Biomimetic nanocoating promotes osteoblast cell adhesion on biomedical implants

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Implantation of dental and orthopaedic devices is affected by delayed or weak implant-bone integration and inadequate new bone formation. Innovative approaches have been sought to enhance implant-bone interaction to achieve rapid osseointegration. The aim of this study was to develop biomimetic polypeptide nanocoatings and to evaluate cell adhesion, proliferation, morphology, and biocompatibility of polypeptide nanocoatings on implant surfaces. A recently developed nanotechnology, i.e., electrostatic self-assembly, was applied to build polypeptide nanocoatings on implant models, i.e., stainless steel discs. Our in vitro tests using human osteoblast cells revealed that substantially more (one order magnitude higher) osteoblast cells were attached to polypeptide-coated, stainless steel discs than to uncoated discs within the first few hours of contact. The developed biomimetic nanocoatings may have great potential for dental and orthopaedic applications.

I. INTRODUCTION

Bone healing is a complicated process involving the coordination and regulation of numerous mechanical, chemical, and biological aspects at the bone-defect site. Biomedical devices have been intensively used to provide mechanical support, fill voids, or enhance biological repair of bone defects. In the case of permanent implantation, events leading to osseointegration take place at the implant-bone interface, and failure in this process may lead to a delay in implant-bone fusion and/or implant loosening. This is still a major complication in dental and orthopaedic surgeries.[1–4] Therefore, it is important to stimulate the implant-bone integration and bone healing process and to rapidly stabilize the implant by creating a fast anchorage between the implant and the surrounding bone tissue.

Stainless steel, titanium, and cobalt alloys have been commonly used as dental and orthopaedic implant materials. The surfaces of these implants (e.g., dental and orthopaedics) are the sites where osseointegration occurs. Optimizing the surface properties of implants can facilitate the adhesion of bone-forming cells and thereby may promote osseointegration. Different approaches can be used for surface modification. Among them, electrostatic self-assembly nanotechnology is one of the simplest and most promising methods for preparation of nanocoatings of controlled thickness and molecular architecture.[5,6] Electrostatic self-assembly, developed in the early 1990s by Decher and co-workers,[6] has attracted great attention in recent years.[7–15] The self-assembly procedure is simple and straightforward, and therefore it has great potential for commercialization. The process simply involves repetitive sequential dipping of a substrate in solutions of oppositely charged polyelectrolytes.[6] The surface charge is reversed after each cycle, and each layer can have a thickness on the order of several nanometers. The physical basis of electrostatic self-assembly is mainly electrostatic attraction.[6] However, other forces, e.g., hydrophobic, van der Waals, and hydrogen bonding, may also play a significant role in certain systems.

Previous reports have demonstrated that cell adhesion, spreading, and proliferation are complex processes that depend not only on cell types and maturation, but also on the nature of implant surfaces with which cells interact.[16–27] In this study, we will examine the feasibility of applying an advanced surface modification approach,

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i.e., electrostatic self-assembly, to significantly change cell behavior on dental and orthopaedic implants. We will evaluate in vitro the effectiveness of polypeptide nanocoatings on stainless steel samples in promoting osteoblast cell adhesion. We will also study the biocompatibility of the modified implants.

II. MATERIALS AND METHOD

A. Sample preparation

Stainless steel plates were purchased (Small Parts Inc., Miramar, FL) and cut into discs, 10 mm in diameter and 0.25 mm in thickness. These discs were used as dental or orthopaedic implant models. All discs were thoroughly cleaned. They were ultrasonicated in 2% sodium dodecyl sulfate (SDS) solution for 30 min, washed in deionized water, and rinsed successively with ethanol-NaOH solution and deionized water. Electrostatic self-assembly was then performed using a computer-programmed dip-coating robot (Riegler & Kirstein GmbH, Potsdam, Germany) at ambient temperature. Positively and negatively charged polypeptides, i.e., poly(L-lysine) (PLL) and poly(L-glutamic acid) (PLGA), were used as coating materials. Stainless steel discs were immersed in PLL solution (1 mg/mL, pH 10.0) for 20 min and then rinsed for 3 min with glycine-NaOH solution. The discs were then immersed in PLGA solution (1 mg/mL, pH 10.0) for 20 min and rinsed with the glycine-NaOH solution for 3 min. Adsorption of one layer of PLL followed by another layer of PLGA is referred to herein as one bilayer. The procedure was repeated to achieve the desired number of bilayers (2, 5, 10, and 20) of PLL and PLGA. The formation of PLL/PLGA multilayer coatings on stainless steel discs was characterized by Fourier transform infrared (FTIR) spectroscopy under reflection mode. All of the stainless steel discs, coated or uncoated (i.e., control), were sterilized with ethylene oxide gas before they were used for cell culture studies.

