

# The Measurement of Temperature With Electron Paramagnetic Resonance Spectroscopy

J. J. Eckburg

J. C. Chato

K. J. Liu<sup>2</sup>

M. W. Grinstaff<sup>3</sup>

H. M. Swartz<sup>2</sup>

K. S. Suslick

F. P. Auteri<sup>4</sup>

Departments of Mechanical and Industrial Engineering, Chemistry, and Internal Medicine, University of Illinois at Urbana-Champaign, Urbana, IL 61801

*An electron paramagnetic resonance (EPR) technique, potentially suitable for in vivo temperature measurements, has been developed based on the temperature response of nitroxide stable free radicals. The response has been substantially enhanced by encapsulating the nitroxide in a medium of a fatty acid mixture inside a proteinaceous microsphere. The mixture underwent a phase transition in the temperature range required by the application. The phase change dramatically altered the shape of the EPR spectrum, providing a highly temperature sensitive signal. Using the nitroxide dissolved in a cholesterol and a long-chain fatty acid ester, we developed a mixture which provides a peakheight ratio change from 3.32 to 2.11, with a standard deviation of 0.04, for a temperature change typical in biological and medical applications, from 38 to 48°C. This translated to an average temperature resolution of 0.2°C for our experimental system. The average diameter of the nitroxide mixture-filled microspheres was  $\approx 2 \mu\text{m}$ . Therefore, they are compatible with in vivo studies where the microspheres could be injected into the microvasculature having a minimum vessel diameter of the order of  $8 \mu\text{m}$ . This temperature measuring method has various potential clinical applications, especially in monitoring and optimizing the treatment of cancer with hyperthermia. However, several problems regarding temperature and spatial resolution need to be resolved before this technique can be successfully used to monitor temperatures in vivo.*

## Introduction

Hyperthermia has become a useful clinical tool in the treatment of tumors, usually in combination with chemotherapy or radiotherapy. For adequate treatment and safety considerations, the tumor tissue temperature should be maintained higher than a certain value (e.g., 42 or 43°C) for 30 to 60 minutes while the surrounding normal tissue should be kept below that value. An important part of the treatment with hyperthermia is the proper monitoring of the actual temperatures produced in the tumor and surrounding tissue (Levin, 1990). There are many techniques available that utilize invasive probes to measure in vivo temperatures. However, with such techniques only the temperature close to the probe (or probes) is monitored. There are also the risks and discomfort for the patient associated with an invasive procedure (Samulski and Fessenden, 1990). There are also several noninvasive temperature measurement techniques currently under investigation. However, they are at different stages of development for clinical use and suffer from various limitations, including poor temperature resolution, inadequate spatial resolution and long data acquisition times (Bolomey and Hawley, 1990).

The objective of this research was to develop a minimally invasive system for measuring temperature fields in vivo using electron paramagnetic resonance (EPR) techniques. This new technique is based on the dramatic change in the EPR spectrum of a suitable paramagnetic species, especially a nitroxide stable

free radical, during the solid to liquid phase transition of its surrounding medium encapsulated in a microsphere (Figs. 1 and 2). The shape of the EPR spectrum can be calibrated to a known temperature. Therefore, when given an in vivo EPR spectrum, it is possible to determine the temperature from the shape of the spectrum. The potential novelty of this system includes the use of 1) the temperature dependence of EPR spectra in a medium that undergoes a solid to liquid phase transition in the desired temperature range, 2) proteinaceous microspheres to provide a constant, appropriate and protective environment for the nitroxide in the medium, and 3) EPR spectroscopy to obtain information in vivo.

## Materials and Methods

The composition of the medium determined the temperature range of the phase transition. Using the nitroxide 3-doxyl-5 $\alpha$ -cholestane, 1 mol percent cholesterol and 99 mol percent methyl docosanoate (a long chain fatty acid ester), we have obtained a mixture that underwent a large and systematic change in the shape of its EPR spectrum between 38 and 48°C (Fig. 2). This formulation was then encapsulated in an albumin microsphere (average diameter  $< 2 \mu\text{m}$ ) using high intensity ultrasound (Fig. 3). With this system we were able to obtain a temperature resolution of 0.2°C in the temperature range of 38 to 48°C.

The albumin microspheres performed two critical functions. First, these proteinaceous microspheres were suitable for use in vivo. They were easily injectable into tissue because they were smaller than the diameter of capillaries and were suspended in water. Second, the nitroxide mixture was encapsulated in the microspheres and, therefore, separated from the local environment of the tissue. This ensured that the shape of the EPR spectrum was affected only by the medium's phase transition temperature range.

<sup>1</sup> Current Address: University of Wisconsin Medical Center, Madison, WI.

<sup>2</sup> Current Address: Dartmouth Medical School, Hanover, NH.

<sup>3</sup> Current Address: California Institute of Technology, Pasadena, CA

<sup>4</sup> Current Address: Cummins Engine Co., Columbus, IN

Contributed by the Bioengineering Division for publication in the JOURNAL OF BIOMECHANICAL ENGINEERING. Manuscript received by the Bioengineering Division December 31, 1993; revised manuscript received November 5, 1995. Associate Technical Editor: K. R. Diller.

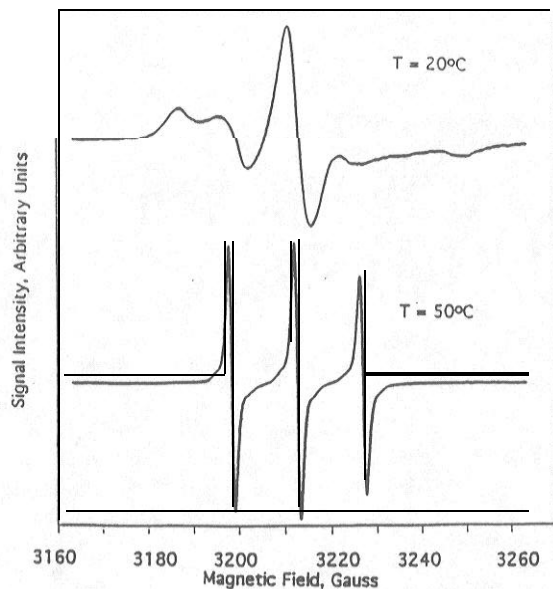


Fig. 1 EPR spectra showing the change in the lineshape from the solid ( $T = 20^{\circ}\text{C}$ ) state to the liquid ( $T = 50^{\circ}\text{C}$ ) state for 7-doxyl stearic acid in methyl nonadecanoate encapsulated in microspheres

The nitroxide, 3-doxyl-5 $\alpha$ -cholestane, is an analog of cholesterol and the doxyl stearic acid nitroxides are fatty acid analogs. Experiments were performed on a Varian E109E X-band (9.2 GHz) spectrometer with a Varian  $TE_{102}$  cavity using an EPR data acquisition system (Morse, 1987). Other experiments were performed on an L-band (1.1 GHz) bridge with a surface probe connected to a Varian E-9 spectrometer.

