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Multifuctional Nanofilm for Stimulating Bone Cell Attachment, Proliferation and Preventing Bacterial Colonization

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Abstract

Osseointegration and infection prevention are of great importance for the long-term success of bone-in-contact implants. In this study, we developed multi-drug loaded polypeptide nanofilms on orthopedic implant models for potential enhanced osseointegration and improved infection prevention. RGD, a cell-adhesive ligand, transforming growth factor beta 1 (TGF-β1), a growth factor, and gentamicin, a common antibiotic, were successfully incorporated into one single polypeptide nanofilm. On one hand, the incorporation of Arg-Gly-Asp (RGD) within polypeptide nanofilms significantly improved osteoblast cell adhesion, proliferation, and viability. The incorporation of TGF-β1 led to enhanced osteoblast cell proliferation at day 5 and improved cell viability as well. However, polypeptide nanofilms with RGD or a combination of RGD and TGF also promoted bacterial growth after 2 days. On the other hand, the incorporation of gentamicin resulted in anticipated antimicrobial properties against *Staphylococcus aureus* (*S. aureus*). However, the incorporation of gentamicin alone had reduced cell adhesion and proliferation. By combining RGD and TGF with gentamicin within a single nanofilm, an ideal surface with overall improved osteoblast adhesion, proliferation, viability, and antibacterial properties was able to be achieved. The developed multi-drug loaded polypeptide nanofilms may provide a promising means for not only enhancing tissue integration but also simultaneously reducing bacterial infection.

Keywords: Polypeptide; Nanofilm; Infection prevention; Adhesion; Proliferation

Introduction

Implanted medical devices are of great importance in improving the life quality of million patients in the U.S. and the demand of implants is continually arising and will increase in the next decades [1,2]. Osseointegration and infection prevention are of critically importance for the long-term success of bone-contacting devices. Surface lacking of cell adhesion and osseointegration or favoring of exogenetic bacterial subsequently leads to failure of implant [3-5]. Since cell and bacterial adhesion are the fundamental and important steps in osseointegration and biofilm formation, respectively, it's crucial to design and engineer device surface to manipulate cell adhesion. Predominant osteocyte adhesion competing with bacteria on artificial material surfaces is the premise for successive device indwelling.

Many efforts have been put to develop functional films on medical devices to reduce the risk of infections and enhance bone healing. Construction of drug-containing films on the implant surface is one of the efficient methods [6,7]. Among various coating approach, the nanofilm based on layer-by-layer (LBL) technique offers one of the greatest advantages--the ability to control the structure and concentration of the incorporated materials in precise-scale by varying the number of coating layers [8]. The advantage to control film properties at a nanoscale level has attracted studies of the multilayer films on planar supports such as implant devices to improve biocompatibility [9-11] and inflammation [12,13]. To achieve priority of osteocyte adhesion battering with bacteria, bacterial non-adhesion or bacterial killing is required in a decisive period for indwelling implant devices [14,15]. Antibacterial coating have developed to inhibit bacterial adhesion and growth by loading with antibiotics and incorporating organic or inorganic antimicrobial agent, such as antimicrobial peptide and nanosilver, as well as fabricating adhesion resistant coatings [12,16]. However, the coating with antibacterial agent or antiadhesive property common possessed negative effects on osteoblast adhesion and growth. For instance, utilizing high content of antibiotic could decrease osteoblast cell attached number, viability and cell function [17,18]. Adhesion resistant coatings by modifying surface physical and chemical properties had a negative on cell adhesion [19]. Meanwhile, the route to improve osteoblast adhesion and proliferation often increase the chance of bacterial colonization on the coating surface [20]. Thus, it is of extraordinary requirement in combining osteoblast-adhesive and bacterial adhesion-protective together and achieving synergistic effects for potential enhancing osseointegration and preventing infection.

In this study, we have developed a multifunctional nanofilm integrating with cell-adhesive and proliferative peptide/protein with bactericidal agent based on LBL nanotechnology. Cell-adhesive peptide was assembled in the nanofilm during coating procedure. Cell proliferative growth factor TGF-beta 1 and bactericidal gentamicin, common-used antibiotic were post-loaded in produced film. These biomolecules incorporated was designed to release in desirable period, decisive period (0~6 h) for implantation, so that reduces the negative influence on their synergistic effects. Cell adhesion, viability, proliferation and antibacterial property were investigated in the present study.

