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Communication

Concentration by centrifugation for gas exchange EPR oximetry measurements with loop–gap resonators

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Abstract

Measurement of the bimolecular collision rate between a spin label and oxygen is conveniently carried out using a gas permeable plastic sample tube of small diameter that fits a loop–gap resonator. It is often desirable to concentrate the sample by centrifugation in order to improve the signal-to-noise ratio (SNR), but the deformable nature of small plastic sample tubes presents technical problems. Solutions to these problems are described. Two geometries were considered: (i) a methylpentene polymer, TPX, from Mitsui Chemicals, at X-band and (ii) Teflon tubing with 0.075 mm wall thickness at Q-band. Sample holders were fabricated from Delrin that fit the Eppendorf microcentrifuge tubes and support the sample capillaries. For TPX, pressure of the sealant at the end of the sample tube against the Delrin sample holder provided an adequate seal. For Teflon, the holder permitted introduction of water around the tube in order to equalize pressures across the sealant during centrifugation. Typically, the SNR was improved by a factor of five to eight. Oxygen accessibility applications in site-directed spin labeling studies are discussed.

Keywords: EPR; Lipoproteins; Loop–gap resonator; Oximetry; Site-directed spin labeling

1. Introduction

Both the loop–gap resonator [1,2] and the methylpentene polymer (TPX) (Mitsui Chemicals) capillary [3–5] were introduced for EPR measurements at the National Biomedical EPR Center of the Medical College of Wisconsin. Loop–gap resonators provide improved sensitivity for EPR experiments under conditions where the sample size is limited. This class of studies includes site-directed spin labeling experiments [6,7]. X-band loop–gap resonators have been optimized for samples of a few microliters, and a Q-band loop–gap resonator was constructed for samples containing only 30 nL [8,9]. The active region of a loop–gap resonator varies in length, depending on the design. It can be as short as 1 mm for Q-band [6,9] or 2.5 mm for X-band [1]. The active length of a typical X-band loop–gap resonator is 5 mm, with an inside diameter of 0.6–1.0 mm, resulting in a sample volume of about 2 μL.

Typically, the X-band sample tubes are capillaries machined from TPX with dimensions of 0.6 mm ID, 0.1 mm wall thickness, and 25 mm length. This plastic is permeable to oxygen, nitrogen, and other gases and is substantially impermeable to water. The capillary is fixed inside the loop–gap resonator with a special Teflon holder. TPX rods from which the capillaries are machined can be purchased from Midland Plastic, (Madison, WI) and TPX capillaries together with the holder can be obtained from Molecular Specialties (Milwaukee, WI).

Flow of dry nitrogen over the TPX capillary allows easy deoxygenation of samples to study lineshapes [4] and to measure spin–lattice relaxation times (T_1) [10]. Also, sample oxygenation can be readily controlled for oxygen accessibility studies. Major progress in spin

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label oximetry was made using the oxygen permeable capillary design [5,11]. The field has recently been reviewed [12].

The three-loop–two-gap resonator developed for 35 GHz Q-band measurements with aqueous samples has a sample loop of 0.65 mm diameter [9]. Because of difficulties in machining a TPX capillary to match this size, a Teflon tube from Zeus (Orangeburg, SC) with an ID of 0.25 mm, wall thickness of 0.075 mm and length of 55 mm was used for Q-band measurements. Teflon is not as favorable as TPX for transport of gases such as N_2, O_2, or air, but because of the thin wall, it works well for this purpose [13].

The combination of a loop–gap resonator and oxygen permeable capillary has been used not only in conventional X-band and Q-band EPR spectroscopy, but also in saturation-recovery EPR [9,14–17], and multiquantum EPR [18–20].

Concentration by centrifugation has been used by other investigators in EPR, but always, to the best of our knowledge, with glass or quartz capillaries. See, for example, the paper by Kurad et al. [21] who use flame-sealed quartz capillaries. With plastic capillaries, which are a necessity for gas-exchange methods, a sealant is used at the end of the tube. We found these seals are easily broken during centrifugation, which may be caused by increasing deformation of the plastic tube as the rotation speed increases. Solutions of this problem are described here.

