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Enhancing the biological response of titanium surface through the immobilization of bone morphogenetic protein-2 using the natural cross-linker genipin

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ABSTRACT

Titanium (Ti) is widely used in orthopaedic and dental implants; however, the surface modification methods used to promote osseointegration require further development. In this study, we developed a simple, cost-effective method for the immobilization of bone morphogenetic protein-2 (BMP-2) on Ti surfaces using a natural cross-linker genipin. We then investigated the surface characteristics, including topography, chemistry, hydrophilicity, coating layer adhesion, and protein (albumin) adsorption. In accordance with ISO 10993-5, the cytotoxicity of the resulting materials was evaluated. Human bone marrow mesenchymal stem cell responses, including adhesion, proliferation, and mineralization, were also evaluated. Immersion in alkaline solution resulted in the formation of a porous Ti surface. The use of the cross-linker genipin for the immobilization of BMP-2 on porous Ti surfaces improved the surface hydrophilicity and protein adsorption, which resulted in a non-cytotoxic coating with good adhesion characteristics. The immobilization of BMP-2 on porous Ti surfaces was shown to significantly increase cell mineralization and bioactivity (*i.e.* Ca/P formation ability), resulting in a biomolecular surface with outstanding potential for bone implant applications.

1. Introduction

The surfaces of bone implants come into direct contact with bone tissue. As a result, surface characteristics, such as the morphology, chemical composition, and wettability, of the implant surface play a crucial role in the healing of tissue surrounding the implant, largely determining the success of bone tissue formation [1–3]. Despite the numerous advantages of titanium (Ti) and its wide applicability in orthopaedics and dentistry, several problems have yet to be resolved. The biologically inert nature of the oxide film that spontaneously forms on the surface of Ti implants can hinder bonding between bone and the implant [4].

Numerous researchers have investigated the modification of Ti surfaces using physical, chemical, or biological methods [5–15]. Physical treatments alter the surface morphology of Ti, resulting in the creation of microstructures at micro-, submicro-, or nanoscales. These microstructures induce protein expression associated with osteogenic differentiation and largely determine cell responses with regard to cell adhesion, proliferation, and differentiation [5–7]. Chemical treatments can be used to modify the morphology of Ti surfaces and even the chemical properties, such as surface wettability (or surface energy) and corrosion resistance [8–11]. Materials with greater surface energy are better able to promote the maturation and differentiation of osteoblasts [11]. Biological treatments are being developed for the immobilization of Ti surfaces using biologically active molecules to enhance biological activity [12–15].

Biological surface treatments are meant to induce osteogenesis and osseointegration *via* signal transduction through multifunctional growth factors or proteins that regulate cell growth and differentiation. The immobilization of bone morphogenetic protein (BMP) can promote osseointegration and even induce heterotopic ossification [14]. BMP-2 is a low molecular weight glycoprotein, classified as a morphogen. BMPs belong to the expanding superfamily of transforming growth factor-β. BMP-2 has been shown to induce the formation of structurally sound orthotropic bone in a variety of experimental systems [15]. Non-

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osteogenic pluripotent mouse cells have been shown to differentiate into osteogenic cells under BMP-2 treatment [16]. Therefore, the application of BMP-2 in the treatment of Ti implants has attracted considerable interest due to its potential in osseointegration.

Glutaraldehyde is able to increase the mechanical strength of scaffolds by cross-linking collagen, gelatin, and chitosan [17,18]. The fact that glutaraldehyde is potentially cytotoxic has led to the development of the natural cross-linker genipin as a potential replacement [19]. Genipin is extracted from the fruit of *Gardenia jasminoides* Ellis and is commonly used in traditional Chinese medicine to soothe headaches and fevers and treat the symptoms of liver diseases, diabetes, and hypertension [20]. Previous studies have reported that genipin produces antioxidant and anti-inflammatory effects and inhibits hepatic apoptosis caused by transforming growth factor β1 (TGF-β1). It has even been shown to protect the nervous system from the toxic effects of the amyloid β protein associated with Alzheimer's disease [21,22].

This study sought to enhance the biological response to Ti surfaces through the immobilization of BMP-2 using the natural cross-linker genipin. This is a simple, cost-effective approach applicable to the mass production of advanced Ti bone implants.

2. Materials and methods

2.1. Sample preparations

Biomedical grade Ti discs (diameter 15 mm, thickness 1 mm) were sequentially ground using SiC papers until #1200 for use as a substrate (designated as Ti samples). Samples underwent 12 h alkaline (5 M NaOH) immersion treatment at room temperature to produce hydroxyl (OH) groups on the ground Ti surface (designated as Ti-OH samples). This was followed by the immersion of some samples in phosphate-buffered saline (PBS) containing genipin (0.05%) for 12 h (designated as Ti-OH–G samples). The negatively charged hydroxyl groups on Ti-OH surfaces enable bonding with the genipin *via* the aldehyde groups (CO). Finally, the Ti-OH–G samples were immersed in PBS containing BMP-2 (10 and 50 ng/ml) for 12 h (respectively designated as Ti-OH–G-B10 and Ti-OH–G-B50 samples). The N-terminals of the BMP-2 interact with the genipin to form amide bonds, resulting in the immobilization of BMP-2 on Ti surfaces.

