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Retrievable micro-inserts containing oxygen sensors for monitoring tissue oxygenation using EPR oximetry

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Abstract

Tissue oxygenation is a crucial parameter in various physiopathological situations and can influence the therapeutic response of tumours. EPR oximetry is a reliable method for assessing and monitoring oxygen levels *in vivo* over long periods of time. Among the different paramagnetic oxygen sensors available for EPR oximetry, lithium phthalocyanine (LiPc) is a serious candidate for *in vivo* applications because of its narrow linewidth and its high signal-to-noise ratio. To enhance the biocompatibility of the sensors, fluoropolymer Teflon AF2400 was used to make cylindrical micro-inserts containing LiPc crystals. This new micro-pellet design has several advantages for *in vivo* studies, including the possibility of being able to choose the implant size, a high sensor content, the facility of *in vivo* insertion and complete protection with preservation of the oxygen sensor's characteristics. The response to oxygen and the kinetics of this response were tested using *in vivo* EPR: no differences were observed between micro-inserts and uncoated LiPc crystals. Pellets implanted *in vivo* in muscles conserved their responsiveness over a long period of time (∼two months), which is much longer than the few days of stability observed using LiPc crystals without protection by the implant. Finally, evaluation of the biocompatibility of the implants revealed no inflammatory reaction around the implantation area.

Keywords: EPR, oximetry, oxygen, biocompatibility

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Evaluation of the oxygen pressure in tissues is critical in physiology, pathophysiology and during certain therapies. Monitoring oxygen levels in different tissues can help in adapting

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therapeutic protocols in various situations, such as radiotherapy, wound healing and ischaemic areas in diabetes. Different methods exist for determining and monitoring oxygen pressure *in vivo* (Gallez *et al* 2004), and can be classified into two groups: (i) direct measurement of oxygen (invasive and non-invasive methods), such as the polarographic oxygen electrode (Eppendorf®), which has, for years, been considered as the 'gold standard' method; and (ii) methods based on the correlation between parameters related to oxygen levels, such as the haemoglobin saturation.

EPR oximetry has been widely used over the last decade to quantify oxygen pressure in the brain (Hou *et al* 2003), heart (Kuppusamy and Zweier 2004), gastrointestinal tract (He *et al* 1999), skeletal muscle (Jordan *et al* 2004, Aragonés *et al* 2008), liver (Gallez *et al* 1998, James *et al* 2002), kidneys (James *et al* 1996), skin (Krzic *et al* 2001) and tumours (Goda *et al* 1996, Gallez *et al* 1999, Jordan *et al* 2000, 2002, 2003). The technique is based on the introduction of an oxygen sensor into the tissue of interest a few hours before the measurement. Several parameters, such as toxicity, oxygen sensitivity and *in vivo* stability, are crucial for guiding the choice of the oxygen sensor. Oxygen sensors can be divided into two groups: (i) soluble sensors such as trityl or nitroxides, and (ii) particulate materials such as India Ink, charcoals and chars (Gallez *et al* 2004, Vahidi *et al* 1994, James *et al* 1997, Lan *et al* 2004, Jordan *et al* 1998). In spite of their interesting properties, including their rapid diffusion and distribution in physiological compartments, soluble sensors have a serious disadvantage for pO_2 monitoring due to their metabolism and elimination. In contrast, particulate paramagnetic materials are generally very inert. Several studies have described repeated *in vivo* pO_2 measurements with these materials over long periods of time, from days to months, including in humans (Swartz *et al* 2004).

Among the particulate spin probes, the phthalocyanine group has been used most for *in vivo* pO_2 measurements. Since the first description of lithium phthalocyanine (LiPc) as a probe for EPR oximetry by Liu *et al* (1993), LiPc crystals have been used for oxygen monitoring in the brain (Hou *et al* 2003) and in tumours (Ilangovan *et al* 2004, Bratasz *et al* 2007, Matsumoto *et al* 2008, Dunn *et al* 2002). Most studies used 'uncoated' LiPc crystals *in vivo*, except in our recent study in which we implanted catheters holding LiPc crystals coated with a Teflon AF polymer (Dinguizli *et al* 2006). In this context, resonators were developed to carry out measurements deeper than 1 cm, which usually represents the limit of depth penetration for *in vivo* measurements using EPR spectrometers operating at 1 GHz (L-Band), due to non-resonant absorption by water. Although LiPc is considered as stable in tissues such as brain and tumours, a loss of oxygen sensitivity has been described in muscles (Liu *et al* 1993). In this regard, the development of biocompatible inserts presents a unique possibility of preserving the oxygen sensitivity of LiPc. Other studies have indeed demonstrated the effectiveness of this coating for preserving responsiveness in tissues (Gallez *et al* 1999, He *et al* 2001).

