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Development of biocompatible implants of fusinite for *in vivo* EPR oximetry

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The development of oxygen-sensitive paramagnetic materials is being actively pursued because of their potential applications for *in vivo* electron paramagnetic resonance (EPR) oximetry. Among these materials, fusinite is of particular interest because of the high sensitivity of the EPR linewidth to the partial pressure pO_2. Although this material has led to a number of very useful results in experimental systems, its potential use in humans is limited by the need to prove that it will not cause deleterious effects. The strategy used in this study to optimize the biocompatibility of the oxygen-sensitive materials was to prepare small silicon implants containing the fusinite. The use of silicon permits the diffusion of oxygen inside the implant while the material does not have contact with the biological environment. Radiosterilization did not affect the pO_2 sensitivity of the material. The feasibility of performing pO_2 measurement was verified *in vivo* by perodically inducing ischemia in the gastrocnemius muscle of mice over a period of 6 weeks.

Keywords: EPR, oximetry, *in vivo*, biocompatibility, ischemia, MRI.

INTRODUCTION

Several carbon-centered radicals such as contained in coals (fusinite) [4], and carbohydrate chars [5] are of particular interest to *in vivo* EPR oximetry [1–3] because of the high sensitivity of their EPR linewidth to the concentration of oxygen and their chemical inertness.

Although these materials have led to a number of useful results on the pathophysiology of ischemic processes [6] and although several oxygen-sensitive particulate materials were found noncytotoxic *in vitro* [7], their potential use in humans still is limited by the need to prove that they will not lead to deleterious effects *in vivo*. The concern arises, paradoxically, because their inertness results in their persistence in tissues. Up to now, India ink is the only such compound which appears to be available for immediate use in human subjects [8–10] because it already has been used extensively in humans. This material, however, has some significant limitations: it can have a multi-component EPR signal and a relatively low density of spins [9]. Other materials present better oxygen sensitivity and a higher spin density; but some of them sometimes possess a variability in the *in vivo* response, and all of them have an unknown long-term tolerance. For this reason, we have developed several different strategies to optimize biocompatible oxygen-sensitive materials. We have carried out studies using different types of particle coatings of fusinite and carbohydrate char as representative types of oxygen sensitive particulate materials (B. Gallez et al., submitted).

The present study is based on the development of biocompatible silicon implants containing fusinite. The use of silicon should permit the diffusion of oxygen inside the implant while the material does not have any contact with the biological environment. Moreover, these implants can be removed from the tissue after the analysis. We checked the *in vitro*

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sensitivity of such implants to the oxygen concentration and the possibility of using γ-radiation to sterilize these implants. We then performed *in vivo* experiments using an ischemia model based on the restriction of the blood in the gastrocnemius muscles of mice. Because it is also often desirable to check the *in vivo* localization of such implants [6], we used the T_2 effect of these materials to visualize them using magnetic resonance imaging.

METHODS

Preparation of the implants

100 mg of finely ground fusinite (obtained from Dr. R. Clarkson, University of Urbana-Champaign) were mixed with 0.2 ml of polydimethysiloxane oil (Dow Corning Medical Fluid 360, 200 cps, Midland, MI) and ±600 mg of silicon paste (Bricobi, Brussels). This mixture was placed in a syringe and extruded in a Silastic® medical grade tubing (Dow Corning, Midland, MI 0.02 in. inner diameter, 0.037 in outer diameter). For *in vivo* purposes, small pieces of ±2 mm were cut. The ends were covered with the silicon paste. It took approximately two weeks to dry the samples at room temperature. The EPR properties (linewidth) were found to be constant in the dried samples. Several samples were irradiated using a ^{60}Co panoramic chamber and received a dose of 25 kGy (a dose commonly used for the radiosterilization of implants).

Calibration curves: linewidth versus pO_2

The calibration measurements of fusinite linewidths versus pO_2 were performed at 9.3 GHz with a Bruker (Rheinstetten) ER-200tt EPR spectrometer equipped with a variable temperature controller B-ST 100/700. We compared silicon implants and the suspension of fusinite in water (100 mg/ml) placed in a gas permeable teflon tube (Norell, Inc., Landisville). Gas with known concentrations of nitrogen and oxygen (obtained using a medical gas mixer Minerve, Esternay®) was equilibrated at 37°C and flushed over the samples, and the spectra were recorded after an equilibration time of at least 15 min. Typical spectrometer parameters were: modulation amplitude less than one third of the peak-to-peak linewidth; incident microwave power, 1 mW; scan range, 10 G.