B. Cell culture techniques

A human osteoblast-like cell line CRL-11372 was purchased from American Type Cell Culture (ATCC; Manassas, VA) and cultured in 1:1 Dulbecco's modified Eagle's medium (DMEM)/F-12 medium supplemented with 10% fetal bovine serum and 0.3 mg/mL antibiotic (G418) in a 75-cm^2 cell culture flask and incubated at 37 °C in a 5% CO_2 atmosphere according to the manufacturer's recommendation. At confluence, cells were detached with trypsin-ethylenediamine tetraacetic acid (EDTA) (0.25% trypsin/0.53 mM EDTA) from the culture plate surface and subcultured at different ratios for six passages. All of the stock solutions used in this study were purchased from ATCC. The stainless steel discs, coated and uncoated, were placed in a 24-well culture plate before adding the osteoblast cells (at passage 3-6) at the density of 30,000 cells/cm^2. The culture medium was changed every other day.

C. Cell adhesion

The adhesion of osteoblast cells on stainless steel discs was tested. After culturing for 1, 4, and 8 h, the discs were removed from the 24-well culture plate and rinsed twice with phosphate buffered saline (PBS). Remaining cells were then detached from the discs by two consecutive trypsinization procedures followed by centrifugation. The supernatant from the centrifuge tubes was discarded, and the remaining cell pellets were resuspended in PBS. The numbers of cells detached were determined by direct counting using a hemocytometer under a light microscope. All counting was run in triplicate. At least three samples (i.e., discs) were used for each type of sample and each time point.

D. Cell spreading and morphology

To examine the effects of implant surface on cell spreading and morphology, cells attached on the stainless steel discs were observed using scanning electron microscopy (SEM) (Hitachi S-4000; Hitachi, Tokyo, Japan). The samples were routinely prepared, dehydrated, and finally dried in the critical-point dryer.

E. Cell proliferation and cytotoxicity

Cell proliferation was observed, and related numbers of cells were counted after culturing osteoblast cells on stainless steel discs for 1, 3, and 5 days. Similar detaching and counting procedures as described in the aforementioned cell adhesion assay were used.

After detachment from the stainless steel discs by trypsinization, cells were suspended in PBS and treated with 1% Trypan blue that stained only dead cells. This technique was used to enumerate the live and dead cells detached from the stainless steel discs after they were cultured for various times. The number of dead cells as well as the total number of cells (dead cells plus live cells) was counted, and the percentage of live cells to the total number of cells was calculated.

F. Statistical analysis

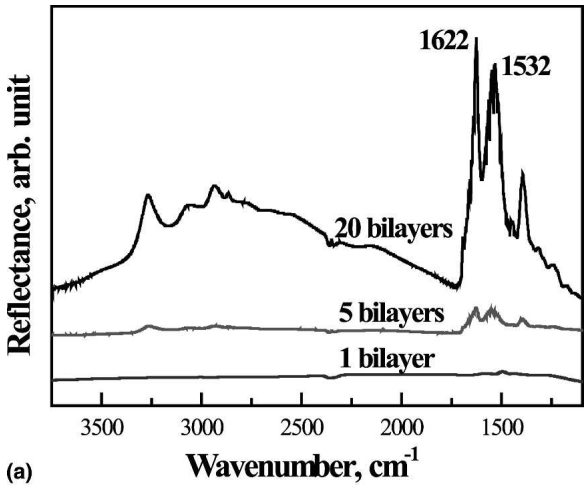
All experiments were performed with at least three samples under the same conditions. The results were reported as the mean ± SD. The Student's t test was used for statistical analysis. A p value <0.05 was considered to be statistically significant.