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Materials and Methods

Materials

Poly-L-lysine (PLL, Mn=150 kDa), poly-L-glutamic acid (PLGA, Mn=50 kDa), Arg-Gly-Asp (RGD, Mn =346.3), Rhodamine B (RhoB), fluorescein isothiocyanate (FITC), gentamicin sulfate salt, transforming growth factor beta 1 (TGF-β1) and FITC-labeled PLL (FITC-PLL, Mn 15~30 kDa) are used as received from Sigma Aldrich (St. Louis, MO). Quant-iT dsDNA high-sensitivity assay kit was purchased from Invitrogen (Carlsbad, CA).

RhoB labeled RGD (RhoB-RGD) and gentamicin (RhoB-gentamicin) were prepared in our laboratory. Quartz slides from SPI supplies (West Chester, PA) were cut into 25 mm × 10 mm × 1 mm, and precleaned with piranha solution (4:1 H_2SO4/H_2O_2). Stainless steel (SS) sheet (Small Parts Inc., FL) was cut into discs of 10 mm in diameter and 0.25 mm in thickness and cleaned via ultrasonication in 2% sodium dodecyl sulphate (SDS) solution for 30 min, washing in deionized water, and rinsing with ethanol-NaOH solution and deionized water. Buffer solution of pH 4.0 was prepared using 10 mM Tris-HCl, 10 mM NaAc, 130 mM NaCl, and 0.1% NaN_3. RGD (0.5 mg/mL), PLL (1 mg/mL), and PLGA (1 mg/mL) solutions were prepared by dissolving RGD, PLL, and PLGA in the buffer solution (pH 4.0). Gentamicin (5 mg/mL), FITC-PLL (1 mg/mL), and TGF-β1 (0.2 mg/mL) were dissolved in phosphate-buffered saline or PBS (pH 7.0, cell culture grade).

Incorporation of RGD in polypeptide nanofilms using layer-by-layer nanoassembly: Polypeptide multilayer nanofilms were prepared at pH 4.0 using a dipping-machine. Briefly, quartz slides or stainless steel discs were dipped in PLL solution for 10 min followed by rinsing with buffer solution for 3 min and briefly air dried. The samples were then dipped in RGD solution for 10 min, rinsed with buffer solution for 3 min, and dried with air. The samples were next dipped into PLGA solution for 10 min, rinsed with buffer solution for 3 min, and dried with air. These three dipping processes, i.e. dipping in PLL, RGD, and PLGA solutions were referred as one deposition cycle. By repeating the deposition cycle, polypeptide multilayer nanofilms, $(PLL/RGD/PLGA)_n$, where n is the number of deposition cycles were prepared. Similarly, $(PLL/PLGA)_n$ films were prepared by dipping in PLL and PLGA solutions alternatively. The formation of polypeptide multilayer nanofilms, where RhoB-RGD was used, on quartz slides was examined using UV-vis spectrometry (Biomate 3, Thermo Scientific, Madison, WI) and confocal laser scanning microscopy (CLSM, Zeiss 510 Meta Confocal Microscope, Thornwood, NY).

Post-incorporation of drugs in polypeptide multilayer nanofilms: First, $(PLL/PLGA)_{10}$, designated as Film, and $(PLL/RGD/PLGA)_{10}$, designated as Film+RGD, were prepared as aforementioned [21]. Next, TGF-β1 and/or gentamicin were loaded into such polypeptide nanofilms at ambient temperature: (i) $(PLL/RGD/PLGA)_{10}$ nanofilms were incubated in TGF-β1 solution for 30 min and obtained nanofilms were designated as Film+RGD+TGF; (ii) Samples of $(PLL/PLGA)_{10}$, $(PLL/RGD/PLGA)_{10}$, and $(PLL/RGD/PLGA)_{10}$ loaded with TGF-β1 were incubated in the antibiotic (i.e. gentamicin) solution for 30 min. Corresponding samples were designated as Film+anti, Film+RGD+anti, and Film+RGD+TGF+anti.

Determination of drug loading and release from multi-drug loaded polypeptide nanofilms: TGF-β1, a protein with an isoelectric point of 8.5, is positively charged at pH 7.0. In this study, FITC-PLL, positively charged at pH 7.0, was used as a substitute for TGF- β1 in the drug loading and release studies. To determine the loading of FITC-PLL and gentamicin into polypeptide multilayer nanofilms, $(PLL/RGD/PLGA)_{10}$ nanofilms were prepared on quartz slides and incubated with FITC-PLL followed with RhoB-gentamicin for the same time period for up to 30 min; at time periods of 5, 10, 20, and 30 min, the samples were rinsed with PBS and dried with N_2 gas followed with UV-vis absorbance measurements. The characteristic peak absorbance corresponding to FITC-PLL and RhoB-gentamicin at 556 and 482 nm, respectively, were recorded.

