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Optimization of a Novel Nitric Oxide Sensor Using a Latex Rubber Matrix

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Abstract: This study present a novel NO sensor made of a spin trap (iron(II)-diethylthiocarbamate complex, FeDTC) incorporated in a latex rubber matrix and works as a trap for NO, which is detectable by Electron Paramagnetic Resonance (EPR). We explored the optimization of our sensors changing systematically two fabrication parameters: the latex rubber matrix temperature of polymerization and FeDTC concentration inside the matrix. The sensor was prepared in four different temperatures: 4, 10, 20 and 40°C. The FeDTC concentration was also varied from 0.975 to 14.8 mM. We observed a variation of the EPR signals from the sensors prepared at different conditions. We found a high stability of the EPR response from our sensor, 40 days at RT. The best sensor was made with a latex rubber matrix polymerized at 10°C and with a FeDTC concentration of 14.8 mM. In vivo tests show good biocompatibility of our sensor.

Key words: Nitric oxide, sensor, FeDTC, latex rubber matrix, optimization

INTRODUCTION

Nitric Oxide (NO), a free radical molecule, has numerous roles in various physiological functions, such as the regulation of blood pressure (Rees et al., 1989), immune response to bacterial infection and nervous systems (Snyder et al., 1998). NO is an important bioactive molecule derived from endogenous or exogenous sources (Kerwin et al., 1995; Moncada et al., 1991). NO can exhibit genotoxicity through the formation of reactive nitrogen species. NO has become one of the most important molecules in the area of biological and medical investigation in recent years (Lee and Pfeifer, 2007).

The detection of NO presents a series of challenges since it is highly reactive, which means it has short lifetimes in living organisms, depending on the environment from ms to minutes, before it reacts and consequently is found in low quantities in biological systems (Vallance et al., 1995; Archer, 1993). On the other hand, iron complexes with dithiocarbamates and porphyrins are used as spin traps due to the affinity between NO and the iron complexes, however these complexes are unstable for long-term measurements.

Recent studies show that latex can cause natural angiogenesis and this capability can be controlled (Oliveira et al., 2003; Frade et al., 2004; Mraí et al., 2004; Nova Biocure, 2007). Latex also shows biocompatibility in mammals, is easy to manipulate (Frade et al., 2001; Neves-Junior et al., 2006a; Neves-Junior et al., 2006b) due to its good mechanical properties. Latex has been used successfully as a matrix in many applications where the entrapment of organic molecules is of interest, as for example NO (Herculano et al., 2006). Biocompatibility is fundamental in in vivo detection since one does not want the sensor to interfere in the biological systems. In a previous work we proposed a sensor (Herculano et al., 2006) based on encapsulating the complex iron(II)-diethylthiocarbamate (FeDTC) in a solid rubber latex matrix. Once the FeDTC trap is embedded in the matrix and in contact with a NO solutions, the NO radical is trapped by FeDTC, which allows its detection by Electron Paramagnetic Resonance (EPR). Apart from latex, two other inorganic matrices are reported in the literature based on the same principle, aluminosilicate zeolite (van Faassen and Vanm, 2006) and SiO (Melo et al., 2004).

In this study, the optimization of this sensor is explored. For that purpose two fabrication parameters have been systematically changed; the latex rubber matrix temperature of polymerization and FeDTC concentration inside the matrix. We do also report an in vivo test on the biocompatibility of this sensor.

