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An implantable Teflon chip holding lithium naphthalocyanine microcrystals for secure, safe, and repeated measurements of pO₂ in tissues

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Abstract

Lithium naphthalocyanine (LiNc) is a crystalline material that has significant potential as a probe for EPR (electron paramagnetic resonance)-based biological oximetry (Pandian et al. *J. Mater. Chem.* 19:4138–4147, 2009a). However, implantation of LiNc crystals in tissues in raw or neat form is undesirable since dispersion of crystals in tissue may lead to loss of EPR signal, while also exacerbating biocompatibility concerns due to tissue exposure. To overcome these concerns, we have encapsulated LiNc crystals in an oxygen-permeable polymer, Teflon AF 2400 (TAF). Fabrication of TAF films incorporating LiNc particles (denoted as LiNc:TAF chip) was carried out using solvent-evaporation techniques. The EPR linewidth of LiNc:TAF chip was linearly dependent on oxygen-partial pressure (pO₂) and did not change significantly relative to neat LiNc crystals. LiNc:TAF chip responded to changes in pO₂ reproducibly, enabling dynamic measurements of oxygenation in real time. The LiNc:TAF chips were stable in tissues for more than 2 months and were capable of providing repeated measurements of tissue oxygenation for extended periods of time. The results

demonstrated that the newly fabricated, highly oxygen-sensitive LiNc:TAF chip will enhance the applicability of EPR oximetry for long-term and clinical applications.

Keywords

Encapsulation; Oxygen permeability; EPR oximetry; Implantable biosensor; Teflon; Lithium naphthalocyanine

1 Introduction

Among the many techniques available for measuring oxygen concentration (oximetry), electron paramagnetic resonance (EPR) oximetry, using paramagnetic crystalline materials, is a powerful technique that allows incessant monitoring of oxygenation in tissues for longer periods (Kulkarni et al. 2007; Springett and Swartz 2007; Swartz and Khan 2005). The particulate-based oximetry probes, such as lithium phthalocyanine (LiPc), lithium octa-*n*-butoxy-naphthalocyanine (LiNc-BuO), or lithium 1,8,15,22-tetraphenoxypthalocyanine (LiPc- α -OPh) crystals, possess EPR linewidths that are highly sensitive to the local oxygen concentration (Ilango et al. 2004; Pandian et al. 2007; Pandian et al. 2006; Pandian et al. 2003). These spin probes can be implanted at the desired tissue site or internalized in cells, enabling accurate measurements of intracellular pO₂ (Bratasz et al. 2006). These probes are stable in tissues, nontoxic, and biocompatible. The measurements can be performed noninvasively and repeatedly over a period of several months at the same site (Pandian et al. 2003). We have recently reported a lithium naphthalocyanine paramagnetic crystalline probe, which has a unique advantage over other probes, in terms of its high oxygen sensitivity (Pandian et al. 2009a, b). Such high sensitivity to oxygen enables accurate pO₂ measurements at low oxygen environments with reasonably high resolution. Apart from its high oxygen sensitivity, LiNc also possesses very suitable characteristics for *in vivo* oximetry, such as chemical inertness, high spin density, and biocompatibility.

However, direct *in vivo* administration of particulate materials, such as LiNc, could lead to biocompatibility concerns as the particles will be in direct contact with tissue. Moreover, dispersion of the particles in tissue could lead to progressive decay of the EPR signal, as well as, hinder the retrieval of the implanted probe, if needed. To alleviate concerns with direct implantation of particulate EPR probes, in previous work along these lines, we and others, have encapsulated the probes, namely LiPc and LiNc-BuO, in biocompatible Teflon and polydimethylsiloxane polymer matrices (Dinguizli et al. 2008; Dinguizli et al. 2006; Eteshola et al. 2009; Meenakshisundaram et al. 2009a; Meenakshisundaram et al. 2009b).

In the present work, we chose Teflon AF 2400 (TAF) as the polymer for encapsulation of LiNc. TAF possesses several favorable properties including inertness, high hydrophobicity, lubricity, mechanical strength, oxygen permeability and biocompatibility. It is moderately soluble in fluorinated solvents at room temperature; thus thin and dimensionally stable chips can be prepared easily through solvent casting. The TAF film is transparent within a wide UV—Visible and IR range enabling analysis of the chip with spectroscopic/optical techniques. TAF has a high fractional free volume (FFV) (Alentiev et al. 1997), which is the reason for its exceptional oxygen permeability (Merkel et al. 2006).

