In Vivo Measurement of Oxygen Concentration Using Sonochemically Synthesized Microspheres

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ABSTRACT Proteinaceous microspheres filled with nitroxides dissolved in an organic liquid have been synthesized for the first time using high intensity ultrasound; these were used to measure oxygen concentrations in living biological systems. The microspheres have an average size of 2.5 pm, and the proteinaceous shell is permeable to oxygen. Encapsulation of the nitroxides into the microsphere greatly increased the sensitivity of the electron paramagnetic resonance signal line width to oxygen because of the higher solubility of oxygen in organic solvents. The encapsulation also protected the nitroxide from bioreduction. No decrease in intensity of the electron paramagnetic resonance signal was observed during 70 min after intravenous injection of the microspheres into a mouse. Measurement of the changes in oxygen concentration in vivo by means of restriction of blood flow, anesthesia, and change of oxygen content in the respired gas were made using these microspheres.

INTRODUCTION

The concentration of oxygen ([0,]) is one of the most important variables in many physiological, pathological, and therapeutic processes. The need for new techniques to measure $[O_2]$ in tissues with high accuracy and sensitivity has been increasingly recognized (Stone et al., 1992). The existing methods for such oximetry are useful but have limitations, especially for use in vivo (Vanderkooi et al., 1991; Chapman, 1991).

Electron paramagnetic resonance (EPR) oximetry is one of the most promising and rapidly developing techniques for measurement of [0,] in tissues in vivo. Initially, the paramagnetic probes for EPR oximetry were almost exclusively nitroxides (Berliner and Wan, 1989; Halpem et al., 1990; Zweier et al., 1991; Rosen et al., 1988; Kalyanaraman et al., 1993; Jiang et al., 1992), but recently several particulate probes have been introduced (Swartz et al., 1991; Swartz et al., 1994; Liu et al., 1993). The major limiting factors for the widespread use of nitroxides are their relatively low sensitivity to oxygen and their susceptibility to bioreduction under biological conditions. To help overcome these limitations, isotopically substituted nitroxides can enhance sensitivity to oxygen (Halpem et al., 1990), and the encapsulation of nitroxides into liposomes or plastic tubing can slow or eliminate reduction (Glockner et al., 1991; Subczynski et al., 1986). Nonetheless, substantial room for improvement still exists. In this report, we describe a technique using high intensity ultrasound to synthesize sonochemically microspheres (d = 2.5 μm) filled with a solution of nitroxides in organic liquids. This approach provides a versatile means to administer the spin probe that increases the sensitivity to 0, by as much as 40-fold and prevents the reduction of the nitroxide in vivo.

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THEORY

Oximetry using nitroxides is based on EPR line broadening caused by Heisenberg exchange between molecular oxygen (which is a stable diradical) and the nitroxide (Hyde and Subczynski, 1989). Pake and Tuttle (1959) defined the relationship between EPR line exchange broadening and radical-radical collision rate as

$$LW = kpw + N, \quad (1)$$

where LW is the EPR line width, k is a proportionality constant, p is the probability that exchange will occur upon each collision ($0 \leq p \leq 1$), ω is the collision rate, and N represents other contributions to the EPR LW that are independent of ω. Thus, the change in the EPR LW caused by different encounter rates can be given as

$$ALW = kp(\Delta\omega). \quad (2)$$

The encounter rate is governed by the Smoluchowski equation

$$\omega = 4\pi R\{D(O_2) + D(SL)\}[O_2], \quad (3)$$

where R is the interaction distance, $D(O_2)$ and $D(SL)$ are the diffusion constants of 0, and the nitroxide, respectively, and [0,] is the effective oxygen concentration in the solution. When [0,] changes, the encounter rate changes accordingly. The net change of the encounter rate is linearly proportional to the change of [0,]:

$$\Delta\omega = 4\pi R\{D(O_2) + D(SL)\}(\Delta[O_2]). \quad (4)$$

Combining Eqs. 4 with 2, we have

$$ALW = 4\pi Rkp\{D(O_2) + D(SL)\}(\Delta[O_2]). \quad (5)$$

It can be seen from above equations that oxygen-dependent changes in LW of the nitroxide should be proportional to the solubility of oxygen in the solvent.

