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Multilevel surface engineering of nanostructured TiO_2 on carbon-fiber-reinforced polyetheretherketone

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ABSTRACT

As an implantable material, carbon-fiber-reinforced polyetheretherketone (CFRPEEK) possesses an adjustable elastic modulus similar to that of cortical bone and is a prime candidate to replace metallic surgical implants. However, the bioinertness and poor osteogenic properties of CFRPEEK limit its clinical application as orthopedic implants. In this work, titanium ions are introduced energetically into CFRPEEK by plasma immersion ion implantation (PIII). Scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS) reveal the formation of nanopores with the side wall and bottom embedded with ~20 nm TiO_2 nanoparticles on the CFRPEEK surface. Nanoindentation measurements confirm the stability and improved elastic resistance of the structured surfaces. *In vitro* cell adhesion, viability assay, and real-time PCR analyses disclose enhanced adhesion, proliferation, and osteo-differentiation of rat bone mesenchymal stem cells (bMSCs). The multilevel structures on CFRPEEK also exhibit partial antibacterial activity to *Staphylococcus aureus* and *Escherichia coli*. Our results indicate that a surface with multifunctional biological properties can be produced by multilevel surface engineering and application of CFRPEEK to orthopedic and dental implants can be broadened and expedited based on this scheme.
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1. Introduction

Orthopedic implants are required when irreparable bone damage occurs due to trauma, disease, or congenital defects [1] and carbon-fiber-reinforced polyetheretherketone (CFRPEEK) is becoming a prime candidate to replace metallic implants [2,3]. Different from typical metallic materials which possess high elastic moduli of over 100 GPa, CFRPEEK has an adjustable elastic modulus close to that of cortical bone (18 GPa) which can mitigate concerns over the risks of osteanabrosis and bone resorption caused by stress shielding as a result of the elasticity mismatch between the implants and human bones [4,5]. In addition to its excellent mechanical properties, CFRPEEK inherits the non-toxicity, good chemical resistance, natural radiolucency, and even MRI (magnetic resonance imaging) compatibility from PEEK [2,3,6–8]. However, although the materials have attracted much attention as orthopedic implants since the 1980s, the bioinertness of CFRPEEK impedes osteointegration after implantation thereby severely hampering clinical adoption [2,3,9].

Surface modification is an effective way to enhance the surface mechanical and biological properties while the advantageous bulk properties of the materials can be preserved. Among the various surface modification techniques, plasma immersion ion implantation (PIII) is a non-line-of-sight method that has been widely applied to microelectronics, aerospace engineering, precision manufacturing, and biomedical engineering. By introducing different elements and functional groups, surface properties such as cytocompatibility, antibacterial activity, and mechanical properties can be selectively tailored [10–13]. Moreover, unique structures with different size can be formed selectively under proper conditions [14–16]. For example, pinnacle-like diamond-like carbon (DLC) coatings were by Wang et al. to reduce bacterial adhesion [14] and silver nanoparticles were formed on titanium and TiO_2 to enhance both the biocompatibility and antibacterial activities [15]. Recently, Qian et al. produced nanocap structures on TiO_2 nanotubes to alter the properties [16].

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It is well known that electrical micro-arcing induced by surface charging during PIII can cause surface damage to semi-insulating semiconductors and insulators [17–19]. However, unlike micro-electronics, a “damaged” surface may be preferred in biomedical engineering because for instance, native bones possess a hierarchical structure on the micro-/nano-scale as well [20–23]. It has in fact been reported that some surface structures impact bacterial adhesion and growth [24,25]. TiO_2 nanotubes have attracted much attention because they can improve the adhesion and proliferation of osteoblasts and stem cells as well as up-regulate osteoblastic levels [22,26–28]. Besides, TiO_2 nanotubes provide an excellent platform for drug delivery for growth factors and antibacterial agents [29,30]. However, there have been few studies concerning TiO_2 nanotube coatings on polymers due to the practical limitation of anodic oxidation.

