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Regulation of the differentiation of mesenchymal stem cells *in vitro* and osteogenesis *in vivo* by microenvironmental modification of titanium alloy surfaces

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ABSTRACT

To mimic the extracellular microenvironment of bone, a bioactive multilayered structure of gelatin/chitosan pair, containing bone morphogenetic protein 2(BMP2) and fibronectin (FN), was constructed onto Ti6Al4V surface via a layer-by-layer assembly technique. The successful fabrication of multilayered structure was confirmed by contact angle measurement, field emission scanning electron microscopy (FE-SEM) and confocal laser scanning microscopy (CLSM), respectively. Bioactive BMP2 released in a sustained manner along with the degradation of multilayered structure. MSCs grown onto the multilayer coated TC4 substrates displayed significantly higher ($p < 0.01$ or $p < 0.05$) production levels of alkaline phosphatase (ALP), mineralization and genes expressions of runt related transcription factor 2 (Runx2), osterix, osteocalcin (OC), osteopontin (OPN), ALP and collagen type I(ColI) compared to the controls after culture for 7 days and 21 days, respectively. More importantly, MicroCT analysis and histological observations demonstrated that the multilayer coated Ti6Al4V implants *in vivo* promoted the bone density and new bone formation around them after implantation for 4 weeks and 12 weeks, respectively. The results indicated that Ti6Al4V coated with biofunctional multilayers was beneficial for osteogenesis and integration of implant/bone. The study therefore presents an alternative to fabricate bio-functionalized Ti6Al4V-based implants for potential application in orthopedics field.

1. Introduction

The natural extracellular microenvironment is a highly complicated network, which is composed of proteins, proteoglycans, soluble signals (growth factors, cytokines, chemokines), physical signals (fibronectin, vitronectin, laminin, collagen, fibrillin) and signals arising from cell/cell interactions [1]. It plays a critical role in the regulation of cellular responses to wound healing, tissue repair and regeneration. The ultimate cell fates, such as cell adhesion, proliferation, migration, differentiation and apoptosis, are then directed by clues in the extracellular microenvironment.

The creation of a proper microenvironment is a relevant aspect that needs to be accounted for the development of new generations of biomaterials. In recent years, many efforts have been devoted to constructing suitable extracellular microenvironments, taking advantage of proper biomaterial design and engineering. These approaches could, in turn, elicit specific biological responses. To construct desirable extracellular microenvironment is essentially important for the development of new generation biomaterials. Naturally derived extracellular matrix (ECM) proteins [2], three-dimensional (3 D) dynamic hydrogel with bioactive motifs [3], lipid bilayers [4], self-assembled monolayers [5], cell sheets [6], cell adhesion molecules [7] as well as calcium apatites [8] were introduced onto surfaces of biomaterials via different techniques to mimic the extracellular microenvironment. Among them, the layer-by-layer (LbL) assembly technique is one of the most simple and efficient approaches for surface modification. Previous studies confirmed that the extracellular microenvironment formed by various polyelectrolyte multilayers demonstrated good biocompatibility and osteoconductivity [9]. In our previous studies, we fabricated hybrid multilayers drug-delivery system with gene-activation capabilities onto titanium and poly (D,L-lactic acid) substrates via layer-by-layer assembly technique, respectively [10–12], implying great potentials to regulate the differentiation of

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MSCs and maintain bone homeostasis. Very recently, Hammond *et al.* demonstrated that the growth factor-eluting multilayers coated 3D β-tricalcium phosphate scaffolds could direct cellular functions and tissue integration [13].

A perfect bone implant should have two characteristics: the first one is to actively recruit osteoblasts or progenitor cells (e. g. MSCs) from the surrounding native tissue and promote cell adhesion; the second one is to provide appropriate bioactive signals (e. g, growth factors or cytokines) to induce cell proliferation and differentiation for new bone formation. Both characteristics are highly related to the extracellular microenvironment. Commercial pure titanium (cpTi) and its alloys were extensively employed in orthopedic field [14], however, with only poor osseointegration between implant and natural bone tissue [15]. Thus, to construct a desired microenvironment onto a titanium-based implant for mimicking the natural extracellular matrix may greatly improve its osseointegration. Previously, we assembled chitosan and gelatin onto the surface of titanium substrates [16]. The results showed that the Chi/Gel multilayer improved the biocompatibility of titanium films. However, the further application of such a system was still limited by its passive response to native tissue.

