Fabrication and Characterization of Collagen-Apatite Composites for Bone Tissue Engineering

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Fabrication and Characterization of Collagen-Apatite Composites
for Bone Tissue Engineering

Changmin Hu, PhD
University of Connecticut, 2018

Abstract

Due to the high demand of scaffolds for treating bone fractures every year, biomimetic coatings and scaffolds resembling the composition and structure of natural bone are of keen interest to bone tissue engineers. In this research, a series of biomimetic coatings and scaffolds have been developed, including biomimetic apatite coatings, biomimetic collagen-apatite composite coatings, intrafibrillar calcified collagen fibrils, intrafibrillar silicified collagen fibrils, and intrafibrillar calcified collagen scaffolds.

First, a crack-free apatite coating was deposited on the surface-treated Ti6Al4V substrate using a biomimetic process, in which the surface-treated titanium substrate was immersed in a modified simulated body fluid (m-SBF). Dual-beam focused ion beam/scanning electron microscopy (FIB/SEM) was used to characterize the cross-sectional microstructures of the ceramic coating. Cross-sectional SEM images and TEM images revealed that crack-free apatite coatings have been achieved by adjusting the pH of the m-SBF. The coating formed demonstrates a gradient porous structure, which is dense close to the substrate and then it becomes more and more porous towards the surface of the coating.

Second, a biomimetic collagen-apatite composite coating was also successfully
formed on the surface-treated Ti6Al4V alloy using collagen-containing m-SBF. During the composite coating formation, collagen and apatite co-precipitate to form the composite coatings. Dual-beam FIB/SEM was used to characterize the cross-sectional microstructures of the composite coatings. The result indicates that the cross-section of the collagen-apatite composite coating also exhibits a gradient porous structure, and that collagen-apatite composite coating is thinner and less porous than the pure apatite coating.

Third, inspired by the structure of natural bone, collagen fibrils with intrafibrillar calcification were prepared for bone tissue engineering. Poly(acrylic acid) (PAA) was used as a sequestration analog of non-collagenous proteins (NCPs) for stabilizing amorphous calcium phosphate (PAA-ACP) nanoprecursors to infiltrate into collagen fibrils. Additionally, sodium tripolyphosphate (TPP) was applied as a templating analog of NCPs to regulate the orderly deposition of minerals within the gap zone of collagen fibrils. The effect of PAA concentration on the intrafibrillar mineralization of reconstituted collagen fibrils was also investigated.

Fourth, silicon is an essential element contributing significantly to the health of bone, and great efforts have been made to produce silica-containing biomaterials. Poly (allylamine) hydrochloride (PAH) was used as an analog of zwitterionic proteins to stabilize silicic acid (SA) to produce fluidic silica (PAH-SA) nanoprecursors, and TPP was used as a templating analog of zwitterionic proteins. Silicified collagen fibrils with core-shell, twisted and banded structures were produced by controlling the zwitterionic proteins analogs. Intrafibrillar silicified collagen fibrils were produced
using PAH-SA as fluidic silica nanoprecursors and TPP as the templating analog to modulate the deposition of silica within the gap zone of collagen fibrils.

Fifth, with the success in reproducing intrafibrillar mineralized collagen fibrils, biomimetic collagen/apatite scaffolds consisting of intrafibrillar mineralized collagen fibrils were prepared via a bottom-up approach, resembling the composition and structure of natural bone from the nanoscale to the macroscale. At the macro-scale, unidirectional macro-pores are aligned across the entire scaffold, and at the micro-scale, each layer is comprised of aligned and well stacked lamella. Finally, at the nano-scale, apatite minerals deposit within the gap zone of collagen fibrils and orient along the long axis of these fibrils. The biomimetic collagen-apatite scaffolds demonstrate a good biocompatibility in vitro, indicating a great potential to be used for bone tissue engineering applications.

In summary, a novel methodology has been developed for the preparation and characterization of biomimetic coatings and scaffolds with a hierarchical structure, demonstrating a great potential for bone tissue engineering.
Fabrication and Characterization of Collagen-Apatite Composites

for Bone Tissue Engineering

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for Bone Tissue Engineering

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1. Introduction

1.1 Background

In the United States, approximately one million bone fractures are treated every year. [1, 2, 3] Various materials have been used, such as autografts, allografts, and artificial grafts, including metals, polymers and ceramics. Autografts have long been considered as the gold standard for bone grafting procedures. However, the amount of bone that can be harvested is limited, and there is a high potential for donor site morbidity. Allografts are alternatives to autografts, but there are concerns of disease transmission and immuno-rejection. [4, 5, 6, 7] Artificial grafts can overcome the resource problem of autografts and disease transmission issues of allografts; however, many bone grafts are not bioactive. A promising strategy to address these problems is bone tissue engineering, where a combination of supporting scaffolds, cells and/or biological signals is used to regenerate defected bone. [4, 8, 9]

Supporting scaffolds play an important role in bone tissue engineering, and the ideal supporting scaffolds for bone regeneration should have the following properties: (1) biocompatibility, that does not induce immunogenic responses; (2) biodegradability, that degrades at a suitable rate to match the regeneration rate of new tissue; (3) osteoconductive, that facilitates the cell attachment and proliferation; (4) osteoinductive, that stimulates the osteoprogenitor cells to differentiate into osteoblast cells and induces new bone formation. [1]

Based on these criteria, bioinspired or biomimetic scaffolds have been prepared, which have demonstrated great promises and attracted great attention from the
biomaterial community. [10, 11] Polymer scaffolds or metal implants were coated with hydroxyapatite, the main inorganic composition of bone, to render these materials with excellent bioactivity. [12, 13] Pek et al. produced the collagen-apatite nanocomposite foams by mixing collagen fibers with apatite nanocrystals, mimicking the composition of natural bone. [14] Xia et al. developed a biomimetic collagen-apatite scaffold with multi-lamellar structure in order to mimic the composition and structure of natural bone. [15]

1.2 Structures and composition of natural bone

Natural bone is a hierarchical composite mainly composed of apatite and collagen. Type I collagen accounts for 95% of the organic material in bone, and has good resorbability and high affinity to cells. [16, 17] Apatite constitutes about 65 wt% of natural bone, exhibiting biocompatibility, osteoconductivity, and bone-bonding ability. [3, 18] Additionally, silicon has been found in active bone growth area, such as the osteoid of the bone of young rats, [19] indicating osteoinductive property of silicon for new bone formation and stimulative for neovascularization without supplements of growth factors. [20, 21]
Figure 1.1. Hierarchical structures of natural bone: starting from sub-nanostructure of collagen molecules and tropocollagen triple helix, to nanostructure of ordered HA crystals, and to sub-microstructure of collagen fibrils and macrostructure (a). The arrangement of HA crystals with collagen fibrils (b). [22]

As shown in Figure 1.1, natural bone is a complex hierarchical composite having multi-scale microstructure. Apatite nanocrystals deposit within the gap zone of collagen fibrils (intrafibrillar mineralization) at an early stage of mineralization and then on the surface of the collagen fibrils (extrafibrillar mineralization) at a later stage. [23, 24, 25, 26, 27, 28] Bundles and arrays of mineralized collagen fibrils further arrange into a multilayered structure rotating across concentric lamellae, which pack densely into compact bone or loosely into spongy bone. [29, 30, 31]

1.3 Intrafibrillar mineralization

In order to mimic the composition and structure of natural bone, extensive researches have been done to replicate the intrafibrillar mineralization process in nature. [32, 33, 34] Non-collagenous proteins (NCPs) are found to play an important
role in regulating the intrafibrillar mineralization of collagen fibrils. [35, 36] Their functions are to sequester calcium and phosphate ions to form liquid amorphous calcium phosphate nanoprecursors, and then template the nucleation and growth of the nanocrystals at the gap zone of the collagen fibrils. [35, 37, 38] According to Kerschnitzki et al., abundant vesicles containing small mineral particles were found in the lumen of blood vessels, in the bone-forming tissue between the blood vessels and the forming bone surfaces, and in the cells associated with bone formation, proving the important role of nanoprecursors in bone formation. [39, 40] As NCPs play an important role in regulating intrafibrillar mineralization, polyanionic polymers such as PAMAM-COOH dendrimer, poly (aspartic acid) and poly (acrylic acid) have been used as the sequestration analog of the NCPs. [32, 41, 42] Liu et al. employed poly (acrylic acid) as the sequestration analog and sodium tripolyphosphate as the templating analog to induce collagen intrafibrillar mineralization, and proved that the intrafibrillar mineralized collagen fibers have improved nanomechanics and cytocompatibility. [43] Based on the success of apatite intrafibrillar mineralization, silica was also deposited within the gap zone of collagen fibrils using poly (allylamine hydrochloride) or choline chloride as the analogs of zwitterionic proteins. [44] Niu et al. produced intrafibrillar silicified collagen scaffolds by incubating collagen scaffolds in the choline-stabilized silica precursor, and the intrafibrillar silicified collagen scaffolds promoted the osteocalcin expression and calcium deposition after transplantation in vivo. [45]
1.4 Implant coating

Titanium and its alloy (Ti-6Al-4V) have been widely used as orthopedic and dental implants because of their good biocompatibility, good corrosion resistance and mechanical strength. [46, 47] However, titanium implants do not have a good bone bonding and integration ability, resulting in implant failures. [48] Thus, coating hydroxyapatite, the main inorganic composition of natural bone, on titanium has been widely applied to impart the bioactivity and osteoconductivity to titanium implants and improve the bone-bonding ability of the implants. [49, 50] After implanting, a bone-like apatite layer forms on the bioactive coating, acting as an intermediate layer connecting new bone and implants. [51] A lot of approaches have been used to form a hydroxyapatite coating on the titanium implant, such as electrodeposition, [52] cold spray, [53] electrostatic spraying, [54] plasma spray, [55] sol-gel, [56] pulse laser deposition [57] and biomimetic approach. [58] The biomimetic process closely mimics apatite formation in vivo, which significantly improves the biological performance of the biomaterial. This coating technique has attracted extensive attention because it is conducted at a condition close to body temperature (37 °C) and pH (7.4), and it is able to co-precipitate with biologically active molecules without compromising their bioactivities. [59]

1.5 Dual beam focused ion beam/scanning electron microscopy (FIB/SEM)

Focused ion beam (FIB) was developed in the middle 1970’s mainly for semiconductor applications. The basic principal is to sputter atoms from the materials using accelerated heavy ions. [60] FIB is able to mill into the bulk of a specimen,
exposing the hidden internal microstructure without causing significant damages. As a result, it has been used to prepare TEM samples, cross-sections, and metal depositions.

Dual-beam focused ion beam/scanning electron microscopy (FIB/SEM) was introduced to include an FIB column for material etching and an SEM column for imaging to facilitate simultaneous milling, imaging, serial sectioning, and many other functionalities (Figure 1.2). [61] Both ion beam and electron beam are focused at the same point, enabling the electron column to monitor the process while the ion beam is milling the specimen. The dual-beam FIB/SEMs not only have all the capabilities as single beam instruments, but also allow a more precise cross-sectioning of heterogeneous, fragile, and/or porous materials and structures, 3-D imaging and preparation of TEM specimens.

The majority of FIB/SEMs are equipped with a Ga liquid metal ion source on the FIB column (GFIB); however, they are limited by the low material removal rate. [62] This problem has been addressed by the introduction of a new generation of dual-beam FIB/SEM, which are equipped with plasma ion source on the FIB column (PFIB). [63, 64] The PFIB offers milling rates up to two orders of magnitude higher than those of GFIB. [65, 66]
Figure 1.2. The specimen is tilted towards the ion beam (left), and a cross-section is milled to be imaged by the electron beam (right). [67]
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[40] M. Kerschnitzki, A. Akiva, A. B. Shohamb, Y. Asscher, W. Wagermaier, P. Fratzl, L. Addad, S. Weiner. Bone mineralization pathways during the rapid growth of


2. Cross-sectional microstructure study of apatite coating on titanium alloy

Abstract

Biomimetic hydroxyapatite (HA) coating has been used to render titanium implants with excellent osteoconductivity and osseointegration in orthopedic and dental applications. However, to date it has not been possible to reveal the fine details of the coating structure or the coating/substrate interface. In this study, a crack-free biomimetic HA coating has been successfully formed on sand-blasted, acid-etched Ti-6Al-4V discs by simply immersing the discs in a modified simulated body fluid (m-SBF). Dual-beam FIB/SEMs with either gallium ion source (GFIB) or xenon plasma ion source (PFIB) were used to prepare the cross-sections and thin transmission electron microscopy (TEM) specimens of the coating, and the capabilities of GFIB and PFIB were compared. Both the cross-sectional SEM and TEM images confirmed that a well bonded biomimetic HA coating has been formed on the Ti-6Al-4V substrate. The coating exhibits a gradient structure with a dense microstructure adjacent to the titanium substrate to facilitate strong bonding, while there is a porous structure at the surface, which is beneficial to bone cell attachment and subsequent new bone integration.

Keywords: Biomimetic HA coating; Cross-section; TEM specimen; Dual-beam FIB/SEM; Gradient structure.
2.1 Introduction

The titanium alloy Ti-6Al-4V (Ti – 6% Al – 4% V (wt.%)) has been used extensively in orthopedic and dental implants because of its good biocompatibility, superior mechanical properties, and good corrosion resistance. [1, 2] However, Ti-6Al-4V implants do not have a good bone-integration ability. [3] Thus, it has become common practice to coat the Ti-6Al-4V implant with bioactive hydroxyapatite (HA) to impart good bioactivity, to induce osteoconductivity, and to promote osseointegration. [4] A variety of different deposition techniques has been used to produce HA coatings on titanium alloy substrates. These include: plasma spraying, sol-gel coating, electrophoretic deposition, and biomimetic immersion. [5-9] The biomimetic coating approach has attracted extensive attention due to its mild conditions (low temperature and neutral pH), ability to produce coatings with good bonding strength, and capability to incorporate bioactive molecules during the coating process. [2, 10-15] The surface morphology of the biomimetic coating has been studied extensively using scanning electron microscopy (SEM). [16-18] However, there are very few publications that describe the fine details of the internal microstructure for these biomimetic coatings, or of the interface between the coating and the alloy substrate.

Cross-sectional studies of the coating microstructure and the coating/substrate interface are essential for investigations of crack formation, coating dissolution, ion diffusion and distribution, and interfacial bonding. [19] For SEM studies the samples are typically produced by fracturing or by cutting, grinding and polishing the coated
substrate. Both approaches have severe limitations. Fracture surfaces often have complex topographies that complicate the interpretation of structural detail, and there can be problems associated with plastic deformation in the substrate leading to interfacial delamination. Cutting, grinding and polishing to produce metallographic-type cross sections often induces significant mechanical damage and limits the useful detail that can be extracted from such samples. [20-22] More serious complications arise if one attempts to study such coatings by transmission electron microscopy (TEM). These studies require extremely thin (typically <100nm) samples free of mechanical damage or other artefacts. Traditional approaches for TEM specimen preparation such as ultramicrotomy, or dimple grinding followed by ion beam milling, are simply not feasible for HA-coated Ti alloy substrates. The mechanical stresses introduced by these approaches would cause significant structural damage both to the coating and to the coating/substrate interface. [23-25]

Focused ion beam (FIB) microscopy is a well-established technique for both imaging and modifying samples on the micro- or nano-scale. The ability of FIB to remove material from specific locations without inducing significant ion beam damage makes it an ideal approach to prepare cross-sections of various materials and, in particular, to produce site-specific cross-sectional TEM specimens. [26] Most modern instruments include both an FIB column and an SEM column to facilitate simultaneous milling and imaging, serial section tomography and many other functionalities. [23] These dual-beam FIB/SEMs are capable of precise cross-sectioning of heterogeneous, fragile, and/or porous materials and structures, and
preparation of TEM specimens. The vast majority of FIB/SEMs are equipped with a Ga liquid metal ion source on the FIB column. While the latest generation of Ga FIB (GFIB) columns is capable of operating over a wide range of probe sizes and beam currents, there are restrictions on the material removal rate that can be achieved; this limits the volume that can be interrogated in a reasonable milling time. [27] This problem has been addressed by the introduction of a new generation of dual-beam FIB/SEMs which utilize an inductively coupled plasma ion source with a heavy inert gas feed (typically Xe) on the ion column. [28, 29] These plasma FIB (PFIB) columns offer milling rates up to two orders of magnitude higher than those that can be achieved using GFIB. [30, 31] They also offer advantages for materials that undergo undesirable chemical interactions with the Ga ions during GFIB milling. However, to date there have been very few studies that consider the relative merits of the GFIB and PFIB approaches for specific complex materials systems.

Here we present a study of the microstructural details in a crack-free biomimetic HA coating deposited onto a Ti-6Al-4V substrate by immersing it in a modified simulated body fluid (m-SBF) at body temperature and pH. Conventional microstructural studies on such coatings have been reported previously. [22] In the current study we have concentrated on the use of dual-beam FIB/SEM techniques to obtain high quality cross-sectional specimens for SEM and TEM experiments. These specimens were prepared using both GFIB and PFIB instruments to reveal the relative advantages of these approaches for such coated samples.
2.2 Materials and methods

2.2.1 Biomimetic HA coating preparation

Ti-6Al-4V discs with a thickness of 2 mm and a diameter of 15 mm were surface treated as described previously. [2, 22] The Ti-6Al-4V discs were sandblasted with 46 grit SiC, followed by acid etching with a mixed solution of HCl and HNO₃ and then with a hot acid mixture of HCl and H₂SO₄. The acid-treated discs were then exposed to UV irradiation for 8 h. Biomimetic HA coating was produced by immersing the surface-treated Ti-6Al-4V in m-SBF. The ion concentrations of m-SBF and human blood plasma are listed in Table 2.1. The initial pH of the m-SBF solution was adjusted to 6.4, 6.5, 6.6, and 6.7 using HCl and HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid), and the coatings formed at different initial pH values were labeled as HA-6.4, HA-6.5, HA-6.6, and HA-6.7, respectively. The coating formation was carried out by soaking the Ti-6Al-4V discs in the m-SBF at a temperature of 37 °C for 24 h. The HA coated Ti-6Al-4V discs were then washed gently with deionized water and air-dried overnight.
Table 2.1 Ion concentrations (mM) of human blood plasma and m-SBF.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Human blood plasma (mM)</th>
<th>m-SBF (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>142.0</td>
<td>109.9</td>
</tr>
<tr>
<td>K⁺</td>
<td>5.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>2.5</td>
<td>7.9</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>103.0</td>
<td>111.2</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>27.0</td>
<td>17.5</td>
</tr>
<tr>
<td>HPO₄²⁻</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

2.2.2 Microstructural characterization

The phase(s) present in the coatings were evaluated by X-ray diffraction (XRD) using a Bruker AXS D2 Phaser diffractometer with CuKα radiation. Data were acquired at 20 values of 5° to 60° using a step size of 0.02° and a scan rate of 0.5 °/min.

