Surface characterization and osteoblast response to a functionally graded hydroxyapatite/fluoro-hydroxyapatite/titanium oxide coating on titanium surface by sol–gel method

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Abstract

Objectives: To improve efficacy of current titanium and its alloys, in bioactivity and speed of osseointegration, of orthopaedic implants.

Materials and methods: A novel triple-layered functional graded coating, consisting of a porous hydroxyapatite (HA) outermost layer, fluoro-HA (FHA) intermediate layer and titanium oxide (TiO₂) innermost layer, was created on a titanium substrate by a multistep sol–gel method. X-ray diffraction analysis showed TiO₂ anatase and apatite crystallization in the coating.

Results: Morphological analysis performed by scanning electron microscopy showed excellent bonding between coating and substrate, with a thickness of ~2 µm. Scratch testing found favourable adhesion strength of the composite coating. In addition, optical microscope images suggested good biocompatibility. Considering that *in vitro* cell response, osteoblasts on the coating exhibited higher cell proliferation and ALP activity compared to pure titanium and HA coating, and demonstrated excellent coating bioactivity.

Conclusions: Current results indicated that the novel TiO₂/FHA/HA coating has promising clinical applications in orthopaedic and dental implantation.

Introduction

Titanium and its alloys have been the main medical materials for dental and orthopaedic implants over the

last three decades, based on their excellent mechanical properties, good corrosion resistance and favourable biocompatibility. Structural and functional connection occurs between the titanium and bone tissue, defined as osteointegration (1,2). However, titanium lacks bioactivity, and requires at least a 3 month load-free healing period for successful osteointegration (3). To reduce healing time, many methods have been investigated to improve bioactivity and speed of osseointegration.

Hydroxyapatite [HA, Ca₁₀(PO₄)₆(OH)₂] coatings on titanium surfaces have been proven to be osteoconductive, reducing time of osseointegration. From the 1980s, HA has widely been used as coating material on the surface of titanium implants by plasma spray, when coating thickness is 50–100 µm. However, after some years, people have found that rapid solubility of the thick HA coating might cause disintegration of the coating layer, and lead to failure of implants (4–6).

To overcome the disadvantage of HA coating, fluoro-hydroxyapatite (FHA, Ca₅(PO₄)₃(OH)_{1–x}F_x) was developed as coating material. FHA is formed by replacing part of OH[–] group in HA with F[–] ions. By incorporation of F[–], dissolution rate and the bioactivity are reduced, although stability increases. Correct amounts of F[–] promote deposition and calcification of Ca and P. By controlling quantities of F[–] incorporation, it is possible to reach an optimal balance between short-term bioactivity and long-term stability (7–10).

TiO₂ film formed on surfaces of titanium in an aqueous environment is very stable and plays an important role in erosion resistance of titanium. Furthermore, TiO₂ has good chemical affinity to both titanium substrate and apatite. Nevertheless, its surface hydroxyl groups such as Ti–OH promote hydroxyapatite formation and bone bonding (11–13).

A much wiser way to design coating for a titanium substrate is to use composite coatings. For example,

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material scientists and engineers have assembled an HA/FHA composite coating on Ti substrate (7,14), which has shown reduced solubility and improved cell activity and functionality. Other studies have demonstrated that HA/TiO₂-coated Ti implants have better long-term stability and improved cell response of osteoblasts *in vitro* and *in vivo* (15,16).

Such composite coating has only solved part of the clinical problem. None fully meets clinical requirements of dental and orthopaedic implants. There are no reports on fabrication and properties of functional graded coatings composed of HA, FHA and TiO₂. The purpose of the study described here was to design and fabricate a novel functional graded coating on the surface of titanium implants composed of nano-HA/FHA/TiO₂, by a sol-gel method (17,18). By such functional graded design of three different coatings, it is possible to reach the optimal balance between short-term bioactivity and long-term stability, which are the two major requirements for ideal dental and orthopaedic implants. Surface and interface microstructure, phase composition and bonding strength of the composite coatings were characterized. Cell responses of osteoblasts to the coatings were investigated.

Materials and methods

Preparation of TiO₂/FHA/HA coatings

Processes of preparing TiO₂ sol, FHA sol and HA sol have been described in more detail in the literature (15,19,20). Whole sol-gel methods began with preparation of TiO₂ sol. Titanium isopropoxide [Ti((CH₃)₂CHO)₄] solution was prepared with absolute ethanol, after which a small quota of distilled water mixed with 2-ethoxyethanol (CH₃CH₂OCH₂CH₂OH, catalyst) was added for hydrolysis, and the mixture was stirred vigorously for 3 min. The mixed sol was aged for 24 h in 0 °C.

