GENERATION OF SILK FIBROIN-CA-P COMPOSITE BIOMIMETIC BONE REPLACEMENT MATERIAL USING ELECTROCHEMICAL DEPOSITION

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Abstract: *Mineralized natural protein based novel bone replacement materials are investigated for tissue engineering. Mineralized silk fibroin composite foams and films display excellent biocompatibility. In this study, the biomimetic and electrochemical mineralization of orderly oriented silk fibroin scaffolds was studied.*

Commercially obtained pure silk woven fabric was boiled in 0.02 M Na_2CO_3 for 20 min. Calcium phosphate was deposited at 37°C for twenty minutes in seven sequential immersion steps, using 250 mM $CaCl_2$ $2H_2O$ and 120 mM K_2HPO_4, containing 0.15 M NaCl and 50mM TRIS-HCl, pH 7.4, followed by electrochemical treatment in modified SBF solution at 40°C at a current density of $-25mA/cm^2$ for 60 min.

The amount of biomimetically deposited Ca-P increased with the number of immersion steps. SEM images and XRD analysis of the Ca-P deposit indicated the initial formation of brushite with its monoclinic crystal structure and characteristic peak at 11.76 2θ, and electrochemical conversion of brushite to hydroxyapatite on silk after electrochemical cathodization as confirmed by XRD and SEM analysis.

Thus, a silk-fibroin-hydroxyapatite composite material prepared as a xenograft consisting of biocompatible components, and easily prepared as an economical bone segment replacement material with highly oriented fibers.

Keywords: *silk-fibroin-hydroxyapatite scaffold, composite biomaterials, bone tissue engineering, bone replacement materials.*

1. Introduction

Bones provide not only calcium, magnesium, and phosphate ions for the vertebrates, but also enable their mobility, protect the visceral organs, and act as sites of blood cell production [1]. As hierarchically organized biological structures, bones consist of hydroxyapatite (HA), Type I Collagen, proteoglycan and water [2]. In the treatment of bone and joint fractures, in addition to metal implants [4], autograft, allograft, xenograft and alloplastic materials [3] are used. Osteoconductive and osteoinductive, nanostructured Calcium phosphate based (Ca-P) biomaterials [5,6] may need to be complemented with polymeric and ceramic composite bone replacement materials, which enable adhesion, growth and development of osteoprogenitor [7]. The osteoconductive scaffold, with sufficient pore size and total porosity allows both the adherence of cells and the delivery of the nutrients and metabolites to them [8].

Biodegradable polyglutamic acid (PGA), polylactic acid (PLA), polylactic-glutamic acid (PLGA), as well as naturally obtained, biodegradable alginate, collagen, fibroin [9], and silk [10] polymers are commonly used. Among these, silk proteins with low biodegradability, high chemical and thermal stability under severe conditions, and connected porosity providing homogeneous cell proliferation [11] can be preferred as the scaffold for mineralization with a wide use in biomedical applications [12-14].

Natural silk is a protein polymer consisting of two proteins: fibroin and sericin. The interest in silk fiber has increased with the removal of adhesive serine protein in crude silk from the silk-fibroin protein, and thus prevention and elimination of an immunological response [12]. Furthermore, experimental studies have shown that the silk is not more thrombogenic than similar biomedical materials. Therefore, in the search for different biocompatible materials, silk fibroin materials have

demonstrated superiority over biodegradable materials such as PLGA, causing inflammation due to acidic hydrolysis products, polyester, with incoherent hydrolysis rates and tissue response profiles, or polystyrene and pHEMA, with thrombogenic properties [12].

Silk-fibroin is a hydrophobic protein with a general essential amino acid sequence of (Gly-Ser-Gly-Ala-Gly-Ala)n and a molecular mass of approximately 400 kDa. The highly repetitive primary sequence of hydrophobic silk protein leads to homogenous β-sheet secondary structure, where the hydrophobic domains with repeated amino acid sequence are assembled into nano-crystals as β-sheet. The hydrophilic links between the hydrophobic domains consist of bulky polar side chains, forming the amorphous part of the secondary structure, giving silk its elasticity [12].

