Transformation of nacre coatings into apatite coatings in phosphate buffer solution at low temperature

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Received 28 July 2006; revised 20 April 2007; accepted 15 May 2007
Published online 9 November 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.31541

Abstract: Nacre coatings were deposited on Ti6Al4V substrates by electrophoretic technique, and subsequently converted into apatite coatings with hierarchical porous structures by treatment with a phosphate buffer solution. The samples were characterized by X-ray diffraction, Fourier transform infrared spectroscopy, scanning electron microscopy, transmission electron microscopy, inductively coupled plasma optical emission spectroscopy, X-ray photoelectron spectroscopy (XPS), and N2 adsorption–desorption isotherms. The results show that the nacre coatings are converted into the plate-like apatite coatings via a dissolution–precipitation reaction, while the organic components of the nacre are reserved. The mesopores with pore size of \( \sim 4.4 \) nm are formed within the plate-like structure, and the macropores are formed among the plate-like structure. Simulated body fluid (SBF) immersion tests reveal that the apatite coatings have a good \textit{in vitro} bioactivity. Bone-like apatite crystals are formed on the surfaces of the apatite coatings after soaking in SBF for 12 h, and fill up the macropores on the coatings with increasing the soaking time. In addition, XPS indicates that a TiO\(_x\) layer and PO\(_4\)\(^{3-}\) ions appear on the substrate surfaces by pretreatment with a H\(_3\)PO\(_4\)/HF solution. The TiO\(_x\) layer and PO\(_4\)\(^{3-}\) ions can induce the formation of apatite crystals, resulting in a composition gradient from the oxide layer to the external apatite layer. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 86A: 510–521, 2008

Key words: apatite coating; nacre; mesopore; titanium oxide; chemical treatment

INTRODUCTION

Natural biomaterials, such as algae, coral, and nacre, have been used for bone graft substitutes and for the correction of bone irregularities because of their biocompatibility and osteoconductive activity.\(^1\) Nacre contains one or more signal molecules, such as bone morphogenetic proteins, capable of activating the osteogenic bone marrow cells and leading to bone formation.\(^2\) After nacre granules are implanted into the femurs of rats for 2 weeks, new bone is formed over most of the granules without an intervening soft tissue layer.\(^3\) Unfortunately, intrinsic shape and size of shell nacre limit its wide applications in bioimplants. The drawback can be addressed by depositing nacre powders on Ti6Al4V substrates, which exhibit excellent mechanical properties under load-bearing conditions.\(^4,5\)

Processes for deposition of biocoatings on bioinert metallic materials include plasma spraying,\(^6\) hydrothermal hot-pressing method,\(^7\) and electrophoresis technique.\(^8,9\) Plasma spraying, one of most common methods for actual clinical application, is performed usually at near 10,000° over the decomposed temperature of nacre.\(^10\) Recently, electrophoretic deposition has attracted many researchers’ interests because of its low cost, simple, rapid, and high reproducible coating method.\(^9\) The thickness and morphology of coatings can be regulated easily by changing the depositing time and applied voltage. The main disadvantage of electrophoretic deposition is the weak bonding strength between substrates and coatings, when compared with plasma spraying or thermal spraying. These coatings must be post-treated by densification at \( \sim 800°\).\(^8\) Such high temperatures will cause decomposition of the nacre coatings; therefore, to develop a simple and effective method without densification at high temperatures still remains scientifically challenging.

Another question is that nacre differs from human bone in mineral composition. The mineral phase of nacre is calcium carbonate in the aragonite form,\(^11\) whereas that of human bone is calcium phosphate in the hydroxyapatite form.\(^12\) Hydroxyapatite can be obtained directly from natural calcium carbonate material by hydrothermal method or by treatment
with a phosphate buffer solution (PBS).\textsuperscript{11,13,14} Calcium carbonate is converted hydrothermally to hydroxyapatite in phosphate solutions by solid-state topolectric ion-exchange reaction, in which the hydroxyapatite retains the orientation of original calcium carbonate.\textsuperscript{13,14} Ni et al. have shown that nacre can be transformed into hydroxyapatite in PBS at room temperature via a surface reaction.\textsuperscript{14}

