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Growth Factor Release from Polyelectrolyte-Coated Titanium for Implant Applications

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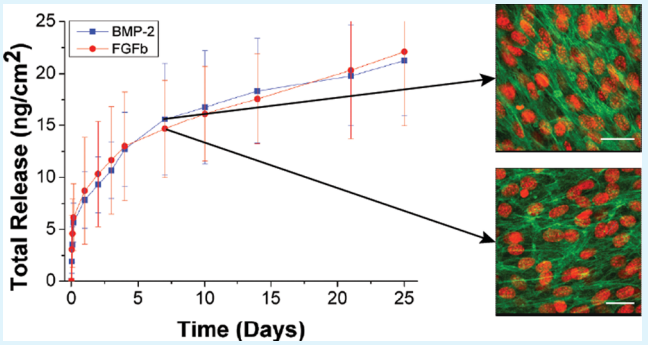
ABSTRACT: Polyelectrolyte multilayer coatings based on poly(methacrylic acid) and poly-L-histidine were formed on anodized titanium surfaces with adsorbed bone morphogenetic protein 2 (BMP-2) or basic fibroblast growth factor (FGFb). These coatings are proposed for use on titanium implanted devices. Coatings were capable of sustained release of growth factor over 25 days, with BMP-2 and FGFb exhibiting approximately identical release profiles. Cell culture on growth factor-eluting surfaces was more effective for preosteoblasts on BMP-2-eluting surfaces than for fibroblasts on FGFb-eluting surfaces. Cell counts at all time points on BMP-2-eluting surfaces were significantly higher than for those on anodized titanium or polyelectrolyte surfaces that did not contain BMP-2. Alkaline phosphatase levels were significantly higher after 21 days on BMP-2-eluting surfaces, indicating increased bone growth.



KEYWORDS: *polyelectrolytes, biomaterials, titanium implants, controlled release, bone morphogenetic protein 2, basic fibroblast growth factor*

INTRODUCTION

Interactions at the interface between the body and an implanted device are essential to the success or failure of the device. As stents, bone scaffolds, hip and other implants become more widely used thanks to longer lifespans in the developed world, it is essential to improve the understanding of biointerfaces and to tailor the interface for a given application. Titanium is a popular choice for implant material given its strength, durability and biocompatibility; however, strong coupling with the surrounding tissue is not achieved, resulting in stress shielding and implant loosening. Interfacial failure can be caused by a combination of insufficient osseointegration of the implant and stress shielding due to the stiffness mismatch between implant and bone.[1,2] Bacterial adsorption on the implant surface and the resulting biofilm formation are also significant causes of implant failure and bacterial infection.[3,4]

If the implanted device surface is incompatible, the device will be rejected. Even if the surface is not incompatible, there can be limited integration of the implant within the body as a result of limited adhesion and growth of desired cells, either as a result of poor surface properties or adhesion of bacteria and other undesired cells and proteins.[5] Significant strides have been made over the past two decades in developing improved biointerfaces.[6−9] However, much is still unknown given the complexity of interactions at the interface and the many parameters (including topography, molecular design, biofouling, cell-material interactions and inter- and intramolecular forces) that control interactions at the interface.

Polyelectrolytes (PEs) are polymers with positively (polycation) or negatively (polyanion) charged repeat units. Weak PEs are those where there is an equilibrium between charged and uncharged side groups near neutral pH. When weak polycations and/or weak polyanions are deposited on a surface in an alternating fashion, electrostatic interactions form a pH-sensitive film. PE films and microcapsules have been used for a variety of applications, including drug delivery,[10−12] microreactors for synthesis of difficult to achieve crystalline nanomaterials[13,14] and encapsulation of corrosion inhibitors for self-healing coatings.[15,16] The properties of a PE film are dependent upon many processing parameters including PE

