



Natural rubber latex coated with calcium phosphate for biomedical application

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Natural rubber latex (NRL) is a flexible biomembrane that possesses angiogenic properties and has recently been used for guided bone regeneration, enhancing healing without fibrous tissue, allergies or rejection. Calcium phosphate (Ca/P) ceramics have chemical, biological, and mechanical properties similar to mineral phase of bone, and ability to bond to the host tissue, although it can disperse from where it is applied. Therefore, to create a composite that could enhance the properties of both materials, NRL biomembranes were coated with Ca/P. NRL biomembranes were soaked in 1.5 times concentrated SBF solution for seven days, avoiding the use of high temperatures. SEM showed that Ca/P has been coated in NRL biomembrane, XRD showed low crystallinity and FTIR showed that is the carbonated type B. Furthermore, hemolysis of erythrocytes, quantified spectrophotometrically using materials (Ca/P, NRL, and NRL + Ca/P) showed no hemolytic effects up to 0.125 mg/mL (compounds and mixtures), indicating no detectable disturbance of the red blood cell membranes. The results show that the biomimetic is an appropriate method to coat NRL with Ca/P without using high temperatures, aiming a new biomembrane to improve guided bone regeneration.

Keywords: calcium phosphate (Ca/P); simulated body fluid; natural rubber latex; biomembrane; guided bone regeneration

1. Introduction

In the field of orthopedic and dental implants, calcium phosphates (Ca/P) has shown promising applications due its inherent structural properties. Hydroxyapatite [Ca₁₀(PO₄)₆(OH)₂] is present in bone and teeth, corresponding to 55% of bone, 96% of tooth enamel, and 70% of dentin compositions.[1]

The high biocompatibility of Ca/P bioceramics is due its mechanical, physical, and chemical similarity to bone mineral phase.[2,3] It has ability to promote osteointegration, -induction, and -conduction without fibrous tissue. However, ceramics are brittle and have low mechanical strength, in additional, HAp possess low biodegradation due its high stability [1] and it disperses into soft tissue area.[3]

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On the other hand, there is a variety of polymers, which are easy to manipulate and their properties can be controlled. Natural rubber latex (NRL) is a colloidal system, in which the rubber particles core are stabilized by anionic shell of phospholipids and proteins.[4,5] It has recently been investigated as a delivery system of proteins,[6] drugs,[7] and nanoparticles,[8] which enhances soft tissue healing and stimulated hard tissue growth in guided bone regeneration (GBR) technique.[9,10]

NRL biomembrane has been used for GBR in tibia, calvaria, and dental alveolus enhancing healing and bone formation with no rejection, fibrous tissue, or even allergies.[10,11] It is known from several works that NRL promotes neoangiogenesis,[4] which is essential to bone healing.[12] In addition, Ca/P has shown to improve the integration between biomaterials and bone by reducing hydrophobicity and resembling them to the mechanical properties of bone, enhancing cell proliferation, differentiation, and mineralization.[13]

Schliephake [14] observed that a mineralized collagen membrane enhanced the repair of calvarial defects in rats. Zhang et al. [15] combined HAp with PCL (poly- ϵ -caprolactone) to overcome the difficulty in remodeling, hardness, fragility, and lack of flexibility of the HAp, observing significantly increased amount of alkaline phosphatase (ALP) and bone phenotypic marker, in a dose-dependent manner with the increase in HAp/PCL ratio.[15]

Several surface treatments of implants have been proposed to incorporate Ca/P in order to ensure greater permanence besides anchoring to bone.[14] Some of these techniques use high temperatures, that limits its applicability, since it may lead to polymers and proteins degradation. Moraes et al. [16] reviewed and stated the effectiveness of Simulated Body Fluid (SBF, solution that mimics blood ions concentration) in predicting bone–biomaterial mineralization and integration, and with changes in its composition it was possible to control and accelerate the apatite phase deposition.

