Published in final edited form as:

Mater Sci Eng C Mater Biol Appl. 2012 December 1; 32(8): 2501–2507. doi:10.1016/j.msec.2012.07.032.

Effects of Protein-Simulated Body Fluid Mixing Methods on Characteristics of Bone-Like Mineral

Ho-Jun Song^a, Yeong-Joon Park^a, Won-Jin Moon^b, Linh N. Luong^c, and David H. Kohn^{c,d,*}

^aDepartment of Dental Materials and MRC for Biomineralization Disorders, School of Dentistry, Chonnam National University, Gwangju 500-757, Korea

^bKorea Basic Science Institute, Gwang-Ju Center, 300 Yongbong-dong, Book-gu, Gwangju 500-757, Korea

^cBiomedical Engineering, University of Michigan, Ann Arbor, MI 48109, USA

^dBiologic & Materials Sciences, University of Michigan, Ann Arbor, MI 48109, USA

Abstract

This study examined effects of protein mixing methods into modified simulated body fluid (mSBF) on the crystalline structure and morphology of bone-like mineral (BLM) coated on poly(lactic-co-glycolic acid) PLGA. Using bovine serum albumin (BSA) as a model protein, four sample groups were prepared: the N-BLM group was coated by soaking substrates in mSBF without BSA; the B-BLM group was coated by soaking in mSBF with BSA added immediately before soaking; the Ca-BLM group was coated by soaking in Ca-mSBF prepared by premixing BSA with a CaCl₂ solution before preparing the mSBF; the P-BLM group was coated by soaking in P-mSBF made by premixing the BSA with a KH₂PO₄ solution. The B-BLM and Ca-BLM groups exhibited densely coated, thick BLM layers, whereas the P-BLM group exhibited loosely connected BLM clusters. The hydroxyapatite (HAp) crystallites of the B-BLM and Ca-BLM groups were aligned along the c-axis, but those of the P-BLM group were disordered and had a lower crystallinity. The alignment to the c-axis of Ca-BLM and the disordered orientation of P-BLM was caused by calcium ions bound to BSA in Ca-mSBF and phosphate ions bound to BSA in P-mSBF, respectively. These results show that the crystallinity and morphology of BLM can be controlled by the mixing of BSA in mSBF. The crystallinity of BLM is closely connected to its solubility. Therefore, the release characteristics of growth factors and cell regulation on BLM could be affected by the changes in the crystallinity of BLM.

Keywords

Bone-like mineral; Poly(lactic-co-glycolic acid); Bovine serum albumin; Simulated body fluid; Coprecipitation

© 2012 Elsevier B.V. All rights reserved

*Corresponding author dhkohn@umich.edu Postal address: 1011 N. University Ave., Room 2213, Ann Arbor, MI 48109-1078 Telephone and fax numbers: Ph: 734-764-2206; Fax: 734-647-2110.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Bone like mineral (BLM) coatings on biomaterials, such as titanium alloys and biodegradable polymers, lead to improved bioactivity and osteoconductivity [1,2]. Osteoinductivity can be achieved by delivering biological factors to local sites, by binding the factors to biomaterials and controlling their release [3,4]. Coprecipitation of BLM and growth factors, which is achieved by incubating a substrate in a simulated body fluid (SBF) containing growth factors, is one strategy for incorporating growth factors into BLM [5,6]. When growth factors are incorporated via co-precipitation, their biological activity remains high because the BLM is coated onto the substrate at physiological temperature, and the loading capacity of growth factors in BLM is higher than that achieved via adsorption [5,7]. In addition, the growth factors are incorporated through the thickness of the BLM by the coprecipitation method, and are released slowly, whereas growth factors adsorbed to a surface undergo only a burst release. The different release profiles also result from the fact that during co-precipitation, growth factors are chemically bound to the three-dimensional crystal lattice of the BLM vs. just weakly adsorbed [5].

