 200 μM and eventually environmental factors negate the advantages of very long scans (numbers of days). For transition ions, the necessary concentrations are highly dependent on the experiment and the metal ion. For cwEPR, a very rough guide would be >100 μM for S = 1/2 ions, octahedral Mn(II), rhombic S = 5/2 Fe(III) and high-spin heme; >200 μM for S = 3/2 Cr(III); and >500 μM for S = 3/2 Co(II) or integer-spin systems. ESEEM and ELDOR-NMR have slightly lower sensitivity whereas ENDOR (cw and pulsed) may have much lower sensitivity. Cryoprotectants are commonly used to maintain the structural integrity of biological macromolecules that would otherwise be susceptible to conformational change or damage from ice crystal
results from a lowering of strains in \( g \) and \( A \), and/or in the zero-field splitting).

**Rapid freeze quench.** Rapid freeze quench (RFQ) sample preparation for EPR (and other techniques) involves the rapid mixing of two or more solutions and freezing the mixture as a spray in a cold, immiscible solvent contained in a cooled EPR tube assembly. Typically, reactants (e.g. enzyme and substrate) are mixed at 2–40 °C and frozen in 2-methylbutane (isopentane) at –110 °C. Reaction times of 10 ms and beyond are accessible with the Center’s equipment. EPR, ENDOR, DEER etc. reveals changes in metal ion geometry and coordination, or in protein folding or conformation using spin labels. The species of interest will be diluted by a factor of either 3 or 4 in the final sample (1.5 or 2-fold dilution with e.g. substrate, and a further 2-fold “dilution” due to aqueous spheres packing in the isopentane matrix) depending on how the experiment is carried out. Therefore, concentrations of starting material for e.g. EPR, ESEEM, ENDOR need to be increased accordingly. For DEER, the final concentration in the aqueous phase should not exceed 0.2 mM, so the stock solution should be 0.3 or 0.4 mM. Generally, cryoprotectants cannot be used with RFQ; fortunately, the freezing is so rapid that they are not necessary, even for DEER. RFQ can be expensive in material, and users should plan on having at least 0.5 ml of sample for a single RFQ time-point, though some of this material is recoverable. A 1 ml sample may allow for up to four time-points.

**Aqueous samples.** Aqueous samples absorb the microwave electric field \( E_1 \) non-resonantly and require that either the sample is precisely positioned in a cavity along an axis of lowest \( E_1 \), or else a resonant structure is used that concentrates \( E_1 \) outside the active (high \( B_1 \)) region of the resonator such as the loop-gap resonator developed at the Center [7]. Both options are available and both are well-suited to the use of very small capillaries (e.g. 1 mm O.D.; Figure 1E) with sample volumes as low as 5 μl. Borosilicate capillaries are deemed necessary for DEER, which is sensitive to long range interactions, but are not generally necessary otherwise (some line-narrowing sometimes formation, and to prevent solute concentration at grain boundaries during freezing that can lead to intermolecular spin-spin interactions. Cryoprotectants are deemed necessary for DEER, which is sensitive to long range interactions, but are not generally necessary otherwise (some line-narrowing sometimes

<table>
<thead>
<tr>
<th>Resonator or cryostat</th>
<th>Experiment</th>
<th>Tube dimensions</th>
<th>Sample height (approx. volume)</th>
<th>Suggested supplier (part number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxford Instr. ESR900 cryostat</td>
<td>X-band cwEPR, cwENDOR</td>
<td>~4 mm O.D.</td>
<td>3.5–4.0 cm (250–300 μl)</td>
<td>Wilmad (707-SQ-250M)</td>
</tr>
<tr>
<td>Bruker EN4118X-MD-4W 4 mm dielectric</td>
<td>X-band pulsed (incl. ENDOR)</td>
<td></td>
<td>&gt;1.3 cm (100 μl)</td>
<td>Wilmad (706-PQ-9.50) or (706-SQ-250M)</td>
</tr>
<tr>
<td>Bruker ER4118X-MS-2 2 mm dielectric</td>
<td>X-band pulsed (not ENDOR)</td>
<td>&lt;2 mm O.D.</td>
<td>&gt;1.3 cm (10 μl)</td>
<td>VitroCom</td>
</tr>
<tr>
<td>MCW EPR Ctr. 4 mm loop-gap</td>
<td>L- &amp; S-band</td>
<td>~4 mm O.D.</td>
<td>&gt;1 cm (100 μl)</td>
<td>Wilmad (707-SQ-250M)</td>
</tr>
<tr>
<td>Bruker ER5106-QTE cavity</td>
<td>Q-band cwEPR/ENDOR (4–70 K &amp; 295 K)</td>
<td>1.6 mm O.D.</td>
<td>&gt;1.4 cm (10 μl)</td>
<td>Bruker, VitroCom or Wilmad (WG-221T-RB)</td>
</tr>
<tr>
<td>Varian Q-band cavity</td>
<td>Q-band cw-EPR (&gt;77 K)</td>
<td>2 mm O.D.</td>
<td>&gt;1.3 cm (10 μl)</td>
<td>VitroCom</td>
</tr>
</tbody>
</table>

**Figure 1.** Sample tubes for EPR. (A) 160 × 4 mm O.D. X-band EPR tube for cwEPR/ENDOR. (B) 100 × 3.8 mm O.D. tube for pulsed X-band EPR, mounted in sample holder. (C) 70 × 4 mm O.D. X-band EPR tube bottom-mounted in pulsed EPR resonator. (D) 100 × 1.6 mm O.D. Q-band EPR/ENDOR tube. (E) 10 × 0.32 mm O.D. capillary for aqueous solution EPR at X- or Q-band. (F) 100 × 1.6 mm O.D. capillary for aqueous EPR at X-band, sealed at the bottom with ChaSeal and containing ~2.5 cm of aqueous sample.
ies (micropipettes) are cheap and work well for reasonably concentrated samples, and are available at the Center. For more challenging applications, clear fused quartz capillaries are available from VitroCom. For weak signals, a flat cell can be used in conjunction with a rectangular cavity, though the loop-gap resonator has largely superseded this approach. Capillaries are sealed at one or both ends with sealing compound (e.g. Cha-Seal; Figure 1F). For gas exchange experiments, TPX capillaries are available from Bruker [8].

Solid samples. The Center has historically had very few requests for single crystal studies on minerals or other materials science studies and we recommend that any potential users contact us directly. On the other hand, we have had many samples of powders for study. At room temperature, a powder sample in an EPR tube is straightforward, though we urge the user to be careful to ensure that the outside of the tube is clean. At cryogenic temperatures, heat transfer is very poor from the sample to the cryostat, and if the powder can be suspended in an inert matrix, such as paraffin oil or glycerol, then heat transfer will be much improved and temperatures reliable. Powders of undiluted paramagnetic complexes may absorb very strongly, and it is recommended that the sample be ground very finely with an inert material, e.g. 1% sample in boron nitride. Polycrystalline material will exhibit incompletely averaged powder spectra; again, fine grinding helps alleviate this. Finally, undiluted paramagnetic complexes may exhibit quite strong intermolecular spin-spin interaction and paramagnetic doping, where possible, will remove this problem (e.g. 1:99 Co(II):Zn(II)).

Safety. It is imperative that the Center is made aware of any safety issues regarding samples at the time of the initial application. Potentially hazardous samples include human material, pathogens, toxins, poisons and oxidants and corrosive compounds, pyrophoric and explosive compounds, and environmental hazards. Sealed samples should be tested thoroughly for leaks that could admit gas at low temperature that can explosively expand upon warming the tube.

References
1. http://www.mcw.edu/EPRCenter.htm