Casein – nanosized nucleator for *in vitro* mineralization

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This work reports the use of nanostructured Casein as nucleator for the *in vitro* formation of apatite-like mineral phase. Protein physical immobilization during the free-radical polymerization of acrylamide monomer was performed. Two loadings of Casein in the carrier hydrogel were investigated. The capacity of the immobilized Casein to induce *in vitro* calcification was followed through incubation in synthetic body fluids with ionic composition similar to human plasma (SBF 1x and 1.5x) for maximum 21 days. Nucleation of calcium phosphate was observed only on the Casein-clusters. The nucleation is followed by homogeneous apatite coating of the whole samples. This study confirms the potential of Casein to be used as nucleator for *in vitro* apatite formation from acellular SBF media.

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1. Introduction

In an approach of mimicking the chemistry of proteins involved in the calcification occurrence, the use of negative functional groups was expected to induce biomimetic mineralization on/in different polymer compounds. In this respect, we investigated the potential of carboxyl- and phosphate- containing polymer substrates to spontaneously form calcium phosphates *in vitro* [1-5]. Casein is the main protein of milk and its potential to deliver micronutrients has recently regained interest [6-8]. The structure and function of this phosphoprotein were extensively studied. Livney [6] has emphasized that Casein has a well known role in providing the body with bioactive essential nutrients such as calcium and phosphate [6-8]. Nevertheless, the affinity of the Casein phosphopeptides (CPP) towards calcium and amorphous calcium phosphate (ACP) is reported in the literature [6,9,10]; CPP are bioactive peptides resulted from the enzymatic hydrolysis of Casein.

The phosphor-seryl moieties [11-13] flanked by glutamate residues [8,11,13] are considered responsible for the capacity of Casein to bind metallic ions (including calcium). Moreover, anticariogenic properties of CPP-ACP nano-complexes were reported [14-18]. Wong and Sissons studied the influence of Casein as macronutrient on the calcium phosphate deposition and growth in plaque mineralisation [19]. To the best of our knowledge, despite well recognised relationship between dietary proteins and bone quality, there are no studies on the spontaneous *in vitro* mineralisation of Casein when physically entrapped in hydrogel carriers. Accordingly, this work investigates the induction of apatite nucleation using Casein nanosized nucleators embedded in a crosslinked polyacrylamide carrier.

2. Materials and methods

Materials

a) for the synthesis of the hybrids:

Acrylamide (AA) and methylene bis acrylamide (MBA) were used as such, one as synthetic monomer and the other one as cross-linking agent, respectively. Cas from bovine milk was dissolved in 1M sodium hydroxide (NaOH), according to its technical datasheet; this solution is further named Casein0 (protein concentration is 2% w/v). Ammonium persulphate (APS) was used as polymerization initiator and tetraethylene amine (TEA) as accelerator. Finally, sodium azide (NaN_3) was used as preservative agent for the protein solution. All the reagents were purchased from Sigma-Aldrich and used as such. MilliQ water was used for the reactions and double-distilled water (ddw) for purification steps.

b) for *in vitro* calcification testing medium

The simulated body fluids (SBF 1x and 1.5x) were prepared using: sodium chloride (NaCl), sodium hydrogen carbonate ($NaHCO_3$), potassium chloride (KCl), dipotassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H2O$), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), calcium chloride ($CaCl_2$), sodium sulfate

(Na_2SO_4). All salts were supplied from Sigma-Aldrich and used as such. MilliQ water was used as solvent. Hydrochloric acid (1N) was purchased from CHIMOPAR Bucharest. TRIS (tris-hydroxymethyl aminomethane) 99+% was from Sigma-Aldrich.

Methods

Immobilization of Casein in the carrier hydrogel

Casein-PAA-MBA hybrids (further identified as samples I and II) were synthesized through the direct physical immobilization of the protein during the polymerization of the monomer. Two protein loadings were used: I with 0.2% Casein and II with 1% Casein. Briefly, a basic free-radical polymerization of AA using MBA as crosslinker, was performed in the presence of Casein. The ratio monomer - crosslinking agent was constant in the two polymerization mixtures (15% MBA with respect to AA). For each composition, the corresponding amount of APS (1.5% molar with respect to the monomer content) was dissolved in 100 μL MilliQ water and the so-formed solution was added to the polymerization mixture. NaN_3 (0.1 % wt with respect to Casein) was added to Casein0 solution to prevent bacterial growth. The reaction mixture containing Casein was vigorously stirred on a vortex at 1100 rpm and then degassed for 15 minutes (using an ultrasound bath ELMA S 30 H (Elmasonic)); the appropriate amount of TEA was added and then the polymerization took place at room temperature, in Petri dishes. After 24 hours, the samples were removed from the Petri dishes and extensively washed with ddw to remove unreacted monomers. After 48 hours in ddw, cylinders of 10 mm diameter were cut from the obtained gels.