Because the solubility of oxygen varies up to two orders of magnitude in different solvents, this phenomenon can be exploited to maximize the line width change in EPR oximetry. Generally, oxygen solubility in organic solvents is much higher than in water (Linke, 1958). Therefore, one expects that the dynamic range of EPR line widths in organic solvents should be much higher, which should result in greater sensitivity to changes in $[O_2]$. Although this might be exploited in vivo by using a lipophilic nitroxide and then making measurements in naturally occurring lipid-rich areas, there would be significant limitations: the heterogeneous nature and the variations in location of lipophilic regions would make calibration of the oximetry system difficult. Furthermore, oximetry of tissues without large amounts of lipids would not be possible. In addition, rapid bioreduction of nitroxides limits the utility of such an approach.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), and all nitroxides were purchased from Molecular Probes (Junction City, OR) except for 2,2,6,6-tetramethylpiperidine-d_{16}-1-^{15}N-oxyl-4-one ("N-PDT), which was obtained from MSD Isotopes (St. Louis, MO).

Generation of microspheres with ultrasound

The synthesis of proteinaceous microspheres with high intensity ultrasound has been described previously (Suslick and Grinstaff, 1990; Grinstaff and Suslick, 1991; Suslick and Flint, 1987). In brief, 2 ml of solution of an organic liquid containing l-5 mM nitroxide and 3 ml of 5% w/v BSA aqueous solution were placed in a glass container. The tip of the ultrasonic horn was positioned at the interface of the organic and aqueous phases. The sample was irradiated with ultrasound (XL-2020, Heat Systems, Farming dale, NY) for 3 min at an initial temperature of 30°C at 20 kHz and -100 W/cm^2. This produces an aqueous suspension of proteinaceous microspheres filled with nitroxides in an organic liquid. The concentration of the nitroxide in the microspheres is the same as the initial concentration in the organic liquid. The microspheres were separated from the remaining protein solution by sequential centrifuging and washing. Yields are highly sensitive to the temperature during irradiation and must be optimized for the specific experimental configuration. Determination of the size distribution of the microspheres was accomplished with an Elzone 180 Particle Counter interfaced to an AT&T 386 computer.

Animals

BALB/c mice weighing 20–25 g were obtained from Charles River Laboratories (Wilmington, MA). Before the experiments, the animals were anesthetized with ketamine (0.12 mg/g body wight, i.p.). The microspheres were introduced into the animal through a syringe with a 26-gauge needle.

Calibration of EPR line width with [0,]

100 μl of the microsphere suspension in water was drawn into a gas-permeable teflon tube (Zeus Industrial Products, Raritan, NJ; 0.623 mm i.d.; wall thickness 0.038 ± 0.004 mm). This teflon tube was folded twice and inserted into a quartz EPR tube open at both ends. Samples were maintained in the EPR cavity at 37 ± 0.2°C with a Varian temperature controller. $[O_2]$ in the perfusing gas was monitored and measured by a Clark-type electrode (Microelectrodes, Londonderry, NH), calibrated by vigorously stirring 100 ml of distilled water equilibrated with 0, or N_2. The quantitative dependence on [0,] of the EPR spectrum was obtained by measuring LW as a function of $[O_2]$ in the perfusing gas, with LW defined as the difference in magnetic field between the maximum and minimum of the first derivative recording of the signal. The middle peak of the three-peak spectrum was used in all experiments because it had the highest signal intensity. The resulting calibration curve was fit by linear regression, which was then used to convert values of LW measured in biological systems into appropriate values of $[O_2]$.

EPR measurements

The spectra in tissues were obtained using an EPR spectrometer constructed in our laboratory with a low frequency (1.2 GHz, L-band) microwave bridge (Nilges et al., 1989). Some calibrations and studies of physicochemical properties of the microspheres were performed using a Varian E-12 EPR spectrometer (9.5 GHz, X-band). Typical settings for the spectrometers included: magnetic field, 3210 Gauss (X-band)/392 Gauss (L-band); incident microwave power, 5 mW; modulation frequency, 100 kHz. Modulation amplitude was set at less than one-third of the EPR line width. Temperature control at X-band was achieved by a Varian gas flow system using an NJO, mixture. At L-band, the temperature of anesthetized animals (37 ± 2°C, measured by a rectal probe) was regulated by an infrared lamp. The EPR spectra on both spectrometers were collected, stored, and manipulated using the software EW (Scientific Software, Normal, IL) installed on IBM compatible computers. Accurate measurement of the LW of the EPR spectra was achieved by using a spectral simulation program designed in our laboratory.