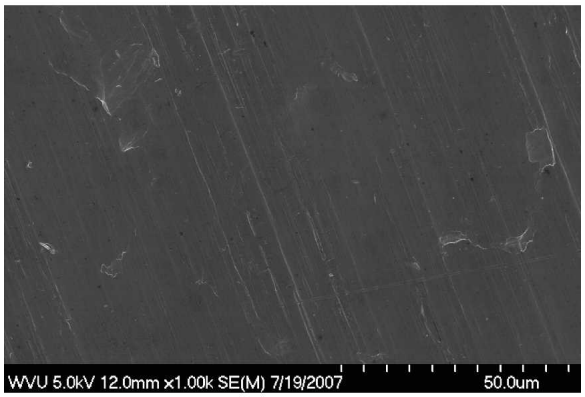
III. RESULTS

A. Polypeptide multilayer nanocoating formation

The formation of PLL/PLGA multilayer nanocoatings was examined by FTIR spectroscopy as well as SEM

(Fig. 1). Figure 1(a) shows that the absorbance intensity increased with increasing number of bilayers. The inset picture in Fig. 1(c) further confirms the formation of polypeptide coatings on stainless steel discs. The surface morphology of the uncoated stainless steel discs and those coated with a 20-bilayer polypeptide nanocoating was shown in Figs. 1(b) and 1(c), respectively. The thickness of a 20-bilayer polypeptide coating was approximately 260 nm based on ellipsometry measurements. In addition, the polypeptide coatings on stainless steel discs were found to be stable in aqueous medium (data not shown).





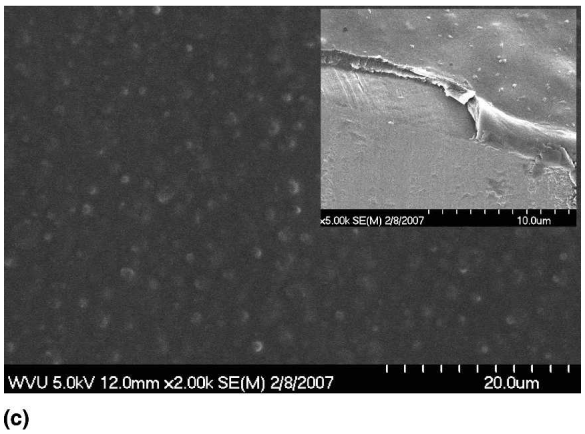


FIG. 1. (a) FTIR spectra of PLL/PLGA multilayer nanocoatings on stainless steel discs and surface morphologies of (b) a control, i.e., uncoated, stainless steel disc, and (c) a stainless steel disc coated with a 20-bilayer PLL/PLGA. The peaks in (a) are as follows: 1622 cm^{-1}, Amide I vibrate (C = O); 1532 cm^{-1}, Amide II vibrate (C = O). The inset shows the PLL/PLGA coating. No treatment except cleaning was performed on the discs. The multilayer nanocoatings were prepared at pH 10.0, and the concentrations of both PLL and PLGA were 1 mg/mL.

B. Cell adhesion and morphology

Figure 2 shows that substantially more osteoblast cells adhered on the stainless steel discs with polypeptide nanocoatings than on the control, i.e., uncoated, samples. At 4 h, one order magnitude more osteoblast cells adhered on the polypeptide-coated samples than on the control samples; 26,400 cell/cm^2 versus 2,900 cell/cm^2.

Figure 2 shows that there was a big difference in cell adhesion at the time periods studied, i.e., 4–8 h. To investigate the effects of number of polypeptide bilayers on cell adhesion, the samples were cultured for 4 h. Figure 3 shows that the number of polypeptide bilayers on the stainless steel discs also influenced cell adhesion. The number of cells adhered at 4 h increased exponentially with an increasing number of bilayers. The number of cells adhered almost doubled on the 20-bilayer samples compared to the 5-bilayer samples; 26,400 cell/cm^2 vs. 13,500 cell/cm^2, respectively.

The SEM osteoblast results are shown in Fig. 4. More cells were adhered on the one layer of PLL-coated samples than on the control and the one bilayer of PLL/PLGA-coated samples. It also seems that the cells spread better on the coated samples than on the control, i.e., uncoated samples (Fig. 4 insets).