Physico-chemical characterization

The success of Casein immobilization in the polymer hybrids was assesses through Fourier transformed infrared spectroscopy (FT-IR). FT-IR spectra were taken on a Jasco 4200 spectrometer equipped with a Specac Golden Gate attenuated total reflectance (ATR) accessory, using a resolution of 4 cm^{-1} and an accumulation of 60 spectra, in the 4000-600 cm^{-1} wave number region.

The stability of the samples in ddw and SBF was assessed through visual inspection and gravimetrically, after 7 and respectively 14 days immersion in each fluid.

Information with respect to hybrids' morphological features, phase segregation and other aspects was obtained through the scanning electron microscopy (SEM) analysis of the gold-coated cross-sections of the samples. The analysis has been performed using a QUANTA INSPECT F SEM device equipped with a field emission gun (FEG) with a resolution of 1,2 nm and with an X-ray energy dispersive spectrometer (EDS).

In vitro calcification testing

The capacity of the Casein-PAA scaffolds (I, II) to induce hydroxyapatite (HA) formation was explored through the incubation of the cylinders in SBF solutions, miming the ionic composition of the human plasma. The preparation of the two test solutions, SBF 1x and SBF 1.5x was performed as recommended by Kokubo for the revised-SBF formulations [20]. The polymer samples were immersed 48 hours in ddw to reach their maximum swelling degree prior the incubation in SBF. Thereafter they were introduced in 50 ml of freshly prepared SBF 1x at 36.5 °C, for 21 days and in SBF 1.5x, for only 14 days. The test media were changed every two days. At the end of the incubation time, the samples were gently washed in a large excess of ddw to remove the residual salts physically deposited onto the samples. Washing was carried out for 24 hours and then the materials were dried at 40 ^oC to constant mass. The success of the calcification was explored through Von Kossa staining, FT-IR, SEM, and EDAX. The same equipments were used as for the physico-chemical characterisation. The specimens for SEM were coated with a thin layer of gold.

3. Results and discussion

This work was aimed at the investigation of the potential of Casein nanograins to induce the nucleation of HA-like mineral phase after immersion in SBF. First, two Casein-PAA-MBA hybrids were synthesized. Since Casein is water insoluble while the carrier is a hydrogel, phase segregation was expected to occur. This is a desired phenomenon since it would further allow the assessment of the calcification occurrence in the presence of Casein when compared to the carrier alone. Then, *in vitro* calcification tests were performed in acellular media following well known procedures. After this, a visual inspection of the materials was performed first. Whitish appearance could be noticed, probably due to both Casein phase segregation and *in vitro* calcification. At the end of the test, the samples incubated in SBF 1.5x were completely white. Von Kossa staining successfully proved the existence of calcium salts in all the samples. It seems that, as expected, the amount of mineral depends on the amount of Casein. Thus, reddish-brown colour was obtained for samples I, while brown-black colours resulted for samples II. The intensity of the colour also depended on the test used. Thus, darker colours corresponded to the use of SBF 1.5x, indicating a higher amount of mineral formed following this test. This is the first qualitative evidence on the occurrence of calcification on the studied materials.

