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Influence of Phosphorylated Peptides on the Growth of Hydroxyapatite

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Phosphorylated peptides, having varying degrees of substitution, were soaked in a simulated body fluid (SBF) solution in order to investigate their ability to induce the formation of a calcium phosphate layer. We had dealt with the effect of phosphorylated peptides and peptides onto synthetic hydroxyapatite by separately considering their influence when they were present in the 1.5SBF solutions. Mineralization was assessed by transmission electron microscopy and FT-IR spectromete. It was demonstrated that the phosphate introduced on the chain of peptides could promoted the higher extent of mineralization and exhibited a guiding role in the mineralization of hydroxyapatite. This was suggested that the phosphate moieties in the peptides were important for its function as a mediator of biomineralization.

Key Words: Hydroxyapatite, 1.5SBF solutions, Phosphorylated peptides, Polypeptide.

INTRODUCTION

Natural bone, on a volume basis, is mainly composed of 50 % mineral composition (hydroxyapatite) and extracellular organic matrix (ECM) which is a hydrated mixture of collagen and noncollagenous matrix proteins (NCPS)[1]. The noncollagenous matrix proteins are mostly acidic proteins, rich in glutamic acid, aspartic acid and phosphorylated serine/threonine residues, with a high capacity for binding calcium ions and hydroxyapatite crystal surfaces, moreover, they can join in the regulation of mineral deposition. However, the preparation and application of noncollagenous matrix proteins in the biological material field is also subject to various limitations. Phosphate is one of the functional groups in noncollagenous matrix proteins and it is more meaningful to find these model compounds with such a functional group to simulate the important role and apply to the modification of biological materials[1,2]. Bradt *et al.*[3], have investigated the interaction of polyaspartate and polyglutamate instead of acid proteins with calcium,and shows that the noncollagenous proteins have a strong effect on the mineralization of collagen. In the present work we have examined the role of the phosphate groups on polypeptide in hydroxyapatite interactions and mineral deposition by comparing the behaviour of nonphosphorylated polypeptide with that of highly phosphorylated polypeptide. These data demonstrate that phosphorylated of polypeptide contributes to the binding of calcium ions and the biomineralization of hydroxyapatite.

EXPERIMENTAL

Phospho-polypeptide: Two gram polypeptide dispersed in 100 mL 0.1 M, pH = 7 phosphate buffer solution, $POCl_3$-CCl_4 solution were simultaneously and gradually added into above solution under vigorous stirring for 1-2 h, pH was adjusted 6-8 with 5 M NaOH went on stirring for 2.5 h, then filtering. The supernatant was added dropwise to 80 % $(NH_4)_2SO_4$ saturated solution and proceeded with centrifugal separation. Then washed for several times with $(NH_4)_2SO_4$ saturated solution, next, freeze-drying, finally the relative high-purity sample was obtained and stored in the refrigerator[4].

Effect of phosphorylated peptides and peptides on the growth of hydroxyapatite: 1.5 SBF solution, which had *ca.* 1.5 times higher ionic concentration than human blood plasma as shown in Table-1[5].

TABLE-1
IONIC CONCENTRATIONS OF SBF AND 1.5 SBF IN COMPARISON WITH THOSE OF HUMAN BLOOD PLASMA

Ion	Concentration (mM)		
	Human plasma	SBF	1.5 SBF
Na^+	142.0	142.0	213.0
K^+	5.0	5.0	7.5
Mg^{2+}	1.5	1.5	2.3
Ca^{2+}	2.5	2.5	3.8
Cl^-	103.0	147.8	221.7
HCO^{3-66}	27.0	4.2	6.3
HPO_4^{2-}	1.0	1.0	1.5
SO_4^{2-}	0.5	0.5	0.8
pH	7.2-7.4	7.4	7.4

100 mg phosphorylated peptides and 100 mg peptides were, respectively added to 30 mL of 1.5 SBF solutions and performed at 36 ± 0.5 ºC in constant water bath[6]. Then sampling at different times, washing and sample preparation. Theparticle morphology and aggregation states were analyzed by Jeol JEM-2010 transmission electron microscope (TEM); the samples obtained after a week was measured by Fourier transformed infrared (FT-IR) transmission spectroscopy (Model Nexus 470) in the range of 4000-400 cm^{-1}.

