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CASEIN – PHEMA: *IN VITRO* FORMATION OF NANOMETRic Ca-P NUCLEI

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This work reports the synthesis of polymeric hybrids based on Casein and 2-hydroxyethyl methacrylate polymers (PHEMA). Protein physical immobilization during the free-radical polymerization was performed. Two strategies were used to generate both films and cylinders. The homogeneity and the stability of the hybrids were assessed as well as their capacity to perform *in vitro* calcification when incubated in synthetic human plasma (SBF) for 14 days. Nucleation islets of calcium phosphate mineral in the form of nanometric nodules (20-30 nm) were observed on the Cas-rich domains. This study reports for the first time the synthesis of Cas-PHEMA materials and it tried to determine if these hybrids could cause a calcification under *in vitro* conditions.

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Keywords: PHEMA, Casein, hybrid, calcification

1. Introduction

Casein (Cas) represents the main protein of milk and its potential in delivering micronutrients has recently regained attention [1-3]. Cas is one of the most extensively studied proteins from the point of view of its structure and functions. Livney [1] has emphasized the fact that this phosphoprotein has a recognized role in providing bioactive nutrients such as calcium and phosphate [1-3]. The affinity of Cas phosphopeptides (CPP) towards calcium and amorphous calcium phosphate (ACP) is well recognized [1,4,5]. CPP are bioactive peptides that can be obtained from by the enzymatic hydrolysis of Cas. The ability of Cas to bind metallic ions (including calcium) is associated to the phosphor-seryl moieties [6-8] flanked by glutamate residues [3,6,8]. The anticariogenic properties of CPP-ACP nano-complexes are reported in several studies [9-13]. In a different work, Wong and Sissons studied the influence of Cas as macronutrient on the calcium phosphate deposition and growth in plaque mineralisation [14]. To our knowledge, despite the well recognised relationship between dietary proteins like Cas and bone quality/density, there are no studies investigating the spontaneous mineralisation ability of Cas when immobilised in polymer carriers. In this context, the present work tried to determine if apatite nucleation occurs *in vitro* on Cas-polymer organic hybrids when poly(2-hydroxyethyl methacrylate) (PHEMA) was selected as a biocompatible carrier.

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2. Materials and Methods

Materials

a) for the synthesis of the hybrids:

2-hydroxyethyl methacrylate (HEMA) and tetraethyleneglycol dimethacrylate (TEGDMA) were used as such, one as synthetic monomer and the other one as cross-linking agent, respectively. PHEMA with a molecular weight of cca. 20 000 grams/moles was used as polymeric component of the final hybrid materials. Cas from bovine milk was dissolved in 1M sodium hydroxide (NaOH), according to its technical datasheet; this solution is further named Cas0 (protein concentration is 2% w/v). Ammonium persulphate (APS) was used as polymerization initiator. Finally, sodium azide (NaN_3) was used as preservative agent for the Cas0 solution. All the reagents were purchased from Sigma-Aldrich; TEGDMA was from Fluka. 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 fluid (SBF) was 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

Synthesis of Cas-PHEMA thin films

Cas-PHEMA hybrids (further identified as samples A'-C' and A-C) were synthesized through the direct physical immobilization of the protein during the polymerization of HEMA. Different protein loadings were used as shown in Table 1. PHEMA (25 mg) was dissolved in the monomer only for samples A-C. Briefly, a basic free-radical polymerization of HEMA was performed in the presence of Cas. The mixture monomer - cross-linking agent was first prepared using 3% TEGDMA molar ration with respect to HEMA. 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 monomer-polymer mixture. NaN_3 (0.1 % wt with respect to Cas) was added to Cas0 solution to prevent bacterial growth. The mixture was vigorously stirred on a vortex at 1100 rpm and then degassed for 15 minutes (using an ultrasound bath ELMA S 30 H (Elmasonic)) prior to injection between vertically situated, silanized glass plates. Films of 1 mm thick were obtained using silicon spacers and the polymerization was performed in a water bath at 60^0C, followed by 2 hours post-polymerization at 80^0C. Cylinders of 10 mm diameter were cut from the obtained films and immersed in ddw for extraction of unreacted reagents.