In the present study, we tested a new design of retrievable polymeric micro-inserts containing LiPc in order to stabilize the responsiveness of the sensor, to increase the content in oxygen sensor in the implant (compared to the films previously described, Dinguizli *et al* 2006), and also to facilitate implantation into and resection from tissues. To characterize this new type of insert, the kinetics of the response and the oxygen sensitivity of the sensor cast in polymeric tubes were measured. We also investigated by *in vivo* EPR the stability of the responsiveness to oxygen during a long *in vivo* residence. Histological studies were performed after implantation of this insert in the gastrocnemius muscle of mice to check the biocompatibility of the inserted pellet.

2. Materials and methods

2.1. Micro-insert preparation

Cylindrical micro-pellets were made using a Teflon® AF2400 tubing (ID: 0.034″) that was filled with LiPc crystals suspended in a polymer solution (1% Teflon AF 2400 w/v in FC 75 3M solvent). LiPc crystals were kindly provided by H M Swartz. The tubing was dried. Pellets of different lengths were cut and then coated using a polymer solution (2% Teflon AF 2400 w/v in FC 75 3 M solvent), and finally dried for 24 h in an oven at 70 °C.

2.2. X-Band EPR measurements

Calibration curves and the kinetics of response for coated and uncoated materials were performed by monitoring the variation in the EPR linewidth when the gas content was rapidly changed from 0 to 21% oxygen. Calibration was performed at 9.3 GHz with a Bruker EMX EPR (Rheinstetten, Germany) spectrometer equipped with a variable temperature controller BVT-3000. Gas with known concentrations of nitrogen and air (obtained by using a gas mixer Aalborg®, Orangenburg, NJ, USA), equilibrated at 37 °C, was flushed over the samples, and the spectra were recorded every minute until equilibration was achieved. The oxygen content in the gas was analysed using a Servomex oxygen analyser OA540. The modulation amplitude was less than one third of the peak-to-peak linewidth, incident microwave power was 50 μW, and modulation frequency was 10 kHz.

2.3. In vivo implantation and measurements

After sterilization in an autoclave (120 °C for 20 min), micro-inserts were implanted using an 18G trocar needle in the gastrocnemius muscle of NMRI male (25 g, $n = 4$) mice under anaesthesia with ketamine/xylazine (60 mg kg^{-1} and 6 mg kg^{-1}, respectively). The loaded trocar was inserted in the muscle, and the micro-inserts were deposited by pushing in the stylus. *In vivo* EPR spectra were recorded using an EPR spectrometer (Magnettech, Berlin, Germany) equipped with a low-frequency microwave bridge operating at 1.2 GHz and a surface-coil resonator. Measurements were made under anaesthesia (1.8% isoflurane in air). In order to assess the responsiveness of sensors *in vivo*, spectra were recorded under normal conditions and under hypoxic conditions obtained by a transitory restriction in blood flow. The modulation frequency was 10 kHz, and the amplitude modulation did not exceed one third of the linewidth of recorded spectra to avoid peak distortion. Linewidth was measured after 10 min of animal stabilisation and was the average of ten accumulations (20 s scans) in the same experimental conditions.

2.4. Histological studies

The animals were sacrificed by cervical dislocation 7 days after implantation. Muscles were excised, and the implant was removed. The tissues were fixed with 10% formalin, embedded in paraffin, and stained with haematoxylin–eosin. Observations were made using optical microscopy (YS2-H, Nikon Corporation, Tokyo, Japan).

3. Results and discussion

Using the method described above, we were able to design micro-inserts holding LiPc crystals. The picture of a typical cylindrical insert is presented in figure 1. The length of the

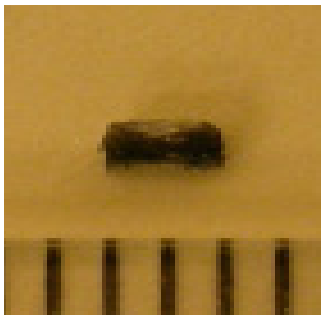

Figure 1. Picture of a micro-pellet composed of a Teflon AF2400 tube filled with an LiPc sensor. This implant was 2 mm long.

micro-inserts can be adapted as a function of the tissue size and the site of implantation. The choice of a cylindrical design is well adapted to easy *in vivo* implantation through trocar needles in various sites, such as the brain or other specific areas. Filling the tubing with LiPc crystals in polymer solution helps to avoid development of microbubbles, which can form micro gas containers and disturb pO_2 measurements or the kinetics of equilibrium. All micro-inserts were systematically checked with a microscope to confirm the absence of microbubbles; inserts with bubbles were not used to avoid the potential problem of oxygen reservoirs disturbing the measurement. Interestingly, we observed a rapid equilibrium with the gas phase and significant responses to small changes in oxygen level *in vivo*. Figure 2 shows the responsiveness of LiPc after inclusion in the polymeric cylinder. Comparison between the kinetics of response of LiPc in pellets and simple crystals (used without any coating) demonstrated that there was no significant difference between these two forms of sensors under *in vitro* conditions (data not shown). It should be noted that the polymer material was carefully chosen for its oxygen permeability. Polymeric coatings should indeed be completely permeable to oxygen as is the case for Teflon AF2400. Teflon AF2400 has a high oxygen permeability compared to other fluoropolymers, and is used in contact lens technology where oxygen permeability is a crucial parameter (Legeay *et al* 1998).

