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Enhanced cytocompatibility of silver-containing biointerface by constructing nitrogen functionalities

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ABSTRACT

Silver (Ag) has recently been introduced into polymeric biomedical implants by plasma immersion ion implantation (PIII) to enhance the antibacterial capability. However, Ag ions and nanoparticles can increase the cytotoxicity and inhibit cellular proliferation and the relationship is time- and dose-dependent. In this study, Ag and N_2 PIII is conducted in concert to produce nitrogen functional groups as well as Ag-containing biointerface. In addition to the creation of nitrogen functionalities, the surface roughness and hydrophilicity are improved in favor of protein adsorption. Compared to the biointerface created by Ag PIII only, the nitrogen functionalities generated by N_2 co-PIII do not affect DNA synthesis and the total protein level but evidently enhance cellular adhesion, viability, and proliferation at the biointerface. The modified surface is observed to upregulate the osteogenesis-related marker expression of bone cells in contact. Our findings suggest that dual Ag and N_2 PIII is a desirable technique to enhance both the cytocompatibility and antibacterial capability of medical polymers.

1. Introduction

Polymers are commonly used in bone tissue engineering [1,2] but when they are implanted in the human body, they can become places for bacteria to adhere and breed resulting in infection and ensuing clinical complications [3–5]. Silver as a broad-spectrum antiseptic can be used to combat bacterial infection [6–10] and the biological safety of silver ions and nanoparticles is still controversial [8,11,12]. It has been revealed that the cytotoxicity and degree of inhibition of cell viability and proliferation depend on the time and Ag dosage [6,12,13]. It is imperative that the cytocompability of a silver-containing biointerface in contact with body tissues be improved from the perspective of antibacterial capability. In our previous experiments, Ag was implanted into various types of medical polymers by plasma immersion ion implantation (PIII) [14,15] to enhance the antibacterial properties while the desirable bulk properties of the materials are preserved [15,16]. One of the important issues of antibacterial polymers is that they must be compatible with cells and tissues [12,17].

The cytocompatibility of biomaterials is typically defined to be the ability of materials to perform with an appropriate host response in a specific application [17–19]. It is recognized that cellular adhesion, proliferation, and differentiation on a silver-containing biointerface depend on silver release and surface physicochemical properties [20]. Our previous work has revealed that nitrogen functional groups including primary, secondary, and tertiary amines possesses some capability to inhibit bacteria growth while promoting the growth and osteogenesis related marker expression of osteoblasts in contact [21,22]. It has also been reported that these amine groups facilitate adhesion of extracellular matrix related proteins fostering the adhesion and proliferation of cells such as osteoblasts, fibroblasts, and so on [23–26]. The objective of this work is to investigate whether simultaneous introduction of Ag and nitrogen functional groups can serve the dual purpose of enhancing the cytocompatibility and antibacterial characteristics of medical polyethylene (PE).

2. Experimental details

2.1. Preparation of the functional biointerface

The 1.0 cm × 1.0 cm × 0.1 cm medical PE slices were inserted into the PIII machine equipped with a silver cathodic arc plasma

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source. The arc was ignited using a pulse duration of 300 μs, repetition rate of 30 Hz, and arc current of 1 A. Ag PIII was conducted by applying an in-phase bias voltage of −5 kV with a repetition rate of 30 Hz and pulse width of 300 μs to the PE samples. In the simultaneous Ag and N_2 PIII treatment, nitrogen gas was introduced into the vicinity of the silver arc discharge plume at a flow rate of 10 sccm (standard cubic centimeter). The dual PIII process was conducted by applying the same bias voltage as the Ag PIII treatment. The treatment time was 10 min and the pressure in the vacuum chamber was $1-2\times10^{-4}$ Torr. The untreated PE control, Ag PIII PE, and Ag and N_2 co-PIII samples were designated as PE control, Ag PIII, and Ag/N_2 PIII, respectively.

2.2. Physicochemical characterization of the functional biointerface

The surface chemical states were determined by X-ray photoelectron spectroscopy (XPS) conducted on the Physical Electronics PHI 5802. Static contact angle measurements using distilled water as the medium were performed by the sessile drop method on a Ramé-Hart (USA) instrument at ambient humidity and temperature. Contact mode atomic force microscopy (AFM) was performed on a Park Scientific Instrument (PSI) Autoprobe Research System to examine the surface morphology in a scanned area of 15 μm × 15 μm.

2.3. Protein adsorption assays

The bovine serum albumin (BSA, Thermo Fisher Scientific Inc.) solution was used to prepare the protein solution with a concentration of 50 μg/mL and then 2 mL of the solution was added to each sample on a 24-well cell culture plate. After incubation at 90% relative humidity at 37 °C for 4, 24 and 48 h, the amounts of BSA adsorbed on the samples were determined by monitoring the residual BSA concentrations in the solutions using a Bio-Rad protein assay (Bio-Rad laboratories).