The nitroxide/fatty acid/cholesterol mixture was encapsulated in microspheres using high intensity ultrasound as described by Suslick and Grinstaff (1990). The high intensity ultrasound created alternating regions of compression and expansion in the liquid mixture. This resulted in acoustic cavitation: the formation, growth and implosive collapse of gaseous bubbles in the liquid. These gaseous bubbles imploded violently in less than a millisecond, heating and compressing its contents to 5500°C and  $> 10^7$  atmospheres. The high temperature and pressure decomposed water into hydrogen atoms and hydroxyl radicals. In the presence of oxygen, the hydrogen atoms formed superoxide,  $\text{HO}_2\cdot$ , which is known to produce disulfide bonds

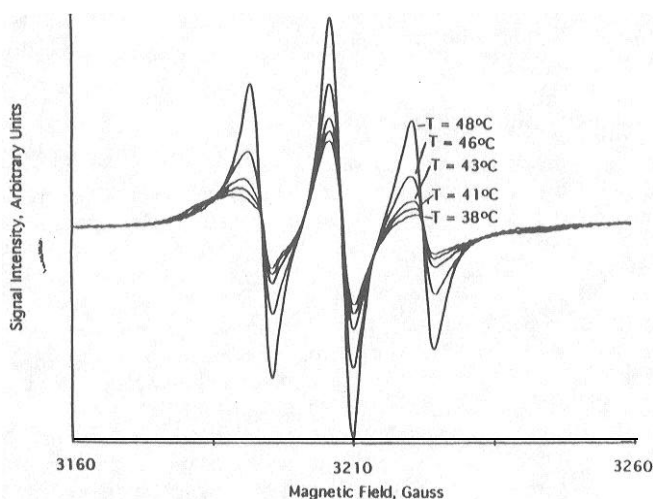


Fig. 2 Temperature dependence of the EPR spectra between 38 and 48°C for 0.8 mM 3-doxyl-5 $\alpha$ -cholestane in 1 mol percent cholesterol and 99 mol percent methyl docosanoate encapsulated in microspheres

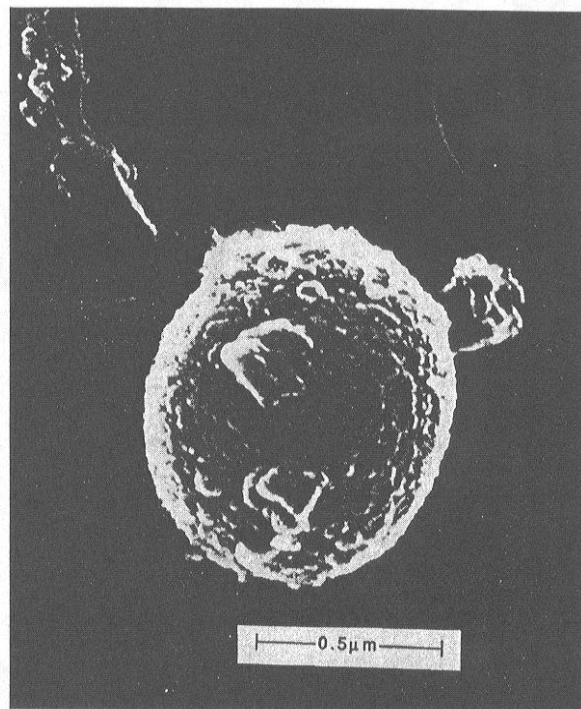


Fig. 3 Electron micrograph of a typical albumin microsphere that encapsulates 3-doxyl 5 $\alpha$ -cholestane in 1 mol percent cholesterol and 99 mol percent methyl docosanoate

between cysteine residues in proteins. Concurrently, the sonication also produced emulsification of the non-aqueous nitroxide/fatty acid/cholesterol mixture in an aqueous bovine serum albumin (BSA) solution. BSA is a large protein (585 amino acids, MW = 65,000) and has a large number of cysteines. After aggregation of the BSA at the emulsion interface, the superoxide produced from the cavitation cross-linked the cysteine residues, completing the microsphere formation (Suslick, 1989, 1990).

For the temperature dependent experiments, a nitroxide/alcohol solution was placed into a glass vial and the alcohol was evaporated off by vacuum. Fatty acid and cholesterol were then added and the mixture was repeatedly heated and vortexed (6 or 7 times) for approximately 30 seconds to ensure adequate mixing. Approximately 1.5 ml of the nitroxide/cholesterol/fatty acid mixture was then placed above 3 ml of a 5 percent aqueous solution of BSA in a glass container, creating a two-phase solution. The preparation of the 5 percent aqueous solution of BSA is described by Grinstaff (1992). A heating rod was set to approximately 70°C, inserted into the glass container and used to selectively melt the top layer of the nitroxide mixture without significantly heating, and consequently denaturing, the lower layer BSA solution. The entire solution was then sonicated using a Heat Systems W375 ultrasonic 0.5 in. Titanium horn at 20 kHz at 200 W/cm<sup>2</sup> for three minutes, producing two separable phases: an aqueous phase with the microspheres and an organic phase made of the leftover nitroxide/fatty acid/cholesterol mixture (Fig. 4). The microsphere solution was then drawn into a gas-permeable teflon tube with an inside diameter of 0.8 mm and a wall thickness of 0.04 mm. This tube was folded and inserted into an open ended quartz tube, which was then inserted into the EPR cavity. The sample was heated with nitrogen or air using a Varian temperature controller containing an electrical heating element. Temperature measurements were recorded using a Luxtron 755 Multichannel Fluoroptic Thermometer and a Luxtron MSA Fluoroptic Temperature Probe. The Fluoroptic Probe does not interact with magnetic fields. The probe was placed inside the quartz tube, in contact with the teflon tube. By experimental design there was no thermal gradient between the microsphere solution in the teflon tube and the Luxtron

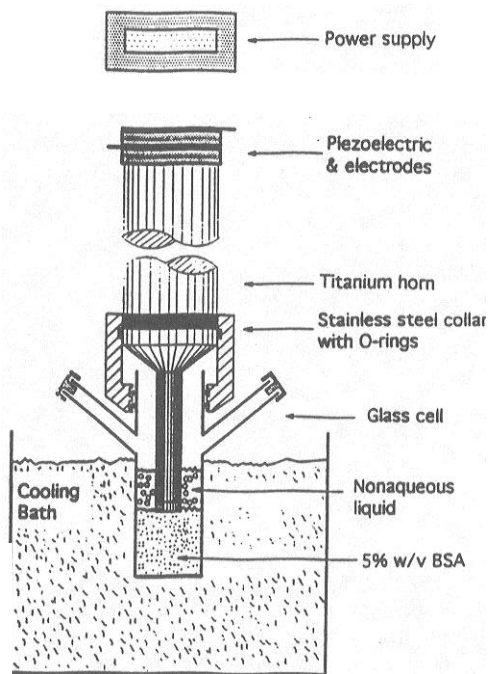


Fig. 4 Experimental apparatus for production of microspheres using

probe. Therefore, it was assumed that the temperature recorded by the probe was essentially that of the microspheres.