Measurement of [0,] in mice in vivo

Adult mice were anesthetized, and 0.15 ml of the microspheres was injected through the tail vein. The animal was placed between the poles of the magnet with the L-band loop gap detector positioned over the area of interest. For measurement of [0,] in the skeletal muscle, 0.1 ml of a suspension of microspheres was used. The leg of the animal was then centered on the detector. When required, blood flow was restricted by a ligature around the upper leg. The composition of the breathing gas was controlled by means of a plastic hood placed over the head of the animal.

RESULTS

Oxygen solubility varies enormously from solvent to solvent. For example, under partial pressure of 159 mmHg of oxygen (i.e., 1 atmosphere of air), the solubility of oxygen in toluene and ethyl ether is 8 and 20 times higher, respectively, than in water (Linke, 1958). As discussed earlier, the broadening of the EPR line width of nitroxides depends on the solubility and diffusion constant of 0, in the solvent. Using 16-doxyl-stearic acid (16-DS) as an example, Table 1 lists the LW of 16-DS in air and in nitrogen when dissolved in several common solvents. Compared with the values in water, the change of LW from nitrogen to air was larger in all organic solvents,

TABLE 1 EPR line width (LW) of 16-DS In various solvents

Solvents	LW in air (G)	LW in N_2 (G)	ΔLW (G)
Water	1.03	0.97	0.06
Ethanol	2.45	1.01	1.44
Hexane	4.41	1.14	3.27
Cyclohexane	2.66	1.08	1.58
Toluene	2.50	1.08	1.42
n-Pentane	3.15	1.16	1.99

Except in water, the concentration of 16-DS was 1.0 mM for all the experiments and the LW of the mid-field peak was used. Because of low solubility, the concentration of 16-DS in water was 0.1 mM.

with a maximum of 50 times larger in hexane versus water. Very similar results were obtained using other nitroxides, including 5-doxyl-stearic acid (5-DS), 7-doxyl-stearic acid (7-DS), 10-doxyl-stearic acid (10-DS), 3-maleimido-proxyl, 3-(3-maleimidopropylcarbamoyl)-proxyl, and 4-(*N*,*N*-dimethyl-*N*-pentadecyl) ammonium-2,2,6,6-tetramethyl-piperidine-1-oxyl iodide (Cat-16).

Aqueous suspensions of proteinaceous microspheres of BSA filled with nitroxides in hexane were synthesized with high intensity ultrasound. High concentrations of microspheres were obtained (1.5 X 10^9 microsphere/ml) with a narrow size distribution (2.5 $\pm$ 1.0 μm). Microencapsulation of other solvents, including toluene, cyclohexane, octanol, and n-pentane was also successful with both human serum albumin and BSA. In the descriptions of the following experiments, microspheres made with various nitroxides and solvents are used to demonstrate this versatility.

To determine whether the shell of the microspheres imposes a barrier to the diffusion of O_2, we used a well established technique that has been used to study the intracellular and extracellular oxygen concentrations (Samuni et al., 1986; Glockner et al., 1989). Two nitroxides were utilized: 7-DS, which is enclosed inside the microsphere, and "N-PDT, which reports oxygenation of the aqueous environment outside of the microspheres because the volume outside microspheres is much larger than inside, and because EPR signal intensity of ^{15}N-PDT from inside microspheres is very weak because of its large line width. Because the EPR spectra of the two nitroxides do not overlap, it is possible to measure simultaneously the rate of oxygenation inside (with 7-DS) and outside (with "N-PDT) the microsphere, using the *LW* of each nitroxide as a measure of $[O_2]$ of each environment. No significant differences were observed in the kinetics of oxygenation (Fig. 1), which demonstrates that 0, can easily pass through the shell of the sphere.

To study whether the nitroxide or organic liquid leaks from the microspheres, the microspheres were mixed with fresh human blood. No changes in EPR signal intensity were recorded for up to 40 min, indicating that no significant leaking of nitroxide or organic liquid (which would carry nitroxide with it) has taken place, because any nitroxide leaked out of the microsphere would change the EPR signal intensity because of the very different line widths of the nitroxide in the two different environments.

The microspheres have good stability at room temperature. When stored at 4°C, the toluene-filled microspheres had <10% degradation after 2000 h. When injected intravenously, no significant decrease in the EPR signal intensity occurred during 70 min of observation in vivo in a mouse. In another experiment, the microspheres were introduced intramuscularly into the leg of a mouse to study the in vivo stability of the microsphere. Five hours after the initial injection, about 14% reduction in the EPR signal intensity was recorded.

