



## Towards the production of natural rubber-calcium phosphate hybrid for applications as bioactive coatings



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### ABSTRACT

This paper assesses the morphological, structural and bio-physicochemical stability of natural rubber (NR) *Hevea brasiliensis* coatings incorporated with microparticles of calcium phosphate-based (CaP) bioactive ceramics. Optical and electronic spectroscopic imaging techniques were employed to successfully evaluate the NR encapsulation capability and the stability of the coating in a biologically relevant media for bio-related application, i.e., simulated body fluid (SBF). The chemical structure of the natural polymer, the microchemical environment at the NR-CaP interface and the morphology of the CaP clusters were fully characterized. Further, the response of the hybrid coating to SBF was evaluated by incubating the samples for 30 days. The hybrid coating formed on Si surface (inert substrate) exhibited both stability and biodegradability in different levels (time dependence), thus opening horizons for applications as coatings for both biomaterials and drug delivery systems.

### 1. Introduction

Natural rubber (NR) obtained from the *Hevea brasiliensis* latex is highly demanded by manufacturing industry because it has superior properties as compared with the synthetic rubber. However, in order to expand the potential for new applications, the development of novel composites combining NR with other materials is a current target of many investigations [1–3]. These hybrid systems usually present new and superior properties to this fine NR. For instance, a NR-graphene composite has been studied in order to obtain a material with high electrical conductivity combining the flexibility properties of the polymer [4]. In another hand, a promising field for application of the NR-based materials is in biomedicine [5–15].

A great potential lies on calcium phosphate-NR composites and hybrids materials, especially for applications as (or associated with) implants. However, prior to achieving commercial products, one must elucidate relevant aspects related to the processing of NR for the production of biomaterials or composites for such biomedical applications. An issue to be clarified includes the possible effects of the ammonia content (used to avoid clotting and it is very important for the preservation of the latex [16]) on the morphology and structure of NR particles present in the cream phase. Additionally, in composites, the

molecular interactions between non-rubber constituents of the NR cream and charged particles were not properly described so far. In the case of CaP charged particles dispersed in NR phase, the suggestion of the homogeneous and random distribution of different molecular groups correlated with two emission bands in the photoluminescence spectrum are not reproducible for attesting the protein-phospholipids heterogeneous distribution models [17–19]. In this way, new techniques and approaches are required to improve the hybridization of the NR looking to fully exploit the polymeric matrix to design a functional material with optimized properties.

Recently, we have described the production of membranes of NR-CaP hybrids formed from casting methods using bioceramic CaP particles dispersed in NR colloidal suspensions [20]. The membranes conjugated the favorable biocompatible properties of CaP with the renewable and low-cost characteristics of NR. Here, we describe the production and characterization of coatings formed from NR-CaP hybrids and their physicochemical stability in the presence of a simulated body fluid (SBF), which has a high relevance towards the development of implants coatings. By applying physico-chemical assays, vibrational and electronic spectroscopic imaging techniques, we assessed morphological and structural aspects of the processed NR prior and after to the incorporation of CaP. The influence of ammonia content on the final

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products was investigated. The organic-inorganic interfaces of the NR-CaP hybrid were characterized by molecular spectroscopy as well. We investigated the functional chemical groups responsible for forming the core-shell structure that traps the bioceramics (CaP). The chemical structure of the natural polymer, the microchemical environment at the NR-CaP interface and the morphology of the CaP clusters were fully described. In view of biomedical applications of the NR-CaP hybrid, we evaluated the response of the NR-CaP coating exposure to SBF by incubation of the samples for 30 days. The remarkable stability of the coating on Si substrate and its surface modifications by exposure to the biological environment, as well as the biodegradability in different levels regarding time dependence, are discussed aiming the possible applications in the biomedical field, such as coatings for biomaterials and drug delivery systems.