Observations of surface morphology and FIB sectioning were performed in two FEI Helios dual-beam FIB/SEMs: a Nanolab 460F1 GFIB and a Xenon PFIB in the UConn/Thermo Fisher Scientific Center for Advanced Microscopy and Materials Analysis (CAMMA). Both instruments are equipped with EasyLift nano-manipulators, and the GFIB also has a flip-stage and a scanning transmission electron microscopy (STEM) detector for improved final thinning of TEM specimens. To reduce charging effects during SEM imaging and FIB milling, a thin conducting layer of Au was sputtered onto the HA coating surfaces using a Polaron E5100 coating unit.

Secondary electron (SE) SEM images of the HA coating surface morphology
were obtained using the Everhart-Thornley and in-lens detectors on the electron columns. All sectioning was performed with the coating surface perpendicular to the optic axis of the ion column (at 52° to the electron column). Prior to cross-sectional milling, a 3-µm-thick Pt layer was deposited onto the chosen region of the HA coating surface to protect the coating from ion beam damage. This Pt deposition was performed in two steps. Firstly, a 1-µm-thick Pt layer was deposited using a 5 kV electron beam (E-beam) to crack the organometallic Pt precursor. A further 2-µm-thick Pt layer was then deposited using the ion beam (I-beam). [32] The ion column accelerating voltages used in this process were 30 kV for the GFIB and 8 kV for the PFIB.

For cross-sectional SEM studies a stepped, wedge-shape was first milled out using a regular cross-section (RCS) pattern, so that the microstructure could be observed on the vertical wedge wall using the electron column. The RCS pattern is a multi-pass scan method used to mill the initial wedge. In this approach, the beam is scanned through the entire pattern and then repeats for a set number of passes. For the GFIB sectioning study, the wedge was 20 µm long, 10 µm wide, and 5 µm deep, giving a wedge angle of ≈ 45°. In the PFIB study, larger wedges with the same angle were produced. In these cases, the dimensions were 60 µm long, 12 µm wide and 6 µm deep. After the wedge was milled, the vertical wedge wall was polished using an ion beam at a reduced beam current to eliminate curtaining and other milling artefacts. Two different pre-set milling patterns were used for the process, namely cleaning cross-section (CCS) pattern, and rectangle (Rec) pattern. The CCS pattern is
processed line by line with each line containing a set number of passes, and the Rec pattern repeatedly scans the beam over a rectangular array of equally spaced positions.

For TEM studies, specimens were produced in a similar manner, but a second wedge was milled on the other side of the protective Pt strap so that the vertical wedge walls defined a pre-thinned FIB lamella. The lamella was then attached to the EasyLift W probe, cut free from the surrounding material and transferred to a Cu omni-grid. Final thinning of the lamellae to electron transparency was performed on the grid using the ion beam with iteratively decreased ion currents to minimize any residual ion damage.

The TEM specimens were examined using an FEI Talos F200X S/TEM operating at an accelerating voltage of 200 kV. This instrument is equipped with a Super-X silicon drift detector (SDD) energy dispersive X-ray spectrometry (EDXS) system, which allows for a rapid acquisition of spectrum images for elemental mapping.

2.3 Results and discussion

Figure 2.1 is a series of SE SEM images showing the surface morphologies of the HA coatings produced by immersing surface-treated Ti-6Al-4V substrates in an m-SBF solution at different initial pH values for 24 h. When the initial pH was 6.4, a thin, crack-free dense mineral coating was formed (HA-6.4, Figure 2.1A). As the coating is very thin, the morphology of the underlying Ti-6Al-4V substrate can be observed clearly. When the initial pH was 6.5, a smooth coating with a few minor cracks due to dehydration shrinkage was formed, as indicated by the black arrows in
Figure 2.1B (HA-6.5). [33] A crack-free coating was formed when the initial pH was 6.6 (HA-6.6, Figure 2.1C). In contrast, the coating produced at an initial pH of 6.7 exhibits mudflat cracking with globules of HA scattered on the surface (HA-6.7, Figure 2.1D). It has been observed previously that the HA-6.5, HA-6.6, and HA-6.7 coatings exhibit a porous and rough coating surface, which has been discovered to be beneficial to cell attachment and the initiation of bone formation and integration. [34]

![SE SEM images of HA coatings formed at different initial pH values: HA-6.4 (A), HA-6.5 (B), HA-6.6 (C), and HA-6.7 (D). The black arrows in (B) indicate cracks formed on the surface. Examples of the corresponding XRD patterns are shown in Figure 2.2. These data indicate that an HA coating can be formed at all initial pH values selected. However, very weak HA peaks together with strong peaks from the hexagonal-close-packed (HCP) α phase of the Ti-6Al-4V substrate were observed for the HA-6.4 coating. This](image_url)
indicates that a very thin layer of HA coating was formed on the Ti-6Al-4V substrate, which is in agreement with the SEM observation in Figure 2.1A. Strong peaks for HA at 2θ=26° ((002) plane) and 31-34° (overlap of (211), (112), and (300) planes) were observed for HA-6.5, HA-6.6, and HA-6.7 coatings, indicating that a thick HA coating has been formed on the Ti-6Al-4V substrate. However, the broad diffraction peak around 31-34° suggests that the HA coatings were not fully crystalline. A preferred c-axis orientation of the HA crystals can be inferred from the high intensity of the (002) peak compared to the (112) peak. [35] Diffraction peaks from the HCP α phase of the Ti-6Al-4V substrate were present in all of the XRD patterns.

![XRD spectra of HA coatings on Ti-6Al-4V substrates after 24 h of](image)

**Figure 2.2.** XRD spectra of HA coatings on Ti-6Al-4V substrates after 24 h of
immersion in m-SBF.

Based on the SEM and XRD data, it was found that the best-quality coatings were HA-6.5 and HA-6.6 coatings. As such, these two coatings were selected for more detailed cross-sectional investigation using FIB techniques. As shown in Figure 2.3, HA-6.5 and HA-6.6 coatings exhibit gradient structures: the HA coating adjacent to the substrate was dense, and it became more and more porous towards the coating surface. It is the first time that we clearly observed a gradient, porous cross-sectional microstructure of the coating. When cross-sections produced are observed by the conventional method (fracturing/grinding), only a dense structure was observed, but the porous gradient structure was completely ruined by the sample preparation process. [22, 36] Moreover, it has been shown that the pore size of HA-6.5 coating at the outermost surface was larger than that of the HA-6.6 coating. Also, the HA-6.5 coating was thicker than HA-6.6 coating during the same immersion time (24 h), which is in agreement with our previous observations.[12]

Figure 2.3. Cross-sectional SE SEM images of HA coatings on Ti-6Al-4V substrates: HA-6.5 (A) and HA-6.6 (B). White arrow in image (A) is a crack induced by the ion beam of PFIB and black arrow in image (A) is a crack between the HA-6.5 coating
and the Ti-6Al-4V substrate.

The formation of the HA coating can be expressed by Equations (1) and (2). As discussed in our previous work, the pH of the m-SBF solution is determined by the initial pH of the solution and the amount of HCO$_3^-$ in the solution. The pH profile of the m-SBF during the coating process has been investigated in details as reported previously. [12, 22] Briefly, at the initial stage of coating formation, the pH of the solution increases due to the decomposition of HCO$_3^-$, as shown in Equation (2). Simultaneously, Ca$^{2+}$ and HPO$_4^{2-}$ ions are adsorbed onto the substrate surface to form HA nuclei, as expressed in Equation (1). Previous research reported that a high concentration of HCO$_3^-$ ions in m-SBF resulted in a dense apatite coating. [2, 22, 37] Thus, in the initial stage of coating formation, a lower pH leads to slower coating formation, resulting in a dense coating adjacent to the substrate. With the decomposition of HCO$_3^-$, the increase in pH and the decrease in the HCO$_3^-$ concentration leads to the formation of a porous HA coating toward the coating surface. When the initial pH of m-SBF was 6.5, more HCO$_3^-$ ions were needed to bring up the pH than for an initial pH=6.6, thus less HCO$_3^-$ ions remained, contributing to the larger pore size observed toward the surface of the HA coating.

$$5\text{Ca}^{2+}+3\text{HPO}_4^{2-}+4\text{OH}^-\rightarrow\text{Ca}_5(\text{PO}_4)_3\text{OH}+3\text{H}_2\text{O}$$  \hspace{1cm} (1)

$$\text{HCO}_3^-\rightarrow\text{CO}_2+\text{OH}^-$$ \hspace{1cm} (2)

As shown in Figure 2.3A, there is a large crack between the HA-6.5 coating and the Ti-6Al-4V substrate (black arrow in Figure 2.3A); this is consistent with the cracks observed on the coating surface, as indicated in Figure 2.1B. Cracks at the
surface are due to the dehydration shrinkage, which significantly reduces the interfacial bonding strength. [21] Thus, the cracks on the surface indicate weak bonding between the HA-6.5 coating and the Ti-6Al-4V substrate. However, no cracks were observed between the HA-6.6 coating and Ti-6Al-4V substrate, indicating a strongly bonded HA coating was formed (Figure 2.3B). This might be because HA-6.6 coating is thinner and denser compared to HA-6.5 coating. Because a well-bonded HA coating formed at an initial pH=6.6, HA-6.6 coating system was selected for further cross-sectional microscopy investigations.

In principle, cross-sections of the coating can provide critical information regarding the interface as well as about the coating itself. However, due to the significant differences between the stiffness and density of the coating and substrate, it becomes extremely difficult to create the cross-sectional samples without damaging the microstructure both at the interface and within the coating itself. FIB instruments are powerful tools for studies of interfaces between materials with such different properties, but there are certain drawbacks and instrumental artefacts. A common artefact produced during FIB milling is the “curtaining” effect in which fluctuations in the ion beam current, position and/or milling efficiency (due to sample inhomogeneities) lead to undulations on the surface of an ostensibly planar FIB cut. This effect can significantly degrade the resolution of images obtained from FIB-cut cross-sections. Ideally, cross-sectional FIB cuts should be free of such curtaining effects. After the initial wedge milling using the RCS pattern, the microstructure of the cross-section was obscured by the curtaining (Figure 2.4). Then CCS and Rec
patterns were applied to obtain cross-sections with reduced curtaining in both GFIB and PFIB, and the efficiencies of these two approaches were compared.

**Figure 2.4.** Gas-assisted deposition of Pt layer at 8 kV and 30 kV ion beam voltage in PFIB.

**Figure 2.5.** SE SEM images of HA-6.6 coating on Ti-6Al-4V substrate from cross-sections prepared using GFIB (A, B) and PFIB (C, D). The cross-sections were polished using CCS pattern (A, C) or Rec pattern (B, D). Cracks indicated by white
arrows in HA coating in image (C) were induced by the CCS process in PFIB.

Figure 2.5 shows the cross-sectional images of HA-6.6 coated Ti-6Al-4V substrates cleaned by Rec and CCS patterns in GFIB and PFIB. First, a 3-µm-thick Pt layer was deposited onto the area of interest by gas-assisted Pt deposition to protect the HA coating surface, allowing the features close to or at the surface to be analyzed without damage. [38] In GFIB, an ion beam with accelerating voltage of 30 kV was used to deposit the Pt-protective layer, however, in PFIB a dense Pt layer was obtained at 8 kV. Increasing the ion beam accelerating voltage to 30 kV in PFIB led to the formation of a porous Pt layer (Figure 2.4). In GFIB, wedges 20 µm in length, 10 µm in width and 5 µm in depth were milled using RCS, and then cleaned up using the CCS and Rec patterns as shown in Figures 2.5A and 2.5B, respectively. In PFIB, wedges 60 µm in length, 12 µm in width and 6 µm in depth were milled using RCS. The images obtained from after cleaning up using CCS and Rec patterns are shown in Figures 2.5C and 2.5D, respectively. Even though much larger wedges were milled using PFIB, the total time for milling and the subsequent clean-up was less than those used in GFIB. As shown in Figure 2.5, in GFIB, the Rec pattern proved to be more suitable for the production of curtaining-free cross-sections than the CCS pattern at the same ion beam current. However, the Rec pattern is as efficient as the CCS pattern in creating a reasonable cross-section using PFIB. When CCS was applied to clean up PFIB cross-sections, cracks were formed in the HA coating (white arrows in Figure 2.5C). Overall, the detailed microstructure of the HA coating and the interface between the coating and the substrate have been well preserved by both GFIB and
PFIB, but GFIB produces a cross-section with higher quality than PFIB, which may be due to the smaller ion beam spot size achievable in GFIB. [27]

Figure 2.6. An extraction sequence of a thin lamella from the bulk in GFIB (A-H).

The thickness of the TEM specimen was confirmed by STEM in GFIB (I).

Figure 2.7. The thin lamella from the site of interest before extraction (A) and after mounted onto copper omni grids (B) in PFIB.

TEM experiments were performed to study the interface between the HA-6.6 coating and the Ti-6Al-4V substrate on the nano-scale. The TEM specimens were
prepared as thin lamellae from the sites of interest following the extraction sequence presented in Figure 2.6. Firstly, a 3-µm-thick Pt strap was deposited onto the area of interest (Figure 2.6A); this served as a protective layer for the HA coating during the milling process. Wedge-shaped trenches were then milled out on either side of the Pt strap (Figure 2.6B), defining a lamella that was then thinned further in preparation for lift-out (Figures 2.6C and 2.7A). Due to the larger beam size and higher beam current in the PFIB, some of the lamellae sustained significant damage during thinning (data not shown), as indicated by the cracks in the HA coating in Figure 2.5C. As a result, for PFIB a thicker lamella was prepared (~4 µm; Figure 2.7) than for GFIB (~900 nm, Figures 2.6C and 2.6H). In both cases, the lamella was cut free at the bottom and on one side, the W EasyLift probe was attached to the Pt strap by depositing additional Pt at the point of contact, the lamella was cut free on the other side and was then lifted out (Figure 2.6D). The lamella was then transferred to a copper omni grid (Figure 2.6E), attached to the grid at two corners by depositing Pt in these locations (Figure 2.6F), and then cut free from the W EasyLift probe (Figure 2.6G). The TEM lamella was finally thinned to electron transparency using the ion beam with iteratively decreasing ion currents. In GFIB, STEM imaging at an electron column accelerating voltage of 30 kV was used to confirm that the specimen was thin enough for TEM analysis (Figure 2.6I).
Figure 2.8. Data obtained from the TEM specimens prepared by GFIB: BF TEM image (A) and HAADF STEM image (B). The inset to (A) is the SAED pattern from the coating. C-F: Compositional maps obtained from the region in (B), showing the distribution of Ca (C), P (D), Ti (E), and Ga (F).
Figure 2.9. Data obtained from the TEM specimens prepared by PFIB: BF TEM image (A) and HAADF STEM image (B). C-F: Compositional maps obtained from the region in (B), showing the distribution of Ca (C), P (D), Ti (E), and Xe (F).
The nano-scale microstructure of the HA coatings on Ti-6Al-4V was investigated by S/TEM analysis of the TEM specimens prepared by both GFIB and PFIB, and examples of the data are shown in Figures 2.8 and 2.9, respectively. In each case, these include a bright field (BF) TEM image, a high angle annular dark field (HAADF) STEM image, and compositional maps for Ca, P, Ti and the appropriate milling ion (Ga and Xe, respectively). The maps were obtained from spectrum imaging experiments in which full EDXS spectra were acquired at each pixel, and then standard-less quantification was performed at each point. Thus, Figures 2.8C-F and 2.9C-F are compositional maps (rather than X-ray maps) and the intensities in these maps are proportional to the measured concentrations of the various species.

The BF TEM and HAADF STEM images showed that the thickness of the coating was about 1.5-2.5 µm, and no structural defects were observed between the HA coating and the Ti-6Al-4V substrate, indicating that a strong bond has been formed at the interface. These images also reveal the details of the gradient structure in the HA coating, which is consistent with the SEM observations in Figures 2.3 and 2.5. A dense coating is formed adjacent to the substrate to facilitate the strong bonding between the coating and the substrate, and there is a highly porous surface that could contribute to coating dissolution and cell attachment. [33] The preferential crystallite alignment was also observed by STEM (Figure 2.8). Previous research reported that the HA crystal dimensions decreased with increasing HCO_3^− ion concentration, and that the crystals were more equi-axed and less needle-like at a higher concentrations. [39] In the initial stage of coating formation, a smaller crystal size and denser coating
was formed adjacent to the substrate due to a higher HCO$_3^-$ ion concentration and a lower pH. [2, 22, 35, 39] As the immersion time increases, the HCO$_3^-$ ions decompose to bring up the pH, thus the dimensions of the HA crystal increased, and crystallites elongated preferentially perpendicular to the surface, which is in accordance with the XRD results (Figure 2.2). [35, 40] The corresponding mineral phase of the coating was evaluated by selected area electron diffraction (SAED) coupled to TEM, and the inset pattern in Figure 2.8A indicates that the HA coating is poly-crystalline, which resembles the apatite structure in natural bone. [33] The compositional maps show that the distributions of Ca and P in the coating are uniform, and thus there is no change in composition associated with the structural gradient. These maps also reveal that there is some implantation of Ga and Xe ion in the TEM specimens prepared by GFIB and PFIB, respectively (Figures 2.8F and 2.9F). Such implantation is typical in FIB analyses,[41] and does not appear to have caused any significant changes in the structure of the coatings. We note, however, that the data from the TEM specimen produced by PFIB (Figure 2.9) appears to be at slightly lower resolution and shows somewhat more residual curtaining than the specimen produced by GFIB (Figure 2.8). This is consistent with there being low-level damage and artefacts due to the coarser, more powerful Xe ion beam in the PFIB.

