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Osteoblast differentiation and phenotype expressions on chitosan-coated Ti-6Al-4V

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ABSTRACT

Chitosan (C), alginate-crosslinked chitosan (CA), and pectin-crosslinked chitosan (CP) were covalently bonded to Ti-6Al-4V surfaces and tested for their biocompatibility. Compared to the clinically treated Ti-6Al-4V surface (Ti64), C, CA, and CP, had higher contact angles and promoted higher cell proliferation, type I collagen deposition, and mineralization after two weeks (all $p < 0.05$). Cells on C, CA, and CP expressed more alkaline phosphatase (ALP) activity compared to those on Ti64 ($p < 0.05$). The swelling ratios and drug release efficacies of CA and CP were significantly higher and lower, respectively, than those of C (both $p < 0.05$). Only cells on CA expressed ALP activity after three weeks of culture. Generally speaking, crosslinking with alginate and pectin changed surface wettability as well as the swelling and drug release properties of the chitosan coatings. Cells on the coatings had higher proliferation, type I collagen deposition, and degree of mineralization compared to those on Ti64.

1. Introduction

Osteointegration is essential for the successful implantation of an orthopedic prosthesis in a human body. Ti-6Al-4V titanium alloy is one of the most commonly used orthopedic materials because of its high mechanical strength, durability, corrosion resistance, and biocompatibility (Head, Bauk, & Emerson, 1995). Surface modifications have been investigated to improve the clinical performances of titanium and its alloys. Researchers have attempted coating Ti and its alloys with collagen (Becker et al., 2002; Bierbaum et al., 2003; Geissler et al., 2000), chitosan (Bumgardner, Wiser, & Elder, et al., 2003; Bumgardner, Wiser, & Gerard, et al., 2003; Greene, Bumgardner, Yang, Moseley, & Haggard, 2008), hydroxylapatite (Aksakal & Hanyaloglu, 2008), and calcium phosphate (Cleries, Fernandez-Pradas, & Morenza, 2000) to improve their cell adhesion, proliferation, and differentiation performances and also to carry desirable compounds on their surfaces.

Chitosan has been reported to enhance bone and cartilage tissue formation because the molecular structure of chitosan is similar to the components of the extracellular matrix of bone cells (Abarrategi et al., 2010; Schwartz, Griffon, Fredericks, Lee, & Weng, 2011). Chitosan, coated on pure titanium surfaces through silane bonding, has been reported to be stable for weeks and to promote serum protein adsorption and the growth of osteosarcoma cells (Bumgardner, Wiser, & Elder, et al., 2003; Bumgardner, Wiser, & Gerard, et al., 2003). Chitosan hydrogels have been crosslinked using natural polymers such as alginate and pectin to further improve their biocompatibility and mechanical properties (Lin & Yeh, 2010a, 2010b). Alginate is a brown seaweed extract that has been shown to elicit little or no immune reaction in nearby tissues (Klock et al., 1997; Tang, Chen, Weir, Thein-Han, & Xu, 2012). *In vitro* and *in vivo* results have shown that alginate is osteogenic (Lin & Yeh, 2010a; Tang et al., 2012; Xing et al., 2013; Yang, Frei, Rossi, & Burt, 2009). Pectin is contained in the primary cell walls of terrestrial plants and is a natural crosslinker of chitosan (Kim, Park, Kim, & Cho, 2003; Li et al., 2011). *In vitro* results have shown that pectin has good biocompatibility and is osteogenic (Kokkonen et al., 2008, 2010, 2012; Lin & Yeh, 2010b). Pectin and alginate are both anionic polysaccharides, which crosslink with chitosan, a cationic polysaccharide, through ionic attraction between the NH_3^+ of chitosan and the COO^- of alginate and pectin.

To the authors' best knowledge, the biocompatibility of Ti-6Al-4V surface modified with alginate- and pectin-crosslinked chitosan has not been investigated. In this study, chitosan was covalently coated on Ti-6Al-4V through silanization and further crosslinked with alginate and pectin. The changes in physical and biological properties after crosslinking were measured and compared to those of the chitosan coating and the Ti-6Al-4V surface used for implants. The physical properties of the coatings that were

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measured included their thickness, wettability, swelling ratio, and drug release efficacy. Mature osteoblasts were cultured on the Ti-6Al-4V surface and the coatings for three weeks. The biological responses of osteoblast cells – including cell proliferation, viability, morphology, type I collagen expression, alkaline phosphatase activity, and mineralization – were assayed.

2. Materials and methods

All chemicals were purchased from ACROS (NJ, USA) unless otherwise specified.