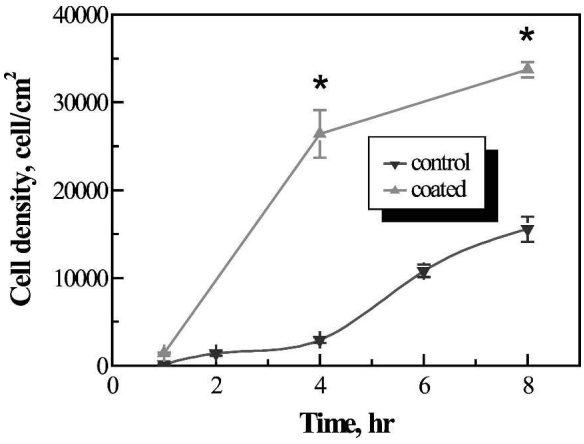


FIG. 2. Cell adhesion to stainless steel discs at 1, 4, and 8 h. Samples of 20-bilayer PLL/PLGA were used, the multilayer nanocoatings were prepared at pH 10.0, and five samples were examined for each testing condition. Data were reported as mean ± SD. *Polypeptide-coated samples are significantly ($p < 0.05$) different from the control samples.

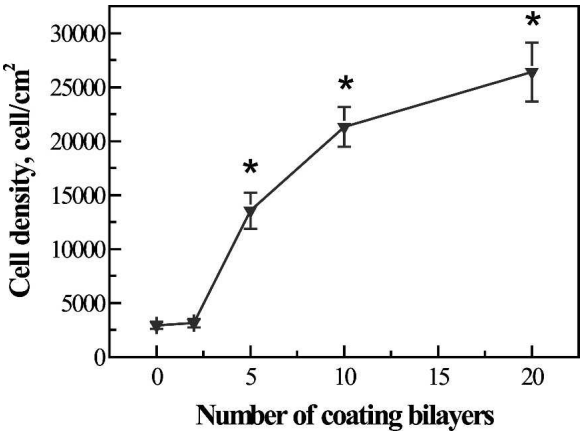


FIG. 3. Cell adhesion versus number of polypeptide bilayers at 4 h. The multilayer nanocoatings were prepared at pH 10.0, and five samples were examined for each testing condition. Data were reported as mean ± SD. *Polypeptide-coated samples are significantly ($p < 0.05$) different from the control samples.

C. Cell proliferation

Figure 5 represents the proliferation of osteoblast cells at 1, 3, and 5 days. In general, cell proliferation was greater for the polypeptide-coated samples than the control ones, whereas the difference diminished at 5 days. Cell proliferation slowed down from 3 to 5 days compared to from 1 to 3 days.

D. Cytotoxicity

The percentages of live cells on polypeptide-coated samples and on control samples were about the same at 1, 3, and 5 days (Fig. 6). This indicates that both the polypeptide-coated and the control stainless steel discs had similar cytotoxicity.

IV. DISCUSSION

It is well known that the chemical composition of implants plays a key role in determining their biocompatibility and applications. Stainless steel, titanium, and cobalt alloys have been widely used in dental and orthopaedic applications due to their advantageous combination of mechanical strength, corrosion resistance, and biocompatibility. Meanwhile, the success of dental and orthopaedic implants depends on the efficient and stable interaction between bone cells (osteoblast cells) and the implants. The quality of cell adhesion to the implant determines osseointegration.[28] The nature of the implant surface, where cells adhere and grow, can directly influence osteoblast cell adhesion, spreading, proliferation, and the osseointegration process.[20,22,29] Previous investigations have demonstrated that most implant osseointegration failure was associated with initial poor interaction between the implant surface and adjacent tissues,[30–32] and surface properties may be one of the most important factors determining implant success. Therefore, control of the events at the implant-bone interface has become a field of major interest in dental and orthopaedic applications.

