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Selective preparation and characterization of nano-hydroxyapatite/collagen coatings with three-dimensional network structure

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ABSTRACT

Nano hydroxyapatite/collagen (HA/COL) coatings were prepared on the surface of carbon/carbon (C/C) composites by a two-step process, i.e. electrochemically assisted co-deposition of calcium phosphate/collagen (CaP/COL) coatings and subsequent soaking CaP/COL coated C/C composites in $Ca(OH)_2$ aqueous solution. Both CaP/COL and HA/COL coatings on the C/C composites exhibit three-dimensional network structure consisting of collagen fibers and uniform CaP covered on the surface of collagen fibers. CaP in the composite coatings was deposited into different forms depending on the concentration, Ca/P molar ratio and pH value of initial electrolyte. The concentration and pH value of initial electrolyte have little impact on the shape of CaP though they significantly influence the size of CaP. Higher Ca/P molar ratio of initial electrolyte leads to spherical amorphous calcium phosphate (ACP)/COL coatings. Plate-like octacalcium phosphate (OCP)/COL coatings were prone to form with low Ca/P molar ratios in the electrolyte. After $Ca(OH)_2$-treatment as the second processing step, OCP/COL coatings on C/C were converted to HA/COL coatings. Their structure and compositions are similar to those of human bone tissue. SBF immersion tests indicate that HA/COL coated C/C composites have better in vitro bioactivity. In vitro cellular biocompatibility tests indicate OCP/COL and HA/COL coated C/C composites have almost the same biocompatibility.

1. Introduction

Carbon/carbon (C/C) composites combine the intrinsic biocompatibility of carbon material and the excellent mechanical properties of fiber reinforced composites. Owing to their similar elastic modulus to that of human cortical bone, bone resorption caused by stress shielding effect can be significantly reduced by using them as bone implants [1]. A successful example is the C/C implants produced by National Science Centre "Kharkov Institute of Physics and Technology"(NSC KIPT, Kharkiv Ukraine), which have been used clinically as bone substitutes for many years with satisfactory results [2].

However, C/C composites cannot bond to living bone directly. Also, they may release carbon debris to the surrounding tissues due to the friction in a surgical procedure. These carbon debris can flow with the body fluid and deposit on the skin, causing "black skin effect" [3]. To solve these problems, some bioactive materials such as calcium phosphate (CaP) and calcium phosphate/collagen (CaP/COL) are coated on C/C composites using plasma spray, chemical electrophoretic deposition, ion beam-assisted deposition, injection and sinter, plasma spraying, biomimetic deposition, and electrochemical deposition [1,3–6]. Some attention has been paid to prepare HA/COL scaffolds or CaP/COL coatings via biomimetic deposition owing to their inherent bioactivity [7–9]. However, it is interesting to note that a little attention has been paid to coat HA/COL on C/C composites using electrochemical deposition.

Electrochemical deposition method has a variety of advantages, such as ease of process control, low preparation temperature and suitability for complex implant geometries. It has been applied for preparation of HA/COL coatings on various substrates. Manara et al. [10] synthesized a biomimetic bone-like composite made of self-assembled collagen fibrils and carbonate HA nanocrystals on Ti plate using electrochemical deposition. They found that the addition of collagen to HA coating led to an increased fibronectin adsorption. Xu et al. [11] prepared HA/collagen composite coatings on Ti substrate by electrochemically assisted co-deposition technique. Their study showed that the HA/collagen composite coatings exhibited better biological properties compared with pure HA and pure Ti. Okamura et al. [12] reported collagen and HA composite coatings formed in electrolytes with a low pH value of 2.1 to 3.3 by electrochemical deposition. Most of the composite chipped off to form the precipitates in the solution during the deposition. Roessler et al. [13] and Schliephake et al. [14] immobilized collagen type I on a HA layer on titanium surface by electrochemical deposition. Then mineralization of this collagen was accomplished using cathodic polarization.

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Using electrolytic deposition, octacalcium phosphate/collagen composite coatings were produced on silicon substrate [15] and on Ti6Al4V substrate [16]. Sun et al. [9] fabricated apatite/collagen composite coatings on the surface of NiTi SMA through electrochemical deposition technique. In comparison with either uncoated or apatite-coated samples, apatite/collagen-coated NiTi SMA showed higher wettability and corrosion resistance. The influence of deposition parameters on morphology, structure and composition of the coatings is not very clear. The in vitro bioactivity and biocompatibility of calcium phosphate/collagen (CaP/COL) coatings are also not systematically investigated.

In our previous work, CaP/COL composites were coated onto C/C substrates using electrochemically assisted co-deposition. The effects of collagen concentration in the electrolyte on morphology, structure and composition of the coatings were investigated. Transformation of CaP/COL into hydroxyapatite/collagen (HA/COL) coatings was also conducted in $Ca(OH)_2$ aqueous solution [8]. However, there are of course other deposition parameters, not systematically investigated in the work, which might influence the morphologies and composition of CaP/COL coatings during deposition of the CaP/COL coatings. During the transformation of CaP/COL into HA/COL coatings, the morphologies of HA/COL coatings was difficult to control, and the transformation mechanism was not clarified. In vitro biological behavior of nano HA/COL coatings were not investigated.

In this study, the effects of Ca and P concentrations, Ca/P molar ratio (designated as $(Ca/P)_e$), and pH value of initial electrolyte on morphology and composition of the coatings were investigated for an electrochemically assisted co-deposition process. HA crystals with a variety of morphologies and dimensions in nano hydroxyapatite/collagen (HA/COL) coatings were prepared on C/C composites by a two-step process, deposition and subsequent $Ca(OH)_2$-treatment of CaP/COL. The aim of this work is selective production of HA/COL coatings with various morphologies and investigation of the formation mechanism of nano HA/COL coatings through $Ca(OH)_2$-treatment on different precursor CaP/COL. In vitro biological behavior of nano HA/COL coatings was investigated by simulated body fluid (SBF) immersion and in vitro cellular biocompatibility tests.