Coating the membrane with Ca/P could also provide a surface for protein adhesion and release. Several works provided methodologies to co-precipitate Ca/P and BSA (bovine serum albumin), enhancing the protein adsorption and controlling its release.[17–19]

One of great advantages of using the biomimetic approach is to tailor the content of Ca/P by changing the biomimetic coating parameter. By changing the initial pH and temperature, Qu and Wei observed differences in the Ca/P formed, such as formation of a porous, dense, or ball-shaped coatings.[20] The ionic strength and carbonate influenced in a 5× SBF solution, by allowing nucleation and reducing the crystallite size, respectively.[21] Hydrogen carbonate content also influenced the formation of A- or B-carbonated apatite.[22] With changes in SBF solution, Aparecida et al. [23] observed different Ca/P coating, such as calcium-deficient hydroxyapatite, amorphous calcium phosphate (ACP), octacalcium phosphate (OCL) or/and carbonated HAp). It is also possible to enhance the nucleation of Ca/P by surface treatment with sodium hydroxide [24] or hydrogen peroxide.[25] These possibilities of manipulating the SBF solution and controlling the Ca/P coating can overcome the problem associated with the high temperature of sintering employed in metal and ceramics.[26]

Since NRL possess anionic particles that are able to nucleate Ca/P system without other surface treatments, when Ca/P is precipitated in supersaturated aqueous solutions, an amorphous phase forms in the early stages of the reaction, being further converted to phases such as ACP, OCL, and hydroxyapatite.[27,28] The composites of these two systems were developed to obtain the optimum properties for producing a scaffold for GBR application.

NRL has been subjected to biomimetic method and characterized by SEM, FTIR-ATR, and XRD. In addition, the hemolytic activity of the materials (NRL, Ca/P, and NRL + Ca/P) was investigated. Hemolytic measurements of complement activity are widely adopted in both clinical and experimental biochemistry.[29,30] The complement activity value usually determines the amount of hemoglobin released after erythrocyte (EA) lysis. In our experiments, we observed that the materials presented less than 10% hemolysis level up to 0.125 mg/mL, according to Fisher et al. (2003).[31] Results showed that the composite has been produced, with the incorporation of β -carbonated Ca/P at NRL with sizes between nano- and micro-scale, aiming a new biomembrane to improve GBR.

2. Experimental methods

2.1. NRL biomembrane

The latex solution used consisted of a mixture of a non-controlled variety of clones extracted from *Hevea brasiliensis*, which was kindly provided by ESALQ-USP, Piracicaba, Brazil. After extraction, ammonia was used to keep the latex liquid, and this material was centrifuged at 8000 g to separate proteins related to allergies that are water-soluble.

The membranes were prepared by pouring 2 mL of NRL in a stainless steel plate with 5.00 ± 0.05 cm diameter, and left at room temperature for 2 days to fully polymerize before use.

2.2. Biomimetic coating

The solutions were prepared by dissolving analytical grade reagents into demineralized water and buffered at pH 7.25 with *tris*-hydroxymethylaminomethane and 1 M hydrochloric acid. All solutions used were 1.5 times concentrated than regular SBF (1.5 $\times$ SBF). Table 1 shows the ionic concentrations of the SBF solution.

The samples were immersed in 1.5 $\times$ SBF solution and maintained at 37 °C for seven days. The solutions were replaced every 24 h. After the immersion period, the substrates were removed from the solutions, washed with demineralized water, and dried at room temperature 37 °C during 4 days.[32]

2.3. X-ray powder diffraction (XRD)

The samples were also characterized by X-ray powder diffraction (XRD), using a Siemens D5005 X-ray diffractometer and a graphite crystal as monochromator to select Cu K α 1 radiation (1.5406 Å), in a step of 0.02° s⁻¹.

Table 1. Ion concentrations (mM) of the 1.5 SBF in comparison with human blood plasma in total amounts.

	Na ⁺	K ⁺	Ca ²⁺	Mg ²⁺	HCO ₃ ²⁻	Cl ⁻	HPO ₄ ²⁻	SO ₄ ²⁻
Blood plasma	142.0	5.0	2.5	1.5	27.0	103.0	1.0	0.5
1.5 SBF	213.0	7.5	3.8	2.3	6.3	223.0	1.5	0.75

2.4. Fourier transform infrared (FTIR)

The membranes were characterized with Fourier transform infrared (FTIR) spectroscopy using a NICOLET 380 (Thermo Electron Co., USA) spectrophotometer in the Attenuated Total Reflectance [33] mode ($4000\text{--}650\text{ cm}^{-1}$) with a resolution of 4 cm^{-1} .