In vivo EPR

Male Balb/ C mice (16–18 g) were used for the studies in muscle. The mice were anesthetized by intramuscular injection of a mixture containing xylazine (10 mg/kg) and ketamine (200 mg/kg). *In vivo* measurements were performed on a homemade EPR spectrometer with a low-frequency microwave bridge operating at 1.1 GHz [11]. Two groups of 5 mice were injected in the gastrocnemius muscle with the silicone/fusinite implants or 50 µl of a slurry containing 100 mg/ml of fusinite. These EPR measurements were started the day after the injection and were repeated for six weeks to determine the reproducibility and the stability of the EPR measurements. Hypoxia was induced by restriction of the blood supply in the muscle. To restrict the blood supply, the base of the thigh was reversibly tied with a thread to restrict flow through the femoral arteries. The muscle under study was placed below the center of an extended loop resonator (1 cm depth sensitivity). The linewidth (calculated using software developed at the EPR Research Center, Dartmouth Medical School, Hanover, NH) was followed with these typical spectrometer parameters: modulation amplitude, 0.32 G; scan range, 5 G; scan time 1 min.

MRI

A Biospec® (Rheinstetten Bruker) imager operating at 4.7 Tesla was used for this study. A T_2-weighted fast spin-echo pulse sequence (a modification of the rapid acquisition with relaxation enhancement (RARE) sequence developed by Hennig [12]) was performed on male NMRI anesthetized mice that weighed 18–22 g with acquisition of sagittal sections, each 1.5 mm in thickness. This provides conventional SE contrast in shorter imaging times than the classical T_2-weighted SE. This reduction in imaging acquisition time is characterized by a reduction factor termed the "RARE factor." In our study, the T_2-weighted fast spin-echo parameters were 1000 ms/ 12.5 ms, TR/ TE and 4, RARE factor. The mice were injected with the fusinite/ silicon implant before the imaging protocol.

RESULTS

Calibration curves: linewidth versus pO_2

The calibration curves of the fusinite in a slurry and contained in the silicon implant are shown in Fig. 1. The greater value of the slope induces a higher sensitivity to pO_2 for the silicon implants than for the fusinite slurry. Typical EPR spectra of the fusinite/ silicon implants, recorded in nitrogen and in air, are shown in Fig. 2.

We checked the influence of γ-irradiation of the fusinite/ silicon implants on the EPR spectra. Because radiosterilization often induces the appearance of stable radicals in solids, as already observed in polymers used for medical implants [13], it was essential to

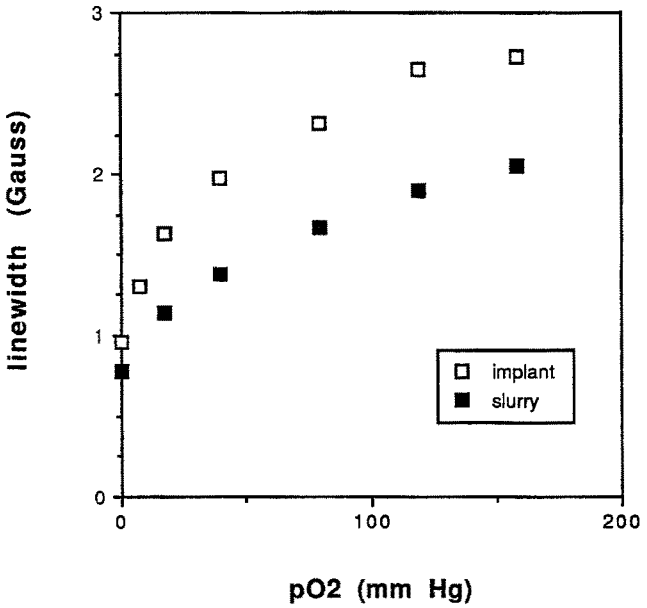

Fig. 1. Calibration curves (peak-to-peak linewidth versus pO_2) of fusinite in a slurry (*top*) and in silicon implant (*bottom*). Note the greater value of the slope meaning a higher sensitivity to pO_2 for the silicon implants than for the fusinite slurry.

check this possibility. No new EPR signal was observed in the spectra of the implants at room temperature and 37°C (*in vivo* conditions). This is particularly important for obtaining readily interpretable EPR spectra *in vivo*. Moreover, the pO_2 sensitivity was unchanged by the γ-irradiation. It should be noticed that this behavior of fusinite was not found in all materials we tested. For instance, a carbohydrate char we prepared by heating showed a completely different pO_2 sensitivity after gamma-irradiation (data not shown).

