

# Surface Constructions of Nano-Micro Structured CaP Biomaterials and Their Biocompatibility and Bioactivity

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## ABSTRACT

In this study, we concentrated our attention to develop various methods for surface nano-micro construction of calcium phosphate (CaP) and their composite coatings, including octacalcium phosphate (OCP), hydroxyapatite (HAp), micropatterned titanium surface by using electrochemical techniques. The physico-chemical and biological properties of the as prepared nano-micro structured biomaterials were characterized by SEM, XRD, FT-IR, Raman, *in vitro* and *in vivo* evaluations respectively. The SEM patterns show a uniform micro-porous morphology consisting of wirelike crystals at nanometer scale. It is suggested that under controlled prepared conditions, the primary CaP nanowires grow and self-assemble to construct an ordered micro-porous nest-like morphology, thus to form a nano-micro two-level structure. The XRD results indicate that the CaP nanowires are orderly arranged with their c-axis preferentially perpendicular to the substrate surface. The Raman and IR spectra affirm that the main component of the coating is well crystallized HAp and OCP under the different preparations respectively. It is demonstrated that the nano-micro structured CaP composite coatings exhibit a preferable biocompatibility, because they are similar to the natural bone in both chemical composition and micro structure on surface.

**Keywords:** electrochemical constructions, CaP composite; nano-micro structure; biological properties

## 1 INTRODUCTION

Bone is an amazing nanostructured composite material consisting mainly of mineral (calcium phosphate) and organic matrix (mainly type I collagen). It has a very complex hierarchical structure and is optimized to achieve a remarkable mechanical performance and biological properties as well [1–3]. In order to heal the diseased bone tissues, both single- and multi-phases synthetic materials were used [4,5]. But single-phase materials usually do not satisfy all the essential requirements for bone growth. Therefore, it is very necessary to develop the multi-phases materials or composite with the chemical composition and structure similar to natural bone. Since the increased osteoblast adhesion on nanomaterials was first reported in 1999 [6], further investigations have exhibited that the

unique properties of nanomaterials may induce novel tissue substitutes with significantly improved performances in both biology and mechanics [5,7–9]. From the bionic point view, the ideal bone substitutes should be made of not only calcium phosphate (CaP) and protein composite, but also special multi-levels of structures mimicking to the natural bone.

Quite a few studies have been reported on the precipitating protein and calcium phosphate composite materials to mimic the basic composition of natural bone. Some researches adopted the different methods to synthesize a bulk collagen/calcium phosphate composite [10]. However, the bulk materials were mechanically weak and consequently only accepted to the non-loading applications. Many approaches were developed to apply calcium phosphate/protein composite coating to the surface of metallic substrates. Such composite-coated implants would have shown an excellent biocompatibility while maintaining a high mechanical performance of metallic substrates. They are able to accelerate bone growth and implant fixation *in vivo*, thereby to shorten healing period. However, the structures of the above composite coating are till far away from that of the natural bone actually.

The aim of this work is to co-precipitate a highly ordered and hierarchically structured, in nano-micro scale, composite coating of calcium phosphate and protein on titanium. The electrochemically-induced deposition is developed in the experiments because it can be performed in a mild condition and more controllable deposition conditions. Some spectroscopic measurements, including X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM) and fourier-transformed infrared spectroscopy (FTIR), were carried out comprehensively to characterize the structure and composition for the prepared composite coatings. And *in vitro* and *in vivo* evaluations were also performed to demonstrate their biocompatibility and bioactivity.

## 2 EXPERIMENTAL

The commercial pure titanium plates with a dimension of 10mm×10mm×2mm were polished with 1000grit SiC paper, etched in a mixture contained 10% HNO<sub>3</sub> and 1% HF and deionized water (v/v) for 60s, and then ultrasonically cleaned consecutively with acetone, ethyl alcohol and deionized water each for 15min.