The release characteristics of growth factors from BLM in-vitro or in-vivo depend on the rate of BLM dissolution and the chemical binding states between the growth factors and BLM. The dissolution rate of BLM is related to its crystalline structure. Therefore, it is important to determine how growth factors affect the crystalline structure of BLM and chemical binding state. Bovine serum albumin (BSA) has been used as a model protein in coprecipitation research. BSA inhibits BLM formation and decreases its crystallinity [5,8,9]. In contrast, Wen et al.[1] reported that BSA coprecipitation increases the crystallinity of BLM and coprecipitated BLM has a dominant HAp crystalline structure with a more preferred orientation to the c-axis compared to BLM without BSA. Therefore, the effect of proteins on the growth and crystal structure of BLM and the specific growth mechanisms are in need of further investigation if general rules for using BLM in drug delivery applications are to be defined. We hypothesize that the chemical interaction between BSA and inorganic ions in SBF affects the crystalline structure of BLM. The chemical interactions between proteins and ions in SBF can be modified by changing the BSA mixing procedure during the process of making SBF. In most studies investigating BSA/apatite coprecipitation, BSA has been simply mixed into the pre-made SBF in [1,5–7,10]. Therefore, this study examined the effects of the BSA mixing procedure on the crystalline structure of BLM coprecipitated with BSA on PLGA substrates.

2. Materials and methods

Poly(lactic-co-glycolic acid) (PLGA) films were prepared using 20 wt% PLGA (85:15 PLA:PGA ratio (Sigma-Aldrich, lot no. MKBG8825V, USA) in a chloroform solution. The PLGA solution was poured over 18×18 mm² square cover glasses, and air-dried for at least 24 h. The films were etched in 0.5M NaOH for 15 minutes followed by ultrasonic rinsing in distilled water. A modified simulated body fluid (mSBF) [5,11], which contains 2× the concentration of Ca²⁺ and HPO₄²⁻ as standard SBF [12], was used to mineralize the films. mSBF contained the following reagents in ultrapure water (Millipore, Milli-Q, USA): 141 mM NaCl, 4.0 mM KCl, 0.5 mM MgSO₄, 1.0 mM MgCl₂, 4.2 mM NaHCO₃, 5.0 mM CaCl₂·2H₂O, and 2.0 mM KH₂PO₄. mSBF was titrated to pH 6.8 using NaOH.

The BLM coating was deposited onto PLGA films by soaking the films in mSBF. The non-coprecipitated bone-like mineral control group (N-BLM) was prepared using mSBF without BSA. To fabricate the BSA-coprecipitated samples (B-BLM), BSA (BioShop, lot no. 9D11406, Canada) was added to mSBF immediately before soaking the specimens, which is designated B-mSBF. An additional two groups were prepared to investigate the effect of the

BSA mixing methods with mSBF on the characteristics of BLM. BSA was premixed with a CaCl_2 solution before preparing mSBF, and then kept at 37°C for 24 hrs. This solution was used instead of CaCl_2 while preparing the mSBF. The Ca-BLM group was fabricated using this variant of mSBF, which is designated Ca-mSBF. The P-BLM group was fabricated using a P-mSBF prepared by premixing BSA with a KH_2PO_4 solution and applying similar procedures to that used for Ca-mSBF. The pH of the BSA-containing CaCl_2 and KH_2PO_4 solutions were 6.0 and 5.6, respectively. The BSA concentration in mSBF was $200\text{ }\mu\text{g/ml}$ for all groups. All specimens were soaked in 15 ml of mSBF for 6 days at 37°C . The solutions were exchanged daily to replenish the ion concentration to the supersaturated levels. For the B-BLM group, new BSA was added to mSBF immediately before soaking the specimens. Four specimens from each group used for each measurement.

Cross-sectional images of the sample groups were observed by field emission scanning electron microscopy (FE-SEM; Hitachi S-4700, Japan). BSA incorporation into BLM was examined by FT-IR spectroscopy (Spectrum 400, PerkinElmer, UK) with an Attenuated Total Reflectance (ATR) accessory. The crystalline structure was analyzed by X-ray diffraction (XRD; PANalytical, X'Pert PRO, Netherlands) with $\text{Cu K}\alpha$ radiation (30mA, 40kV) at a scan speed of $0.067^\circ/\text{s}$ from 10° to 80° . The intensities and the values of full width half maximum (FWHM) of XRD peaks were analyzed by ANOVA with Tukey post-hoc tests to determine effects of mixing method. The morphology and structure of the crystallites were examined by high resolution transmission electron microscopy (HRTEM; Techni F20, Philips, Netherlands) operating at 200 kV. The TEM specimens were prepared by cutting the BLM films coated on PLGA in cross-sectional direction using a focused ion beam (FIB; FEI Co., Quanta 3D, Netherlands). Therefore, the cross-sectional plane of each BLM film, perpendicular to the BLM growth direction, was analyzed by TEM. The chemical binding states were analyzed by X-ray photoelectron spectroscopy (XPS; Multilab 2000, VG, UK). The XPS spectra were obtained for all groups and BSA powder using monochromatic $\text{Al K}\alpha$ radiation with a take-off angle of 90° . High-resolution narrow scanning was performed for the C 1s, N 1s, Ca 2p and P 2p peaks.