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pair, molecular weight[17−19] and number of layers.[20] Therefore, the properties of a PEM can, in principle, be tailored precisely for a given application. In addition to PE films, microcapsules based on PEs have been used for controlled release for biological applications.[12,21,22]

Given the biocompatibility of many PEs, PE coatings have been used to improve the biocompatibility of implanted devices. Tryoen-Tóth et al. demonstrated that PE coatings terminating in poly(sodium 4-styenesulfonate) (PSS), poly(L-glutamic acid) (PGA) and poly(L-lysine) (PLL) show good biocompatibility for osteoblast-like cells.[23] Thin films of chitosan, PGA and PLL were used by Park et al. to adjust the surface wettability of titanium. It was found that increased wettability could enhance osteoblast activity, but this enhancement is dependent on titanium microtopography.[24] A PE coating of hyaluronic acid (HA) and chitosan was developed by Chua et al. to confer antibacterial properties.[25] When arginine-glycine-aspartic acid was immobilized on this coating, osteoblast adhesion was also significantly improved as compared to pristine titanium.[26] Improved implant anchoring in rats was observed on titanium allow implants coated in either chitosan/gelatin or chitosan/HA.[27] Additionally, Brunot et al. reported enhanced fibroblast activity on titanium coated with PSS/poly(allylamine hydrochloride) (PAH) multilayers.[28] Other PE coatings that have been shown to improve cell adhesion include chitosan/heparin[29] and protamine sulfate/PSS.[30] A number of recent reviews highlight the ability of polyelectrolyte multilayers to control cellular function.[31−33]

PE films have also been used on implant surfaces for the controlled release of biologically relevant molecules such as drugs and growth factors. Macdonald et al. demonstrated the coating of a polymer scaffold with LbL-deposited poly(β-aminoester) and chondroitin sulfate, a complex capable of delivering microgram scale amounts of bone morphogenetic protein 2 (BMP-2).[34] Cross-linked PLL/HA coatings on a porous ceramic also showed microgram level release of BMP-2 from porous ceramic scaffolds.[35] However, over 60% of release was observed in the first day. Subsequent studies of the cross-linked PLL/HA coating on titanium surfaces showed cross-link density dependent release of BMP-2 as well as long-term stability (>1 year) when stored at 4 °C.[36]

The goal of this work is the controlled long-term release of growth factors from PE coatings so as to improve osseointegration and durability of titanium implants. Morphogens are biomolecules that act as a spatial regulator and can dictate cell behavior and tissue development through concentration gradients. Growth factors play key roles in regulating osteoblast behavior and osteoid and bone formation. As such, they were selected as molecules of interest for encapsulation and controlled release.[37,38]

BMP-2 is a key player in osteoblast behavior.[39−41] However, in vivo, osteoblasts are not the only cells relevant to patient recovery following the implantation of a medical device. For example, fibroblasts are essential for the formation of fibrous tissue, which often must be reformed following implantation. Interestingly, Hughes-Fulford and Li found that FGFb is capable of inducing greater osteoblast proliferation than BMP-2, while BMP-2 plays a critical role in stimulating mineralization.[42] Other studies have confirmed BMP-2's role in enhancing differentiation and extracellular matrix mineralization.[43−45] Therefore, the release of BMP-2 as well as basic fibroblast growth factor (FGFb) from PE coatings was evaluated.

Previously, we demonstrated microgram levels of release of a model polypeptide from a PE coating.[18] In this work, that coating is further characterized and the release of BMP-2 and FGFb is measured. We also evaluate the performance of the model polypeptide by comparing its release behavior to those of BMP-2 and FGFb. Additionally, preosteoblasts and fibroblasts were cultured on growth factor-containing coated titanium to determine the effect of these levels of growth factor release on cell proliferation and differentiation.