To determine the extent of nitroxide reduction in ascorbic acid or fresh whole blood, the microspheres (approximately 0.1 ml) were added to an oxygen depleted solution of ascorbic acid or heparinized blood (approximately 1 ml), vortexed and drawn into the teflon tube. Nitrogen was perfused over the sample while in the EPR cavity to prevent oxidation of the ascorbic acid, which would destroy its reducing potential. Heparin was added to the fresh whole blood to prevent clotting. The extent of reduction was determined by recording the signal intensity as a function of time.

For the *in vivo* experiments, approximately 0.2 ml of the microspheres, containing approximately 1 mM 7-doxyl stearic acid in cyclohexane, were injected into the tail vein of an anesthetized mouse and measurements were recorded at room temperature at L-band using a surface probe (Nilges et al., 1989) over the lower abdomen of the mouse. Again, the extent of reduction was determined by recording the signal intensity as a function of time.

One dimensional, linear magnetic field gradient coils were also used in an L-band experiment with the microspheres. The coils were made in our laboratory. They allowed the simultaneous recording of EPR spectra of separate spin-labeled probes in the same magnetic field by altering the magnetic field positions for resonance, as explained in the Theory section. The experiment illustrated the line shape distortion due to a magnetic field gradient that occurred for large sample volumes (approximately 0.5 ml). A small plastic vial containing the microspheres was placed on the surface probe and the EPR signal was recorded twice, once with the gradient on and once with it off.

Preliminary experiments were performed with the doxyl stearic acid nitroxides. However, in experiments involving the albumin microspheres, the temperature dependent EPR spectra changes were severely limited due to the strong affinity of the nitroxides to the albumin microspheres. Our search for an inexpensive, available nitroxide that was lipid soluble and would not bind tightly to the albumin microspheres led us to 3-doxyl-5 $\alpha$ -cholestane.

## Theory

Electron paramagnetic resonance specifically occurs in paramagnetic molecules, i.e., molecules with unpaired electrons (e.g., free radicals, transition metals, and synthetic stable free radicals). Consider the electron as a small negatively charged object possessing a quantum mechanical property that is analogous to spinning on its axis. This "spin"  $S$  is limited to  $2S + 1$  discrete states. For an unpaired electron,  $S = \frac{1}{2}$ , so there are only two possible spin states, considered as spins in opposite directions. The value of  $S$  taken along a specified direction ( $M_s$ ) is then  $+\frac{1}{2}$  or  $-\frac{1}{2}$ . The spin states of an electron are usually labeled by their values of  $M_s$ . In the presence of an applied magnetic field, the quantum nature of the unpaired electron restricts it to two possible orientations (and hence two different energy states), parallel ( $M_s = +\frac{1}{2}$ ) or anti-parallel ( $M_s = -\frac{1}{2}$ ) to the field with an energy difference,  $\Delta E$ , as described in Eq. (1). This is known as Zeeman splitting. Electromagnetic radiation, with a frequency corresponding to the difference in energy between these two energy states, can induce transitions of the electron between these energy states. EPR spectroscopy uses the existence of these two different energy states in a magnetic field to give an absorption spectrum based on these transitions (Swartz and Swartz, 1983).

The basic equation for an energy level transition (resonance absorption) for an unpaired electron is:

$$\Delta E = h \cdot \nu = g \cdot B \cdot H, \quad (1)$$

where

$\Delta E$  = the energy difference between the two energy states

$h$  = Planck's constant

$\nu$  = frequency of electromagnetic radiation

$g$  =  $g$  factor

$B$  = Bohr magneton

$H$  = resonance magnetic field

Since  $h$ ,  $g$ , and  $B$  are constants,  $\nu/H_r = g \cdot B/h = \text{constant}$ , and resonant absorption by an unpaired electron can occur at various combinations of magnetic field strength and microwave radiation frequency (Swartz and Swartz, 1983). In addition, for resonance to occur, the magnetic component of the electromagnetic radiation must be polarized perpendicular to the static magnetic field. For reasons of sensitivity, frequencies in the microwave region of the electromagnetic spectrum are used. In conventional X band spectroscopy,  $\nu = 9.5$  GHzertz and  $H_r = 3400$  Gauss (Swartz et al., 1972).

The  $g$  factor in Eq. 1 is a universal constant and is characteristic of the electron ( $g_e = 2.00232$ ), provided that  $H_r$  is the magnetic field at the electron. However, an applied external magnetic field may generate an internal magnetic field which will add to or subtract from the external field. For convenience,  $H_r$  is defined as the external magnetic field; any local magnetic fields are accounted for by allowing the  $g$  factor to vary:

$$g_{\text{eff}} = h \cdot \nu / B \cdot H_r, \quad (2)$$

where  $g_{\text{eff}}$  = "effective"  $g$  factor. The effective  $g$  factor can, therefore, be considered as a characteristic quantity of the molecule in which the unpaired electron is located.

The main source of the local magnetic fields is an orbital magnetic moment caused by a mixing of excited states into the ground state. For most molecules the admixture of excited states is anisotropic, i.e., the magnitude of the induced local field (hence the deviation of  $g$  from  $g_e$ ) depends on the molecule's orientation with respect to the applied external magnetic field (Swartz et al., 1972).

In addition to the local fields induced by the applied external magnetic fields that lead to different  $g$  factors, there are also local fields that are permanent and do not depend on an external field. These permanent local fields are created by the presence

of other magnetic moments, usually those of magnetic nuclei that have net spins in the molecule. This interaction of an unpaired electron with a nuclear magnetic moment is called nuclear hyperfine splitting. The spin of a nucleus is characterized by the quantum number  $I$ . There are  $2I + 1$  possible nuclear spin states and, therefore, resonance may be observed at  $2I + 1$  values of the external magnetic field (Swartz et al., 1972).  $I = 1$  for nitrogen (nitroxides are used in this research). This accounts for the occurrence of resonance at three different values of the external magnetic field (e.g., see Fig. 1,  $T = 50^\circ\text{C}$ ). Similar to the  $g$  factor, nuclear hyperfine splitting is also anisotropic.