Fluoro-hydroxyapatite sol was composed using suitable quantities of ammonium fluoride (NH₄F) in P-containing solution. The [P]/[F] molar ratio was 6 for the purpose of appropriate composition of Ca₁₀(PO₄)₆(F·OH). After stirring for 24 h, the solution was added gently to a Ca(NO₃)₂·4H₂O solution to yield FHA sol.

To prepare HA sol, controlled amounts of triethyl phosphate P(C₂H₅O)₃ and Ca(NO₃)₂·4H₂O (molar ratio Ca:P = 1.67) were dissolved in absolute ethanol. After vigorous stirring for 6 h, HA gel was prepared by heating it to 80 °C and cooling for 2 h. Then, the sol was aged for 48 h to make conventional HA sol (19). To make a porous HA coating, organic porosifier agent (polyvinyl butyral) was added in accordance with the literature (20).

Disc specimens of commercial pure Ti were ground with silicon carbide papers of 500, 800 and 1200 grit,

and ultrasonically cleaned in acetone and ethanol for 5 min. TiO₂, FHA and HA films were obtained in given sequence (TiO₂-FHA-HA) by dip-coating in respective sols at 2 cm/min withdrawal speed. Procured coatings were dried at 80 °C for 10 min, then heat-treated at 500 °C for 1 h in an oven. Specimens with functional graded coating composed of HA/FHA/TiO₂ were made as test group, and pure titanium as well as conventional HA coating was used as controls.

Characterization of coatings

Surface and interface morphology of coatings were investigated using a field emission scanning electron microscope (FE-SEM, JEM-5900LV, JEOL; Tokyo, Japan) and an optical microscope. Crystal structures of the coatings were analysed by X-ray diffraction (XRD, X'Pert Pro MPD X, Philips, Eindhoven, The Netherlands, Cu-K α , λ = 0.15406 nm) and fluorine content was determined by X-ray photoelectron spectroscopy (XPS, PHI 5600; Physical Electronics, Chanhassen, MN, USA). To ensure measurement accuracy, two samples in each group were used and two different areas on each sample were analysed.

Bonding strength

Coating adhesion to Ti substrates was evaluated using WS-2003 MicroScratch Tester, with a Rockwell diamond indenter, 0.2 mm tip radius. The test is described in detail in the literature (21). MicroScratch Tester has a load ranging from 0 N up to 15 N, scratching speed 3 mm/min, and loading speed 10 N/min. Five individual tests were performed on every specimen and average values of critical load were analysed.

In vitro cell assessment

Cell culture. Osteoblast-like cells were extracted from crania of newborn SD mice within 3–5 days of birth (all surgical procedures conformed to the Guide for Animal Care and Use Committee Standards). Cells were washed three times in HANK'S solution containing streptomycin 100 μ /ml and ampicillin 100 μ /ml, then collected by centrifugation. Under standard incubation conditions of 5% CO₂, 37.1 °C, cells were incubated for up to 7 days in osteoblast-inducing conditioned media, containing DMEM, dexamethasone, vitamin C and β -sodium glycerol-phosphate).

Attachment of osteoblasts

To determine the effect of coating-promoted cell attachment, osteoblasts morphology was observed by SEM and optical microscope (22). The culture media were

removed and samples were fixed with 2.5% glutaraldehyde in PBS for 90 min at 4 °C. After dehydration with graded ethanol (70%, 90% and 100%), the samples were sputter-coated with Au-Pd and examined in SEM.

Osteoblast proliferation

MTT colorimetry assay was used to study cell proliferation (23). Five hundred microlitres cell suspension and 50 µl medium were inoculated to a 24-multiwell plate containing samples with different coatings. Pure titanium substrate was tested for comparison. Following incubation for 72 h, suspensions were removed to further 96-multiwell plates, then colorimetric measurement of formazan dye was made using a spectrophotometer at 490 nm.

Alkaline phosphates activity

Level of ALP activity was measured at 5, 10 and 15 days using a phosphate detection kit as previously reported (24). By using the micro-plate assay mode, measurements were taken at 620 nm and a standard curve for calculation of ALP was plotted. Measured ALP activity was finally normalized to total protein content, determined using a commercial Micro BCA protein assay kit, following Coomassie brilliant blue staining as described elsewhere (25).