Cas-PHEMA blocks

The reaction mixture was prepared starting with the dissolution of PHEMA (25 mg) in HEMA-TEGDMA. Then, the same above-mentioned procedure was applied to incorporate Cas0. The reaction mixture was then vigorously stirred on a vortex at 1100 rpm and then poured into 2 mL polymerization vials. The solutions were degassed for 15 minutes prior to polymerization. The reaction occurred 6 hours at 60^0C, followed by 2 hours post-polymerization at 80^0C. Then, polymer cylindrical blocks were removed from the vials and immersed in distilled water to extract unreacted reagents. Cas-PHEMA hybrids were further identified as samples A-E (see Table 1).

Control PHEMA scaffolds (further denoted A0-E0) were synthesized following the same protocol like for the Cas-containing hybrids but using MilliQ water to replace Cas0 (see Table 1).

Table 1. Cas loading in the polymerization mixtures (for 0.5 mL HEMA).

Samples	CAS0, ml	Water, ml
A'	1,25	0,25
B'	0,625	0,875
C'	0,375	1,125
A	0,5	-
B	0,45	-
C	0,4	-
D	0,3	-
E	0,2	-
A0	-	0,5
B0	-	0,45
C0	-	0,4
D0	-	0,3
E0	-	0,2

Physico-chemical characterization

The success of Cas immobilization in the polymer hybrids was performed 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, eventual 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 Cas-PHEMA scaffolds (A-E) to induce HA formation was explored through the incubation of the hybrid cylinders in SBF solution, miming the ionic composition of the human plasma. The preparation of the SBF was performed as recommended by Kokubo for the revised-SBF formulation [15].

The polymer samples were immersed 48 hours in ddw to reach their maximum swelling degree prior to incubation in SBF. Thereafter they were introduced in 50 ml of freshly prepared SBF, at 36.5°C, for 2 weeks. The SBF was changed every three days. After two weeks, the test was stopped and 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^{o}C to constant mass. The success of the calcification was explored through SEM and FT-IR. 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

Given the fact that this work aimed the synthesis of Cas-PHEMA hybrids in the context of investigating the potential of the phosphoprotein to induce calcification when immersed in SBF, the study followed three main steps. First, the possibility to physically immobilise Cas during the polymerisation was assessed. In a first step, the compatibility of the two components of the hybrids was preliminary verified through film synthesis. Cas is water insoluble while HEMA is a water

soluble monomer and the corresponding homopolymer is hydrophilic. This in why phase segregation could appear when purifying the hybrids and thus the homogeneity of the thin films or its lack would offer an overview on the initial compatibility of the two organic components. Then, based on these findings, blocks with improved homogeneity and stability in ddw and SBF were synthesized. The third step consisted in the evaluation of the capacity of the hybrids to induce calcification *in vitro*, under physiological conditions.

a. Cas-PHEMA films

The first three compositions (A'-C') films presented a two-sides appearance; a visual evaluation allowed noticing that one side was glassy and the other one had a compact aspect.

To obtain more information on the homogeneity of these materials, ATR FT-IR spectra of the two sides of each sample were recorded and compared with PHEMA and Cas controls. PHEMA displays the typical signals for O-H stretching vibration at approximately 3407 cm^{-1}, the C-H stretching vibrations at 2928 cm^{-1} and 2840 cm^{-1}, respectively, and the C=O vibration at 1716 cm^{-1}. The spectrum of Cas 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. Distinctive signals for amide I (1648 cm^{-1}) and amide II (1530 cm^{-1}) are present in the spectra corresponding to the compact surface of the films; these signals are the main characteristic vibrations of proteins. Also, the shifting of the peak at approximately 3407 cm^{-1} in PHEMA to 3314 cm^{-1} in the compact side states for the presence of N-H vibrations from Cas. On the other hand, the spectra corresponding to the glassy side presented all the characteristic peaks of PHEMA: the O-H- extended peak, the C-H stretching peaks and the C=O specific peak at 1716 cm^{-1}; the amide I and II signals are not detectable and just a little left shifting of the O-H vibration to 3393 cm^{-1} could be assigned to a modest contribution of N-H from Cas. Figure 1 is representative with this respect.