With a high durability and superior properties of biodegradability [12-14], silk has advantages over other polymers, such as PGA and PLA, which release acidic by-products as they are resorbed through metabolic pathways. Silk, on the other hand, retains strength over a long time, which can be an advantage in bone tissue engineering, where slow degradation and load bearing capacity are required. [12-14]. Native silk has good toughness and ductility with a strength-to-density ratio of up to ten times that of steel [12-14]. The mechanical properties, porosity and pore size of the three-dimensional porous silk fibroin scaffolds can be easily adjusted by varying the concentration of silk-fibroin protein and porogenin [15]. However, when native silk is compared with the materials regenerated from silk-fibroin solution in terms of dry tensile strength of silk film and elongation at break (0.5–0.6 GPa vs. 0.02 GPa and 10–40% vs. <2% respectively) native fibers with appropriate secondary and hierarchical structures demonstrate superior mechanical properties [12-14].

Calcium phosphates are traditionally used as bone graft materials, due to their close compositional resemblance to the bone mineral. However, the biomimetic deposition of HA on fibrous polymer requires the use of expensive polyhydroxyacids [16-19]. On the other hand, although the use of alkaline treatment may increase the pH to form HA, the samples may need to be immersed in alkaline solution for hours and sometimes for days [20,21].

The aim of this study was to deposit carbonated hydroxyapatite (CHA) on silk scaffolds using electrochemical methods, which are expected to provide both a quick, practical and economical alternative method, conducted at physiological temperatures without the use of any polyhydroxyacids and growth factors [19-22]. The deposition of Ca-P on silk fibroin protein using the electrochemical method has been conducted out in this study for the first time as an important alternative to the biomimetic method, which, in this study has been used as the control method. With the precipitation of biologic HA using electrochemical coating processes [19] on the porous silk fibroin scaffolds, enhancement of both osteoconductive and osteoinductive properties is anticipated.

2. Material and Method

In the study, Merck grade chemicals were used. Solutions prepared were (a) carbonate, containing 0.02M Na_2CO_3, (b) calcium, containing 0.15M NaCl, 50mM TRIS-HCl, 250 mM $CaCl_2$ $2H_2O$ buffered at a pH of 7.4, (c) phosphate, containing 0.15 M NaCl, 50 mM TRIS·HCl, 120 mM Na_2HPO_4 buffered at a pH of 7.4, (d) alkali, containing 0.5M NaOH, and (e) simulated body fluid (SBF), containing 0.15 M NaCl, 50 mM TRIS·HCl, 250 mM $CaCl_2$ $2H_2O$, and 120 mM Na_2HPO_4 buffered at a pH of 7.4 were prepared and sterilized. Six pure silk-fibroin fabric scaffolds with dimensions of 5x3 centimeters were used.

Preparation and characterization of silk scaffolds: Pure silk-fibroin scaffolds were boiled in carbonate solution (a) for 20 minutes to remove dirt and oil, and rinsed in distilled water. Silk fibroin fabric surfaces were characterized using Scanning Electron Microscopy (Zeiss, EVO 40).

Mineralization of silk-fibroin scaffolds using the cyclic biomimetic method: Pure silk-fibroin scaffolds were then placed in 50-ml of calcium solution (b) at 37°C for 20 minutes, followed by immersion in 50 ml. of phosphate solution (c) at 37°C for 20 minutes under constant stirring [20]. This procedure was cyclically repeated seven times using fresh solution at each cycle. Ca-P layer was expected to gradually deposit on the fabric surface [20]. The data obtained from the biomimetically mineralized scaffolds was used as the control and compared with the data obtained using the electrochemical method.

