

## Nitric Oxide Release Using Natural Rubber Latex as Matrix

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Nitric oxide (NO) is a diffusible messenger that has been involved in numerous physiological processes ranging from vasodilatation and antimicrobial properties to wound healing. The beneficial effects can be attributed to the role NO plays in angiogenesis, inflammation and tissue remodeling. In the present work, a polymeric device for the sustained site specific delivery of nitric oxide using a latex rubber matrix from *Hevea brasiliensis* which encapsulates the spin trap iron(II)- diethyldithiocarbamate complex (FeDETC) was developed. The release profiles of NO from latex rubber matrix were studied and stability studies were carried out. Electron Paramagnetic Resonance (EPR) signal of NO was detected in the membrane exposed to ambient atmosphere at room temperature (25 °C) even after 350 hours. FTIR spectroscopy data indicated that NO-FeDETC retained its structural and spectroscopic properties upon encapsulation in the latex matrix. The NO delivery system developed in this work as a membrane, presented high stability.

**Keywords:** nitric oxide, latex membrane, biomaterials, drug delivery system

### 1. Introduction

Nitric oxide (NO) is an important cell-signaling molecule whose role in a variety of cellular processes, such as vascular relaxation<sup>1-4</sup>, neurotransmission, platelet aggregation and immune regulation<sup>5</sup>. Over recent years it has become apparent that NO has important effects on bone remodeling.

Natural rubber latex (NRL, *cis*-1,4-polyisoprene) extracted from *Hevea brasiliensis* has been widely used as raw material in the manufacturing of gloves, condoms, balloons, and other medical and dental devices. Typically for those applications, the processing of NRL was basically the same as those found in the tire's industry. However recently, several new biomedical applications for NRL have been proposed using a different manufacturing process (NRLb)<sup>6-11</sup>. Of special interest, NRL has shown to stimulate angiogenesis, cellular adhesion and the formation of extracellular matrix<sup>12-14</sup>, promoting the replacement and regeneration of tissue<sup>15-17</sup>. In fact, NRLb is now commercialized in Brazil and other 60 countries as a band-aid curative (BIOCURE<sup>®</sup>) for the treatment of ulcers in diabetic patients among other applications<sup>17</sup>.

Nitric oxide (NO) is an important physiological cellular signaling molecule that functions in a diverse range of cellular processes playing many biological roles as a major defense molecule against intercellular bacteria, tumor cells and aiding in wound repair. NO is produced by intracellular enzyme nitric oxide synthase (NOS), but can permeate cell membranes to reach extracellular spaces and adjacent cells<sup>18</sup>.

Many methods for quantification of NO in biologic media have been reported, including colorimetry, fluorometry, electrochemical methods, and electron paramagnetic resonance (EPR)<sup>19</sup>. Among these techniques the electron paramagnetic resonance (EPR) using diethyldithiocarbamates complexes (FeDETC) as spin trap is very promising technique due to their sensitivity, selectivity, and possibility for application in vivo. In FeDETEC the electron-donating group on the dithiocarbamate ligand is favorable for enhancing the sensitivity of NO detection due to the stabilization of the Fe(II) complex form. However, these complexes are unstable for long-term measurements<sup>20</sup>.

To overcome the instability at long-term measurements FeDETC-NO complexes, we developed in a previous work a sensor<sup>6-7</sup> based on the encapsulation of FeDETC in NRL matrix. NRL may be an interesting material for applications as wound healing due to their biocompatible properties and ease of handling due to its good mechanical properties<sup>8,21-23</sup>.

The increasing evidence that NO plays a key role in wound healing suggests that the NRL-FeDETC matrix may be an interesting biomaterial for in situ delivery of NO for wound healing. It is well known that Diabetes Mellitus patients show impaired wound healing, posing a major clinical challenge. It has been shown that the production of multiple growth factors is impaired in patients with diabetes are directly related to the low NO production<sup>24-26</sup>.

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In this work, we propose a NO release system based on the encapsulation of FeDETC in a NRL matrix for the sustained and controlled delivery of NO for future applications in medicine as wound healing. To characterize the release behavior of NO by this system, the NRL-FeDETC system was exposed to ambient atmosphere (humidity 60%, 25 °C) and the EPR signal of NO in the membrane was monitored as function of time.