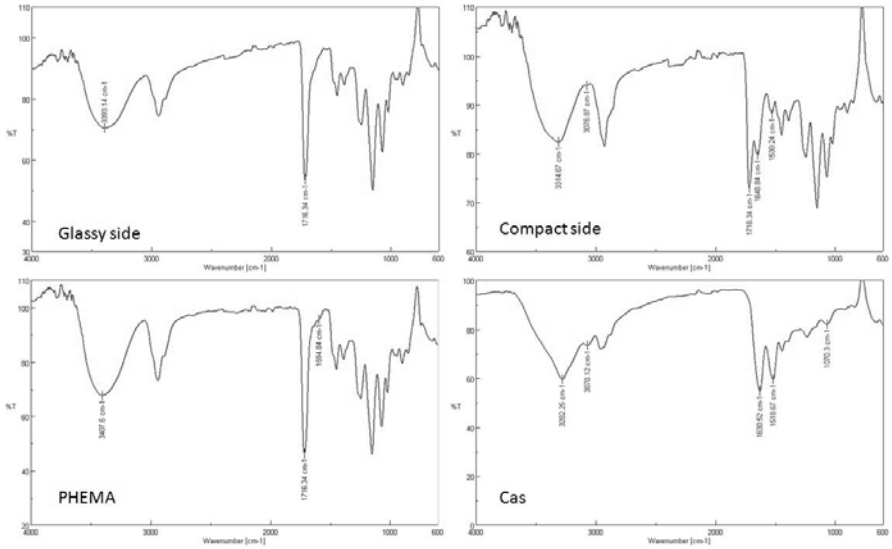

Fig. 1. FT-IR spectra recorded on the two sides of sample C'; PHEMA and Cas were used as control. The glassy side displayed the typical vibrations for PHEMA: the vibration at 3393 cm^{-1} is assigned to O-H stretching and it appears shifted to the rio-ght when compared to control PHEMA (3407 cm^{-1}). The compact side of the sample displayed a stronger right shifting (3314 cm^{-1}) of the O-H vibration probably due to its coupling with N-H vibrations from Cas. Moreover, amide I (1648 cm^{-1}) and amide II (1530 cm^{-1}) signals are characteristics for Cas.

These results indicated that the procedure used to obtain the Cas-PHEMA films initially lead to phase separation between the homopolymer and a hybrid protein-polymer layer, probably due to the water content used in the initial mixture (see Table 1). Based on this decisive evidence films prepared without adding supplementary water were prepared (A-C). Their homogeneity was

convenient and FT-IR spectra on the two sides of the materials proved the existence of Cas-PHEMA hybrid combining the spectral features of both components. These compositions were further selected for preparing hybrid blocks.

b. Cas-PHEMA blocks

The visual inspection of the obtained materials was performed first. Solid hybrids with different appearance depending on the Cas and water content, respectively, were obtained. Higher Cas loading corresponded to more macroscopically visible two phases as displayed in Figure 2. More glassy, yellowish and more homogeneous hybrids are obtained when decreasing the Cas content. On the other hand, PHEMA control samples are homogeneous; their appearance also depends on the water amount used during the synthesis. The samples proved stability in ddw.

FT-IR spectra successfully confirmed the homogeneous presence of Cas in all A-E blocks. Amide I and II were considered the main evidence for the presence of the protein. The second evidence was the right shift of the vibration at 3407 cm^{-1} in PHEMA due to the N-H vibration from the protein aminoacids.

The morphology of the hybrids was further studied through SEM. Samples A0-E0 were used as control. The homopolymers were all homogeneous. Moderate roughness with different surface features was due to the polymerization mould and to the removal of the water used during the polymerization.

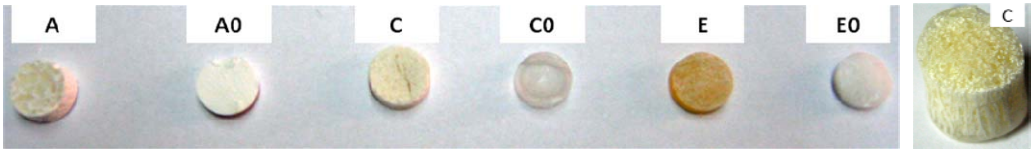

Fig.2. Cross-section of the cylindrical block samples A, C and E and of their corresponding control samples.