In this work, TiO_2 nanotube-like multilevel structures are produced on CFRPEEK by titanium plasma immersion ion implantation (Ti-PIII). Micro-arcing occurring during high frequency, long pulse, and long implantation time PIII produces special nanopores with TiO_2 walls on the CFRPEEK surface. The osteogenic properties of the structured CFRPEEK surface are evaluated using rat bMSCs and the antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* is assessed *in vitro*. A model is proposed to discuss the formation mechanism of the multilevel structure.

2. Materials and methods

2.1. Sample preparation

Biomedical grade carbon-fiber-reinforced polyetheretherketone (CFRPEEK) with 30% (v/v) carbon fibers was used in this study. The samples were machined into different dimensions. Square samples ($10 \times 10 \times 1$ mm^3) were used for surface characterization, immersion tests, and *in vitro* studies on 24-well tissue culture plates. Square samples with a different size ($20 \times 20 \times 1$ mm^3) were used in the real-time PCR tests. All the samples were polished on one side to a near mirror finish and ultrasonically cleaned in acetone, ethanol, and ultra-pure water prior to PIII. Titanium ions were implanted into the CFRPEEK samples using a filtered cathodic arc source housing a 99.99% pure titanium rod with a diameter of 10 mm. Before PIII, the chamber was evacuated to a pressure of 5×10^{-3} Pa. By applying a pulsed negative high voltage to CFRPEEK sample on the sample stage, Ti ions were implanted and the sample stage was continuously rotated during PIII to obtain uniform ion implantation. Table 1 lists the important parameters and sample designation. The sample after undergoing Ti-PIII at 30 kV for 120 min is designated as Ti-120.

2.2. Surface structure and chemical characterization

The surface and cross-section of the CFRPEEK samples were examined by field-emission scanning electron microscopy (FE-SEM, Hitachi S-4800, Japan) at different magnification without applying a conductive coating. The surface chemical states and elemental depth profiles were determined by X-ray photoelectron spectroscopy (XPS, Physical Electronic PHI 5802 equipped with a monochromatic Al K_α source) in City University of Hong Kong. The C, O, and Ti profiles were acquired by XPS in conjunction with argon ion bombardment at a sputtering rate of about 4 nm/min. Nanoindentation was performed at loadings of 1.3 mN and 3.0 mN and the results of at least eight indents were averaged to improve the statistics.

2.3. Ion release

Two pieces of each sample were incubated in 5 mL of phosphate buffered saline (PBS 1 M) for different immersion time (7, 14, 21, and 28 days) at 37 °C without stirring. At a prescribed time, the solution was withdrawn and analyzed by inductively-coupled plasma atomic emission spectroscopy (ICP-AES, JY 2000-2, France) analysis to determine the amount of released titanium.

Table 1
Main conditions in titanium plasma immersion ion implantation of Ti-120.

Parameters	Description
Cathode source	99.99% pure Ti
Voltage pulse duration (μs)	500
Pulsing frequency (Hz)	7
Ion implantation voltage (kV)	−30
Ion implantation time (min)	120
Pressure (Pa)	5×10^{-3}

Table 2
Primer pairs used in real-time PCR analysis.

Gene	Primers (F = forward, R = reverse)	Amplicon
COL-I	F: CTGCCCAGAAGAATATGTATCACC R: GAAGCAAAGTTTCCTCCAAGACC	198 bp
Runx2	F: TCTTCCCAAAGCCAGAGCG R: TGCCATTCGAGGTGGTCG	154 bp
BMP-2	F: TGGGTTTGTGGTGGAAGTGGC R: TGGATGTCCTTTACCGTCGTG	154 bp
ALP	F: CGTCTCCATGGTGGATTATGCT R: CCCAGGCACAGTGGTCAAG	209 bp
OCN	F: GCCCTGACTGCATTCTGCCTCT R: TCACCACCTTACTGCCCTCCTG	103 bp
OPN	F: CCAAGCGTGGAAACACACAGCC R: GGCTTTGGAACTCGCCTGACTG	165 bp
β-actin	F: CACCCGCGAGTACAACCTTC R: CCCATACCCACCATCACACC	207 bp