2.5. SEM

The surface morphology of the NRL membrane was observed using SEM model Zeiss® EVO 50 (20 KV) at a take-off angle of 35° (using gold as conductor material) and analyzed by freeware ImageJ v1.47 downloaded from the NIH website (<http://rsb.info.nih.gov/ij>). Image was set as 8-bit and submitted to binary conversion for analysis. Three aleatory areas were analyzed to perform the size estimation and distribution.

2.6. Hemolytic assay

The hemolytic activity of the materials (NRL, Ca/P, and NRL + Ca/P) was investigated according to Onuma et al. [34]. Blood, collected in heparinized-tubes from human, was centrifuged at 700 g for 10 min. The materials were washed four times with phosphate buffer solution (PBS) pH 7.4 by centrifugation at 700 g for 5 min and resuspended in the same buffer. Serial dilutions (starting with 1:5) of samples were used to determine the percentage of hemolysis. The final volume in each well was $100\text{ }\mu\text{L}$. Then $100\text{ }\mu\text{L}$ of 1% (v/v) suspension of EA was added to each well. The wells containing complement-free buffer were used as standard with a zero degree of hemolysis (0%); to attain 100% hemolysis, and $100\text{ }\mu\text{L}$ of Triton X-100 1% (v/v) was used instead of the buffer. The plate was incubated at 37°C for 1 h in Shaker Incubator (Quimis, Brazil) and then absorbance at 540 nm was determined in each well in an Epoch Microplate Spectrophotometer (BioTek, USA). Less than 10% hemolysis was regarded as nontoxic effect level in our experiments.[31] The experiments were performed in triplicate.

3. Results and discussion

Recently, NRL biomembrane has been investigated and used for GBR in dental alveolus [11] and cranial healing.[10,35] It is used as a barrier against the adjacent tissue, in which the results show an enhancement of healing with reduced time, and angiogenic effect. Incorporation of Ca/P should enhance the treatment, due its osteoconductive and osteoinductive properties.

Surface deposition of Ca/P was showed to increase ALP, bone formation, and expression of bone markers of human fetal osteoblast cells in PCL membranes.[26] At *in vivo* conditions, it was observed to enhance the repair of 8-mm-diameter calvarial defects in rats.[14]

The SEM (Figure 1(a)) shows the NRL biomembrane without Ca/P, while Figure 1(b) shows that the biomimetic method was successful to create Ca/P at its surface. It is also possible notice some particles which resembles hexagonal form (arrow in Figure 1(b)).[3,36] Hydroxyapatite crystallizes in the hexagonal (or monoclinic) phase, which was observed to stimulate osteoblast proliferation.[37]

Analysis of the mineral loaded at the surface reveals that the percentage of the area occupied is around of 14.9%, although there are many different sizes at the surface, indicating differences in maturation stages (Figure 2(a)). Analysis of the size