RESULTS AND DISCUSSION

Characterization of phospho-polypeptide: The obtained phospho-polypeptide samples was proceeded to determining of organic phosphorus in molybdenum-antimony antispectrophotometric method after digestion with potassium sulfate, the result was 3.119 mg/g. If the average molecular weight of polypeptide chain was 3000, then each mole of phospho-polypeptide contained 0.301 mol of phosphorus.

The infrared spectra of phospho-polypeptide and polypeptide plotted in Fig. 1 showed the three significant strong peaks in the 1411, 1089 and 614 cm^{-1}, which was stretching vibration of P=O, asymmetric stretching vibration of P-O and symmetric stretching vibration of P-O in the tetrahedral PO_4^{3-}, respectively. While the relatively characteristical adsorption peaks around 1657, 1528 and 1300 cm^{-1} almost disappeared due to the weak adsorption intensity in the phospho-polypeptide.

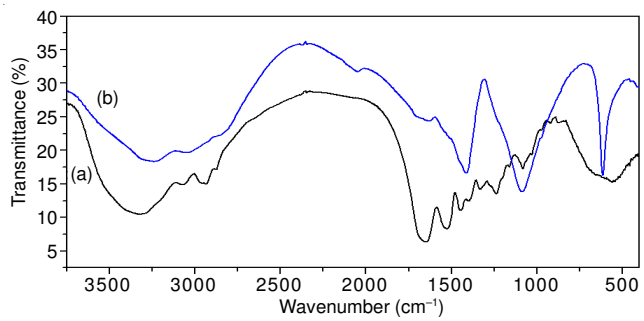


Fig. 1. (a) IR of polypeptide; (b) IR of phosphor-polypeptide

Synthesis of hydroxyapatite in 1.5 SBF solutions: Fig. 2 showed the biomineralization process after immersing phosphorylated peptides in 1.5 SBF solutions for 1 h, 2, 4 and 7 days, respectively, in which representative stages were presented. At first, when the phosphorylated peptides added into 1.5 SBF solutions just after 1 h, the reation system had already been in the incipient precipitation stage and the growth of HAP had a certain of orientation and formed mineral particles of 5 nm in diameter (Fig. 2a). From then on, these tiny mineral particles started to be increasingly formed. However, this trend of orientation was not obvious at the beginning with the increasing of time, but we still could observe this threnđ. Until the sixth day, the accumulation of HAP was severe, so we could not see the growth direction of the HAP from electron micrographs of (d).

Fig. 3 showed the TEM images of HAP with peptides immersion in 1.5 SBF solutions for different time. As can be shown in Fig. 3(a), after 20 h, the reation system started to precipite and the HAP particles (< 5 nm in diameter) had more

