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Original article

Mineralization and osteoblast behavior of multilayered films on TiO_2 nanotube surfaces assembled by the layer-by-layer technique

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ABSTRACT

In this paper, the multilayer films of poly-L-lysine (PLL) and DNA were created on TiO_2 nanotube surfaces using the layer-by-layer (LBL) self-assembly technique. Chemical compositions of the assembled multilayered films were investigated by X-ray photoelectron spectroscopy. Biological properties of the multilayered films were evaluated by the biomimetic mineralization and osteoblast cell culture experiments. The results indicated that PLL and DNA were successfully assembled onto TiO_2 nanotube surfaces by electrostatic attraction. Moreover, the samples of assembled PLL or/and DNA had better bioactivity in inducing HA formation and promoting osteoblast cells adhesion, proliferation and early differentiation.
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1. Introduction

Titanium (Ti) and its alloys are widely used in the orthopedic and dental fields because of their remarkable biological and mechanical properties. However, after implantation *in vivo*, these Ti materials cannot establish bone bonding and only develop mechanical fixation with the bone [1]. How to improve the osteointegration between an implant and surrounding natural bone tissue, in turn to extend the lifetime of the implant, still remains unresolved. The biological behaviors of cells are highly regulated by the local chemical and topographical microenvironment [2,3]. Previous studies confirmed that a nano-porous rough structure can enhance the specific surface area and surface energy of a material, which played a role in promoting the proliferation and differentiation of osteoblasts and regulating the secretion of growth factors and cytokines [4–6]. However, the biological activity resulted from this modification is limited, requiring further modifications to the material.

Layer-by-layer self-assembly technology is based on the electrostatic attractions between multiple self-assembly film systems. This technique has important applications in electronic biosensors, cell engineering and nanotechnology. This is a simple and effective method for constructing a bioactive surface that has the following advantages: no special requirement for chemical structures or active functional groups on material surface; the construction of the layered structure does not require additional conditions, such as energy supply; various biocompatible water-soluble polymers (especially various bioactive biological macromolecules, such as proteins and nucleic acids) can be used for assembly [7].

Poly-L-lysine (PLL) shows good biocompatibility and contains functional groups such as amino and carboxyl groups. Jessel *et al.* [8,9] deposited two types of plasmids (plasmids containing the green fluorescent protein gene sequence and a transcription factor gene sequence) at various locations in multilayer films of PLL and polyglutamic acid to regulate the expression timing of the two plasmids in cells. DNA is an important genetic substance and is also an anionic polyelectrolyte. DNA molecules contain a large number of phosphate groups; the high affinity between phosphate and calcium ions was shown to favor calcium deposition during the osteogenic process [10]. Using layer-by-layer self-assembly technology, DNA, as a polyanion, can successfully form a layered structure on a material's surface. Blacklock *et al.* [11] revealed that a multilayer film of polypeptide TAT/DNA containing disulfide bonds was degraded within 24 h in a reductive environment.

Our preliminary studies demonstrated that PLL and DNA could achieve layer-by-layer self-assembly on Ti surface and effectively improve the biological properties of the Ti surface

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[12,13]. Cell-nanotopography interactions are believed to represent a promising management to precisely control seed cell function and differentiation in bone tissue engineering, because bone itself has a structural hierarchy at the first level in the nanometer range [5,14,15]. If PLL and DNA can achieve self-assembly on a nano-porous rough surface, the bioactivity of Ti and its alloys should be further improved. However, further study is required to confirm whether PLL and DNA can form multilayer films through layer-by-layer self-assembly on a nano-porous rough surface and whether these films could enhance biological activity. This study fabricated PLL/DNA multilayer films through self-assembly on the surface of TiO_2 nanotubes, and evaluated the physical chemical properties and biological properties of the material.

2. Experimental

2.1. Materials

Commercial pure titanium (CP-Ti, 99.7%) was cut into ∅10 mm × 1.5 mm. Poly-L-lysine (PLL, (M_W: 30–70 kDa)), *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] (HEPEs, free acid, high purity grade) and Gold view I were purchased from Sigma (USA). Deoxyribonucleic acid (DNA, fish sperm, sodium salt) was obtained from AppliChem (Germany).

2.2. Fabrication of TiO_2 nanotubes

Titanium disks were polished with silicon carbide sandpaper of no. 240, 400, 800, 1000 and 1500 grits in series, and then washed in an ultrasonic cleaner with distilled water, acetone, ethanol and distilled water sequentially and finally dried in air at room temperature.

The samples were pre-processed using a previously reported method [16]. Briefly, the titanium disks were anodic oxidized in a 2 mol/L H_3PO_4 + 0.15 mol/L HF electrolyte solution at 20 V for 1 h, followed by heating at 450 °C to produce anatase (TiO_2) nanotubes. The obtained samples were labeled as T. T was then processed in a 5 mol/L NaOH solution at 80 °C for 6 h, followed by ultrasonic cleaning and drying. This dried sample was labeled as TNT.

