The effects of *Anodonta cygnea* biological fluids on biomineralization of chitosan membranes

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ABSTRACT

The use of chitosan membranes, a low-cost, biocompatible material with promising capabilities in biomedical applications has already been fairly investigated in other fields with good results. Here, the aim was the *in vitro* mineralization assays accomplished with the chitosan membranes incubated in the control calcium and phosphate solutions on “Ussing” chambers. Biological fluids from *Anodonta cygnea* were added to the control solutions under different physical–chemical parameters such as pH, synthetic and natural organic compounds. Some organic compounds analyses in the solutions as well as the chitosan membrane SEM-EDS, XRD and FTIR observations were carried out at the end of experimental period. From the experiments it was shown that the chitosan membrane may act as a selective epithelium with preferred ionic movements. Particular mineral deposits, in the chitosan membrane, with hexagonal hydroxiapatite crystals, occurred only at pH 6 with HCL or succinic acid. Biological fluids of *A. cygnea* induced an increase in number and size of hexagonal crystals forming specific rosaceous structures, especially at pH 6 with succinic acid. It seems that proteins, GAGs and lipids can be involved on this process, since there is a significant reduction on its contents after experiments. So, further applied research with these materials can be a promising subject.

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1. Introduction

Chitosan has recently been appointed as a biomaterial with a wide variety of biomedical and industrial applications [1]. It is a linear polyaminosaccharide composed of (1–4)-linked-D-glucosamine and N-acetyl-D-glucosamine, commonly derived at a low-cost from chitin, a waste material from the seafood industry [2]. Important properties like antimicrobial and antitumoral activity, haemostatic and accelerated wound healing, biodegradability, low toxicity and biocompatibility have made chitosan a promising biomaterial of the twenty-first century [3].

Different forms of chitosan-based biomaterials have recently been developed for biomedical purposes, including scaffolds, nanocomposites, tubes, sponges, coatings, microspheres, wound dressings and membranes, which are especially useful for potential

applications in bone repair and regeneration. Among the most current topics in this field is the adaptation of *in vitro* biomineralization experiments to resemble *in vivo* conditions. In fact, biomineralization is the process by which organisms convert ions in solution into solid minerals. It is also the result of the cellular activity that makes possible the necessary transformations that lead to crystal formation and growth. In order to occur, some requirements are needed: specialized microenvironments where mineral supersaturation may be achieved, a supply of ions and macromolecules, removal of waste products and balanced physical–chemical parameters (e.g., pH, osmolarity). For many reasons the biomineralization phenomenon has been a point of interest for some scientific and technological areas, e.g., in medicine, understanding the deposition of calcium salts in bone cells as part of the growth and repair process is essential in order to develop prosthesis and osteogenic implants or to avoid pathological calcification of organs and medical devices.

The organic matrix of molluscan shells is thought to be an essential biomineralization mediator [4–6]. The matrix may control crystal nucleation, polymorph selection, crystal orientation and/or crystal growth inhibition [4]. Calcium carbonate/phosphate crystals are well regulated, theoretically through an ionotropic or

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stereochemical mechanism [7–10]. According our studies [9–14], the freshwater mussel *Anodonta cygnea* is a good model to study biomineralization processes due to have large volume biomineralization compartments with easy access to their organic matrixes. In these animals, biomineralization events occur in two different biomineralization compartments, each with a specific biological fluid, haemolymph and extrapallial fluid, involved in mineral production at the microspherules (mineral concretions) level [9,10] and in the shell calcification respectively. Although the fluids act at different levels of calcification, their organic and inorganic composition is very similar and balanced either by transport across the mantle barrier or by mantle cellular synthesis. Proteins, glycosaminoglycans (GAGs) and hexosamines (chitin) are the major organic constituents known to be important for biomineralization [9,10].

In this view our aim was to gain further knowledge about the basic principles of biomineralization and to evaluate the influence of bivalve biological fluids, which are involved in *in vivo* biomineralization events.

2. Experimental

2.1. Membrane preparation

The chitosan membrane was obtained and treated as already described by Mano [15].