These anisotropies are the basis for the EPR spectral changes with temperature. The magnitude of the anisotropies depends on the angle between the axis of the molecule and the applied external magnetic field. When the nitroxide molecule is immobilized, as in a solid medium, the spectrum is broadened, reflecting the various external magnetic field values where resonance occurs. When the nitroxide is freely rotating, as in a low viscosity liquid medium, the anisotropies are averaged out and the spectrum is narrowed (Fig. 1).

Linewidths, peakheight ratios, or any other components of the spectrum could be used to calculate a temperature dependence of the spectrum. In principle, the more features of the spectrum that can be used, the better the temperature resolution. Computer simulation of the whole spectrum could, therefore, provide the greatest temperature resolution if the unavoidable noise could be sufficiently reduced. Excluding computer simulation, the mid-field to high-field peakheight ratio was the parameter which gave the best temperature resolution for the spectra in Fig. 2. Neither of the other two peakheight ratios nor any of the peak-to-peak linewidths of the three hyperfine splittings provided better temperature resolution.

"Order" parameters and rotational correlation times are parameters that have been used to assess the relative motions of nitroxides in biological systems and hence, may also serve as temperature dependent parameters. Calculations of two correlation times,  $T_c$  and  $R$ , (Swartz, 1985) did not provide better temperature resolution. It was impossible to determine the "order parameter"  $S$  (Capaldi, 1977) from the spectrum because the high-field end of the parallel hyperfine component was not discernible. In addition, the perpendicular hyperfine component did not change (Eckburg, 1993a).

## Results and Discussion

**Temperature Dependence and Sensitivity in Microspheres.** Figure 2 shows the temperature dependence of the EPR spectra between  $38$  and  $48^\circ\text{C}$  for  $0.8$  mM 3-doxyl-5 $\alpha$ -cholestane in 1 mol percent cholesterol and 99 mol percent methyl docosanoate encapsulated in the proteinaceous microspheres. Figure 5 shows a plot of the mid-field to high-field peakheight ratio versus temperature for the spectra in Fig. 2. During the increase in temperature the resolution, defined as the minimum discernible change, using the peakheight ratio shown in Fig. 5 was  $0.2^\circ\text{C}$ . Similar but somewhat different spectra were obtained when the temperature was lowered from  $47$  to  $37^\circ\text{C}$ , resulting in a hysteresis effect below approximately  $43^\circ\text{C}$  as shown in Fig. 5 and discussed below. The average temperature resolution decreased to  $0.4^\circ\text{C}$  when the temperature was lowered from  $47$  to  $37^\circ\text{C}$ .

The hysteresis was avoidable if the temperature range in the application of this system was kept at less than approximately  $3^\circ\text{C}$ . It is, however, potentially problematic, and its discussion is warranted.

For thermally induced phase transformations, two independent factors can be identified: (1) the magnitude of the enthalpy change in the system undergoing the process and (2) the rigid crystal lattice in a solid, which is normally unaffected by temperature unless heated to its melting point. When heated, the

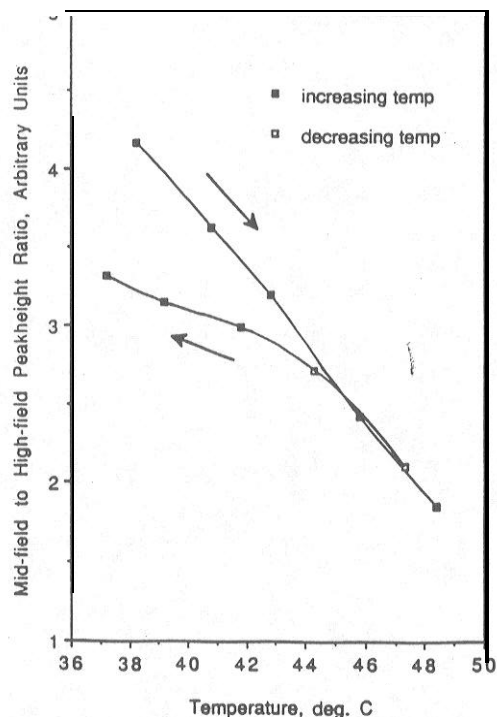


Fig. 5 Temperature dependence of  $0.8$  mM 3-doxyl-5 $\alpha$ -cholestane in 1 mol percent cholesterol and 99 mol percent methyl docosanoate encapsulated in microspheres

individual molecules of a solid will be prevented from "melting" by the overall structure of the crystal. Beyond a distinct temperature, the system must pass into a metastable situation. At the end of this, the enthalpy potential has increased to a point sufficient to overcome the lattice energies and the solid "melts." On retracing the process in the opposite direction, hysteresis will arise if the "lattice" stabilities of the two phases (solid and liquid) do not coincide. In this case the solidifying will have to await the buildup of an enthalpy potential sufficient to overcome the "lattice" forces (e.g., hydrogen bond formation and breakage) of the liquid phase (Yalpani et al., 1985). That is, crystallization or solidification is not a homogeneous, but a nucleated process.

Below a certain temperature (which varied for each fatty acid system), hysteresis was not observed when the temperature was increased and decreased within approximately three degrees. This range is still useful in the application of this system in treatments with hyperthermia where the temperature distribution for a finite amount of microspheres inside a finite volume of tissue will be narrow and the fatty acid used in the mixture can be chosen such that the midpoint of its phase transition is higher than any of the tissue temperatures. Heating and cooling the microspheres through several phase transition cycles should allow for reproducible and, therefore, predictable hysteresis (cf. House and Kemper, 1990). Even better, narrow temperature ranges below the midpoint of the phase transition should eliminate the hysteresis altogether (cf. Black and Dixon, 1981).