The electrolytes contained Ca(NO<sub>3</sub>)<sub>2</sub>•4H<sub>2</sub>O and NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> with different concentrations depending on the

preparation of OCP or HAp. Type I collagen (Sigma, Haoyang Biological Manufacture, Inc.) was initially dispersed in 0.01M acetic acid solution at 4°C with a concentration of 1mg/ml. Then this collagen solution was added to the above Ca-P solution. The pH was adjusted to 4.2 by adding 0.1M NaOH solution. All reagents used were of analytical grade.

The electrochemical depositions were carried out in a three-electrode electrochemical cell by the PGSTAT30 electrochemical workstation in Galvanostatic mode. The CaP/protein coating was prepared at a constant cathodic current density of about 0.5mA/cm<sup>2</sup> for 20min. In order to compare the effect of collagen on the composite coating, the CaP coating obtained in the electrolyte without collagen was as a control sample, keeping all other deposition parameters in the same.

TiO<sub>2</sub> nanotube array film was fabricated by electrochemical anodizing of titanium sheets in 0.5 wt % HF electrolyte with Pt counter electrode under certain voltage for 1 h. The as-prepared amorphous TiO<sub>2</sub> nanotubes were calcinated at 450 °C to form anatase phase under air ambient for 2 h, then treated with a methanolic solution of hydrolyzed 1 wt % PTES for 1 h and subsequently heated at 140°C for 1 h. Then the superhydrophobic film is selectively exposed to UV light for 20 min through a copper grid (photomask) to photocatalytically cleave the fluoroalkyl chain. Finally, the nano OCP coating was selectively electrochemically deposited on the superhydrophilic micropatterned TiO<sub>2</sub> surface.

For SEM analysis were performed by a scanning electron microscope (LEO-1530) at 20kV, and the corresponding structural characterization of the prepared coatings was examined using X-ray diffraction pattern (PANalytical X'pert PRO) with Cu-K $\alpha$  radiation source at 40kV and 30mA. Fourier transform infrared spectroscopy (Nicolet-740) was employed to analyze the chemical composition of the coatings. The chemical state of the coating surface was detected by using X-ray photoelectron spectroscopy (VG MultiLab 2000, Mg-K $\alpha$  source, Thermo). The charging shift was referred to the C1s line emitted from saturated hydrocarbon at a binding energy (BE) of 284.8 eV. Wide scan (0–1100 eV BE) and high-resolution (C1s) spectra were acquired.

Osteoblast-like cells were used in cell cultures experiments for evaluation of biocompatibility and bioactivity. The titanium plates, the CaP coatings (obtained without collagen) and the CaP/protein coatings (obtained with collagen) were used for the *in vitro* cell culture. Each type of materials has three parallel samples. The samples sterilized were inoculated with osteoblast-like cells at a density of 1×10<sup>5</sup> cells/cm<sup>2</sup>. After 6 hours incubation, the samples were gently washed three times with cold 0.1 M phosphate buffer, fixed by 2.5% glutaraldehyde for 2h at 4°C, washed with 0.1M phosphate buffer (4°C) three times for 15min per time, then dehydrated through a series of graded alcohols. The samples were dried in a critical point

drier (HCP-2 HITACHI, Japan) and sputtered with a thin gold film then examined by SEM.

The two groups of prepared micro-nano structured CaP coating implants with a diameter of 4mm and 3mm high were implanted in the thighbone tissue of New Zealand White rabbits. At 5, 9 and 12 weeks after implantation, the surrounding tissues of the implants were histological examination and radiographic analysis.