Statistical analysis of data

Statistical analyses of data were carried out using one-way ANOVA, followed by Student's *t*-test. *P*-value <0.05 was considered statistically significant.

Results

Coating phase and morphology

X-ray diffraction patterns of the functional graded coatings at different stages of multilayer formation are shown in Fig. 1. Coatings have identical XRD patterns of apatite besides strong diffraction peaks of Ti. No impurity phase was detected. A distinct anatase phase appears in XRD patterns after the innermost TiO₂ layer was assembled. After the intermediate FHA layer, a weak 002 peak appeared just to the right of the TiO₂ anatase peak, and distinct 211 peaks appear near 32° in 2θ, which are the main peaks of apatite. The 002 and 211 peaks suggest formation of apatite. After full assembly of the functional graded coating, 002 peak of apatite became higher and anatase peak became weaker, dividing the two peaks from each other; intensity of 211 apa-

Figure 1. X-ray diffraction patterns of pure TiO₂, TiO₂/FHA, TiO₂/FHA/HA coating.

tite peaks also increased. Results indicate that TiO₂ anatase and two kinds of apatite were acquired on the functional graded coating.

After the innermost TiO₂ and intermediate FHA coatings were assembled, XPS survey spectra were shown in Fig. 2. This demonstrates that main elements in the coatings are Ca, P and O. F1s peak was observed at ~690 eV, indicating presence of fluorine in the composite coating. Since there was no obvious peak of other fluorine compounds (such as CaF) in XRD, the only possible source of fluorine was FHA. Figure 3 is XPS after the whole TiO₂/FHA/HA coating was assembled. Obviously reduced intensity of F1s peak was observed. This is because the outermost HA layer is free of fluoride and fluoride of the intermediate layer is difficult to detect, since XPS can only detect elements in a few nanometers beneath the surface.

Figure 2. X-ray photoelectron spectroscopy profile of TiO₂/FHA coating.

Figure 3. X-ray photoelectron spectroscopy profile of TiO_2 /FHA/HA coating.

Scanning electron microscope images of functional graded coatings at different stages of multilayer formation on the Ti substrate are presented in Fig. 4. When the titanium surface was coated with a single layer of TiO_2 , initial machining scratches on the Ti surface could still be seen (Fig. 4a). FHA coating was then deposited on top of the TiO_2 coating, resulting in a surface a little rough and irregular (Fig. 4b). Conventional sol-gel-derived HA coating without porosifier agent exhibited dense and smooth surface morphology (Fig. 4c), while final functional graded TiO_2 /FHA/HA coating, in which the outermost HA layer was processed with porosifier agent, had regular micro-pores on the surface (Fig. 4d). Cross-sectional viewing (Fig. 4e) illustrated that thickness of the TiO_2 /FHA/HA coating was approximately 2 μm , every layer bonded with each other tightly without any sign of void. No interface between coatings and

Figure 4. Scanning electron microscopy images of different coatings deposited on Ti: (a) TiO_2 coating surface; (b) TiO_2 /FHA coating surface; (c) TiO_2 /FHA/HA coating surface; (d) TiO_2 /FHA/HA coating surface, outset HA layer processed with porous agent; (e) Cross-section of TiO_2 /FHA/HA coating.

titanium substrate was obvious. Additionally, there was no delamination nor splitting present in the interfaces, suggesting tight bonding of the coating.

Bonding strength by scratch test

Figure 5 shows scratch test results of samples at different stages in the process of composite coating assembly. When the surface coating layer was separated from the underlying layer or substrate, certain audio emission signals could be detected by the apparatus. Such signals are presented as peaks in the smooth line. Height of peaks suggests intensity of the audio emission signal when the coating is separated, but is not relevant to the bonding strength. For every smooth line, the first emerging peak was considered to be the critical load under which the coating would begin to breakdown. The lowest black line indicates the adhesion strength of the innermost TiO_2 coating. Some continuous peaks emerge near 11 N, revealing the critical load under which adhesion between TiO_2 and Ti substrate would fail. Just above this, the red line is the test after FHA coating is assembled outside the TiO_2 coating. The critical load dropped to about 9 N, which might be the critical load under which the FHA layer would be separated from TiO_2 layer. Following the first peak near 9 N, there are some continuous peaks on higher loads, which might indicate separation of the TiO_2 layer from the Ti substrate. Above the red line, the green line is the test after the outermost porous HA layer is assembled. Critical load further dropped to about 6 N, which might be attributed to weakness of porous HA. After the first peak, there are many continuous peaks, which might suggest breakdown