2.4 Conclusions

A crack-free biomimetic HA coating was formed by immersing a surface-treated Ti-6Al-4V substrate in an m-SBF solution at an initial pH of 6.6. Both GFIB and PFIB were used to create curtaining-free cross-sections and TEM specimens of the
coating, and the capabilities of these two tools were compared. Although GFIB and PFIB share a lot of similarities, there are also significant differences, including the optimal voltage of ion-beam-induced Pt deposition, the TEM sample preparation process, and the efficiency and the quality of the cross-sectional cuts produced. Particularly, the GFIB is more beneficial for preparing the high-quality final finish of the sample, while the PFIB efficient for creating a large cut of the sample. Also, both SEM and TEM observations confirmed that a well-bonded HA coating has been formed on the Ti-6Al-4V substrate. The coating demonstrates a unique gradient structure, with a dense layer adjacent to the substrate to facilitate a strong bonding, and a porous structure at the surface to allow for bone cell attachment.
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3. Cross-sectional microstructure study of collagen-apatite composite coating on titanium alloy

Abstract

A biomimetic bone-like collagen-hydroxyapatite (Col-HA) composite coating was formed on a surface-treated Ti-6Al-4V alloy substrate via simultaneous collagen self-assembly and hydroxyapatite nucleation. The coating process has been carried out by immersing sand-blasted, acid-etched and UV irradiated Ti-6Al-4V alloy in type I collagen-containing modified simulated body fluid (m-SBF). The surface morphology and phase composition of the coating were characterized using scanning electron microscopy (SEM), X-ray diffraction (XRD), and Fourier transformation infrared spectroscopy (FTIR). More importantly, dual-beam FIB/SEMs with either gallium ion source (GFIB) or xenon plasma ion source (PFIB) were used to investigate the cross-sectional features of the biomimetic Col-HA composite coating in great details. As a result, the cross-sectional images and thin transmission electron microscopy (TEM) specimens were successfully obtained from the composite coating. Both the cross-sectional SEM and TEM results confirmed that the Col-HA coating had a similar microstructure to pure hydroxyapatite coating that consists of a gradient coating at the cross-section, with a dense layer adjacent to the interface between the titanium substrate and the coating, while a porous structure at the coating surface.

Keywords: Collagen-hydroxyapatite coating, Dual Beam FIB, TEM sample, cross-section, microstructure
3.1 Introduction

Ti-6Al-4V (Ti-6%Al-4%V (wt.%)) alloy has been widely used as load-bearing implants due to its excellent mechanical properties, corrosion resistance and biocompatibility. [1, 2] However, there is still concern of the poor bone-implant integration due to the formation of a fibrous tissue layer surrounding the Ti-6Al-4V implants. [3] Thus, a layer of hydroxyapatite (HA) coating is usually deposited on the surface of the Ti-6Al-4V implants to improve their bioactivity, osteoconductivity and osteointegration. [4-6] HA, the main component of natural bone, exhibits excellent biocompatibility, osteoconductivity and bone-bonding ability. [7] A variety of deposition techniques, such as plasma spray process, hot isostatic pressing, electro-deposition, sol-gel coating, and biomimetic method have been applied to successfully deposit HA coatings on the Ti-6Al-4V implants. [5, 8-13] Collagen, the most abundant protein in bone, is effective in accelerating cellular adhesion and promoting cell proliferation. [14, 15] Thus, a composite coating comprised of both collagen and HA will better mimic the composition of natural bone and thereby improve the bone-implant integration.

Collagen/HA (Col-HA) composite coating has been deposited onto the surface of Ti-6Al-4V substrates via electrolytic deposition, spin coating, and biomimetic immersion over the past few years. [16-18] Due to the sensitivity of collagen, there are limited methods available to deposit Col-HA composite coatings. For example, plasma spraying and thermal spray, which are two widely used techniques to deposit HA coatings, are impractical to deposit collagen composite coating due to the high
operation temperatures. [3] Biomimetic coating method, on the other hand, is a promising method due to its low cost, easy operation, low temperature, and the ability to incorporate biomolecules such as proteins and collagens. [7, 17, 19, 20] Thus, Col-HA composite coatings have been successfully deposited on the Ti-6Al-4V substrates. [7, 17, 18, 21]

The reliability of the coating and the bonding strength between coating and the substrate are very important for the success of the implantation. [3] Because most of the implant failures result from the debonding between the coating and the substrate, as well as the dissolution of the coating. [22, 23] Cross-sectional studies of the microstructure of the coating and the interface between the coating and the substrate are essential to investigate the coating stability and bonding strength. [5, 24] However, traditional techniques for producing cross-sections, such as coating fracturing or coating cutting, grinding and polishing, suffer from losing microstructure details or resulting in the debonding of the coating due to the plastic deformation. [5, 7, 25]

Meanwhile, focused ion beam (FIB) microscopy has been widely used as a modern tool for structural microanalysis and micro-machining of materials. The most important application of FIB is to prepare cross-sectional milling and transmission electron microscopy (TEM) sample, since the microstructure of the sample is maintained without damage. [26] The most advanced FIB instrument incorporates both an FIB column and a scanning electron microscopy (SEM) column in a single system, allowing simultaneous milling and imaging, serial sectioning and many other functionalities. [27] The dual beam FIB/SEM is capable of preparing cross-sections
and TEM specimens made of heterogeneous, fragile, and porous materials and structures. [5] The most widely used FIB/SEM is equipped with gallium liquid ion source (GFIB), however, is limited with slow removal rate. [28] Thus, a dual beam FIB/SEM system with plasma ion source (PFIB), which offers two orders of magnitude higher milling rate than that of GFIB, was used in the current study. [29, 30] In our previous work, a comparison study has been conducted to investigate the microstructure of the biomimetic HA coating on Ti-6Al-4V substrates using dual beam FIB/SEM. The capabilities of GFIB and PFIB were also compared. [5] However, due to ion bombardment and beam heating effects of FIB, the cross-sectioning the sample using FIB is challenging when the sample involves polymer, which has a low heating conductivity. [31]

In the present work, a biomimetic Col-HA coating was deposited on the surface-treated Ti-6Al-4V alloy through simultaneous collagen self-assembly and HA crystal nucleation. Dual-beam FIB/SEM techniques were used to produce high quality cross-sections and TEM samples to study the cross-sectional microstructures of the composite coating and the interface between the coating and the substrate. Finally, the microstructure of the composite coating was compared with pure HA coating. Additionally, a comparison between GFIB and PFIB in characterization of Col-HA composite coatings was conducted.

3.2 Materials and methods

3.2.1 Biomimetic Col-HA coating formation

Ti-6Al-4V discs with a thickness of 2 mm and a diameter of 15 mm were
polished using 240 grit silicon carbide abrasive paper (LECO Corporation, USA), and
then treated by sandblasting and acid etching (SLA) techniques, followed by UV
irradiation for 8 h. SLA is a widely used surface treatment technique for Ti-based
implants. [5] Briefly, Ti-6Al-4V discs were sandblasted with 46 grit SiC, and then
acid etched by a mixed acid solution of HCl and HNO₃, followed with a hot acid
mixture of HCl and H₂SO₄ for 5 min. After the surface treatment, the Ti-6Al-4V discs
were immersed in the m-SBF containing collagen (0.05 g/L) to produce biomimetic
Col-HA coatings. Type I collagen was extracted from rat tails and purified based on
the protocol reported by Rajan et al.. [32] The m-SBF was prepared as reported
previously (Table 3.1), [5] and the initial pH of the solution was adjusted to 6.75 by
During the coating process, urea with a concentration of 0.5 mol/L was added to the
m-SBF containing collagen solution to slow down the collagen polymerization
process. [7] The coating formation was carried out by soaking the surface-treated
Ti-6Al-4V discs in the collagen-containing m-SBF solution at a temperature of 37 °C
for 24 h, as shown in Figure 3.1. As a control, the HA coating was formed by
immersing the surface treated Ti-6Al-4V substrates in the m-SBF solution with an
initial pH of 6.6 at 37 °C for 24 h. Such formed Col-HA and HA coated Ti-6Al-4V
discs were then washed gently with deionized water and dried in air for 24 h.
Table 3.1. Ion concentration (mM) of human blood plasma and m-SBF.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Human blood plasma (mM)</th>
<th>m-SBF (mM)</th>
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<td>Na⁺</td>
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<td>HCO₃⁻</td>
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</tr>
<tr>
<td>HPO₄²⁻</td>
<td>1.0</td>
<td>3.1</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

3.2.2 Materials characterization

The phases of the Col-HA and HA coated Ti-6Al-4V alloys were evaluated by X-ray diffraction (XRD) using a Bruker AXS D2 Phaser Diffractometer with CuKα radiation. The diffraction data was collected using a step size of 0.02° and a scan speed of 0.5 °/min, and the 2θ range is 5° to 60°. The Col-HA and HA coatings were scratched from the Ti-6Al-4V alloys, and the coatings were then characterized using attenuated total reflection-Fourier transformation infrared spectroscopy (ATR-FTIR). A Nicolet Magna 560 FTIR spectrophotometer (Artisan Technology Group®, IL) with an ATR setup was used to collect the infrared spectra in the range of 4,000-400 cm⁻¹.

The surface morphology and surface roughness of the Ti-6Al-4V substrates, Col-HA coatings and HA coatings on the substrate were observed using FEI Helios dual-beam FIB/SEMs: a Nanolab 460F1 GFIB and a Xenon PFIB in the UConn/Thermo Fisher Scientific Center for Advanced Microscopy and Materials
Analysis (CAMMA). The milling and observation of the cross-sections of the Col-HA coating on Ti-6Al-4V alloy were also performed in the Helios dual-beam FIB/SEMs. Both GFIB and PFIB are equipped with EasyLift nano-manipulators to facilitate TEM sample preparation, and the GFIB also equipped with a flip-stage and a scanning transmission electron microscopy (STEM) detector for improved final TEM sample thinning process. Before SEM imaging and FIB milling, both the Col-HA and HA coatings were sputter coated with a conducting layer of Au using a Polaron E5100 coating instrument.

The applications of GFIB and PFIB to prepare the cross-sections and TEM samples have been described previously, [5] and the detailed process is as following: SEM images were obtained using through-lens detector (TLD) or Everhart-Thornley detector (ETD) in the electron columns. All the sectioning process was performed with the coating surface normal to the ion column, and 38° to the electron column. Firstly, a 2-µm-thick Pt strap was deposited on the coating surface at the site of interest, with the aim to protect the coating from ion beam damage. The Pt layer was deposited using either electron beam or ion beam to crack organometallic Pt precursor. [33] The ion beam voltages used in the Pt deposition were 30 kV in GFIB and 8 kV in the PFIB.

Secondly, a stepped, wedge-shape was milled out using regular cross-section (RCS) pattern, so that the microstructure of the coating and the interface between the coating and the substrate could be exposed on the vertical wedge wall, and be observed by SEM column. In the GFIB, the size of the wedge was 20 µm in length,
10 µm in width and 5 µm in depth, and in the PFIB, a large wedge was milled, and the dimensions were 100 µm in length, 10 µm in width and 5 µm in depth.

Thirdly, after the initial wedge was milled, the vertical wedge wall was polished by GFIB and PFIB using an ion beam with reduced accelerating current to reduce the curtaining effects and other milling artefacts. Three different pre-set milling patterns were used for the polishing process: RCS pattern, cleaning cross-sectional (CCS) pattern, and rectangle (Rec) pattern.

For the TEM sample preparation, 3-µm-thick Pt strap was deposited at the site of interest, and then two wedges were milled on both sides of the Pt strap, producing a pre-thinned FIB lamella. A U-shape was cut on the vertical wedge wall of the pre-thinned lamella, leaving 2 small pieces on the surface still attached to the bulky substrate. Then this lamella was welded to the EasyLift W probe, cut totally free from surrounding material and transferred to a Cu omni-grid. Finally, the lamella was further thinned to electron transparency on the grid using reduced ion current to minimize ion damage.

The TEM samples were examined using an FEI Talos F200X S/TEM operating at an accelerating voltage of 200 kV in the UConn/Thermo Fisher Scientific CAMMA. This instrument is equipped with a Super-X silicon drift detector (SDD) energy dispersive X-ray spectrometry (EDS) system, allowing for a rapid acquisition of spectrum images for elemental mapping.

3.3 Results and discussion

Collagen-HA (Col-HA) composite coatings have been obtained by a biomimetic
coating process, in which Col-HA coatings were deposited on the Ti-6Al-4V substrates by immersing them in the collagen molecules containing m-SBF at 37 °C and initial pH of 6.75 for 24 h (Figure 3.1). Figure 3.1 shows the process of the formation of biomimetic Col-HA coatings on the surface-treated Ti-6Al-4V substrates. Ti-6Al-4V substrates were polished using a 240 grit silicon carbide abrasive paper, and the scratches were observed due to the silicon carbide abrasive paper (Figure 3.1A). Then the polished substrates were sand-blasted with 46 grit silicon carbide and acid treated (SLA treatment, Figure 3.1B). The acid treatment produced a uniform rough surface, which consists of sharp peaks at the microscale level, resulting in higher surface areas. [35, 36] The acid treated Ti-6Al-4V substrate was then irradiated using UV light at a wavelength of 254.8 nm for 8 h, resulting in a photocatalytic chemical reaction. The surface chemistry alteration by photochemical reaction decontaminates hydrocarbon and increase hydrophilicity by converting Ti⁴⁺ sites to Ti³⁺ sites. [7, 35, 37] Thus abundant Ti-OH or Ti-O⁻ groups are formed on the surface of the substrate when in contact with SBF solution, imparting the surface with negative charge and high surface energy. The negative charge and high surface area of the pre-treated surface will attract and absorb Ca²⁺ to the surface, contributing to the precipitation and deposition of coating on the Ti-6Al-4V substrate. [25] Then the Ti-6Al-4V substrates with a bioactive surface were immersed in the collagen-containing m-SBF (Figure 3.1C).
Figure 3.1. Illustration of the formation process of biomimetic Col-HA composite coatings on the surface-treated Ti-6Al-4V substrates: Surface morphology of the Ti-6Al-4V substrate after polishing using silicon carbide abrasive paper (A) and SLA treatment (B), and coating formation by immersing surface-treated Ti-6Al-4V substrate in the collagen-containing m-SBF solution (C).

Figure 3.2 shows the surface morphologies of the Col-HA coatings on the Ti-6Al-4V substrates which is produced as described above. After 24 h of immersion in the collagen-containing m-SBF, a crack-free homogeneous coating was formed on the surface of the treated Ti-6Al-4V substrates at 37 °C with an initial pH of 6.75 (Figures 3.2A and 3.2B). The coating demonstrates a porous and rough surface with numerous globules, and the roughness of the surface was clearly observed when the Col-HA coated Ti-6Al-4V substrate was tilted 52° in the Dual Beam FIB/SEM (Figure 3.2C). Compared with the HA coatings on the Ti-6Al-4V substrates, the globule size of Col-HA coating was significantly smaller than that of HA coating (Figure 3.3). In our previous research, a crack-free HA coating was formed at an initial pH of 6.6 (Figure 3.3A). [5] In contrast, higher initial pH was required to induce Col-HA composite coating, which is in accordance with the report by Xia et al. [7]
Figure 3.2. SE SEM images of Col-HA composite coatings on Ti-6Al-4V substrates.

Col-HA coated Ti-6Al-4V alloy was titled 52° (C).

Figure 3.3. SE SEM images of the surface morphologies of HA (A) and Col-HA (B) coatings on Ti-6Al-4V substrates.

Figure 3.4 shows the XRD spectra and ATR-FTIR patterns of HA and Col-HA coatings formed after 24 h of immersion. Characteristic peaks of HA phase at 2θ=26.7° ((002) plane), 31.7-34.3° (overlap of (211), (112), and (300) planes), and 53.8° ((004) plane) were observed for Col-HA and HA coatings. The XRD spectra indicate that the crystalline HA presents in both the Col-HA and HA coatings. Further, two additional peaks at 28.9° ((102) plane) and 50.2° ((213) plane) were only observed in the HA coating. [38, 39] However, the broad diffraction peak around 31.7-34.3° indicates that the HA phase in both the Col-HA and HA coatings are poorly crystallized. Weaker HA peaks together with strong peaks from the hexagonal phase of the Ti-6Al-4V substrate were observed for the Col-HA coatings. In comparison, the intensity of the Ti peaks
detected in the HA coating are much lower (Figure 3.4A). This indicates that a thinner Col-HA coating was formed on the Ti-6Al-4V substrate compares with the HA coating. Diffraction peaks for the hexagonal phase of the Ti-6Al-4V substrate were observed in all of the XRD spectra.

![Figure 3.4. XRD (A) and ATR-FTIR (B) spectra of HA and Col-HA coated Ti-6Al-4V substrates.](image)

HA and Col-HA coatings on the Ti-6Al-4V substrates were scratched from the substrates and ATR-FTIR was also applied to characterize the coatings. Typical characteristic peaks of the HA phase were shown in Figure 3.4B. The P=O stretching mode (~1019.5 cm\(^{-1}\)) and P-O bending mode (~597.8 cm\(^{-1}\) and ~560.6 cm\(^{-1}\)) indicate the phosphate ions, and the peak at 873.8 cm\(^{-1}\) is due to the joint contribution from carbonate and HPO\(_4^{2-}\) ions. [40] The peaks at 1448.0 cm\(^{-1}\) and 1414.5 cm\(^{-1}\) were assigned to the CO\(_3^{2-}\) groups. The absorption peak at 1638.4 cm\(^{-1}\) was attributed to the stretching vibration of amide I, which is the characteristics of collagen, indicating the presence of collagen in the coating. [20, 41]
Figure 3.5. SE SEM images of the cross-sections of Col-HA composite coatings on Ti-6Al-4V substrates prepared using GFIB (A1, A2, A3) and PFIB (B1, B2, B3). The cross-sections were polished using Rec pattern (A1, B1), CCS pattern (A2, B2), and RCS pattern (A3, B3). White arrows in B1, B2, and B3 indicate the cracks between Pt layer and Col-HA coatings.

