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Enhancing Protein Incorporation in Calcium Phosphate Coating on Titanium by Rapid Biomimetic Co-Precipitation Technique

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***Abstract*—**Calcium phosphate coating (CaP) has been employed for protein delivery, but the typical direct protein adsorption on the coating led to low incorporation content and fast release of the protein from the coating. By using bovine serum albumin (BSA) as a model protein, rapid biomimetic co-precipitation between calcium phosphate and BSA was employed to control the distribution of BSA within calcium phosphate coating during biomimetic formation on titanium surface for only 6 h at 50°C in an accelerated calcium phosphate solution. As a result, the amount of BSA incorporation and release duration could be increased by using a rapid biomimetic co-precipitation technique. Up to 43 fold increases in the BSA incorporation content and the increase from 6 h to more than 360 h in release duration compared to typical direct adsorption technique were observed depending on the initial BSA concentration used during co-precipitation (1, 10 and 100 $\mu g \cdot ml^{-1}$). From x-ray diffraction and Fourier transform infrared spectroscopy studies, the coating composition was not altered with the incorporation of BSA by this rapid biomimetic co-precipitation and mainly comprised octacalcium phosphate and hydroxyapatite. However, the microstructure of calcium phosphate crystals changed from straight, plate-like units to curved, plate-like units with increasing BSA content.

***Keywords*—**Biomimetic, Calcium Phosphate Coating, Protein, Titanium.

I. Introduction

BIOMIMETIC approach which mimics the mineralization process of bone by soaking the samples in aqueous solutions containing ionic composition closed to that of human plasma was recently received interest for coating implants to enhance the bioactivity [1]-[3]. The biomimetic coating typically consisted of small crystals resembling the apatite crystal in bone which could be readily degraded by osteoclasts compared to large crystals in high temperature coating such plasma spray which is highly stable in body environment.

Due to its low processing temperature, this biomimetic technique could be employed for bioactive molecules delivery for example protein, growth factor or drug by co-precipitating them with the coating and being incorporated in the crystal lattice [4]-[9]. Unlike incorporating technique by the typical direct adsorption on the preformed coating which bioactive molecules were superficially deposited on its surface, the release rate from the biomimetic coating was lower and the duration was longer [4].

Although having many benefits, this biomimetic coating process tended to be slow and needed long soaking times in order of several days to produce sufficient amount of coating. Recently, a simple biomimetic process comprising pretreating the titanium with sodium hydroxide and followed by soaking in accelerated calcium phosphate solution which contained highly supersaturated calcium and phosphate ions was developed [10]. It was shown to be able to produce bioactive coating comprising octacalcium phosphate (OCP) and hydroxyapatite (HA) as main phases rapidly in few hours which provided a fast and low cost coating process. In this study, the potential of using this rapid biomimetic coating technique to incorporate protein in the coating was; thus, investigated. Coating composition, coating microstructure, protein incorporated content and protein releasing behavior were studied and compared with the coating which incorporated protein by typical direct adsorption technique.

II. Materials and Methods

A. Sample Preparation

Titanium sheet (cpTi gr2, Pengfa Industry Inc.) was cut into 25 mm diameter disc and cleaned by acetone and deionized water using ultrasonic cleaning bath (Crest CP1100D). The disc was alkaline pretreated by soaking in 5 M sodium hydroxide (NaOH) solution at 70°C for 24 h. It was then cleaned by deionized water and left to dry at room temperature.

B. Protein Incorporation in Calcium Phosphate Coating

Bovine Serum Albumin (BSA, Sigma-Aldrich)) was employed as a model protein in this study. Protein was incorporated in the calcium phosphate coating (CaP) by two techniques. Firstly, the typical direct adsorption, designated AD, was done by placing the alkaline pretreated titanium disc in accelerated calcium phosphate solution (Na^+ 154 mM, Cl^- 201.7 mM, Ca^{2+} 3.87 mM and HPO_4^{2-} 2.32 mM; pH 7.3) at 50°C for 6 h to produce calcium phosphate coating on titanium surface [10]. It was then soaked in protein solution having the concentration of 1, 10 and 100 $\mu g \cdot ml^{-1}$ at 50°C for 6 h. Secondly, the pretreated titanium discs were subjected to the biomimetic co-precipitation coating process by submerging in the mixture of protein and accelerated calcium phosphate

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solution using protein concentration of 1, 10 and 100 μg·ml^{-1} at 50°C for 6 h, designated CO.