3. Results and discussion

BLM layers were coated onto the PLGA substrates, and BLM clusters were grown on the initial BLM layers (Fig. 1). Large-sized BLM clusters aggregated with $10\text{--}20\text{ }\mu\text{m}$ sized particles were observed on BLM layers of approximately $3\text{ }\mu\text{m}$ thickness for the N-BLM group. Thicker BLM layers of $5\text{--}6\text{ }\mu\text{m}$ thickness were grown under the B-mSBF and Ca-mSBF conditions, with BLM clusters grown on Ca-BLM, but not B-BLM. The BLM clusters were grown in a disordered manner on a thinner BLM layer of $1\text{ }\mu\text{m}$ thickness under the P-mSBF conditions. These results indicate that the growth mechanisms of BLM depend on the conditions under which proteins are mixed into mSBF, resulting in differences in thickness and density of the mineral. Specifically, premixing BSA with phosphate ions results in a thinner, less organized mineral compared to premixing with calcium ions or no premixing. In magnified SEM images (Fig. 1, inset), the edges of the BLM crystals in the BSA-coprecipitated groups were more rounded compared with that of N-BLM group, which agrees with other reports [1,5–6].

Phosphate, carbonate and amide peaks were observed via FT-IR spectroscopy (Fig. 2). The carbonate peaks (874 , 1410 , and 1450 cm^{-1}) showed that BLM had a carbonated apatite crystalline structure. The amide peaks (1536 and 1654 cm^{-1}) originating from BSA were observed on all 3 BSA-coprecipitated BLM groups. The peak intensities of the PO_4^{3-} peaks (561 , 601 , and 1025 cm^{-1}) of the B-BLM and Ca-BLM groups were higher than those of the control mineral and mineral created by premixing with phosphate ions. When ATR is used in FT-IR measurements, the penetration depth of IR into the sample is typically $0.5\text{--}2\text{ }\mu\text{m}$

[13]. Therefore, the density and the flatness of specimen affect the intensity of FT-IR spectra. The high intensity of FT-IR peaks of the B-BLM and Ca-BLM groups suggests that these groups have a higher density of BLM crystallites and less roughness of the BLM layer, as confirmed by SEM (Fig. 1).

The XRD patterns of all groups were assigned to a HAp structure (Fig. 3). The peak denoted as A is a combination of (211), (112), and (300) peaks, due to their wide peak widths. The intensity ratio ($I_{(002)}/I_{(A)}$) and FWHM values of the (002) peaks were calculated (Fig. 4). The mean $I_{(002)}/I_{(A)}$ value of the B-BLM group was 2-fold higher than that of the N-BLM group, and the $I_{(002)}/I_{(A)}$ of Ca-BLM was 3-fold higher and significantly greater than that of N-BLM. These results indicate that the B-BLM and Ca-BLM groups grew with a preferred orientation to the c-axis of the crystallites, and BSA affects the growth direction of BLM. On the other hand, the $I_{(002)}/I_{(A)}$ value of Ca-BLM was significantly greater than that of the P-BLM group ($p < 0.05$), even though both groups were coated using a BSA-incorporated mSBF. The Ca-BLM group was coated with BSA premixed with a CaCl_2 solution, whereas the P-BLM group was coated with BSA premixed with a KH_2PO_4 solution. Some calcium ions were bound to BSA in Ca-mSBF, whereas phosphate ions were bound to BSA in P-mSBF before formation of BLM. The growth mechanism of BLM on PLGA could be changed by these different chemical conditions of Ca-mSBF and P-mSBF. The $I_{(002)}/I_{(A)}$ value of the B-BLM group was closer to that of the Ca-BLM group than that of P-BLM group. Therefore, the B-BLM and Ca-BLM groups were likely coated under similar chemical conditions.