Since mesoporous silica materials were first synthesized using organic templates in 1992,\textsuperscript{15,16} a variety of mesoporous materials and related synthesis strategies have been developed.\textsuperscript{17–19} Recent studies\textsuperscript{20–22} show that the bone-forming bioactivity of bioactive materials is associated not only with their chemical composition and chemical structure, but also with their textural properties (pore size, pore volume, and pore structure). The appropriate porosity allows the ingrowth of bone to achieve full integration with the living bones.\textsuperscript{23} Pores in the microporous (<2 nm) or mesoporous (2 nm < pore size < 50 nm) structures can promote cell adhesion, adsorption of biologic metabolites, and resorbability at controlled rates to match that of tissue repair.\textsuperscript{24} A great mesopore volume and a wide mesopore size distribution create favorable local conditions that lead to the nucleation and growth of carbonate apatite.\textsuperscript{25}

Since China has a vast resource of nacre throughout the country, the synthesis development of bone substitutes using nacre has been undertaken.\textsuperscript{26,27} The major concern of the present work is to deposit nacre coatings on Ti6Al4V substrates by electrophoretic technique, and to convert the nacre coatings into apatite coatings by treatment with PBS. This method of preparing the apatite coatings has four innovations: (a) hierarchical porous structures of the apatite coatings are fabricated without using any structure-directing agents; (b) the organic components of nacre are reserved as the nacre coatings are converted into the apatite coatings; (c) functional graded apatite coatings on Ti6Al4V substrates are formed by the chemical treatment; (d) the apatite coatings assist the formation of a biologically active apatite layer on their surfaces, which makes them potential candidates to be used on implants.

**EXPERIMENTAL PROCEDURES**

**Preparation of specimen**

The solid raw material was the nacre of *Corbicula fluminea*, collected from Zhejiang province in China, composed of 98.1 wt % mineral phases and 1.9 wt % organic components. Nacre powders were obtained by the following procedure.\textsuperscript{23} Briefly, the shell of *C. fluminea* was cleaned of macroscopic impurities in tap water using a brush, and the nacre was separated from the shell by shaving off the outer layers including periostracum and prismatic layer. Then the nacre was sonicated for 5 min, washed with deionized water, and air-dried. Finally, the nacre obtained was ground into powders in a mortar for 40 min.

Titanium alloys (Ti6Al4V), 15 \( \times \) 15 \( \times \) 0.9 mm\textsuperscript{3} in size, were used for substrate materials. Before deposition, the substrates were abraded with 1000-grit SiC paper and washed with pure acetone and deionized water in an ultrasonic cleaner. Acid treatment was performed by soaking these substrates in a 1.0 mol/L \( \text{H}_2\text{PO}_4 \) – 1.5 wt % HF solution for 20 min at room temperature, to form a layer of \( \text{TiO}_x \) gel on their surfaces. After the acid treatment, the substrates were gently washed with deionized water, and dried at room temperature in an air atmosphere.

To deposit the nacre powders on the substrates, an electrophoretic cell using titanium alloys as cathode and a graphite plate as anode was mounted, with two electrodes about 10 mm apart. The electrophoretic process was carried out at 90 V for 2 min. Prior to deposition, the nacre powder suspensions with 1.25 g of solid powders in 250 mL of ethanol were prepared, and then dispersed ultrasonically for 30 min.

After electrophoretic deposition, the nacre coatings were added into a beaker with PBS (\( \text{NaH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4 \) of pH 7.4. The beaker was then placed in a bio-cultivating box kept at 37°C for 1–9 days. To maintain the pH value at 7.4, the PBS was replaced every day. At the end of the experiment the obtained apatite coatings from the nacre coatings were washed with deionized water, and dried in a convection oven at 37°C for 48 h. To obtain easily enough amounts of apatite to measure its mesoporous structure by using N\textsubscript{2} adsorption–desorption isotherms, the nacre powders were immersed into PBS for 1 day without depositing on the substrates.