## 2. Experimental procedures

### 2.1. Separation of the natural rubber cream

Samples of natural rubber latex (NR) were collected from *Hevea brasiliensis* trees (clone RRIM 600) located at Estância Regina farm, São Paulo, Brazil (see Fig. 1), immediately transferred to propylene tubes containing different ammonia content, which led to final concentrations of 0.3%, 0.5%, 0.7%, 0.9% w/w (denoted sample as NR 0.3A, NR 0.5A, NR 0.7A and NR 0.9A, respectively) and stored under refrigeration at 4 °C to avoid microbial contamination. Initially, aliquots of 1.5 mL of each sample with different ammonia content were added to a 2 mL microcentrifuge tube and then centrifuged for 90 min at 24 °C in an Eppendorf 5418 R centrifuge. For each sample, four different centrifugation speeds were employed: 2000g, 6000g, 10000g and 14000g. After centrifugation, the microtubes were photographed with a Nikon D3200 camera to compare the effect of centrifugation speed in phase separation. Fig. 1 shows the typical appearance of centrifuged NR latex: cream phase (A) contains the concentrated natural rubber particles; serum fraction (B) with high water ratio, as well as rubber particles and non-rubber components; and (C) contains the lutoids [21]. After separation, a part of the resulting upper phase (cream) from the samples centrifuged at 2000g was collected with a spatula and re-dispersed in deionized water for the analysis of the particle size through the dynamic light scattering (DLS). Dispersions were obtained at ~0.011% (w/w) in deionized water, and DLS measurements were performed on a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK).

NR samples were investigated by Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy to verify if their structures are modified by ammonia content and centrifugation process. ATR-FTIR spectra were recorded with OPUS software on a Bruker Vertex 70v spectrometer. The system was equipped with a Global source used to Middle Infrared Region (MIR) and a detector DLaTGS. ATR-FTIR spectra were recorded in the range of 4000–1400  $\text{cm}^{-1}$  with a resolution of 2  $\text{cm}^{-1}$ . The background was measured before testing each sample. For each sample, one spectrum was recorded on five different regions. Each spectrum was obtained from 256 successive scans. The final ATR-FTIR spectra are the average of five spectra acquired at different regions of the sample. This first assessment provided the parameters to enrich the cream fraction with NR, and to decrease the amount of water in the dried NR (after centrifugation).

### 2.2. Production of NR-CaP hybrid coatings

NRs were extracted by centrifugation at 14,000g for 90 min. Then NR cream was dried at 45 °C for 24 h, and then re-suspended in chloroform for 2 days. Powders of a CaP-based bioactive ceramic (cell viability on CaP coatings was described elsewhere by our group [22]) containing  $\text{Ca}_5\text{P}_8$  (47.8 wt%),  $\text{Ca}(\text{PO}_4)_3\text{OH}$  (9.5 wt%),  $\text{CaCO}_3$  (36.3 wt%), and Ca (6.3 wt%) were mixed in the NR cream solution (in chloroform) under magnetic stirring for 30 min.

In addition, silicon substrates of  $5 \times 5 \text{ mm}^2$  with polished surfaces were sequentially cleaned in an ultrasonic bath with acetone, isopropyl alcohol and ultrapure water (for 15 min each step), and finally dried at 50 °C for 10 min. NR and NR-CaP suspensions in chloroform (30  $\mu\text{L}$ ) were controllably deposited on silicon substrates and the substrates were dried for 12 h at 60 °C, thus generating coating samples Si/NR and Si-NR/CaP. The charge effects were evaluated through determination of the polar component of the free energy  $\gamma_s^p$  using the followed liquids: ultra-pure water ( $\gamma^p = 51 \text{ mJ/m}^2$ ,  $\gamma^d = 21.8 \text{ mJ/m}^2$  and  $\gamma = 72.8 \text{ mJ/m}^2$ ), formamide ( $\gamma^p = 18.5 \text{ mJ/m}^2$ ,  $\gamma^d = 39.5 \text{ mJ/m}^2$  and  $\gamma = 58 \text{ mJ/m}^2$ ), ethylene-glycol ( $\gamma^p = 19 \text{ mJ/m}^2$ ,  $\gamma^d = 29 \text{ mJ/m}^2$  and  $\gamma = 48 \text{ mJ/m}^2$ ), di-iodomethane ( $\gamma^p = 0 \text{ mJ/m}^2$ ,  $\gamma^d = 50.8 \text{ mJ/m}^2$  and  $\gamma = 50.8 \text{ mJ/m}^2$ ) and hexadecane ( $\gamma^p = 0 \text{ mJ/m}^2$ ,  $\gamma^d = 27.47 \text{ mJ/m}^2$  and  $\gamma = 27.47 \text{ mJ/m}^2$ ). From measurements of contact angles, we obtain the plot of  $0.5\gamma_L(1 + \cos\theta)$ .  $(\gamma^d)^{-1/2}$  versus  $(\gamma^p/\gamma^d)^{1/2}$  for the determination of the polar component of free energy  $\gamma_s^p$ .