EXPERIMENTAL SECTION

Materials. Titanium foil (99.5% Ti) was acquired from Alfa Aesar. Poly(methacrylic acid sodium salt) (PMAA, Mn ≈ 5400, PDI = 1.8), poly-L-histidine hydrochloride (PH, molecular weight ≥ 5000), phosphine buffered saline (PBS, pH = 7.4), α-modified Eagle's medium (α -MEM), Dulbecco's modified Eagles medium (DMEM), gentamicin, ascorbic acid, glucose, bovine sera albumin (BSA), Triton X-100 and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma-Aldrich. Fetal calf sera and calf sera were obtained from PAA. A 4% paraformaldehyde solution was obtained from BOSTER Biological Technology Ltd. Pronase and measuring buffer for cell counting Casitron were purchased from Roche Diagnostics. The stains Alexa Fluor 488 and TO-PRO-3 were purchased from Invitrogen. Recombinant human bone morphogenetic protein 2 (BMP-2), recombinant human basic fibroblast growth factor (FGFb), the respective enzyme linked immune sorbent assay (ELISA) development kits and the ELISA buffer kit were acquired from Peprotech. The alkaline phosphatase (ALP) test kit was purchased from Rolf Greiner Biochemica GmbH. MC3T3-E1 preosteoblast cells were a gift from Ludwig-Boltzmann Institute of Osteology, Vienna, and NIH3T3 fibroblast cells were purchased from ATCC.

Titanium Preparation. Titanium foil was cleaned in 1.5 M sulfuric acid and then rinsed in deionized water, ethanol, acetone and again in water. Anodization took place in 165 g L^{-1} sulfuric acid at a potential of 30 V for 5 min. Anodization under these conditions results in a porous oxide structure with pores ranging in size from 40 to 200 nm in diameter.[18]

PE Coating Preparation. Coating of titanium foil with the PE was achieved by first immersing the anodized titanium specimen in a 0.1 mg mL^{-1} solution of growth factor (either BMP-2 or FGFb) in water for 15 min. The PE coating was then formed on top of this adsorbed layer by immersing the plate in a 1 mg mL^{-1} PMAA in water solution for 15 min and then in a solution of 1 mg mL^{-1} PH in water for 15 min. Specimens were washed three times in water between each adsorption step to remove weakly adsorbed material. Alternating layers of PMAA and PH were formed until 10 layers (five bilayers) were achieved. This specimen is denoted as $(PMAA/PH)_5$-BMP-2 for the BMP-2-containing coating or as $(PMAA/PH)_5$-FGFb for the FGFb-containing coating. As a control for cell culture, PE coatings without growth factor were also prepared. For these coatings, PH was adsorbed to anodized titanium as the first step, then five bilayers of PMAA/PH were formed on top of this PH layer. These control specimens are denoted as $(PH/PMAA)_{5.5}$. PMAA and PH have been shown previously to be biocompatible.[46,47]

Quartz Crystal Microbalance Analysis. Coatings were characterized using scanning electron microscopy (SEM) and quartz crystal microbalance (QCM) analysis. QCM analysis was performed using a Q-Sense E4 on titanium sensors at a flow rate of 50 μL min^{-1} and a temperature of 22 °C.

Growth Factor Release. To evaluate the release of BMP-2 and FGFb from $(PMAA/PH)_5$ coatings, specimens were immersed in PBS. Five specimens were monitored per condition. One milliliter aliquots were taken from the solutions regularly, with the aliquot volume replaced with fresh PBS. After the release study was completed, coatings were removed from the anodized titanium substrates to determine the amount of growth factor that remained in the coatings. Aliquots were promptly frozen at −20 °C. The amount of growth factor released was quantified using enzyme linked immune sorbent

assay (ELISA). ELISA was performed in accordance with the instructions provided with the respective Peprotech ELISA development kits. Aliquots from the release studies were thawed and returned to room temperature immediately prior to their use in the assay.