2.2. Biological sample collection

Freshwater bivalves (*A. cygnea*) were collected from the clay bottom of Mira lagoon (Aveiro, Portugal). The animals were kept in laboratory in dechlorinated and aerated water for a minimum of 1 day period before the extraction of fluids (haemolymph and extrapallial fluid). For fluids sampling, the animals were considered healthy if they showed active ventilation and powerful valve closing or water ejection upon disturbance. For each experiment, approximately 5 ml of haemolymph and 5 ml of extrapallial fluid were extracted with a needle syringe from two animals. After that, each fluid was centrifuged at 4000 rpm, 5 min. (Kubota KR-20000T, Rotor RA-3R) to remove cellular debris. Subsequently, the supernatant was treated separately in order to be used in each experiment.

2.3. Kinetic of chitosan membranes

All further experimental sets, which were replicated four times, were carried in “Ussing chambers” separated by a chitosan membrane into two compartments: one filled with a phosphate solution and other with a calcium solution (Fig. 1). The chitosan membranes were previously washed with alcohol, air dried and than washed again in distilled water immediately before the insertion between the chambers. In this set of experiences the phosphate chamber was filled with 4 ml of sodium phosphate (20 mM, pH 7.5/HCl) and the calcium chamber with 4 ml of calcium chloride (20 mM, pH 7.5/HCl). The chambers were closed and kept 6 h at room temperature. At the end of this period, the solutions of each individual chamber were collected and stored until posterior analysis. These experiments with synthetic solutions were considered as a control situation for further experimental sets. Time and number of replicates, temperature and final collection and storage procedures were the same in all assays to be described.

Calcium and phosphate ions quantifications in solutions were accomplished at time 0 and 6 h after by atomic absorption spectroscopy and PiBlue™ Phosphate Assay Kit (Bioassay Systems) respectively.

Fig. 1. Photography and schematic view of experimental system with ‘Ussing chambers’ (UC) separated by a chitosan membrane (CM).

2.4. Biological fluids effects

The chitosan membranes were previously treated during 30 min with haemolymph or extrapallial fluid extracted from *A. cygnea* mantle. Then, the membranes were inserted between the two chambers according the anterior scheme, already explained (Fig. 1). In the subsequent experimental sets, the addition of haemolymph or extrapallial solutions was only used in the calcium chamber, since the fluid influence on the calcium phosphate precipitation were only evident in the calcium chamber. So, as in the anterior experiments the phosphate chamber was filled with 4 ml of sodium phosphate (20 mM) however the calcium chamber was filled with a biological fluid (haemolymph or extrapallial) with 100 µl of calcium chloride to reach a final concentration of 20 mM of calcium. These calculations were made based on previous knowledge on calcium concentration in the biological fluids [16].

The determination of organic compounds concentration on experimental fluids was performed at time 0 and after 6 h. Proteins were quantified according Bradford [17] and total GAGs were quantified according Gold [18,19]. For total lipids concentration, a liquid–liquid extraction [20] was carried out followed by quantification using a Total Lipid Kit (Far Diagnostic). The presence of iron ions was detected by atomic absorption spectroscopy (Varian SpectraAA 220FS).

2.5. pH effect

Similar experiments, with calcium and phosphate solutions, were also carried out adjusting the pH solution with HCl (1 M) at pH 6, 7 and with NaOH (1 M) at 9, for both chambers in order to evaluate the pH effects. Other experiments at pH 6 with synthetic

Table 1
Phosphate and calcium variations on the solutions from the first experimental set ($n = 12$).

	$t = 0$ h		$t = 6$ h	
	Phosphate chamber	Calcium chamber	Phosphate chamber	Calcium chamber
Phosphate (mmol/L)	20	0	16.16 ± 3.48	0.03 ± 0.01
Calcium (mmol/L)	0	20	0.058 ± 0.01	18.75 ± 0.56

organic acids, as succinic and citric acids, were carried out with and without haemolymph and extrapallial fluid of *A. cygnea* additions, in order to study the effects of specific organic acids. In these cases, the biological fluids were only added to the calcium chamber while the succinic and citric acids were used in both chambers for pH balance.

2.6. Scanning electron microscopy (SEM) imaging

Membrane pieces from both sides of all experiences were gold-coated (FINE-COAT Ion sputter JFC-1100) and glued to aluminium stubs for SEM observations using JEOL JSM-35C scanning electron microscope (SEM) operated at 10 Kv.

2.7. Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD)

In all experiments, membrane pieces were analyzed by FTIR. Unlike SEM imaging, FTIR analyses were performed only in the side of membrane that was in contact with the calcium solution (calcium chamber).

