Controlled release of BMP-2 by chitosan/γ-PGA polyelectrolyte multilayers coating on titanium alloy promotes osteogenic differentiation in rat bone-marrow mesenchymal stem cells

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A B S T R A C T

The excellent biocompatibility and mechanical properties of titanium alloys have led to their wide-scale application in orthopedic implants. Appropriate surface treatment of these alloys can greatly improve cellular attachment as well as interactions between implants and tissue. This paper reports on the preparation of polyelectrolyte multilayers (PEM) coating comprising chitosan and gamma-poly(glutamic acid) on a Ti6Al4V substrate via spin-coating. Bone morphogenetic protein 2 was then loaded directly into the coating. Following sample preparation, we performed in vitro studies using SD rat bone-marrow mesenchymal stem cells (rBMSCs) to investigate the biological effects of the coating. Our results demonstrate that this chitosan/γ-PGA PEM coating is highly stable and does not have any adverse effects on rBMSCs. The proposed coating proved highly effective in carrying and enabling the gradual release of BMP-2. At day 14, the coated samples presented osteogenic differentiation far exceeding that of the non-coated samples. After co-culturing for 21 days, the calcium content in the PEM coating group was double that of the non-coated group, which is a clear indication of enhanced cellular mineralization.

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1. Introduction

Many large bony defects caused by trauma, aging, or exercise-related injuries are resistant to self-healing, and therefore require grafts and implants to be restored. Due to their excellent mechanical properties, metals are considered particularly effective implant materials. However, poor cellular attachment and osteo-integration can cause metal implants to fail. To develop a successful implant, one must consider not only physical and chemical properties, but surface characteristics as well. Indeed, due to the fact that these devices come into direct contact with human tissue and interact with cells, surface properties are considered a critical issue in the development of medical implants [1,2].

Pure titanium and titanium alloys are widely used in orthopedic implants. When these materials are oxidized, a thin layer of compact titanium oxide forms on the surface, which provides a barrier against corrosion and helps prevent the leakage of heavy metals from the interior of the device [3–5]. A variety of surface treatment methods have been developed to enhance early-stage osteoblast attachment and growth, including chemical etching, physical sand blasting, and the application of surface coatings. Surface coatings have been produced with both inorganic and organic materials. However, hydroxyapatite, which is the major inorganic constituent of human bone, is most frequently used in the coating of metal implants, as this material can enhance the integration of bone tissue [6–9]. Several types of natural and artificial polymers have also been developed for this purpose, and biological elements, such as hormones and proteins, have been combined with the coatings to achieve in situ cellular regulation [10–12].

Polyelectrolyte multilayers (PEM) were first proposed by Iler in 1966 [13]. Since that time, numerous researchers have established fundamental theories related to these materials as well as advanced methods to achieve layer-by-layer application. PEM coatings are assembled through the application of polymer layers, alternating between positively and negatively charged materials. The stability of the coating is largely determined by the static attractive force, the strength of hydrogen bonds, hydrophobic/hydrophilic characteristics, and covalent bonding [14–16]. By adjusting manufacturing processes, a number of properties can be specified, including thickness, mechanical strength, composition, and surface stability [17–21]. In the field of biomedicine, PEM coatings have also been applied in antibacterial coatings, tissue engineering, and drug delivery systems. The chemical modification of

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implant surfaces has been shown to greatly enhance osteoblast and stem cell differentiation [22–25].

Bone morphogenetic proteins (BMPs) are glycoproteins with a low molecular weight. BMPs belong to the transforming growth factor-beta (TGF-beta) superfamily, and have been shown to promote the morphogenesis of bone and cartilage tissues by inducing and modulating the growth of osteoblasts and chondrocytes. Indeed, excellent osteo-inductive properties have led to the wide clinical application of BMPs, particularly BMP-7 and BMP-2, which require only two weeks to induce the growth of osteoblasts and chondrocytes. Indeed, excellent osteomorphogenesis of bone and cartilage tissues by inducing and modulating the growth of osteoblasts and chondrocytes. Moreover, the direct injection of BMPs into the human body is associated with high dosages and medical costs. Although many researchers are working to develop carrier systems for BMPs [26], few studies have investigated on controlled release of BMP-2 using PEM [27–29]. The release profile of BMP-2 depends largely on the constituents of the PEM, the preparation process, and the immersion environment.