Fig. 2 shows typical EPR spectra of microspheres equilibrated with N_2 and air. In this case, the nitroxide was 3-maleimido-proxyl and the solvent hexane. In air, the *LW* increased from 1.16 to 4.96 Gauss. The line height decreased

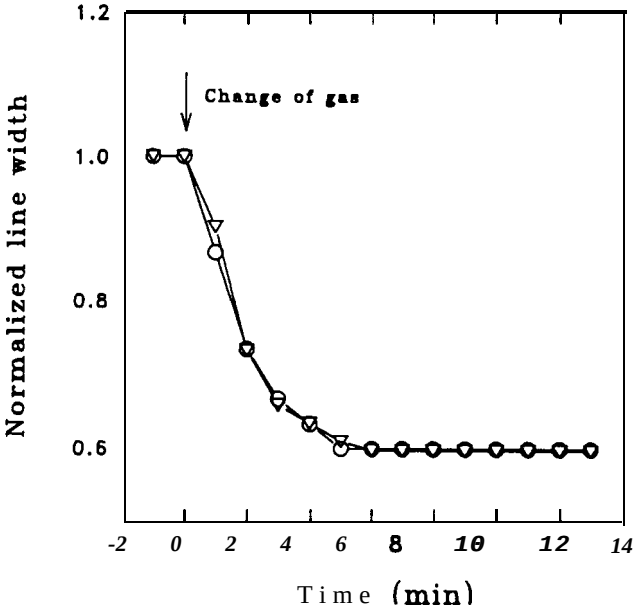

FIGURE 1 Kinetics of oxygenation inside and outside the microspheres. An aqueous solution containing ^{15}N-PDT and microspheres filled with 7-DS in cyclohexane was drawn into a gas-permeable teflon tubing that was inserted in the quartz EPR tube. The perfusing gas was changed from nitrogen to air. The EPR line widths of "N-PDT (0) and 7-DS (∇) were measured simultaneously every 60 s.

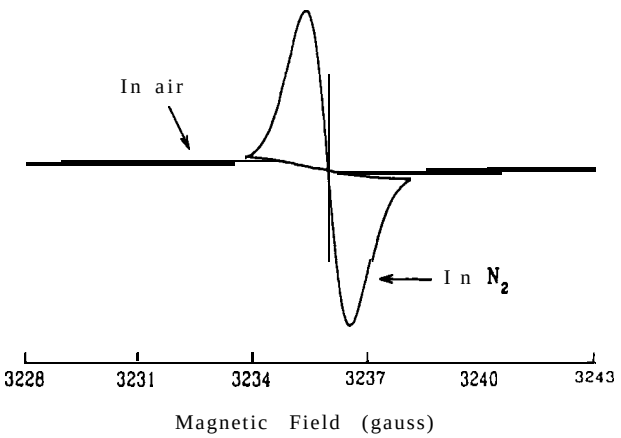

FIGURE 2 EPR spectra of microspheres filled with 2 mM 3-maleimido-proxyl in hexane equilibrated with N_2 and air. The corresponding line width, which is used to calculate [O,], changed from 1.16 to 4.96 Gauss. This change is 60 times larger than the change in aqueous solution for this nitroxide.

by a factor of 14. A typical calibration of EPR *LW* of the microspheres to [0,] is given in Fig. 3, showing a linear relationship as described in Eq. 5. Included in Fig. 3 is the calibration in water for the purpose of comparison.

The potential usefulness of the microspheres for measurement of [0,] in biological systems is demonstrated in the following illustrative experiments. Fig. 4 shows the results of a study in which microspheres were injected intravenously, and $[O_2]$ was measured in lower half of the body of a mouse before and after a large dose of Ketamine, a general