Cell Culture, Cell Counting and Staining. MC3T3-E1 preosteoblast and NIH3T3 fibroblast cell lines were used to evaluate the biocompatibility of coatings. MC3T3-E1 cells were cultured on $(PMAA/PH)_5$-BMP-2, $(PH/PMAA)_{5.5}$ and anodized titanium surfaces, whereas NIH3T3 cells were cultured on $(PMAA/PH)_5$-FGFb, $(PH/PMAA)_{5.5}$ and anodized titanium surfaces. $(PH/PMAA)_{5.5}$ and anodized titanium act as controls for both cell line experiments. Titanium specimens were sterilized using UV light, and each specimen was placed in one well of a six well plate. MC3T3-E1 cells were cultured in α-MEM with 4.5 g L^{-1} glucose, 10 vol % fetal calf sera, 10 μg mL^{-1} Gentamicin and 50 μg mL^{-1} ascorbic acid. NIH3T3 cells were maintained in DMEM with 4.5 g L^{-1} glucose, 10 vol % calf sera and 10 μg mL^{-1} Gentamicin. Approximately 5.76×10^4 cells per well (6×10^3 cells cm^{-2}) were suspended in culture medium, dispersed over the specimen and cultured for 3, 5, 7, 14, or 21 days in an incubator (Binder) at 37 °C in a humidified atmosphere containing 5% CO_2. Fresh medium was given every second or third day of culture.

Cell counting after 3, 5, and 7 days of culture was performed with a Casy Model TT cell counter (Casy Technologies, Roche Diagnostics). The titanium specimens with cells were first placed in new six well plates to avoid counting cells on the surface of the cell culture wells. Cells on the specimen were detached with 500 μL Pronase-EDTA solution in PBS. The detached cells were transferred to a 1.5 mL microcentrifuge tubes. The specimens were washed three times with 250 μL PBS. This PBS was also transferred to the microcentrifuge tubes. Cell/Pronase-EDTA/PBS suspensions were then centrifuged at 650 g for 10 min. The supernatant was removed and cells were redispersed in 200 μL PBS. Subsequently, 10 mL of the Casitron solution and 50 μL of the cell suspension were mixed. The number of cells in the resulting liquid was determined with a CASY Model TT cell counter. Cell counts were performed on three replicates per specimen and three specimens per condition.

Cells cultured for 7 days were also stained and imaged with confocal fluorescence microscopy. Cells were fixed for 15 min in 4% paraformaldehyde, washed in PBS, permeabilized with 0.1% Triton X-100 at 4 °C for 15 min and washed three times in PBS. NIH3T3 cells were additionally blocked in 3% BSA for 30 min. F-actin was stained with Alexa Fluor 488 and cell nuclei were stained with TO-PRO-3. Confocal micrographs were obtained with a Leica TCS SP confocal scanning system (Leica) with a 100× oil immersion objective (numerical aperture 1.4).

Alkaline Phosphatase Enzyme Activity. Preosteoblast differentiation was characterized with alkaline phosphatase (ALP) enzyme activity after 1, 2 and 3 weeks. The cell-seeded titanium surfaces were placed into new wells, washed with PBS, air-dried under laminar airflow for 30 min and frozen at −20 °C for 1 h. After the cells were freezed, they were lysed with a 0.5% Triton X-100 solution for 20 min at room temperature. Then, 8 or 4 μL of the lysed product was added to 200 μL of the ALP enzyme working reagent (ALP Test Kit DiaSys Diagnostic Systems, Germany). The ALP enzyme working reagent was incubating at 37 °C prior to mixing with the lysed product. Immediately after the ALP enzyme working reagent was mixed with the lysed product, the absorbance of these samples was measured at 405 nm at 37 °C for 10 min using a plate reader. Three specimens were measured for each condition at every time point, and the absorbance of these specimens was measured in triplicate. Results are expressed in units of U L^{-1} and are normalized to a specimen surface area of 1 cm^2.