Figure 5. Scratch test results of samples at different stages in the process of composite coating assembly.

of inner two layers at higher load. Uppermost blue line reveals the critical load of conventional sol-gel-derived HA coating, which is in the order of 6.5 N. Adhesion strength was estimated following specification of the ASTM G171-03 norm. Adhesion strength = Critical load/Surface area of scratching tip. Results have shown that adhesion strength of the coatings was 47.7 MPa for porous HA-FHA interface, 50 MPa for conventional HA-Ti interface, 71.6 MPa for FHA- TiO_2 interface and 87.5 MPa for TiO_2 -Ti interface.

Cell responses

Figure 6 shows optical microscope images of osteoblasts after 1, 3 and 5 days culture on titanium substrate and composite TiO_2 /FHA/HA coating. Comparing the images, osteoblasts can be seen in closer contact and packed more densely to the TiO_2 /FHA/HA coating surface than to Ti substrate. These results demonstrate that TiO_2 /FHA/HA coating had better affinity to osteoblasts.

Figure 7 is of SEM images of osteoblasts after culture for 1 day (a), 3 days (b), 5 days (c) and 7 days (d) on composite TiO_2 /FHA/HA coating. As shown in Fig. 7a, after culture for 1 day, cells were firmly attached to the porous coating surface; these were of spindle shape and well spread, and they grew favourably across the coating surface. Also many cells were randomly attached to the surface; these spread poorly and remained round. Osteoblasts had spread further after culture for 3 days (Fig 7b). Subsequently, after 5 days, the porous surface coating became indistinct and unclear due to more and more cell attachment (Fig. 7c). At 7 days (Fig. 7d). Osteoblasts had smooth and flat morphology, close contacted with each other, and they completely covered surfaces of the composite coating.

Proliferation of osteoblasts was detected by MTT assay after culture for 1, 3, 5 and 7 days, as shown in Fig. 8. Results indicate that cell numbers on composite TiO_2 /FHA/HA coating were significantly higher ($P < 0.05$) than those on HA after 1 and 3 days, while there was no significant difference between composite coating and HA coating after culture for 5 and 7 days ($P > 0.05$). Furthermore, cell numbers on titanium substrate were significantly lower ($P < 0.05$) than those on coated samples, at all stages.

Figure 9 shows ALP activity of cells cultured for 5, 10 and 15 days. Cells on composite TiO_2 /FHA/HA coating had significantly higher ALP activity than those on HA coating, and those on HA coating had significantly higher ALP activity than pure titanium substrate ($P < 0.05$). This finding indicates the improved activity and functionality of cells on composite TiO_2 /FHA/HA coating.

Figure 6. Optical microscopy images of osteoblasts cultured on titanium substrate for 1 day (a), 3 days (c) and 5 days (e), and on TiO₂/FHA/HA coating for 1 day (b), 3 days (d) and 5 days (f). Black areas at corners of photographs are borders and shadows of samples. All the images were taken at the same magnification.

Discussion

In this work, we aimed to enhance of both bioactivity and long-term stability of titanium implants by using a functional graded composite HA/FHA/TiO₂ coating. Previous research has shown that HA, FHA and TiO₂ coating have their individual advantages and disadvantages (4,6,9,15,26,27). However, no study previously has involved fabrication and properties of functional graded coating composed of HA, FHA and TiO₂, which may exert their respective superiorities into a composite. The sol-gel route used here can be considered to be the most convenient method to produce a uniform phase and good microstructure of the resultant coating as described in the literature (8,15,28), which focusses mainly on double-layer coatings (HA/FHA, HA/TiO₂ and FHA/TiO₂).

In the present study, we adopted a modified multi-step sol-gel method to fabricate the novel functional graded TiO₂/FHA/HA coating. Functional graded materials exist widely in the human body – for example, bone and teeth. Their mechanical and morphological

properties gradually change from surface to the centre. We designed the TiO₂/FHA/HA triple layer as a functional graded coating. From inner to outer regions, bioactivity of the coating layer increased to facilitate osteointegration. While from outer to inner, stability of the coating layer also increased to ensure long-term success. TiO₂ was used here as the innermost layer, due to its chemical affinity to both titanium substrate and apatite, which ensures good adhesion. Also, its surface hydroxyl groups such as Ti-OH promote hydroxyapatite formation and bone bonding; it is one of the best erosion preventers (29–32). FHA coating was used as an intermediate layer because of its moderate bioactivity and better long-term stability compared to HA coating. Fluoride release from the coating will hopefully promote new bone formation. Porous HA coating was used as the outermost layer because of its satisfactory bioactivity and rapid dissolution rate (33–35).