2.4. Cellular attachment, viability and proliferation assays

A mouse osteoblastic cell line (MC3T3-E1, China Infrastructure of Cell Line Resources) was used to assess influence on cell behavior. The cells were incubated in a minimal essential medium (MEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37 °C under 5% CO_2.

1 mL of the 2×10^5 cell/mL suspension of the MC3T3-E1 cells was applied to each sample. After incubation for 2 h, the samples were washed three times with Dulbecco's phosphate-buffered saline (PBS, Invitrogen) and then a cell counting kit (CCK-8 kit, DOJINDO Molecular Technologies, Inc.) was used to determine the quantity of cells on the sample. To conduct the cell viability assay, the cells were incubated for 24 h using the aforementioned method and the live and dead cells on the samples were visualized by staining the cells with a double staining kit (DOJINDO Molecular Technologies, Inc.) after rinsing three times with the PBS solution. The percentage of dead cells was calculated by counting the live and dead cells by fluorescence microscopy. In the cell proliferation assay, 1 mL of the 5×10^4 cell/mL cell suspension was applied to each sample. After incubation for 3, 7 and 10 days, the number of live cells on the samples was counted using the CCK-8 kit.

2.5. Cell lysis, total DNA and protein, and marker genes expression analysis

1 mL of the 5×10^4 cell/mL cell suspension was added to each sample and after incubation for 4 or 9 days, the samples were washed three times with the PBS solution. The cells were lysed by freezing and thawed for three cycles in 200 μL of 1% triton X-100 in ultrapure water. The prepared cell lysate was centrifuged for 10 min at 4 °C and the supernatants were stored at −20 °C prior to analysis of the DNA, total protein content, and alkaline phosphatase (ALP) activity assay. The cell culture medium corresponding to cell lysis was also centrifuged for 10 min at 4 °C and the supernatant was stored at −20 °C prior to determination of the osteocalcin secretion content.

The DNA content in the cell lysate was assessed using Quant-iT™ dsDNA Assay Kit (SKU# Q-33120, Invitrogen). Quantification of proteins was performed on a micro-BCA protein assay kit (Thermo Fisher Scientific Inc.). Briefly, a 150 μL aliquot of the cell lysate supernatant was mixed with 150 μL of the Micro BCA™ working reagent on a 96-well cell culture plate and incubated for 2 h at 37 °C. The absorbance of the solution at 570 nm was obtained on a plate reader (Bio-Rad Laboratories Inc.). Analysis of DNA and total cellular protein was performed on the same samples used for the ALP activity assay and osteocalcin assay. The ALP activity of the cell lysate supernatants was assessed using p-nitrophenylphosphate as the substrate by an ALP activity kit (Wako Pure chemical industries Ltd.) and quantification of osteocalcin in the supernatants of the cell culture medium was performed using a mouse osteocalcin EIA kit (Biomedical Technologies Inc.). The ALP activity and osteocalcin level were normalized to the total cellular protein content.

2.6. Statistical analysis

The SPSS version 19.0.0 (Chicago, IL, USA) software was used in the statistical analysis. The data were expressed as means ± SD, and $p<0.05$ was considered statistically significant. The least significant difference (LSD) test was used to determine differences among the groups.

3. Results

3.1. Physicochemical characteristics

XPS is conducted to determine the surface composition of Ag PIII and Ag/N_2 PIII. As shown in Fig. 1A, Ag PIII generates a great deal of C═C bonds and also some oxygen-containing functional groups probably due to interaction with ambient oxygen. Fig. 1B shows that dual Ag and N_2 PIII produces nitrogen functionalities in addition to C—O and C═C bonds on the surface [16]. According to our previous experiments, Ag can be found to a depth of several hundred nanometres with a zero valence state indicating that it does not bond with other elements in the polymer [16]. AFM and static contact angle results (Table 1) show that after PIII, both Ag PIII and Ag/N_2 PIII have larger root mean square (RMS) roughness and better hydrophilicity but the degree of roughness is similar.

3.2. Protein absorption

Fig. 2 shows that Ag PIII and Ag/N_2 PIII possess better BSA protein absorption ability than the PE control. In only several hours, a large amount of BSA protein is observed to adsorb rapidly on the PIII samples but not so on the PE control. Moreover, the amount of BSA is constantly larger on both Ag PIII and Ag/N_2 PIII

Table 1
Surface roughness and measured water contact angles of PE control, Ag PIII, and Ag/N_2 PIII, ($n=4$).

Samples	RMS roughness	Contact angles
PE control	202 ± 1.0	87.7 ± 5.0°
Ag PIII	326 ± 4.3	55.4 ± 5.6°
Ag/N_2 PIII	328.5 ± 1.1	54.5 ± 4.7°