Another very important issue is the stability of the oxygen sensor. As already stated (Liu *et al* 1993, Gallez and Mäder 2000), LiPc is known to lose its responsiveness to oxygen in muscles. Interestingly, we observed a significant difference between uncoated LiPc crystals and the LiPc embedded in micro-inserts that we designed. Figure 3 shows an example of the loss of responsiveness of uncoated LiPc in mice muscle over time. During the first 10 days, pO_2 values were around 20 mm Hg, which is in agreement with other studies. The apparent oxygen pressure then decreased below 10 mm Hg. After 20–30 days, the sensors were still able to respond to induced ischaemia, but the degree of responsiveness was significantly reduced compared to the initial response just after implantation in the tissue. In contrast, LiPc containing micro-inserts gave repetitive and comparative values without any loss of sensitivity for up to two months after implantation (duration of the study) as shown in figure 4. The value recorded after four weeks of residence in the muscle (12 mm Hg) was a little bit lower than after one or two weeks (around 20 mm Hg). However, this measurement could probably be considered as an outlier as the pO_2 recorded two months after implantation came back to the initial values of 20 mm Hg. This could also be due to the limited number of animals.

The reason for this preservation of responsiveness is still speculative. However, we can suggest several hypotheses: (i) since the sensitivity of LiPc to oxygen is strongly dependent

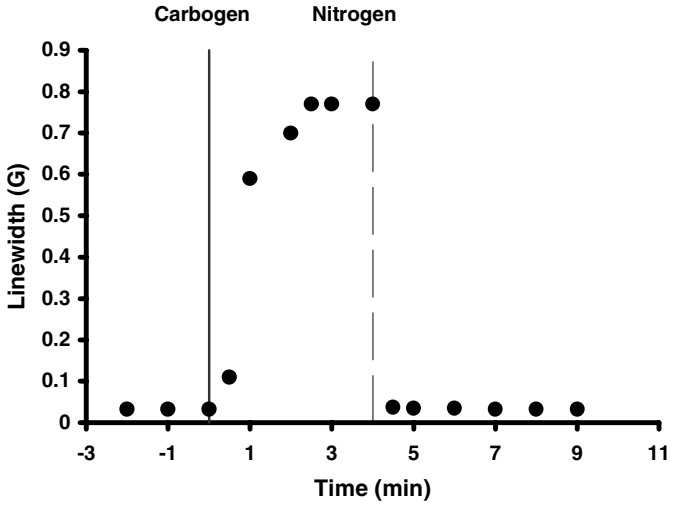

Figure 2. Kinetics of the response of LiPc inserted in the micro-pellet to variations in oxygen. The challenge consisted of switching nitrogen to air, and then air to nitrogen in the cavity of an X-Band EPR spectrometer. Note the rapid response of the micro-inserts to changes in the oxygen environment.

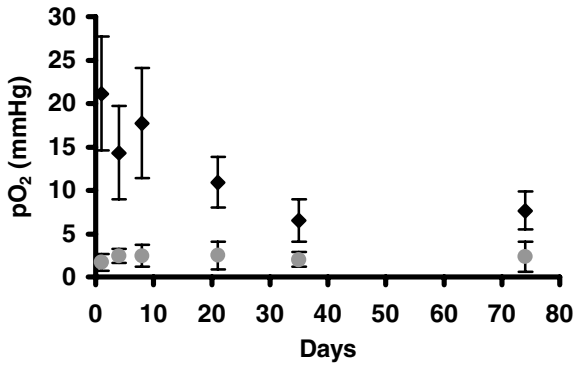

Figure 3. *In vivo* responsiveness of uncoated LiPc after implantation in the gastrocnemius muscle. EPR measurements were carried out with a 1 GHz EPR spectrometer on normal muscles, and on ischaemic muscles after a transient interruption of the blood flow. The pO_2 values were obtained in four different animals (mean $\pm$ standard deviation). Note the decrease in the degree of responsiveness over time.

on the crystal structure (Bensebaa *et al* 1992), it is possible that minor changes or damage to the crystal architecture might occur after a few days *in vivo* in tissues with a high mechanical stress, such as muscles, thus affecting responsiveness; (ii) the implantation of LiPc crystals can induce an inflammatory reaction leading to the recruitment of a high number of inflammatory cells consuming high levels of oxygen that might interfere with the oxygen measurement (Crokart *et al* 2005); (iii) the inflammatory response after insertion of oxygen sensors may be responsible for a tissue reaction leading to release of substances that 'poison' the response of the sensors or to the formation of impermeable capsules surrounding the sensors. Our protocol induces minimum tissue damage and trauma during sensor implantation, which means rapid stabilization of the implant in tissues, which is required before any measurement.