Very interesting, the presence of the protein in the studied hybrids leads to complex structures, consisting in two types of domains: granular areas attributed to Cas particles and a continuous PHEMA matrix. Figure 3 is representative with this respect. It is visible that the amount of Cas influences the composition and the properties of the materials. Thus, the richer Cas-containing sample, A, presents a morphology consisting in a granular construct, with a certain porosity induced by the Cas agglomerates separated by PHEMA ribbon-like structures (see the figures 3 and 4). Cas smallest particle-like features have dimensions of about 1-2 μm but clusters with higher dimension are also formed through agglomeration. It can be concluded that variable ratio between Cas and PHEMA allows to modify the structure of the material. The samples with the lowest Cas content in this study, samples D and E, presents a more homogeneous aspect consisting in nests of PHEMA embedding the Cas particles (panels c and d in Figure 3 and panel a in Figure 5). The distribution of PHEMA changes from rare ribbon-like bridges between Cas agglomerates to more circular “nests” filled with Cas. Nevertheless, in some domains PHEMA seems to form a coating layer on the protein particles (see panel c in Figures 4 and panel c and Figure 5).

Another important parameter, the porosity of the materials, is also influenced by the amount of protein used. There are two reasons for the modest porosity observed: 1) the water removed during the freeze-drying and 2) the fact that Cas agglomerates. The porosity was not quantitatively assessed. However, it may be seen that the dimensions of the pores are modest and variable with the ration Cas:polymer.

c. Cas-PHEMA *in vitro* calcification potential

In a first step, it should be mentioned that all the samples maintained their appearance after the SBF incubation (stating for the stability in SBF). Then, SEM identified only few isolated nucleation areas on the whole polymer surface of the PHEMA-control materials, composed by

small nuclei (see Figure 6). Further, EDAX detected phosphorus but did not detect calcium (probably because P comes from two sources, the matrix contains a phosphoprotein while negligible amount of Ca-P nuclei were formed) and Ca. Moreover, FT-IR did not detect the phosphate absorption that could be associated to eventual calcium phosphates formed following the incubation in SBF. These results are easily explained through the limited sensitivity of the employed methods (FT-IR and EDAX) and the very small amount of formed mineral. Based on this evidence it can be affirmed that the studied PHEMA control samples (A0-E0) do not spontaneously calcify when immersed in SBF. It should be also mentioned that the calcification potential of hydrogels is widely studied since, on one hand it is a disturbing phenomenon when aiming soft tissue engineering and, on the other hand, it is a desired process when intended for bone regeneration. The biomimetic mineralization of different polymer constructs was extensively explored as a suitable method for the enhancement of the integration of these materials by the natural hard tissues. Among the most studied, PHEMA–based scaffolds did not lead to unequivocally results when investigated from the point of view of their self-calcification capacity. Depending on the chemistry and on the structure of the scaffold, the mineralization of PHEMA is observed or not [16, 17]. The isolated presence of few mineral nuclei is not a strong evidence for the calcification potential of a matrix. Thus, in this context, our results should be resumed as a lack of calcification for the TEGDMA-cross-linked PHEMA samples obtained using water during the polymerization.

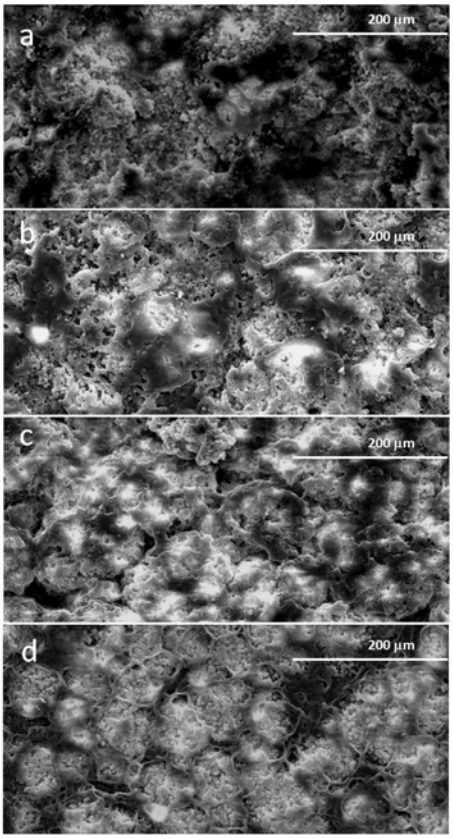

Fig.3. SEM images of the cross-section of Cas-PHEMA hybrids with decreasing Cas content: a - A, b – C, c – D, d-E.