Chitin is a natural abundant polymer found in crustaceans, insects, and fungus. Pretreated and purified chitosan is widely used in biomedicine as a drug carrier, tissue engineering scaffold, and dressing for wounds. Gamma-poly(glutamic acid) (γ-PGA) is a natural polypeptide polymer that is also widely used in biomedical applications [30]. This polymer was first observed in the cellular wall of Bacillus anthracis. Presently however, most commercially-available γ-PGA is produced through the fermentation of Bacillus subtilis. Chitosan possesses a positive charge and γ-PGA possesses a negative charge. BMP-2 is positively charged and therefore able to be immobilized on negatively charged polymers. One previous report used a composite hydrogel of gamma-poly(lactic acid) and chitosan to obtain the CT (cycle of threshold) value of each gene. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 5′–3′), was used as an internal standard. Table 1 lists the primer sequences that were used in rBMSCs gene expression tests. To enable a comparison of differences in gene expression between cells cultured on Ti-PEM and Ti, we calculated the minus delta-delta-CT (−ΔΔCT), as follows:

\[ \Delta CT = CT \text{ of target gene} - CT \text{ of reference gene} \]

\[ -\Delta \Delta CT = -[\Delta CT \text{ of } (\text{Ti-PEM}) - \Delta CT \text{ of } \text{Ti}] \]

ANOVA was conducted in conjunction with the Scheffe test to compare the significance of differences among groups. P values of <0.05 were considered statistically significant.

3. Results and discussion

Fig. 2 presents surface and cross-sectional micrographs of the chitosan/γ-PGA PEM coating. The thickness of the PEM was 5–8 μm, and the surface presented a neat appearance with roughness of <1 μm. Fig. 3 presents the results of PEM degradation tests, whereby 50% of the PEM remained on the substrate after immersion in SBF at

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>GAPDH</td>
<td>GAAAGCTTCTGGCTGATGG</td>
<td>GTAGGCCATGGCTTACCA</td>
</tr>
<tr>
<td>Runx-2</td>
<td>AACCACCCGATCCTGACTCA</td>
<td>CTCCATGAGCGTCAACACA</td>
</tr>
<tr>
<td>COL1</td>
<td>ACGGCAGCTGCTTACTGG</td>
<td>GCCCTGAGCTTACGACG</td>
</tr>
<tr>
<td>OCN</td>
<td>ACTGAGCTGCTTACTGG</td>
<td>AATGTCTGCTTACTGG</td>
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<tr>
<td>PicoGreen®</td>
<td>PicoGreen®</td>
<td>PicoGreen®</td>
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Fig. 1. Composition of polyelectrolyte multilayers coating on Ti6Al4V substrate.
37 °C for a period of 21 days. No morphological differences were observed between samples loaded with BMP-2 and samples which did not contain BMP-2; i.e., the degree and rate of degradation in both samples were the same. The PEM coating would undergo complete dissolution within a period of 45 days \textit{ex vivo}. The release of BMP-2 from PEM was tested using a BMP-2 ELISA Kit based on an antibody specific for human BMP-2. Fig. 4 presents the burst release profile of BMP-2 from the PEM coating, whereby approximately 50% of the loaded BMP-2 was released within the first 3 h. During this short period, the PEM film remained stable and the BMP-2 underwent rapid diffusion from the coating. After 3 h, the rate of release slowed; however, >90% of the loaded BMP-2 was released within 48 h, during which time the PEM film underwent partial degradation. All of the BMP-2 was released within 5 days. The isoelectric point of BMP-2 is 8.5; therefore, it would carry a positive charge in a buffer with a pH of 7.4. We applied negatively charged \( \gamma \)-PGA as the outermost layer to take advantage of the static attractive force in moderating the release of BMP-2. This study achieved the sustained release of BMP-2 from the PEM surface coating at a concentration suitable for enhanced bone restoration [12,22].