**Soaking in simulated body fluid**

The *in vitro* assessment of *in vivo* bone-forming bioactivity is typically carried out by soaking biocoatings in simulated body fluid (SBF) and monitoring the formation of apatite on the coating surfaces over time.\textsuperscript{28} SBF was prepared by dissolving reagent grade chemicals of \( \text{NaCl, NaHCO}_3, \text{KCl, K}_2\text{HPO}_4, \text{3H}_2\text{O}, \text{MgCl}_2, \text{6H}_2\text{O}, \text{CaCl}_2, \text{Na}_2\text{SO}_4, \) and \( \text{CH}_3\text{OH}_2\text{CNH}_2 \) into deionized water, and buffering it at pH 7.40 with hydrochloric acid at 37°C. Each apatite coating was soaked in 25 mL of SBF for predetermined time, and placed in a bio-cultivating box at a constant temperature of 37°C. SBF was completely replaced every day. After being soaked, the specimens were washed with deionized water and dried at room temperature in an air atmosphere.

**Characterization**

The crystalline phases of the coatings were examined with X-ray diffraction (XRD, D/max-II B, Japan) using Cu K\( \alpha \) radiation. Morphological observations of specimens were performed by transmission electron microscopy (TEM, CM200/FEG, Philips) and scanning electron micros-
copy (SEM, S-4800, Hitachi) equipped with an energy dispersive spectrometer (EDS). Fourier transform infrared spectra (FTIR, VECTOR22, Bruker) were collected at room temperature using the KBr pellet technique. X-ray photoelectron spectroscopy (XPS, PHI5700 ESCA) of specimens was obtained by using an aluminum anode (Al Kα = 1486.6 eV radiation) at a pressure of 2 × 10⁻⁷ torr. The number of binding energy peaks was determined by the deconvolution process. The binding energies of the atoms were calibrated against a C 1s of 284.6 eV. N₂ adsorption–desorption isotherms were measured with an automatic surface area and porosity analyzer (AUTOSORB-1-C, Quantachrome) at 77 K. The pore-size distributions were derived from the desorption branches of the isotherms using the Barrett-Joyner-Halanda (BJH) method. Apatite coatings converted from nacre coatings were immersed in 25 mL of SBF per one piece of specimen at 37 °C for 0–96 h. After the predetermined soaking time, the concentrations of phosphorus and calcium in SBF were measured by using inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 5300DV, Perkin-Elmer).

RESULTS

Microstructure

Figure 1(a) shows the morphology of Ti6Al4V substrates soaked in a 1.0 mol/L H₃PO₄ – 1.5 wt % HF solution for 20 min. The chemical etch produces a layer of TiOₓ gel with a rough topography when compared with the original sample appearance. The TiOₓ layer with the thickness of ~2 μm [Fig. 1(b)] is found to be amorphous as evidenced by the absence of the sharp characteristic peaks in the XRD pattern. XPS spectrum in Figure 2 indicates the presence of TiOₓ gel, too. Ti and O originate from TiOₓ, and C originates from common organic contamination adsorbed to the substrate surface. The P 2s and P 2p peaks, originating from PO₄³⁻, are also detected on the surface treated with a H₃PO₄/HF solution. How-
ever, the characteristic peaks of F, originating from the HF, are barely detectable on the treated surface.

Figure 3 shows the top-view SEM micrographs of nacre coatings before and after soaking in PBS at 37°C for different days. A perfectly crack-free nacre coating is deposited on the substrate by electrophoresis technique, as shown in Figure 3(a). The higher magnification in Figure 3(b) reveals that the nacre powders in range from 0.2 to 1 μm are stacked loosely together due to the weak electrostatic bonding during the electrophoretic deposition. After soaking the nacre coating in PBS for 1 day, a uniformly crack-free coating is obtained too [Fig. 3(c)]. A striking difference in the crystal morphology appears when compared with the nacre coating. The obtained crystals with plate-like structures aggregate to form macropores with the diameter of ~1 μm [Fig. 3(d)]. After soaking for 9 days, the coating acquires a sponge-like, smooth surface [Fig. 3(f)].