2.3. Preparation of polyelectrolyte multilayer films

The assembling process was similar to the previous study [13]. The isoelectric point of TiO_2 is 4.2–5.5 [17] and the isoelectric points of PLL and DNA are 9.74 and 4–4.5, respectively [13]. So PLL was first assembled on the TNT surface. TNT was immersed in 1 mg/mL PLL/HEPEs buffer (20 mmol/L HEPEs, 75 mmol/L NaCl, pH 7.4) for 15 min, cleaned and dried to acquire sample TNT/P. Subsequently, TNT/P was incubated in 1 mg/mL DNA/HEPEs buffer (20 mmol/L HEPEs, 75 mmol/L NaCl, pH 7.4) for another 15 min and then cleaned and dried to obtain sample TNT/P/D. Finally, TNT/P/D was incubated in the PLL/HEPEs buffer again and cleaned and dried, resulting in sample TNT/P/D/P.

2.4. Surface characterization

All samples were dried in a vacuum desiccator under room temperature for at least 12 h before the measurements. The morphologies of T and TNT were observed by scanning electron microscopy (SEM, FEI Quanta 200, The Netherlands). The chemical compositions of the self-assembled surfaces determined by X-ray photoelectron spectroscopy (XPS, XSAM800, Kratos Ltd, Britain), and the data were processed using Kratos VISION 2000.

2.5. Biomimetic mineralization

Because of rapid deposition, the self-assembled samples were slant immersed in centrifuge tubes with a double concentration of simulated body fluid (2SBF) at 37 °C [18,19]. The 2SBF was changed every 2 days to ensure the freshness and constant ionic concentration of the solution. The samples were retrieved from the solution at day 2 and day 7 and then cleaned and dried in air. The crystal structure of samples were measured by X-ray diffraction (XRD, X′pert pro-MPD, PANalytical, The Netherlands) using a Cu-*K*α radiation in the regular range $2\theta = 20°–50°$ with a step rate of 0.01°/s. The morphology of samples was observed by SEM at an accelerating voltage of 20 kV.

2.6. Osteoblast culture

Osteoblast cells were isolated from newborn (2–4 days) rats, as previously described [20]. Cells were cultured in α-minima essential medium (Gibco), supplemented with 10% fetal bovine serum (FBS, Gibco). Then, the cultured third-generation of osteoblasts was digested with Trypsin-EDTA to acquire a cell suspension. The density of the cells in the suspension was adjusted to 2.5×10^4 cells/mL. The samples were sterilized and placed into a 24-well plate with 1 mL of the cell suspension in each well. The plates were then placed in a 37 °C, 5% CO_2 cell incubator. The culture medium was renewed every two days. All culture experiments have been done in triplicates: each experimental point is presented as the mean of three measures on three titanium disks.

2.7. Cell viability assay

Alamar blue, a fluorescent indicator dye, was used for assay of cell viability. In brief, at day 3 and day 7, the samples were rinsed with PBS and 300 μL of dye solution (10% v/v FBS/10% Alamar blue/80% M199) was added to each well. After incubating at 37 °C for 4 h, 200 μL of solubilization/stop solution was added into a 96-well plate. The relative cell number was determined by measuring light absorbance (OD) at a wavelength of 570 nm with an automatic micro-plate (ELISA) reader (Molecular Devices, Sunnyvale, CA). The data was normalized to the results of the blank samples. The absorbance of the blank well without material extracts was regarded as 100%, and the percentage of absorbance for each well was calculated.

2.8. Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) was employed to evaluate the early differentiation ability of the cells. After 7 days, cells were washed 3 times in PBS and incubated in Triton X-100 (1% w/v). Then the 24-well plate was placed into a refrigerator to be frozen at −80 °C for 2 h. After running three freeze–thaw cycles to homogenize the solutions, 50 μL of the solutions was added into a 96-well plate for the ALP activity test. ALP substrate solution (200 μL; 4-NPP, ELPN-500, Bio-Assay Systems) was added to each well, and the solutions were mixed at room temperature. The absorbance of each solution was measured at a wavelength of 405 nm with an ELISA Reader.

2.9. Cell morphology

After culture for 3 and 7 days, the cells on the samples were washed with PBS. Subsequently, cells were fixed with 2.5% glutaraldehyde in 0.8 mol/L PBS for 12 h at 4 °C and then thoroughly washed with PBS. The fixed and washed samples were dipped in Rhodamine123 (Sigma) solution containing 1% PBS for 30 min at room temperature, washed with abundant water for

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