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MATERIALS AND METHODS

The latex used in this work consisted of a mixture of different clones extracted from *Hevea brasiliensis* in the Escola Superior de Agricultura Luiz de Queiroz (ESALQ), Universidade de São Paulo. After the extraction, ammonia was used to keep latex liquid and after this the material was centrifuged at 8000 g. For the FeDETC solution iron(III) chloride hexahydrate (FeCl₃·6H₂O), dimethylformamide (DMF, C₉H₁₈ON) and sodium dietyldithiocarbamate (DETC, C₇H₁₆NNaS₂·3H₂O) were used. DMF and DETC were obtained from Acros Organics (Belgium). The solution was prepared using 12 mg of iron chloride and 20 mg of DETC in 3 mL of DMF under magnetic agitation during 10 min. The entrapment of FeDETC in the matrix was obtained by mixing the latex milk with FeDETC solution inside a small glass dish. An elastic membrane resulted from leaving this mixture in air for one day. The temperature at which the latex milk with FeDETC is transformed into a rubber was varied from 4 to 40°C, using a Marconi MA 033 temperature controlled bath. We did also prepare several sensors varying the FeDETC concentration in the latex milk, from 0.925 to 14.8 mM. NO was generated in an aqueous solution by mixing NaNO₂ 10 mM (250 μl), deionized water (750 μl) and Na₂S₂O₄ (145 mg) in an Eppendorf tube of 1.5 mL. In the presence of sodium dithionite (Na₂S₂O₄) nitrite is reduced to NO. The saturated concentration of NO in this solution is 2.2 mM.

Electron Paramagnetic Resonance (EPR) experiments were done in a computer interfaced Varian E-4 X-band spectrometer at room temperature. For EPR measurements, the sensors were removed from the solution, dried and inserted in a quartz tube. For all sensors, the only signal observed was of the NO:FeDETC complex.

Our sensors were inserted in the Choroidal Anti-Neptic Membrane (CAM) of chick embryos in vivo, in order to test its biocompatibility (Seidritz et al., 2004; Nikiforidis et al., 1999). Tests were made with membranes before and after NO immersion. For these tests the sensor was immersed in a 2.2 mM NO solution for 2 h.

RESULTS AND DISCUSSION

As already mentioned the main concern of this study was to optimize our sensor. In a previous work we have shown the signal stability, reproducibility, reusability and sensitivity of latex based NO sensor (Herculano et al., 2006). In Fig. 1, we show the EPR signal of the FeDETC: NO inside of different matrices (Latex, SiOx) and in solution (T = 77K and T = 300K). Notice the similarities of the spectra measured in the matrices at room temperature with the one in the solution measured at 77K.

![Fig. 1: Typical EPR spectra from FeDETC: NO in solution and inside the Latex matrix and SiOx matrix. For the spectra in solution (dashed lines) the measurement temperatures were 300K and 77K.](image)

According to the literature (Lloyd et al., 1990; Matsuyama et al., 2000) Thermally Induced Phase Separation (TIPS) is a method to make microporous membrane. This procedure is perhaps the most versatile and simplest membrane preparation technique. Thus for the optimization of the sensor, we decided to vary the temperature of the latex matrix during polymerization as a way to control the membrane pore sizes. Then we have prepared the latex rubber matrix in four different polymerization temperatures: 4, 10, 20 and 40°C.

In Fig. 2, the EPR signal amplitude is presented against time for samples where the latex matrix was polymerized at 4, 10, 20 and 40°C. All sensors were immersed in a saturated NO solution for 2 h prior to starting the measurements. These spectra were taken after leaving the sample in the dark for 24 h after immersion in the NO solution. It can be seen in the insert of Fig. 2a that the EPR line shape becomes more symmetric as the polymerization temperature increases. This result indicates an increase in the rotation freedom of the NO:FeDETC complex, or in other words an increase in pore sizes (Yave et al., 2005; Lloyd et al., 1991). As can be seen in Fig. 2b the best signal intensity occurs for polymerization temperature of 10°C. We do also observe in Fig. 2b that the signal intensity first increases and for longer times (Fig. 4) it decreases. We do not understand in details these behavior, however one possible explanation, is that since the pores are expected to be small and not very well connected, that it takes some time for NO to reach all possible trapping sites (Herculano et al., 2006).