2.2. Surface characteristics

Surface characteristics were evaluated using methods similar to those employed in previous reports [9,23]. The surface topography, chemistry, and functional groups of the test samples were examined using field emission-scanning electron microscopy (FE-SEM), X-ray photoelectron spectroscopy (XPS), and attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR), respectively. The dimensions of the surface topographies were averaged from three FE-SEM micrographs using the Image-Pro® Plus image analysis software. The adhesion ability of coatings was examined using a cross-cut tape test in accordance with ASTM D3359 standards. Surface hydrophilicity was analyzed by measuring the contact angle of a droplet of pure water on samples using a contact angle goniometer. The measurement pertaining to contact angle was performed in triplicate.

2.3. Biological responses

In this study, we investigated protein adsorption, cytotoxicity, and cell responses, including adhesion, proliferation, and mineralization. Biological responses were analyzed using methods similar to those employed in previous reports [9,23], as outlined below. For further details, please refer to the experiment procedures described in previous reports.

2.3.1. Protein adsorption

Bovine serum albumin (BSA) was used as a model protein. Bicinchoninic acid (BCA) was used for the quantitative evaluation of protein adsorption. In this assay, 200 μl of albumin solution (5 mg/ml in PBS) was placed on the surface of test samples. Following incubation at 37 °C for various durations (1 and 12 h), non-adherent protein was mixed with BCA and maintained at 37 °C for 30 min. The quantity of albumin (%) was determined using a Thermo Scientific Multiskan FC microplate photometer at 570 nm. All measurements were performed in triplicate at each point in time.

2.3.2. Cytotoxicity assay

Cytotoxicity assays were performed in accordance with ISO10993-5 specifications. L929 cells from mouse fibroblast cell line were selected to study the cytotoxicity of extracts from the test samples. The test samples were maintained in Dulbecco's modified Eagle's medium (DMEM) in an incubator under 5% CO_2 at 37 °C for 24 h. Then, the extracts were used to treat a cell monolayer for 24 h. Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay, and optical density (OD) was measured using microplate photometer (wavelength 570 nm). Higher OD values represent greater cell viability. The base medium, DMEM, was used without extracts as a blank control; DMEM treated with 10% dimethyl sulfoxide was used as a positive control (PC); and a biomedical zirconia sheet was used as a negative control (NC). A reduction in cell viability to <70% of the blank control was used as an indication of potential cytotoxicity.

2.3.3. Cell adhesion

Human bone marrow mesenchymal stem cells (hMSCs) were transduced using the gene for green fluorescent protein (GFP) through retroviral delivery. GFP-labeled hMSCs were cultured on test samples at a density of 5×10^4 cells/specimen. The culture medium contained RPMI-1640 medium supplemented with 5% fetal bovine serum and 10% horse serum. During 6-h cell incubation, cells adhering to the test samples were observed *in situ* using a fluorescence microscope. In parallel tests, cells adhering to the test samples were sequentially fixed, dehydrated, and dried using a critical point dryer. The samples were then coated with a thin film of platinum, whereupon cell adhesion morphology was observed using FE-SEM.

2.3.4. Cell proliferation

A MTT assay was used to evaluate cell proliferation. hMSCs were seeded on the test samples (5×10^4 cells/cm^2) for 1 or 3 days and then treated with MTT in culture medium at 37 °C for 4 h. Purple formazan product from the MTT assay was dissolved using isopropanol before measuring absorbance at 570 nm, wherein greater OD was an indication of superior cell viability. All measurements were performed in triplicate at each point in time.

2.3.5. Cell mineralization

Cell mineralization capacity was evaluated using Alizarin red S staining for the detection of calcium compounds. hMSCs were cultured in a normal medium for 24 h before switching to an osteogenic medium containing DMEM supplemented with ascorbic acid, β-glycerophosphate, and dexamethasone. Afterwards, the osteogenic medium was changed every two days. After 21 days of osteogenic incubation, the cells were fixed and then stained using Alizarin red S in distilled water at room temperature. The quantification of stained cells was performed using cetylpyridinium chloride in sodium phosphate for 1 h with shaking at room temperature. The OD values were subsequently measured using a microplate photometer at 550 nm; higher OD values represent more extensive cell mineralization. The mineralization test results were expressed as percentage of Ti group (note: Ti group was used as 100%). All measurements at each time point were performed in triplicate.