2. Materials and methods

Handling samples with a volume of only a few microliters or less, especially in the case of samples with high viscosity and density, such as liposome suspensions, creates certain problems. We propose an approach that allows for an increase in the sample volume of up to about 25 μL (for X-band) and about 3 μL (for Q-band) for easy handling and transfer into a TPX or Teflon capillary. After transfer, the sample can be concentrated by centrifugation of the capillary, so that the pellet at the bottom of the capillary matches the length of the loop–gap resonator active region. We designed special plastic (Delrin) holders to be positioned in the Eppendorf microcentrifuge tube into which the capillary can be inserted.

Fig. 1A shows schematic drawings of the plastic holder and TPX capillary in an Eppendorf microcentrifuge tube. Before centrifugation, the sample is sucked into the capillary or tube and the end is sealed with Baxter Miniseal wax B4425.1. This sealant is no longer commercially available, but can be found in many laboratories. Other tube sealants can be used including two that we evaluated: Critoseal (Fisher Scientific) and X-Sealant (Bruker Biospin). The latter handled particularly well in

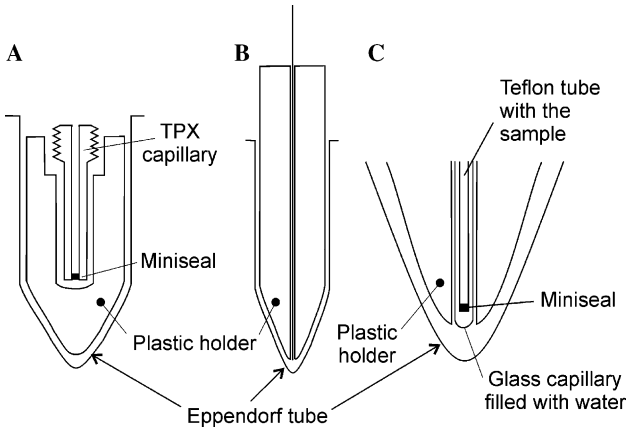

Fig. 1. (A) Schematic drawing of the plastic holder for centrifugation of an EPR sample in a TPX capillary. The depth of the small hole in the plastic holder is less than the length of the capillary, allowing tight contact of the capillary with the bottom of the hole during centrifugation. (B and C) Schematic drawings of the centrifugation assembly for the Teflon tube. The plastic holder with a glass capillary (ID 0.60 mm, OD 0.84 mm, and length 50 mm) sealed at one end is positioned in an Eppendorf tube. The Teflon tube is positioned inside this glass capillary which is filled with water. Aqueous material on both sides of the wax seal was found to be necessary to keep the seal in place during centrifugation.

sealing the Teflon tube. Liposome suspensions are then centrifuged at 16,000*g* for 15 min at 4 °C. The plastic holder for the TPX capillary (Fig. 1A) is designed so that the capillary and the seal are pushed towards the bottom of the holder during centrifugation, which keeps the sample inside the capillary. For this purpose, the TPX capillary fits loosely inside the plastic holder and slides easily.

Figs. 1B and C illustrate the plastic holder that was developed for use with Q-band samples. Teflon tubes from Zeus with the next bigger or the next smaller diameter cannot be used because of difficulty with tuning the resonator that was used. The Teflon tube is rather flexible. It must be introduced into the resonator through a carefully made centering and support assembly, because small deviations from the center create tuning problems.

The three-loop–two-gap resonator used at Q-band can be tuned only when the Teflon tube is filled with aqueous sample and inserted. The 55-mm-long Teflon tube that was used contains 2.7 μL of the sample. After the dilute sample is drawn into the Teflon tube, the tube is sealed with Miniseal wax and placed inside a glass capillary that contains water, which in turn is placed in a plastic holder that fits into an Eppendorf tube for centrifugation. Aqueous material on both sides of the wax seal was found to be necessary to keep the seal in place during centrifugation. The levels of water inside and outside the Teflon tube must be the same to equilibrate the forces acting on the seal. This procedure is illustrated in Figs. 1B and C.