RESULTS AND DISCUSSION

Polyelectrolyte Coating Formation. $(PMAA/PH)_5$ coatings were formed on BMP-2 or FGFb adsorbed to the anodized titanium surface. The build-up of $(PMAA/PH)_5$ was the same with both growth factors. Mass adsorption of $(PMAA/PH)_5$ is given in Figure 1. The amount of PMAA and PH deposited per

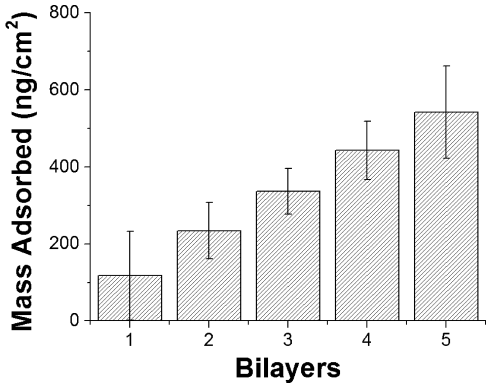

Figure 1. Adsorbed mass amounts from quartz crystal microbalance analysis of the build-up of polyelectrolyte multilayer coatings.

step is fairly constant. Here, 30 ± 9 ng cm^{-2} PMAA is deposited per step, whereas 79 ± 13 ng cm^{-2} PH is deposited per alternating step. In total, this results in approximately 540 ng cm^{-2} of PE coating on top of the growth factor. Based on QCM, the amount of BMP-2 that can be deposited on a titanium surface is approximately 800 ng cm^{-2}, 550 ng cm^{-2} for FGFb.

Growth Factor Release. BMP-2 and FGFb release results are given in Figure 2a. The release profiles are almost identical for BMP-2 and FGFb. Figure 2b shows the percentage of total growth factor released at each time point. Here, 29% of BMP-2 and 31% of FGFb release occurs in the first day, but the release rate decreases substantially after that. Thus, 58% of BMP-2 and 53% of FGFb release is achieved after 1 week. After 25 days, 79% of BMP-2 is released as compared to 80% of FGFb over the same time. In total, 27 ± 4 ng cm^{-2} BMP-2 or 28 ± 8 ng cm^{-2} FGFb can be contained in the PE coating. Sustained release of growth factor entrapped within the PE coating on the titanium surface was achieved.

The PE multilayer coatings formed on the surfaces are relatively thin (14 nm of PE, 35 nm for BMP-2-containing coatings, 34 nm for FGFb-containing coatings). In fact, the mass of growth factor that is deposited is greater than the amount of PE that is covering it, according to QCM. This makes it particularly surprising that extended release can be achieved from these coatings. However, there does appear to be a mismatch between the amount of growth factor deposited on the anodized titanium according to QCM and the amount of growth factor that can be recovered from the coating after deposition of PE layers. The difference is an order of magnitude and could be related to continued desorption of either BMP-2 or FGFb during the deposition and washing steps for the formation of the PE multilayer coating. Alternatively, the QCM sensor surface could differ significantly from the anodized titanium surfaces used for release studies and cell culture.

Release rates of BMP-2 and FGFb are fairly constant after day 7. In comparing release of BMP-2 or FGFb with PL-FITC, the model compound used in previous work,[18] it can be seen that the release profile is quite similar. Figure 3 shows percentage release over 25 days for BMP-2, FGFb and PL-FITC. PL-FITC tracks closely with the growth factors for the first few days but demonstrates a lower relative amount of polypeptide release after the initial burst. The greatest difference in release can be seen in the absolute amount of material release. Approximately 400 ng cm^{-2} PL-FITC can be released over 25 days, which far exceeds the amount of BMP-2