We have developed a procedure for the encapsulation of LiNc crystals in the Teflon AF 2400 matrix. We studied the stability of the responsiveness of the chip to oxygen after sterilization, and after long-term residence *in vivo*. The results demonstrated good biostability and biocompatibility, without any adverse effects on the oxygen sensitivity of this probe.

2 Materials and methods

2.1 Materials

Lithium naphthalocyanine (LiNc) was synthesized as reported (Pandian et al. 2009a). Fluorinert FC-40, Fluorinert FC-77, hexafluorobenzene (HFB), Teflon AF 2400 (TAF) were purchased from Sigma (St. Louis, MO, USA).

2.2 Preparation of chips doped with LiNc microcrystals

The LiNc particle-loaded membranes were fabricated by solution-casting on precleaned glass slides (flat or concave). Prior to casting, LiNc particles were dispersed manually, as uniformly as possible, into a 2.5 wt. % of TAF 2400 in 200 μ L fluorinert FC-77 solvent. The FC-77 used for dissolution of the TAF 2400 was allowed to evaporate at room temperature. The LiNc particle-loaded TAF membrane was kept in an oven at 75°C overnight to assure complete evaporation of solvent. Smaller pieces of various sizes (from 1 to 2 mm) were cut from a larger LiNc:TAF chip. A pure TAF film, without LiNc crystals (Fig. 1(b)), was fabricated for comparison to the LiNc:TAF chips. Fabrication of the pure TAF film was carried out as described for LiNc:TAF chips, but without incorporating LiNc microcrystals.

2.3 Autoclaving

Autoclaving of the LiNc:TAF chip was performed using a standard bench-top autoclave unit (Tuttnauer®/Brinkmann™ Benchtop Autoclave, New York, Model: 3850M). Chips were autoclaved at 121°C for 1 h at 1 atm pressure (wet cycle using steam) followed by exhaust drying for 15–20 min.

2.4 EPR measurements

The EPR spectroscopic measurements were carried out using an X-band (9.8 GHz) spectrometer (Bruker Instruments, Karlsruhe, Germany). The LiNc:TAF chips were calibrated for EPR oximetry as reported (Pandian et al. 2009a). EPR imaging was used to determine the nature of spin distribution in LiNc:TAF chip. LiNc:TAF chips, approximately 1–2 mm in size, were vacuum-shielded in a 4-mm EPR quartz tube. Two dimensional images of the surface of the chip were obtained using an X-band EPR imager (Bruker Eleksys E580 spectrometer, Billerica, MA). EPR Image acquisition was performed using the following parameters: field of view 4 mm, magnetic field gradient 44.3 G/cm and modulation amplitude 0.4 G.

2.5 Surface evaluation of LiNc:TAF chips using atomic force microscopy (AFM)

The LiNc:TAF chip surface was studied using a multimode atomic force microscope (Nanoscope III, Digital Instruments, Santa Barbara, CA). Distilled water was used to clean the sample surface. Wet samples were dried under vacuum and contact-mode AFM measurements were performed.

2.6 Responsivity of LiNc:TAF chip to changes in pO₂

The time-response of LiNc:TAF chip to changes in pO₂ was examined by switching the gas flowing across the sample from room air to nitrogen. Switching between the flowing gases was achieved using a manual three-way valve. The two input lines to the valve were connected to gas cylinders containing pure nitrogen and room air. The output of the valve was connected to the EPR tube containing the sample. The magnetic field corresponding to the peak of the EPR spectrum at anoxic condition (100% N₂) was used for acquisition. The input gas was switched manually between room air and nitrogen at periodic intervals.

2.7 Animal studies

Female C3H mice procured from the Frederick Cancer Research Center, Animal Production Unit (Frederick, MD, USA) were used. The animals were received at 6 weeks of age and housed five per cage in climate-controlled rooms and allowed food and acidified water *ad libitum*. The animals were on average 50-days old at the time of experimentation and weighed 22–28 g. Experiments were conducted according to the principles outlined in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council. Mice were anesthetized with an isoflurane (1.5%)-air mixture delivered through a nose cone. LiNc:TAF chip (2 mm×2 mm), in the form of thin film, was implanted subcutaneously in the gastrocnemius muscle of the right hind leg. All the *in vivo* EPR measurements were made at least 48 h after the implantation.