The temperature resolutions given above, while not yet determined *in vivo* or *in vitro*, compare very well with the sensitivities of other thermographic techniques (Eckburg, 1993b). For electromagnetic radiometry, Bolomey and Hawley's review (1990) cited studies claiming temperature sensitivities of  $0.1^\circ\text{C}$  at the body's surface but only of the order of a few degrees at a depth of 1 cm. For X-ray tomodensitometry, the same review cited studies reporting temperature sensitivities of  $0.25$ – $0.5^\circ\text{C}$  *in vitro* in muscle tissue extracts but difficulties in obtaining adequate data *in vivo*. Using the spin-lattice relaxation time constant,  $T_1$ , of the proton as the temperature dependent param-

eter for NMR thermography, Bolomey and Hawley's (1990) review reported a study claiming a 1-2°C temperature resolution. Using the temperature dependence of molecular diffusion for NMR thermography, Le Bihan et al. (1989), reported a 0.5°C resolution in a polyacrylamide gel phantom but problems in obtaining good resolution *in vivo*. Kuroda et al. (1990) claimed temperature sensitivity within 1°C *in vitro* in various tissue extracts when using the temperature dependence of the proton chemical shift, 6, for NMR thermography. For electrical impedance tomography, Conway et al. (1985). Conway (1987) and Griffiths and Ahmed (1987) claimed 1°C temperature sensitivities *in vivo* and 0.2°C in phantom solutions. Guerquin-Kern et al. (1985), reported temperature changes of a few degrees when imaging homogeneous media using active microwave imaging as the thermographic modality.

**Temperature Dependence of the Nonencapsulated Mixture.** Figure 6 shows the temperature (increasing from 37 to 47°C) dependence of the EPR spectra for 1.4 mM 3-doxyl-5 $\alpha$ -cholestane in 1 mol percent cholesterol and 99 mol percent methyl docosanoate. Except for some broadening of the low-field, hyperfine splitting portion of the spectrum, the shape of the spectra in Fig. 6 for the nitroxide/fatty acid/cholesterol mixture not encapsulated in microspheres looks similar to the shape of the spectra in Fig. 2, where the mixture was encapsulated. This result suggested that most of the nitroxide, 3-doxyl-5 $\alpha$ -cholesterol, was inside the microspheres in the cholesterol/fatty acid mixture. The observed broadening is likely to be due to nitroxide attached to the microsphere or on the outside in the aqueous environment. A nitroxide in different environments (e.g., inside the fatty acid/cholesterol mixture, attached to the BSA microsphere or in the surrounding aqueous medium) will give rise to different spectra. This is partly the result of a change in rotational mobility due to a different solvent viscosity or being bound or unbound to a large molecule or surface. A less viscous solution (or an unbound nitroxide) will produce narrower lines because of the averaging out of the  $g$  factor and nuclear hyperfine splittings. The spectrum will also change because of the effect of the polarity of the solvent on both the  $g$  value and the nuclear hyperfine splitting. In non-polar (e.g., fatty acid) solvents the unpaired electron delocalizes from the nitrogen atom. This produces a decrease in the nuclear hyperfine splitting when compared to an aqueous solution. In addition, the  $g$  factor increases when going from aqueous (polar) solutions to nonpolar solutions, resulting in a slight shift of the spectrum (Bacic et al., 1988).

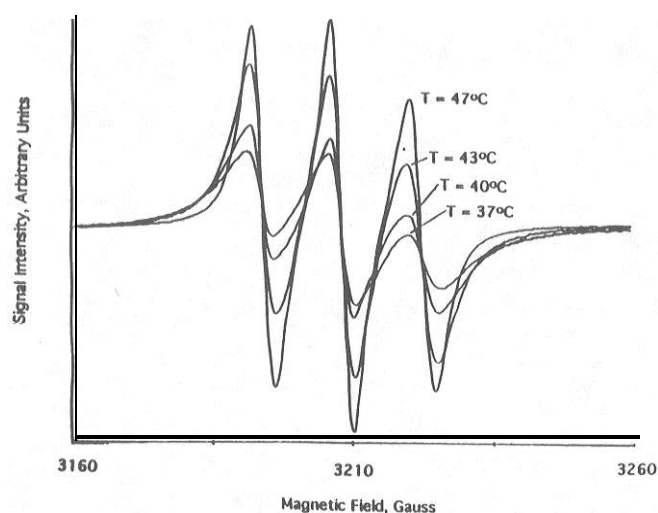


Fig. 6 Temperature dependence of the EPR spectra between 37 and 47°C for 1.4 mM 3-doxyl-5 $\alpha$ -cholestane in 1 mol percent cholesterol and 99 mol percent methyl docosanoate not encapsulated in microspheres

**Retention of the Nitroxides in the Microspheres.** Figure 7 is a plot of the EPR spectra showing the difference in signal intensity between the nitroxide (7-doxyl stearic acid) in the aqueous phase surrounding the microspheres and the nitroxide in the microspheres alone. The spectra labeled 1, 2, 3, and 4 represent the number of "washings" the spheres received before centrifuging. There was a gradual reduction in signal intensity originating from the surrounding aqueous phase after each successive washing of the microspheres. This indicated that a significant part of the EPR signal originated from the nitroxide either inside the microsphere or attached to it (i.e., the BSA). It also demonstrated the fact that the doxyl stearic acids do not "leak" out of the microsphere into the surrounding aqueous media. This was expected since the doxyl stearic acids are lipid soluble and, therefore, possess a much greater affinity for the fatty acid mixture and BSA than for the surrounding aqueous solution. Other experimental results (Eckburg, 1993c) using the water soluble nitroxide, tempo, did not show the gradual reduction in signal intensity originating from the aqueous phase after each successive washing of the microspheres, indicating either that the water soluble nitroxide did "leak" out of the microsphere, or that rupture of the microspheres occurred. This effect was also expected since tempo has a higher affinity for the surrounding aqueous solution than for the fatty acid mixture or BSA.

**Effect of Oxygen.** Figure 8 is a plot of the mid-field peak-to-peak linewidth versus time for 7-doxyl stearic acid in cyclohexane either inside the microspheres or without them. The line broadening or narrowing shown was due to the molecular oxygen's paramagnetic properties. The oxygen ground state has two unpaired electrons and a very rapid rate of relaxation. Therefore, oxygen can effectively relax other paramagnetic species (Swartz and Glockner, 1991). Linewidth broadening occurred when the perfusing gas was switched from nitrogen to air, and linewidth narrowing occurred when the air was switched to nitrogen. The similar slopes indicate that the microspheres provided no significant barrier to the diffusion of oxygen or nitrogen. This result was not surprising since even uniform coatings of polymers allow gas diffusion between chains (Suslick, 1992).