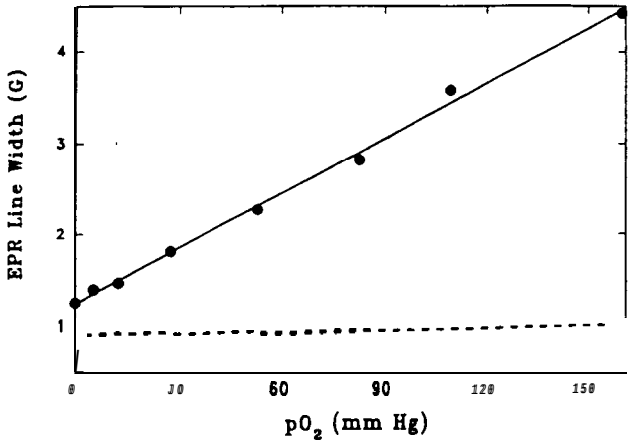

FIGURE 3 Calibration with $[O_2]$ of the microspheres filled with 1 mM 16-DS in toluene. As a comparison, the dashed line shows the calibration of 16-DS in water. The line width of the central hyperfine line was used in these and all other figures.

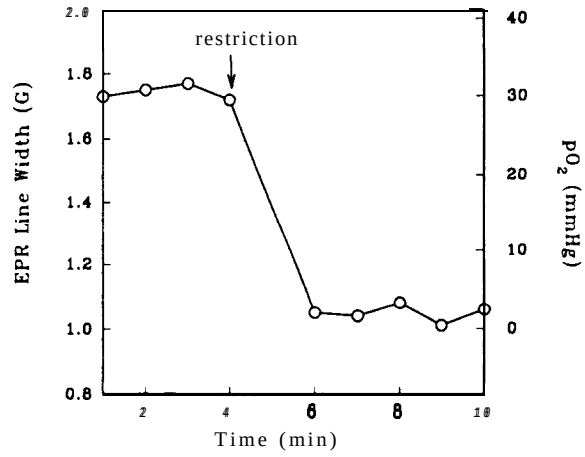

FIGURE 5 [0,] in the skeletal muscle of a mouse with and without constriction of blood flow. The constriction was achieved by means of a ligature around the upper leg. The response to decreases of $[O_2]$ was indicated by a narrowed line width and increased line height. The microspheres were filled with 5 mM 10-DS in cyclohexane.

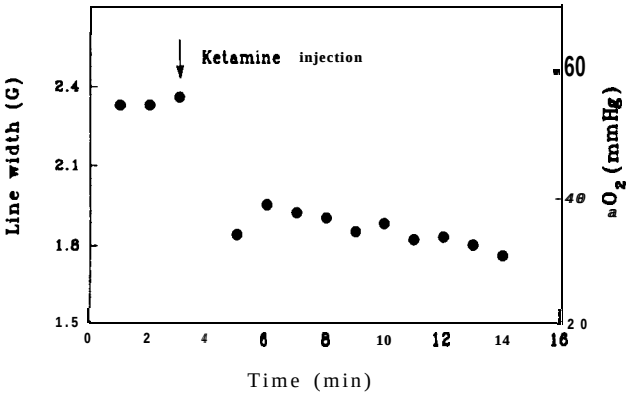

FIGURE 4 Effect of anesthetic on the $[O_2]$ within a mouse. EPR spectra were recorded from the lower half of the body of the animal. The temperature of the mouse was maintained at 37 ± 2°C with an infrared lamp. The microspheres were filled with 5 mM 7-DS in hexane.

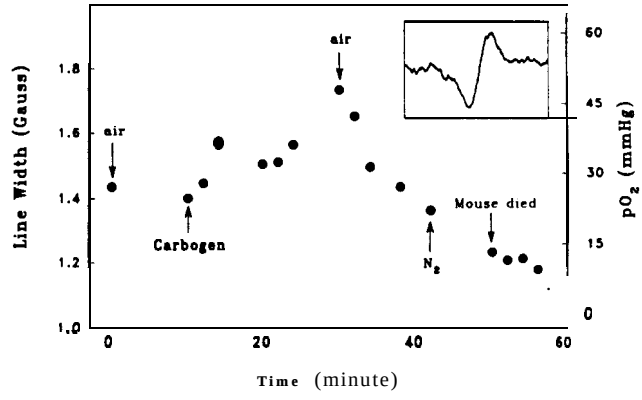

FIGURE 6 The change of $[O_2]$ in the skeletal muscle of a mouse upon the variation of 0, content in the respiration gas. 100 μl of microspheres of 5 mM 16-DS in cyclohexane were injected. The inset shows a typical spectrum of the middle line of the nitrogen hyperfine line. The animal died shortly after breathing N_2. Carbogen: 95% 0, with 5% CO,

anesthetic. Fig. 5 shows a substantial change in $L W$ and $[O_2]$ in the gastrocnemius muscle when the blood flow was restricted. Fig. 6 indicates [0,] in skeletal muscle as the 0, content of the respiration gas for a mouse was altered.

