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Assessment of bone morphogenic protein and hydroxyapatite–titanium dioxide composites for bone implant materials

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ABSTRACT

The HAp–TiO_2 coating, made by micro-arc oxidation (MAO), is successfully grown on the Ti surface in one step; BMP-2 growth factor is physically immobilized on the HAp–TiO_2 coating. The BMP-2/HAp–TiO_2 hybrid system outperforms systems of pure Ti, BMP-2/Ti, and HAp–TiO_2 by promoting cell adhesion increasing filopodia and polygonal lamellipodia extension, and increasing cell viability. Additionally, cell proliferation and alkaline phosphatase (ALP) activity both indicate that the BMP-2/HAp–TiO_2 hybrid system is more successful. According to the characteristics of mechanical support from HAp–TiO_2, the BMP-2/HAp–TiO_2 hybrid system can maintain a stable release rate of BMP-2 to promote bone cell growth and rapidly heal the bone–implant interface. The new BMP-2/HAp–TiO_2 hybrid system developed in this study can be expected to contribute in the application of orthopedic and dental implants.

1. Introduction

Owing to its excellent strength-to-weight ratio, corrosion resistance, and satisfactory biocompatibility with the living body, titanium (Ti) is the best material for preparing orthopedic and dental implants [1]. The naturally grown, dense, and chemically stable amorphous TiO_2 layer (a-TiO_2) on Ti surfaces exhibits average biocompatibility [2]. However, the response from the host bone cell to a-TiO_2 is not always satisfactory because a fibrous layer may form on the bone–implant interface, causing implantation failure [3]. a-TiO_2 is far less biocompatible than crystalline TiO_2 (c-TiO_2) [4]. Consequently, c-TiO_2 does not exhibit the drawbacks of a-TiO_2 and activates positive bone growth [5]. The bioactive material hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$), with excellent biocompatibility and osteoconductivity, is another candidate for implants [6]. However, the biodegradability and poor intrinsic mechanical properties of HAp lead to a high failure rate for implanted HAp prosthetics. A hybrid bioactive ceramic system, composed of HAp and c-TiO_2, has been widely investigated [7–11] for its promotion of cell affinity from HAp and strong mechanical connection between the HAp and TiO_2 films. In order to achieve a highly bioactive structure, many surface-modification techniques, such as electrochemical deposition [12,13], biomimicry [14,15], glow discharge [16,17], and micro-arc oxidation (MAO) [3,18] have been adapted to fabricate such biocompatible coatings. Among these methods, MAO [18–22] is considered the most ideal method for fabricating bioactive HAp/c-TiO_2 hybrid films by a one-step process because it requires simple equipment to form a crystal oxide film can be performed in various electrolyte solutions, and results in a high film growth rate and strong film adhesion. In our previous studies [18,21,23–26], we successfully demonstrated many types of TiO_2 coatings for anatase-phase (A-TiO_2), rutile-phase (R-TiO_2), strontium-doped ($SrTiO_2$), HAp–TiO_2, and strontium-HAp TiO_2 (Sr-HAp–TiO_2) in surface modifications of Ti-based bone implants. Our various MAO–TiO_2 coatings have distinct levels of biocompatibility, biochemical stability, and mechanical properties, allowing for material selection based on specific requirements. The HAp–TiO_2 coating in particular exhibits a very favorable combination of biochemical stability and good mechanical properties [27] and can induce the formation of bone-like apatite, which has the best bioactivity both *in vitro* and *in vivo* [28]. Based on these characteristics of HAp–TiO_2, the reinforcement of its biocompatibility is regarded as another important issue.

Annually, 2.2 million bone-grafting procedures are performed worldwide in the fields of orthopedics, neurosurgery, and dentistry to repair bone defects [23]. These data indicate the huge demand for bone implants and the importance of fabrication of highly compatible surface coatings. Bone grafting uses viable bone cells, osteoinductive substances, and osteoconductive scaffolds to facilitate osteogenesis in cases of fractures with delayed union or non-union, as well as bone voids requiring reconstruction. Bone morphogenic protein (BMP-2) delivery systems have been developed to enhance osteoblast function

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[29]. BMPs were first described by Urist in 1965, when he found a demineralized bone matrix implanted at ectopic sites in rats that induced bone formation [30]. Several studies have shown that BMP-2 is an osteoinductive potential growth factor [31–33], requiring suitable carriers for incorporation. It was reported that functionalized BMP-2 immobilized onto electrochemically anodized Ti nanotubes and amine-terminated anodized Ti promotes osteoblast adhesions better than BMP-2 on non-functionalized anodized Ti or bare Ti [34]. HAp is known to be a suitable carrier for incorporating and retaining the activity of growth factors and promoting the biological activity of osteoinduction [27,35]. HAp enables retention of the growth factor at the site of implantation, enhancing its local concentration. BMPs cannot chemically bind to the carrier, entrapping physically onto carriers instead [36]. Therefore, the design of a carrier with a controllable release rate for BMPs is indispensable. An ideal carrier would prolong the releasing time of BMPs at a constant rate, render BMPs more efficient, and help to create the chemotactic gradient necessary for growth.

We report the development of BMP-2-immobilzed HAp–TiO_2 coatings on Ti surfaces and estimate their effectiveness and reliability in the early stages of cell growth (cell adhesion, proliferation, and ALP activity). BMPs are unique because they trigger the differentiation of mesenchymal cells to become osteoblasts and promote the differentiation of functions of the osteoblast [37]. Therefore, we hypothesize that osteoinductive BMP-2 on a Ti surface can induce rapid differentiation of MC3T3-E1 cells by constant release, and that osteoconductive HAp–TiO_2 with sufficient structural consistency can serve as a scaffold for the further growth of bone cells on and into its porous structure, followed by extracellular matrix deposition and mineralization, as shown in Fig. 1. Therefore, this study serves as a model that successfully accomplishes both osteoinduction and osteoconduction *in vitro*.