We used Scanning Electron Microscopy (SEM) to observe the pore distribution in latex membranes polymerized at different temperatures: 4, 10, 20 and 40°C. It was found that for temperatures below 40°C no pores structures are observed.
Fig. 2: (a) EPR spectra for samples made at different polymerization temperatures. These spectra were taken 24 h after immersion in the NO solution and (b) Peak-to-peak amplitude of the EPR spectra for different samples.

Fig. 3: SEM for different surface of same sample around 40°C. Notice that a difference size pores of the membrane.

In Fig. 3, SEM was used to observe the pore distribution in a latex membrane polymerized at 40°C. Figure 3a and b were taken from the same membrane. As can be seen, the sample is heterogeneous in the micrometer range. Figure 3a shows in detail a porous area in the membrane, while in Fig. 3b, no pores are observed. In Fig. 3a its possible to observe pore sizes with different diameters from 0.2 to 1.2 μm. The average pore size in Fig. 3a is (~0.63±0.14) μm. SEM images of different samples do not reproduce the observations made in Fig. 3. This is however in contradiction with our EPR results. We have made at least 8 samples in the same conditions and found similar EPR responses. The origin of this contradiction is not clear at the moment, however one must remember that SEM is probing a small surface area, while EPR the whole sample. Other techniques such as AFM (Atomic Force Microscopy) (Lin and Meier, 1996) and BET (Brunauer-Emmett-Teller) (Zhu et al., 2003) shall be used to clarify this point.

Another important parameter, which concerns the sensitivity of the sensor, is the density of spin traps (Melo et al., 2004) incorporated into the matrix. In this case where the matrix is latex, FeDETC has two functions. Since DETC contains sulphur, it acts as a crosslinking agent, which accelerates the polymerization, or in other words the rubber formation. In fact, as soon as DETC is dropped into the latex milk, rubber is formed in an uncontrolled way at room temperature. This effect is less pronounced as the latex milk temperature is below room temperature. The other function of FeDETC is to trap NO. The FeDETC concentration was varied from 0.975 to 14.8 mM. However, the latex solution volume was kept...
0.1 mL. In Fig. 4, the EPR signal amplitude against time is presented for samples with different FeDETC concentrations. As can be seen, the EPR signal amplitude increases with the increase in FeDETC concentration, but non-linearly. The strongest EPR signal intensity was found for a FeDETC solution concentration of 14.8 mM. We have tried higher FeDETC concentrations in the latex solution, however for concentrations higher than 14.8 mM, the sensors became very heterogeneous (in the millimeter range) and irreproducible.

Ten sensors were with the same parameters described for the best sensor, to test reproducibility. All ten sensors behaved in a similar way, the EPR signal intensity varied in the range of 10%, attesting the good reproducibility of our preparation method.

One of the most important factors in the evaluation of a material’s biocompatibility is its acceptance by the host (Mnue et al., 2004). As an in vivo test for our sensor, we used the developing chick embryo (Seidlitz et al., 2004; Nikiforidis et al., 1999). The chick embryo and its Chorioallantoic Membrane (CAM) is the one of the most popular assay tissues to study angiogenesis. CAM is a highly vascular structure lining the inner surface of the egg shell (Borges et al., 2003). CAM is easy to use and low cost. In these initial tests, not shown, we used both membranes before and after immersion in a 2.2 mM NO solution for 2 h. In both cases the chick embryo developed well, showing no rejection of our sensor.

CONCLUSION

We report the optimization of a NO sensor based on latex. Two fabrication parameters were varied: the latex membrane polymerization temperature and FeDETC concentration inside the matrix. Our best sensors were made at a polymerization temperature of 10°C and FeDETC concentration of 14.8 mM. The samples were observed to be heterogeneous using electron microscopy. In vivo tests were performed using chick embryo and its chorioallantoic membrane, it was observed that the incorporation of FeDETC and NO has no effect on the biocompatibility of latex. Our results show that the fabricated sensors are simple to prepare, easy to use, stable and reproducible.

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