Figure 4 shows the cross-section SEM micrographs of nacre coatings soaked in PBS at 37°C for 1 day. Some large gaps within the TiOx gel are observed in Figure 1(b), demonstrating the weak bonding strength between the substrate and the oxide layer. It is interesting to find that the gaps disappear and the TiOx layer bonds tightly to both the substrate.
and the coating after treatment with PBS for 1 day [Fig. 4(a,b)]. Moreover, there exists a composition gradient of apatite from the oxide layer to the external apatite layer [Fig. 4(a)].

**XRD measurement**

Figure 5 shows the XRD patterns of nacre coatings before and after soaking in PBS at 37°C. Calcium carbonate has three forms, including calcite, vaterite, and aragonite. Its stable phase at atmospheric pressure is calcite, whereas the phase of the mineral in nacre is aragonite with no other phases (JCPDS file no. 76-0606), as shown in Figure 5(a). The XRD pattern has the very sharp peaks characteristic of a well-crystallized mineral. After soaking in PBS for 1 day, the nacre coatings are converted mainly to the apatite coatings with some unreacted aragonite [Fig. 5(b)]. The broad peaks are due to a defective, low crystallinity of the formed apatite, whose crystallographic structure is quite similar to that of biological apatite. This low crystallinity is usually observed in the organic-mediated crystallization of an apatite. After soaking for 9 days, the nacre coatings are converted completely into the apatite coatings [Fig. 5(c)].

**Infrared spectroscopy**

The FTIR spectrum in Figure 6(a) indicates further the characteristic peaks of CaCO$_3$ in the aragonite form, corresponding to CO$_3^{2-}$ at 1480 ($\nu_3$), 1080 ($\nu_1$), 860 ($\nu_2$), 714 ($\nu_4$) cm$^{-1}$, and C=O groups of carbonate ions at 1790 cm$^{-1}$. Previous research shows that the organic matrix of nacre contains many proteins, including fibrous proteins, proteoglycans, and calcium binding proteins, which perform a function similar to that of collagen present in bone, and tend to induce the bone formation. The strong FTIR bands at around 2920, 3430, and 2520 cm$^{-1}$ are attributed to the C—H stretching modes, the OH and/or NH stretching modes, and the OH groups of carboxylic acid, respectively. However, the intense absorption bands in the range 1660–1100 cm$^{-1}$ due to organic components are overlapped by the absorption band of carbonate ions in the $\nu_3$ region, which have been demonstrated by Balmain et al. using the nacre decalcified by EDTA and acetic acid. Figure 6(b,c) show the characteristic absorption bands of the nacre coatings after soaking in PBS for 1 day and 9 days,
respectively. The FTIR spectra show the absorption bands at 563, 604, 1030 cm\(^{-1}\) corresponding to the \(\text{PO}_4^{3-}\) ions, and broad band at around 3420 cm\(^{-1}\) corresponding to OH group.\(^{13,36}\) OH\(^{-}\) and \(\text{PO}_4^{3-}\) in the apatite lattice can be substituted by the \(\text{CO}_3^{2-}\) ion, known respectively as A-type and B-type substitute.\(^{37}\) The FTIR spectrum in Figure 6(c) indicates the characteristic bands of B-type \(\text{CO}_3^{2-}\) substitution at 1420 cm\(^{-1}\) (\(v_3\)) and at 872 cm\(^{-1}\) (\(v_2\)).\(^{37}\) However, the characteristic bands of B-type \(\text{CO}_3^{2-}\) substitution are overlapped by those of the unreacted nacre if the soaking time is 1 day in PBS [Fig. 6(b)]. In addition, the absorption band at 2920 cm\(^{-1}\) attributed to the C–H stretching modes is detected in Figure 6(b,c). The presence of this characteristic band suggests that the organic matrix of nacre is reserved partly as the mineral phase of nacre is transformed into apatite. The organic matrix can stimulate bone cells and bone stem cells.\(^{26,27}\)