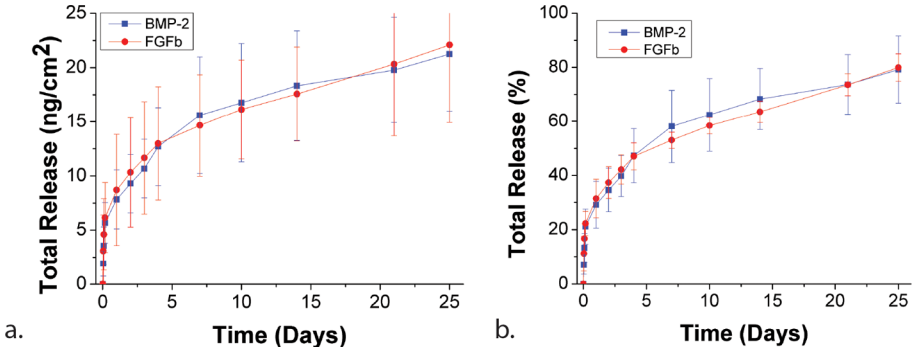

Figure 2. Release of bone morphogenetic factor 2 (BMP-2) and basic fibroblast growth factor (FGFb) from polyelectrolyte multilayer coatings. a. Amount of release was determined using ELISA. b. Percent release of BMP-2 and FGFb from polyelectrolyte multilayer coatings. Error bars represent standard deviation, n = 5, for each data point.

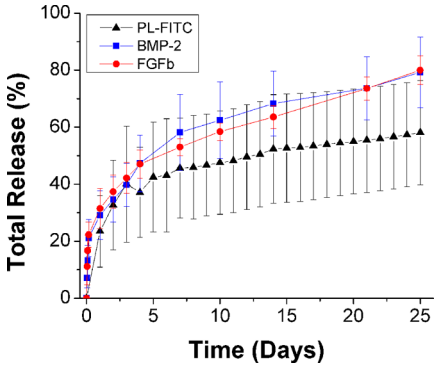

Figure 3. Comparison of release profiles of BMP-2 and FGFb to FITC-labeled poly L-lysine (PL-FITC). PL-FITC release data from Peterson et al.[18] Error bars represent standard deviation, n = 5, for each data point.

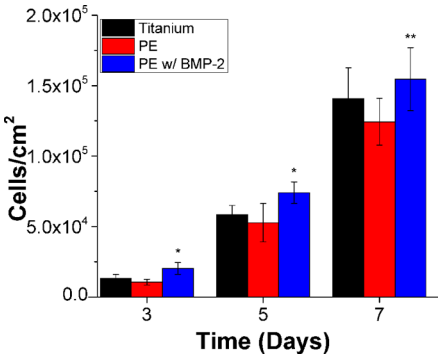

Figure 4. Cell counts for MC3T3-E1 preosteoblast cells seeded on different surfaces. Initial cell seeding of 6×10^3 cells cm^{-2}. Error bars represent standard deviation. Three samples were tested per condition and time point, two measurements were conducted per sample. * indicates $p < 0.05$, ** indicates $p < 0.10$.

or FGFb that can be released. These results suggest that, in terms of percentage release, PL-FITC appears to be a fair analogue. However, PL-FITC is not a great model for BMP-2 or FGFb, in terms of total release. The total amount of PL-FITC release is far greater, which results from the greater initial loading, whereas the percentage release relates more to how the molecule is released from the coating. PL-FITC was selected because of its similar molecular weight, since it was postulated previously that release from these coatings was primarily diffusion-controlled. Additional information about the pH dependent release behavior of $(PMAA/PH)_5$ can be found in ref 18.

Comparing the properties of BMP-2 and FGFb can perhaps elucidate the release mechanism from $(PMAA/PH)_5$ coatings. BMP-2 has higher molecular weight (26 vs 17.2 kDa). Although both proteins contain beta sheets, FGFb is composed entirely of a beta-sheet structure, whereas BMP-2 has a cysteine-knot motif and two double-stranded beta sheets.[48,49] The isoelectric points of BMP-2 and FGFb (pH = 8.5 and 9.2) are in the same range, although small differences in release profiles should still be apparent if release is controlled primarily by electrostatics due to the interactions with the PE multilayers. The effectively identical release profiles indicate that release is diffusion-controlled.