2.1. Coating the polymer on the titanium alloy surface

Medical grade Ti-6Al-4V ELI (ASTM F136) was purchased from Titanium Industries Asia Inc. – Taiwan Branch (Taipei, Taiwan). The alloy was cut into two sizes (1 cm × 1 cm × 1 mm and 5 cm × 5 cm × 1 mm) to be used in different tests. The surfaces of the Ti-6Al-4V plates were treated as described by (Bumgardner, Wiser, & Gerard, et al., 2003) before being coated with chitosan. Briefly, the plates were submerged in a 95% (v/v) ethanol solution (pH 4.5) and 2% (v/v) of 3-isocyanatopropyltriethoxysilane was added to the solution. The pH was maintained at 4.5–5.5 with 1 M NaOH or 10 M acetic acid. Ten minutes later the plates were rinsed with ethanol and heated at 110 °C for 10 min. The plates then were submerged in a 2% (v/v) glutaraldehyde solution at pH 4.3 overnight. The plates were rinsed with de-ionized water. Chitosan (96% deacetylation, M.W. = 300 kDa, Charming & Beauty, Taiwan) was dissolved in 1% acetic acid to make a 2% (w/v) chitosan solution. For the 1 cm^2 and 25 cm^2 plates, 200 μL and 4 mL, respectively, of the chitosan solution was homogeneously spread on the treated Ti-6Al-4V surface and dried at 40 °C in an oven for 8 hr. The dry coatings were neutralized in 10 N NaOH for 5 min and washed in deionized water. Sodium alginate and pectin (~70% methoxylation) were dissolved in deionized water to make 2% (w/v) solutions. These two solutions were used to crosslink the coated chitosan with 200 μL and 4 mL crosslinkers for the 1 cm^2 and 25 cm^2 chitosan coatings respectively. The crosslinker was homogeneously spread across the top of the chitosan coating. One hour later, the coatings were washed three times in deionized water to remove the unbound crosslinker. The alginate solution on the chitosan coating was hard to be completely removed by simple rinsing due to its high viscosity. Hence the alginate-crosslinked chitosan was submerged in 10% (w/v) $CaCl_2$ solution overnight, while the other coatings were kept in deionized water. The alginate-coated chitosan was rinsed several times with water to remove $CaCl_2$ and the debris of the residual alginate from the coating. The chitosan coated Ti-6Al-4V plates will be referred to as C, alginate-crosslinked chitosan coating as CA, and pectin-crosslinked chitosan coating as CP. The Ti-6Al-4V surfaces that were prepared following the guidelines in ASTM F86-12: *Standard Procedure for Surface Preparation and Marking of Metallic Surgical Implants* were used as positive control and referred to as Ti64. The control plates were wet ground with 80 grit SiC paper and ultrasonically cleaned for 10 min each in de-ionized water, ethanol, acetone, and ethanol again. They were passivated in 30% nitric acid for 30 min and rinsed in de-ionized water.

2.2. Peeling strength, thickness of the coating and contact angle measurements

The peeling test was performed on all three coatings to verify whether the bonding strength between the coatings and the metal surface changed after crosslinking. ASTM D903-98(2010) *Standard Test Method for Peel or Stripping Strength of Adhesive Bonds* was used for this test. The coating was partially peeled, and then its free end was folded back to a 180° angle. There was approximately 2 cm of separation between the free end of the coating and the metal. The metal surface was secured with a Vice-Grip on the crosshead of a tensile tester (Kotsao, Taiwan) while the free end of the coating was secured with using another Vice-Grip. The crosshead moved upward at a rate of 6 mm/min until the coating had almost peeled off the metal. Data from the coatings torn during the test were not used to calculate the results. Load (N) vs. peeling distance (mm) was then plotted, and the data points after the load reached a plateau were averaged and used as the peeling strength of the particular sample. The peeling strengths from six successful tests were averaged for each coating ($n = 6$).

The thickness of the coating was measured using a Surface Roughness Tester (Mahr S2, Mahr GmbH, Göttingen, Germany) ($n = 6$). Only half of the metal sample was coated with polymer while the other half was left uncoated. The probe of the Tester moved against the surface from the uncoated side to the polymer-coated side. The change in the probe's altitude was used to determine the thickness of the coating. The contact angle was then measured ($n = 4$) to assess the wettability of the surfaces. Deionized water (5 μL) was placed on the coating, and the contact angle was recorded using a FACE Contact Angle Meter (Kyowa Interface Science, Japan).

2.3. Swelling ratio

Dry 1 cm × 1 cm coated sample plates were weighed, and their weights were recorded (W_0) ($n = 5$). They were placed in a beaker filled with 100 mL of phosphate buffered saline (PBS, pH = 7.4). At different sampling times for up to 60 min, the plates were taken out of the beaker. The excess water on the plates was removed by gently tapping the plates on low-lint tissue (Kimwipes, Kimberly-Clark, WI) before their final weights were recorded (W_f). The polymer coating on each plate was carefully removed by a pair of tweezers, and the weight of the bare metal was measured (W_{Ti}). The swelling ratios of the coatings were calculated as $[(W_f - W_{Ti}) - (W_0 - W_{Ti})/(W_0 - W_{Ti})]$.

2.4. Drug release efficacy

The model drug used to test the release efficacies of the coatings was pentoxifylline (PTX, $C_{13}H_{18}N_4O_3$, Sigma, MO). PTX has been used clinically to reduce inflammation and fibroblast proliferation and subsequent fibrosis (Berman B, 1989).

Each coated sample (5 cm × 5 cm) ($n = 6$) was submerged in 7 mL of 2 mg/mL PTX solution and placed on a shaker (50 rpm). After 48 h, the absorbance of the solution was read at 274 nm (μQuant microplate reader, Biotech, USA), and the concentration of PTX, C_f (mg/mL), was determined using a standard curve. The amount of PTX loaded in each coating was W_L (mg) = 2 (mg/mL) × 7 (mL) − $C_f V_f$. The final volume of the PTX solution was V_f (mL). Each PTX-loaded sample was then placed in 10 mL PBS. At different sampling times, 100 μL of the PBS was removed, and the absorbance was read to calculate the amount of PTX in the solution at that time (W_t (mg)). The amount of PTX released was divided by the total amount of PTX loaded into the coating to obtain the release efficacy (W_t/W_L).

2.5. Cell culture

Osteoblast cells (7F2, CRL-12557, ATCC, VA) were cultured in α-MEM (Invitrogen, Carlsbad, CA), supplemented with 10% (v/v) fetal bovine serum + 100 IU/ml penicillin + 100 μg/ml streptomycin + 1 mM sodium pyruvate (all cell culture reagents were purchased from Invitrogen), and maintained in a CO_2 incubator (5% CO_2, 95% humidity, 37 °C; Astec, Fukuoka, Japan).