To address this challenge, we fabricated ECM-like networks onto Ti6Al4V substrates by embedding BMP2 and FN into chitosan/gelatin mulitlayers. BMP2 was used as the intermediate layer and fibronectin as the top layer (Fig. 1). We intended to regulate the general cascade responses of cell adhesion (by FN) and differentiation (by BMP2) when cells began to contact with a biomaterial. BMP2 is a well known growth factor for maintaining bone homeostasis *in vivo* [17], and it has been widely used for bone repair and regeneration in the clinics [18]. Previous reports demonstrated that BMP2 could stimulate the proliferation, migration and osteogenic differentiation of mesenchymal stem cells [19,20]. Moreover, BMP2 was proved to induce osteogenesis *in vivo* [21,22]. On the other hand, as one of the major ECM proteins, fibronectin plays a key role in guiding initial cell adhesion to biomaterials [23–25]. It is well known that the growth factors (e. g. BMP2) are susceptible to denaturation and degradation when exposed to biological fluids with various chemicals and enzymes, leading to the loss of their bioactivities. Nevertheless, previous studies confirmed that LbL mutlilayers could retain the bioactivities of fragile drugs/proteins in mild aqueous conditions [26,27]. Meanwhile, the LbL mutlilayer was confirmed to be a desired platform for controlled drug/gene delivery [28]. We hypothesized that -once implanted *in vivo*- TC4 substrates, coated with BMP2-containing multilayers, could control the delivery of the factor and, regulate the adhesion and differentiation potency of the MSCs at the same time, ultimately improving the integration of the implant within the surrounding living tissues.

2. Materials and methods

2.1. Materials

Ti6Al4V disks (diameter: 15 mm; thickness: 1 mm) and rods (diameter: 3 mm; length: 13 mm) were provided by Northwest Institute for Non-ferrous Metal Research, China. Alizarin red S sodium salt was provided by Alfa Aesar Co. (Tianjin, China). Chitosan, gelatin, 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fibronectin, p-nitrophenyl phosphate and bicinchoninic acid (BCA) assay kit were obtained from Sigma Chemical Co. (MO, USA). Bone morphological protein 2 (BMP2), mouse recombinant receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (m-CSF) were

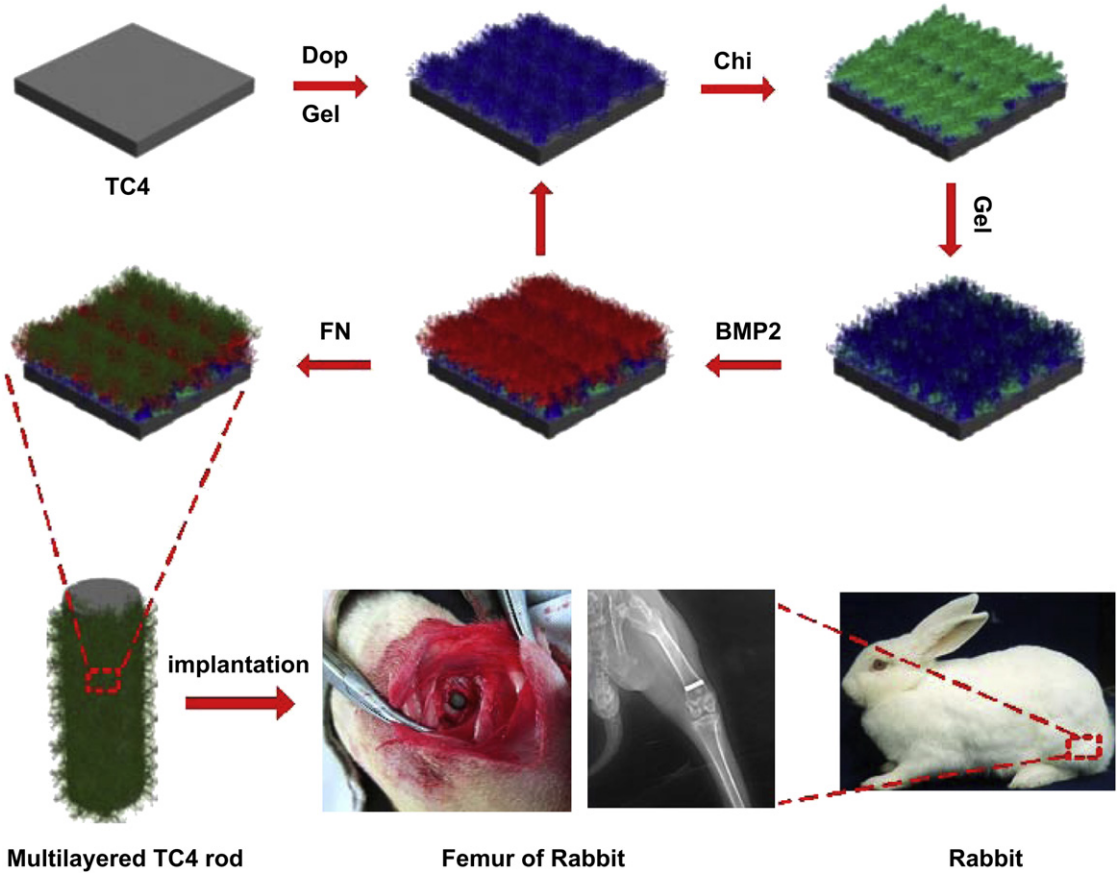

Fig. 1. Schematic illustration of fabrication of biofunctional multilayer coated TiAl6V4 rod and its implantation in rabbit femur. The inserted image is the representative radiographs of implants after implantation for 4 weeks.