For the FTIR analyses, a Bruker Equinox Fourier transform spectrometer was used coupled with a Bruker microscope that collects the IR beam with Cassegrain objectives. Analysis of clay films deposited on a CaF_2 disk was carried out by transmission, using a circular diaphragm with a 60 mm diameter aperture. Each spectrum was processed using the OPUS program ([®]Bruker). Spectra of atmospheric CO_2 and H_2O were recorded independently for subtraction purposes.

The XRD spectra were performed on a Panalytical Xpert MPD with a Cu vial.

2.8. Statistic analyses

For all experimental situations, the differences in organic and inorganic contents were established between the control values and the mean of ($n = 12$) quantitative measurements of samples collected 6 h after. These differences were tested for statistical significance by multiple Paired t -tests. The significance level for all statistical analyses was set at 0.05.

3. Results and discussion

The first set of experiments with NaHPO_4 and CaCl_2 bathing only, induced a clear deposit of CaPO_4 on the chitosan membrane side directed to the calcium chamber while in the other side the membrane stayed free of any mineral deposit. In fact, the SEM and Energy Dispersive Spectroscopy (EDS) observations showed a calcium phosphate deposit of around $1 \mu\text{m}$ with a smooth surface without any apparent external crystalline structures (Fig. 2). There was also a significant decrease on calcium and phosphate contents from the respective solutions (Table 1). Curiously, these ionic changes were not compensated by equivalent amounts on the opposite sides. So, it is possible to suggest that the main volume of phosphate and calcium ions were precipitated on the membrane under mineral deposits in the calcium chamber side. It is also possible to point out that the chitosan membrane presented a selective permeability relatively to both ions mainly to the phosphate. Since

Fig. 2. SEM images and respective EDS spectra of the membrane phosphate side (A) showing only the chitosan membrane (CM) surface without any deposit and calcium side (B) with a layer of amorphous calcium phosphate (ACP).

Table 2Phosphate and calcium variations on the solutions with membranes embedded in haemolymph ($n = 12$).

	$t = 0$ h		$t = 6$ h	
	Phosphate chamber	Calcium chamber	Phosphate chamber	Calcium chamber
Phosphate (mmol/L)	20	0	11.4 ± 5.37	0.05 ± 0.01
Calcium (mmol/L)	0	20	0.07 ± 0.01	16.65 ± 0.85

Table 3Phosphate and calcium variations on the solutions with membrane embedded in extrapallial ($n = 12$).

	$t = 0$ h		$t = 6$ h	
	Phosphate chamber	Calcium chamber	Phosphate chamber	Calcium chamber
Phosphate (mmol/L)	20	0	10.86 ± 5.16	0.06 ± 0.03
Calcium (mmol/L)	0	20	0.05 ± 0.01	17.23 ± 1.00

the PO_4^{3-} ion has a higher molecular size than the Ca^{2+} ion and the chitosan membrane has positively charged pores, it is possible to speculate that this fact induces phosphate ion movements from the phosphate chamber towards the calcium chamber where it precipitates on the membrane under CaPO_4 .

In the second experimental set with membranes previously impregnated on haemolymph and extrapallial fluid from *A. cygnea*, and bathed in the chambers by the same inorganic solutions, above mentioned, similar calcium phosphate deposits were observed by SEM in the membrane on the calcium chamber side of the membrane (Fig. 2). A significant decrease on the calcium and phosphate ion content was detected, with haemolymph and extrapallial respectively (Tables 2 and 3). However, these ionic variations with impregnated membrane were higher than in the experiments without biological fluids impregnated membranes (first set) suggesting an active role to specific organic molecules in the bivalve fluids.

In the third experimental set where the haemolymph or extrapallial fluids from *A. cygnea* were added to the calcium chamber, the chitosan membrane also presented on the same side similar calcium phosphate layer features by SEM and EDS (Fig. 2). In this case, specific organic compounds were measured in these fluids at time zero and 6 h after, showing a significant decline on total protein, Gag and lipid contents (Fig. 3). According to these results it is possible to suggest a direct contribution of these elements in order to form a biomineral layer.