To determine drug release from multi-drug loaded polypeptide nanofilms, FITC-PLL and gentamicin loaded $(PLL/RGD/PLGA)_{10}$ nanofilms on SS discs were incubated in 1.5 mL PBS (pH 7.0). A 0.4 mL solution was taken out (0.4 mL fresh PBS was subsequently added) to determine RhoB-RGD, RhoB-gentamicin, and FITC-PLL concentrations at predetermined time points using UV-vis spectrometry. Independent samples were prepared to determine each type of drugs, and gentamicin was not labeled while determining the release of RhoB-RGD, and similarly, RGD was not labeled while determining the release of RhoB-gentamicin.

Osteoblast cell adhesion and proliferation: CRL-11372 human osteoblast cell line (American Type Culture Collection or ATCC, Manassas, VA) was cultured in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12, 1:1 medium (DMEM: F-12 medium, ATCC) with 10% fetal bovine serum (ATCC) in a 5% CO_2 and 95% air atmosphere incubator at 37°C. Cells (1×10^5 cells/well) were seeded in a 24-well plate. A $(PLL/RGD/PLGA)_{10}$ or $(PLL/PLGA)_{10}$ coated SS disc loaded with or without gentamicin and/or TGF-β1 (i.e. Film, Film+anti, Film+RGD, Film+RGD+anti, Film+RGD+TGF, and Film+RGD+TGF+anti) was placed in each well and incubated at 37°C in a 5% CO_2 humidified incubator. After 2 h culturing, osteoblasts adhered to the surface of polypeptide coated SS discs were gently rinsed with PBS, treated with 1 mL Trypsin/EDTA solution at 37°C for 5 min. Detached cells were centrifuged at 1,200 rpm for 7 min, and re-suspended in 1 mL distilled water with 1% Triton X-100. Next, the cell suspensions underwent three freeze-thaw cycles. The Triton lysates were stored at -80°C until further use for DNA content tests using Quant-iT dsDNA assay kit. A DNA standard curve was obtained. A 30 µl aliquot of cell suspension was added to 100 µL working solution of the assay kit, and incubated for 10 min at room temperature in the dark. Then, dsDNA content was measured using a fluorescence microplate reader (µQuant, Bio-Tek, Winooski, VT) with an excitation filter 365 nm and an emission filter 450 nm. Similarly, osteoblasts, at a density of 2×10^5 cells/well, were incubated for 1, 3, and 5 days on the coated SS discs, and their dsDNA contents were determined.

The effect of RGD release from polypeptide multilayer nanofilms on cell adhesion was also investigated. SS discs of $(PLL/RGD/PLGA)_{10}$ nanofilms (i.e. Film+RGD) were incubated in PBS at 37°C in a water bath. After 2, 8, 12, and 24 h incubation, discs were taken out and placed in wells. Osteoblast cells at a cell density of 1×10^5 cells/well were then cultured on the discs for 2 h. The DNA contents were determined as abovementioned using the Quant-iT dsDNA assay kit. $(PLL/PLGA)_{10}$ nanofilms (i.e. Film) were used as controls, and normalized cell adhesion was calculated as the ratio of (DNA content of sample) / (DNA content of control).

Cell Viability on multi-drug loaded polypeptide nanofilms: MTT assay was used to determine cell viability using an *in vitro* toxicology kit (Sigma-Aldrich, St. Louis, MO). Osteoblasts were seeded in a 24-well plate (1×10^5 cells/well) and incubated at 37°C for 24 h on polypeptide coated SS discs including samples of Film,

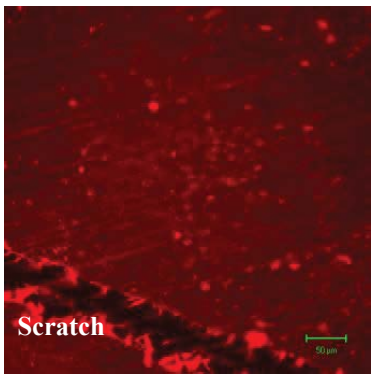
Film+RGD, Film+RGD+TGF, and Film+RGD+TGF+anti. 200 µl of MTT solution was added to each well. After incubating for 2 h, 200 µL MTT solubilization solution was added to each well. The dissolved solution was transferred to another 96-well plate, and immediately the absorbance at nm was recorded using a micro-plate reader (µQuant, Bio-Tek, Winooski, VT). The background absorbances of multiwell plates were measured at 690 nm and subtracted from the nm measurements. Triplicate samples were run.