2.8 *In vivo* EPR measurements

The EPR oximetry measurements were carried out on anesthetized mice using L-band (1.32 GHz) spectrometer (Magnetech, Berlin, Germany) and a topical (surface loop) resonator. Anesthesia was maintained during the measurements with continuous delivery of 1.5% isoflurane mixed with air using a veterinary anesthesia system (Vasco Anesthesia, Pro Tech Medical Inc., Hazel Crest, IL). The flow rate of the breathing gas mixture was maintained at 2.0 L/min. A thermistor rectal probe was used to monitor body temperature. The body temperature was maintained at 37±1°C using an infrared lamp. In order to verify that the implanted LiNc:TAF chip was able to report changes in pO₂ *in vivo*, blood flow to the gastrocnemius muscle was temporarily restricted, above the location of the chip, using an elastic band. EPR measurements were made in the constricted state of the muscle (less than 5-min duration), and then the constriction was removed.

3 Results and discussion

3.1 Fabrication of LiNc:TAF chips

LiNc microcrystals were encapsulated in TAF-2400 using a solvent evaporation approach, which we have previously used for the encapsulation of LiPc (Eteshola et al. 2009). Figure 1 (b) shows a TAF film without LiNc microcrystals for comparison with LiNc:TAF film. The native TAF film was colorless and optically transparent, whereas embedding LiNc in TAF resulted in a dark purple color (Fig. 1(c)). The distribution of LiNc in the polymer was dependent on the mass of the crystals and the concentration of the polymer solution. To make sure of total coverage of the encapsulated LiNc probes within the TAF-2400 polymeric matrix, multiple coatings (typically 2 or 3) were applied. LiNc microcrystals were highly insoluble in solvents, such as FC-40, FC-77 and hexafluorobenzene (HFB), which were all excellent solvents for the TAF polymer. As a result, we were able to fabricate LiNc:TAF chips using FC-77 solvent, without any risk of losing LiNc particles due to dissolution. This matters as oxygen sensitivity of particulate probes, such as LiNc, is dependent on intact crystal structure, so solubility of the probe in the polymer will have an adverse impact on the oxygen-sensing properties of the probe.

Figure 1(d) shows a representative X-band EPR image (3 mm×3 mm) of LiNc embedded in TAF-2400 membrane. The image shows uniform intensity (red) confirming the uniform distribution of LiNc spins within the TAF matrix.

3.2 Surface study of LiNc:TAF chips

Figure 2 shows typical AFM image of LiNc:TAF chips with one (single) or multiple layers of TAF coating. The AFM images showed that the single- and multiple-coated LiNc:TAF chip surfaces were intact, without defects, bubbles, or cracks. Figure 2(c) shows a comparison of

root-mean-square (RMS) roughness of the single- and multiple-coated LiNc:TAF chips. Average surface roughness (RMS) values (expressed in mean $\pm$ SD, $n=10$), suggested that the roughness of single as well as multiple coated LiNc:TAF surfaces were not significantly different. Corresponding surfaces of the clear TAF film also showed similar surface profiles, indicating that the encapsulation of LiNc particulate probes did not significantly affect the integrity of the surfaces (data not shown). Since there was no significant difference in the roughness between single and multiple coatings, a single coating of TAF may have fully covered the crystals. Therefore, multiple coatings of TAF almost certainly cover the LiNc crystals completely, avoiding direct contact between tissues and LiNc, and precluding biocompatibility issues that might arise from tissue-particle contact.

3.3 Effect of oxygen on the EPR linewidth of LiNc:TAF chips

The LiNc:TAF chip exhibited a single-line EPR spectrum which was similar to that of uncoated (neat) LiNc microcrystals. The EPR linewidth of the spectrum of LiNc:TAF chip was highly dependent on the oxygen concentration in its surrounding environment: 670 mG under anoxic (0% oxygen) conditions, 6.3 G in room air (20.9% oxygen or 159 mmHg) at 1 atmospheric pressure. The anoxic peak-to-peak width of LiNc:TAF chip, as well as linewidth at varying oxygen concentrations did not significantly change relative to uncoated LiNc particulates (data not shown). That encapsulation of LiNc probes in TAF did not significantly alter their oxygen sensitivity under *in vitro* conditions is demonstrated by the plot of linewidth versus oxygen partial pressure (Fig. 3). The slopes of the linewidth vs. pO_2 calibration lines for LiNc:TAF and LiNc neat crystals were nearly identical (35.33 mG/mmHg and 35.93 mG/mmHg, respectively), and oxygen sensitivity of the LiNc:TAF chip was large and constant up to 300 mmHg of oxygen, thereby enabling the measurement of the pO_2 over a wide range of oxygen concentrations with high sensitivity. This signified that any small change in pO_2 can be measured with reasonable resolution using LiNc:TAF chips. Oxygen sensitivity of embedded LiNc was unchanged in chips made with any of the solvents (FC-40, FC-77 or HFB) used to dissolve TAF (data not shown).