Mineralization of silk-fibroin scaffolds using the electrochemical method: Mineral deposited on the silk-fibroin scaffolds using the biomimetic method was characterized as described in the following paragraph. The mineralization process was further continued using the electrochemical cathodic method in order to transform the mineral deposit to HA using a three electrode electrochemical cell, consisting of a bioactivated titanium rod as the active electrode, standard silver/silver chloride electrode (in a saturated KCl) as the reference electrode and platinized stainless steel electrode as the counter electrode. The titanium electrode had to be bioactivated by immersion in alkaline solution (d) at 50°C for 2 minutes, and rinsed using deionized water. Afterwards, the silk-fibroin fabric was wrapped tightly around the active metal electrode. The direction of the fibers were oriented parallel and perpendicular to the axis of the titanium electrode, analogous to the tubular orientation of the collagen fibers in compact bone. Electrochemical deposition was conducted using SBF fluid (e) at 40°C with constant cathodic current density of -25mA/cm^2 for 60 minutes.

Characterization of the mineral deposit: Ca-P coated silk-fibroin fabric scaffolds were air-dried, and the crystallographic properties of the mineral deposits were determined using X-Ray Diffraction (XRD) (Philips PW 3710, Cu-Kα radiation source, 40 kV, 30mA) at a scattering angle of 4-60°, a step size of 0.02 2θ, and a stripping angle of 1°. The morphological characteristics of the silk-fibroin

scaffolds, cut at a slice thickness of 1 mm, and gold-coated were examined using Electron Scanning Microscope (SEM) (Zeiss, Evo40) and FE-SEM (JEOL JSM 7000F FEI).

3. Results and Discussion

In this study, a Ca-P-silk fibroin composite material, with silk fibers oriented along the axis of a graft used in segmenter defects was obtained. Brushite was precipitated on the silk-fibroin fabric using cyclic biomimetic mineral deposition method and further converted to CHA using the electrochemical cathodic deposition method.

3.1. Mineral Formed by Cyclic Biomimetic Method:

The precipitation of mineral obtained using the cyclic biomimetic immersion method could be monitored with the naked eye. The mineral layer thickness increased with each cycle of calcium and phosphate biomimetic deposition. XRD analysis of the precipitate obtained by cyclic biomimetic mineralization indicated brushite formation as defined by the typical peak at 11.76 2θ (Figure 1).
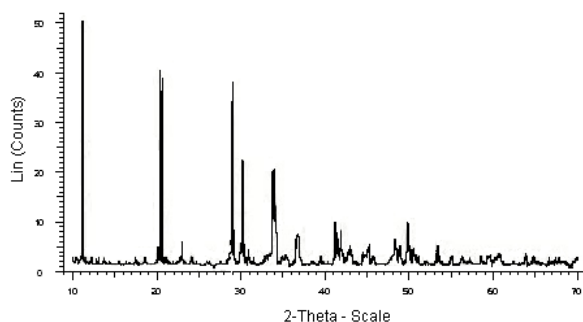

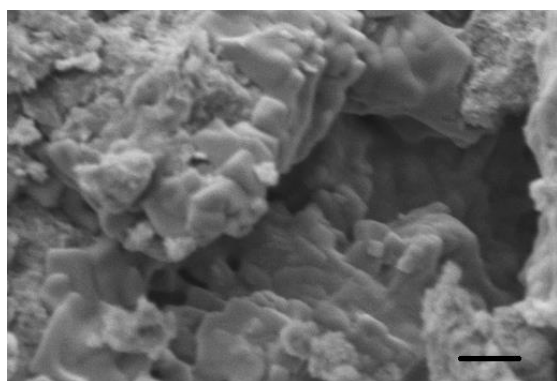**Figure 1.** The XRD spectrum of brushite mineral deposited using the biomimetic method.

Figure 2. SEM image of brushite mineral containing monoclinic crystals deposited using the biomimetic method Scale bar = 2 micrometers.

The brushite crystals in the SEM images, in agreement with the XRD results, were monoclinic and appeared as plates (Figure 2) [21]. Ca/P ratios obtained varied between 0.5-1.