The FWHM values of the XRD (002) peak of the B-BLM and Ca-BLM groups were lower than that of the N-BLM group but the differences were not significant. However, the FWHM of the P-BLM group was significantly higher than that of the B-BLM and Ca-BLM groups ($p < 0.05$). There was no significant difference in the FWHM of peak A in all groups. In general, a narrow XRD peak indicates high crystallinity. Therefore, the crystallinity of the c-axis of the P-BLM group was lower than that of the other groups, whereas that of the B-BLM and Ca-BLM groups did not decrease by coprecipitating BSA with BLM. The XRD patterns therefore suggest that the crystallinity of BLM is affected by the BSA mixing procedure, and this may explain contradictory trends in crystallinity when proteins are coprecipitated with BLM [1,5,8,9].

TEM images of the N-BLM, B-BLM and Ca-BLM groups (Fig. 5) show the cross-sectional structure of the BLM layers grown on PLGA relative to the SEM images (Fig. 1). The TEM image of the P-BLM group shows the cross-sectional structure of the BLM cluster coated on the BLM layer. The nano-wire shaped crystallites were caused by matching the crystalline planes of the sheet-like crystallites composing the BLM with the electron beam. The TEM images of Ca-BLM showed that these crystallites were aligned considerably to the same direction from bottom to top, while those of the N-BLM and P-BLM groups were grown randomly. The TEM-level ultrastructure of B-BLM was more similar to Ca-BLM than to N-BLM or P-BLM. The lattice images of the BLM crystals had HAp crystalline structure (HRTEM in inset of Fig. 5(a) and (c)). These images indicated that the (100) crystalline planes of the HAp structure were aligned to the transverse direction of the BLM crystal. These lattice planes were observed more clearly in the HRTEM image of Ca-BLM. The c-axis of the HAp structure, a hexagonal structure, is perpendicular to the a-axis ((100) crystalline plane). The XRD patterns of the Ca-BLM (Fig. 3) showed a high intensity (002) peak, which indicates that the BLMs were grown along the c-axis. Therefore, the alignment of crystallites shown in the TEM image of Ca-BLM (Fig. 5(b)) was due to the preferential growth of BLM to the c-axis.

Fig. 6 shows high resolution XPS spectra of C 1s (a), N 1s (b), Ca 2p (c) and P 2p (d). The intensity of N 1s for N-BLM was approximately zero compared with those of coprecipitated groups because N-BLM does not contain BSA. The shape of C 1s for N-BLM was different with those of coprecipitated groups, whereas the shape of C 1s for the coprecipitated groups was similar to that of BSA. Therefore, it is concluded that C 1s peaks of N-BLM originated from carbonate or another C-O functional group in mineral and C 1s peaks of BSA-coprecipitated groups originated from BSA. For XPS spectra of Ca and P, the peak intensities of the BSA-coprecipitated groups decreased and their peak energies shifted to a lower energy than that of the N-BLM group. The binding energy shift of the Ca 2p and P 2p spectra indicates that Ca or P ions are covalently or ionically bonded with a functional group of BSA in the coprecipitated specimens [14]. The XPS peak energies were similar for the Ca-BLM and P-BLM groups, even though their crystalline structures were different, as shown via XRD and TEM. The chemical conditions between BSA and inorganic ions in mSBF differed according to the BSA mixing procedure. On the other hand, XPS results indicated that the chemical binding states between BSA and BLM in the Ca-BLM and P-BLM groups were similar. Therefore, the BSA mixing procedure only affects the growth mechanism of the BLM crystallites and both calcium and phosphate ions were chemically bound with BSA in the resulting BLM.