## 2. Materials and Methods

Natural rubber latex (NRL) used in the present study was commercial high-ammonia natural rubber latex (ESALQ-USP, Piracicaba, Brazil) of about 60% dry rubber content (DRC). After extraction, ammonia was used to keep the latex liquid. The deproteinization of natural rubber latex was performed by centrifugation at 8,000 g. The cream fraction after centrifugation was re-dispersed to make 60 wt. (%) of dry rubber content latex and was washed twice by centrifugation to prepare the deproteinized natural rubber latex and then reduce the cytotoxic protein content on NRL.

Ferric chloride hexahydrate ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 99.5%), dimethylformamide (DMF, 99.0%, Acros Organics) and sodium diethyl-dithiocarbamate (DETC, 99.0% Acros Organics) were used as received. The FeDETC solution was prepared by using 12 mg of  $\text{FeCl}_3$  and 20 mg of DETC in 3 mL of DMF under magnetic stirring for 10 minutes at room temperature (25 °C).

In this work, the encapsulation of FeDETC in NRL membranes was attained after mixing 2 mL of natural rubber with FeDETC (2 mL). The NRL-FeDETC solution was cast on a Petri glass dish at room temperature (25 °C) for 48 hours. This methodology provided elastic NRL-FeDETC membranes with thickness of  $(0.512 \pm 0.005)$  mm.

NO was generated in an aqueous solution by mixing  $\text{NaNO}_2$  10 mM (250  $\mu\text{L}$ ), deionized water (750  $\mu\text{L}$ ) and  $\text{Na}_2\text{S}_2\text{O}_4$  (145 mg) in an eppendorf<sup>®</sup> tube of 1.5 mL. In the presence of sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) nitrite is reduced to NO. The saturated concentration of NO in this solution was 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 membranes were removed from the solution, dried at room temperature (25 °C) for 48 hours and inserted in a quartz tube and the signal of the NO:FeDETC complex was observed. To maximize signal to noise ratio various spectra were summed up, typically at least 10. To avoid sample repositioning induced errors for the stability measurements, the sample was kept inside the resonant cavity. A reference sample with a known and stable amount of spins was used before each measurement.

The rate of NO diffusion from NRL membranes was measured by Fourier transform infrared (FTIR) spectroscopy over 300 hours. The spectra were performed at room temperature (25 °C) by using a NICOLET 380 (Thermo Electron Co., USA) spectrophotometer in the attenuated total reflectance (ATR) mode ( $4000\text{--}500\text{ cm}^{-1}$ ) with resolution of  $4\text{ cm}^{-1}$ .

## 3. Results and Discussion

The main component of NRL is cis-1,4-polyisoprene with a high degree of long chain branching generally associated with the presence of non-hydrocarbon groups distributed along the chains. One of the most important of the non-hydrocarbon group's presents in NRL is the aldehyde group, which is thought to be responsible for cross-linking between the chains in NRL<sup>27</sup> and their concentration varying from clone to clone<sup>28</sup>. Other compounds, such as lipids, neutral glycolipids and phospholipids, amount to 1.4-3.2%. NRL contains 0.95% protein that upon centrifugation about 27.2% of this stays in the rubber fraction<sup>29</sup>. Figure 1 illustrates the chemical structure of isoprene polymer from NRL.

FT-IR spectra of natural rubber before FeDETC encapsulation are presented in Figure 2. Initially, the absorption band observed at  $572\text{ cm}^{-1}$  corresponds to the C-C-C deformation of NRL backbone. The cis-1,4-polyisoprene absorption band of strong amplitude corresponding to =CH out of plane bending is observed at  $836\text{ cm}^{-1}$ ; the trans isomer has no absorption at this wavelength. A closer inspection of the infrared spectra reveals that it has absorbance bands at  $1240\text{ cm}^{-1}$ , corresponding to O-P-O asymmetric stretching of phospholipids indicating the presence of associated phospholipids at the rubber chain, since free fatty acids and free phospholipids were removed by centrifugation<sup>28,30</sup>. The absorptions bands at  $1375\text{ cm}^{-1}$  ( $\text{CH}_2$  deformation),  $1394\text{ cm}^{-1}$  and  $1432\text{ cm}^{-1}$  and  $1494\text{ cm}^{-1}$  are characteristics of  $\text{CH}_2$  deformation and  $\text{CH}_3$  asymmetric stretching, respectively. The absorption band at  $1647\text{ cm}^{-1}$  correspond to C=C stretching in cis-1,4-polyisoprene. The  $\text{CH}_2$  symmetric stretching vibrations are observed at the region  $2852\text{ cm}^{-1}\text{--}2925\text{ cm}^{-1}$ . The  $\text{CH}_3$  asymmetric stretching in FT-IR of NRL membranes is observed at  $2961\text{ cm}^{-1}$ . These FT-IR correlations for NRL are consistent with the earlier works<sup>28-30</sup>. In addition, Figure 2 also shows a broad peak at approximately  $3200\text{--}3500\text{ cm}^{-1}$ . This absorption band might be related to the presence of a hydroxyl group, which appears to be generated after the hydrolytic ring opening of epoxy group's formed during the casting of NRL membranes.