2.4. Cell culture

The bone mesenchymal stem cells (bMSCs acquired from Cells Resource Center, Shanghai Institutes of Biological Science, Shanghai, China) were isolated from the bone marrow of six-week-old male Fisher 344 rats. The bMSCs were cultured in the α-minimum essential medium (α-MEM, Gibto-BRL, USA) with 10% fetal bovine serum (FBS, Hyclone, USA), 1% antimicrobial of penicillin, and streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The α-MEM was refreshed every 3 days during cell culturing. The experiments were carried out with the bMSCs before passage five. All the samples were sterilized with 75% alcohol for 3 h and rinsed twice with sterile PBS before cell seeding.

2.5. Cell adhesion

The bMSCs were seeded on the samples on a 24-well plate at the density of 5×10^4 cells per well. After 1, 3, and 24 h, the samples were taken to another 24-well plate. The samples were rinsed twice with PBS and fixed with 3% glutaraldehyde overnight. Ethanol with different concentrations of 30, 50, 75, 90, 95, 100, and 100% v/v was used sequentially to dehydrate the samples for 10 min. The samples were finally dehydrated in a series of hexamethyldisilizane (HMDS) ethanol solution and the dried samples were sputter coated with platinum for SEM (Hitachi S-3400, Japan) observation.

2.6. Cell proliferation and viability

The alamarBlue™ (AbD Serotec Ltd, UK) assay was employed to quantitatively determine the cell proliferation and viability on the samples. The bMSCs were seeded on the samples (four replicates) on 24-well plates at the density of 2.5×10^4 cells per well. After 1, 4 and 7 days, the culture medium was replaced by 0.5 mL of the fresh medium with 5% alamarBlue™ in each well. After incubation for 4 h, 100 μL of the medium was transferred to a 96-well plate for measurement. The amount of reduced alamarBlue™ was determined by an enzyme-labeling instrument (BIO-TEK, ELX 800) at wavelengths of 570 nm and 600 nm. The operation and calculation of cell proliferation followed the instruction of the alamarBlue™ assay.

2.7. Alkaline phosphatase activity

The bMSCs were seeded on the samples (four replicates) on 24-well plates at a density of 1×10^4 cells per well (cultured for 7 days) or 0.5×10^4 cells per well (cultured for 14 days). In the quantitative alkaline phosphatase (*ALP*) assay, after culturing for 7 and 14 days, a Bio-Rad protein assay kit (Bio-Rad, USA) was used to calculate the total protein content and the results were adjusted with a series of BSA (Sigma) standards by measuring the optical density (OD) values of the absorbance at 570 nm. After incubation with p-nitrophenyl phosphate (Sigma) at 37 °C for 30 min, the *ALP* activity was calculated and adjusted with a series of 4-Nitrophend NaOH (0.02 M) solutions by measuring the OD values at 405 nm. The *ALP* levels were normalized to the total protein content and described as μM/mg total proteins.

2.8. Quantitative real-time PCR

The expression of osteogenesis-related genes was analyzed using quantitative real-time reverse-transcriptase polymerase chain reaction (real-time PCR). Three pieces of each sample were placed on the cell culture dish (6 cm diameter, Nunc, Denmark). The bMSCs were seeded on the dish at a density of 1×10^5 cells per dish (cultured for 7 days) or 0.5×10^5 cells per dish (cultured for 14 days). The total RNA was extracted using TRIZOL reagent (Invitrogen, USA) and the complementary DNA (cDNA) was reverse-transcribed from 1 μg of total RNA using a PrimeScript I Strand cDNA Synthesis kit (TaKaRa) according to the manufacturer's protocols. The forward and reverse primers for different genes are listed in Table 2. The expression of selected genes, including Type I collagen (*COL-I*), runt-related transcription factor 2 (*Runx2*), bone morphogenetic protein 2 (*BMP-2*), alkaline phosphatase (*ALP*),

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