The EPR spectrum of LiNc:TAF chip was also non-saturable up to 30 mW (data not shown), in principle allowing the signal-to-noise ratio to be substantially improved by increasing microwave power levels. On the other hand, LiPc embedded in polymer membranes saturates at 1 mW (Liu et al. 1993). In live animals, motion or breathing frequently causes signal interruptions at low microwave power, so non-saturation of LiNc:TAF at typical incident microwave powers, used for *in vivo* measurements, is a real advantage.

3.4 Time-response of LiNc:TAF chip to changes in pO_2

An important characteristic of any oxygen-sensing material is reasonably quick response to changes in oxygen concentration (responsivity) and reproducibility in successive measurements. We compared the response of the LiNc: TAF chip to that of neat LiNc crystals to changes in pO_2 via EPR spectroscopy. Figure 3(a) inset shows the $t_{1/2}$ response time of EPR absorption to cycles of rapid switching of the equilibrating gas, between 100% nitrogen and room air, at a constant flow rate of 2 L/min as reported (Pandian et al. 2006). It was observed that $t_{1/2}$ of LiNc:TAF was reasonably good (oxygenation 2 s; deoxygenation 35 s) and highly reproducible in successive gas switching cycles (Fig. 3(a) inset). The reproducibility also implied that oxygen was not adsorbed irreversibly onto the LiNc or TAF matrix. The uncoated LiNc (in crystalline form) exhibited $t_{1/2}$ values of 0.25 s for oxygenation and 5 s for deoxygenation, thereby demonstrating that the crystalline probe was capable of responding to changes in oxygenation almost instantaneously. Responsivity of LiNc:TAF chip to changes in pO_2 was moderately slower, but largely similar to the time-response of unencapsulated LiNc. However, deoxygenation time was significantly longer with LiNc: TAF chip (35 s) than with neat LiNc crystals (5 s). We attribute the difference to the physical barrier created by the TAF

coating to the free flow of oxygen in and out of the channels of the LiNc crystal structure. While TAF is exceptionally permeable to oxygen (Merkel et al. 2006), diffusion through TAF layer takes time, so it is not surprising that oxygen responses of LiNc:TAF chip was slower than oxygen responses of uncoated crystals. We have observed similar asymmetry in oxygenation/deoxygenation rates of other polymer-coated lithium phthalocyanine spin probes (Meenakshisundaram et al. 2009a; Meenakshisundaram et al. 2009b). Neat LiNc crystals may be preferable to the LiNc:TAF chip in oximetry applications monitoring rapid fluctuations in pO_2 levels, particularly when accurate measurement of rapid decreases in pO_2 is important.

3.5 Autoclaving

Sterilization of any biomaterial implant is crucial to minimizing infection and inflammation at the site of which leads to failure of the implant. We studied the effect of autoclaving on the LiNc:TAF chip. Oxygen calibration and sensitivity of LiNc:TAF chips were recorded before and after autoclaving. Comparison of the results, before and after treatment showed that the anoxic linewidth of LiNc:TAF did not change after autoclaving, nor did the autoclaving produce significant changes in oxygen sensitivity (data not shown). The fact that the oxygen-sensing performance of LiNc:TAF did not change after autoclaving indicated that autoclaving did not degrade the oxygen permeability of TAF. Spin density of LiNc:TAF was calculated before and after autoclaving, by comparing the AUC of the LiNc:TAF spectra with the spectral AUC of a known spin density standard (TAM; Kutala et al. 2004). Spin density did not change significantly (Fig. 3(b)). Thus, LiNc:TAF chips maintained their EPR/oxygen sensitivities and remained stable (without any loss of active paramagnetic spins) after sterilization by autoclaving.

3.6 Stability in tissues

In order to assess the stability of LiNc:TAF in tissues, we implanted the encapsulated probes in the gastrocnemius muscle tissue of mice and performed repeated measurements of pO_2 in the same animals over a period of 2 months using L-band EPR spectroscopy. The pO_2 of the leg muscle, as reported by the implanted chip, was recorded periodically for up to 2 months after implantation of the chips (Fig. 4). The results demonstrated the ability of the implanted LiNc:TAF chip to provide repeated measurements of *in vivo* pO_2 from the same tissue site over time. In order to verify that the chip responded to changes in *in vivo* pO_2 , blood flow to the upper leg was constricted with an elastic band (for approximately 5 min) and the constriction was released. Sharpening of the EPR spectrum (narrowing linewidth) during interruption of blood flow to the leg was used as an indication of reduced tissue oxygenation and responsiveness of the implanted chip to change in tissue pO_2 (Fig. 4). The tissue pO_2 also returned to baseline values when the constriction was removed (data not shown).