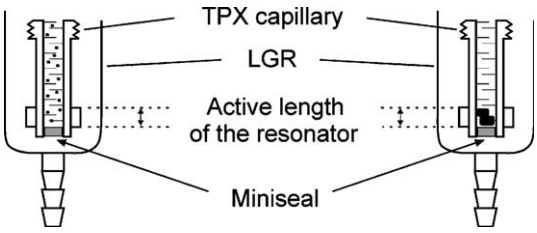

Fig. 2. Schematic drawings indicating positions of the sample relative to the active length of the resonator before and after centrifugation. (Left) Before centrifugation, sample is distributed over the full length of the TPX capillary, ~25 mm. (Right) After centrifugation, sample is pelleted at the capillary bottom to a length of ~5 mm.

Schematic drawings in Fig. 2 for a TPX capillary and the X-band loop–gap resonator indicate positions of the sample relative to the active length of the resonator before and after centrifugation. The same principle applies for the Teflon tube and three-loop–two-gap resonator used at Q-band.

The oxygen concentration in both TPX and Teflon capillaries is controlled by equilibrating the sample with a nitrogen–oxygen mixture adjusted using a flowmeter (Matheson Gas Products, Model 7631 H-604). The gas is also used for temperature control.

Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids (Alabaster, AL). *n*-Doxylstearic acid spin labels (*n*-SASL, $n = 5$ and 16) were purchased from Molecular Probes (Eugene, OR).

3. Results and discussion

The paramagnetic sample that is distributed along the entire length of the capillary (typically 25 mm for the TPX capillary and 55 mm for a Teflon tube) is pelleted at the capillary bottom. The typical inside diameter of the TPX capillary used with the X-band loop–gap resonator is 0.5–0.6 mm, so that the capillary holds 2–3 μL per cm of capillary length. The sample can be concentrated (pelleted) to the typical active length of the X-band loop–gap resonator (5 mm), giving approximately a fivefold increase in EPR signal intensity. Exact positioning of the concentrated sample within the active length of the resonator is necessary.

The length of the Teflon capillary that was used for Q-band (55 mm) was determined by convenience in sample handling. In our hands, the length of the pellet is about 7 mm. Since the length of the active volume of the resonator is 1 mm, the issue of sample positioning does not arise.

EPR spectra recorded at X-band from a TPX capillary containing a dimyristoylphosphatidylcholine (DMPC) liposome suspension (~3% w/w) labeled with 16-doxylstearic acid spin label (16-SASL) (16-SASL/DMPC = 1/100) before and after centrifugation are shown in Figs. 3A and B. Both spectra were recorded at the same spectrometer settings (gain, microwave power, modulation amplitude), indicating that the signal-to-noise ratio increased after centrifugation by a factor of ~4.5. EPR spectra recorded at Q-band from a Teflon tube containing a DMPC liposome suspension (~3% w/w) labeled with 5-doxylstearic acid spin label (5-SASL) (5-SASL/DMPC = 1/100) before and after centrifugation are shown in Figs. 3C and D. The increase in EPR signal amplitude after centrifugation depends on the initial sample concentration and on the time of centrifugation. As can be seen from Figs. 3C and D, for a liposome suspension containing 3.4 mg DMPC in 100 μL, the EPR signal amplitude increased about eight times. Of course, the observed increase in signal-to-noise can alternatively be traded for a reduced acquisition time.

Another advantage was found for liposome suspensions. In less dense preparations, these suspensions exhibit a tendency for irregular clumping along the sample tube. The sample can be distributed unevenly along the tube, with spots of practically pure buffer and spots of concentrated sample. This was a particular complication during measurements with a loop–gap resonator of small (1 mm) active length. The sample position seemed