### 3 RESULTS AND DISCUSSION

The typical SEM micrographs of the OCP/protein composite coating and OCP coating (control) are shown in Fig. 1. In the deposition process of the present of collagen, the morphology of the thin bluish layer on titanium, appears at the beginning as a precursor, is very similar to the amorphous calcium phosphate phase. The uniform OCP/protein composite coating formed on top of the thin precursor exhibits a highly hierarchically porous structure. Such coating exhibits a unique nano-micro two levels structure. From the high magnification SEM image, the basic nanostructure is of fiber-like crystals with an average

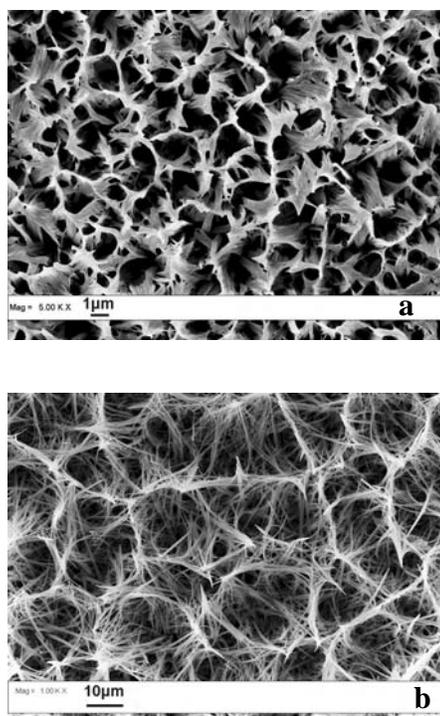


Fig. 1. SEM morphologies of the nano-micro structured OCP coatings electrochemically synthesized in the presence of collagen (a) and in the absence of collagen (b) in 0.028M [Ca<sup>2+</sup>], 0.017M [PO<sub>4</sub><sup>3-</sup>] and 0.333 mg/ml collagen.(pH 4.2)

width diameter ranged in 20–60nm. The fiber-like crystals mostly aggregate parallel to their length and form the

crystal bunch. The microstructure with an ordered porous topography in several micrometers is constructed by the crystal bunches. Compared the different types of the coatings, it is indicated that in the presence of collagen, the dimensions of crystals and pores are all reduced distinctly, and the morphology of the coating displays more ordered crystal arrangement and larger surface area.

Fig.2 shows a typical SEM micrographs of the nano-micro structured hydroxyapatite/collagen composite coating constructed by the electrochemical deposition. A uniform topography of the coating can be observed at a low SEM magnification. The coating exhibits a special nano-micro two level structure, i.e., wire-like nano crystals in the first structure and an ordered nest-like micro topography in the second structure. It is displayed that the nano crystals grow preferentially with the long axis parallel to the normal of the substrate surface.

The sharp peaks of the XRD patterns indicated that this special nano-micro two level structured coating was octacalcium phosphate (OCP)/ collagen and hydroxyapatite (HAp)/collagen composite coating respectively with high crystallinity. The strongest diffraction peak of the composite coatings are ascribed to (002) crystal face, which means crystal grains in the coating is preferentially arranged in [002] direction or c-axis.

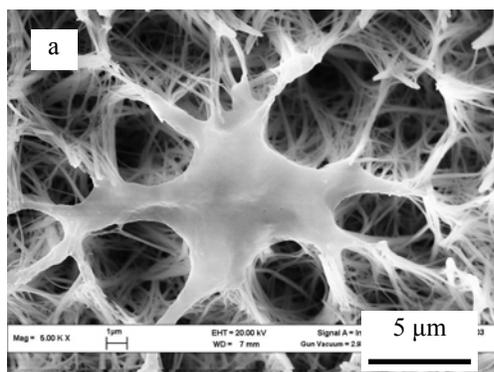


Fig. 2 SEM micrographs of the nano-micro structured hydroxyapatite/collagen composite coating constructed by electrochemical deposition in  $2.08 \times 10^{-4}$  M  $\text{CaCl}_2$ ,  $1.25 \times 10^{-4}$  M  $\text{NaH}_2\text{PO}_4$  and 0.1M  $\text{NaCl}$  (pH 6.30) solution.