FIB was then used to mill the Col-HA coated Ti-6Al-4V substrate to study the details of cross-sectional microstructure of the coating and the interface between the coating and the substrate. As described in our previous work, [5] a 2-µm-thick dense Pt protective layer was deposited onto the surface of Col-HA coating at the area of interest by gas-assisted Pt deposition, protecting the surface of the coating from ion damage. [42] The ion beam accelerating voltages were 30 kV for GFIB and 8 kV for PFIB. Then RCS pattern was used to mill a stepped, wedge-shape trench, exposing the cross-sections of the Col-HA coated Ti-6Al-4V substrates and the interface between the coatings and substrates. By applying RCS pattern, which is a multi-pass scan method, the beam scanned through the entire pattern and then repeats for four passes. Then Rec, CCS, and RCS patterns with reduced current (40 pA for GFIB and
24 pA for PFIB) were applied to reduce the “curtaining” effect, which is one of the main drawbacks and artefacts of FIB milling. The curtaining effect is a result from the fluctuations of the ion beam current and position, and the inhomogeneity of sample, [5] which significantly reduces the resolution of the cross-sectional images after the initial wedge cutting. Thus, eliminating curtaining effect is usually followed after the initial wedge cutting using RCS pattern. Rec pattern is a pre-set milling pattern, in which beam repeatedly scans over a rectangular array of equally spaced positions. The CCS pattern is processed line by line with each line containing a set number of passes.

Figure 3.5 shows the cross-sectional images of Col-HA coated Ti-6Al-4V substrates polished by Rec, CCS and RCS patterns using a reduced ion beam current of 40 pA in GFIB (Figure 3.5 (A1-A3)) and an even lower ion beam current of 24 pA in PFIB (Figure 3.5 (B1-B3)). With stepwise polish of Rec, CCS and RCS patterns in reduced ion beam current, the curtaining effect on the cross-sectional images of Col-HA coatings can be attenuated in both GFIB and PFIB, disclosing more details of the coating cross-sections. Whereas it is also obvious that even low ion beam current can cause curtaining effect on the cross-sectional images of Col-HA coating. This issue is predominant in PFIB mainly owing to its larger ion beam spot size compared to that of GFIB. The cracks between Pt protective layer and Col-coating (marked by white arrows in Figure 3.5 (B1-B3)) were generated by ion beam of PFIB. [5] Nevertheless, the cross-sectional details of Col-HA coating have been preserved after a serial of milling in both GFIB and PFIB, indicating their capability of revealing inner coating microstructures.
In a previous study, our research group for the first time managed to clearly observe a gradient, porous HA coating using dual beam GFIB and PFIB. [5] In this work, a similar characteristic was observed in Col-HA coatings. Figure 3.6 shows a cross-sectional comparison of Col-HA and HA coatings. Both coatings demonstrate a relatively dense structure adjacent to the interface of the coating and the substrate, whereas they become more and more porous towards the coating surfaces. The thicknesses of Col-HA and HA coatings were 1-1.5 μm and 1.5-2.5 μm, respectively. The cross-sectional SEM images also show that Col-HA coating exhibits a less porous structure compared to the HA coating possibly owing to the fact that collagen molecule polymerization occurred simultaneously with the HA nucleation. During this process, the collagen molecules penetrated into the coating, and therefore filled some of the pores in the coating, resulting in a less porous structure.

\[ \text{Figure 3.6. SE SEM images of the cross-sections of Col-HA (A) and HA (B) coatings on the Ti-6Al-4V substrates.} \]
**Figure 3.7.** TEM sample preparation sequence from Col-HA coated Ti-6Al-4V alloy using GFIB (A-K). The TEM sample was confirmed to be electron transparency by STEM in GFIB (L).

**Figure 3.8.** TEM sample preparation sequence of Col-HA coated Ti-6Al-4V alloy using PFIB (A-K). Col-HA coated Ti-6Al-4V sample was mounted on TEM grid holder for preparing a TEM sample using PFIB (L).

To study the coating inner structure and the interface between coating and
substrate at a nanoscale, TEM samples were prepared from Col-HA coating using both GFIB and PFIB. A representative extraction sequence of TEM sample preparation in GFIB is shown in Figure 3.7. Briefly, a 3-μm-thick Pt protective layer was firstly deposited onto the area of interest (Figure 3.7B). Then two wedge-shaped trenches were milled out along with either edge of the Pt stripe and further thinned and cleaned (Figures 3.7C and 3.7D). The sample stage was then tilted to allow a 52° angle between ion beam and coating cross-section in order to completely cut through the TEM lamella (Figure 3.7E). The W Easylift probe was then moved adjacent to the top of the TEM lamella, and an extra Pt layer was deposited to connect the probe and TEM lamella (Figures 3.7F and 3.7G). After being completely free from the pristine sample, the TEM lamella was lifted out and welded onto a copper omni-grid (Figures 3.7H and 3.7I). The W Easylift probe was then removed after cutting free from the TEM lamella (Figure 3.7J), which was finally thinned using ion beam to be electron transparent (Figures 3.7K and 3.7L). A similar process was conducted on the same Col-HA sample in PFIB to achieve a TEM lamella (Figure 3.8).

The nanoscale microstructure of the TEM specimens fabricated using GFIB and PFIB were investigated using S/TEM and EDS. In each case, the representative images including a bright field (BF) TEM image, a high angle annular dark field (HAADF) STEM image, and EDS mappings for Ca, C, Ti and the milling ion (Ga and Xe, respectively) are shown in Figures 3.9 and 3.10, respectively. The BF and HAADF images confirm that the thickness of the Col-HA coating is 1-1.5 μm, and it consists of a gradient structure with a more porous layer on the surface, and a denser
structure towards the interface of the coating and the substrate. By comparing the Ca, C, and Ti maps, it is clear that a uniform, defect-free interface between the substrate and the coating was formed. There was very limited amount of milling ions (Ga or Xe) detected within the coating that underneath the Pt protective layer. The porous Pt layer on the Col-HA coating was due to the deposition voltage (30 kV) chosen in PFIB (Figure 3.10B), which is in accordance with our previous work. [5] There are many studies reported that a porous surface topography is beneficial to cell adhesion, proliferation as well as drug loading and delivery, [43-45] while a dense interface could facilitate a strong bonding between the coating and the substrate, which is an important parameter for in vivo surgical handling. [7, 46] Moreover, our previous result suggested that with the incorporation of collagen protein molecules in hydroxyapatite coating could significantly enhance the biocompatibility of the coating. [7] Therefore, the Col-HA coated Ti-6Al-4V could be used as a hard tissue replacement material, and the disclosure of the cross-sectional details in this work provides an insight on the great potential of utilizing Col-HA composite coating in clinical applications.
**Figure 3.9.** Characterization of the TEM specimens prepared by GFIB: BFTEM (A) and HAADF STEM image (B). C-F: Compositional maps obtained from the region in (B), showing the distribution of Ca (C), C (D), Ti (E), and Ga (F).

**Figure 3.10.** Characterization of the TEM specimens prepared by PFIB: BFTEM (A)
and HAADF STEM image (B). C-F: Compositional maps obtained from the region in (B), showing the distribution of Ca (C), C (D), Ti (E), and Xe (F).

3.4 Conclusions

A biomimetic collagen-containing hydroxyapatite composite coating was successfully fabricated on the surface of Ti-6Al-4V by simply immersing the surface-treated Ti-6Al-4V substrates into a collagen-containing m-SBF. The cross-sectional microstructure of the Col-HA coating has been mainly investigated by a dual-beam FIB/SEMs with either gallium (GFIB) or xenon plasma (PFIB) ion source and the capabilities of these two systems were compared. Both techniques were able to acquire cross-sectional images and obtain TEM samples from the Col-HA coating with good quality. It was revealed that the Col-HA coating consists of a dense layer adjacent to the interface between the coating and the substrate, while a porous structure towards the surface. Compared to GFIB, PFIB is more time efficient even at a lower ion beam voltage and current. However, GFIB process induces less damage to coating and the curtaining effect could be better attenuated.
References


4. Fabrication of intrafibrillar calcified collagen fibrils

Abstract

Bone is an organic-inorganic hierarchical biocomposite. Its basic building block is mineralized collagen fibrils with both intrafibrillar and extrafibrillar mineralization, which is believed to be regulated by non-collagenous proteins (NCPs) with polyanionic domains. In this study, collagen fibrils with both intrafibrillar and extrafibrillar mineralization were successfully prepared and the mechanism of biomineralization was proposed. To achieve intrafibrillar mineralization, polyacrylic acid (PAA) was used to sequester calcium and phosphate ions to form fluidic PAA-amorphous calcium phosphate (PAA-ACP) nanoprecursors. At the presence of sodium tripolyphosphate (TPP), PAA-ACP nanoprecursors were modulated to orderly deposit within the gap zone of collagen fibrils. The effect of PAA concentration on the intrafibrillar and extrafibrillar mineralization of reconstituted collagen fibrils was investigated. It was found that with the decrease of PAA concentration, the inhibitory effect of PAA on mineralization and the stability of ACP nanoprecursors decreased. As a result, more minerals were deposited both within and on the surface of the collagen fibrils.

Keywords: Intrafibrillar mineralization; extrafibrillar mineralization; non-collagenous proteins; amorphous calcium phosphate nanoprecursors; hierarchical structure
4.1 Introduction

Bone is an outstanding organic-inorganic nanocomposite, which is made of mineralized collagen fibrils. It consists of seven hierarchical levels of organization, ranging from the nanometer scale to the millimeter scale. Nanoscale hydroxyapatite (HA) crystals are embedded within the interstices of the collagen fibrils and aligned along the long axis of collagen fibrils, which are in turn present in bundles or arrays. [2, 3] These aligned mineralized collagen fibrils are arranged into a multilayered cylindrical structure rotating across the concentric lamellae and forming osteons. [4] Finally, the osteons are packed densely into compact bone or loosely into a spongy bone. [5, 6] Since mineralized collagen fibrils are the primary unit of the complex bone structure, it is important to understand the mineralization mechanism of collagen fibrils in order to replicate biomineralization of collagen fibrils in vitro.

Biomineralization of collagen fibrils includes oriented HA crystals within and on the surface of self-assembled collagen fibrils. Triple helical type I collagen molecules self-assemble into collagen fibrils in a quarter-staggered manner, which leads to 67 nm periodicity with alternating gap and overlap zones. [7, 8] During the mineralization of collagen fibrils, HA crystals first form within the gap zone, and then spread through the fibrils, leading to arrays of HA nanocrystals embedded within and oriented along the collagen fibrils, namely intrafibrillar mineralization. [9-11] Following intrafibrillar mineralization, HA crystals grow on the surface of the collagen fibrils, leading to extrafibrillar mineralization. [9] It has been reported that intrafibrillar mineralization of collagen fibrils attributes to the high mineral content in
bone and the improvement of the mechanical and biological properties of the collagen matrix. [5, 6, 12-14] Meanwhile, extrafibrillar mineralization of collagen fibrils plays an important role in improvement of mineral content and mechanical properties. [13] The HA minerals in the bone contribute to better biocompatibility, osteoconductivity and osteointegration. [15, 16]

Substantial research have been done to deposit HA crystals onto the surface of collagen fibrils (extrafibrillar mineralization) and within collagen fibrils (intrafibrillar mineralization), [17-19] and it has been widely accepted that collagen matrix cannot induce intrafibrillar mineralization by itself. Anionic non-collagenous proteins (NCPs) are found to play an important role in modulating HA crystal nucleation and the alignment of apatite deposited within collagen fibrils. [20-23] Owing to the limited availability and high cost of NCPs, polyanionic electrolytes, such as polyacrylic acid (PAA) and polyaspartic acid (PAsP), are often used to mimic the functional groups of NCPs. Their functions are to sequester calcium and phosphate ions to form fluid amorphous calcium phosphate nanoprecursors (polymer induced liquid precursors, PILP). [21, 24, 25] It has been identified recently that bone mineralization initially nucleates from an amorphous phase and then grows into crystals. [15, 26-29] The advantages of using fluid amorphous precursors are that they are moldable and infiltrate easily into collagen fibrils. [10, 27] However, polyanionic macromolecules induced amorphous precursors alone could not reproduce highly ordered intrafibrillar mineralization. Inorganic polyphosphate (sodium tripolyphosphate, TPP) is normally used to replicate the template function of
phosphoproteins, which modulate HA deposition due to its excellent binding capacity for calcium ions and its strong affinity to collagen fibrils. [14, 24, 30]

With considerable progresses in unveiling mechanisms of biomineralization of collagen fibrils in vivo and in vitro replicating the process, most of the recent research has been focused on the biomineralization of 3-D constructs through a PILP process. For example, collagen scaffolds, turkey tendon, eggshell membrane and demineralized human dentine have been biomineralized with intrafibrillar and extrafibrillar minerals via polyanionic macromolecules-induced liquid precursors. [19, 22, 29, 31-34] Dai et al. have proved that the presence of polyaspartic acid mimicking NCPs is essential for inducing intrafibrillar mineralization. [35] Meanwhile, Jee et al. have revealed that oriented HA crystals in turkey tendon can be formed via the PILP process, in which polyanionic polyaspartic acid is used to mimic the role of NCPs to facilitate the formation of fluid amorphous nanoprecursors. [19] Li et al. have also used PAA to mimic NCPs to stabilize amorphous calcium phosphate precursors to mineralize eggshell membrane with intrafibrillar mineralization. [34]

Although many studies have been conducted to investigate intrafibrillar mineralization via the PILP process to mimic the structural organization in natural bone or teeth, little work has been done to investigate the concentration of polyanionic polymers on the formation of intrafibrillar or extrafibrillar minerals, and there is no report on the bottom-up process to produce intrafibrillar mineralized collagen fibrils. In this study, we actively investigated the effect of polyanionic polymer
concentrations on the intrafibrillar mineralization of collagen fibrils using a bottom-up approach in order to develop better understanding of the mineralization process in natural bone.

4.2 Materials and methods

4.2.1 Materials

Type I collagen was extracted from rat tails, purified based on the protocol reported by Rajan et al. [36] Polyacrylic acid (PAA, Mw 2000 Da) and sodium tripolyphosphate (TPP) were purchased from Sigma-Aldrich. All chemicals were of analytical chemical grade.

4.2.2 Preparation of intrafibrillar and extrafibrillar mineralized collagen fibrils

Based on our previously established protocol for biomimetic collagen apatite scaffolds, [37] intrafibrillar and extrafibrillar mineralized collagen fibrils were fabricated at different PAA concentrations. Briefly, PAA, the sequestration analog, was added to a modified simulated body fluid (m-SBF) to form stabilized amorphous calcium phosphate (ACP) nanoprecursors (PAA-ACP). The m-SBF was prepared as reported previously: NaOH, K₂HPO₄·3H₂O, MgCl₂·6H₂O, CaCl₂ were added in deionized water (DIW) in the order as they are listed, and the ion concentrations were adjusted according to Table 4.1. [38-40] Concentrations of PAA in PAA-ACP nanoprecursors were 0, 0.4, 0.8, 2, and 5.6 mg/mL, and the pH of each PAA-ACP nanoprecursor was adjusted to 7.2 by addition of HEPES (4-(2-hydroxyapatiteethyl)-1-piperazineethanesulfonic acid) and NaOH. Type I
collagen stock solution (4.5 mg/mL) was diluted to a given concentration (0.2 mg/mL) using PAA-ACP nanoprecursors. TPP (1.2 wt%), the templating analog, was added to the collagen solution to template the hierarchical arrangement of hydroxyapatite within collagen fibrils. The ion concentrations of all chemicals in the suspensions are listed in Table 4.1. All of the reactions were taken under 4 °C, and then transferred to a water bath at 37 °C for 24 hr.

Table 4.1. Ion concentrations of suspension and body plasma

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4.2.3 Characterization

PAA-ACP nanoprecursors with a PAA concentration of 0.4 mg/mL were added to the collagen solution, and TPP (1.2 wt%) was then added to template minerals deposition within collagen fibrils. The pH of the solution was adjusted to 7.2 using HEPES and NaOH. After pH adjustment, the solution was dropped onto Formvar carbon coated copper grid, rinsed with DIW, dried under room temperature, and then observed using transmission electron microscopy (TEM, JEOL JEM-2010) at an
accelerating voltage of 80 kV. Five TEM images were analyzed using Image J software to achieve the average size of ACP nanoprecursors and amorphous calcium phosphate nanoparticles.

In order to examine the mineralization of collagen fibrils, intrafibrillar and extrafibrillar mineralized collagen fibrils using PAA-ACP nanoprecursors at different PAA concentrations were deposited on copper grid and observed using TEM. The corresponding mineral phase was evaluated by selected area electron diffraction (SAED) coupled to TEM. The surface morphology of the mineralized collagen fibrils using PAA-ACP nanoprecursors at different PAA concentrations was also imaged using field emission scanning electron microscopy (FESEM, JEOL JSM-6335F, Japan).