Thiol-substituted compounds, as FeDETC, tend to give rise to very weak absorptions in IR spectrum. The higher mass of sulfur, results in the characteristic group frequencies occurring at lower frequencies. The C-S stretching vibrations of FeDETC tend to give rise to very weak absorptions in IR spectrum. The principal characteristics of FT-IR absorption bands of NRL-FeDETC membranes are shown in Figure 4. In the IR spectra of NRL-FeDETC the CN stretching frequencies are observed at  $1494\text{ cm}^{-1}$ ,  $1358\text{ cm}^{-1}$  and  $1270\text{ cm}^{-1}$ . The absorption band at  $1210\text{ cm}^{-1}$  corresponds to CN stretch of tertiary amine from FeDETC (Figure 3). The C-S stretching frequency appears close to  $990\text{ cm}^{-1}$  and is characteristic for FeDETC (Figure 3)<sup>31</sup>. The absorption band for the Fe-S was not observed in NRL-FeDETC. However, our density functional (DFT) calculations of Fe-S vibrations in FeDETC (not shown) indicate that this absorption band appears to be active in the IR region between  $350\text{--}240\text{ cm}^{-1}$ . These calculated results are found to be in excellent agreement with

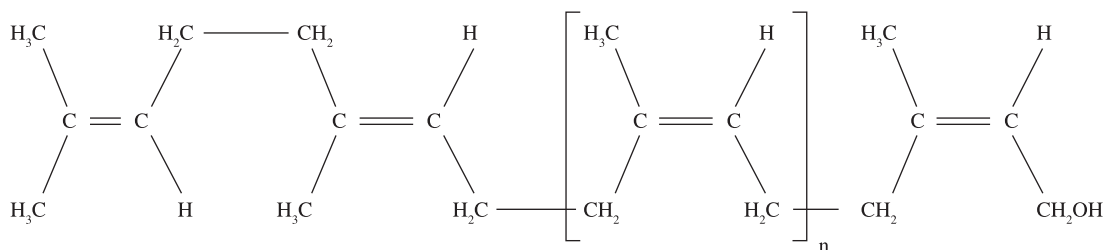
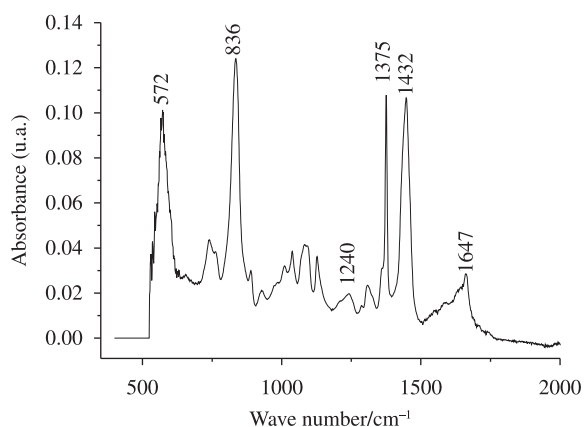
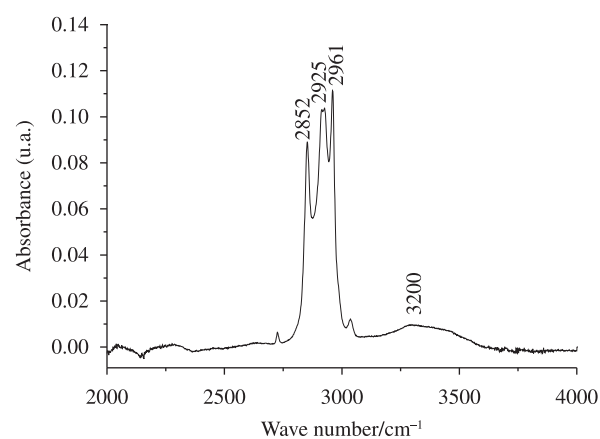


Figure 1. Chemical structure of cis-1,4-polyisoprene from natural rubber.