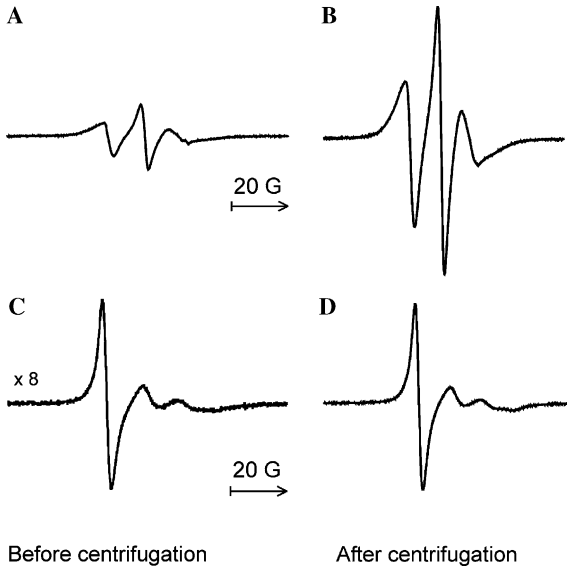

Fig. 3. (A and B) EPR spectra from a suspension of DMPC liposomes (3.4 mg DMPC/100 μL) containing 1 mol% of 16-SASL recorded at X-band with a loop–gap resonator, active region 5 mm in length. (A) Before centrifugation, the sample is distributed over the full length of the TPX capillary, ~25 mm. (B) After centrifugation, the sample is pelleted on the capillary bottom to a length of ~5 mm. (C and D) EPR spectra from a suspension of DMPC liposomes (3.4 mg DMPC/100 μL) containing 1 mol% of 5-SASL recorded at Q-band with a loop–gap resonator, active region 1 mm in length. (C) Before centrifugation, the sample is distributed over the full length of the Teflon tube, ~55 mm. (D) After centrifugation, the sample is pelleted on the capillary bottom to a length of ~7 mm.

almost unpredictable. Pelleting the sample inside the capillary eliminated this difficulty.

This paper contributes to loop–gap resonator technology. Methods are described for increasing the concentration of spin-labeled proteins and lipids in proteolipid samples by formation of pellets at the tip of plastic capillaries through centrifugation. The main "technical" problem is preservation of the integrity of the wax seal at the end of the capillary. Two solutions have been found. In the first, centrifugal force of the sealed capillary against the end of the Eppendorf tube preserves the seal. In the second, the sample is presumed to be essentially aqueous. A water bath envelops the capillary, resulting in canceling forces on either side of the seal during centrifugation. In both of the geometries described here, use of plastic capillaries facilitates gas exchange across the capillary walls, which is valuable in the techniques known as spin label oximetry. There seems to be no disadvantages in formation of pellets of proteolipid samples when using spin labels to obtain information on proteins or lipid dynamics. Caution is advisable, however, when using the methods of this paper in the study of water-soluble proteins because critical protein–protein interactions may be disrupted.

The technique described here involving the concentration of membrane proteins within an EPR sample tube has a number of applications for SDSL studies where material is limited. The ability to concentrate the typical 5–10 μL of sample into the 0.03–2 μL active region of a resonator within the actual sample tube is felt to be a significant advance in membrane–protein sample handling. The signal-to-noise ratios dramatically increase because the entire sample is contained within the resonator. This is advantageous especially for under-labeled protein samples, which is often the case in spin labeling studies of membrane proteins.

In addition to enhancing the signal-to-noise ratios, this technique improves the sample homogeneity. For example, in samples where the membrane fraction settles to the bottom of the sample tube during the course of data collection, the EPR spectrum can change over time due to the settling of the labeled proteins. If the sample is not completely contained within the active region of the resonator, the signal may be lost due to settling below the active region and be erroneously skewed toward the spectrum of the spins located in the soluble fraction.

This technique will also be beneficial to the study of binding proteins or substrates to membrane proteins. By concentrating only the membrane-bound fractions of labeled substrate, the signal intensity of the bound fraction will be highly enhanced and better differentiated from the unbound fraction of substrate. In addition, in double label studies where one spin label is located within the membrane protein and a second label is located on the binding protein or substrate, spectra would be greatly enhanced if the unbound excess substrate were removed from the sample by pelletting of the membrane-bound fraction within the sample tube.

Finite-element design of a loop–gap resonator for use at 94 GHz (W-band) was presented in poster format [22]. The structure was evaluated for aqueous samples using a Teflon tube from Zeus of the next available smaller size (0.203 mm ID). It therefore seems certain that the methodology of this paper can be extended from Q-band to W-band.

Acknowledgments

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