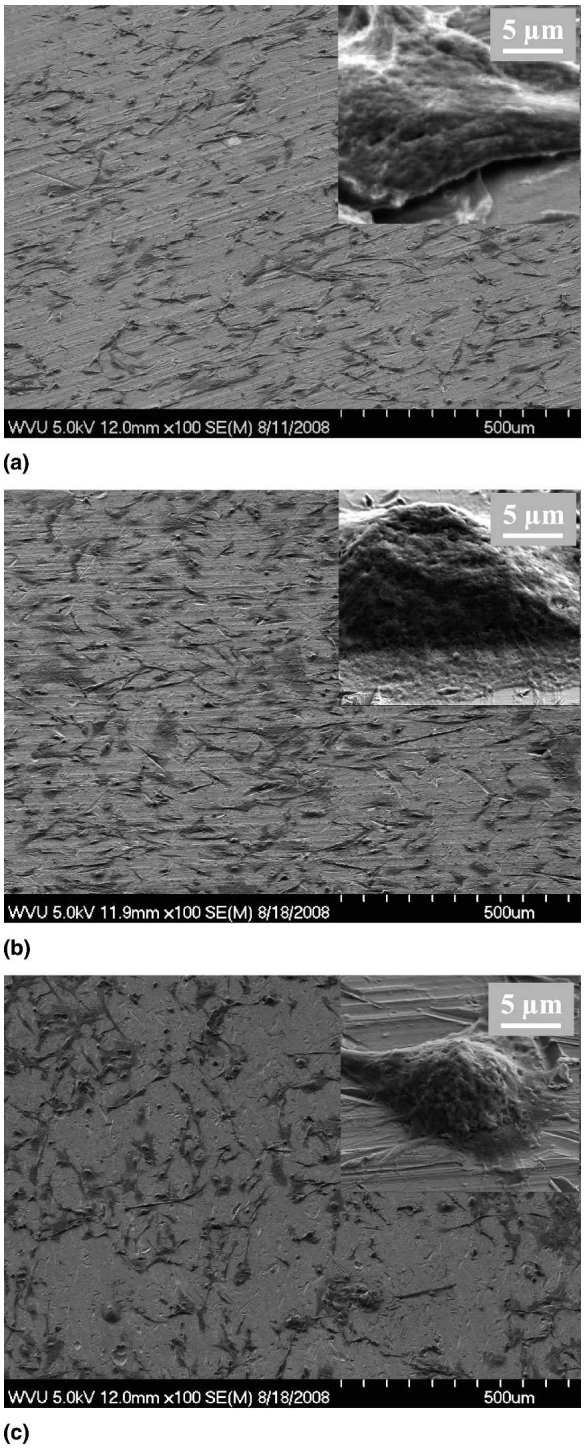


FIG. 4. Scanning electron micrographs of (a) control samples, i.e., bare stainless steel discs, (b) one layer of PLL-coated samples, and (c) one bilayer of PLL/PLGA-coated samples. Samples were cultured for 4 h.

The osseointegration capability, fixation, and stability of dental and orthopaedic implants can be improved by

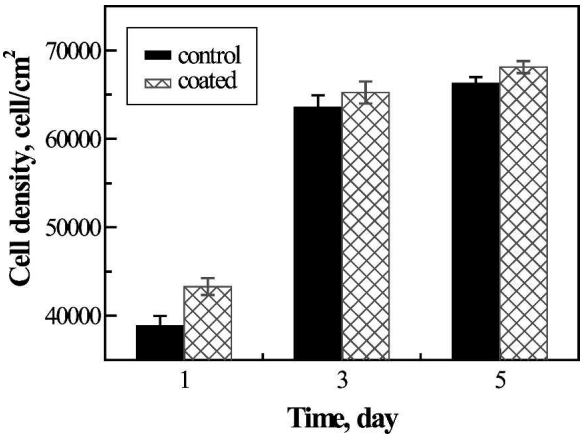


FIG. 5. Osteoblast cell density at 1, 3, and 5 days. Samples of 20-bilayer PLL/PLGA were examined. The multilayer nanocoatings were prepared at pH 10.0, and 3–5 samples were examined for each testing condition. Data were reported as mean ± SD.