Fig. 5 presents results of MTT assays of rBMSCs cultured for 4, 7, and 14 days. On days 4 and 7, no obvious difference in viability was observed between cells grown on Ti and cells grown on Ti-PEM. On day 14 however, the viability of cells on Ti appears to have increased, whereas the viability of cells on Ti-PEM decreased. The rBMSCs attached to the surface of the bare Ti6Al4V substrate continued proliferating for 14 days, which demonstrates the remarkable biocompatibility of the Ti6Al4V substrate. The presence of PEM did not appear to affect cell viability within one week. Fig. 6 (A), (B), and (C) presents fluorescence micrographs of cells on Ti and Ti-PEM after cultivation for 4, 7, and 14 days. These images correspond to the results of MTT tests. Overall, these findings indicate that the PEM coating contains small ridges and grooves which provide additional surface area for cell attachment. Fig. 7 summarizes the ALP activity of rBMSCs on Ti and Ti-PEM after cultivation for 7, 14, and 21 days. Higher ALP activity is an indication of improved osteodifferentiation by stem cells [11]. Very little difference in ALP activity was observed between the samples on day 7; however, on day 14, we observed a pronounced increase in the ALP activity of cells cultured on Ti-PEM. The use of PEM to carry BMP-2 was shown to promote the differentiation of rBMSCs to bone tissue.

By day 21, the differentiated cells had matured sufficiently to begin subsequent stages of differentiation, as indicated by a drop in ALP activity. Mature osteocytes trigger a process of bio-mineralization resulting in the precipitation of calcium phosphate in the form of apatite. Fig. 8 presents images of cells stained using alizarin red s after being cultured...
for 21 days. Differentiated rBMSCs on both Ti and Ti-PEM produced apatite precipitate; however, cells stimulated by BMP-2 (cultured on Ti-PEM) produced a greater quantity of apatite. Furthermore, through calcium quantification, rBMSCs on Ti-PEM precipitated a higher concentration of calcium than did those on Ti (Fig. 9). This effect was particularly pronounced on day 14, when the difference was as high as 7×. Runx2, COL1 and OCN genes are closely relative to the process of mesenchymal stem cells differentiation into osteocyte. rBMSCs cultured on Ti-PEM had significantly higher expression of these important genes than on Ti for 14 and 21 days (Fig. 10). However, even cells cultured on Ti had begun the differentiation process by day 21, such that the differences became less pronounced. These results demonstrate that loading BMP-2 within a chitosan/γ-PGA PEM coating on Ti6Al4V substrate is effective in promoting the early stage differentiation of mesenchymal stem cells.

Fig. 6. Fluorescence image of cells stained with Hochest33258 after being cultured for (A) 4 days, (B) 7 days, and (C) 14 days (100×).

Fig. 7. ALP activity of rBMSCs after cultivation for 7 days, 14 days, and 21 days (P < 0.05 is considered statistically significant).
4. Conclusions

This paper reports on the preparation of chitosan/\(\gamma\)-PGA polyelectrolyte multilayers coating on Ti6Al4V substrate for use as a sustained carrier of BMP-2. The proposed method involves spin coating 30 layers of polymer (alternating between positively and negatively charged materials) to a Ti6Al4V substrate, with negatively charged \(\gamma\)-PGA as the outermost layer. This coating was then loaded with bone morphogenetic protein 2. The resulting film measured 5–8 \(\mu\)m and required >45 days to completely decompose ex vivo. An initial burst release comprising 50% of the loaded BMP-2 occurred within the first 3 h, whereupon the release of BMP-2 continued at a slower rate over a period of 5 days. In vitro investigations using rBMSCs demonstrated that the coating did not affect the biocompatibility of the Ti6Al4V substrate. The results of alkaline phosphatase activity tests, calcium quantification, and gene expression further revealed that the release of BMP-2 from PEM triggered differentiation in rBMSCs within a few days. These findings demonstrate that the chitosan/\(\gamma\)-PGA PEM coating is effective in carrying and gradually releasing BMP-2 in order to trigger the early stage differentiation of mesenchymal stem cells.

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