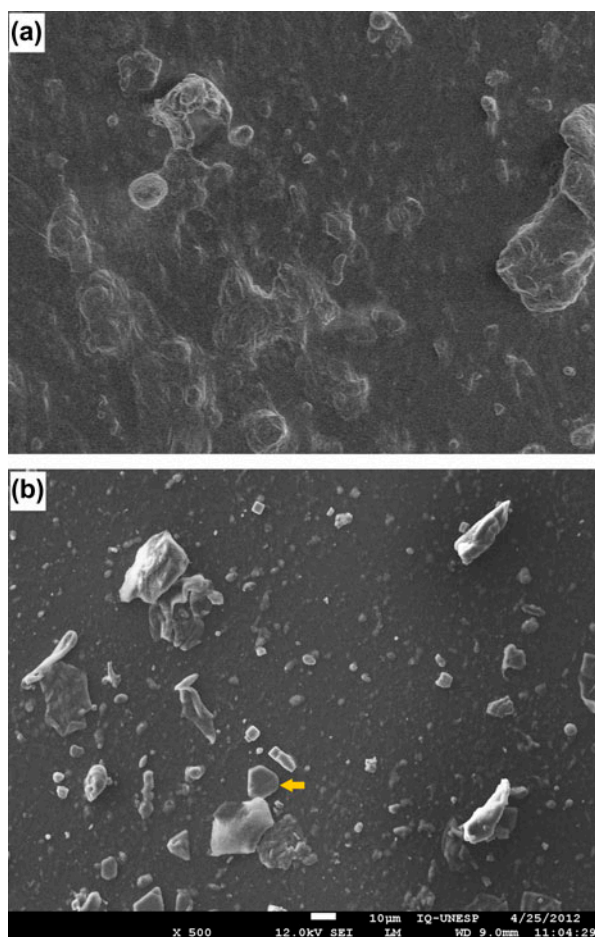


Figure 1. Representative micrograph of SEM: NRL (a) and NRL submitted to biomimetic method with Ca/P at surface (b).

distribution indicates that the predominant size is between 0.0343 and 1.0, with a low amount for higher sizes (Figure 2(b)), with few particles reaching up to 1080.727 μm (Figure 2(c)).

Due NRL biomembrane is an amorphous polymer it is not detected by XRD equipment (Figure 3). When subjected to biomimetic method is possible to observe a sharp peak around 20.5°, 22.5°, and 40°.[1,32]

The low intensity and broad peaks of XRD confirm the small sizes of Ca/P by SEM or the presence of a disordered phase.[38] It has also observed significant peak broadening and decrease in intensity of XRD, associated with decrease in Ca/P crystallite size.[39,40] Bone mineral phase also exhibited lower peak intensity compared to synthetic Ca/P.[41] This suggests that this method produced Ca/P similar to biological bone.

ACP phase has been applied in biomedical field due to its bioactivity, cell adhesion, biodegradation rate, and osteoconduction.[28,42,43]