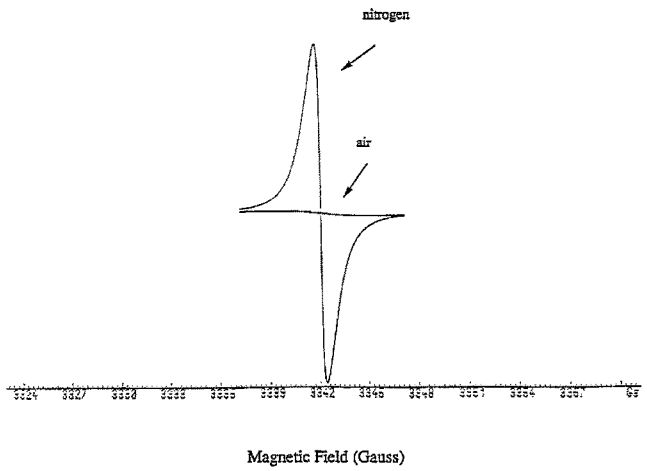

Fig. 2. Typical EPR spectra of the fusinite/silicon implants recorded in nitrogen and in air.

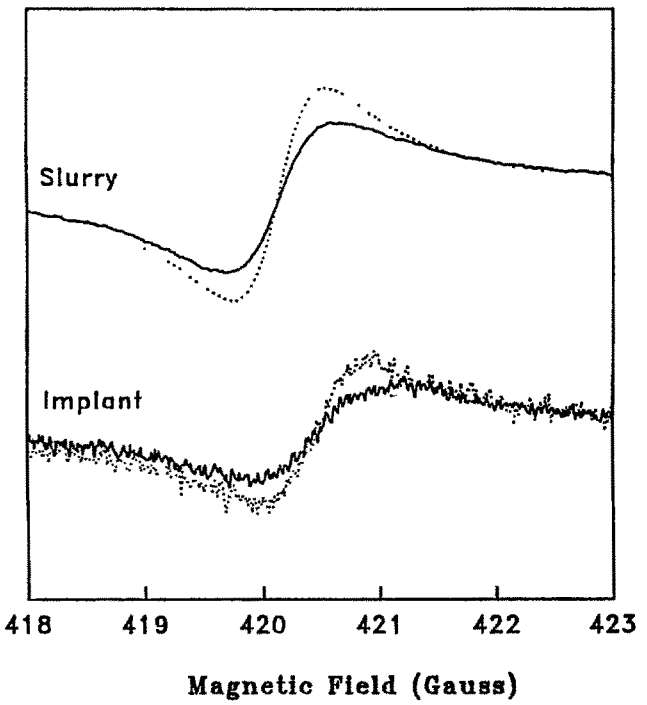

Fig. 3. Typical EPR spectra recorded *in vivo* in the gastrocnemius muscle of mice before (*solid line*) and after (*dotted line*) restriction of the blood supply. Note the larger difference in the variation of the linewidth using the implants rather than using the slurry. However, note the lower signal-to-noise ratio in the case of the implant.

In vivo EPR

Typical *in vivo* EPR spectra are shown in Fig. 3. The results are summarized in Table 1. Using the silicon/fusinite implants, an increase in the sensitivity was observed *in vivo* in the response of the fusinite to the pO_2, similar to that observed *in vitro*. We did not observe any change in the pO_2 response over the observation period of the mice. This is important in view of the potential value of EPR oximetry in making repeated measurements of pO_2 for a prolonged period, for example in the monitoring of radiotherapy [10]. Using the calibration curves, the measured pO_2 was approximately 8 mm Hg and 0 mm Hg for the normal and hypoxic muscle. These data are consistent with previously published data [4]. The only disadvantage of using the implants is the lower signal-to-noise ratio

Table 1. Linewidth (mGauss; mean ± SD) recorded *in vivo* in the gastrocnemius muscle of mice.