DISCUSSION

Several different types of EPR oximetry probes have been designed and used, including free nitroxides, nitroxide-containing liposomes, and solid-state materials such as lithium phthalocyanine, fusinite, and India ink (Swartz et al., 1991, 1994; Liu et al., 1993). For example, Subczynski et al. (1986) introduced the use of an implantable O_2-permeable capsule to enclose light paraffin oil containing a nitroxide. The dimensions of the capsule, however, are large, the locations in which it can be used are limited, and it requires the physical insertion of the tube into the animal. In contrast, the microsphere encapsulation of nitroxide in organic solvents provides several unique features and combines many desirable features into a single system. Compared with free nitroxides, the encapsulation of nitroxides into microspheres increased the sensitivity to oxygen by nearly 40-fold and greatly reduced the rate of bioreduction of nitroxide in tissues.

Compared with isotopically substituted nitroxides, which increase the sensitivity to oxygen by decreasing the intrinsic line width, or by enhancing certain spectral features (Halpern et al., 1990), the encapsulation technique offers greater improvement in oxygen sensitivity. As can be seen from Table 1, the effects of oxygen on the $L W$ for nitroxides in lipophilic solvents in microspheres can be quite large: for example, the line width for 16-DS in hexane is 1.14 (in NJ to 4.41 Gauss (in air); under the same conditions, the line width of perdeuterated Tempone (^{15}N-PDT) in water changes from 0.18 to 0.28 Gauss. With an intrinsically narrower EPR line width, however, isotopic substitution also increases the EPR signal

intensity, which is not provided by the microspheres. Signal intensity, indeed, is the major problem associated with the microspheres, and at all but the lowest $[O_2]$, it is the necessary trade-off for the increase in sensitivity to oxygen. Difficulties in recording spectra with good SIN ratio were experienced in some of the experiments reported here. For example, the increase in *LW* of 16-DS in hexane translates into a loss of signal height by a factor of 30–200 (Table 1). Encapsulation of *substituted* nitroxides into microspheres could produce microspheres with high oxygen sensitivity and higher signal heights.

In certain aspects, microspheres are similar to liposomes: both can protect nitroxides from bioreduction, and both can be injected as aqueous suspensions intravenously or directly into tissues. Microspheres offer the added benefit of substantial increases in oxygen sensitivity. Although the microsphere was stable in vivo for up to 70 min, it will be metabolized over extended periods of time. Therefore, it is intended for short term [0,] measurement, although the particulate probes might be better for much extended times, such as weeks and months.

One potential concern of using microspheres is the possibility of leakage of nitroxide from the microsphere into the media, thereby exposing the nitroxides to bioreduction and possibly altering the calibration to [O,]. Based on the results presented here from studies in vivo, this does not seem to be a major problem; the microspheres with 7-DS were stable in vivo for over 70 min, and no reduction was observed. This probably occurred because the nitroxides chosen are very lipophilic and, therefore, have a very high partition coefficient for the encapsulated organic solvents versus the surrounding media.

Another potential concern is the leakage, as well as release of the organic liquid from the microspheres. Based on our experimental results, significant leakage does not seem to occur in a short time while the microsphere is intact. However, trace amounts of the leaked solvents could potentially affect the tissue oxygenation. In addition, when the microspheres eventually are metabolized, the encapsulated medium is released and becomes a potential source of toxicity to the tissue. One way to solve this problem is to use chemically inert solvents, such as peffluorocarbons (which have already been clinically used as blood substitutes) or to use organic liquids with low toxicity (such as fatty acids).

The microspheres can be made with different proteins, nitroxides, and organic liquid. In addition to BSA, we are able to produce microspheres from other proteins, such as human serum albumin. In addition, we have been able to use nearly any immiscible organic liquid, including toluene, octanol, hexane, cyclohexane, and n-pentane. Any lipophilic nitroxide can be enclosed into the microsphere. This versatility provides the capability to synthesize microspheres with specific components to suit particular needs. It also suggests that nontoxic, biocompatible microspheres could become candidates for clinically usable oximetry. The modest life time in vivo (in contrast to particulate probes that persist in tissue almost indefinitely) would be an advantage for short term oxygen measurements in a clinical setting.

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