Published in final edited form as:

Langmuir. 2010 November 16; 26(22): 16625–16629. doi:10.1021/la101851f.

Electrically Polarized Biphasic Calcium Phosphates: Adsorption and Release of Bovine Serum Albumin

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Abstract

In this study, we applied electrical polarization technique to increase adsorption and control protein release from biphasic calcium phosphate (BCP). Three different biphasic calcium phosphate (BCP) composites, with hydroxyapatite (HAp) and β -tricalcium phosphate (β -TCP), were processed and electrically polarized. Our study showed that stored charge was increased in the composites with the increase in HAp percentage. Adsorption of bovine serum albumin (BSA), as a model protein, on the poled, as well as unpoled surfaces of the composites, was studied. The highest amount of BSA adsorption was obtained on positively poled surfaces of each composite. Adsorption isotherm study suggested a multilayer adsorption of BSA on the BCP composites. The effect of electrical polarization on BSA release kinetics from positively charged BCP surfaces was studied. A gradual increase in percent BSA release from positively charged BCP surfaces with decreasing stored charge was observed. Our study showed that the BCP based composites have the potential to be used as drug or growth factor delivery vehicle.

Introduction

Calcium phosphates (CPs) are widely used bioceramics in bone-tissue engineering due to their excellent bioactivity and compositional similarities to bone.¹⁻³ Among various CPs, hydroxyapatite [HAp, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] and β -tricalcium phosphate [β -TCP, $\text{Ca}_3(\text{PO}_4)_2$] are most commonly used ones. The high degradation rate of β -TCP makes it difficult to match with the new bone growth rate.⁴ Some research has been reported on biphasic calcium phosphates (BCPs), consisting of HAp and β -TCP, in which degradation rate can potentially match the new bone growth rate.⁵⁻⁸ The principal difference in the crystal lattice between HAp and β -TCP is the presence of a hydroxyl group (OH^-) along the columnar channel parallel to c-axis in the hexagonal unit cell of HAp. Electrical polarization can be utilized to induce surface charge in HAp. Due to the presence of this hydroxyl group.⁹⁻¹¹ Surface charge generation in HAp based bioceramics by electrical polarization to induce acceleration of bone growth has received much attention from the researchers, both *in vitro* and *in vivo*, on electrically polarized HAp surfaces.¹²⁻¹⁵ Thus, electrical polarization as a surface modification technique can also be applied to a BCP, having HAp in its composition, to achieve acceleration in bone growth.

Incorporation or adsorption of different drugs, DNA, bone morphogenetic proteins (BMPs) on CP based biomaterials to prevent post-surgical infection or to accelerate bone growth

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Supporting Information **Available:** Experimental details including XRD patterns and TSDC thermograms of the BCP composites. This material is available free of charge via the Internet at <http://pubs.acs.org>.

have received much attention due to the potential use as drug carrier or delivery agent of these materials.¹⁶⁻²⁰ Although CP based biomaterials are osteoconductive, these materials lack intrinsic osteoinductivity.²¹ Thus, adsorption of BMPs on CP is usually applied to enhance the osteoinduction. Retention of protein on CPs for sufficiently long time or controlled release is highly crucial to induce bone growth. Any burst release of the adsorbed drugs or growth factors is a prime concern.^{22, 23} In this study, bovine serum albumin (BSA), as a model protein, was adsorbed on the electrically polarized BCP surfaces. Objective of this study was to understand protein adsorption and release behavior on and from electrically polarized BCPs, respectively to investigate the potential use as drug or bone morphogenic protein (BMP) carrier or delivery agent.

Experimental Section

Sample Preparation

Three different compositions of BCPs containing β -TCP and HAp were prepared. The compositions were β -TCP 75 wt% + HAp 25 wt%, β -TCP 50 wt% + HAp 50 wt% and, β -TCP 25 wt% + HAp 75 wt%. From here on, these composites will be termed as HAp 25, HAp 50, and HAp 75, respectively. The resulting dried powders after wet milling were uniaxially pressed to obtain disk samples [12 mm (ϕ) $\times$ 1 mm (h)] and then sintered at 1200 °C for 2 h.