4.3 Results

To investigate the effect of PAA concentration in PAA-ACP nanoprecursors on the biomineralization of collagen fibrils, different PAA concentrations (0, 0.4, 0.8, 2, 5.6 mg/mL) were used to induce PAA-ACP nanoprecursor formation. PAA-ACP nanoprecursors were added to the type I collagen stock solution and TPP was used to modulate mineral arrangement in the collagen fibrils. Figure 4.1 shows the TEM images of unstained collagen fibrils with different PAA concentrations in PAA-ACP nanoprecursors. An increasingly distinct banding pattern (D-bandings) in electron-dense nucleation sites can be observed in collagen fibrils with decreasing PAA concentration, implying that the mineral content formed within the gap zone of
collagen fibrils increased with the reduction of PAA concentration (Figure 4.1). At a PAA concentration of 5.6 mg/mL, vague D-periods are observed (Figure 4.1A), indicating electron-dense minerals deposited, poorly reproducing the D-band pattern of naturally mineralized collagen fibrils normally observed in bone. With the decrease of PAA concentration, the intrafibrillar mineralization becomes more obvious and homogeneous (Figure 4.1A-D), which is attribute to the higher mineral content formed within the collagen fibrils. The inserted SAED result of the mineralized collagen fibrils formed at the PAA concentration of 2 mg/mL (Figure 4.1B) does not exhibit a ring pattern, which indicates that the minerals formed within the collagen fibrils are either amorphous or nanocrystallined. The control is the sample with no PAA in the collagen-containing calcium phosphate solution. As such, no D-band is observed, suggesting that minerals are not deposited in specific nucleating sites within collagen fibrils. Both SEM and TEM results reveal that HA minerals (white arrow, Figure 4.2B) only formed on the surface of collagen fibrils (Figure 4.2). SAED result (Figure 4.2A, insert) exhibits rings of typical low crystalline apatite. These results collectively suggest that ordered mineralization within collagen fibrils cannot be achieved without the presence of NCPs analogs.
Figure 4.1. TEM images of unstained mineralized collagen fibrils with different PAA concentrations: (A) 5.6 mg/mL, (B) 2 mg/mL (insert: SAED result), (C) 0.8 mg/mL and (D) 0.4 mg/mL.
**Figure 4.2.** TEM image (A) and SEM image (B) of mineralized collagen fibrils without PAA. SAED result for mineralized collagen fibrils without PAA (Insert, A). White arrow in (B): HA minerals on the surface. The cracks on collagen fibrils were induced by high voltage employed during SEM operation.

Figure 4.3 shows FESEM images of mineralized collagen fibrils with different PAA concentrations. Vague D-banding is observed at a PAA concentration of 5.6 mg/mL (Figure 4.3A, white arrow). This suggests that few minerals have deposited within collagen fibrils. The D-banding becomes more distinguished with the decrease of the PAA concentration, indicating more minerals are incorporated into the collagen fibrils (Figures 4.3A-D), which is in accordance with the TEM result (Figure 4.1). White dotted arrow and white arrow heads in Figure 4.3D show minerals formed on the surface of collagen fibrils (extrafibrillar mineralization) with low PAA concentration in PAA-ACP nanoprecursors. The inset image in Figure 4.3D is the enlarged image indicated by white dotted arrow, and white thin arrows show minerals aligned on the surface of collagen fibrils with an almost 67 nm spacing distance. This suggests that this specific site is the entering sites for calcium phosphate nanoclusters (Figure 4.3D). Moreover, intrafibrillar and extrafibrillar mineralized collagen fibrils are arranged into bundles or fused to form collagen fibers (Figure 4.3).
Figure 4.3. FESEM images of mineralized collagen fibril bundles with different PAA concentrations (white arrows indicate D-banding): (A) 5.6 mg/mL, (B) 2 mg/mL, (C) 0.8 mg/mL (the insert is the enlarged image of the spot indicated by the white arrow), and D) 0.4 mg/mL (white dotted arrow and white arrow heads show minerals on the collagen surface), minerals aligned on the surface of collagen fibrils with an almost 67 nm spacing distance (white dotted arrow and thin arrows in insert image). The insert is the enlarged image of the spot indicated by the white dotted arrow.

4.4 Discussion

Since mineralized collagen fibrils are the primary unit of the complex bone structure, investigation of the intrafibrillar and extrafibrillar mineralization of collagen fibrils has become essential. It is widely accepted that biomineralization is well regulated by NCPs, such as DMP 1, [41] osteonectin, osteocalcin and bone
sialoprotein. Two functional motifs in NCPs have been found to play an important role in regulation of nucleation, dimension and order of HA deposition within natural bone. First, acidic sequestrating motif of NCPs binds to calcium to form calcium-bound organic materials, and thereby inhibits the precipitation of calcium phosphate. This in turn forms stabilized amorphous calcium phosphate nanopresursors. PAA was used in our study to replicate the sequestration functional groups in NCPs. The −COO⁻ groups of PAA attract calcium ions from solution, which further recruit phosphate ions into the fluid droplets to form supersaturated amorphous calcium phosphate nanoprecursors (Figures 4.4 and 4.5). Electron dense nanoprecursors were observed in Figure 4.4, and amorphous calcium phosphate nanoparticles were also shown, indicating nanophase separation has formed in the PAA-ACP nanoprecursors. The mean size of nanoprecursors is 37.61±6.46 nm. Amorphous calcium phosphate nanoparticles were observed in the PAA-ACP nanoprecursor droplets with a mean size of 6.70±1.04 nm measured by Image J. The small size of ACP nanoprecursors and ACP nanoparticles makes it possible for PAA-ACP to infiltrate into collagen fibrils. It has been reported that matrix vesicles (MVs) play a crucial role in bone mineralization. During bone mineralization, MVs with a diameter of 40-200 nm are secreted during osteoid formation, and observed at sites where mineralized tissue is synthesized de novo. As a result, MVs are considered the initial mineralization site. MV membranes enriched with acidic phospholipids have high affinities for calcium cations, which can induce the formation of stable calcium phosphate-phospholipid complex. Obviously,
each polymer stabilized amorphous calcium phosphate nanoprecursor has a similar function as an MV, so PAA-ACP is regarded as an analog to MV. [42, 43]

Besides, templating motif of NCPs regulates the alignment of HA within collagen fibrils through its excellent binding capacity for calcium ions and collagen fibrils. [22] Due to the low cost and availability of the chemical, sodium tripolyphosphate (TPP) has been used as an analog to NCPs in templating the HA deposition within collagen fibrils in the current study. [24] Highly negatively charged TPP accumulates in the gap zone of collagen fibrils dominated by highly positive net charge through electrostatic attraction. [8, 11, 24] The high binding capacity of TPP to calcium and collagen makes it possible to act as an ionic bridge to attract PAA-ACP nanoprecursors to enter the gap zone of collagen fibrils (Figure 4.5). [24] PAA-ACP nanoprecursors were found to accumulate in the close vicinity of collagen fibrils and in contact with collagen fibrils, demonstrating the attraction of PAA-ACP to the collagen fibrils (Figure 4.4A). Once the PAA-ACP nanoprecursors penetrate the gap zone of collagen fibrils via capillary action and electrostatic attraction, the desorption of calcium from PAA is expected because TPP is a much stronger pentanionic chelating agent to ACP comparing to the carboxylate groups of PAA. [44] When a high concentration of TPP (2%) is added to PAA-ACP nanoprecursors, white precipitates are formed in the solution (data not shown). This also suggests that TPP has attracted calcium ions from PAA-ACP and induced the aggregation of ACP in the gap zone of collagen fibrils (see Figure 4.4A). The free polyanionic macromolecules would exit the gap zone and re-enter the collagen and PAA containing m-SBF
solution to continuously recruit free calcium and phosphate ions to form PAA-ACP nanoprecursors and re-infiltrate the fibrils to form ACP minerals. [35] These ACP minerals are then crystalized under the influence of the amino acid side chains of collagen fibrils. [33, 45, 46] A large fraction of charged amino acid side chains in the gap zone helps to recruit and bind calcium and phosphate ions, and the resulting calcium-phosphate prenucleation cluster become crystallized HA with time. [8, 34, 46] Thus the gap zone of collagen fibrils serves as the entering (as inferred from the inset image in Figure 4.3D) and mineralization sites, which forms the D-banding of collagen fibrils (Figure 4.5). This is in agreement with the finding by Nudelman et al., who reported that the gap region is the infiltrating and nucleating site for the intrafibrillar mineraliation of collagen fibrils. [11]

**Figure 4.4.** TEM images of collagen solution containing PAA-ACP nanoprecursor droplets on copper grid. (A) Low magnification TEM image showing PAA-ACP nanoprecursor droplets that were formed in the close vicinity of the collagen fibrils. White arrow shows the collagen fibrils that have been removed. (B) & (C) High magnification images of PAA-ACP nanoprecursor droplets. (C) One electron dense
PAA-ACP nanoprecursor droplet highlighted in the white circle consisting of many amorphous calcium phosphate nanoparticles.

Fibrils with intrafibrillar and extrafibrillar mineralization are fabricated using PAA as a sequestration analog to induce amorphous calcium phosphate nanoprecursors and TPP as a templating analog, and the mechanisms involved have been illustrated in Figure 4.5. [47, 48] The rate of collagen mineralization is dependent on the concentration of PAA. The lower the concentration of PAA, the weaker the inhibition of PAA for ACP nanoprecursor precipitation, the faster the mineralization and crystallization process, and the more minerals deposited. [10, 49, 50] When the PAA concentration is high, vague D-banding has been observed, implying that only a small number of minerals are formed in the gap zone of collagen fibrils (Figures 4.1A and 4.2A). This is due to that the ACP nanoprecursors are highly stabilized by PAA (Figures 4.1 and 4.3). With the decreasing concentration of PAA, PAA-ACP precursors became less stable and crystallization process could be quick enough to form both intra- and extra-minerals, which can be proved by the increase of the mineral amount and formation of extrafibrillar mineralization (Figures 4.1, 4.2 and 4.3).
Figure 4.5. Schematic illustration of the intrafibrillar mineralization process induced by PAA as a sequestration analog and TPP as a templating analog.

4.5 Conclusions

Self-assembly of collagen fibrils and intrafibrillar and extrafibrillar mineralization were achieved using PAA as sequestration analog and TPP as a templating analog. The effect of the PAA concentration on intrafibrillar and extrafibrillar mineralization was realized by adjusting the concentration of NCPs analog during the biomineralization process. Thus, this study demonstrates a promising approach to produce intrafibrillar mineralized collagen fibrils using a bottom-up approach.
References


[38] Z. Xia, X. Yu. Biomimetic collagen/apatite coating formation on Ti6Al4V


5. Intrafibrillar silicification of collagen fibrils

Abstract

Inspired by the silicification process in nature, intrafibrillar silicified collagen fibrils were successfully fabricated using a biomimetic one-step collagen self-assembly/silicification approach, in which collagen self-assembly and intrafibrillar silicification occurred simultaneously. To the best of our knowledge, it is the first time that intrafibrillar silicified collagen fibrils were formed via a one-step simultaneous approach, where collagen fibrils served as a templating matrix, and poly(allylamine) hydrochloride and sodium tripolyphosphate acted as the respective positive and negative analogs of zwitterionic proteins. By tailoring zwitterionic proteins analogs, silicified collagen fibrils with different microstructures, including core-shell, twisted and banded structures, were achieved. The intrafibrillar silicified collagen fibrils demonstrated significant improvement of osteoblasts proliferation compared with apatite mineralized collagen fibrils. These findings open a new avenue for preparation of silicon-containing hierarchical biocomposites for biomedical needs.

Keywords: Biomimetic; intrafibrillar silicification; hierarchical structure; zwitterionic proteins; one-step approach
5.1 Introduction

Silicon is an essential element contributing significantly to the health of bone and cartilage. [1] It has been discovered that silicon-containing organic-inorganic biocomposites with unique hierarchical structures exist in numerous natural biological systems, such as bone, fish scales, diatom frustules, and spicules of glass sponges. [2-5] For example, the long spicules of *Hyalonema sieboldi* demonstrate a perfect hierarchical structure, remarkable optical properties, and excellent durability and flexibility. [6] More importantly, silicon-containing biomaterials have been found to be osteoinductive for new bone formation and stimulative for neovascularization without supplements of growth factors. [7, 8] Due to the unique properties of the silicon-containing biomaterials, great enthusiasm has been generated and extensive effort has been made to replicate these materials to be used in different biomedical applications. [6, 9, 10]

In recent years, effort has been directed to develop an in-depth understanding of the biosilicification mechanism so as to replicate the process *in vitro* and thereby fabricate organic-inorganic hybrid biomaterials with a hierarchical structure. Chemical analyses of glass sponge and diatom have shown that fibrillar collagen can serve as templates for silicification, while the highly zwitterionic proteins, such as silaffins, silicateins, and long-chain polyamines, are actively involved in the interplay with collagen and the mediation of silicification process. [6, 11] The zwitterionic proteins exhibit both positive charges introduced by the quaternary ammonium groups and negative charges by the phosphorylated serine residues. [12] The zwitterionic
nature of these proteins enables them to form aggregates, which controls the silicification process and silica morphology. [13] Polyamines have been found to induce silica precipitation \textit{in vitro} in the presence of polyanions, where polyamines and polyanions act as positive and negative analogs of zwitterionic proteins, respectively. [3] Despite all the successes in fabrication of silicon-containing biomaterials, an in-depth understanding of the mechanism of biomimetic silicification process has not yet been achieved.

A variety of silicified collagen hybrids have been produced, such as silica-collagen xerogels, and silicified collagen hydrogels with different silicon sources (silica and silicates). [14-18] Typically, silica is formed on the surface of collagen fibrils (extrafibrillar silicification), resulting in a weak bond between silica and collagen. [14, 15, 19] However, the recent successes in intrafibrillar calcification of collagen fibrils have shed light on the understanding of intrafibrillar silicification (forming silica within collagen fibrils), as intrafibrillar calcification and silicification of collagen fibrils may share a similar mechanism. In our previous work, intrafibrillar calcified collagen fibrils have been achieved by a one-step collagen self-assembly and calcification approach. [20, 21] In the present study, we also propose to produce intrafibrillar silicified collagen fibrils via a one-step collagen self-assembly/silicification (OCSS) approach, where collagen self-assembly and silicification occur simultaneously with the aid of positive and negative analogs of zwitterionic proteins. To test this hypothesis, a systemic study was conducted to investigate the effect of zwitterionic proteins analogs on the OCSS approach, and an
*in vitro* cell culture study was also conducted to test the biocompatibility of the intrafibrillar silicified collagen fibrils.

5.2 Materials and methods

5.2.1 Materials

Type I collagen was extracted from rat tails, purified based on the protocol reported by Rajan et al. [22] Poly(allylamine) hydrochloride (PAH, Mw = 15,000 Da) and sodium tripolyphosphate (TPP) were purchased from Sigma-Aldrich. All chemicals were of analytical chemical grade.

5.2.2 Preparation of hydrolyzed silicic acid

A 3 wt% hydrolyzed silicic acid stock solution was prepared as followed: Silbond® 40 (40% hydrolyzed tetraethyl orthosilicate (TEOS); Silbond Corp., Weston, MI, USA), absolute ethanol, deionized water (DIW) and 37% HCl were mixed at a molar ratio of 1.03:199:6.11:0.03 for 1 hour at room temperature to hydrolyze TEOS into orthosilicic acid and its oligomers. [23]

5.2.3 Preparation of PAH-stabilized silica (PAH-SA) precursors

PAH-stabilized silica (PAH-SA) precursors were prepared by the following procedure: A solution with a PAH concentration of 0.267 mM was mixed with 3 wt% silicic acid at a 1:1 volume ratio to obtain 1.5 wt% silicic acid stabilized by PAH. After stirring for 1 min, the mixture was centrifuged at 3000 RPM and 25 °C for 10 min. The PAH-stabilized silica (PAH-SA) precursors were then collected. [24]
5.2.4 Controlled biomimetic silicification of collagen fibrils via an OCSS approach

Buffer solution was used to avoid sudden pH changes and prepared based on our established protocol, [20, 25] where 184.8 mM NaCl, 6.3 mM K$_2$HPO$_4$·3H$_2$O and 100.7 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid) were added to DIW in the order listed above.

*Collagen-silica (Col-SA) fibrils: Type I collagen stock solution (4.5 mg/mL) was diluted to a given concentration (0.3 mg/mL) using buffer solution at 4 °C. Then different amounts of silicic acid solution (3 wt%) were added dropwise to the diluted collagen solution immediately with vigorous stirring. The amount of silicic acid added was controlled at a SA/Col ratio of 1:2, 1:1, 2:1, 3:1 and 4:1. After mixing the diluted collagen solution with the silicic acid solution and stirring for 5 min, the mixtures (pH = 6.4) were incubated at 25 °C for 10 min, and then incubated in a water bath at 37 °C for 24 h.

*Collagen-poly(allylamine) hydrochloride-silica (Col-PAH-SA) fibrils: Type I collagen stock solution (4.5 mg/mL) was diluted to a given concentration (0.3 mg/mL) using the buffer solution at 4 °C. Then different amounts of PAH-SA precursors were added dropwise into the diluted collagen solution immediately with vigorous stirring. The amount of PAH-SA precursors was controlled at a SA/Col ratio of 1:1, 4:3 and 2:1. Higher ratios of SA/Col were not used since precipitates formed rather quickly when the ratios of SA/Col were 3:1 and 4:1. After mixing the diluted collagen solution with PAH-SA precursors and stirring for 5 min, Col-PAH-SA mixtures (pH = 6.4) were incubated at 25 °C for 10 min, and then transferred to a water bath at 37 °C.
for 24 h.

*Intrafibrillar silicified Collagen (Col-inSA) fibrils: Type I collagen stock solution (4.5 mg/mL) was diluted to a given concentration (0.3 mg/mL) using the buffer solution at 4 °C. TPP (1.2 wt%) was added to this diluted collagen solution and stirred for 5 min. Different amounts of PAH-SA precursors were then added dropwise into this collagen-TPP solution with vigorous stirring. The amount of PAH-SA precursors was controlled at a SA/Col ratio of 1:1, 4:3 and 2:1. After stirring for 5 min, Collagen containing mixtures (pH = 6.8) were incubated at 25 °C for 10 min, and then transferred to a water bath at 37 °C for 24 h.