	Normal	Hypoxic	Δ linewidth
Slurry	944 ± 43	769 ± 41	175
Implant	1238 ± 117	886 ± 37	352

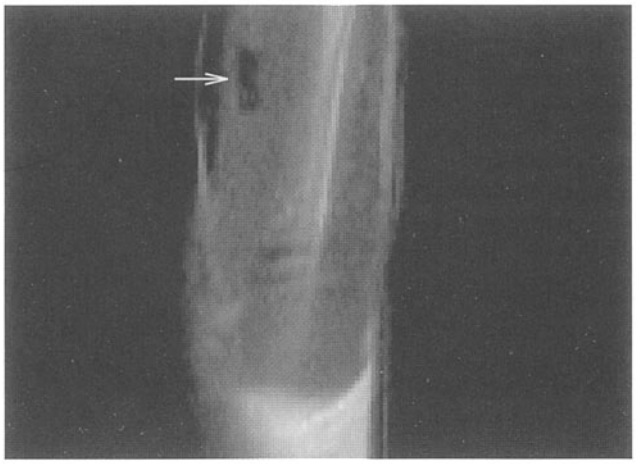

Fig. 4. Typical image obtained in the muscle of a mouse injected with a fusinite/ implant using the RARE sequence (1000 ms/ 12.5 ms; TR/ TE, 4; RARE factor).

of the EPR signal, as shown in Fig. 3. However, the overall quality of the spectrum still is sufficient to make an accurate measurement of the linewidth and consequently of the pO_2.

MRI

Because it is also often desirable to check the *in vivo* localization of the oxygen-sensitive probe, we wanted to check the feasibility of using MRI for such a purpose. Bacic et al. [6] used the T_2* effect of fusinite for visualizing charcoal by magnetic resonance imaging. We used the RARE sequence because it provides a better signal-to-noise ratio in the image and still reflects susceptibility effects of the paramagnetic probe. As shown in Fig. 4, the parameters used (1000 ms/ 12.5 ms; TR/ TE, 4; RARE factor) in the sequence provided a good localization of the implant. These parameters were optimized for measurements in the muscle and they are likely to be modified for the localization of this implant in other tissues because of different intrinsic T_1 and T_2.

DISCUSSION

In vivo EPR oximetry has led to a significant increase in understanding pathophysiological processes related to the pO_2 [1–6, 13, 14]. Combined with functional MRI and the quantitative measurements of the perfusion and diffusion [15], *in vivo* EPR appears to be a powerful tool in the study of pathologies associated with ischemic processes as well as a predictive tool for the evaluation of possible treatments. Obviously, the next step will be to apply this kind of research directly to humans. Up to now it has appeared that the first human studies would need to be performed using India ink as the oxygen-sensitive paramagnetic probe because of its established biocompatibility. Although other materials have more optimal properties in terms of spin density and oxygen sensitivity, their immediate application to humans is limited because of the need for extensive research in order to determine that the paramagnetic compounds are well tolerated (absence of acute and long-term toxicity). An alternative is to incorporate these probes in thin biocompatible implants. Silicon is the most widely used material for catheters and implants. Although some controversy exists about the potential health risk associated with long-term silicon implants [16], there appears to be little grounds for concern in proposing such implants for oximetry in humans. First, the aim is diagnostic and therapeutic use (their use will be limited to the measurement of the pO_2 in potentially pathological tissues such as tumors). Second, the size of such implants is very small when compared to the large implants used for breast implants and third, the type of silicon-containing material to be used is different. Finally, it is likely that such implants will be left in place for relatively short periods of time and then removed after the period necessary for the diagnosis or monitoring of the treatment.

The device presented here permits an accurate, precise, and reproducible measurement of pO_2 in tissues without any alteration over the time that was studied (6 weeks). Moreover, these implants showed a higher sensitivity to changes in pO_2 than the native fusinite. This oxygen sensitivity was especially higher in the range of low pO_2 (Fig. 1), which often is the most interesting area for the determination of pO_2 in tissues. For example, readings around $pO_2 = 5$ mm Hg are the data needed to characterize the potential radiosensitivity of most tumors since that is the critical value associated with a change in radiosensitivity. This sensitivity to changes in pO_2 might be improved by using other solvents in the implants, i.e., oils or perfluoroalkanes which have higher oxygen solubility. This type of result was obtained by Liu et al. [17] using nitroxides contained in albumin microspheres. It is also likely that other oxygen-sensitive materials can be used in this kind of device and that the characteristics presented here are not limited to the fusinite we used in this study. Finally, we demonstrated that these implants can also be easily sterilized using gamma irradiations that may be essential for their use *in vivo*.

In conclusion, we have presented here a convenient way to use oxygen-sensitive materials for *in vivo* EPR oximetry which should greatly reduce the time required for approval for its use in human subjects.

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