Polarization and Thermally Stimulated Depolarization Current Measurement

The BCP composites were electrically polarized in air at 400 °C for 1 h under a 5 kV/cm electric field. The samples were first heated at a rate 10 °C/min from room temperature to the polarization temperature (T_p), 400 °C, and soaked for 1 h before applying the 5 kV/cm d.c. electric field (E_p). The electric field was applied for 1 h at T_p . The samples were then cooled to room temperature under the same electric field. Stored static current in the composites as a result of polarization was determined from the thermally stimulated depolarization current (TSDC). TSDC thermograms are provided in supporting information. To calculate the stored electrical charge from the TSDC spectra, following equation was used.¹⁰

$$Q_p = \frac{1}{\beta} \int J(T) dT \quad (1)$$

Where Q_p is the stored charge, β is the heating rate and $J(T)$ is the current density. From here on, unpoled, positively poled, and negatively poled surfaces will be termed as U-poled, P-poled, and N-poled respectively.

BSA Adsorption and Release Study

To investigate the adsorption behavior of BSA protein (Sigma-Aldrich, St. Louis, MO) on the polarized surfaces of the BCP composite, U-poled, P-poled, and N-poled samples from each of the three compositions were kept separately in a 10 ml BSA solution containing 5 mg BSA/ml at a pH 7.4. Adsorption was carried out at room temperature for two different time intervals, 6 h and 12 h. Protein measurement was carried out using Micro BCA™ protein assay kit (Pierce, Rockford, IL).²⁴ P-poled surfaces of the BCP composites obtained after 6 h BSA adsorption were used to study the BSA release behavior since the highest adsorption was observed on the P-poled surfaces compared to U-poled and N-poled surfaces of each composition.

BSA Protein Secondary Structure Analysis by FTIR Spectroscopy

BSA secondary structures were analyzed on the U-poled, P-poled and N-poled surfaces of the BCP composites after 6 h adsorption to investigate the percent denaturation after adsorption on the polarized surfaces. BSA secondary structures were analyzed after release from the P-poled surfaces at different time intervals in the amide I region of the IR spectra. The FTIR spectra were recorded using a Nicolet 860 spectrometer, with an ATR cell having a diamond crystal.

Results and Discussions

XRD analysis (given in supporting information) confirms the presence of only HAp and β -TCP phases, and no change in the β -TCP and HAp ratio in the fabricated composites after sintering compared to starting powder. The characteristics peaks of HAp and β -TCP in the composites match well with JCPDS # 09-0432 (HAp) and 09-0169 (β -TCP). The bulk densities of HAp25, HAp50, and HAp75 composites are $97.02 \pm 1.8\%$, $95.68 \pm 2.5\%$, and $97.86 \pm 2.0\%$ of theoretical density, respectively. After sintering, semi-quantitative phase percentage calculation was carried out from the peak intensity ratio of the corresponding HAp and β -TCP strong peak in the XRD pattern. A 17%, 46%, and 70% HAp were found in HAp25, HAp50, and HAp75, respectively, instead of 25%, 50%, and 75% HAp, respectively. Grain size measurement shows a decreasing trend with the increase in HAp percentage, though the grain size difference among the BCPs is not significant. Grain size of HAp25, HAp50, and HAp75 composites are $1.67 \pm 0.14 \mu\text{m}$, $1.45 \pm 0.09 \mu\text{m}$, and $1.37 \pm 0.17 \mu\text{m}$ respectively. Table 1 presents the stored charge in the BCP composites obtained from TSDC measurement. Current density in the composites gradually increased as the percentage of HAp is increased. Our results suggest polarizability of BCP composites is a function of HAp percentage. The observed trend can be explained on the basis of the hydroxyl group's presence in the crystal lattice of HAp. It is believed that the presence of the hydroxyl group in the crystal lattice of HAp causes the polarization.^{10, 11}