Analysis of mesoporous structure

Figure 7 shows the TEM image of apatite coatings converted from nacre coatings by treatment with PBS for 1 day. The image in Figure 7 reveals many light-shaded spots within the plate-like apatite, indicating the presence of mesoporous structures. The mesopores are basically in random arrangement without precise orientation ordering. Figure 8 shows the nitrogen adsorption–desorption isotherm and the corresponding pore size distributions of apatite powders converted directly from nacre powders. The curve in Figure 8(a) is identified as Type IV isotherms with Type H3 hysteresis loops.\(^{38}\) The Type H3 loop, which does not exhibit any limiting adsorption at high \(P/P_0\), is attributed to aggregates of apatite particles giving rise to slit-shaped pores. This result is in good agreement with that of TEM observations in Figure 7. The BJH pore-size distribution plot in Figure 8(b) shows that most of the pore sizes are distributed around 4.4 nm.

DISCUSSION

Nacre coatings deposited by electrophoresis technique

The main problem concerned with electrophoretic deposition is the weak bonding strength between bio coatings and substrates. In the present work, we found that nacre powders were deposited difficulty on the substrates without chemical pretreatment, and the deposited nacre coatings slid easily from the substrate surfaces. Acid pretreatment with a \(\text{H}_3\text{PO}_4/\text{HF}\) solution is one of the important method to address this drawback. A roughly microtopography (Fig. 1) is created after chemical pretreatment, resulting in a micromechanical interlocking bonding.

Another problem concerned with electrophoretic deposition is cracked coatings due to drying shrinkage during drying and sintering stage.\(^{39}\) Wei et al. have solved this problem by using unagglomerated hydroxyapatite nanoparticles, which are obtained from the "gel-like" hydroxyapatite by an Ostwald ripening approach, that is, by boiling and/or ambient aging.\(^{40}\) Another method to produce a thick, uni-
form, and crack-free coating is repeated deposition process. The deposition of the next layer fills up the cracks, which appear after drying of previous layer, and the interface of layers may effectively hinder crack propagation along depth of coating. In the present work, it is worth noting that uniform and crack-free coatings [Fig. 3(a)] are obtained without pretreatment of nacre powders by Ostwald ripening approach or repeated deposition process. The possible reason may be that the presence of organic component reduces the surface energy of nacre powders, resulting in dispersed particles and crack-free coatings after electrophoretic deposition.

Conversion of nacre coatings to apatite coatings

After soaking in PBS for 1 day or 9 days, apatite coatings are obtained from nacre coatings (Figs. 5 and 6). Dissolution–precipitation mechanism can be employed to discuss the conversion mechanism. Previous studies have demonstrated that after soaking nacre powders in PBS, the calcium ions are dissolved from calcium carbonate grains, and react with $\text{PO}_4^{3-}$ ions to form calcium phosphate phases. The apatite is precipitated at the active sites on the surface as the activity product exceeds its thermodynamic solubility product. The reactions can be expressed as follows:

\[
\text{CaCO}_3 \rightarrow \text{Ca}^{2+} + \text{CO}_3^{2-} \quad (1)
\]
\[
5\text{Ca}^{2+} + 3\text{PO}_4^{3-} + \text{OH}^- \rightarrow \text{Ca}_5(\text{PO}_4)_3(\text{OH}) \quad (2)
\]

After soaking nacre coatings in PBS, the other reactions take place simultaneously by the following equations:

\[
\text{CO}_3^{2-} + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{OH}^- \quad (3)
\]
\[
\text{HCO}_3^- \rightarrow \text{CO}_2 + \text{OH}^- \quad (4)
\]
\[
\text{H}_2\text{PO}_4^- \rightarrow \text{HPO}_4^{2-} + \text{H}^+ \quad (5)
\]
\[
\text{HPO}_4^{2-} \rightarrow \text{PO}_4^{3-} + \text{H}^+ \quad (6)
\]