FT-IR analysis. The study further continued with the FT-IR investigation. ATR FT-IR spectra were recorded before and after the incubation tests in SBF 1x and SBF 1,5x and they do confirm the presence of phosphate groups on all the analyzed samples. Fig. 1 and 2 are

representative with this respect. The spectrum of Casein presents, on one hand strong O-H and N-H vibrations due to the high number of -OH and $–NH_2$ groups from the contained aminoacids residues, and, on the other hand, strong amide I (at 1630 cm^{-1}) and amide II (at 1518 cm^{-1}) vibrations characteristics to the amide groups of the protein chain. On the spectra of the protein-hydrogel hybrids, typical signals for O-H and two spikes amide stretching vibration at approximately 3407 cm^{-1}, and 3310 cm^{-1}, respectively are observed. C-H stretching vibrations are visible at 2928 cm^{-1} and 2840 cm^{-1}, respectively, while a combination of C=O vibrations and amide vibration from both the substrate and the immobilized protein are observed in the wavenumber interval 1730-1500 cm^{-1}. Distinctive signals for phosphate vibration modes appear on the spectra of the samples incubated in the acellular solutions, close to 1034 cm^{-1}.

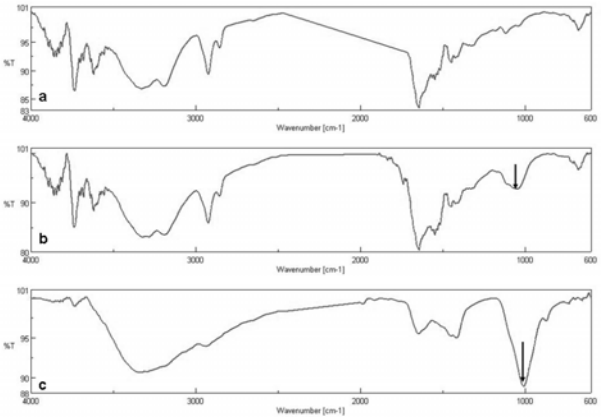

Fig. 1. FT-IR spectra recorded before (a) and after immersion of sample I for 21 days in SBF 1x (b) and for 14 days in SBF 1.5x (c), respectively. Phosphate vibrations - indicated by arrows.

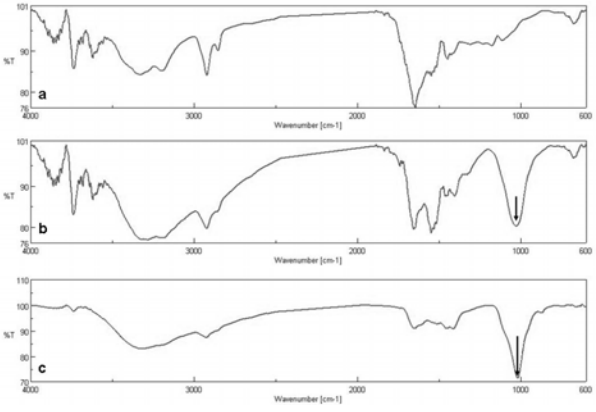

Fig. 2. FT-IR spectra recorded before (a) and after immersion of sample II for 21 days in SBF 1x (b) and for 14 days in SBF 1.5x (c), respectively. Phosphate vibrations - indicated by arrows.

On one hand, following the comparison between the spectra of the two samples, one may say that the formation of phosphates is stronger on the sample with higher Casein content (II). On the other hand, it can be noticed that the incubation in SBF 1.5x was followed by stronger phosphate vibrations when compared to the response obtained after the incubation of the samples for 21 days in SBF 1x. Moreover, the spectra obtained after the incubation in SBF 1.5x indicate the formation of a thick phosphate layer, the signals corresponding to the hybrid becoming less intense when compared to O-H and phosphate vibrations. These are very important findings supporting the initial hypothesis that Casein is an efficient nucleator for calcium phosphates when immersed in SBF-like solutions, in physiological conditions. However, further analysis is needed to prove this behaviour.

SEM analysis. The study continued with the morpho-structural characterisation of the *in vitro* tested samples through SEM. In a first step, it should be mentioned that all the samples maintained their appearance after the SBF incubation (stating for the stability in SBFs). The two materials presented, as expected, phase segregation. The presence of the protein in the studied hybrids leads to complex structures, consisting in two types of domains: granular areas attributed to Casein particles and a continuous hydrogel matrix. Casein granular areas consisting in nanosized globules (Fig. 3) are homogeneously distributed in the bulk of the carrier hydrogel (see Figs. 4). Of course, the richer the sample in Casein, the more Casein clusters.