In order to understand the pH influence on the precipitation process, a fourth experimental set was carried out where calcium and phosphate solutions differed only on pH values, that were set to 6(HCl), 7(HCl) and 9(NaOH). Observations by SEM and EDS demonstrate that, in all these assays, calcium phosphate precipitation occurred only on the calcium chamber side of the membrane. At pH 7 and 9 a coat of amorphous calcium phosphate, similar to the other sets, was formed (Fig. 2), but at pH 6, on this amorphous layer, well defined, calcium phosphate hexagonal crystal formations were observed (Fig. 4B). These results suggest that an alkaline environment induced a rapid precipitation that does not allow an external habit under a crystalline form, while an acidic environment favours a slower precipitation where a more structured form could be constructed.

Although with the same pH 6, the different acids used to set this pH value revealed different results. With pH 6(HCl) (Fig. 4A), single crystal forms were observed in great numbers. On the other hand, when pH 6(succinic acid) was used, few crystalline forms were constructed (Fig. 4B). Additionally, with citric acid no crystalline formation was observed as in the control set of experiments.

It was observed that haemolymph and extrapallial fluid have in their constitution organic molecules like proteins, GAGs and lipids which contribute to the biomineralization process in the chitosan membranes. When these fluids were introduced in the fifth experimental set maintaining the pH 6, some differences in crystalline forms were denoted comparatively to the others already described.

Fig. 3. Variation of total proteins (A), total GAGs (B) and total lipids (C) between times 0 and 6 h after ($n = 12$).

Fig. 4. SEM images of crystalline calcium phosphate (CCP) formed at pH 6(HCl) (A) and at pH 6(succinic acid) (B) growing over an amorphous calcium phosphate (ACP) layer.

So, at pH 6(HCl) and pH 6(succinic acid) plus both fluids (Fig. 5) the crystals presented an increased size than without fluids (Fig. 4) and were better organized. At pH 6(succinic acid) the crystals have a “*rosaceous*” form and at pH 6(HCl) the crystals exhibit a typical hexagonal crystalline formation. At pH 6(citric acid) with biological fluids the results were very similar to the control, i.e., no crystalline formation was constructed.

In summary, the results suggest that the synthetic solutions with HCl and succinic acid induced dispersed crystalline structures while the citric solution did not produce any crystals. Moreover, the addition of organic fluids promoted an intense aggregation and an increase of calcium phosphate crystalline structures only when there is a natural tendency to form crystals (HCl, succinic acid).

So, as in shell construction on *A. cygnea*, specific molecules of respective biological composites should nucleate and be responsible for the modulation of calcium phosphate polymorphic structures determining the crystalline shape and acting as glue stabilizing crystal structure (Figs. 6 and 7).

3.1. Nature of mineral layers

The X-ray analyses of the deposited mineral layers on the chitosan membranes showed precipitation of calcium phosphate as a crystalline structure assigned to hydroxyapatite. Surface area observed by electron microscopy shows major surface of amorphous phases and crystalline phases displaying the hexagonal shapes. The crystals under the hexagonal forms are mainly observed on the pH 6.0 with or without any organic fluids.

The studied materials by both FTIR and XRD techniques confirm an excellent crystallinity of hydroxyapatite. FTIR spectra of the samples C1, C2, S1, S3, Ct1 and Ct3 show a single ν_3 asymmetrical vibration corresponding to tetrahedral molecule of PO_4^{3-} ions at about 1010 cm^{-1} . The narrowness vibration is assigned to the P–O stretching band (Td symmetry) Fundamental vibrational frequency (ν_3) of HPO_4^{2-} anion is correlated with both vibration planes at 1010 cm^{-1} and $1070\text{ (1080)}\text{ cm}^{-1}$. The band at 1070 cm^{-1} occurs as a shoulder at the P–O stretching band (samples C1, C2, C3, S1, S3, Ct1, Ct2 and Ct3).

A tetragonal penta-atomic molecule (e.g., PO_4^{3-}) exhibits four different vibrations (i) the symmetric stretching (ν_1), (ii) the symmetric bending (ν_2), (iii) the asymmetric stretching (ν_3) and (iv) the asymmetric bending (ν_4). Thus, the FTIR spectra collected of the samples studied display a typical asymmetric stretching (ν_3).

However, the C–O vibration planes related to chitosan may be well observed in this wave region when hydroxyapatite is poorly crystallized. In our case, the asymmetric vibration of PO_4^{3-} ions could be also due to vibration planes of chitosan, where the shoulder at 1070 cm^{-1} could be also assigned to the C–O stretching [21].