5.2.5 Preparation of silicified collagen fibrils via a two-step approach

Firstly, collagen fibrils without mineralization were produced by the following procedure: type I collagen stock solution (4.5 mg/mL) was diluted to a given concentration (0.3 mg/mL) using the buffer solution at 4 °C, followed by incubation at 25 °C for 10 min and then in a water bath at 37 °C for another 24 h to precipitate collagen fibrils. Secondly, the collagen fibrils without mineralization were silicified by incubating in silica precursors, which was described below. *Col-SA fibrils via a two-step approach (T-Col-SA):* Silicic acid was added dropwise to the collagen fibrils at a SA/Col ratio of 4:1, and then incubated at 37 °C for another 24 h. *Col-PAH-SA fibrils via a two-step approach (T-Col-PAH-SA):* PAH-SA precursors were added to the self-assembled collagen fibrils at a SA/Col ratio of 4:3, and then incubated at 37 °C for 24 h. *Col-inSA fibrils via a two-step approach (T-Col-inSA):* TPP was dissolved in the self-assembled collagen fibrils at a concentration of 1.2 wt%, and
PAH-SA precursors (SA/Col = 4:3) were added after 5 min of stirring. The mixed solution was then incubated at 37 °C for 24 h.

5.2.6 Materials characterization

*Transmission electron microscopy (TEM):* Silicified collagen fibrils and collagen fibrils without mineralization were dropped onto Formvar carbon coated copper grids, rinsed with DIW and then dried at room temperature. TEM (JEOL, JEM-2010) was used to examine the silicification of collagen fibrils at an accelerating voltage of 80 kV. All samples were examined without staining.

*Field emission scanning electron microscopy (FESEM):* FESEM (JEOL JSM-6335F, Japan) was used to characterize the surface morphology of the silicified collagen fibrils. Different silicified collagen fibrils were sputter-coated with gold and examined using FESEM operating at 8-10 kV.

*Thermogravimetric analysis (TGA):* TGA was performed using a Q500 TGA analyzer (TGAQ-500, TA Instrument, New Castle, DE, USA). About 20 mg freeze-dried silicified collagen fibrils were placed in a platinum pan and heated from 30 to 900 °C at a rate of 10 °C/min in air to determine the amount of minerals in the silicified collagen fibrils.

*Attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR):* The freeze dried silicified collagen fibrils were characterized using ATR-FTIR. A Nicolet Magna 560 FT-IR spectrophotometer (Artisan Technology Group®, Illinois, USA) with an ATR setup was used to collect infrared spectra in the range 4,000-400 cm⁻¹ at a 4 cm⁻¹ resolution, 32 scans.
5.2.7 *In vitro* cell culture and proliferation

Mouse calvaria 3T3-E1 (MC3T3-E1, ATCC, USA) cell line, an osteoblast precursor cell line, was used to determine the cytocompatibility of various samples. MC3T3-E1 cells were plated in a culture flask (FALCON, USA) containing 10 mL of α-minimum essential medium (α-MEM; Gibco, Invitrogen, Inc., USA), which was supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen, Inc., USA) and 1% penicillin–streptomycin (Gibco, Invitrogen, Inc., USA). In a humidified atmosphere of 5% CO₂ under 37 °C that maintained by an incubator (NAPCO, USA), cells were further passaged when reaching 80-90% confluence. The fibrous membranes of 5.5 mm in diameter were collected and cross-linked with 1 wt% EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide). Collagen-apatite membrane with an apatite content of about 67 wt% was produced using a co-precipitation approach, which serves as a control. The cross-linked fibrous membranes were sterilized and placed in a 96-well plate.

To observe cell proliferation and viability using alamarBlue assay, cells were seeded on various samples (four replicates) in 96-well plates at a cell density of 8,000 cells per well in 100 μL cell culture medium. The cells were cultured for 14 days and the cell culture medium was refreshed every 2 days. After incubating for 1 day, samples that attached with cells were transferred to a new 96-well plate to leave behind the cells escaped from samples. Phenol-red-free cell culture medium with 10% alamarBlue (Thermo Scientific, USA) was added to each well. After culturing for another 4 h, 100 μL of the culture solution from each well was transferred to a new
96-well plate to measure the absorbance at wavelengths of 570 nm and 600 nm by a microplate reader (BioTek, USA). The samples were then rinsed against PBS twice to remove residue alamarBlue, and 100 μL fresh cell culture medium was added to each well and refreshed every other day. After culturing for 4, 7, 10, and 14 days, cell viabilities were investigated according to the procedure described above. Percentage of reduction of alamarBlue was calculated according to the instruction provided by the assay.

5.2.8 Statistical analysis

Statistically significant differences (p) between various groups were measured using two-way RM ANOVA analysis of variance that carried out by GraphPad Prism 5.

5.3 Results and discussion

5.3.1 Col-SA fibrils

Silicified collagen fibrils with a core-shell structure are successfully produced using a one-step approach. When silicic acid is mixed with collagen molecules with a SA/Col ratio of 3:1 or 4:1, a thick layer of dense silica is coated on the collagen fibrils, forming a core-shell structured Col-SA hybrid (Figures 5.1A1, 5.1A2, 5.1B1 and 5.1B2). This result is in accordance with the reports by Eglin et al. and Ono et al., where hollow silica fibrils were produced with silica coated onto the surface of templating collagen fibrils. [26, 27] Some Col-SA fibrils also demonstrate a twisted structure, which is due to the presence of negatively charged silanol groups of silica.
coating on the surface of Col-SA hybrid (Figures 5.1B1, 5.1B2, and 5.1C). [9, 28]
When the SA/Col ratio is low (1:2, 1:1, or 2:1), silicified collagen fibrils with core-shell structures are not formed (Data not shown). When the SA/Col ratio is 3:1, the distance between two adjacent protrusions is 66.9±5.8 nm (n = 30, Figures 5.1A1 and 5.1B1), which is identical to the distance of the gap zone present in collagen fibrils (67 nm), indicating the templating function of collagen fibrils. Negatively charged silicic acid preferentially accumulates at sites of highly positively charged gap zone of collagen fibrils, leading to the formation of protrusions with a periodicity of about 67 nm (Figure 5.1D). [15, 29] When the SA/Col ratio is 4:1, the shell thickness of the Col-SA core-shell structure increases, and the periodicity of the distinguished protrusions increases to 82.7±21.0 nm (n = 40, Figure 5.1A2 and 5.1B2). This is due to that with the increase of the SA/Col ratio, more and more silica precipitates on the surface of collagen fibrils, resulting in an increase in shell thickness and a decrease in electrostatic attraction between silicic acid and the gap zone of collagen fibrils. As a result, the surface of the coating was smoothened, and some adjacent protrusions fused together, leading to a longer distance between two adjacent protrusions (Figures 5.1A2 and 5.1B2).
Figure 5.1. TEM images (A1 and A2), FESEM images (B1 and B2) of Col-SA fibrils with different SA/Col ratios: 3:1 (A1, B1), 4:1 (A2, B2). ATR-FTIR spectra (C) of Col-SA fibrils (Stars indicate the peaks for Si-O-Si, and triangle shows the peak for Si-OH). Schematic illustration (D) of the process to form Col-SA fibrils, and the TEM image shows Col-SA fibrils with a SA/Col ratio of 3:1.

5.3.2 Col-PAH-SA fibrils

By controlling the silica precursors, twisted silicified collagen fibrils with bandings are produced (Figure 5.2). A long-chain polyamine (poly(allylamine) hydrochloride (PAH, Mw = 15,000 Da)) was used to stabilize silica by the formation of hydrogen bonds with silanol groups of silicic acid, which reduces the availability of silicic acid from forming polysilicic acid, and thereby leads to the formation of fluidic PAH-SA nanoprecursors. [4] Rope-like, twisted silicified collagen fibrils are formed via a one-step approach by mixing PAH-SA precursors and collagen molecules.
(Figures 5.2A-D). This result is in agreement with the reports by Desimone et al. and Heinemann et al., [9, 28] where twisted fibrils were formed due to the presence of negatively charged silanol groups of silica (Figure 5.2D). The average distances of two adjacent bandings for Col-PAH-SA fibrils are 143.3±12.1 nm, 145.8±7.5 nm, and 145.0±11.8 nm for SA/Col ratio of 1:1, 4:3 and 2:1, respectively (Figures 5.2A-C, n = 30). All the banding distances are significantly larger than that of the typical D-banding of collagen fibrils (67 nm). This may be due to that the amine groups of PAH in PAH-SA precursors interact with carboxyl groups of collagen molecules during the self-assembly of collagen fibrils, where PAH is inserted between two adjacent collagen molecules as a space linker, leading to a longer distance between adjacent gap zones (Figure 5.2E). In PAH-SA precursors, silica is stabilized by PAH through ionic bonding and/or hydrogen bonding. [4] Subsequently, PAH-SA precursors infiltrate into collagen fibrils along with PAH during the collagen fibril formation process. PAH is then separated from silicic acid, leading to the precipitation of silica at the C-terminus of collagen molecules, where the potential energy is the lowest. [29] This explains why the banding in Figure 5.2 is larger than that of the typical collagen D-banding because of the longer distance between adjacent gap zones of the fibrils and the precipitation of electron-dense minerals at the C-terminus of collagen molecules. The FTIR result also confirms the attachment of PAH onto collagen fibrils (Figure 5.2D). The 528 cm⁻¹ peak of FT-IR is assigned to the amine hydrogen bonding of PAH, indicating the attachment of PAH onto the silicified collagen fibrils (Figure 5.2D).
Figure 5.2. TEM images of Col-PAH-SA fibrils with different SA/Col ratios: 1:1 (A), 4:3 (B), and 2:1 (C). ATR-FTIR spectra (D) of Col-PAH-SA fibrils (Stars indicate the peaks for Si-O-Si, triangle shows the peak for Si-OH, and arrow indicates amine hydrogen bonding). Schematic illustration (E) of the process to form Col-PAH-SA fibrils, and the TEM image shows Col-PAH-SA fibrils with a SA/Col ratio of 4:3.

5.3.3 Col-inSA fibrils

As shown in Figure 5.3, intrafibrillar silicified collagen fibrils were produced using PAH and TPP as the positive and negative analogs of zwitterionic proteins, respectively. Compared to Col-PAH-SA fibrils, the morphology of collagen fibrils in the Col-inSA group exhibits a normal structure instead of twisted fibrils (Figure 5.3). [20] This may be due to the competition between TPP and PAH to interact with collagen molecules during the collagen self-assembly process. As a penta-anion, TPP
has a stronger electrostatic attraction to the gap zone of collagen fibrils than PAH, leading to prohibition of further bonding of collagen molecules with PAH. Instead, fluidic PAH-SA precursors infiltrate into the collagen fibrils and precipitate within the gap zone (Figure 5.5). Clear D-bandings are observed using TEM due to electron-dense silica precipitating within specific sites of collagen fibrils (Figures 5.3 and 5.4). As shown in Figure 5.3, the D-banding becomes more and more obvious with the increase of SA/Col ratios, and silica nanoparticles are also formed on the surface of collagen fibrils. Similar to silaffins and long-chain polyamines in diatoms, PAH and TPP have been used as respective positive and negative analogs of highly zwitterionic proteins to promote silica formation in vitro. [30] In details, PAH mimics the positively charged groups of lysine residues in silaffins, while TPP mimics the negatively charged groups introduced by phosphorylation of serine residues. [3, 31] Similar to the mechanism of calcium phosphate intrafibrillar mineralization, TPP accumulates in the gap zone of collagen fibrils due to electrostatic attraction between the highly negatively charged TPP and the positively charged gap zone of collagen fibrils. [20, 29] TPP further attracts fluidic PAH-SA nanoprecursors to infiltrate into gap zones of collagen fibrils via electrostatic attraction and capillary action (Figures 5.5 and 5.6). [4, 32, 33] PAH promotes silica precipitation in the presence of TPP in the gap zone of collagen fibrils, leading to intrafibrillar silicification of collagen fibrils (Figure 5.6). [31, 34]
**Figure 5.3.** TEM images (A1, B1, and C1) and FESEM images (A2, B2, and C2) of Col-inSA fibrils with different SA/Col ratios: 1:1 (A1, A2), 4:3 (B1, B2), and 2:1 (C1, C2).

**Figure 5.4.** ATR-FTIR spectra (A) and TGA spectra (B) of Col-inSA fibrils with different SA/Col ratios. (Stars indicate the peaks for Si-O-Si, triangle shows the peak for Si-OH, and arrow indicates amine hydrogen bonding.)
Figure 5.5. The initial stage of PAH-SA precursors interacted with collagen fibrils in the presence of TPP as a regulator. Low (A) and high (B) magnification of the fluidic silica precursors infiltrated into the collagen fibrils during the collagen self-assembly process. White arrows in B: Vague D-banding can be observed.

Figure 5.6. Schematic illustration of the intrafibrillar mineralization process of Col-inSA fibrils. TEM image shows Col-inSA fibrils with a SA/Col ratio of 4:3.

5.3.4 Silicified collagen fibrils via a two-step approach

Moreover, silicification of collagen fibrils via a one-step approach was compared with a two-step approach. In a two-step approach, collagen fibrils were first self-assembled, and then silica precursors were added to the self-assembled collagen...
fibril suspension to enable the silicification of the precipitated collagen fibrils. Compared T-Col-SA fibrils with Col-SA fibrils at the same SA/Col ratio (SA/Col=4:1), silica particles rather than a continuous coating are deposited on the surface of collagen fibrils (Figures 5.7A1 and 5.7A2). Silica particles may be absorbed onto the collagen fibrils due to electrostatic attraction, or through hydrogen bonding between silanol groups of silica and amine or hydroxyl groups of collagen. [6] In the T-Col-PAH-SA fibrils, silica particles are only formed on the surface of collagen fibrils, while twisted silicified collagen fibrils are formed in Col-PAH-SA fibrils using one-step approach (Figures 5.7B1 and 5.7B2). The comparison between the T-Col-PAH-SA and the Col-PAH-SA fibrils indicates that in the one-step approach PAH and silica are well integrated into the collagen self-assembly process and that the silicification and self-assembly of collagen fibrils occur simultaneously. In contrast, intrafibrillar silicification was observed in the Col-inSA fibrils formed via both the two-step and the one-step processes (Figures 5.7C1 and 5.7C2). In both the two-step and the one-step approaches, highly negatively charged TPP accumulates in the positively charged gap zone of collagen fibrils through electrostatic attraction, which further attracts fluidic PAH-SA precursors to infiltrate into collagen fibrils. [4, 29, 31] PAH and TPP function as the positive and negative analogs of zwitterionic proteins, respectively, which mediate the biosilicification process in nature systems. Similarly, the accumulation of PAH-SA and TPP in the gap zone of collagen fibrils enables the PAH-SA precursors to aggregate and form silica within collagen fibrils. [3, 13]

Two kinds of polyanions were used: phosphate ions and TPP, but they have
different functions. TPP inhibits the incorporation of PAH during the collagen self-assembly process (Figures 5.7B2 and 5.7C2), and it also functions as a regulator to guide precise deposition of silica within collagen fibrils (Figures 5.5B, 5.7B1 and 5.7C1). Phosphate ions, being one kind of kosmotrope anions, were used to favor collagen self-assembly. [16] The comparison between T-Col-PAH-SA and T-Col-inSA fibrils indicates that, even though both phosphate ions and TPP induce phase separation of PAH-SA precursors, [34, 35] phosphate ions do not function as a regulator to attract PAH-SA to infiltrate into collagen fibrils (Figure 5.7B1), but TPP does (Figure 5.7C1).

![Figure 5.7](image)

**Figure 5.7.** Col-SA fibrils produced using either a two-step process (T-Col-SA (A1), T-Col-PAH-SA (B1), and T-Col-inSA (C1)) or a one-step approach (Col-SA (A2), Col-PAH-SA (B2), and Col-inSA (C2)).
5.3.5 In vitro cell culture

Different mineralized collagen fibrous membranes were tested for their ability to support cell growth in vitro. An osteoblastic cell line MC3T3-E1 was used. Collagen-apatite (Col-Ap) matrices have been widely used as scaffolds for bone tissue engineering, thus the Col-Ap fibrous membrane was used as the control. [36-42] Cell proliferation and viability on different fibrous membranes were quantified using alamarBlue assay. The Col-inSA group supports a higher proliferation rate of MC3T3-E1 cells, especially at the late stage of incubation, compared with Col-Ap, Col-SA and Col-PAH-SA (Figure 5.8). This result indicates that the intrafibrillar silicified collagen fibrous membrane has a better biological response to osteoblast cells compared with Col-Ap. It is well-established that silicon-induced cell responses are dose-dependent. [43, 44] As shown in the TEM and SEM images of Col-inSA fibrils (Figure 5.3), silica is wrapped up by collagen fibrils, demonstrating dense and obvious D-banding structures, allowing gradual release of silicon. [45] Therefore, intrafibrillar silicified collagen fibril can serve as a carrier and gradually release silicon ions from the collagen fibrils to positively impact cell activities instead of burst release causing negative cell responses. [46]
Figure 5.8. Proliferation of MC3T3-E1 cells on mineralized collagen membranes: Collagen-apatite (Col-Ap, control), Col-SA (SA/Col=4:3), Col-PAH-SA (SA/Col=4:3), and Col-inSA (SA/Col=4:3) over the course of 14 days. Data is presented as mean ± standard error. Statistically significant differences (p) between various groups were measured using two-way RM ANOVA analysis of variance, and *: p<0.05, **: p<0.01, ***: p<0.001.