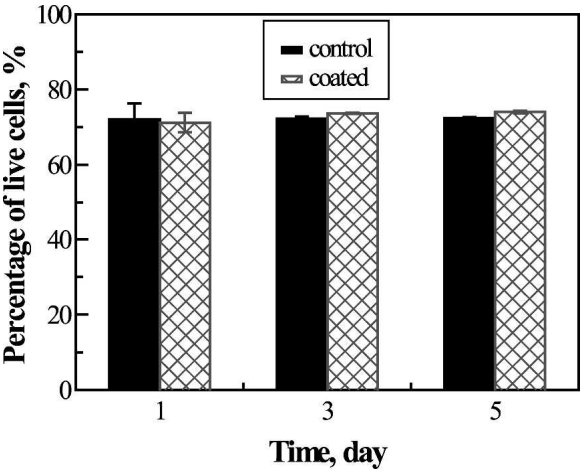


FIG. 6. Percentage of live cells at 1, 3, and 5 days. Samples of 20-bilayer PLL/PLGA were examined. The multilayer nanocoatings were prepared at pH 10.0, and five samples were examined for each testing condition. Data were reported as mean ± SD.

creating a surface that stimulates implant-bone integration. Various techniques have been developed to modify the surface properties of dental and orthopaedic implants to promote osseointegration.[21,33–39] For instance, blasting has been used to increase the surface roughness of titanium alloys,[34,35] and it was found that blasting of titanium alloys favored in vitro osteoblast differentiation. Further thermal oxidation of blasted surfaces enhanced early stage osteoblast cell attachment.[36]

In this study, our objectives were (i) to develop a universal approach for improving cell adhesion and (ii) to investigate the effects of polypeptide nanocoatings on osteoblast cell behavior. We developed polypeptide nanocoatings using a recently developed nanotechnology, i.e., electrostatic self-assembly. This nanotechnology allows us to prepare nanocoatings on substrates of any shape at room temperature in aqueous media; the process is environmentally friendly and cost-effective. Also, we can control the structure and thickness of the coatings at the molecular level; the thickness of the polypeptide nanocoating of a 20-bilayer sample was approximately 260 nm. We found that stainless steel discs with polypeptide nanocoatings exhibited greater cell adhesion (Figs. 2 and 3) than the control samples, i.e., uncoated stainless steel discs, and more cells adhered with increasing polypeptide coating layers (Fig. 3) as the sample surface changed from partial coverage to complete coverage with polypeptides. Substantially more, one order higher, osteoblast cells adhered on the polypeptide-coated samples at the early time point, e.g., 4 h (Fig. 2). It is believed that the initial interaction, i.e., cell adhesion, of bone cells with implants influences all subsequent responses and may determine osseointegration.[28,40] Meanwhile, cell adhesion to implants has been long recognized as a complex process that plays a key role in wound healing, cell growth, morphogenesis, immune response, and osseointegration.[41–46] Therefore, the early stage adhesion of osteoblast bone-forming cells to our developed polypeptide-coated implants could be desirable for implantation.

Cell morphology and proliferation were both investigated in this study to assess the relative biocompatibility of the polypeptide nanocoatings in vitro. We found that more cells were adhered on the one layer of PLL-coated samples than on the control and the one bilayer of PLL/PLGA-coated samples. This is probably because PLL is positively charged, and the surfaces of most cells are negatively charged, and as a result more cells would attach to the PLL-coated surfaces. Polypeptide-coated samples were noncytotoxic compared with the controls (Fig. 6). The increase in cell adhesion on polypeptide-coated samples may be due to the possible smoother surface roughness of the polypeptide nanocoatings and also the nature of polypeptides; polypeptides are part of cell membranes.

In future studies, we will investigate the effects of our developed polypeptide nanocoating on the healing of a femur facture using an in vivo rat model that we recently developed.[47]

V. CONCLUSIONS

Polypeptide nanocoatings were successfully prepared on stainless steel discs using the recently developed electrostatic self-assembly nanotechnology. Compared with other coating techniques, the advantages of electrostatic self-assembly include fewer polymers to build the coating and more control over the structure of the nanocoating.[5,6] We found that polypeptide nanocoatings on stainless steel discs substantially promoted the adhesion of osteoblast cells within the first several hours of contact, and the adhesion of osteoblast cells increased with the

number of polypeptide bilayers. Moreover, the polypeptide nanocoatings were biocompatible. The approach applied, i.e., electrostatic self-assembly, and the use of polypeptides as nanocoating materials, may offer an exciting opportunity for developing advanced dental and orthopaedic implants.

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