The ν_1 vibration shifts at about 870 cm^{-1} (sample C3). However, the band vibration assigned as a shoulder at about $883\text{--}890\text{ cm}^{-1}$ (samples C1, C2, S1, S3, Ct1, Ct2 and Ct3), should be also related to C–O band of chitosan [21].

Sample S2 show a ferric hydroxyphosphate complexation corresponding to a fundamental monodentate asymmetrical vibration (ν_3) at 980 cm^{-1} , 1051 cm^{-1} and 1118 cm^{-1} . Probably goethite [FeO(OH)] was precipitated later or joined with phosphate.

Fig. 5. SEM images of calcium phosphate crystals with a ‘rosaceous structure’ (RS) formed at pH 6(HCl) (A) and at pH 6(succinic acid) (B) with a typical hexagonal structure (HS) plus *A. cygnea* fluid. Both crystals grew over an amorphous calcium phosphate (ACP) layer.

Fig. 6. FTIR spectra of chitosan membrane in the calcium chamber side. (A) C1, C2 and C3 represent pH 6(HCl), pH 7(HCl) and pH 9(NaOH), respectively. (B) S1, S2 and S3 refers to pH 6(succinic acid), pH 6(succinic acid/haemolymph) and pH 6(succinic acid/extrapallial), respectively. (C) CT1, CT2 and CT3 refers to pH 6(citric acid), pH 6(citric acid/haemolymph) and pH 6(citric acid/extrapallial) respectively.

Fig. 7. XRD of the chitosan membrane at pH 6(HCl/haemolymph) in the calcium chamber side. The diffraction peaks are compatible with a solid phase composed by hydroxyapatite. The vertical axis represents X-ray intensity. The horizontal axis records angles in degrees (2θ) with time steps of 60 s between 10° and 90° .

The OH^- -band is well observed at 3500 cm^{-1} and 3400 cm^{-1} in the samples studied. However, the OH^- -bands at 3500 and 3400 cm^{-1} are not observed in samples C2, S1, S3, Ct1 and Ct3 studied, probably due to $\text{Ca}(\text{OH})_2$ impurities.

The band at 2900 cm^{-1} is assigned to $-\text{CH}_2$, often used as reference band for chitosan [22]. The band at 1633 cm^{-1} is assumed to water, indicating the interaction of the amine on the chitosan and phosphates in the solution [23]. Both bands at 1525 and 1379 cm^{-1} are assumed to CO_3^{2-} , being well highlighted in samples S2 and Ct2.

The vibration planes observed in the FTIR spectra suggest the presence of hydroxyapatite (samples C1, C2, S1, S3, Ct1 and Ct3) and Fe-hydroxyapatite (sample S2 and Ct2). The presence of Fe^{2+} ion in haemolymph and extrapallial, 16.0 and $15.0\text{ }\mu\text{M}$ respectively, can explain the fact to form Fe-hydroxyapatite. However, the selective presence of Fe^{2+} only in the deposit of haemolymph samples can suggest any specific mechanism of complexation, probably based on particular biomolecules which can induce preferred precipitation on the chitosan membranes.

4. Conclusions

Considering the present results it is possible to suggest three distinct and interesting points. One is that the chitosan membrane may function as a selective epithelium where preferred ionic movements and subsequent specific biomineralization occur in only one face of the membrane. This particular behaviour might be due to the charge effects between the ions and the membrane pores.

The second aspect concerns the properties of the mineral deposit obtained in varied conditions. For general situations, it is possible to indicate that the chitosan biomineralized surface is composed by an amorphous mineral layer where intrinsic hydroxyapatite crystals may be formed. Additionally, under specific cases, these crystals may exhibit specific hexagonal forms induced by a low pH 6.

A third point is relative to the organic compounds which seem to have a clear effect by ionotropic and/or epitaxial effects over the hydroxyapatite crystals inducing accentuated hexagonal shapes, or forming a very typical and intense rosaceous shape when influ-

enced by the proteins, GAGs and lipids from the haemolymph or extrapallial fluids of *A. cygnea*, mainly under the action of succinic acid.

So, these properties of the chitosan membrane to assume those physical–chemical behaviours, under very particular conditions and processes, may be used in several fields on the development of biological, bioengineering or even medical applications. In fact, these biological fluids with natural biomineralizing components should be also thoroughly researched in this sense, as adequate mineral nucleators and modulators.

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