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Study of collagen/γ-PGA polyelectrolyte multilayers coating on plasma treated 316 L stainless steel substrates

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ABSTRACT

316 L stainless steel has been widely used in orthopaedic and dental implants due to its good corrosion resistance, good biocompatibility and strong mechanical strength. However, limited cell attachment, osseointegration failure and bacterial infection of 316 L stainless steel are the main challenges for its clinical application, and these problems can lead to severe implant failure. Among all solutions, surface modification is one of the most promising techniques without damaging the structural integrity of the base metal. In this research, polyelectrolyte multilayers (PEM) coating was applied on plasma treated 316 L stainless steel. PEM coating was fabricated composing two biocompatible materials: collagen and γ-poly-glutamic acid. In addition, a chitosan barrier was applied at the 11th layer to seal and control the drug release rate. Bone morphogenetic protein 2 (BMP-2) and basic fibroblast growth factor 2 (FGF-2) were loaded at the 1st and 11th layers of PEM. Plasma treatment was found to enhance the hydrophilicity and adhesion of PEM coating on 316 L stainless steel. The degradation rate of PEM was ~80% on day 70. Releases of FGF-2 and BMP-2 from PEM were 58% and 50% after 768 h. PEM coating with dual growth factors was demonstrated with good biocompatibility and promoted cell proliferation of rat bone mesenchymal stem cells (rBMSCs). Furthermore, alkaline phosphatase activity and mineralization of rBMSCs were also enhanced with the addition of BMP-2 and FGF-2. In conclusion, the extreme mechanical properties of 316 L stainless steel was greatly reduced by PEM coating while the BMP-2 and FGF-2 loaded further endowed osteoconductivity and osteoinductivity of the metal substrate.

1. Introduction

Skeleton is made of organic and inorganic matters which are mainly composed of 30 wt% of collagen, 65 wt% of hydroxyapatites and other matters. Bone is continuously modeled and remodeled with the contribution of different cells, which are osteoblasts, osteocytes, and osteoclasts. Mesenchymal stem cells (MSCs) are multipotent, which can self-renew and differentiate into variety of cells such as adipocytes, osteoblasts, and chondrocytes; consequently, these cells form fat, bones, and cartilage [1,2]. Thus, orthopaedic biomaterial research is trying to approach multifunctionality such as osteoinductive and osteoconductive properties [3].

Growth factors have been introduced to stimulate new bone tissue regeneration which is able to trigger and motivate MSCs to differentiate into osteoblasts. Bone morphogenetic protein-2 (BMP-2) can accelerate new cartilage formation and bone generation as well as the wound healing (Cheng 03). Meanwhile, basic fibroblast growth factor (FGF-2) has also been well documented to function on the cell proliferation, neovascularization, and collagen synthesis [4]. Low dose FGF-2 has been reported to have synergistic effect on BMP-2, in which together these two growth factors promote biological activity and healing [5]. However, *in vitro* delivery of growth factors always faces the same disadvantage of rapid release and absorption [4,6,7]. Consequently, the inappropriate dose release can cause undesired adipogenesis and form cyst-like bone [8]. Generally, there are two main methods for growth factor delivery, which are the use of porous structure material and biodegradable polymer. Porous structure materials are widely reported in many research fields due to the high surface area for drug loading and delivery.

In this study, we designed a collagen/γ-poly glutamic acid (γ-PGA) polyelectrolyte multilayers (PEM) coating together with a middle chitosan barrier on plasma treated 316 L stainless steel (SS). The

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advantages of using PEM as a delivery medium are well drug release controlling by layer-by-layer of PEM, which have various biodegradation rates, and the ability of carrying multiple factors at different layers [9]. In this corporation of PEM, multiple drugs delivery has demonstrated excellent abilities to improve biological response. BMP-2 delivered by PEM is able to stimulate MSCs differentiation into cartilage or other new bone generation and FBF-2 is able to accelerate cell proliferation and collagen synthesis, respectively [10,11]. On the other hand, plasma surface modification technique has been investigated its improvement on cell viability [12] and vascular regeneration [13] due to some hydroxyl group on the surface of material which can increase hydrophilicity for cell attachment [14]. As a result, the surface of SS has been treated by plasma treatment in order to provide an excellent adhesion surface for PEM. The surface morphology, physical and mechanical properties were characterized by scanning electron microscope (SEM), contact angle analyzer, white light interferometry, and nanoindentation. The biological activity was evaluated by the cell viability and osteogenic differentiation of rat bone marrow mesenchymal stem cells (rBMSCs). This current collagen/γ-PGA PEM coating can modify the surface properties of SS with reduced mechanical properties and better biological properties, in which this PEM coating endows SS more possibilities to be used in orthopaedic applications.

2. Materials and methods

2.1. Materials

Chitosan (Mw = 300 kDa), type I collagen and γ-PGA (Mw = 3840 kDa) were purchased from Charming & Beauty Co., Ltd. (Taiwan), Victory Biotech (Taiwan) and Vedan Enterprise Co., Ltd. (Taiwan). Minimal Essential Medium – Alpha (α-MEM), phosphate buffered saline (PBS) and fetal bovine serum (FBS) were provided by Gibco (Thermo Fisher Scientific Corporation, USA). Tetracycline, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (USA). Hoechst 33,258 was purchased from Sigma-Aldrich (USA). All other chemicals were reagent grade and obtained from Showa (Japan) or Sigma-Aldrich (USA) if not specified.