**Nitroxide Reduction in Ascorbic Acid and in Blood.** Ascorbic acid, which is present both intra- and extracellularly in blood, can reduce nitroxides (Iannone, 1989). The use of a high concentration (4.7 mM) of ascorbic acid resulted in a relatively rapid rate of reduction (<40 minutes) of the nitroxide 16-doxyl stearic acid in cyclohexane encapsulated in the microsphere. The high concentration also resulted in a decrease in the average size of the microspheres, indicating their breakup (Eckburg, 1993d). In fresh whole blood and *in vivo* in a mouse, however, the reducing effect of ascorbic acid was not significant over a 40 minute period for 16-doxyl stearic acid in cyclohexane encapsulated in microspheres. Our results suggest the potential use of these nitroxide-filled microspheres *in vitro* and *in vivo*.

**Effect of Cholesterol.** The use of cholesterol in the solutions in the microspheres served two purposes: to broaden the temperature range of the solid to liquid phase transition (a pure compound has a narrower phase transition temperature range than a mixture of two or more substances), and to accelerate the rate of approach of the EPR changes with temperature to their equilibrium values. Subzynski and Kusumi (1986) investigated the effects of very small amounts of cholesterol on gel-phase phosphatidylcholine membranes. They performed temperature drop experiments across the pretransition temperature and measured the rate of approach to the equilibrium value of the parallel hyperfine splitting,  $T_{11}$  (a measure of nitroxide mobility), of 5-doxyl stearic acid. They found that small amounts of cholesterol dramatically accelerated the rate of approach. The membrane reached equilibrium after 10 hours with

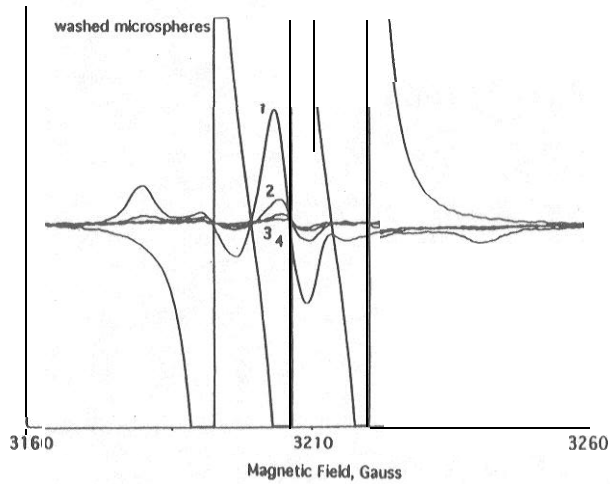


Fig. 7 Comparison of the EPR spectra of the aqueous phase surrounding the microspheres and the microspheres themselves. The decrease in signal intensity of the aqueous phase after each washing and the relatively large signal intensity from the washed (4 times) microspheres indicates that the nitroxide does not leak out of the microsphere.

no cholesterol added, after three hours in the presence of 0.01 mol percent cholesterol and less than 10 minutes with 0.03 mol percent cholesterol. They reported that this effect was not due to a simple impurity effect (which tends to accelerate the kinetics) because these effects were observed by using much higher levels of "impurities," i.e., 0.25 mol percent of 5-doxy stearic acid. The absence of a significant difference in the spectral response to temperature for cholesterol concentrations varying from 1 to 15 mol percent compared well with the results of Kusumi et al. (1986). They reported that 5-doxy stearic acid mobilities, when plotted as a function of mole fraction of cholesterol in the fluid

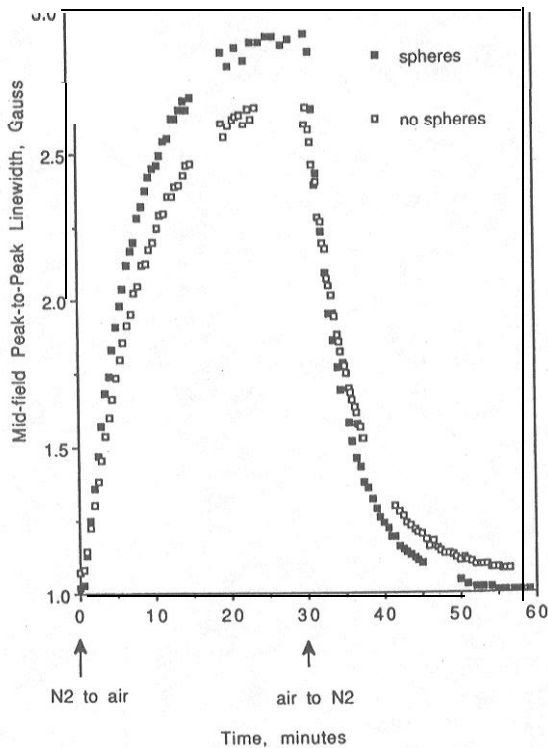


Fig. 8 Time dependence for 7-doxy stearic acid in cyclohexane perfused with air (increasing linewidth) or nitrogen (decreasing linewidth), with and without microspheres

phases of three different types of choline-cholesterol membranes. were similar in the cholesterol mole fraction range of 0 to 50 percent. In most of the experiments we performed, a value of approximately 1 mol percent cholesterol was used in the fatty acid/cholesterol mixtures. This provided a measurable spectral change with temperature with only a small amount of time needed to equilibrate after each temperature change.

**Effect of Magnetic Field Gradient at L Band.** For the eventual *in vivo* application of this system, measurements need to be performed on a lower frequency, e.g., an L band ( $\approx 1$  GHz), spectrometer, as opposed to the more frequently used X band (a9 GHz) unit. The lower frequency allows a greater depth of penetration of the microwave energy, and, therefore, enables the measurement of *in vivo* temperatures using an appropriate surface probe. A detailed description of the surface probe is given in Nilges et al. (1989). In addition to a greater depth of penetration, nonresonant absorption of the microwave power by water, which makes up approximately 75 percent of tissues, is less at lower frequencies.

Figure 9 shows the lineshape distortion of the EPR spectra, recorded using an L band spectrometer, due to a one dimensional linear magnetic field gradient (approximately 15 Gauss/cm) for approximately 0.5 ml of 1.2 mM 7-doxy stearic acid in 1 mol percent cholesterol and 99 mol percent ethyl palmitate encapsulated in microspheres.

A magnetic field gradient is required for spatial resolution between two different samples. For paramagnetic species with only one absorption peak with an inherently small linewidth, only a small gradient is needed to completely separate two samples of that species. However, for our nitroxides in the fatty acid/cholesterol mixture, whose spectra have three absorption peaks and large (from 1 to 10 Gauss) linewidths, larger gradients were needed to separate the spectra of two different samples. For example, assume we wanted to completely separate the spectra between two samples that are 8 mm apart (8 mm spatial resolution) with peak-to-peak first derivative linewidths of approximately 3 Gauss and a magnetic field gradient of 60 Gauss/cm was available. To prevent distortion of the lineshapes, very small sample widths must be used in the direction of the gradient. If a 1 percent lineshape distortion was allowed, a sample width of 0.1 mm or less must be used. For a 5 percent distortion allowance, 0.2 mm is the limit (Smimov et al., 1993).