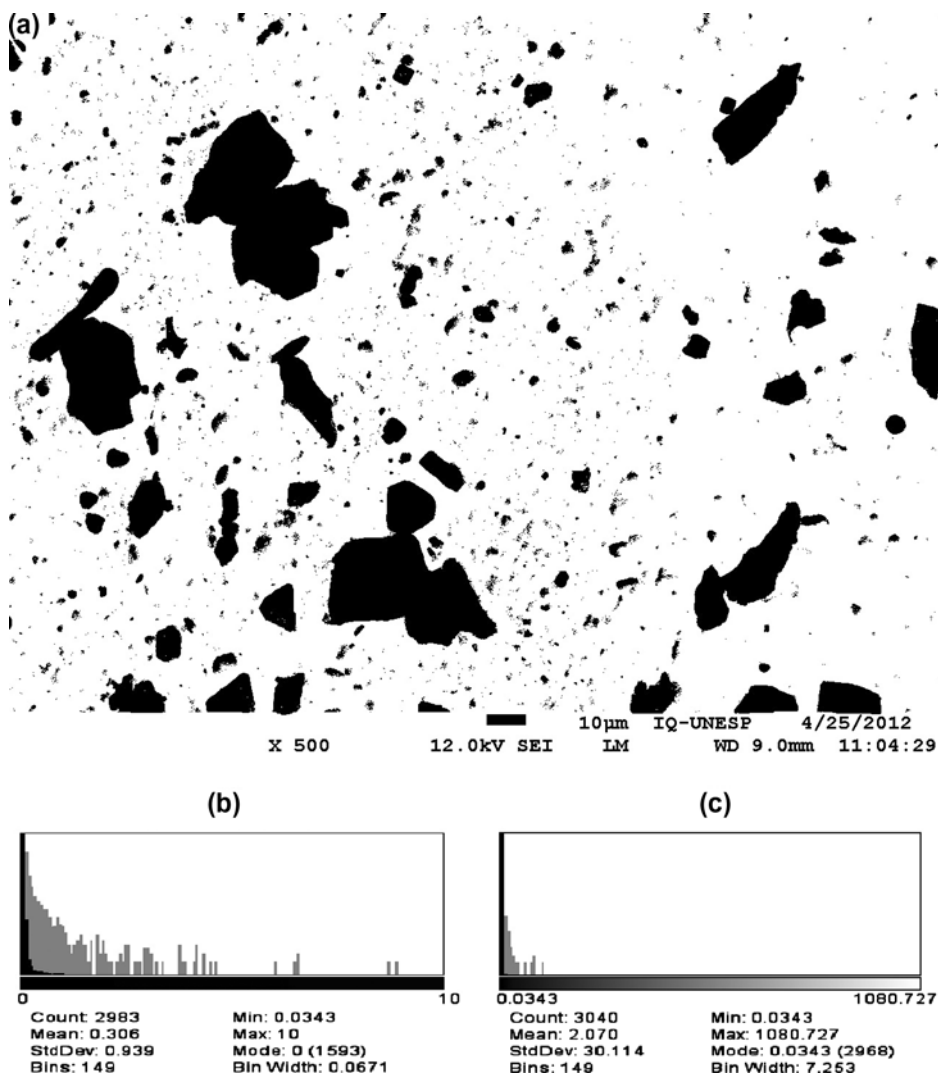


Figure 2. Binary mask of Figure 1(b) for Ca/P occupied area and size distribution analyzes (a); particle size distribution of Ca/P at membrane surface from minimum size until 10 µm (b) and until maximum size (c).

Studies on the preparation of calcium phosphates of interest biological phases, which are the synthetic prototypes of bone mineral, have shown the precipitation of initial solid phase from a calcium phosphate solution depends on the degree of supersaturation.[28]

The precursor ACP phase, transforms to thermodynamically more stable Ca/P phase or will be taken place by an autocatalytic solution-mediated crystallization process. On the other hand, the first solid to form in low-supersaturated solutions is hydroxyapatite with Ca/P ratio of 1.67 obtained without precursor phases. Therefore, ACP is considered as a 'mandatory precursor to calcium phosphates', and Ca/P of biological interest can be formed in dilute solutions without going through this precursor.[28]

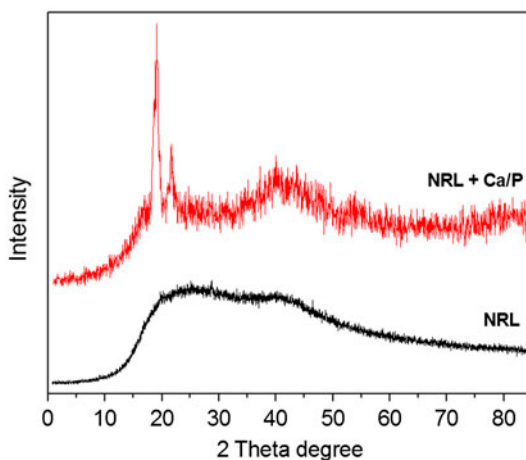


Figure 3. X-ray diffraction pattern of NRL and NRL + Ca/P by biomimetic method.

The pH value also affects the initial solid phase in the precipitation of calcium and phosphate ions. It is known that the ACP phase is the first-formed deposit *in vitro*, at neutral pH and supersaturated solution.[28,44]

Transition mechanism of ACP to Ca/P of biological interest at physiological pH has been described as following: firstly ACP dissolution, then a transient OCP solid phase reprecipitation through nucleation growth, and finally hydrolysis of the transient OCP phase into the thermodynamically more stable calcium phosphate phase.[28,44]

The presence of amorphous phase gives a biodegradable character, OCP was more osteoconductive than HAp due to its higher sorption.[1] Type B-carbonated HAp has a composition similar to bone and tooth tissue,[3] since natural bones present the mineral apatite phase with low crystallinity.[2] Chesnutt et al. (2007) observed that the surface with amorphous Ca/P has showed to be more osteoconductive to cell growth in the chitosan coated by biomimetic method.[45,46]

It seems that different Ca/P synthesis methods results in differences in Ca/P characteristics produced Ca/P by hydrothermal,[40] chemical, and biological route, obtaining different Ca/P ratio, size, and crystallinity.