Figure 1 (a) and (b) show the variation in BSA adsorption on unpoled, P-poled, and N-poled surfaces of the composites after 6 h and 12 h, respectively. After 6 h, the highest amount of BSA adsorption is observed on P-poled surfaces, and the lowest on the N-poled surfaces among all the composites. After 12 h, no increase in adsorption on the P-poled surface of HAp75 composite is observed, but significant increase in the adsorption on the P-poled surface of HAp25 and HAp 50 composites is observed. BSA adsorption on the P-poled surfaces of all the composites after 12 h is almost same. Significant increase in adsorption after 12 h on the U-poled and N-poled surfaces of all composites is noticed. Among the P-poled surfaces of the three compositions, BSA adsorption was gradually increased with the increasing stored charge or percent HAp content. At pH 7.4 net charge of BSA is negative as its isoelectric point is 4.7. This explains the highest BSA adsorption on the P-poled surfaces and lowest adsorption on the N-poled surfaces is due to strong electrostatic attractions and repulsions, respectively. Thus, depending on the net charge of a specific protein at a certain pH, P-poled or N-poled surface can be applied to achieve high protein adsorption.

Contact angle values (Table 2) show that surface property change by the application of electrical polarization results in increased wettability of the BCP surfaces. In our earlier works we have shown that contact angle depends on many factors such as chemistry, surface roughness, grain size, and polarity.^{25, 26} For calcium phosphate, contact angle decreases with decreasing grain size.²⁶ Bigger grain size of the composites explains large contact angle values obtained on the unpoled surfaces. Increased wettability is observed with increasing HAp percentage in the BCP composites. No direct relationship between total adsorbed protein and wettability due to compositional variation is seen. Surface charge of

the BCPs and net charge of protein with respect to solution pH are the dominating factors evident from the adsorption behavior of protein on the unpoled and poled surfaces.

Figure 2 shows the adsorption isotherm of BSA on the P-poled surface of the HAp75 composite, where the adsorbed amount of BSA, Q (mg/cm²), is plotted against the BSA concentration in solution at equilibrium, C (mg/ml). Data was analyzed with both Langmuir (2) and Freundlich (3) equations.

$$\frac{C}{Q} = \frac{1}{bQ_0} + \frac{C}{Q_0} \quad (2)$$

$$\log Q = \log K_F + \frac{1}{n} \log C \quad (3)$$

where Q is the adsorption capacity (mg/cm²), C is the concentration of BSA in solution at equilibrium (mg/mL), Q_0 is the saturation adsorption capacity (mg/cm²), b is an empirical parameter, n is the Freundlich constant, and K_F is the binding energy constant. The parameters for Freundlich and Langmuir isotherms obtained from linear fitting of BSA adsorption on the P-poled surface of this composite are compared in Table 3.

Adsorption isotherm study on the positively poled HAp75 shows an initial slope indicating high affinity for BSA, and a plateau value corresponding to the maximum BSA adsorption indicates the adsorption saturation. Both Freundlich and Langmuir models have been utilized to describe the BSA adsorption isotherm on the P-poled surface of HAp75 composite. The Langmuir model is applicable to monolayer adsorption on the homogeneous support surface with equivalent adsorption sites, while the Freundlich model is applicable to multilayer adsorption on the heterogeneous surface with non-identical adsorption sites.²⁷ The regression coefficient, R^2 , obtained from Freundlich model is much closer to one than that of Langmuir model (Table 2), suggesting multilayer BSA adsorption on the P-poled surface of the HAp75 composite and the non-applicability of the Langmuir model to this system.

Figure 3 shows the BSA release up to 48 h from the P-poled surfaces of the composites after 6 h adsorption. No initial burst release of BSA is observed; moreover, initial release (up to 2 h) rate from all P-poled surfaces is almost the same. Initially there is a gradual increase of BSA release from the P-poled surface of all the composites. A plateau value is reached in all cases after the initial period, 12-hour in this case. Figure 3 also shows that protein release is increased with a decrease in HAp percentage in the BCPs. Protein adsorption and release results indicate that compositional designing of BCPs along with the application of electrical polarization can potentially be used to achieve desired protein adsorption and/or release depending on the application.