### 2.3. Incubation in simulated body fluid

The SBF medium was obtained with the dissolution of 0.2 g KCl, 8.0 g NaCl, 0.2 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.05 g  $\text{NaH}_2\text{PO}_4$ , 1.0 g  $\text{NaHCO}_3$ , 0.1 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  and 1.0 g glucose in 1 L of ultrapure water at a constant pH (7.45). We evaluated two protocols for this study aiming an optimization and reproducibility of the results and this protocol was chosen to prevent early nucleation and precipitation of salts. Both the coatings (NR and NR-CaP) and the SBF medium were sterilized in UV for 15 min before the experiments. A control clean Si substrate (without coating) was also used. All substrates were weighted in an analytical balance before each experiment. Samples were then immersed in wells containing 5 ml of SBF and kept at 37.5 °C for different periods of time, i.e. 1, 15 and 30 days. After every time intervals, the substrates were collected, gently cleaned in ultrapure water, dried in a dissector for 24 h, and finally measured in the balance. Coatings for Si, Si/NR and Si/NR-CaP were labeled as function of time (#1, #15 and #30), totalizing nine groups of surfaces. After the removal of the samples from SBF, the medium just for samples #30 (with 30 days of immersion) was evaporated up to the volume of 0.5 mL, then dripped on glass slides and dried at 37 °C. These three new samples (SBF/Si, SBF/Si/NR and SBF/Si/NR-CaP) were analyzed in regard to their composition. The SBF immersion experiments were repeated at least 5 times, and the results are provided as mean values and standard deviation. For each day (1, 15 and 30), samples of Si, NR and NR-CaP were compared by the Kruskal-Wallis statistical approach.

### 2.4. Physical and chemical characterizations

Confocal Raman spectroscopy and Raman spectral mapping were employed to probe the phase of the encapsulated particles of the hybrid NR-CaP through XYZ profiles of the vibrational bands. The spectra and the Raman spectral mapping were obtained by using a microscope WITec *alpha* 300 equipped with a linear stage, piezo-driven, objective lens Nikon 20 $\times$  (NA = 0.46) and 100 $\times$  (NA = 0.8) and polarized laser with 514 or 632 nm wavelengths. The Raman light was detected by a high-sensitivity, back-illuminated spectroscopic CCD after being dispersed by a 600 grooves/mm grating. The organic-inorganic interface was investigated by Raman mappings from XY depth profiles in two planes (Z = 0 and Z = 2.6  $\mu\text{m}$ ) carried out in a region of 30  $\times$  30  $\mu\text{m}^2$  with 60 points per line and 60 lines per image. The integration time for each point was 0.5 s and all mapping were performed at room temperature.

Both NR samples and dried SBF were analyzed by scanning electronic microscopy (SEM) and energy dispersive X-ray spectrometry (EDS) in the large-field scan. The experiments were carried out in the electron microscope Quanta-450 (FEI) with a field emission gun, a 100 mm stage, and X-ray detector model 150, Oxford. An overlapping of areas of adjacent images acquired independently after x-y

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