2.2. Plasma treatment and PEM coating on 316 L SS

316 L SS (INTAI, Taiwan) disc had a diameter and thickness of 6 mm and 1 mm (labeled as SS). The disc was then polished with 2000 Cw sandpaper. The plasma treatment of SS discs were carried out in gas plasma system (OP-300, Plasma Technology Systems, US). The pressure was brought to 10^{-2} Torr. The plasma reactor was loaded with oxygen at 50 V for 5 min. PEM coating was applied by spin coating technique. Spin coating was conducted with two different speed, initially started from 2000 rpm for 5 s and increased to 4000 rpm for another 5 s. The as-prepared substrate was set on a spin coater and coated with 100 μL of 5 mg/mL collagen solution (in 0.5 M acetic acid). The negatively charged layers were acquired from 100 μL of 5 mg/mL γ-PGA solution. Collagen and γ-PGA were coated alternatively with collagen as the odd layers (except the 11th layer) and γ-PGA for the even layers for a total of 20 layers (labeled as SS-PEM without plasma treatment or plasma-PEM with plasma treatment). At the 11th layer, 100 μL of 5 mg/mL chitosan solution (in 1 mM acetic acid) was applied as the barrier layer. In addition, 20 μL each of 2.5 μg/mL of BMP-1 and FGF-2 was loaded to the 1st collagen layer and 11th chitosan layer, respectively.

2.3. Materials characterization

The surface morphology, wettability, surface roughness and mechanical property of the coated substrates were characterized. The surface morphology and PEM film thickness was observed using scanning electron microscope (SEM; S-3000H, Hitachi/ USA). For the measurement of the film thickness, half of the substrate was covered by adhesive tape prior to PEM coating which the thickness could be measured by a peel-off method. The specimens were sputter-coated with a 100 Å layer of gold-palladium for SEM imaging. The contact angle of the surface was measured using the sessile drop method with a contact angle analyzer (FTA1000, First Ten Angstroms, UK). The surface roughness of the specimens was analyzed by a white light interferometer (7502, Chroma, The Netherlands). Nanoindentation was performed with a nanoindenter (TI 700 Ubi, Hysitron, USA) equipped with a Berkovich pyramid tip to find out the hardness and reduced Young's modulus of the coating. The indentation force was 600 μN while the indentation depths were ~120 nm and ~240 nm for specimens without and with PEM coatings.

2.4. Coating adhesion test

PEM coating on SS and plasma specimens was investigated followed ASTM D3359-17 testing standard [15]. A cross-cut tester was first used to create 11 lines on the coating while a second cut was made intersecting the first pattern at 90°. The results of the test were evaluated as indicated in ASTM D3359-17.

2.5. Degradation

The degradation of the PEM coating was examined by soaking SS-PEM and plasma-PEM specimens in PBS at 37 °C for 10 weeks. SS-PEM and plasma-PEM specimens were weighed at day 0 initially and weighed again every week. The weight loss was calculated according to the following equation,

$$\text{Weight loss} = \frac{W_0 - W_W}{W_0} \times 100\%$$

where W_0 was the original mass at day 0 of the dried PEM coating, and the Ww was the dried mass at certain timepoint after soaking.

2.6. Apatite formation

The bone-bonding ability of the PEM was evaluated by examination of apatite formation on its surface in simulated body fluid (SBF). Two times concentrated SBF was prepared by doubling the concentration of conventional SBF as reported in the literature to accelerate the formation of apatite [16]. The surface morphology of the coating after SBF soaking was imaged by SEM while the chemical composition of the newly formed apatite was characterized using X-ray fluorescence (XRF; SEA6000VX, SII NanoTechnology, JAPAN).

2.7. Growth factor release

Release of BMP-2 and FGF-2 was performed by soaking plasma-PEM specimens in PBS at 37 °C and measured by the enzyme-linked immunosorbent assay (ELISA). Each specimen was soaked in 1 mL PBS in 37 °C incubator for a period of 32 days. At each timepoint (every 3 h on day 0 and 1, 2, 4, 8, 16 and 32), the PBS was collected for later ELISA measurement and replenished with fresh PBS. The amount of BMP-2 and FGF-2 released was measured using commercially available ELISA kit (BMP-2 and FGF-2 Standard ABTS ELISA kits, PeproTech, USA) according to the manufacturer's protocol. The absorbance was read at 405 nm using a microplate reader (Tecan, Sunrise remote F039300, Austria).

2.8. rBMSCs proliferation, ALP activity & mineralization

rBMSCs was used for studying the biological responses of PEM coating. rBMSCs were isolated from the rat femurs using a standard protocol and characterized with flow cytometry (results not reported here). rBMSCs were cultured in Minimal Essential Medium - Alpha (α-