Based on the as-prepared pattern of superhydrophilic-superhydrophobic property, we are able to realize a direct and guide the selective deposition of bioactive calcium phosphate onto the  $\text{TiO}_2$  nanotube film by a facile electrochemical deposition. Fig. 3 shows the ESEM image of micropatterned OCP crystals selectively deposited onto  $\text{TiO}_2$  nanotube array film by electrochemical deposition. It is of interest to note that the OCP crystals with nano size were preferentially deposited on superhydrophilic regions generated by photocatalytic lithography. On the center of the superhydrophobic region, one can clearly see several

micro concaves of the substrate without the deposition of belt-like nano OCP crystals. This is due to the superhydrophobic regions covered with the PTES monolayers can strongly prevent from any aqueous contact the electrolyte solution.

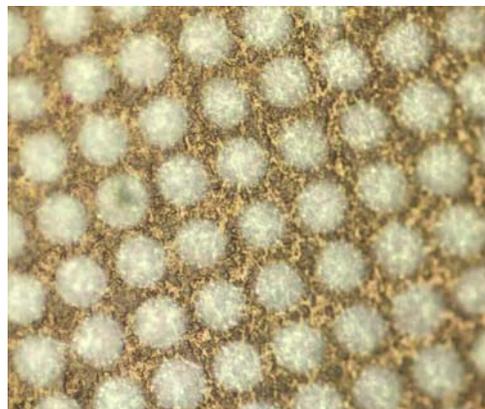
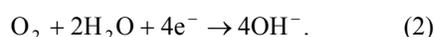
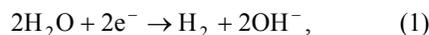


Fig. 3 Typical SEM image of the patterned OCP thin films selectively deposited in predefined superhydrophilic regions on  $\text{TiO}_2$  nanotube films by electrochemical deposition.

When a current passes through the electrodes, at first, the following electrochemical reactions occur at titanium electrode:



The two products ( $\text{H}_2$  and  $\text{OH}^-$ ) at the interface of electrode/solution play a vital role in the CaP precipitation process in  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$  containing solution.  $\text{OH}^-$  generation at the cathode surface makes the following acid-base reaction possible:



The rise of the local pH in the vicinity of titanium electrode surface will increase the supersaturation of calcium phosphate, their precipitates are following formed on the surface of the titanium cathode. There are four calcium phosphate phases usually formed from solution:  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  (HAp),  $\text{Ca}_2(\text{PO}_4)_3 \cdot n\text{H}_2\text{O}$  (TCP),  $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$  (OCP), and  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  (DCPD). During the electrochemical process, the pH value at the electrode/solution interface could influence greatly the composition of the precipitated products. The increasing of pH at the electrode/solution interface may depend mainly on the current density. When the cathodic current density is controlled at a low value ( $0.5\text{mA}/\text{cm}^2$ ), the interfacial pH value is lower in favor of OCP formed, and the higher pH induces the HAp formation. Furthermore, the solution concentration, the solution temperature and the deposited time also influence the crystal phase of the coatings. When

adding collagen into the electrolyte, the protein and calcium phosphate mineral can be co-precipitated on the electrode by the charge drive and the molecular interaction between protein and mineral.

In this work, various novel nano-micro two level structure of CaP/protein coatings are constructed on the cathode. At beginning of ED process, the uniform CaP (OCP and HAp) nano fibers, in the nano-level structure, form and grow on the Ti surface under an appropriate electrochemical condition. Then the micro-level porous structure consisting of CaP nano fibers is possible to form, based on the template of hydrogen bubbles generated at the cathode interfere. At first the hydrogen bubble in random distribution occurs on the titanium surface under an appropriate cathodic polarization. If the bubbles are small enough, they cannot be dislodged by the force of buoyancy, and a relatively stable gas template is possible to be built on the surface. The CaP nano crystals cannot be precipitated at sites occupied by hydrogen bubbles because of no mass transport through this gas phase, and it can only precipitate at the other locations and grow around the bubble template. When the hydrogen bubble is large enough, it is dislodged from the surface, but a special porous structure of CaP with nano-micro two level dimension has already formed.