As the results reveal, the novel-composite coating worked successfully. XRD detected TiO₂ anatase and apatite crystallization in the coating and suggested good

Figure 7. Scanning electron microscopy morphology of the osteoblasts cultured on $\text{TiO}_2/\text{FHA}/\text{HA}$ coating after (a) 1 day; (b) 3 days; (c) 5 days and (d) 7 days.

Figure 8. Cell proliferation on $\text{TiO}_2/\text{FHA}/\text{HA}$ composite coating, HA coating and uncoated Ti after culturing for 1, 3, 5 and 7 days.

Figure 9. ALP levels of cells on $\text{TiO}_2/\text{FHA}/\text{HA}$ coating, uncoated titanium substrate and HA coating after culturing for 5, 10 and 15 days.

bonding between the coating and the substrate as no obvious interface or voids were visible. The scratch test confirmed favourable adhesion strength of the composite coating. The surface of the composite coating was regularly porous and the innermost TiO_2 had best bonding strength of 87.5 Mpa, significantly higher than conventional sol-gel-derived HA coating. After the middle layer of FHA was assembled, adhesion strength was reduced to 71.6 MPa, and further dropped to 47.7 MPa after the outmost porous HA layer was added. However, reduced adhesion strength meant delamination of the outermost layer, not of the whole coating layer. It is reasonable that porous HA layer would break down under relatively lower load, while the middle FHA layer is stronger, and inner TiO_2 is most stable and difficult to break. It is important to know that scratch testing is widely used in measurement of ultra-thin coatings. Numerical value of adhesion strength obtained by scratch testing is hardly comparable between different studies due to the nature of critical load and too many variables (for example, different apparatus, material friction and different coating/substrate surfaces) (36,37). However, in the same study, it is readily comparable between different coatings. A further point is that the composite coating was very thin (2–3 μm) compared to conventional plasma-sprayed HA coating (50–100 μm), thus, any gap occurring between implant and bone tissue is minimal even if the whole coating layer should break down; it might not cause any catastrophic result (38,39).

In the present study, optical microscopy images demonstrated that $\text{TiO}_2/\text{FHA}/\text{HA}$ coatings had better affinity to osteoblasts than to pure titanium. This is

attributed to the outermost porous HA layer, which is believed to have good osteoconductivity (40,41). Although bonding strength of porous HA is relatively low, it may not cause failure of implants as sufficient bonding strength of inner layers has been obtained.

In vitro osteoblast responses in this study indicate beneficial effects of TiO₂/FHA/HA composite coatings. Cell proliferation on the coatings (both HA and TiO₂/FHA/HA) were significantly higher than on uncoated titanium substrate, confirming that both coatings have the ability to improve proliferating activity of the cells.

Compared to conventional HA coating, cell numbers on the functional graded coating were significantly higher after 1 and 3 days, while no significant difference was observed after 5 and 7 days. This indicates that the composite coating specially promoted proliferation of osteoblasts in early phases after application, which might be attributed to the porous surface structure as well as to fluoride release (42). In later phases, osteoblasts became arranged together closely and their growth was inhibited; thus, the level of cell proliferation on two coatings became similar. TiO₂/FHA/HA composite coating had significantly higher ALP expression levels than pure HA coating, confirming effectiveness of porous surface design, which is only possible in the composite coating. Single layers of porous HA coating are too weak and unstable to obtain long-term success for dental and orthopaedic implants.

In conclusion, a novel functional graded TiO₂/FHA/HA coating was successfully assembled by a modified multistep sol-gel method. SEM and scratch testing showed excellent bonding between coating and substrate and XRD detected TiO₂ anatase and apatite crystallization. Microscopy images confirmed that TiO₂/FHA/HA coating had good affinity to osteoblasts. Those on the coating exhibited higher cell proliferation and ALP activity compared to pure Ti and HA, which suggested excellent *in vitro* bioactivity. Thus, this functional graded coating may integrate both short-term bioactivity and long-term stability into one, and has promising prospects in surface modification of future dental and orthopaedic implants.

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Conflicts of interests

The authors have declared that no competing interests exist.

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