Based on differences in BLM crystalline structure with BSA mixing procedures, growth mechanisms are inferred. When Ca-mSBF and P-mSBF were prepared, the same concentration of BSA was pre-mixed with different amounts of CaCl_2 (5 mM) and KH_2PO_4 (2 mM). Therefore, it is possible that there are different amounts of bound vs. free BSA between Ca-mSBF and P-mSBF. These different concentrations of free BSA could affect the crystalline structure of BLM. However, it can be inferred that the amount of free BSA is highest in B-mSBF which was prepared before BSA mixing. On the other hand, the amount of free BSA in P-mSBF may be higher than in Ca-mSBF because the concentration of KH_2PO_4 was higher than that of CaCl_2 . Thus, if free BSA plays a role in controlling BLM crystalline structure, then P-BLM would be more similar to B-BLM than Ca-BLM. However, P-BLM was different from B-BLM. The denaturation of BSA in CaCl_2 and KH_2PO_4 solutions could be a cause of the difference in BLM crystallinity. However, BSA-containing CaCl_2 and KH_2PO_4 solutions used in this study had pH 6.0 and 5.6, respectively. Brahma et al. [15] reported that BSAs are highly aggregated and irreversibly form a partially unfolded dimeric intermediate at a pH of 4.2, which is more acidic compared with the pH of BSA-containing CaCl_2 and KH_2PO_4 solutions. Therefore, it can be concluded that the chemical binding state between BSA and inorganic ions in mSBF plays the most important role in controlling BLM formation.

The following mechanism is proposed to explain the effect of protein mixing on the crystalline characteristics of BLM (Fig. 7). In HAp crystals of a hexagonal structure, calcium ions are rich on the *ac* (or *bc*) face (C-sites), parallel to *c*-axis, while phosphate ions or hydroxyl groups are rich on the *ab* face (P-sites), perpendicular to the *c*-axis [16]. This indicates that Ca ions bind to C-sites more readily, while phosphate ions bind to P-sites when HAp crystallites are grown from SBF. In Ca-mSBF, calcium ions combine with carboxyl groups (COO^-) of BSA. These calcium ions binding with BSA will be precipitated to form BLM and can be bound to C sites more easily than P-sites. Therefore, BSA will be accumulated at C-sites and restrict the growth of *ac* (or *bc*) faces of HAp. It is inferred that this growth scenario controls the orientation of HAp crystallites and leads to alignment of BLM layers to the *c*-axis in the Ca-BLM group. However, phosphate ions combine with NH_3^+ groups in BSA when premixed within the P-mSBF group, and bind to P-sites more easily than C-sites. Therefore, a rich BSA content on *ab* face may hinder the growth of BLM to the *c*-axis. The low crystallinity of the *c*-axis and the disordered orientation of P-BLM group, as shown via XRD and TEM, are attributed to this growth mechanism. When BSA

was mixed with pre-made mSBF, BSA or its complex could bind to calcium ions in mSBF because BSA, having an isoelectric point (pI) of 4.7, is negatively charged under physiological conditions. Several studies have suggested that the carboxyl groups of BSA or collagen combine with Ca^{2+} through electrostatic interactions [10,17,18]. Therefore, the growth mechanism of B-BLM is similar to that of Ca-BLM.

The solubility of BLM coatings plays an important role in regulating the delivery of biological factors coprecipitated into BLM, because growth factors are chemically bound to the three-dimensional crystal lattice of the BLM [5]. This study showed that the crystallinity of BLM can be controlled by changing the mixing method of BSA into mSBF. The solubility of carbonated apatite increases with increasing broadening of the (002) XRD peak, that is a decrease in crystallinity [19,20]. Therefore, P-BLM coated using P-mSBF is likely more soluble than B-BLM and Ca-BLM because P-BLM has a lower crystallinity, and BSA coprecipitated via this method is expected to be released more rapidly.

This study showed that a model protein incorporated into bone-like mineral via simple mixing and premixing with calcium ions does not result in a decrease in crystallinity, although some studies have found that BSA decreases the crystallinity of BLM [5,8,9]. The crystallinity of BLM coated using coprecipitation is affected by the chemical binding state of BSA with inorganic ions and its content in mSBF. Also, the cross-sectional SEM images (Fig. 1) did not show that BSA inhibits BLM formation. However, the change in crystallinity of BLM with BSA mixing method could be explained by BSA restricting the growth of BLM in certain orientations. Depending on the concentration of a protein in SBF, it can act as a promoter of mineralization, inhibitor or both [21]. Therefore, even though a BSA concentration of 200 $\mu\text{g/mL}$ did not affect BLM formation in this study, it can be inferred that BSA could act as inhibitor of BLM formation, if a high enough concentration was used for coprecipitation.