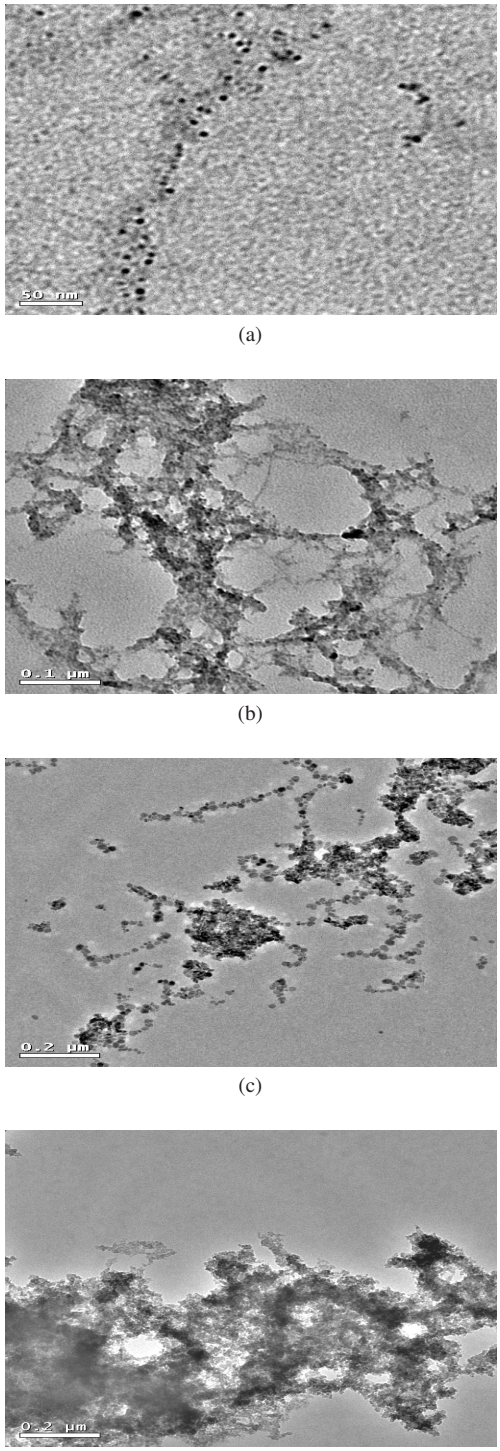
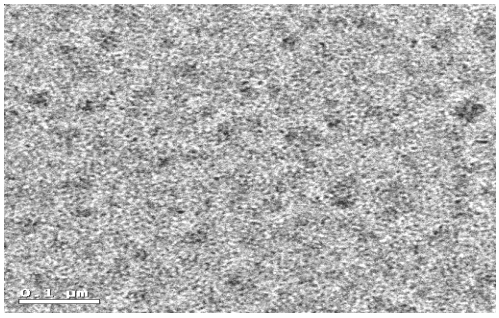


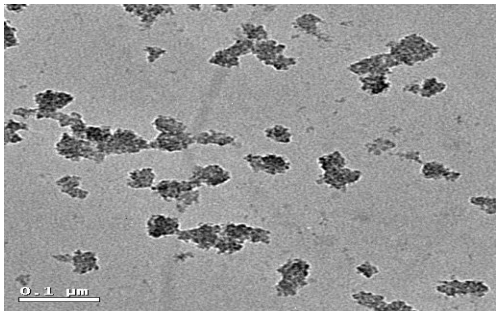
Fig. 2. TEM images of HAP with peptides immersion in 1.5 SBF solutions for different time (a) one hour (b) two days (c) four days (d) six days

evenly distributed, but no orientation occurred, moreover, with little agglomeration. There was more and more serious accumulation as time passes (Fig. 3(b-d).

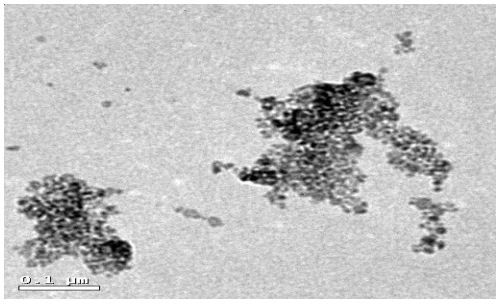
Comparing with the above two sets of experiment, it is concluded that the phosphate introduced on the chain of peptides could exhibit a guiding role in the mineralization of hydroxyapatite, moreover, the growth rate of hydroxyapatite particles was significantly faster with the presence of phosphorylated peptides in reaction system. As for phosphorylated