Cell Proliferation and Differentiation. Cell counts for preosteoblasts on titanium, PE coated titanium and BMP-2 containing PE coated titanium are shown in Figure 4. The coating containing BMP-2 demonstrated higher cell counts than the control surfaces ($p < 0.05$ for days 3 and 5, $p < 0.10$ for day 7). Preosteoblast activity for the first week of culture focuses on replication. By the ninth day of culture, growth decreases as the cells begin to exhibit osteoblast functions including production of alkaline phosphatase and deposition of an extracellular matrix. Mineralization of this extracellular matrix begins 16 days after culture.[50] Preosteoblast culture results are very promising for application of the described surface modification for titanium implants. Cell proliferation was highest on BMP-2-eluting surfaces at all times. This effect is less apparent by day 7.

Because BMP-2 regulates differentiation rather than proliferation,[42,44] the increase in cell proliferation is caused by other changes in the environment. Surface roughness of dry specimens prior to cell culture was characterized using atomic force microscopy (AFM). PE coatings have RMS roughness of 60 ± 11 nm, as compared to 78 ± 10 nm for BMP-2-eluting PE coatings. However, roughness is not the whole story, as the smoother titanium surfaces (54 ± 4 nm) perform better than the PE coatings. It appears that the chemistry of the PE coatings may somewhat inhibit cell proliferation, whereas the increased roughness of the BMP-2 eluting coatings allows for this less desirable chemistry to be overcome.

Cell counts for fibroblasts on FGFb-containing PE coated titanium as well as the two controls are shown in Figure 5. Titanium specimens perform consistently better, with higher cell counts for all time points. This behavior is not statistically significant at a threshold of $p < 0.10$ for days 5 and 7 but is statistically significant ($p < 0.05$) for day 3.

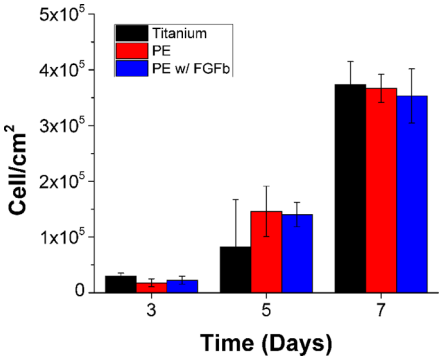

Figure 5. Cell counts for NIH3T3 fibroblast cells seeded on different surfaces. Initial cell seeding of 6×10^3 cells cm^{-2}. Error bars represent standard deviation. Three samples were tested per condition and time point, two measurements were conducted per sample.

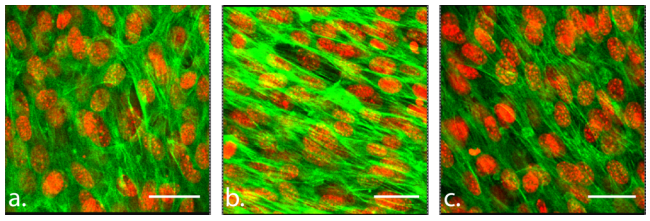

Figure 6. Fluorescent confocal laser scanning microscopy of stained MC3T3-E1 cells after 7 days on a. anodized titanium b. polyelectrolyte coating c. polyelectrolyte coating containing BMP-2. Scale bars represent 50 μm.

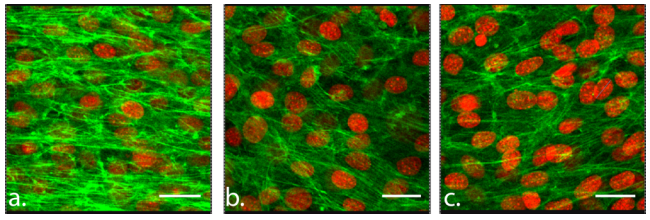

Figure 7. Fluorescent confocal laser scanning microscopy of stained NIH3T3 cells after 7 days on a. anodized titanium b. polyelectrolyte coating c. polyelectrolyte coating containing FGFb. Scale bars represent 50 μm.