3.2. Mineral Formed by the Electrochemical Deposition Method:

Using the electrochemical cathodic deposition method, the brushite crystals were converted to CHA. The XRD analysis of the mineral layer indicated presence of both the brushite peak observed at 11.76 2θ and the XRD peaks at 31.8 and 32.18 2θ (ICSD 00-019-0272) indicating CHA formation (Figure 3).
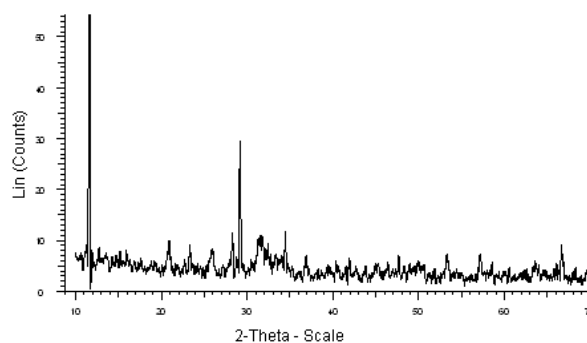

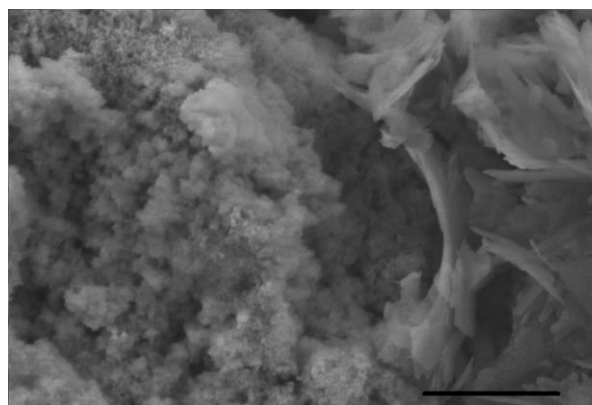**Figure 3.** The XRD spectra of mineral deposited using the electrochemical deposition method.

Figure 4. SEM image of mineral deposit showing the transformation to hydroxyapatite using the electrochemical deposition method. Scale bar = 2 micrometers.

The CHA peaks in the XRD spectra indicated conversion of brushite to CHA as a result of the electrochemical treatment in SBF for 60 minutes. SEM images of the mineral layer confirmed the XRD results indicating presence of brushite, amorphous calcium phosphate as particles and hydroxyapatite as apical crystals (Figure 4) [21]. Although the Ca-P ratio varied between 1-1.3, implying OCP formation, the XRD data was not noisy as observed in an OCP XRD spectrum. On the contrary, it displayed only the sharp brushite peaks and the emerging peaks for poorly crystallized biological CHA at 31.8 and 32.18 2θ.

In this study, silk-fibroin fabric scaffold was shaped to form tubular structures of osteonal cortical bones, designed for the treatment of segmental defects, aimed to guide and orient the cells and associated structures in three dimensions. As foam and film silk scaffolds have

previously demonstrated osteogenic differentiation of hMSCs, mineralization and bone formation, the aim was to form a base material that would further lead to the development of a biomimetic, cortical three dimensional bone scaffold with osteon-like structures, covalently bound cell signaling factors and adequate porosity for cellular attachment [20].

Brushite, not firmly held on to the silk fibers, could be obtained in powder form in high crystallinity, with its characteristic 100% peak at 11.76 2θ (Figure 1). The compound precipitated on the silk-fibroin fabric surface by the electrochemical method appears to display a weaker brushite peak, and indicates formation of a low crystallinity compound, such as biological HA, with peaks of (211) and (112) at almost equal intensity (ICSD 00-019-0272, reference XRD spectrum for CHA) [19]. Brushite, formed as a precipitation product and as a highly water soluble mineral, may be expected to transform into CHA through an amorphous calcium phosphate intermediate phase and be deposited on the silk-fibroin scaffold (Figs. 2 and 4) [19,21]. It has been shown that using the electrochemical cathodic method, hydrogen phosphate ions and water are reduced, forming phosphate and hydroxide ions by electron withdrawal, and thus generating the ions necessary for CHA formation.