Electrostatic attraction and repulsion act as the driving force for adsorption of protein on ionic surface.^{28,29} BSA release is increased with the decrease in HAp percentage as well as the decrease in the charge density of the composites. This can be explained in terms of strong electrostatic interactions between the positive entities (H^+ , Ca^{2+}) present at the surface and the negative terminals ($-COO^-$) of the BSA.³⁰ Increased charge density is achieved by increased number of hydroxyl group (OH^-) alignments present in the crystal lattice achieved by electrical polarization. This results in increased number of protons (H^+) at one surface, termed as positive surface, and increased number of oxide ion (O^{2-}) at other

surface, termed as negative surface. In unpoled state the hydroxyl groups (OH^-) of HAp crystal lattices are oriented in a random way.¹¹ Electrical polarization does not cause any change to the orientation of other cations and anions (Ca^{2+} , PO_4^{3-} , HPO_4^{2-}) present at the surface. It becomes more difficult for a protein to be released in the solution breaking strong electrostatic attractive forces with the composite surface. The electrostatic attraction between protein molecules and the surface becomes weaker due to increased distance as the layer number increases. Thus, initial protein release occurs from the distant layers, loosely attached to the surface. The lowest amount of BSA adsorption on the N-poled surfaces of the composites could be due to electrostatic repulsion²⁸ between the oxide ions (O^{2-}) present in the negatively poled surface and the negative terminals ($-\text{COO}^-$) of the BSA.³⁰ Figure 4 shows a simple schematic presentation of the differences in protein adsorption on the U-poled, N-poled and P-poled surfaces of a BCP composite, showing the adsorbed layers as a result electrostatic interactions.

Amide I region is most commonly used for secondary structure analysis of proteins. A broad amide I IR band ($1600\text{--}1700\text{ cm}^{-1}$) is produced by the overlapping absorptions of different secondary-structure components of the protein at different positions. Curve fitting procedure is most commonly used technique to decompose the amide I band into various components of the secondary structure.³¹ Table 4 shows the individual band assignments for the Gaussian curves underneath the IR spectra in the amide I region.^{32–34} Table 5 and 6 present the percent content of α -helix and β -sheet of the secondary structure of BSA in the amide I region for both after 6 h adsorption and release from the P-poled surfaces of the composites. A decrease in the α -helix content is observed after adsorption on all surfaces (U-poled, P-poled, and N-poled) of the BCP composites. After adsorption, 79–89%, 43–48%, and 58–67% α -helix content of BSA is retained on the U-poled, P-poled, and N-poled surfaces of the composites, respectively. This observation is in line with our previous study.³⁵

After BSA adsorption on the surface of each composition, low α -helix percent content on the P-poled surfaces compared to the native BSA in solution could be due to strong electrostatic interaction between BSA and the P-poled surface. This strong electrostatic interaction might have caused the greater distortion in the secondary structure. Percent decrease in the α -helix content on the N-poled surfaces is due to strong repulsive electrostatic interaction between the BSA and the N-poled surfaces. The lowest decrease in α -helix content on the U-poled surfaces is due to the lowest electrostatic interactions between BSA and the U-poled surfaces compared to poled surfaces. No further denaturation in protein conformation is observed after release in PBS. Moreover, some increase in α -helix content is observed immediately after release. The α -helix content After 6 h adsorption on the P-poled surface of HAp75 composite was 19% but the α -helix content found to be 28% in solution after 30 minute release from the same surface (Table 5 & 6). This increase in the α -helix content could be interpreted due to relaxation of strong electrostatic interactions caused by the detachment from the charged surface. No further significant denaturation in BSA is observed up to 48 h release in 7.4 PBS at 37°C.

Our results show that surface charge due to electrical polarization in the BCPs is controlled by HAp percentage. The highest protein adsorption is observed on P-poled surfaces of each composite when net charge of protein is negative at the solution pH. High protein adsorption can also be achieved on the N-poled surface by lowering the solution pH, which can change the net charge of protein from negative to positive. No initial burst release of BSA indicates these compositionally designed BCP charged surfaces could potentially be used for local application of cell adhesive proteins or bone morphogenic proteins (BMPs). Our results also show although protein adsorption is increased with increasing stored charge or HAp content, release is increased with decreasing stored charge or HAp content in the BCP composites.