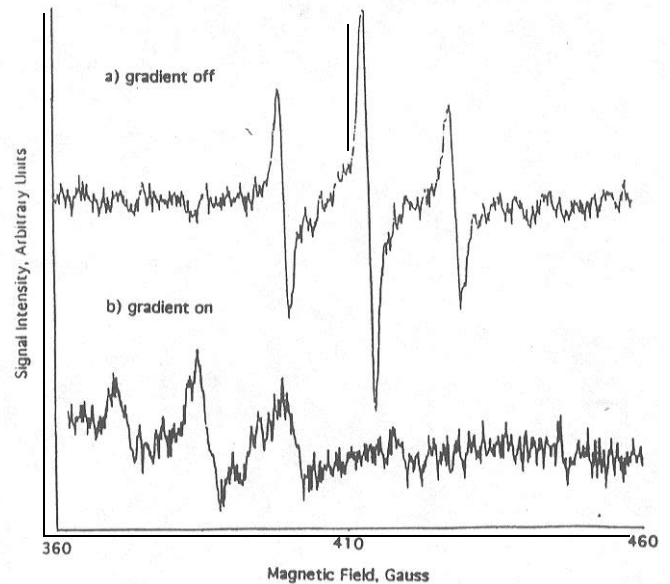


Fig. 9 L-band EPR spectra for an encapsulated nitroxide/fatty acid mixture illustrating the lineshape distortion in the presence of a one-dimensional magnetic field gradient of 15 Gauss/cm, (a) gradient off, (b) gradient on



There also is a decrease in signal intensity when going from X to L band. This, in combination with the poor signal to noise ratio of the spectrometer used in our experiments, meant larger sample volumes were required at L band to produce a detectable signal. In the presence of a linear magnetic field gradient, a large sample volume distorted the signal because each "end" of the sample rested in a different magnetic field, and hence, a different resonance condition existed for each "end" of the sample. This resulted in the very significant spectral lineshape distortion illustrated in Fig. 9 for a small plastic vial of microspheres containing approximately 0.5 ml of a 1.2 mM 7-doxyl stearic acid in 1 mol percent cholesterol and 99 mol percent ethyl palmitate. The magnetic field gradient used was only 15 Gauss/cm.

It therefore appears that further development of temperature dependent systems with narrower linewidth spectra and/or enhanced signal processing techniques (which would decrease the required sample volume) will be required to achieve the desired spatial resolution with this technique.

## Conclusions and Recommendations

We have developed a system that has the potential to be used for minimally invasive *in vivo* temperature measurements in living systems. Using the nitroxide, 3-doxyl-5 $\alpha$ -cholestane in 1 mol percent cholesterol and 99 mol percent methyl docosanoate encapsulated in proteinaceous microspheres, we have shown a large and, consequently, easily measurable change in the EPR spectral lineshape between 38 and 48°C (Fig. 2). This technique has potential clinical applications, especially in optimizing the treatment of cancer with hyperthermia. This technology can be used over a temperature range between 10 and 70°C simply by changing the composition of the nitroxide's medium. The system described above was developed to be used with the hyperthermia treatment, hence the 38 to 48°C temperature range. Using the mid-field to high-field peakheight ratio as the temperature dependent parameter, the temperature resolution for this system was 0.2°C (Fig. 5).

Encapsulation of the mixture in the microspheres provided two critical functions for use *in vivo*. First, the microspheres were widely distributed since they were smaller than the diameter of capillaries and were suspended in water. Second, the microspheres separated the mixture from the surrounding environment, thereby protecting the nitroxide from bioreduction and ensuring that the shape of the EPR spectrum was affected primarily by the medium's phase transition temperature range.

Experiments were carried out at L band with a linear magnetic field gradient to determine the potential spatial resolution of our system. The decrease in signal intensity when going from X to L band, in addition to the poor signal to noise ratio of the spectrometer, required very large sample volumes. The use of a surface probe at L band, which included an exponential drop in the signal intensity with distance from the probe (Nilges et al., 1989), also contributed to the need for larger sample volumes. The result was very significant spectral lineshape distortion (Fig. 9).

In order to increase the temperature resolution, computer simulation of the EPR spectra for the temperature measurement system was attempted. Unfortunately, initial attempts to increase the temperature resolution by simulating the spectra for the temperature measurement system between 3X and 48°C were unsuccessful (Eckburg, 1993e; Eum, 1993).

Additional efforts should continue to improve the spatial resolution of this system. This can be achieved through development of improved computer simulation, a temperature dependent system with narrower linewidth spectra, or enhanced signal processing techniques. This will provide the groundwork for the ultimate goal of this research: the ability to measure and map *in vivo* temperatures. It is anticipated that many of the principles and technologies currently available in EPR imaging

(Eaton et al., 1991) and NMR imaging may be very useful and applicable to this system.

## Acknowledgments

This research was supported, in part, by NIH grants RR-01811 (University of Illinois EPR Research Center), 5 T32 CA 09067 (Radiation Oncology Training Program) and HL 25934 (University of Illinois School of Chemical Sciences), by NSF grants CTS-89-13619 (Thermal Tomography using Electron Spin Resonance Techniques) and CHE-89.15020 (Chemical Effects of Ultrasound), and by the Bioengineering Faculty of the University of Illinois at Urbana-Champaign.