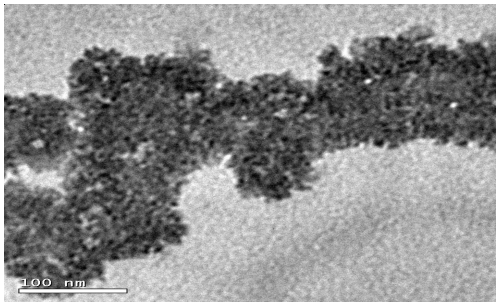
(a)



(b)



(c)



(d)

Fig. 3. TEM images of HAP with the phosphorylated peptides immersion in 1.5 SBF for different time (a) 20 h (b) 2 days (c) 4 days (d) 6 days

peptides, due to its highly phosphorylated post-translational modification, could bind calcium ions with high affinity and at the same time aggregated at the mineralization front. Therefore, the phosphate introduced on the chain of peptides might be essential for dicating the crystal orientation relative to the peptides.

The FTIR spectra of HAP after 4 days immersion showed the typical spectral features in their respective places (Figs. 4 and 5). As shown in Fig. 4b, the typical peaks of phosphate bands in HAP at the position of 1113, 558 and 917 cm^{-1} and

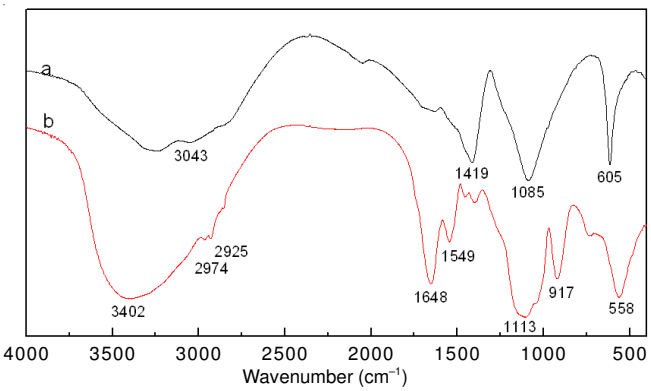


Fig. 4. (a) IR of phospho-polypeptide; (b) IR of HAP in the presence of phospho-polypeptide

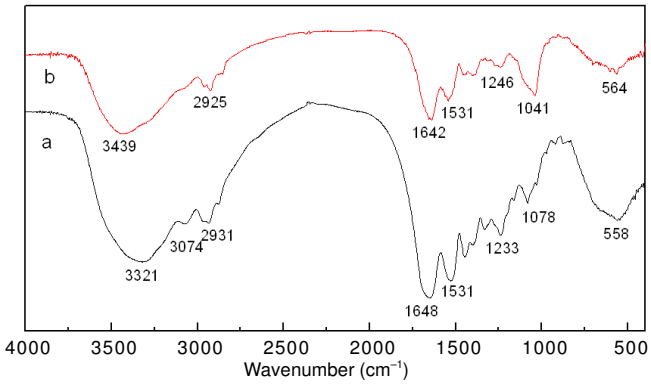


Fig. 5. (a) IR of polypeptide; (b) IR of HAP in the presence of polypeptide

the characteristic peaks of amide bond in phosphorylated peptides was signed to the 1648 and 1549 cm^{-1}. Fig. 4a exhibited FT-IR spectrum of phosphorylated peptides. Compared with the spectrum of Figs. 4 and 5 showed the FT-IR spectra of hydroxyapatite for peptides immersion in 1.5 SBF solutions. The characteristic peaks of amide band in peptides around 1648 and 1531 cm^{-1} occurred the red shift and to some extent were weakened because of the formation of the bonding between peptides and hydroxyapatite.

Conclusion

From the synthesis of hydroxyapatite in 1.5 SBF solutions with the presence of phosphorylated peptides and peptides, we had come to a conclusion that the oriented crystal growth of hydroxyapatite was modulated by the binding of the phosphate introduced on the chain of peptides to particular faces and the growth rate of hydroxyapatite particles was significantly faster in phosphorylated peptides system. Thus, the phosphorylation for polypetide was indeed a key factor in the mineralization of hydroxyapatite.

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