2. Materials and methods

2.1. Electrochemically assisted co-deposition of CaP/COL coatings

The C/C composites blocks were fabricated using chemical vapor infiltration process in Northwestern Polytechnical University in China. C/C samples (10.5 mm × 10.5 mm × 2.6 mm) were cut from the block and were polished with Nos. 400, 800, 1200 and 1500 abrasive paper. The final dimensions of the C/C samples were 10 mm × 10 mm × 2 mm. Then the C/C composites samples were cleaned ultrasonically in turn by acetone, alcohol, and deionized water. C/C composites were initially treated at 50 mA/cm^2 by ultrasound-assisted anodic oxidation treatment [17]. Then the surface-treated C/C composites were conditioned with 0.5 mol/L NaOH aqueous solution for about 10 min and washed with deionized water.

Electrochemically assisted co-deposition was carried out in a two-electrode electrochemistry system controlled by the CS310 electrochemical workstation. The surface-treated C/C samples served as cathode while graphite electrode served as anode. Type I collagen (Worthington) were dissolved in 500 mmol/L acetic acid solution and stored at 4 °C. Then the collagen containing solution was added to the electrolyte consisting of $NH_4H_2PO_4$ and $Ca(NO_3)_2$ aqueous solutions at a concentration of 500 mg/L. The pH value of the electrolyte was measured using a pH 211 acidity meter. The initial pH value was adjusted to 4.1 or 4.3 by sodium hydroxide aqueous solutions and nitric acid solution. Electrochemically assisted co-deposition was conducted at a current density of 2.0 mA/cm^2 for 60 min and the temperature was controlled at 33 ± 1 °C.

2.2. Conversion of CaP/COL to HA/COL coatings

The electrochemically assisted co-deposition was carried out in an electrolyte containing 12.5 mmol/L $Ca(NO_3)_2$ and 25.0 mmol/L $NH_4H_2PO_4$. The pH value of the electrolyte was adjusted to 4.1. After deposition, the CaP/COL coatings coated samples were immersed in a 1.0 mmol/L $Ca(OH)_2$ aqueous solution at 35 ± 1 °C for 120 h. The CaP/COL coatings were converted into nano HA/COL coatings which is similar to the natural human bone. The treated samples were then washed using distilled water and dried in air.

2.3. SBF immersion tests and in vitro biological behavior of the coatings

A SBF with ion concentrations approximately equal to those of human blood plasma has been used widely for to assess the in vitro bioactivity of bioceramics and biocoatings. The SBF was prepared by dissolving reagent grade chemicals of NaCl, $NaHCO_3$, KCl, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2$, Na_2SO_4 and $(CH_2OH)_3CNH_2$ into deionized water, and buffering it at pH 7.40 with hydrochloric acid [18]. Each HA/COL coated C/C sample was soaked in 35 mL of SBF and kept at 37 °C. The samples were removed from SBF solution and washed with deionized water after 1, 3, 7 and 14 days of immersion. Then they were dried at room temperature.

To observe the morphology of MC3T3-E1 cells cultured on CaP, OCP/COL and HA/COL coated C/C samples, 700 μL suspension with a concentration of 5 × 10^4 cells/mL were added. The samples with cells were incubated at 37 °C. After seeding for 24 h, the cells were fixed by glutaraldehyde. Then, acetonitrile replacement, vacuum drying and gold spraying were applied on these samples. The cells on the samples were examined using a scanning electron microscope (SEM) (Hitachi S3400, Japan).

In order to evaluate in vitro biocompatibility of the coatings, MC3T3-E1 cells were cultured in alpha Minimal Essential Medium (MEM-α, Hyclone), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution in a humidified atmosphere at 5% CO_2 and 37 °C. They were seeded at a density of 2 × 10^4 cells/mL onto the CaP, OCP/COL, HA/COL coated, and uncoated C/C composites. The incubation was carried out for 7 days and the culture medium was changed every two days. After 1, 3 and 7-day culture, the cells were incubated in a tetrazolium salt solution, 3-[4, 5-dimethylthiozol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) for 4 h at 37 °C. Then the medium was removed and dimethylsulfoxide (DMSO) was added to each well. After complete solubilisation of the dark-blue crystal of MTT formazan, the cell viability was measured at 490 nm on a spectrophotometer. The proliferation rate of cells was quantified by measuring the optical density (OD) [19].

To evaluate the functionality of the cultured cells on OCP/COL and HA/COL coatings, intracellular ALP activity expression were analyzed. Cells were seeded on each sample at a density of 5 × 10^4 cells/mL and cultured for 7 and 14 days. The culture medium was changed twice a week. ALP activity were determined by ALP assay kit (Biovision Inc., Mountain View, CA), following the manufacturer's protocols.

Statistical analysis was carried out on the cellular tests using one-way analysis of variance (ANOVA) at an average of 3–5 replicates. $P < 0.05$ are considered statistically significant.

2.4. Materials characterization

A JEOL JSM-6700 scanning electron microscope (SEM) was employed to observe the surface morphology of the coatings. Elemental analysis was conducted by energy-dispersive X-ray spectroscopy (EDS) (INCA System, Oxford Instruments, United Kingdom). A Philips X'Pert PRO MPD X-ray diffraction (XRD) instrument was used to analyze the crystal structure of the coatings. The instrument was operated with a Cu Kα radiation (λ = 0.15406 nm) source at 40 kV and 35 mA. Tecnai F30G^2 field emission transmission electron microscopy (TEM) was used to examine the structure, crystal size and diffraction pattern of