Conclusions

The unique superiority of these charged BCP surfaces is that no other chemical agents were used to create surface charge. Surface charge, as well as materials composition or chemistry of BCPs, played a significant role on BSA adsorption. P-poled surfaces showed highest BSA adsorption over U-poled and N-poled surfaces. BSA release from the P-poled surfaces was decreased with increasing charge or HAp content in the BCPs. These electrically polarized BCPs could have a potential use for orthopedic and dental application to enhance the local healing. Adsorption and release of protein on these materials can be tailored varying the composition along with the application of electrical polarization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge financial support from National Institute of Health (NIH), NIBIB (Program manager Dr. Albert Lee) under the Grant No. NIH-R01-EB-007351. The authors also thank Dr. Subhadip Bodhak for experimental help, and Joe Edgington for helping to take the XRD patterns.

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(a) BSA adsorption after 6-hour

(b) BSA adsorption after 12-hour

Figure 1.

Adsorption of BSA on the U-poled, P-poled and N-poled surfaces of the BCP composites after 6 h (a), and 12 h (b). Statistical analysis indicates that BSA adsorption on different surfaces are significant (** $P < 0.05$, $n = 5$).

Figure 2.
Adsorption isotherm of BSA on the P-poled surface of HAp75 composite at pH 7.4.

Figure 3.
BSA release from the P-poled surfaces at pH 7.4 and 37°C.

Figure 4.

Schematic presentation of BSA adsorption on P-poled (a), N-poled (b), and U-poled (c) surfaces of a BCP composite. BSA adsorption takes place onto the surface as a result of electrostatic interactions between surface positive charges and BSA molecules. These electrostatic interactions become weaker as the layer number increases. For simplicity, only three layers are shown here.

Table 1
Calculated stored charge and current density in the composites

Composition	Current density	Stored charge
HAp 75	$9.35 \times 10^{-10} \text{ A/cm}^2$	$2.38 \text{ } \mu\text{C/cm}^2$
HAp 50	$7.52 \times 10^{-10} \text{ A/cm}^2$	$1.59 \text{ } \mu\text{C/cm}^2$
HAp 25	$5.72 \times 10^{-10} \text{ A/cm}^2$	$1.11 \text{ } \mu\text{C/cm}^2$

Table 2
Contact angle values (in degree) of deionized water on the unpoled and poled surface of the BCP composites

	HAp25	HAp50	HAp75
U-poled	68.17 $\pm$ 1.84	65.4 $\pm$ 1.34	63.7 $\pm$ 2.1
P-poled	62.45 $\pm$ 1.26	60.35 $\pm$ 1.15	56.8 $\pm$.38
N-poled	57.72 $\pm$ 1.58	55.7 $\pm$ 1.78	53.37 $\pm$ 1.37

Table 3
Langmuir and Freundlich parameters for BSA adsorption on the P-poled surface of HAp75 composite

	<u>Freundlich parameters</u>		<u>Langmuir parameters</u>		
	K_F	$1/n$	R^2	Q_0	b
HAp75	1.20	0.825	0.983	9.95	0.140
					0.895

Table 4
Qualitative band assignments for FTIR spectra of BSA in the amide I region (1600-1700 cm⁻¹)

Band position (cm ⁻¹)	Assignment
1610±4	Side chain
1618±3	Self association
1628±2	β-Sheet
1635±3	β-Sheet
1642±2	Unordered
1651±4	α-Helix
1665±5	Unordered
1680±5	Unordered
1693±2	β-Sheet

Table 5
Secondary structure (%) of BSA in aqueous solution, and on the unpoled and poled surfaces after 6 h adsorption

		α -Helix	β -Sheet
BSA in aqueous solution		42	31
HAp75	U-poled	37	17
	P-poled	19	28
	N-poled	28	17
HAp50	U-poled	35	33
	P-poled	18	24
	N-poled	23	21
HAp25	U-poled	33	24
	P-poled	20	32
	N-poled	24	36

Table 6
Secondary structure of BSA in PBS after release at different duration from the P-poled surfaces

	α -Helix	β -Sheet
After 30 min from the HAp75	28	25
After 12 h from the HAp75	23	16
After 36 h from the HAp75	21	25
After 48 h from the HAp75	19	23
After 48 h from the HAp50	20	18
After 48 h from the HAp25	24	17