On day 7, cells were stained and imaged for each surface condition. Results for preosteoblasts are shown in Figure 6 and results for fibroblasts are shown in Figure 7. In all cases, cells were healthy and formed a thick cell layer after day 7.

ALP enzyme activity was used as a measure of cell differentiation for MC3T3-E1 preosteoblasts. Titanium, PE coated titanium and BMP-2 containing PE coated titanium specimens were cultured with preosteoblasts for 1, 2 and 3 weeks for ALP enzyme activity analysis. Results are shown in Figure 8. Activity after 1 week is in the same range for all conditions. After 2 and 3 weeks, ALP activity is lowest for anodized titanium specimens and highest for PE coatings containing BMP-2. The enhanced ALP activity on BMP-2-eluting surfaces is statistically significant ($p < 0.05$) for the 3 week time point. This indicates that the amount of BMP-2 eluted was appropriate for enhancing cell differentiation. The results for BMP-2-eluting surfaces are very promising in terms of cell propagation and differentiation. These coatings should result in improved osseointegration of an implanted titanium device.

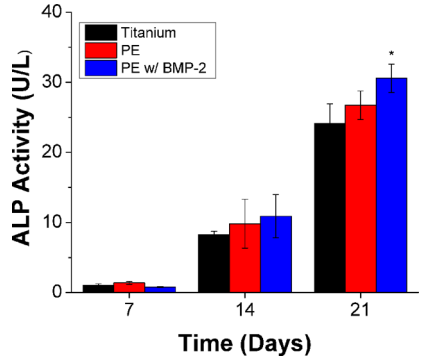

Figure 8. ALP activity after 1, 2 and 3 weeks for MC3T3-E1 cells cultured on different surfaces. ALP activity is normalized to 1 cm^2 of specimen surface area. Error bars represent standard deviation. Three samples were tested in triplicate per condition and time point. * indicates $p < 0.05$.

No effect on fibroblast cell count was observed with FGFb-containing coatings. This could indicate that an insufficient amount of FGFb was released to increase cell proliferation. Alternatively, the PE coating and/or anodized titanium may not be ideal for adhesion of fibroblasts. Brunot et al. previously observed that fibroblast viability was greater on titanium surfaces than on polyelectrolyte multilayer surfaces.[28] Hallab et al. showed that metal surfaces exhibited enhanced fibroblast proliferation and adhesion as compared to polymer surfaces.[51] However, they also noted that twice as much extracellular matrix was deposited on polymer surfaces despite the lower fibroblast proliferation and adhesion. Future work will investigate FGFb-eluting coatings in greater detail.

CONCLUSIONS

PE multilayer coatings based on poly(methacrylic acid) and poly-L-histidine can be formed on anodized titanium surfaces with adsorbed bone morphogenetic protein 2 (BMP-2) or basic fibroblast growth factor (FGFb). These coatings are capable of sustained release of growth factor, with BMP-2 and FGFb exhibiting approximately identical release profiles. The release profiles of growth factors and PL-FITC, a polypeptide proposed as a model compound in a previous study, are similar, with good agreement at short times but somewhat less sustained release. PL-FITC is therefore a fair analogue for BMP-2 and FGFb.

Cell culture on growth factor-eluting surfaces is more effective for osteoblasts on BMP-2-eluting surfaces than for fibroblasts on FGFb-eluting surfaces. No effect on fibroblast cell count was observed with FGFb-containing coatings. However, osteoblast cell count and ALP activity (i.e., differentiation) is significantly increased on the surfaces releasing BMP-2.

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Notes

The authors declare no competing financial interest.

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