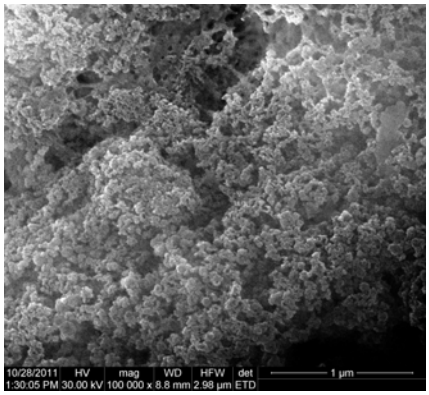

Fig. 3. SEM morphology of the Casein nanosized grains organized in coral-like clusters. Nanometric pores are slso visible.

Then, SEM identified only few isolated nucleation areas on the whole hybrid surfaces after incubation in SBF 1x (see Fig. 4). Both the distribution of the Casein nanostructured areas and the protein amount strongly impact on the formation of calcium phosphate (Ca-P) mineral. Thus, in Fig. 4 one may notice that sample I contains lower amount of smaller Casein clusters when compared to sample II. Nevertheless, few Ca-P nodules are observed; they do not completely coat the materials.

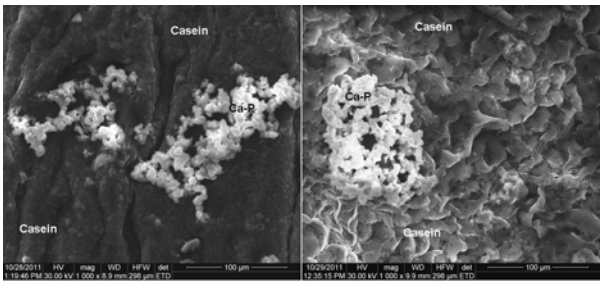

Fig. 4. SEM morphology of the Casein phase segregation in the hybrids. Ca-P mineral is formed onto the surfaces after 21 days incubation in SBF 1x. Left – sample I; right – sample II.

Further, EDAX detected Ca and P with different ratios, corresponding to different areas in the hybrid (Fig. 5). Thus, small amount of Ca and P are detected all over the materials (panel a), with Ca:P > 1 on the Casein rich areas (panel b), and with Ca:P close to 1.6 on the mineral deposits (panel c). This evidence further confirms the formation of HA on the Casein rich areas. The values of Ca:P ratios from the Casein clusters not coated yet with mineral are due to the retention of these ions without the organization of the mineral phase in nodules; this will follow at a later stage.

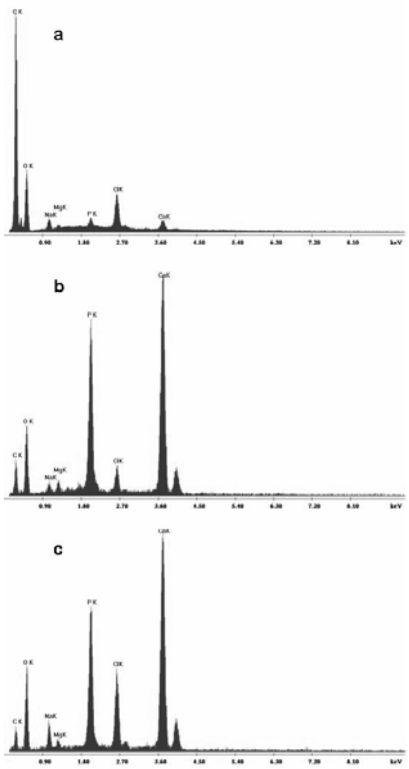

Fig. 5. EDAX spectra indicating different Ca:P ratios corresponding to different analysed areas on the hybrid I incubated in SBF 1x. a – overall spectrum; b - Ca:P > 1 on the Casein rich areas; c – Ca:P close to 1,6 on the mineral nodules.

More detailed observation confirmed the formation of calcification nuclei on the Casein granular clusters (Fig. 6). Again, it was observed that sample II leads to more nucleation areas when compared to the sample with lower amount of Casein.

The study further continued with the investigation of the *in vitro* calcification of the samples immersed in SBF 1.5 x for only 14 days. The increased ionic composition of this fluid results in faster results with respect to the estimation of the calcification potential of the Casein-containing samples. On the other hand, without being coated by a continuous layer of calcium phosphate, all the samples containing Casein presented significantly more important calcification after the SBF 1.5x - incubation with respect to the incubation in SBF 1x. Thus, the two Casein-containing samples presented after the treatment with SBF 1.5x morphologies consisting in granular constructs, with certain porosity induced by the Casein and HA agglomerates organized in ribbon-like structures (see the Fig. 7). This statement is also based on SEM investigation since it was not possible to observe the calcification details by eye. Remarkably, it is also in agreement with the previously presented Von Kossa and FT-IR results.