In Figure 4 FTIR-ATR spectrum of NRL shows their characteristic peaks at 3283 cm^{-1} (OH stretching), 3038 cm^{-1} (–CH stretching), 2959 cm^{-1} and 2917 cm^{-1} (CH_2 asymmetric stretching), 2849 cm^{-1} (CH_2 symmetric stretching), 1740 cm^{-1} (C=O stretching), 1646 cm^{-1} (C=C stretching), 1446 cm^{-1} (CH_2 deformation), 1375 cm^{-1} (CH_3 asymmetric deformation), 1307 cm^{-1} (CH_2 wagging), 1242 cm^{-1} (CH_2 twisting), 1126 cm^{-1} (C–C stretching), 1080 cm^{-1} (CH_2 twisting), 1038 cm^{-1} (CH_3 rocking), 931 cm^{-1} (C–C stretching), 889 cm^{-1} (CH_3 wagging), 832 cm^{-1} (=CH out-of-plane bending), and 738 cm^{-1} (CH_2 rocking). The proteins in NRL can be identified due to the peaks around $1620\text{--}1700\text{ cm}^{-1}$ (amide I) and $1520\text{--}1580\text{ cm}^{-1}$ (amide II).[4,47,48]

In Figure 4 is possible to observe absorptions related to the carbonated Ca/P, type-B carbonated, due the absence of the peak at 1582 cm^{-1} and 1562 cm^{-1} (related to A-type carbonated), and due to the presence of the peak at 1446 cm^{-1} and 844 cm^{-1} , this may explain the low crystallinity in XRD. Due to the instrumentation ranging, it is not possible observe before 650 cm^{-1} to state about ν_2 and ν_4 vibrational

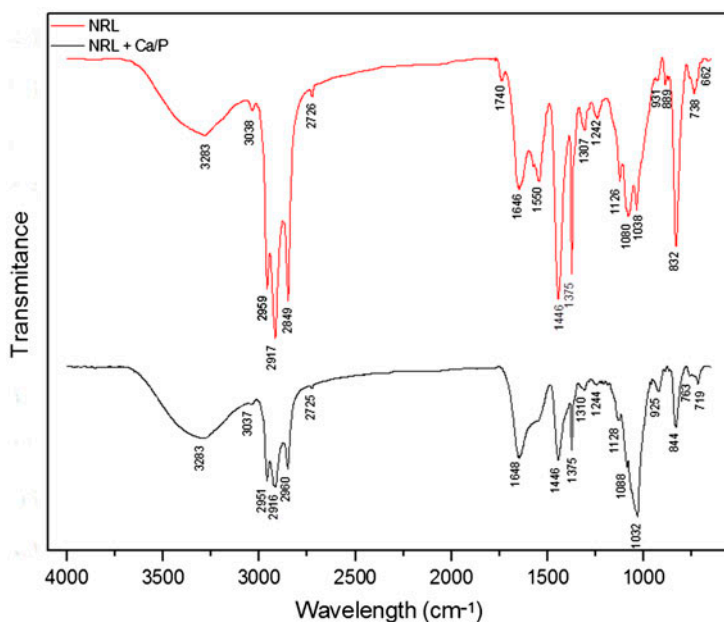


Figure 4. FTIR-ATR analysis of NRL and NRL submitted to biomimetic method.

modes of P–O, although, peaks at 1032 cm^{-1} and 1088 cm^{-1} are related to ν_3 and at 925 cm^{-1} related to ν_1 vibrational modes of P–O from PO_4^{3-} . Due to the Ca/P present at surface, a reduction of same peaks from NRL is observed.[32] It is also observed the reduction of the amide II band related to proteins, appeared as a shoulder. As proposed by Bonewald et al. [49] it is possible to measure the carbonate:mineral ratio from unsubtracted area of the carbonate ($890\text{--}850\text{ cm}^{-1}$) and phosphate (from 1200 to 900 cm^{-1}), and the mineral:matrix ratio from the unsubtracted area from phosphate and protein amide I ($1720\text{--}1590\text{ cm}^{-1}$). The carbonated:mineral ratio is 0.03 and the mineral:matrix is 2.27 (Table 2).