The methods of mixing BSA with mSBF could be applied to other, more biologically relevant growth factors because proteins have ionizable chemical groups such as carboxyl and amino groups. Among the growth factors with a similar pI to BSA, are acidic fibroblast growth factor (aFGF) and epidermal growth factor (EGF) which have pIs of 5.6 and 4.6, respectively. However, the effect of each protein used for coprecipitation on the crystallinity and formation rate of BLM will be different because the size, the isoelectric point, and the chemical state of proteins are different.

5. Conclusions

BSA-coprecipitated with bone-like mineral was coated onto PLGA substrates using different methods of mixing BSA with mSBF. The co-precipitated mineral formed by adding BSA directly to mSBF or pre-mixing with CaCl_2 exhibited densely coated, thick BLM layers with crystallites aligned to the c-axis. In contrast, mineral formed by pre-mixing BSA with KH_2PO_4 exhibited loosely connected BLM clusters and had a lower crystallinity. The alignment to the c-axis of Ca-BLM and the disordered orientation of P-BLM were caused by calcium ions bound to BSA in Ca-mSBF and phosphate ions bound to BSA in P-mSBF, respectively. This study showed that the crystallinity and morphology of BLM on PLGA can be controlled by the mixing of BSA in mSBF, and these material characteristics of BLM could affect the controlled delivery of therapeutic molecules from BLM.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0070054, HJS) and NIH DE013380 and DE015411 (DHK).

References

- [1]. Wen HB, de Wijn JR, van Blitterswijk CA, de Groot K K. Incorporation of bovine serum albumin in calcium phosphate coating on titanium. *J Biomed Mater Res.* 1999; 46:245–252. [PubMed: 10380003]
- [2]. Murphy WL, Kohn DH, Mooney DJ. Growth of continuous bonelike mineral within porous poly(lactide-co-glycolide) scaffolds in vitro. *J Biomed Mater Res.* 2000; 50:50–58. [PubMed: 10644963]
- [3]. Murphy WL, Peters MC, Kohn DH, Mooney DJ. Sustained release of vascular endothelial growth factor from mineralized poly(lactide-co-glycolide) scaffolds for tissue engineering. *Biomaterials.* 2000; 21:2521–2527. [PubMed: 11071602]
- [4]. Morandian-Oldak J, Wen HB, Schneider GB, Stanford CM. Tissue engineering strategies for the future generation of dental implants. *Periodontology 2000.* 2006; 41:157–176. [PubMed: 16686932]
- [5]. Luong LN, Hong SI, Patel RJ, Outslay ME, Kohn DH. Spatial control of protein within biomimetically nucleated mineral. *Biomaterials.* 2006; 27:1175–1186. [PubMed: 16137760]
- [6]. Liu Y, Layrolle P, de Bruijn J, van Blitterswijk C, de Groot K. Biomimetic coprecipitation of calcium phosphate and bovine serum albumin on titanium alloy. *J Biomed Mater Res.* 2001; 57:327–335. [PubMed: 11523027]
- [7]. Yu X, Qu H, Knecht DA, Wei M. Incorporation of bovine serum albumin into biomimetic coatings on titanium with high loading efficacy and its release behavior. *J Mater Sci Mater Med.* 2009; 20:287–294. [PubMed: 18763021]
- [8]. Xie J, Riley C, Chittur K. Effect of albumin on brushite transformation to hydroxyapatite. *J Biomed Mater Res.* 2001; 57:357–365. [PubMed: 11523030]
- [9]. Dorozhkin SV, Dorozhkina EI. The influence of bovine serum albumin on the crystallization of calcium phosphates from a revised simulated body fluid. *Colloids Surf A.* 2003; 215:191–199.
- [10]. Wu Y, Yang B, Lu J, Gu Z, Zhang X. The Influence of Bovine Serum Albumin on the Biomimetic Calcium Phosphate Coating of Bioactivated Titanium. *Key Eng Mat.* 2007; 330–332:581–584.
- [11]. Murphy WL, Mooney DJ. Bioinspired Growth of Crystalline Carbonate Apatite on Biodegradable Polymer Substrata. *J Am Chem Soc.* 2002; 124:1910–1917. [PubMed: 11866603]
- [12]. Kokubo T, Hiroaki T. How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials.* 2006; 27:2907–2915. [PubMed: 16448693]
- [13]. Mirabella, FM, Jr.. *Practical Spectroscopy Series v. 15; Internal reflection spectroscopy theory and applications.* Marcel Dekker, Inc.; New York: 1993. p. 17-52.
- [14]. Feng B, Chen J, Zhang X. Interaction of calcium and phosphate in apatite coating on titanium with serum albumin. *Biomaterials.* 2002; 23:2499–2507. [PubMed: 12033597]
- [15]. Brahma A, Mandal C, Bhattacharyya D. Characterization of a dimeric unfolding intermediate of bovine serum albumin under mildly acidic condition. *Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics.* 2005; 1751:159–169.
- [16]. Kandori K, Tsuyama S, Tanaka H, Ishikawa T. Protein adsorption characteristics of calcium hydroxyapatites modified with pyrophosphoric acids. *Colloids Surf B.* 2007; 58:98–104.
- [17]. Rhee SH, Lee JD, Tanaka J. Nucleation of Hydroxyapatite Crystal through Chemical Interaction with Collagen. *J. Am. Ceram. Soc.* 2000; 83:2890–2892.
- [18]. Combes C, Rey C, Freche M. In vitro crystallization of octacalcium phosphate on type I collagen: influence of serum albumin. *J Mater Sci Mater Med.* 1999; 10:153–160. [PubMed: 15348163]
- [19]. Baig AA, Fox JL, Young RA, Wang Z, Hsu J, Higuchi WI, Chhetry A, Zhuang H, Otsuka M. Relationships Among Carbonated Apatite Solubility, Crystallite Size, and Microstrain Parameters. *Calcif Tissue Int.* 1999; 64:437–449. [PubMed: 10203421]
- [20]. Bayraktar D, Tas AC. Chemical Preparation of Carbonated Calcium Hydroxyapatite Powders at 37°C in Urea-containing Synthetic Body Fluids. *J Eur Ceram Soc.* 1999; 19:2573–2579.
- [21]. Combes C, Rey C. Adsorption of proteins and calcium phosphate materials bioactivity. *Biomaterials.* 2002; 23:2817–2823. [PubMed: 12059033]