5.4 Conclusions

We have presented a one-step collagen self-assembly/silicification approach in preparation of intrafibrillar silicified collagen fibrils. Highly negatively charged TPP first accumulates in the positively charged gap zone of collagen fibrils due to electrostatic attraction, which further attracts positively charged fluidic PAH-SA precursors to enter the collagen fibrils. As the positive analog of the zwitterionic proteins in silicification, PAH promotes silica precipitation in the presence of negative analog, TPP. Thus the accumulation of PAH-SA and TPP in the gap zone of collagen fibrils facilitates the formation of silica within gap zone of collagen fibrils, resulting
in intrafibrillar silicification. Moreover, the structure of the silicified collagen fibrils can be manipulated by varying silica precursors, polyanions and SA/Col ratios to produce silicified collagen fibrils with a core-shell, twisted, or banded structure. The intrafibrillar silicified collagen fibrils possess better cell compatibility compared with the collagen-apatite fibrils. Thus, this approach provides a facile method to produce intrafibrillar silicified collagen fibrils for biomedical applications.
References


6. Intrafibrillar calcified collagen scaffolds

Abstract

A biomimetic collagen-hydroxyapatite (Col-HA) scaffold resembling the composition and structure of natural bone from the nano- to macro-scale has been successfully prepared for bone tissue engineering. We have developed a bottom-up approach to fabricate hierarchically biomimetic Col-HA scaffolds with both intrafibrillar and extramembrillar mineralization. To achieve intrafibrillar mineralization, polyacrylic acid (PAA) was used as a sequestrating analog of non-collagenous proteins (NCPs) to form a fluidic amorphous calcium phosphate (ACP) nanoprecursor through attraction of calcium and phosphate ions. Sodium tripolyphosphate (TPP) was used as a templating analog to regulate orderly deposition of HA within collagen fibrils. Both X-ray diffraction and Fourier transform infrared spectroscopy suggest that the mineral phase was HA. Field emission scanning electron microscopy, transmission electron microscopy and selected area electron diffraction indicate that hierarchical collagen-HA scaffolds were formed with both intrafibrillar and extramembrillar mineralization. Biomimetic Col-HA scaffolds with both intrafibrillar and extramembrillar mineralization (IE-Col-Ap) were compared to Col-HA scaffolds with extramembrillar mineralization only (E-Col-Ap) as well as pure collagen scaffolds in vitro for cellular proliferation using MC3T3-E1 cells. Pure collagen scaffolds had the highest rate of proliferation, while there was no statistically significant difference between IE-Col-Ap and E-Col-Ap scaffolds. This approach renders a promising
Col-HA scaffold, which bears high resemblance to natural bone in terms of composition and structure.

**Keywords:** Intrafibrillar mineralization; bottom-up approach; hierarchical scaffolds; amorphous calcium phosphate nanoprecursors
6.1 Introduction

In the United States, approximately one million bone fractures are treated every year. [1-3] Autografts have long been considered as the gold standard for bone grafting procedures. However, the amount of bone that can be harvested is limited and there is a high potential for donor site morbidity. Allografts are alternatives to autografts, but there are concerns of disease transmission. [4-7] Thus a promising strategy to address these problems is bone tissue engineering, where a combination of a supporting scaffold, cells and stimuli is used to regenerate bone. [4, 8, 9]

There are many different types of scaffold materials, but biomimetic scaffolds have attracted great attention from researchers. [10, 11] Natural bone is a composite of hydroxyapatite (HA) and collagen fibrils. HA constitutes 65 wt% of natural bone, exhibiting biocompatibility, osteoconductivity, and bone-bonding ability. [3, 12] Collagen, the most abundant protein in bone, has good resorbability and high affinity to cells. [13, 14] Inspired by the composition of natural bone, scaffolds for bone repair consisting of collagen and apatite have attracted extensive attention of researchers in recent years. [10, 11]

Recently, great efforts have been made to produce collagen-apatite composites resembling the composition and structure of natural bone from the nano- to the macro-scale. The conventional strategies to produce bone substitutes include coating a collagen scaffold with biomimetic apatite, physically mixing apatite nanocrystals with collagen fibrils, and biomimetic co-precipitation of collagen and apatite in which
apatite is deposited on the surface of collagen fibrils. [3, 12, 15-17] Collagen/apatite scaffolds with improved mechanical properties were achieved by either coating collagen scaffolds with HA or physically mixing pre-synthesized apatite with type I collagen. [3, 18] Recently, our research group employed a self-assembly approach to form extracellular mineralized collagen fibrils in a collagen-containing modified simulated body fluid (m-SBF). [13, 16] The materials characterization confirmed that the scaffold has a composition closely mimicking that of natural bone, and our in vitro cell culture and in vivo animal tests suggested that this structure has excellent osteoconductivity and is suitable for bone repair. [1, 15, 16, 19] Although these biomimetic collagen/apatite scaffolds successfully mimic the composition of natural bone, they do not recapitulate the structure of bone at all levels, especially at the nanostructural level. [20]

In natural bone, HA nanocrystals deposit within the gap zone of collagen fibrils (intrafibrillar mineralization) at the early stage of mineralization and then on the surface of collagen fibrils (extrafibrillar mineralization) at a later stage. [10, 21-25] Bundles and arrays of mineralized collagen fibrils further arrange into a multilayered structure rotating across concentric lamellae, which pack densely into compact bone or loosely into spongy bone. [26-28] Both intrafibrillar and extrafibrillar mineralization are essential for the functions of natural bone. Intrafibrillar and extrafibrillar mineralization of collagen fibrils have been reported to contribute to the high mineral content in bone and the improvement of mechanical and biological properties in collagen matrix. [29-33] Thus, biomimetic mineralization must
recapitulate both intrafibrillar and extrafibrillar mineralization of collagen fibrils to mimic natural bone.

Extensive research has been conducted on the mechanism of intrafibrillar mineralization, and it has been proposed that non-collagenous proteins (NCPs) play an important role in the mineralization process. Chen et al. investigated the effect of NCPs (osteocalcin (OC) and bone sialoprotein (BSP)) on intrafibrillar and extrafibrillar mineralization of collagen fibers. [21] Both BSP and OC are present on the surface of collagen fibrils, but only OC is present within the intrafibrillar space of collagen fibrils. [21] BSP is too large to be accommodated by the gap zone of collagen fibrils due to the size exclusion rule for NCPs - molecules larger than a 40 kDa protein are completely excluded from collagen fibrils while those smaller than 6 kDa can freely diffuse into all spaces within collagen fibrils. [34] Moreover, OC would potentially be located at the boundary between the gap and the overlap region of collagen fibers, further confirming the observations by Nudelman et al.[25] Nudelman et al. proposed that the net charges of each band play crucial roles in the deposition and maturation of HA. Rodriguez et al. further proved that osteopontin, the most abundant NCP in bone, can induce intrafibrillar mineralization of collagen and modulate osteoclastic activity in vitro. [35]

With a better understanding of the mechanism for intrafibrillar mineralization of collagen, biomimetic collagen/apatite scaffolds mimicking the natural structure of bone from nano- to macro-scale have been investigated. Collagen scaffolds, turkey tendons, eggshell membranes and demineralized human dentines have been subjected
to either intrafibrillar or extrafibrillar mineralization via a polymer-induced liquid-precursor (PILP) process. [36-42] In the PILP process, an electrolyte is used to form a fluidic amorphous calcium phosphate (ACP) precursor. Polyanionic electrolytes, such as poly (aspartic acid) (PAsP), poly (acrylic acid) (PAA) and poly (vinyl phosphonic acid) (PVPA), are often used to mimic the functional groups of NCPs due to the limited availability and high cost of NCPs. [43-45] Collagen tapes were immersed in PAsP-stabilized ACP nanoprecursors, and hydroxyapatite initially precipitated within collagen fibrils, and then a mineral coating formed on the surface of the collagen tape. [43, 44] In a separate study, human demineralized dentine was incubated in ACP precursors, which were stabilized by poly(amidoamine) (PAMAM), another candidate to mimic NCPs. [40] Intrafibrillar HA mineralization was achieved, which mimicked the arrangement of natural minerals in human dentine at the nanometer scale. Thus, a bioinspired intrafibrillar mineralization process can be achieved via employing polyanionic electrolytes, which are analogs of NCPs. [36-40]

However, a two-step process is normally required to produce a collagen/HA scaffold with intrafibrillar mineralization. Typically, a collagen scaffold is produced, and then incubated in a precursor solution to induce mineral infiltration into collagen fibrils. There are no reports on a bottom-up approach to fabricate scaffolds with a bone-like composition and hierarchical structure. In the bottom-up approach, materials assemble from the nanoscale, such as molecules, ions and atoms, to form a macroscopic structure. [46] In this study, we employed a bottom-up approach to produce intrafibrillar and extrafibrillar mineralized collagen-apatite scaffolds from the
collagen molecules and ACP nanoprecursors instead of the aforementioned two-step process. The structure and biological performance of the intrafibrillar and extrafibrillar mineralized collagen-apatite scaffolds were compared with that of extrafibrillar mineralized scaffolds produced via our previous co-precipitation method.

6.2 Materials and methods

6.2.1 Materials

Type I collagen was extracted from rat tails, purified based on the protocol reported by Rajan et al. [47] Polyacrylic acid (PAA, Mw 5000 Da) and sodium tripolyphosphate (TPP) were purchased from Sigma-Aldrich. All chemicals were of analytical chemical grade.

6.2.2 Fabrication of extrafibrillar mineralized collagen-apatite (E-Col-Ap) scaffolds

Based on our previously established protocol, E-Col-Ap scaffolds were produced by the following procedure: A 6X simulated body fluid, called modified simulated body fluid (m-SBF) was prepared as reported previously: [48-50] NaCl, K$_2$HPO$_4$·3H$_2$O, MgCl$_2$·6H$_2$O, CaCl$_2$ were added in deionized water (DIW) in the order as they are listed, and the ion concentrations were adjusted according to Table 6.1. A collagen stock solution (4.5 mg/mL) was diluted to 2.2 mg/mL by the addition of m-SBF. The pH of the collagen containing m-SBF solution was adjusted to 7.2 by addition of HEPES (4-(2-hydroxyapatiteethyl)-1-piperazineethanesulfonic acid), NaHCO$_3$ and NaOH. A two-temperature process was used to form a mineralized
collagen hydrogel: The solution was incubated at 25 °C for 1 h and then transferred to a water bath at 37 °C for 24 hr. Subsequently, the collagen-apatite hydrogel was allowed to undergo unconfined self-gravity driven compression (self-compression) at room temperature for 20 min. The resulting self-compressed gel was transferred to a custom-made mold and subjected to unidirectional freezing in a freeze-drier (Free Zone®, Labconco, USA) precooled to -25 °C. The freeze-dried scaffolds were subsequently cross-linked with 1 wt% N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) for 24 h. Cross-linked scaffolds were then rinsed thoroughly in DIW, followed by rinsing with a 5% glycine solution to react with residual active carboxylic acid groups. [51, 52] The scaffolds were then rinsed again with DIW, and freeze-dried for a second time to completely remove all the moisture within the scaffold while maintaining the structure of the scaffold.

6.2.3 Fabrication of intrafibrillar and extrafibrillar mineralized collagen apatite (IE-Col-Ap) scaffolds

Based on our previously established protocol for the biomimetic intrafibrillar mineralized collagen fibrils, [16, 53] IE-Col-Ap scaffolds with a lamellar structure were fabricated via the following procedure: PAA, the sequestration analog, was added to a m-SBF solution to form stabilized ACP nanoprecursors (PAA-ACP). The m-SBF was prepared as described above. [48-50] The concentration of PAA in PAA-ACP nanoprecursor was 800 ug/mL. Type I collagen stock solution (4.5 mg/mL) was diluted to a given concentration (2.2 mg/mL) using PAA-ACP nanoprecursors. TPP (1.2 wt%), the templating analog, was added to the collagen stock solution to
template the orderly arrangement of hydroxyapatite within collagen fibrils. The pH of the solution was then adjusted to 7.2 by addition of HEPES, NaHCO$_3$ and NaOH. All of the reactions were taken place at 4 °C, and then transferred to a water bath at 37 °C for 24 h. The self-compression process, unidirectional freeze-drying and cross-linking process were conducted following the same procedure as described above.

6.2.4 Materials characterization

A small piece of collagen-apatite hydrogel (IE-Col-Ap and E-Col-Ap hydrogel) prepared by the two-temperature process was placed on a copper grid and observed using transmission electron microscopy (TEM) at an accelerating voltage of 80 kV and selected area electron diffraction (SAED) coupled to TEM (TEM, JEOL JEM-2010). [54] The cross-linked dry scaffold (IE-Col-Ap and E-Col-Ap scaffold) was grinded into tiny pieces in liquid nitrogen, dispersed in ethanol, deposited onto a copper grid, dried in air, and then observed using both TEM and SAED coupled to the TEM. The surface morphology of the freeze-dried collagen-apatite scaffold was imaged using field emission scanning electron microscopy (FESEM, JEOL JSM-6335F, Japan).

Freeze dried IE-Col-Ap and E-Col-Ap scaffolds were characterized using attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR). A Nicolet Magna 560 FT-IR spectrophotometer (Artisan Technology Group ®, Illinois, USA) with an ATR setup was used to collect infrared spectra in the range of 4,000-400 cm$^{-1}$ at a 4 cm$^{-1}$ resolution, 32 scans. X-ray diffraction (BRUKER AXS D2 Phaser) was also performed on the mineralized collagen scaffolds from 10° to 50° at a
step size of 0.02° and a scan rate of 0.5 °/min with CuKα radiation. Thermogravimetric analysis (TGAQ-500, TA Instrument, USA) was performed from 30 to 900 °C with a heating rate of 10 °C/min in air to determine the mineral content in the collagen composite scaffolds.

6.2.5 In vitro cell culture

The ability of E-Col-Ap, and IE-Col-Ap scaffolds to support cell proliferation was monitored through the reduction of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) dye. A pre-osteoblastic cell line, mouse calvaria 3T3-E1 (MC3T3-E1), was used for the study. Cells were grown in alpha modified eagles medium (α-MEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C under an atmosphere of 5% CO₂. The medium was changed every other day until the culture reached 90% confluence, at which point cells were harvested and seeded onto pure collagen, E-Col-Ap, and IE-Col-Ap scaffolds at 7,500 cells per scaffold (n ≥ 4). Scaffolds were cylindrical with a diameter of 3.5 mm and a height of 1 mm.

For the proliferation study the medium was replaced every other day. Proliferation was assessed by replacing the medium at 1, 3, and 7 days with a working solution of α-MEM supplemented with 10% MTT solution (5 mg/ml). The treated scaffolds were incubated for 4 hours at 37 °C. The medium was removed and dimethyl sulfoxide was added to dissolve the formed formazan crystals. Aliquots of 150 µl were measured using a microplate reader (Biotek) at a wavelength of 560 nm. All data are represented as the mean ± standard error. Statistical analysis was
conducted using a student $t$-test with two tails, and differences were considered significant if $p < 0.05$.

6.3 Results and discussions

Figure 6.1 shows the TEM images of mineralized collagen fibrils in the IE-Col-Ap and E-Col-Ap hydrogels. At the initial stage of IE-Col-Ap scaffold preparation, a hydrogel consisting of intrafibrillar and extracellular mineralized collagen fibrils was formed using a two-temperature process (Figure 6.1A). In Figure 6.1A, a clear D-banding pattern is observed in the unstained collagen fibrils, indicating electron-dense minerals have deposited within specific sites of collagen fibrils, reproducing the D-banding pattern of naturally mineralized collagen fibrils normally observed in bone. [22] The SAED result of intrafibrillar minerals does not demonstrate a ring pattern, but only a diffraction spot (Figure 6.1A, inset), indicating that the minerals deposited are nanocrystalline. This specific alignment of minerals within collagen fibrils was regulated by TPP, a templating analog of NCPs. [37] Highly negatively charged TPP accumulates within positively charged gap zones of collagen fibrils through electrostatic attraction, which further attracts fluidic PAA-ACP nanoprecursors to enter the gap zone of collagen fibrils via electrostatic attraction. [25, 44, 53, 55] Thus, TPP functions as an ionic bridge between collagen fibrils and PAA-ACP nanoprecursors due to the high affinity of TPP to collagen and calcium. [53] A large fraction of the charged functional groups in the gap zone recruit and bind with calcium and phosphate ions from the PAA-ACP nanoprecursors to precipitate electron-dense minerals within the gap zone, which results in the observed
D-banding of collagen fibrils. [42, 55, 56] In extrafibrillar mineralized collagen fibrils, D-banding is not observed. Instead, minerals are only formed on the surface of collagen fibrils (Figure 6.1B). The SAED result of extrafibrillar mineralized collagen fibrils exhibits a ring pattern of typical low crystalline hydroxyapatite (Figure 6.1B, inset).

![Figure 6.1](image)

**Figure 6.1.** TEM images of a small piece of IE-Col-Ap (A) and E-Col-Ap (B) hydrogel fabricated using the two-temperature process. Inset images in A and B: SAED results. The mineralized collagen fibrils were unstained.

Then IE-Col-Ap and E-Col-Ap hydrogels were self-compressed and frozen radially to form the hierarchical scaffold as shown in Figure 6.2. Both IE-Col-Ap and E-Col-Ap scaffolds exhibit a multi-lamellar structure from the macrostructure to the microstructure level. At the macrostructure level, unidirectional macro-pores are aligned across the entire scaffold (Figures 6.2A and 6.2B). At the microstructure level, each layer is comprised of aligned and well stacked lamellae with a thickness of
several hundred nanometers (Figures 6.2A1 and 6.2B1). In each lamella, mineralized collagen fibers are partially aligned along the freezing direction of the scaffold, as indicated by double headed arrows shown in Figures 6.2A2 and 6.2B2. In the extrafibrillar mineralized scaffold, minerals are observed to be randomly dispersed on the surface of collagen fibrils (Figure 6.2B2, black circle). The shear stress during the gravity self-compression and unidirectional freezing processes leads to the alignment of macro-pores, a multi-lamellar structure, and partially aligned mineralized collagen fibrils of the Col-HA scaffold (Figure 6.2). [16]
**Figure 6.2.** SEM images of IE-Col-Ap (A, A1, A2) and E-Col-Ap (B, B1, B2) scaffolds. Double headed arrows in A2 and B2 indicate the freezing direction of self-compressed hydrogels. Black circle in B2 shows the randomly dispersed minerals on the surface.