ICP-OES analyses reveal that the formed apatite coatings after soaking in PBS for 9 days are calcium-deficient with an average Ca/P molar ratio of 1.36. The general formula of calcium-deficient apatite, $\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$, with $0 < x < 2$, should be replaced by $\text{Ca}_{10-x-y/2}(\text{HPO}_4)x_y(\text{PO}_4)_{6-x-y}(\text{CO}_3)_{y/2}(\text{OH})_{2-x}$, after $\text{PO}_4^{3-}$ ions in the apatite lattice are substituted partly by $\text{CO}_3^{2-}$ ions (Fig. 6). The average Ca/P molar ratio for the apatite coatings converted from the nacre coatings by treatment with PBS for 1 day is 1.93 due to the presence of unreacted nacre powders [Fig. 5(b)]. The formation reaction of the apatite coatings is expressed as follows:

\[
(10 - x - y/2)\text{Ca}^{2+} + x \text{HPO}_4^{2-} + (6 - x - y)\text{PO}_4^{3-} + y \text{CO}_3^{2-} + (2 - x)\text{OH}^- \rightarrow \text{Ca}_{10-x-y/2}(\text{HPO}_4)x_y(\text{PO}_4)_{6-x-y}(\text{CO}_3)_{y/2}(\text{OH})_{2-x} \quad (7)
\]

The conversion mechanism of the calcium-deficient apatite coatings from the nacre coatings is schematized in Figure 9. After soaking nacre coatings in PBS, calcium ions are dissolved from nacre powders. At the same time, $\text{PO}_4^{3-}$ ions that are transformed from $\text{H}_2\text{PO}_4^-$ and $\text{HPO}_4^{2-}$ ions in PBS enter into the coating. The released $\text{Ca}^{2+}$, $\text{PO}_4^{3-}$, and $\text{HPO}_4^{2-}$ ions increase the degree of supersaturation of the soaking solution with respect to apatite; thus apatite crystals deposit on the substrate surface. The mesoporous structure in plate-like apatite [Figs. 3(d) and 7] is also attributed to the dissolution–precipitation reac-

![Figure 8](https://example.com/figure8.png)

**Figure 8.** (a) Nitrogen adsorption–desorption isotherm and (b) BJH pore size distribution of apatite powders converted from nacre powders by treatment with PBS for 1 day.
(a) the apatite nanoparticles deposit on the nacre powder surface and aggregate, resulting in the formation of slit-shaped pores without precise orientation ordering; (b) the Ca$^{2+}$, CO$_3^{2-}$ ions are released from the inner nacre powder layer by using the slit-shaped pores as ion-transferred channels. The dissolution–precipitation reaction is proved by the different morphologies of the nacre coatings and apatite coatings. Figure 3(b,d) show that the grain-like nacre powders disappear, and the plate-like apatite crystals form after soaking in PBS for 1 day. The plate-like crystals become larger with time [Fig. 3(e)], and then turn into a sponge-like, smooth structure after soaking for 9 days [Fig. 3(f)]. The change of the morphologies reveals that nacre powders are dissolved into the solution and apatite crystals are formed simultaneously.

**Oxide layer on substrate surface**

To improve micromechanical interlocking and physicochemical bonding between biocoatings and substrates, the substrate surfaces are treated with a H$_3$PO$_4$/HF solution. The reactions are expressed as follows:

\[
2Ti + xO_2 \rightarrow 2TiO_x \quad (8)
\]

\[
TiO_2 + 6HF \rightarrow [TiF_6]^{2-} + 2H_2O + 2H^+ 44 \quad (9)
\]

\[
2Ti + 6HF \rightarrow 2TiF_3 + 3H_2 \uparrow \quad (10)
\]

\[
2V + 10HF \rightarrow 2VF_5 + 5H_2 \uparrow \quad (11)
\]

\[
2Al + 6HF \rightarrow 2AlF_3 + 3H_2 \uparrow \quad (12)
\]

The presence of V and Al may be harmful to the biocompatibility of Ti6Al4V substrates. Fortunately, after acid pretreatment, the V and Al originating from the substrates are barely detected in Figure 2(a). The main reason is that the V and Al on the substrate surfaces are oxidized into V$^{5+}$ and Al$^{3+}$ ions, respectively, and then released into the solution, as shown in Eqs. (11) and (12).