Highlights

- Bone-like minerals coated on PLGA using different methods of mixing BSA with mSBF.
- Pre-mixing BSA with CaCl_2 provides a thick mineral layer aligned to the c-axis.
- Mineral formed by pre-mixing BSA with KH_2PO_4 has a low crystallinity.
- Chemical binding BSA with inorganic ions in mSBF affects crystallinity of mineral.

Fig. 1. Cross-sectional FE-SEM images of: (a) non-coprecipitated N-BLM group, (b) BSA-coprecipitated B-BLM, (c) Ca-BLM, and (d) P-BLM groups.

Fig. 2. FT-IR spectra of non-coprecipitated N-BLM group and BSA-coprecipitated B-BLM, Ca-BLM, and P-BLM groups measured using the ATR accessory.

Fig. 3. X-ray diffraction patterns of the non-coprecipitated N-BLM group and BSA-coprecipitated B-BLM, Ca-BLM, and P-BLM groups.

Fig.4.

(a) $I_{(002)}/I_{(A)}$ values and (b) FWHMs of (002) peaks calculated from the XRD patterns. * indicates significantly different ($P < 0.05$).

Fig. 5. TEM images of: (a) non-coprecipitated N-BLM, (b) BSA-coprecipitated B-BLM, (c) Ca-BLM and (d) P-BLM groups. HRTEM images of N-BLM and Ca-BLM are shown in the insets.

Fig. 6. High resolution XPS spectra of (a) C 1s, (b) N 1s, (c) Ca 2p, and (d) P 2p for non coprecipitated N-BLM group, and BSA-coprecipitated B-BLM, Ca-BLM, and P-BLM groups. C 1s (a) and N 1s (b) include XPS spectrum of BSA.

Fig. 7. Schematic demonstrating the growth of HAp crystallites in BSA containing Ca-mSBF and P-mSBF. The HAp crystallites in Ca-mSBF are growing preferential to the c-axis, while those in P-mSBF are growing randomly.