There are several Ca/P synthesis methods, such as hydrothermal, precipitation, sol–gel, plasma spraying, but these methods may include high temperature for processing, which may modify or even degrade polymeric materials. While these techniques usually provide a crystalline Ca/P, the biomimetic coating method overcomes this temperature problem, allowing incorporations of intricate shapes, and use of growth factors.[29]

The production of Ca/P of high crystallinity should be used in fast biodegradable biopolymers, since it creates a barrier between the scaffold and the surrounding environment.[38] Biomimetic method is more like bone mineralization, since both start with the formation of transient amorphous and poorly crystalline.[38] The isotropic and less brittle amorphous mineral components are supposed to contribute resistance to crack propagation.[38]

Recently, Ca/P was incorporated into NRL by sol–gel method, and also produced hexagonal $\text{Ca}(\text{PO}_4)_3$ and monoclinic Ca_5P_8 . [27] In this context, this article presented an alternative methodology to produce Ca/P in NRL biomembranes by biomimetic approach. This methodology allowed the controlled release of BSA.[18,19]

Table 2. Assignment of the main peaks in the FTIR spectra of NRL and NRL + Ca/P.

Wavenumber (cm ⁻¹)	Assignment
<i>NRL</i>	
3283	OH stretching
2959-2849	CH ₂ stretching
1646	C=C stretching
1446	CH ₂ deformation
1375	CH ₃ asymmetric deformation
832	=CH out-of-plane bending
<i>Proteins in NRL</i>	
1620-1700	amide I
1520-1580	amide II
<i>NRL + Ca/P</i>	
1032	v3 P-O
1088	v3 P-O
1446	B-type
925	v1 P-O
844	B-type
1520-1580 (shoulder)	amide II

The allergies related with NRL are well known, but the latex used in this experiment was centrifuged to reduce its protein content, particularly the allergenic proteins.[50] Its centrifugation produces three main parts: the rubber phase, C-serum, and the bottom fraction. The C-serum and the bottom fraction contains the water-soluble proteins, Hev b 2, 4–10, which has been shown to be the most allergenic among latex proteins. The sensitivity to Hev b 1 and 3 (water-insoluble proteins from rubber particles) has been particularly associated with spina bifida patients, with latex-sensitive adults far less commonly sensitive.[51]

The explanation for the mineralization is that NRL is an anionic [5] colloidal dispersion in which the rubber particles are stabilized by proteins and phospholipids.[52] Observed negative charge groups enhance the mineralization. Ca/P also presents high affinity to bind with proteins, which may explain the reduction of the peak of amide II in FTIR-ATR, proteins with isoelectric point higher than 8.0 adsorb proteins due the electric interaction between PO₄ from Ca/P and NH₄ of proteins, for acid proteins Ca²⁺ interact with carboxylic group of proteins.[1] Another explanation is that during drying process, the calcium present in the serum binds to proteins and phospholipid, and subsequently condensate at rubber surface,[53] then phosphate ions stabilize calcium.[54]

Nascimento et al. [27] stated that NRL particles could interact with the proteins in latex, which could explain the shift of the emission bands related to tryptophan in the evaluated NR-Ca/P composites; also that Ca/P grains are surrounded by NR particles.[27]

The interactions between the materials (Ca/P, NRL, and NRL + Ca/P) and membranes have also been studied by hemolysis experiments. The release of hemoglobin was used to quantify the membrane-damaging properties of the materials. As 100 and 0% values we used Triton X-100 and phosphate-buffer-treated EA, respectively. EA were incubated with five different concentrations of each material in the range of 0.031–0.5 mg/mL for 1 h (Figure 5). Under these conditions, NRL, Ca/P, and NRL + Ca/P showed no hemolytic effects up to 0.125 mg/mL indicating no detectable disturbance of the red blood cell membranes. However, it was found considerable