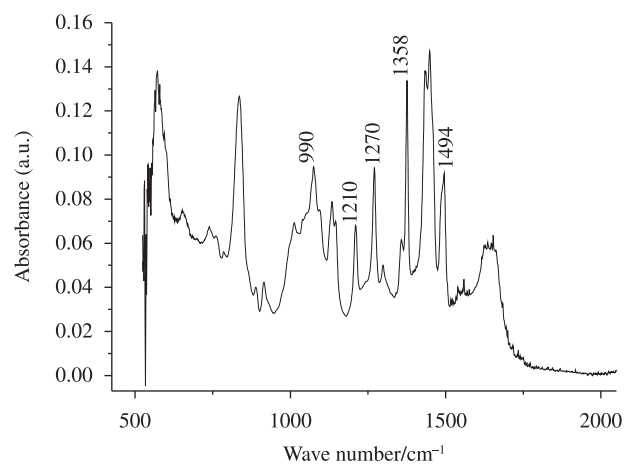


(a)

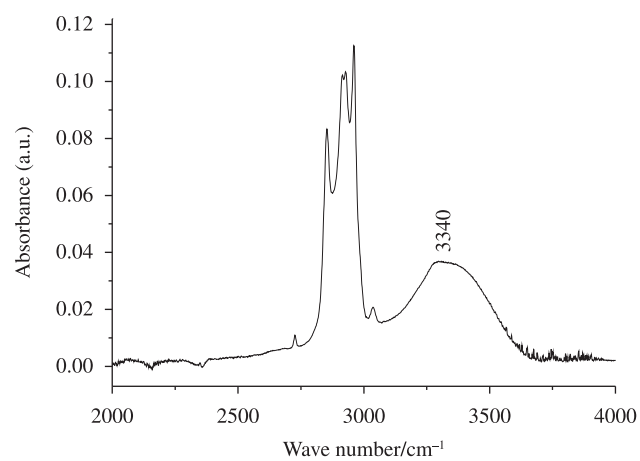


(b)

— Natural rubber latex



(a)

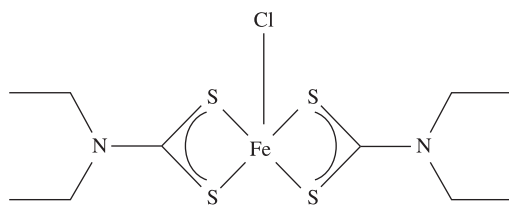


(b)

— Natural rubber latex + FeDETC

**Figure 2.** FT-IR spectra of the natural rubber membranes in potassium bromide disc. The characteristic bands for cis-1,4-polyisoprene are indicated with their wave numbers.

**Figure 4.** FT-IR spectra of the natural rubber membranes after complexation of Fe-DETC. FeDETC concentration: 14.8 mM<sup>7</sup>.



**Figure 3.** Illustration of the molecular structure of FeDETEC.

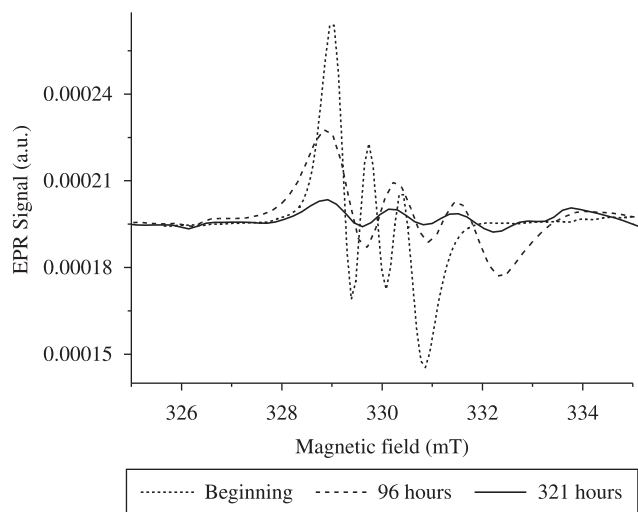
the Fe(III) spin crossover behavior studied by infrared spectroscopy and might be assigned with  $\nu$  (Fe-S) vibrations<sup>32</sup>.