***In vitro* antibacterial properties of multi-drug loaded polypeptide nanofilms:** *In vitro* antimicrobial activity of polypeptide multilayer nanofilms was examined using two assays: zone of inhibition (ZOI) test [22] and a bacterial killing assay [23]. For the ZOI assay, (PLL/RGD/PLGA)n, n=5, 10, and 20, were prepared on SS discs and incubated in gentamicin (5 mg/mL, PBS buffered) for 30 min at room temperature. A clinical isolate *S. aureus* was grown overnight in Trypticase soy broth, then centrifuged, washed and diluted with PBS to a concentration of ~1 × 10^8 colony forming units (CFU)/ml. *S. aureus* was inoculated onto Mueller Hinton (MH) agar plates, the SS discs with gentamicin loaded $(PLL/RGD/PLGA)_n$ nanofilms were immediately gently pressed onto the plates and the plates were inverted and incubated at 37°C for 24 h. The diameters of the ZOI (including diameters of discs) were recorded. For the bacterial killing assay, tubes containing 10 mL of MH broth were inoculated with the clinical *S. aureus* isolate at a concentration of 1 × 10^7 CFU/ml. Polypeptide coated SS discs were immersed in tubes and incubated at 37°C on an orbital shaker rotating at 50 rpm for up to 9 days. Optical density (OD) at 610 nm was measured at pre-determined time points. Inoculated tubes with bare disc were used as controls. The percentage of bacterial killing was calculated and defined as {1 – [(OD of sample – OD of MH broth) / (OD of control – OD of MH broth)]} × 100%.

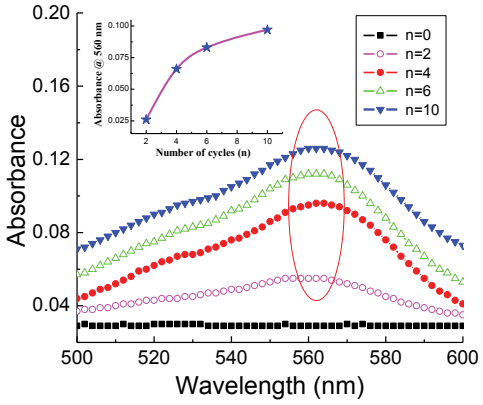
Results

Incorporation of multiple drugs in polypeptide multilayer nanofilms

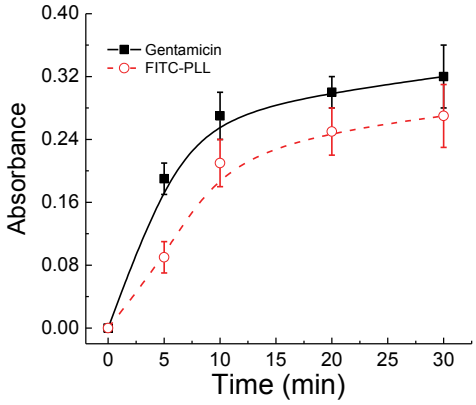
Three drugs (i.e. RGD, gentamicin, and FITC-PLL) were successfully incorporated into polypeptide multilayer films using different drug-incorporation strategies. Among them, RGD (a cell-adhesive ligand) was immobilized as one of the film components by alternative deposition of PLL, RhoB-RGD, and PLGA on quartz slides. The deposition of RhoB-RGD in the $(PLL/RGD/PLGA)_{10}$ nanofilms was observed under CLSM (Figure 1A), and the absorbance intensity of RhoB-RGD increased with increasing number of deposition cycles (Figure 1B). Gentamicin (an antibiotic drug) and FITC-PLL (a protein drug model) were loaded post-preparation of the polypeptide nanofilms. In another word, after $(PLL/RGD/PLGA)_{10}$ nanofilms were



(A)



(B)



(C)

Figure 1: (A) CLSM image of a $(PLL/RhoB\text{-}RGD/PLGA)_{10}$ nanofilm. (B) Assembly of (PLL/RGD/PLGA)n nanofilms on quartz slides. The inset shows the absorbance at 560 nm in the UV-vis spectra which represents the characteristic absorbance of RhoB-RGD. (C) Gentamicin and FITC-PLL loading *vs.* incubation time in $(PLL/RGD/PLGA)_{10}$ nanofilms. The absorbance of FITC-PLL and RhoB-gentamicin in the nanofilms on quartz slides were recorded at 556 and 482 nm, respectively; RGD was not labeled.

prepared, gentamicin and FITC-PLL were loaded via electrostatic attraction between the drug molecules and the film components. The loading of gentamicin or FITC-PLL increased with increasing loading time within the first 30 min (Figure 1C).