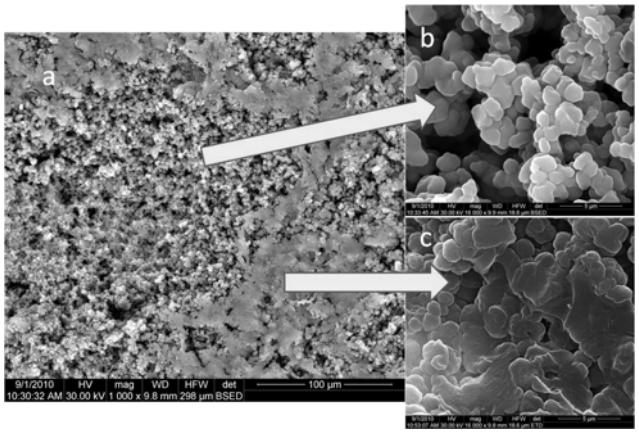

Fig.4. SEM morphology of sample A: a – general view at 1000x; b – detail of the Cas zone at 16000 x; c - detail of the PHEMA-coated Cas grains at 16000 x

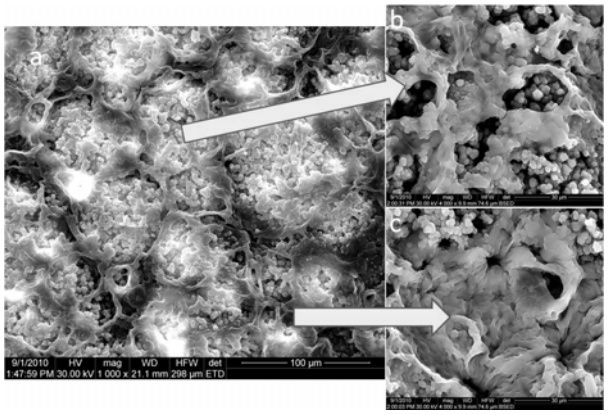

Fig.5. SEM morphology of a cross-section of sample E: a –general view at 1000x; b – detail of the Cas-PHEMA zone at 4000x; detail of a PHEMA richer zone at 4000x; few pores are also visible.

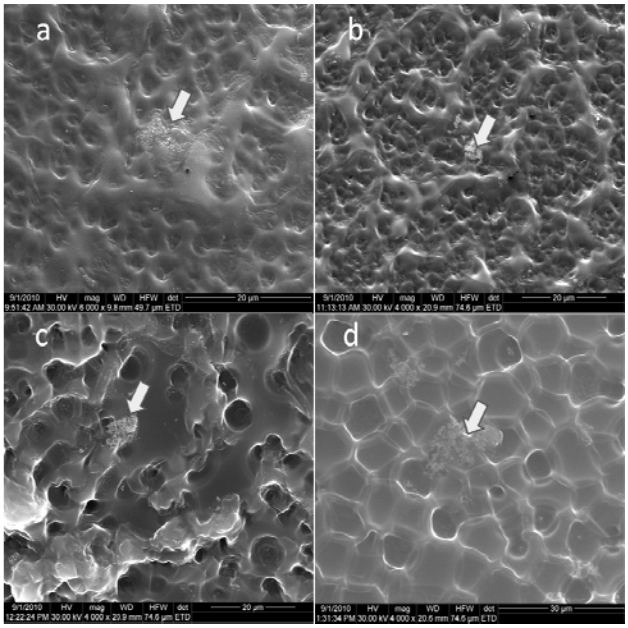

Fig.6. SEM images presenting PHEMA surfaces (a-A0, b-B0, c-C0 and d – E0) after the incubation in SBF. Calcified small areas (indicated by white arrows) are accidentally observed.

On the other hand, without being coated by a continuous layer of calcium phosphate, all the samples containing Cas presented significantly more important calcification after the SBF-incubation with respect to their controls. This statement is based on SEM analysis since it was not possible to observe the calcification by eye. The mineral phase identified by SEM consisted in globular mineral nuclei with maximum dimensions of the constitutive features of around 20-30 nm (Figure 7). With a magnification of 800000 x we could observe nuclei with diameters even < 10 nm. These structures are formed preferentially on the surface of PHEMA-coated Cas particles. The nanometric nodules are uniformous in size and they emerge from the polymer surface and agglomerate in clusters of even 10 μm long. It seems that the dimension and the density of the mineral nodules is increasing with decreasing the ratio Cas:PHEMA (from A to E). Figure 7 is representative with this respect. EDAX spectra successfully identified Ca and P in these mineral structures (Figure 8). However, no further quantitative evaluation was possible through EDAX since phosphorus from Cas is also detected by this determination; this is why the Ca/P ratios are <1. These results confirm the phospho-calcium nature of the mineral structures formed, without establishing their composition. However, when compared with the *in vitro* behaviour of the control samples, it is obvious that the presence of Cas certainly enhanced calcification phenomena when the protein was immobilized in PHEMA matrix.