The high resolution spectrum of Ti 2p region in Figure 2(b) is decomposed into several contributions. The main doublet peaks are attributed to Ti$^{4+}$, indicating that TiO$_2$ is the main constituent of the oxide layer. The Ti$^{3+}$ peaks corresponding to Ti$_2$O$_3$ are also observed in Figure 2(b). The TiO$_x$ layer not only promotes effectively the adherence of the outer coating because of its rough topography, but also acts as an improved chemical barrier against in vivo release of metal ions from the substrates. Moreover, the TiO$_x$ exhibits an excellent bioactive property to induce the growth of apatite, which is demonstrated by this experimental result.

After soaking nacre coatings in PBS, Ca$^{2+}$ and PO$_4^{3-}$ ions are released from the nacre coatings and PBS, respectively [Eqs. (1), (5), and (6)], and then enter into the oxide layer of substrates. At the same time, the TiO$_x$ layer is dissolved partially into the solution due to the corrosive attack of hydroxyl groups:

\[
TiO_2 + OH^- \rightarrow HTiO_3^- \quad (13)
\]

These negatively charged samples are combined with alkali ions in PBS, resulting in the formation of Ti$-$OH groups. The Ti$-$OH groups formed initially
are combined with positively charged Ca$^{2+}$ ions to form amorphous calcium titanate. As the calcium titanate becomes positively charged due to the accumulation of calcium ions, it combines with negatively charged phosphate ions to form apatite. In addition, the PO$_4^{3-}$ ions adsorbed on the substrate surface due to treatment with a 1.0 mol/L H$_3$PO$_4$ – 1.5 wt % HF solution, are effective for apatite nucleation. The PO$_4^{3-}$ ions can absorb Ca$^{2+}$ to form calcium phosphate phases as the activity product of apatite exceeds its thermodynamic solubility product. Therefore, a composition gradient of apatite is formed from the oxide layer to the external apatite layer, and no crack in oxide layer is observed in Figure 4 when compared with that in Figure 1(b).

**Bioactivity in SBF**

It is believed that the prerequisite for biocoatings to bond to living bone is the formation of a biologically active apatite layer on the surfaces of the apatite coatings in the body. Therefore, the in vitro bioactivity of the apatite coatings, which are converted from nacre coatings, can be evaluated by examining apatite formation on their surfaces in SBF. SEM images of the apatite coatings before soaking in SBF show a plate-like or sponge-like structure [Fig. 3(d,f)]. The deposition of bone-like apatite is observed after soaking the apatite coatings in SBF for 12 h [Fig. 10(a,e)], and the EDS analyses reveal that the apatite formed is also calcium-deficient with an average Ca/P molar ratio of 1.48. The macropores among the plate-like apatite on the surfaces of coatings [Fig. 3(d,f)] are filled with the newly formed apatite, and the surfaces of coatings become smooth with the increase of soaking time in SBF (Fig. 10). High-resolution SEM images in Figure 10 (the insets) show that the apatite crystals formed in SBF are also plate-like with thin edges (~50 nm in thickness), whose morphology is similar to that of the apatite crystals observed on bioactive glasses after soaking in SBF.

Figure 11 shows Ca and P concentrations in SBF as a function of soaking time for the apatite coatings converted from the nacre coatings. As can be seen, the Ca and P concentrations in SBF decrease obviously in the first 48 h, and then stabilize at a certain value or experience a little increase. The soaking time, in which Ca and P concentrations reach a stable value, may be used as one of the criteria for the evaluation of the in vitro bioactivity of biocoatings. The shorter the soaking time is, the better bioactivity the biocoatings have. The changes in the Ca and P concentrations for both the apatite coatings converted from the nacre coatings by treatment with PBS for 1 day and 9 days follow the similar trend. Taken together, SEM, EDS, and ICP-OES investigations demonstrate the rapid in vitro bone-forming bioactivity of the apatite coatings.