## References

- Bacic, G., Walczak, T., Demsar, F., and Swartz, H. M., 1988, "Electron Spin Resonance Imaging of Tissues with Lipid-Rich Areas," *Magnetic Resonance in Medicine*, Vol. 8, pp. 209-219.
- Black, S. G. and Dixon, G. S., 1981, "AC Calorimetry of Dimyristoyl-phosphatidylcholine Multilayers: Hysteresis and Annealing near the Gel to Liquid-Crystal Transition," *Biochemistry*, Vol. 20, pp. 6740-6744.
- Bolomey, J. C., and Hawley, M. S., 1990, "Noninvasive Control of Hyperthermia," *Clinical Thermology*, Gautherie, M., ed., Springer-Verlag, Subseries Therapy, Vol. 3, Chpt. 2.
- Capaldi, R. A., ed., 1977, *Membrane Proteins and their Interaction with Lipids*, M. Dekker, New York, p. 62.
- Conway, J., Hawley, M. S., Seagar, A. D., Brown, B. H., and Barber, D. C., 1985, "Applied Potential Tomography (APT) for Noninvasive Thermal Imaging during Hyperthermia Treatment," *Electronics Letters*, Vol. 21, pp. 836-838.
- Conway, J., 1987, "Electrical Impedance Tomography for Thermal Monitoring of Hyperthermia Treatment: an Assessment Using In Vitro and In Vivo Measurements," *Clin. Phys. Physiol. Meas.*, Vol. 8 (A), pp. 141-146.
- Eaton, G. R., Eaton, S. S., and Ohno, K., eds., 1991, *EPR Imaging and In Vivo EPR*, CRC Press, Inc., Boca Raton, FL.
- Eckburg, J. J., 1993, "Non-invasive Temperature Measurements Using Electron Paramagnetic Resonance Spectroscopy," Ph.D. thesis, Dept. of Mechanical and Industrial Engineering, University of Illinois at Urbana-Champaign, a: 27-31; b: 7-23; c: 33, 59; d: 59-68; e: 80-87.
- Eum, J.S-M., 1993, "The Temperature Relationships of Electron Paramagnetic Resonance and Nuclear Magnetic Resonance Spectra," M.S. thesis, Dept. of Mechanical and Industrial Engineering, University of Illinois at Urbana-Champaign.
- Griffiths, H., and Ahmed, A., 1987, "Applied Potential Tomography for Non-invasive Temperature Mapping in Hyperthermia," *Clin. Phys. Physiol. Meas.*, Vol. 8 [Suppl A], pp. 147-153.
- Grinstaff, M. W., 1992, "The Sonochemical Synthesis of Inorganic and Biological Materials," Ph.D. thesis, University of Illinois, Urbana, IL.
- Guerquin-Kern, J. L., Gautherie, M., Peronnet, G., Jofre, L., and Bolomey, J. C., 1985, "Active Microwave Tomographic Imaging of Isolated, Perfused Animal Organs," *Bioelectromagnetics*, Vol. 6, pp. 145-156.
- House Jr., J. H., and Kemper, K. A., 1990, "Phase Transitions and their Thermal Hysteresis in Anhydrous Potassium Acetate," *Thermochimica Acta*, Vol. 162, pp. 325-331.
- Iannone, A., Hu, H., Tomasi, A., Vannini, V. and Swartz, H. M., 1989, "Metabolism of Aqueous Soluble Nitroxides in Hepatocytes: Effects of Cell Integrity, Oxygen and Structure of Nitroxides," *Biochim. Biophys. Acta*, Vol. 991, pp. 90-96.
- Kuroda, K., Miki, Y., Nakagawa, N., and Tsutsumi, S., 1991, "Non-invasive Temperature Mapping Based upon NMR Procedure," *Symposium on Bioheat and Mass Transfer*, Athens, Greece.
- Kusumi, A., Subczynski, W. K., Pasenkiewicz-Gierula, M., Hyde, J. S., and Merkle, H., 1986, "Spin-label Studies on Phosphatidylcholine-cholesterol Membranes: Effects of Alkyl Chain Length and Unsaturation in the Fluid Phase," *Biochimica et Biophysica Acta*, Vol. 854, pp. 307-317.
- LeBihan, D., Delannoy, J., and Levin, R. L., 1989, "Temperature Mapping with MR Imaging of Molecular Diffusion: Application to Hyperthermia," *Radiology*, Vol. 171, pp. 853-857.
- Levin, R. L., 1990, "Measurements, Instrumentation, and Imaging," *Biothermal Engineering, Report on a Workshop held at Allerton House of the University of Illinois at Urbana-Champaign, April 22-25, 1990*, Chato, J. C. and Oleson, J. R., University of Illinois at U-C.
- Morse II, P. D., 1987, "Data Collection from EPR Spectrometers Using the IBM PC," *Biophys. J.*, Vol. 51, p. 440a.
- Nilges, M. J., Walczak, T., and Swartz, H. M., 1989, "1 GHz In Vivo ESR Spectrometer Operating with a Surface Probe," *Phys. Med.*, Vol. 2, pp. 195-201.
- Samulski, T. V., and Fessenden, P., 1990, "Thermometry in Therapeutic Hyperthermia," *Methods of Hyperthermia Control*, Gautherie, M., ed., Springer-Verlag.
- Smirnov, A. I., Norby, S. W., Clarkson, R. B., Walczak, T., and Swartz, H. M., 1993, "Simultaneous Multi-site EPR Spectroscopy In Vivo," *Magnetic Resonance in Medicine*, Vol. 30, pp. 213-220.
- Subczynski, W. K., and Kusumi, A., 1986, "Effects of Very Small Amounts of Cholesterol on Gel-phase Phosphatidylcholine Membranes," *Biochimica et Biophysica Acta*, Vol. 854, pp. 318-320.

- Suslick, K. S., 1989, "The Chemical Effects of Ultrasound," *Scientific American*, Vol. 260(2), pp. 80-86.
- Suslick, K. S., 1990, "Sonochemistry," *Science*, Vol. 247, pp. 1439-1445.
- Suslick, K. S., and Grinstaff, M. W., 1990, "Protein Microencapsulation of Nonaqueous Liquids," *J. Am. Chem. Soc.*, Vol. 112(21), pp. 7807-7809.
- Suslick, K. S., 1992, personal communication.
- Swartz, H. M., Bolton, J. R., and Borg, D., eds., 1972, *Biological Applications of ESR*, Wiley, New York.
- Swartz, H. M., and Swartz, S. M., 1983, "Biochemical and Biophysical Applications of Electron Spin Resonance," *Methods of Biochemical Analysis*, D. Glick, ed., Wiley, New York, Vol. 29, pp. 207-323.
- Swartz, H. M., 1985, "Use of Electron Spin Resonance to Study Complex Biological Membranes," *NATO ASI Proceedings on Physical Methods on Biological Membranes and Their Model Systems*, Conti, F., Blumberg, W. E., deGier, J., and Pocchiari, F., eds., Plenum Press, New York, pp. 39-53.
- Swartz, H. M., and Glockner, J. F., 1991, "Measurement of Oxygen by EPRI and EPRS," *EPR Imaging and In Vivo EPR*, Eaton, G. R., Eaton, S. S., and Ohno, K., eds., CRC Press, Inc., Boca Raton, FL, pp. 261-290.
- Yalpani, M., Scheidt, W. R., and Seevogel, K., 1985, "Thermochromic Transformations of 2,6-Diorgano-1,3,5,7-tetraoxa-2,6-dibora-4,8-octalindiones: A Model for Solid-State Phase Transformations and Hysteresis," *J. Am. Chem. Soc.*, Vol. 107, pp. 1684-1690.