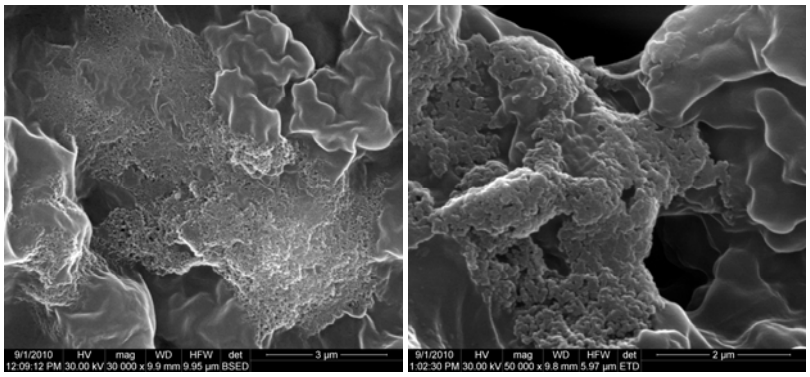

Fig.7. SEM images showing mineral nucleation areas formed on sample C (left) and E (right) after14 days incubation in SBF.

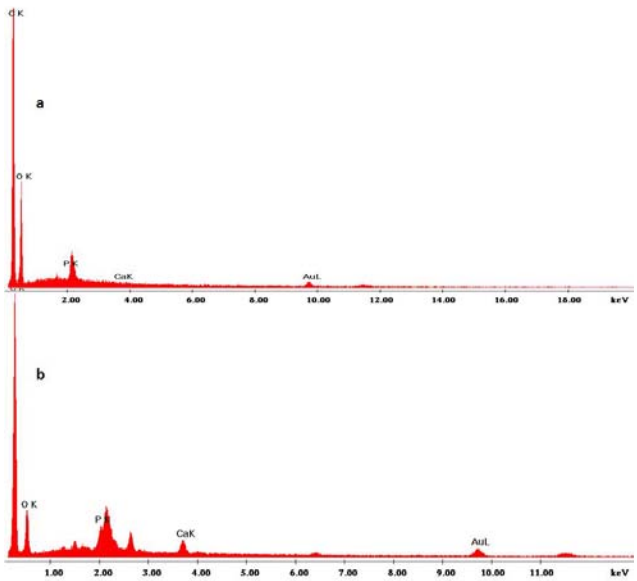

Fig.8. EDAX spectra indicating the presence of P before (a) and after (b) the SBF incubation of sample C. Ca is detected only after the SBF treatment (b) thus proving the successful in vitro calcification.

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 acidic groups and multiple phosphorylation sites plays an important role [18]. This is why the effect of both acidic and phosphate containing polymers on the induction of calcification raised different unequivocal opinions [19-25]. Thus, it seemed interesting to us to compare our results with an older study on the *in vitro* mineralization of PHEMA containing physically embedded alkaline phosphatase [26]. In this situation, the *in vitro* incubation of the organic hybrid in a SBF enriched with β-glycerophosphate lead to the formation of multiple mineral noduli (calcospherites) with dimensions of abour 10 μm. These mineral structures were homogeneously spread onto the surface of the material and further stimulated the adherence of osteoblast-like cells [26]. In this context, we considered that the main idea resulting from our research is that the presence of Cas in the here-studied organic hybrids can be associated with the induction of a calcium-phosphate mineral nucleation. These results are consistent with other recent data of our group reporting HA formation when the mineral nucleates and grows *in vitro*, in the presence of this protein [27].

4. Conclusions

This work represents, to our knowledge, the first attempt to investigate the *in vitro* calcification potential of Cas when the protein was physically immobilized in a synthetic polymer matrix. The Cas-PHEMA hybrids form nanometric Ca-P nuclei after 14 days incubation in SBF under physiological conditions. The nucleation observed justifies further studies on this topic, including the deeper characterisation of the mineral formed and the use of different polymer carriers for Cas.

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