Works have demonstrated that natural rubber lattices may be vulcanized by using diethyldithiocarbamates<sup>33</sup>. However, their amplitudes are essentially dependent of their concentration on latex membranes and a quantitative study about the FeDETC influence on latex vulcanization was not possible in this work. No significant differences have been found in other IR spectral region of NRL-FeDETC relatively to NRL suggesting no chemical interaction between FeDETC and NRL.

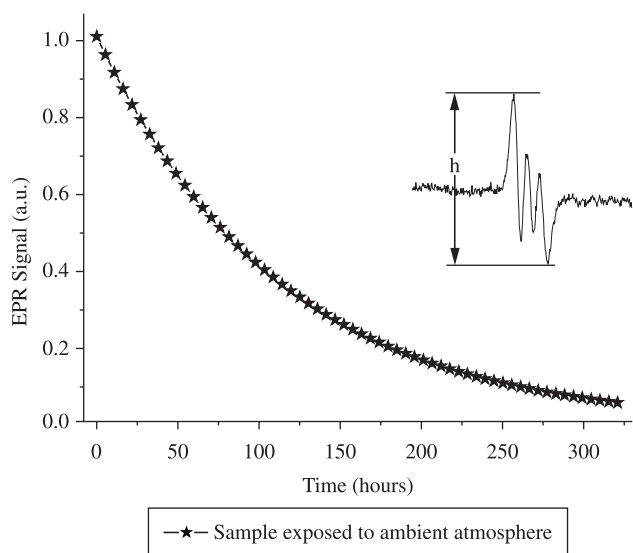
Another important parameter, which concerns the sensitivity of the NO drug delivery system, is the density of spin traps<sup>34</sup> incorporated into the polymer matrix. In this case where the polymer matrix is NRL, FeDETC could have two functions. Since DETC contains sulphur, it acts as a crosslinking agent, which accelerates the polymerization, or in other words the rubber formation. In fact, as soon as DETC is dropped into the latex solution, rubber is formed in an uncontrolled way at room temperature (25 °C). The other function of FeDETC is to trap NO. As already mentioned, to maximize signal to noise ratio various spectra were summed up, typically at least 10. As shown in Figure 5, the EPR signal amplitude decreases with time.

In Figure 6 the normalized EPR signal amplitude (per membrane mass unit) just after the membrane was taken from the NO solution is plotted as a function of time. As can be seen, the signal decreases with time until it vanishes. For these measurements the membrane was immersed in a 2.5 mM NO solution for 2 hours, and then left in ambient atmosphere. Notice the small decrease in signal amplitude even after 350 hours for samples left in ambient atmosphere.

According to the literature<sup>6,7</sup>, NRL associated to FeDETC-NO are easy to manipulate, allow species detection and concentrations



**Figure 5.** Typical EPR spectra from FeDETC:NO in Latex matrix at room temperature. These spectra were taken several hours after immersion in the NO solution. Notice that signal amplitude decrease with the time.



**Figure 6.** EPR signal amplitude as a function of time. The insert graph shows a typical EPR spectrum for our NO delivery system.

measurements with few sample contamination. These characteristics indicate that latex rubber may be a good choice for a NO delivery system, with superior mechanical properties. The samples are very stable: the signal amplitude of NO could be detected even after 350 hours exposed to ambient atmosphere. As NO is an early mediator of the inflammatory process associated to tissue regeneration and the NRL presented interesting biological properties associated to healing, this new drug delivery system is a promising biomaterial.

#### 4. Conclusion

We propose a novel NO delivery system made of a spin trap (iron(II)-diethyldithiocarbamate complex, FeDETC) encapsulated in a NRL matrix. The rate release of NO in this system was observed by EPR, exposing the sample to ambient atmosphere. The results indicate

the sustained delivery of NO from NRL matrix for up to 350 hours. FTIR spectroscopy showed that NO/FeDETC when encapsulated in NRL has its properties and structure preserved which is relevant for its possible application as a biomaterial.

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