The pO_2 of mouse gastrocnemius muscle tissue, under normal blood-flow conditions as reported by the implanted LiNc:TAF chip was 14.2 ± 1.3 mmHg, which was within the range of previously-reported pO_2 values for this muscle using TAF-coated LiPc (15.5 ± 1.5 mmHg; Eteshola et al. 2009), PDMS-coated LiNc-BuO (15.6 ± 2.9 mmHg; Meenakshisundaram et al. 2009a; Meenakshisundaram et al. 2009b), neat LiNc-BuO (19.6 ± 2.1 mmHg; Pandian et al. 2003), neat LiNc (15.55 ± 1.59 ; Pandian et al. 2009a, b) and neat LiPc (18 ± 4 mmHg; Ilangoan et al. 2004).

4 Summary and conclusions

We have developed and evaluated an oxygen-sensing implant, the LiNc:TAF chip. The chip was fabricated by encapsulating LiNc particulates in TAF 2400 using solvent-evaporation techniques. The chips were robust and capable of withstanding harsh sterilization conditions (autoclaving). AFM and EPR imaging demonstrated that LiNc crystals were uniformly

distributed and completely coated in the TAF polymer matrix. The encapsulated LiNc crystals in the chip exhibited oxygen sensitivities that were not significantly different from neat LiNc crystals. The response time of LiNc: TAF chip to changes in pO_2 was increased in TAF chips relative to neat crystals, albeit within reasonable limits for successful applicability. The chips were stable in tissues more than 2 months, and were capable of repeated and realtime measurements of tissue oxygenation for an extended period. Overall, we have identified the LiNc:TAF chip as a highly sensitive oxygen sensor for secure, safe, and repeated measurements of local oxygen concentration in tissues with high resolution and as a promising oxygen-sensing probe for the successful application of EPR oximetry in the clinic.

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Fig. 1. Microscopic images of TAF film holding LiNc microcrystals. (a) Molecular structure of lithium naphthalocyanine (LiNc) radical. (b) Microscopic image of TAF film without LiNc particulates. (c) Microscopic image of LiNc microcrystals encapsulated in TAF. (d) X-band EPR image (3 mm×3 mm) of LiNc spin probes embedded in the TAF matrix. The uniform dark color of LiNc-containing TAF film shows that the LiNc particulates are evenly distributed in the TAF matrices. The EPR image (d) shows bright and dark regions corresponding to the distribution of active LiNc spins within the TAF matrix

Fig. 2. Surface analysis of LiNc:TAF by AFM. Representative AFM images of TAF films containing LiNc crystals, with one coating of polymer **(a)** and multiple coatings of polymer **(b)**. The *darker shade of color* denotes features with smaller heights, whereas the *brighter color* in the images represents taller features on the sample surface. **(c)** Quantitative comparison of single- and multiple-coated LiNc:TAF film surfaces. Average surface roughness (RMS) values (expressed as mean $\pm$ SD, $n=10$) suggested that the roughness of single as well as multiple coated TAF surfaces were not significantly different

Fig. 3.

Oxygen response of LiNc:TAF chips. **(a)** Effect of oxygen concentration (pO_2) on the peak-to-peak EPR linewidth of LiNc particulates encapsulated in TAF 2400 (TAF) film, The *inset* shows a comparison of oxygen-response time ($t_{1/2}$) of pure LiNc crystals (BLACK) with LiNc encapsulated in TAF (GREY). **(b)** Spin density evaluation of TAF-encapsulated LiNc spin probes before and after autoclaving. The linewidth increased linearly with pO_2 in the range 0 to 160 mmHg (corresponding to 0–20.9% oxygen at 1 atmospheric pressure) with an anoxic linewidth of 0.67 G and slope (oxygen sensitivity) of 35.33 mG/mmHg. The response of neat LiNc crystals to changes in local oxygen content was more rapid than the response of TAF-encapsulated probes. Autoclaving did not have any adverse effect on the spin density of LiNc

encapsulated in TAF, which showed that the films could be sterilized before implantation and *in vivo* applications

Fig. 4.

Long-term stability and response to oxygen. TAF-encapsulated LiNc was implanted in the gastrocnemius muscle of mice and pO₂ was monitored using L-band EPR spectroscopy more than 2 months. The graph shows repeated measurements of pO₂ from four animals, in the normal (*filled symbols*) and constricted (*open symbols*) of the muscle. Normal muscle pO₂ is represented as mean±SD, whereas constricted muscle pO₂ values from individual animals are shown. The data show that the encapsulated particulates were stable and responsive to oxygen in live tissue for up to 2 months