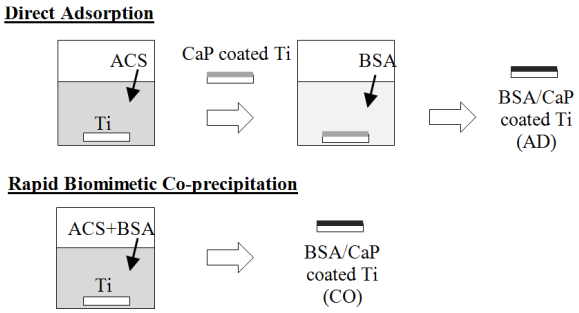

Fig. 1 Process diagram showing protein incorporation in the calcium phosphate coating used in this study

C. Coating Characterization

Phase composition of the coating was evaluated by x-ray diffractometer (Rigaku TTRAX III) in thin film mode using a step angle of 0.02 and speed of 5° min^{-1} in the range of 2-35 °2θ. JCPDS files were used to identify the peaks of main components in the coating. Functional group of the coating was analyzed by FT-IR imaging microscope (Perkin Elmer Spectrum Spotlight 300) using micro attenuated total reflectance (micro-ATR) technique. Microstructure of the samples was studied by using a scanning electron microscopy (JEOL JSM-5410). All the samples were gold sputtered prior to the observation.

D. Total Protein Content

Total protein content in the calcium phosphate coating was measured by placing coated titanium disc in 0.05 M hydrochloric acid for 30 min at room temperature to dissolve the coating. The solutions were then filtered and analyzed for protein content by micro bicinchoninic acid protein assay kit (Pierce, USA) using UV-VIS spectrophotometer (Biotek ELx808) at the wavelength of 570 nm. The content was expressed as weight of protein per weight of coating.

E. Protein Releasing Study

The amount of protein released from the coating was measured by placing coated titanium disc in 2.5 ml of phosphate buffered saline (PBS) for up to 15 d. At each specified period, an aliquot solution of 0.5 ml was collected and new PBS was added to maintain the total volume. The collected solution was analyzed for protein content by micro bicinchoninic acid protein assay kit (Pierce, USA) using UV-VIS spectrophotometer (Biotek ELx808) at the wavelength of 570 nm.

III. Results and Discussion

Fig. 2 shows the XRD patterns of the coatings. Regardless of the initial concentration of BSA solution bath used, both direct adsorption and rapid biomimetic co-precipitation coatings similarly contained peaks at 5° and 26° which belonged to octacalcium phosphate (OCP) and hydroxyapatite (HA) respectively [4], [11]. Peaks at 16° and 31-33° could be assigned for both OCP and HA. No differences in these characteristic diffraction peaks between protein incorporated calcium phosphate coating and pure calcium phosphate coating or between two protein incorporation techniques were observed. Fig. 3 shows FT-IR spectrum of all calcium phosphate coatings. All samples displayed the spectra bands at 1028 and 1102 cm^{-1} (υ_3 PO_4^{3-} bending vibration) and at 1658 and 3403 cm^{-1} (adsorbed water) which are the characteristic peaks for both OCP and HA [4], [11]. However, amide II peak of BSA at 1539 cm^{-1} arising from the combination of C–N stretching and N–H bending vibrations of the protein backbone [4], [5], [12] was detected for both protein incorporated coatings, but not for pure calcium phosphate coating. The intensity of this amide II peak increased with increasing protein content. This result indicated that the protein was successfully incorporated in the coating produced by both techniques. However, it was only clearly seen for AD-10, AD-100, CO-10 and CO-100 samples, but not for AD-1 and CO-1 samples. This was due to the limited amount of BSA in the coating of AD-1 and CO-1 samples. In addition, the intensity of hydroxyl peak at 1658 cm^{-1} tended to increase with increasing protein content as well. This was likely due to the amplification effect of another amide I peak of BSA at 1652 cm^{-1} representing the stretching vibrations of C=O bonds in the backbone of the protein [4], [5], [12] which coincided with this hydroxyl peak. Comparing between two techniques at similar initial protein bath concentration used, rapid biomimetic co-precipitated samples showed greater amide peak intensity than direct adsorption samples indicating greater amount of incorporated protein in the coating produced by rapid biomimetic co-precipitation technique.

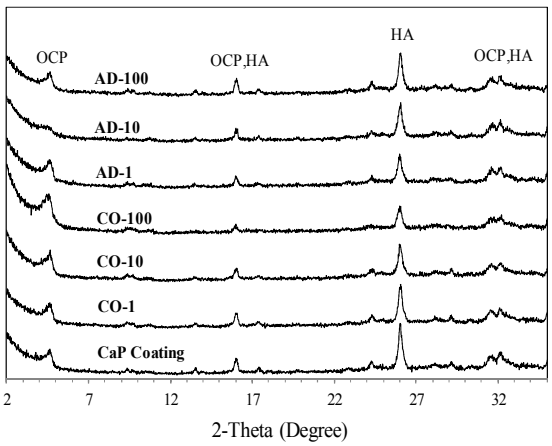

Fig. 2 XRD patterns of BSA incorporated calcium phosphate coatings produced by direct adsorption technique (AD) and rapid biomimetic co-precipitation technique (CO). The concentrations of protein solution used were 1 μg·ml^{-1} (CO-1, AD-1), 10 μg·ml^{-1}(CO-10, AD-10) and 100 μg·ml^{-1} (CO-100, AD-100)