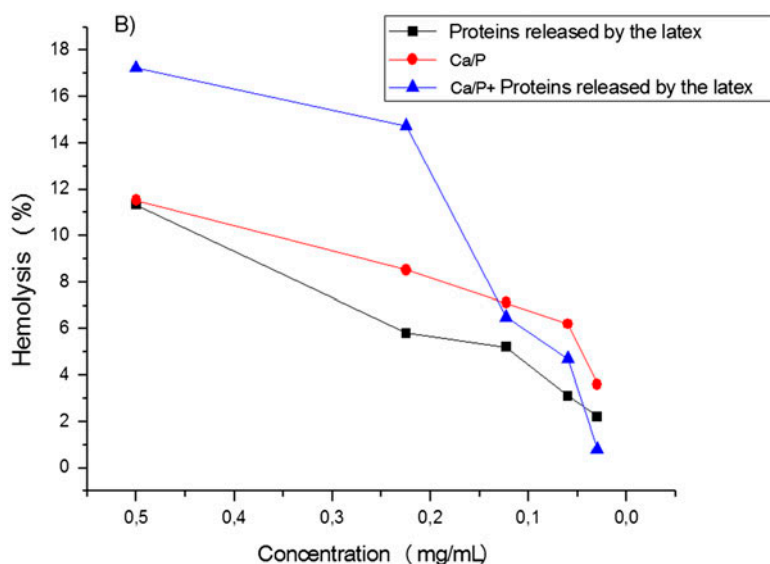


Figure 5. Percentage release of hemoglobin of human red blood cells after 1 h incubation with different concentrations of the materials at 37 °C ($n = 3$).

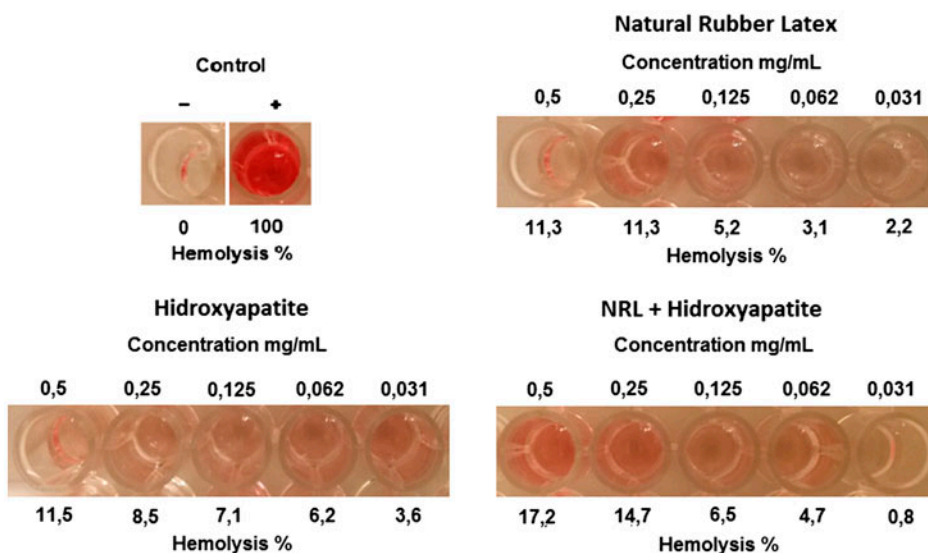


Figure 6. Increase of hemolysis as a function of materials concentrations.

amounts of hemolysis at concentrations of ≥ 0.25 mg/ml to NRL and NRL + Ca/P caused 11.3 and 14.7% hemolysis, Figure 6.[31]

Incorporation of Ca/P in NRL should enhance bone healing, since it was noticed that Ca/P is able to enhance hydrophobicity¹¹ and augment mechanical strength of the scaffold (creating a scaffold with properties close to bone).[38] Ca/P should also be

used in NRL to control the release of substances, since NRL is also used as delivery system for drugs and proteins and [55] noticed that a mineralized layer mitigates the burst release.

4. Conclusions

Analysis of the results sufficiently proved the successful loading of Ca/P particles onto the membrane as showed by SEM with different sizes ranging with different maturation stages. FTIR shows that the phase of hydroxyapatite is the carbonated type-B, also proved by its low crystallinity in XRD. In addition, the materials (NRL, Ca/P, and NRL + Ca/P) presented no cell damaging effects up to 0.125 mg/mL, where the hemolysis level was less than 10%.

Disclosure statement

No potential conflict of interest was reported by the authors.

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