Freeze-dried IE-Col-Ap and E-Col-Ap scaffolds were grinded into tiny pieces, and observed using TEM (Figure 6.3). At the nanostructure level of the IE-Col-Ap
scaffold, minerals are arranged within collagen fibrils and a clear D-bandning pattern is observed (Figure 6.3A). Minerals are also observed on the surface of the collagen fibrils, as indicated by white arrow in Figures 6.3A. As demonstrated by SAED (inset of Figure 6.3A), the typical ring pattern for HA is observed, indicating that the minerals formed within collagen fibrils are HA. An arc-shape diffraction pattern of the (002) growth face is observed in Figure 6.3A, implying that the mineral phase is oriented along the long axis direction of collagen fibrils. [22, 57] In contrast, the minerals within the E-Col-Ap scaffold are randomly dispersed on the surface of collagen fibrils (Figures 6.2B2 and 6.3B). The ring pattern observed from SAED is consistent with the typical ring pattern for HA which implies that the minerals formed are HA (inset of Figure 6.3B).

![Image](image_url)

**Figure 6.3.** Freeze-dried IE-Col-Ap (A) and E-Col-Ap (B) scaffolds were ground into small pieces in liquid nitrogen, and one of these small pieces were observed using TEM. Inset images in A and B: SAED results. White arrows in A and B indicate the extrafibrillar minerals.
Figure 6.4 shows the XRD spectra of IE-Col-Ap and E-Col-Ap scaffolds. IE-Col-Ap and E-Col-Ap scaffolds exhibit similar XRD spectra (Figures 6.4A and 6.4B). The broad peak at about 20° (2θ) is attributed to collagen. The peak at 26.2° (2θ) corresponds to the (002) plane of HA. The peak at 29.4° is assigned to the (210) plane. The broad peak from 30.7-35° is an overlap of three major reflections (112), (211) and (300). The peak at about 39.6° corresponds to (310) plane of HA. The broad peaks of 30.7-35° indicate that the apatite obtained is at the nanosized level and poorly crystalline. [16]

![Figure 6.4](image)

**Figure 6.4.** XRD spectra of IE-Col-Ap (A) and E-Col-Ap (B) scaffolds.

Figure 6.5 shows the FT-IR spectra obtained from IE-Col-Ap and E-Col-Ap scaffolds. Both IE-Col-Ap and E-Col-Ap scaffolds show similar spectra. The broad peak around 3400 cm\(^{-1}\) is associated with the absorption of water in the scaffolds. Both spectra show typical peaks of collagen: the peak around 1660 cm\(^{-1}\) is attributed to the C=O stretching vibration (amide I), while those at 1549 cm\(^{-1}\) and 1188 cm\(^{-1}\) arise from angular deformation of N-H bond (amide II) and vibration of C-N bond (amide III). All of these peaks are typical major peaks for collagen. Peaks between
500-600 cm\(^{-1}\) are assigned to anti-symmetric bending vibration of the PO\(_4^{3-}\) groups, and peaks at 950 cm\(^{-1}\) and 1036 cm\(^{-1}\) are identified as the stretching vibration of PO\(_4^{3-}\) groups. Moreover, the peaks at 1410 cm\(^{-1}\) and 875 cm\(^{-1}\) are typical vibration bands of carbonate apatite, which is similar to the mineral phase in natural bone. [58]

![FT-IR spectra of IE-Col-Ap (A) and E-Col-Ap (B) scaffolds.](image)

**Figure 6.5.** FT-IR spectra of IE-Col-Ap (A) and E-Col-Ap (B) scaffolds.

Figure 6.6 shows TGA measurements of E-Col-Ap and IE-Col-Ap scaffolds. Both E-Col-Ap and IE-Col-Ap scaffolds have a mineral content of approximately 30 wt%. In the E-Col-Ap scaffolds, the first 7 \% of weight loss occurs between 30-150 °C (peak at 71.2 °C), which corresponds to the evaporation of physisorbed water on the scaffold. Decomposition of collagen molecules occurs between 200-650 °C (peaks at 308.9 °C and 544.5 °C), leaving a residual 30 wt\% which are the minerals obtained (Figure 6.6B). In the IE-Col-Ap scaffolds, the initial weight loss (10 wt\%) occurs between 30-150 °C (peak at 50 °C), and the decomposition of collagen molecules occurs between 150-800 °C (peaks at 227.7 °C, 306.6 °C and
576.2 °C), which yields approximately 30.6 wt% of minerals (Figure 6.6A). From Figure 6.6 the decomposition of collagen molecules in E-Col-Ap scaffolds started at a higher temperature than that of IE-Col-Ap scaffolds. This may be due to the extrafibrillar HA minerals existing on the surface of collagen fibrils, which in turn protects the collagen fibrils from decomposition. However, a higher temperature was needed to fully decompose the collagen in the IE-Col-Ap scaffolds, which may be due to the association of intrafibrillar minerals with collagen fibrils.

**Figure 6.6.** TGA of IE-Col-Ap (A) and E-Col-Ap (B) scaffolds.

Compared to E-Col-Ap scaffolds fabricated in our previous study using a co-precipitation method, similar composition and crystallinity of HA minerals were obtained. However, oriented HA minerals were formed within collagen fibrils via this bottom-up approach, which closely mimics the organization of mineralized collagen fibrils in natural bone.

Substantial research has been performed on the mechanism of intrafibrillar mineralization of collagen fibrils. [25, 43, 44, 56, 59] Two functional groups in NCPs
have been found to play vital roles in regulation of nucleation, dimension, and order of HA deposition within natural bone. Acidic sequestrating motifs of NCPs bind to calcium and inhibit the precipitation of calcium phosphate, forming stabilized-ACP nanoprecursors. [60] The templating groups of NCPs regulate the deposition of HA within gap zone of collagen fibrils through its excellent binding capacity for calcium and its strong affinity for collagen fibrils. [37] Fluidic ACP nanoprecursors infiltrate and are retained within the gap zone of collagen fibrils through electrostatic attraction, capillary action and size exclusion. [61] Then the infiltrated ACP minerals mature into HA within collagen fibrils.

However, it is still not clear why the polymer-induced liquid precursor droplets are more likely to infiltrate collagen fibrils instead of precipitating on the surface of collagen fibrils. If only size exclusion and electrostatic attraction played a role in mineralization, then more minerals should have deposited on the surface of collagen fibrils since there is more room on the surface for nucleation than within the collagen fibers. The electrostatic attraction is presumably the same, since charged groups are located on both the interior and exterior of collagen fibers. [35, 56] Thus, more minerals should have deposited on the surface of collagen fibrils. However, this was not observed in our study. Herein, we propose that there are other driving forces playing crucial roles in directing the deposition and maturation of HA minerals within collagen fibrils: the stereochemical match between the interior of collagen fibrils and ACP precursors. On one hand, the charged functional groups of collagen molecules are located and oriented spatially within collagen fibrils to increase the likelihood of
calcium and phosphate ions binding to the fibrils, leading to the nucleation of ACP within collagen fibrils. This has been confirmed by Xu et al. by applying all-atom Hamiltonian replica exchange molecular dynamics simulation. [56] On the other hand, calcium ions induce conformation changes of many proteins. [21, 62] Bound calcium ions to dentin matrix protein 1 (DMP 1) induce a conformation change resulting in self-assembly into elongated dimers. [63] Osteocalcin also undergoes a conformation change when calcium binds. The conformation change resembles the interatomic locations of calcium in the HA lattice. [21, 64] Thus, conformation changes of proteins occur after binding with calcium to form ACP nanoprecursors, which leads to the re-arrangement of calcium and phosphate ions into a structure resembling HA. This re-arrangement of calcium and phosphate ions decreases the activation energy for the transformation of ACP into HA. Moreover, the conformation change helps NCPs interact specifically and spatially with certain domains of collagen sequences, which leads to the spatial deposition of minerals within collagen fibrils. [65] Thus, we propose that HA minerals form within collagen fibrils due to two factors: (1) The stereochemical match of NCPs with the interior functional groups of collagen fibrils, and (2) the conformation change of NCPs decreases the activation energy of nucleation within collagen fibrils and regulates HA orientation. [65, 66] Meanwhile, the work by other research groups also proposed the stereochemical template function of collagen fibrils, which is conducive to nucleation, growth and development of apatite crystals within collagen fibrils. [67, 68] A similar mechanism has been proposed that specific molecular recognition between DMP1 and apatite surface aids
in the controlled growth of HA crystals with oriented crystallography. [63] Thus, PAA-ACP nanoprecursors penetrate the gap zone of collagen fibrils via capillary action and electrostatic attraction. Subsequently, both spatial interactions between collagen molecules and ACP nanoprecursors along with preferential association of NCPs on certain HA crystallographic planes play important roles in the orderly alignment of HA within collagen fibrils. [65, 69]

**Figure 6.7.** Proliferation of MC3T3-E1 cells on collagen, E-Col-Ap, and IE-Col-Ap scaffolds over the course of 7 days. Data is represented as the mean ± standard error. # denotes statistical significance (\( p < 0.05 \)) between collagen scaffolds and experimental scaffolds.

The biocompatibility of the scaffolds was assessed through the proliferation of MC3T3-E1 cells on E-Col-Ap and IE-Col-Ap scaffolds compared to cell proliferation on pure collagen scaffolds which were used as a control, and the results are depicted in Figure 6.7. All scaffolds supported cell growth over the course of 7 days. Cell
proliferation was the greatest on collagen scaffolds and statistically significant from mineralized scaffolds. The difference in mineralization strategies did not result in a statistically significant difference in proliferation between E-Col-Ap and IE-Col-Ap. Despite the lower rate of cell proliferation on E-Col-Ap and IE-Col-Ap scaffolds compared to collagen scaffolds, the mineralized scaffolds are a suitable material for bone tissue engineering. The *in vitro* performance of a material is not always indicative of its *in vivo* performance. Calcium phosphate materials are well known for their excellent biocompatibility and *in vivo* performance, but these materials have been shown to have inhibitory effects on osteoblasts *in vitro*. [70-73] Lyons *et al.* observed inhibited cell proliferation on a biomimetic collagen-calcium phosphate scaffold compared to a collagen-glycosaminoglycan scaffold *in vitro* but observed a better response *in vivo* with the collagen-calcium phosphate scaffold. [73] Minardi *et al.* also observed inhibited cell proliferation of hBM-MSC on a collagen HA composite *in vitro* compared to a pure collagen scaffold. [71] The presence of the biomimetic apatite phase was found to support the expression of osteogenic genes better than the pure collagen scaffold. [74] E-Col-Ap scaffolds fabricated with the described technique have previously been shown to have excellent *in vivo* performance. [15] Future studies to compare the *in vivo* performance of IE-Col-Ap to E-Col-Ap scaffolds will be conducted to determine the role of intrafibrillar mineralization on the scaffolds bone forming ability.

### 6.4 Conclusions

A biomimetic Col-HA scaffold mimicking the composition and structure of
natural bone has been successfully prepared via a bottom-up approach. Compared with E-Col-Ap scaffolds prepared using the co-precipitation approach in our previous study, IE-Col-Ap scaffolds showed similar multi-lamellar structure and mineral composition, which were confirmed by FESEM, XRD and FT-IR examinations. However, the TEM and SAED results indicated that HA minerals in IE-Col-Ap scaffolds were deposited within collagen fibrils and oriented along the long axis of these fibrils, which bears higher resemblance to natural bone than the scaffold fabricated using the co-precipitation approach. The biomimetic Col-HA scaffolds demonstrated good biocompatibility in vitro with comparable cellular proliferation for both E-Col-Ap and IE-Col-Ap scaffolds. This biomimetic IE-Col-AP scaffold can be a promising biomaterial for bone tissue engineering applications.
References:


[23] X. Wang, F. Cui, J. Ge, Y. Wang. Hierarchical structural comparisons of bones


[52] X. Duan, C. McLaughlin, M. Griffith, H. Sheardown. Biofunctionalization of


7. Conclusions and future work

7.1 Conclusions

The objective of this study was to prepare and characterize biomimetic collagen-apatite composite coatings and scaffolds for enhanced bone repair and regeneration. In Chapter 2, a crack-free apatite coating was produced using a biomimetic approach. Biomimetic apatite was deposited on the surface-treated Ti-6Al-4V substrates by soaking them in the modified simulated body fluid (m-SBF) for 24 hours. The apatite morphology could be adjusted by controlling the pH of the m-SBF solution. Dual beam FIB/SEM was used to mill the cross-sections and prepare TEM foils. Both cross-sectional SEM images and TEM observations confirmed that a well-bonded HA coating has been formed on the Ti-6Al-4V substrate, and that the biomimetic apatite coating possessed a unique gradient structure, with a dense structure adjacent to the substrate to facilitate a strong bonding, and a porous structure at the surface to allow bone cell attachment.

Furthermore, building on the success of preparation of biomimetic apatite coatings, biomimetic collagen-apatite composite coatings were successfully fabricated on the Ti-6Al-4V substrates to better mimic the composition of natural bone. The coating was also formed by simply immersing the surface-treated Ti-6Al-4V substrate into a collagen-containing m-SBF. The cross-sectional microstructure of the collagen-apatite coating has been investigated using dual beam FIB/SEM. It has been revealed that the collagen-apatite coating also consists of a gradient structure with a
dense a dense layer adjacent to the interface and a porous structure towards the surface. Compared with apatite coating, collagen-apatite composite coating formed is thinner and less porous than the apatite coating.

The cross-sectional microstructure of both the apatite coating and the collagen-apatite composite coating has been investigated using dual beam FIB/SEM with either gallium (GFIB) or xenon plasma (PFIB) ion source, and the capabilities of these two dual beam FIB/SEM systems were also compared. Although GFIB and PFIB share a lot of similarities, there are also significant differences, including the optimal voltage of ion-beam-induced Pt deposition, the TEM sample preparation process, and the efficiency and the quality of the cross-sectional cuts produced. Particularly, the GFIB is more beneficial for preparing the high-quality final finish of the sample, while the PFIB is more efficient for creating a large cut in the sample.

Inspired by the composition and structures of natural bone, intrafibrillar calcification of collagen fibrils have been successfully produced using poly (acrylic acid) as a sequestration analog and sodium tripolyphosphate as a templating analog. First, the effect of the sequestration analog on the intrafibrillar mineralization has been investigated. Based on the success of intrafibrillar calcification of collagen fibrils, intrafibrillar silicification of collagen fibrils has been successfully produced by a one-step collagen self-assembly/silicification approach, in which both the collagen fibril self-assembly and the intrafibrillar silicification occurred simultaneously. As the positive analog of the zwitterionic proteins in silicification, poly (allylamine hydrochloride) promotes silica precipitation in the presence of a negative analog, such
as sodium tripolyphosphate. The structure of the silicified collagen fibrils can be manipulated by varying silica precursors, polyanions and silica/collagen ratios to produce silicified collagen fibrils with a core-shell, twisted, or banded structure. Further, the mechanism of the collagen silicification has also been explored. The intrafibrillar silicified collagen fibrils possess better cell compatibility compared with the collagen-apatite fibrils.

A collagen-apatite scaffold consisting of intrafibrillar mineralized collagen fibrils has been successfully prepared via a bottom-up approach, closely mimicking the composition and structure of natural bone. Such prepared scaffolds demonstrate a hierarchical structure at nano-, micro- and macro-levels, bearing striking resemblance to natural bone. The results indicated that at the nanoscale level, the apatite minerals deposited within the gap zone of collagen fibrils and oriented along the long axis of these fibrils; At the microscale level, the aligned thin multi-lamella formed a thick layer; At the macroscale level, the scaffolds consisted of interconnected macropores throughout the entire scaffold. This biomimetic collagen-apatite scaffold with high resemblance to natural bone can be a promising biomaterial for future bone tissue engineering applications.

7.2 Future work

Great progresses have been made to produce intrafibrillar calcification and silicification of collagen fibrils, but there are still many challenges remaining, for examples: (1) the apatite mineral content in the intrafibrillar mineralized collagen-apatite scaffolds is much lower than that in the natural bone; (2) the lack of
Silicon-containing biomaterials have been found to be osteoinductive for new bone formation and stimulative for neovascularization. Intrafibrillar silification of collagen fibrils have been successfully developed in our study. In order to impart the osteoinductivity to the collagen-apatite scaffolds, a scaffold with both intrafibrillar apatite and silica have been produced. Figure 7.1 shows the TEM images of intrafibrillar mineralized collagen fibrils in the collagen-apatite, collagen-silica and collagen-apatite-silica hydrogels. At the initial stage of scaffold preparation, a hydrogel consisting of apatite and/or silica mineralized collagen fibrils was formed using a two-temperature process (Figure 7.1). In Figure 7.1, clear D-banding pattern are observed in the unstained collagen fibrils, indicating electron-dense minerals have deposited within the gap zone of collagen fibrils, reproducing the D-banding pattern of naturally mineralized collagen fibrils normally observed in bone. As discussed above, sodium tripolyphosphate was applied as the templating analog to direct the deposit of calcium phosphate (Figure 7.1A) and silica (Figure 7.1B) within gap zone of collagen fibrils, and then composite minerals of calcium phosphate and silica were deposited within the collagen fibrils (Figure 7.1C).

Further characterization needs to be conducted to prove the application of the intrafibrillar collagen-apatite-silica composite scaffolds in bone tissue engineering. In vitro cell culture studies are essential for initial assessing the biocompatibility of the material, because they help predict how the material will interact with cells in vivo. Cell culture studies will be conducted using MC3T3-E1 cells, an osteoblast precursor.
Intrafibrillar silica, apatite and apatite/silica mineralized collagen scaffolds will be prepared to assess the viability and proliferation of cells, which will be monitored through the use of MTT dye.

**Figure 7.1.** TEM images of a small piece of hydrogel composed of intrafibrillar apatite (A), silica (B) and apatite/silica (C) mineralized collagen fibrils. The mineralized collagen fibrils were unstained.
Appendice A: List of publications

Publications


Conference presentations


2. Changmin Hu, Mark Aindow, Mei Wei*. A comparison of using plasma FIB and
Ga FIB to study porous hydroxyapatite coating on Ti6Al4V alloy. *2016 MRS Fall Meeting & Exhibit*, USA, MA, 2016.