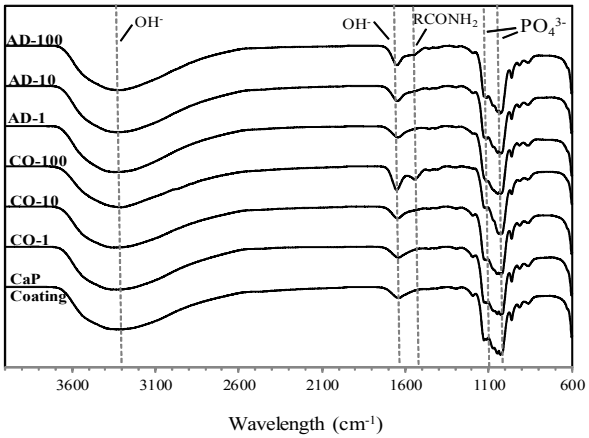

Fig. 3 FT-IR patterns of BSA incorporated calcium phosphate coatings produced by direct adsorption technique (AD) and rapid biomimetic co-precipitation technique (CO). The concentrations of protein solution used were 1 μg·ml^{-1} (CO-1, AD-1), 10 μg·ml^{-1} (CO-10, AD-10) and 100 μg·ml^{-1} (CO-100, AD-100)

Figs. 4 and 5 show the microstructure of protein incorporated calcium phosphate coating produced by direct adsorption and rapid biomimetic co-precipitation techniques respectively. Both types of coating were homogenous and porous comprising sharp and interconnected plate-like crystals vertically grown on the surface of titanium. In the case of direct adsorption coating, no difference in microstructure was observed among different protein incorporated content. In contrast, calcium phosphate crystals in rapid biomimetic co-precipitation coating changed from straight plate-like units to curved, plate-like units with increasing BSA content and the microstructure also tended to be less porous. This change in morphology was due to the interruption of calcium phosphate crystal initiation and growth process by protein molecules [13]. This effect was more pronounced with increasing protein concentration.

Table I shows a comparison of BSA incorporated content in the calcium phosphate coating between direct adsorption and rapid biomimetic co-precipitation techniques. The BSA incorporated content in the coating increased with increasing initial BSA concentration. For equivalent initial BSA concentration used in the process, BSA incorporated content in rapid biomimetic co-precipitation coating was significantly greater than that of direct absorption coating, about 2, 10 and 43 times for initial BSA concentration of 1, 10 and 100 μg·ml^{-1} respectively. Comparing to previously reported biomimetic coating which employed soaking time of 48 h [4], the BSA incorporation content in rapid biomimetic co-precipitation coating in this study was equivalent to that report when using initial protein concentration of 1 and 10 μg·ml^{-1}, but six times greater when using protein solution of 100 μg·ml^{-1}. This signifies the advantage and efficiency of this rapid biomimetic co-precipitation coating in terms of shorter coating time, but greater protein incorporation efficiency.

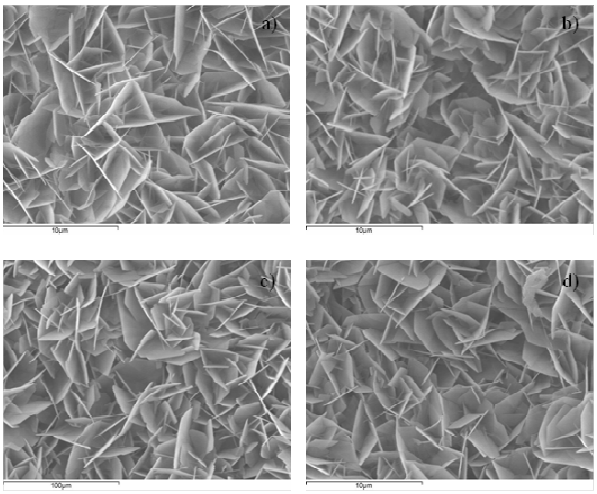

Fig. 4 SEM micrographs of BSA incorporated calcium phosphate coatings produced by direct adsorption technique. The concentrations of protein solution used were: a) 0 μg·ml^{-1}; b) 1 μg·ml^{-1}; c) 10 μg·ml^{-1}; d) 100 μg·ml^{-1} (Magnification × 5,000, Scale bar = 10 μm)