The quick formation of bone-like apatite crystals is attributed to the chemical composition of the apatite coatings, the porous structure, and the reserved organic components ascribed to nacre. First, calcium-deficient apatite assists in the immediate nucleation and growth of biologically equivalent apatite after soaking in SBF. ICP-OES analyses reveal that the carbonated apatite coatings converted from the nacre coatings in PBS are calcium-deficient. The calcium ions in SBF are attracted easily on the surfaces of apatite coatings by the negatively charged ions including H$_2$PO$_4^-$ and HPO$_4^{2-}$ because of the presence of the Ca$^{2+}$ deficiencies. The apatite is precipitated as the activity product exceeds its thermodynamic solubility product. This speculation can be demonstrated by the experimental result that the decrease rate of Ca concentrations in SBF is bigger than that of P concentrations (Fig. 11). The bone-forming activity of calcium-deficient apatite is better than that of stoichiometric hydroxyapatite. No apatite crystals deposit on the surface of stoichiometric hydroxyapatite unless the soaking time is 2 days or more in SBF. Second, the high specific surface area and porous structure of the apatite coatings help to accelerate the kinetic deposition process of apatite and enhance bone-forming bioactivity. The apatite coatings with mesopores within the plate-like apatite (Fig. 7) and macropores among the plate-like apatite [Fig. 3(d)] have bigger surface area than those without porous structure. It becomes easy to attract the calcium and phosphorus ions in SBF on the surface of calcium-deficient apatite coatings and to increase the local ions concentrations, due to the increased surface area in contact with the solution. Moreover, the porous structure can provide sufficient space for cell migration, adhesion, and the ingrowth of new bone tissue. Third, the organic components of nacre might create a favorable environment to induce the formation of carbonated apatite because of the presence of functional groups (–NH$_2$ –OH). As the nacre coatings are converted into the apatite coatings, the organic components are reserved partly (Fig. 6); therefore, we cannot ignore their important role in increasing the in vitro bone-forming bioactivity. Previous in vitro studies have demonstrated that the bioactivity of nacre depends on its organic components, which are the source of signal molecules that can stimulate bone cells and bone stem cells. Moreover, the proteins of organic matrix play an important role in determining the distribution of electrical charges at the interface regions, and therefore regulate bone ingrowth and remodeling.

**CONCLUSIONS**

Apatite coatings with hierarchical porous structures were successfully fabricated by a two-stage
application route. In the first stage, uniform and crack-free nacre coatings were deposited on Ti6Al4V substrates by electrophoresis technique. In the second stage, nacre coatings were transformed into apatite coatings at low temperatures by treatment with PBS. The apatite coatings have a superior in vitro

Figure 10. SEM images of nacre coatings soaked in PBS for 1 day followed by soaking in SBF for (a) 12 h; (b) 3 days; (c) 6 days; (d) 9 days. SEM images of nacre coatings soaked in PBS for 9 day followed by soaking in SBF for (e) 12 h; (f) 3 days; (g) 6 days; (h) 9 days. The insets show high-resolution images.
bone-forming bioactivity based on the three reasons: (i) the obtained apatite coatings are calcium-deficient, which assist in the immediate nucleation and growth of biologically equivalent apatite after soaking in SBF; (ii) the hierarchical porous structures of the apatite coatings help to enhance bone-forming bioactivity; (iii) the reserved organic matrix of nacre might induce the formation of carbonated apatite.

The weak bonding strength of electrophoretic deposition is improved by chemical method. A TiOx layer is formed on the substrate surface by chemical etching of a 1.0 mol/L H3PO4 – 1.5 wt % HF solution. This oxide layer with a rough topography increases the micromechanical interlocking bonding between the coating and substrate. Moreover, there exists physicochemical bonding because of the presence of a composition gradient from the oxide layer to the external apatite layer after treatment with PBS.

The apatite coatings converted from the nacre coatings may be promising for bone graft substitutes, although further work is required to determine the in vivo bioactivity of the apatite coatings.

References


Figure 11. Ca, P concentrations in SBF as a function of soaking time for apatite coatings converted from nacre coatings by treatment with PBS for different day: (a) 1 day; (b) 9 days.