Cyclic biomimetic mineralization has formed simple Ca-P compounds in monoclinic crystal structure (Fig. 2). Since more complex Ca-P compound formation is both time and temperature dependent, using electrochemical mineralization, brushite can be converted to CHA in a short time interval. CHA formation at 40°C using electrochemistry shows that the reduction of both water and dissolved carbon dioxide on the cathodic electrode generates high amounts of hydroxide and carbonate ions, and that CHA, which requires high alkalinity can be formed by depositing and precipitating these ions on silk fibers [19,22].

The binding of HA to collagen fibers occurs through the attachment calcium to specific amino acid side groups, such as aspartic acid and polyglutamic acid [16,17], thus increasing HA deposition with amino acid concentration [20]. Silk-fibroin mineralization depends on the ionic bonding between calcium ions and hydrophilic structures in the chain. Silk fibroin has a hydrophobic content of 79%, with long heavy hydrophobic blocks and short hydrophilic blocks in the primary chain and a hydrophilic block each on both ends of the chain. The repeated GAGAGS peptide sequences are frequently seen in the beta-sheet structure, forming crystallized regions in silk fiber and silk films [12]. High NaCl concentration in the silk solution results in binding of these ions to water, which would otherwise be expected to surround the hydrophilic blocks, resulting in the interaction of the hydrophobic blocks with each other leading to protein folding and formation of beta-sheet structure [12].

The scaffold produced in the film-form are two dimensional, with cells remaining on the surface, while in the foam-form scaffolds, the new tissue generated by the cells appears to remain on the foam surface, with necrotic regions formed in the material [8,20]. Therefore, the tubular, silk-fibroin protein fabric scaffold with 3D interconnected porosity and pore size adequate for cortical bone formation may be more advantageous in bone regeneration (Fig. 5). The porosity created by fabric fiber, ranging between 10 to 100 microns, is large enough for the attachment, settlement and differentiation of bone precursor cells. As silk-fibroin protein-HA composite materials have demonstrated improvement in osteoconductive and osteoinductive properties in cell culture and tissue engineering studies, and osteogenic differentiation of hMSCs [8,10,20], we expect the porosity introduced in this study to promote a higher rate of new bone formation.
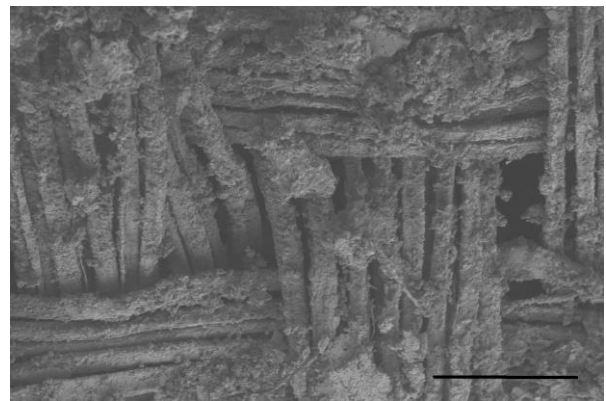

Figure 5. The silk-fibroin-hydroxyapatite composite biomaterial with interconnected porosity that potentially may enable cellular attachment, differentiation and proliferation.

4. Conclusions

In this study, the brushite, deposited on silk-fibroin scaffolds using the sequential cyclic biomimetic mineral coating method, was transformed to carbonated hydroxyapatite under physiological conditions using the electrochemical cathodic coating method. Silk-fibroin-HA, i.e. polymer-ceramic composite biomaterials, shaped as the osteons of compact bone can be used in the treatment of segmental defects. More detailed study on the widespread use of silk-fibroin-HA composite osteobiological materials is aimed.

A silk-fibroin-CHA composite biomimetic material was thus obtained under physiological conditions within 60 minutes.

5. References

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