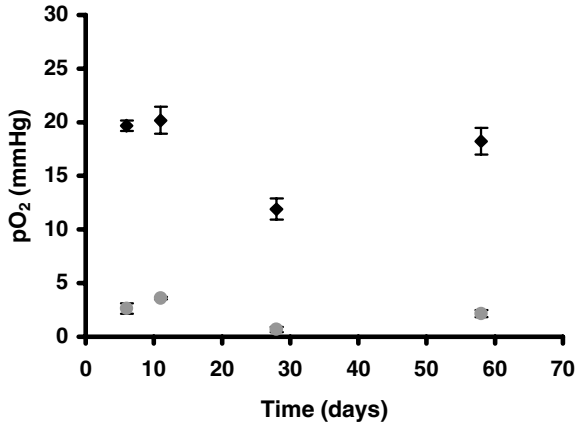

Figure 4. *In vivo* responsiveness of LiPc embedded in micro-inserts after implantation in the gastrocnemius muscle. The EPR measurements were carried out with a 1 GHz EPR spectrometer on normal muscles, and on ischaemic muscles after a transient interruption of the blood flow. The pO_2 values were obtained in four different animals (mean ± standard deviation). Note that the responsiveness to change in oxygenation is well preserved up to two months after implantation.

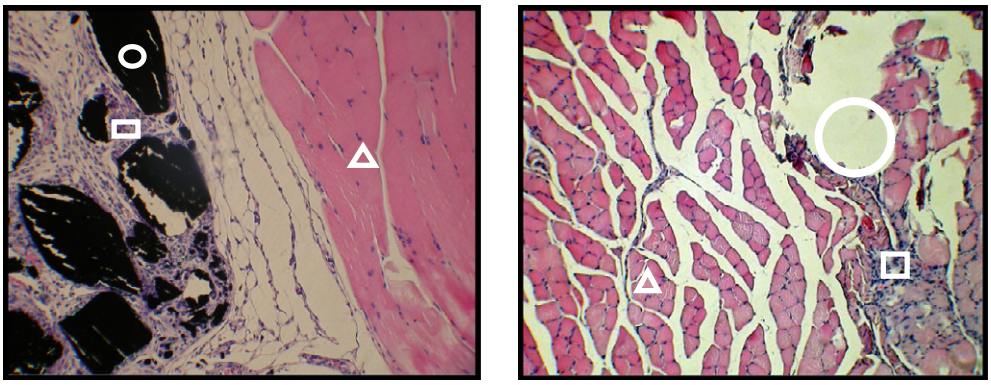

Figure 5. Histological aspect of mouse muscles one month after implantation of the oxygen sensors. Left (100 ×): tissue implanted with uncoated LiPc crystals. The circle shows an agglomeration of LiPc crystals; the square shows an area of local inflammatory reaction; and the triangle indicates the healthy muscle. Right (100 ×): tissue implanted with the micro-insert. The big circle indicates the empty place after removal of the implant; the square shows a very weak local inflammatory reaction; and the triangle indicates the muscle.

Our histological studies cannot discriminate between these different hypotheses. However, we did observe a reduced inflammatory response (figure 5) after implantation of micro-inserts compared to uncoated crystals.

One month after implantation, we noted that the regions surrounding the LiPc crystal implantation site had a larger number of macrophages and inflammatory cells compared to the micro-inserts of LiPc. This could be due to the cylindrical design, avoiding traumatic reactions, or to the biocompatible nature of Teflon AF2400, or both. It should be noted that standardized tests of biocompatibility performed previously (Dinguizli *et al* 2006) demonstrated a lack of reaction against this type of polymer. Assessing the reasons for the improvement in the characteristics of our oxygen sensor is beyond the scope of the present study. However, we

may note three important improvements: (1) longer stability of responsiveness to oxygen, (2) lesser or absent inflammatory response and (3) ease of implantation.

4. Conclusion

Biocompatible polymeric micro-inserts containing LiPc have considerable potential as oxygen sensors in EPR oximetry, especially for measurements over long periods of time. The fact that this form of pellet is retrievable is a main advantage for their potential use *in vivo*. Moreover, the cylindrical shape of the pellets is suitable for implantation with catheters. Two important goals were achieved in this work: (i) enhancement of the biocompatibility of the oxygen sensor and (ii) stabilization of its oxygen sensitivity over long periods of time.

Acknowledgments

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