2. Materials and methods

2.1. Preparation of recombinant human bone morphogenetic protein-2 (rhBMP-2)-immobilized HAp–TiO_2 on Ti surface using MAO

Commercially available pure-Ti (Grade II) thin plates of dimensions 15 mm × 15 mm × 1 mm were polished with waterproof abrasive paper to remove most of the native oxide layer. The polished substructure was cleaned by sonication in acetone, ethanol, and distilled water. The specimens were air dried before use as substrates.

A HAp–TiO_2 coating was formed by placing each prepared Ti thin plate, which was used as the anode, and a stainless steel plate, which was used as the cathode, in an electrolytic tank. The aqueous electrolyte solution was a mixture of 0.20 M $(CH_3COO)_2Ca\cdot H_2O$ and 0.12 M $NaH_2PO_4\cdot 2H_2O$ at an anodic potential of 480 V, obtained by applying a DC field for 10 min. All MAO processing was conducted in a cooling bath to maintain the temperature of the electrolyte solution at 25 °C. A magnetic stirrer was used to homogenize the electrolyte solution and to eliminate air bubbles generated on the Ti surface during the process. Finally, the MAO-treated specimens were rinsed in distilled water, dried at 40 °C for 24 h in an oven, and stored in a desiccator. Table 1 presents the MAO HAp–TiO_2 parameters in this investigation.

To immobilize rhBMP-2 on pure-Ti and HAp–TiO_2 specimens, rhBMP-2 (0.5 μg/mL) was added to phosphate-buffered saline (PBS). The proteins were coated onto both the pure Ti and the HAp–TiO_2 coated specimens by immersing them for 2 h in rhBMP-2-modified PBS at room temperature. Following adsorption, the specimens were left to dry as described above.

2.2. Morphological and microstructural characterization of pure-Ti and experimental specimens

The crystal structures of the pure-Ti and HAp–TiO_2 coated specimens were elucidated using X-ray diffraction (XRD) with Cu-Kα radiation (λ = 1.5405 nm) at a scan rate of 4° min^{-1} in the range between 20° and 60°. The surface morphology of the pure-Ti, HAp–TiO_2, BMP-2/pure-Ti, and BMP-2/HAp–TiO_2 coated specimens were determined using a scanning electron microscope (SEM). Attenuated total reflectance–Fourier transform infrared spectrometer (ATR–FTIR) measurements were made on an analyzing ZnSe crystal. The ZnSe crystal was carefully cleaned with analytical-grade ethanol before and after the analysis. The analyzed spectra were obtained using an ATR–FTIR spectrometer in the 4000 to 650 cm^{-1} spectral domain with a spectral resolution of 4 cm^{-1}.

2.3. In vitro cell culture and osteoblast activity of pure-Ti, HAp–TiO_2, BMP-2/pure-Ti, and BMP-2/HAp–TiO_2 coated specimens

A murine pre-osteoblast cell line (MC3T3-E1) was obtained from the RIKEN Cell Bank (Tsukuba, Japan). The MC3T3-E1 pre-osteoblast cells were cultured in 10% α-MEM (89% α-MEM with 10% fetal bovine serum and 1% penicillin/streptomycin for neutralization). MC3T3-E1 cells were plated in cell culture dishes and allowed adhering for 2 days at 37 °C in an incubator containing 5% CO_2.

The 12-well plates containing pure-Ti, HAp–TiO_2, BMP-2/pure-Ti, and BMP-2/HAp–TiO_2 coated specimens were seeded with MC3T3-E1 cells at a density of 1×10^5 cells mL^{-1} to perform cell

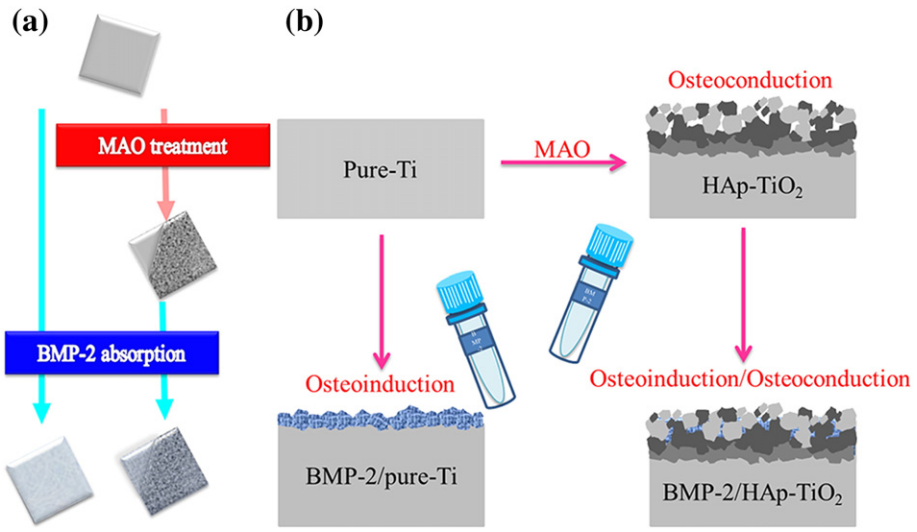

Fig. 1. (a) Flow chart of experiment on multi-functional coatings. (b) Cross-sections of BMP-2 immobilized on Ti before and after MAO treatment.