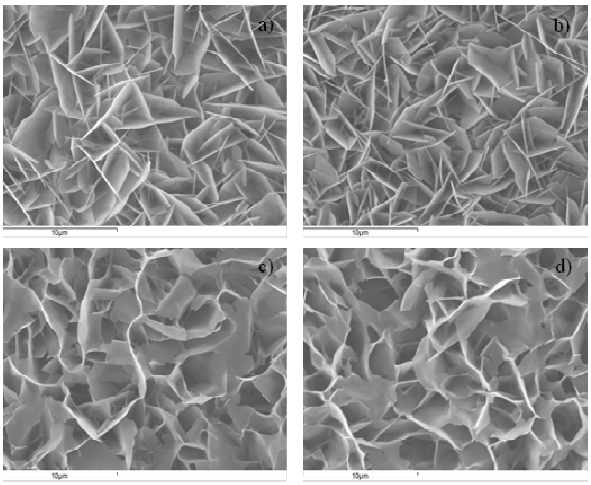

Fig. 5 SEM micrographs of BSA incorporated calcium phosphate coatings produced by rapid biomimetic co-precipitation technique. The concentrations of protein solution used were: a) 0 μg·ml^{-1}; b) 1 μg·ml^{-1}; c) 10 μg·ml^{-1}; d) 100 μg·ml^{-1} (Magnification × 5,000, Scale bar = 10 μm)

TABLE I
TOTAL PROTEIN CONTENT IN THE CALCIUM PHOSPHATE COATING

Techniques	Concentration of Initial Protein Solution Bath Used (μg·ml^{-1})	Measured Total Protein (μg·mg^{-1})
Direct Adsorption (AD)	1	0.4±0.2
	10	0.9±0.4
	100	2.3±1.2
Rapid Biomimetic Co-Precipitation (CO)	1	0.7±0.1
	10	8.6±1.0
	100	98.9±13.6

Protein releasing study in Figs. 6 and 7 showed that the release mechanism of BSA from both coatings was composed

of two stages including burst release and sustained release. For equivalent initial BSA concentration used in the process and release time, BSA release content from rapid biomimetic co-precipitation coating was significantly greater than that of direct absorption coating. Limited released protein was detected for AD-1 and AD-10 samples. This could be related to the low amount of incorporated protein content of those two samples as shown in Table I. The release duration of direct adsorption samples was completed at only approximately 6 h (AD-100). In contrast, rapid biomimetic co-precipitation samples could release BSA at much longer duration for example more than 360 h for CO-100 sample. The longer release duration of rapid biomimetic co-precipitation coating was attributed to the greater efficiency of protein incorporation and the protein is not merely adsorbed superficially on the coating surface by electrostatic interaction, but incorporated into the crystal lattice and also bonded strongly to Ca^{2+} ions within the lattice [4], [7].

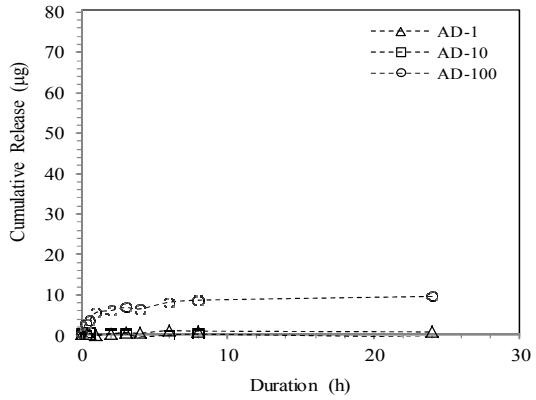

Fig. 6 Cumulative release of BSA from protein incorporated calcium phosphate coating produced by direct absorption technique in phosphate buffered saline at 37°C

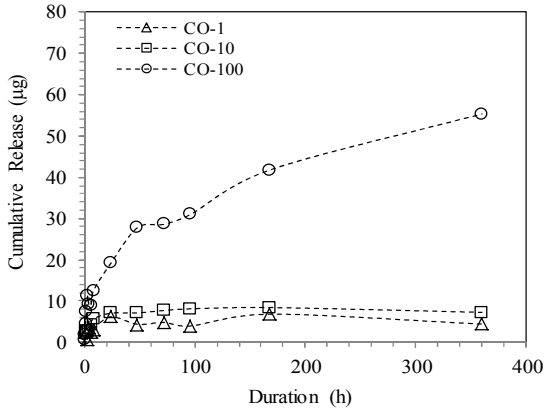

Fig. 7 Cumulative release of BSA from protein incorporated calcium phosphate coating produced by rapid biomimetic co-precipitation technique in phosphate buffered saline at 37°C

IV. Conclusion

It could be concluded that a rapid biomimetic co-precipitation technique could increase the BSA incorporation efficiency and its release duration without altering the phase composition or chemical structure of the calcium coating excepting the microstructure. This technique could be employed as a fast and low cost coating process for protein delivery and applied to other bioactive agents for medical applications.

Acknowledgment

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