The biocompatibility and bioactivity of the nano-micro structured coating were evaluated in the *in vitro* and *in vivo* test. The cell culture test exhibits an excellent cell attachment on CaP/protein coating. It may be related to the nano-micro porous structure which is something similar to natural bone, and is possible to provide sufficient spaces for anchoring cells. And the presence of protein in the composite coating should provide the biomimic composition to improve the cellular responses to materials. The experiments of cell culture have demonstrated that the CaP/protein composite coating exhibits a well micro environment to the cell responses. In the *in vivo* test, after certain weeks of implantation in different animals tissues, none of the coating implants (CaP/protein coating and CaP coating) shows a severe inflammatory reaction. Two implants only exhibit some signs of a normal inflammatory reaction. At the early weeks in different animals tests, the CaP/protein coating all displays slighter inflammatory reaction than CaP coating. And in the rabbit test, the new bone is formed surround all the implants after 12 weeks implantation. All the biological evaluations have demonstrated that the electrochemically prepared nano micro structured CaP coated implants are nontoxic and show excellent biocompatibility and bioactivity.

## 5 CONCLUSION

An electrochemically-induced deposition process has been developed to successfully construct an unique ordered porous structured CaP/protein composite on titanium substrate. It is indicated that the composite coating of CaP possesses a novel two levels of structure in nano-micro scale. Such hierarchical structure is formed due to the

adsorption of protein and the hydrogen bubble template on the metal surface during the ED process. The CaP/protein composite coating with an special ordered nano-micro structure exhibits an excellent biocompatibility and bioactivity due to its mimic structure and composition to natural bone. we have also developed an unconventional approach to construct superhydrophilic-superhydrophobic micropattern on the nanotube structured TiO<sub>2</sub> films by using electrochemically self-assembly and photocatalytic lithography. Furthermore, this micropatterns was further developed, as a novel template, to fabricate a define micropatterned CaP coatings by electrochemical deposition.

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## References

- [1] R.Lakes, Materials with structural hierarchy, Nature, 361,511-515,1993.
- [2] J.D. Hartgerink, E.Beniash, S.I. Stupp, Self-assembly and mineralization of peptide-amphiphile nanofibers, Science, 294,1684-1688, 2001.
- [3] P.Fratzl, H.S.Gupta, E.P.Paschalis, P.Roschger, Structure and mechanical quality of the collagen-mineral nano-composite in bone, J Mater Chem, 14,2115-2123, 2004.
- [4] Puleo DA, Nanci A. Understanding and controlling the bone-implant interface. Biomaterials, 20, 2311-2321,1999.
- [5] R.Murugan and S.Ramakrishna, Development of nanocomposites for bone grafting, Comp Sci Tech, 65, 2385-2406, 2005..
- [6] Webster TJ, Siegel RW, Bizios R. Osteoblast adhesion on nanophase ceramics. Biomaterials, 20, 1221-1227, 1999.
- [7] H.N.Liu and T.J.Webster, Nanomedicine for implants: A review of studies and necessary experimental tools, Biomaterials, 28, 354-369, 2007.
- [8] T.J.Webster, C.Ergun, R.H. Doremus, R.W.Siegel, R. Bizios, Enhanced functions of osteoblasts on nanophase ceramics, Biomaterials, 21,1803-1810, 2000.
- [9] K.M. Woo, J..H Jun, V.J. Chen, J. Seo, J.H.Baek, H.M. Ryoo, G.S.Kim, M.J. Somerman, P.X.Ma, Nano-fibrous scaffolding promotes osteoblast differentiation and biomineralization, Biomaterials, 28, 335-343, 2007.
- [10] M.C.Chang, T.Ikoma, M.Kikuchi, J.Tanaka, Preparation of a porous hydroxyapatite/collagen nanocomposite using glutaraldehyde as a crosslinkage agent. J Mater Sci Lett, 20, 1199-1201, 2001.