The mineral phase identified by SEM consisted in globular mineral structures with maximum dimensions of the constitutive features of around 30-40 nm (Fig. 7). These structures are formed preferentially on the surface of Casein agglomerates.

It was noticed again that the dimension and the density of the mineral nodules is increasing with increasing the amount of Casein in the hybrids.

EDAX indicated again Ca:P ratios close to 1,59-1,64 on the mineral phase. This information and the observed morphology correspond to HA.

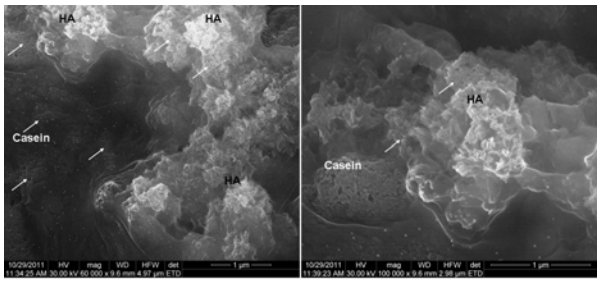

Fig. 6. SEM images of the HA nucleation on Casein rich granular domains (after incubation of sample I in SBF 1x).

Based on these evidences it can be affirmed that the studied Casein-containing samples calcify when immersed in both SBF 1x and SBF 1.5x.

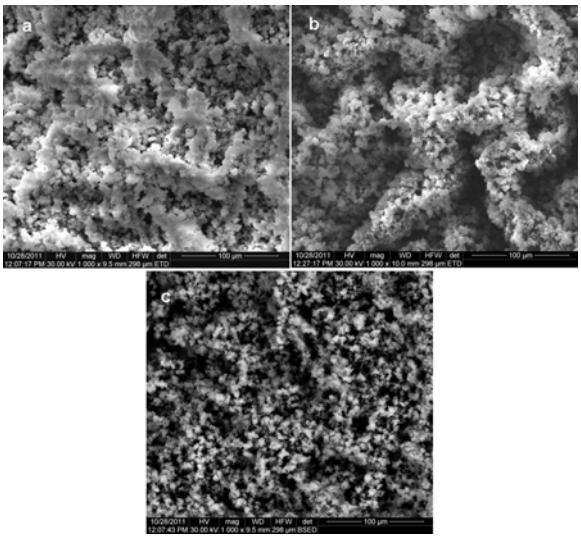

Fig. 7. SEM images of HA-coated Casein formed after the treatment with SBF 1.5x – ribbon-like constructs are formed, with intrinsic porosity. a – sample I; b – thicker ribbons on sample II; c – BSED image corresponding to the image (a) and showing the homogeneous distribution of Ca-P phase.

However, we did not investigate here the calcification kinetics. The mineral structures were homogeneously spread onto the surface of the materials and could further stimulate the adherence of osteoblast-like cells, essential for osseointegration of bone grafting materials. These findings are very interesting since the influence of phosphorous-containing organic scaffolds on the mineralization occurrence is under debate. Despite extensive studies, the mechanism governing the biomineralization control by phosphorylated glycoproteins is not controlled, nor completely understood. However, it is generally recognized that the presence of carboxylic groups and multiple phosphorylation sites plays an important role [17]. In this context, we considered that the main idea resulting from our research is that the presence of Casein in the studied hybrids can be associated with the induction of HA formation.

4. Conclusions

This work reports one of the first attempts to investigate the *in vitro* calcification potential of Casein nanogranules when the protein is embedded in a synthetic polymer (PAA-MBA). It was clearly indicated by Von Kossa staining, FT-IR and SEM that Casein clusters induce the formation of HA. The calcification was enhanced by increased Casein concentration and, nevertheless, by the use of SBF 1.5x. The incubation in SBF 1x allowed to demonstrate that HA nucleation occurs on the Casein rich areas and then it covers the hybrid. The results justify further studies on Casein potential as *in vitro* calcification nucleator.

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