Drug release from multidrug-loaded polypeptide multilayer nanofilms

In vitro drug release from polypeptide multilayer nanofilms incorporated with three drugs, i.e. RGD, gentamicin, and FITC-PLL, was studied. Each drug had a burst release followed with a sustained release (Figure 2). The release of gentamicin at 2 h reached 65 μg/mL; the minimum inhibitory concentration (MIC_{90}) of gentamicin is 0.5 μg/mL [24] against *S. aureus*. The total amounts of RGD, FITC-PLL, and gentamicin released from $(PLL/RGD/PLGA)_{10}$ polypeptide multilayer nanofilms were 43, 63, and 171 μg/mL, respectively.

Osteoblast cell activities on multidrug-loaded polypeptide nanofilms

Compared to the non-coating samples (Figure 3A and 3B) and $(PLL/PLGA)_{10}$ nanofilm (i.e. Film) samples(Figure 3C and 3D), more osteoblasts were adhered on the sample of $(PLL/RGD/PLGA)_{10}$ nanofilms (Figure 3E and 3F). The positive effect of RGD incorporation on cell adhesion was confirmed by the comfortable shape of cell on the sample of $(PLL/RGD/PLGA)_{10}$ nanofilms (Figure 3F).

Osteoblast cell activities on multidrug-loaded polypeptide nanofilms

Compared to the $(PLL/PLGA)_{10}$ nanofilm (i.e. Film) samples, less osteoblasts were adhered on the sample of $(PLL/PLGA)_{10}$ nanofilms loaded with gentamicin (i.e. Film+anti) while significantly more osteoblasts on the samples of RGD modified nanofilms including $(PLL/RGD/PLGA)_{10}$ nanofilms (i.e. Film+RGD) and $(PLL/RGD/PLGA)_{10}$ nanofilms loaded with gentamicin, TGF, and a combination of TGF and gentamicin (i.e. Film+RGD+anti, Film+RGD+TGF, and Film+RGD+TGF+anti) (Figure 4A). Compared to the $(PLL/RGD/PLGA)_{10}$ nanofilms (i.e. Film+RGD), further modifications of $(PLL/RGD/PLGA)_{10}$ nanofilms with gentamicin (i.e. Film+RGD+anti), TGF (i.e. Film+RGD+TGF), or TGF and gentamicin (i.e. Film+RGD+TGF+anti) led to decreased osteoblast adhesion, although not significant (Figure 4A). It is interesting that the incorporation of gentamicin overall had resulted in the decrease of osteoblast adhesion (Film+anti *vs.* Film, Film+RGD+anti *vs.* Film+RGD, and Film+RGD+TGF+anti *vs.* Film+RGD+TGF). However, samples with modifications of both RGD and gentamicin (i.e. Film+RGD+anti and Film+RGD+TGF+anti) still had significant higher osteoblast adhesion compared to Film+anti (Figure 4A).

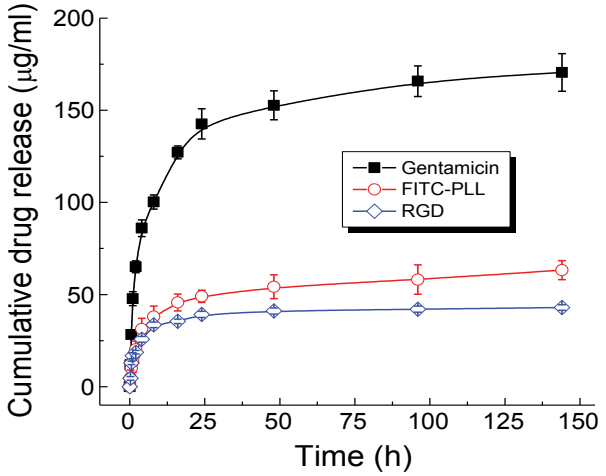


Figure 2: Release profiles of gentamicin, RGD, and FITC-PLL from $(PLL/RGD/PLGA)_{10}$ nanofilms loaded with FITC-PLL and gentamicin.

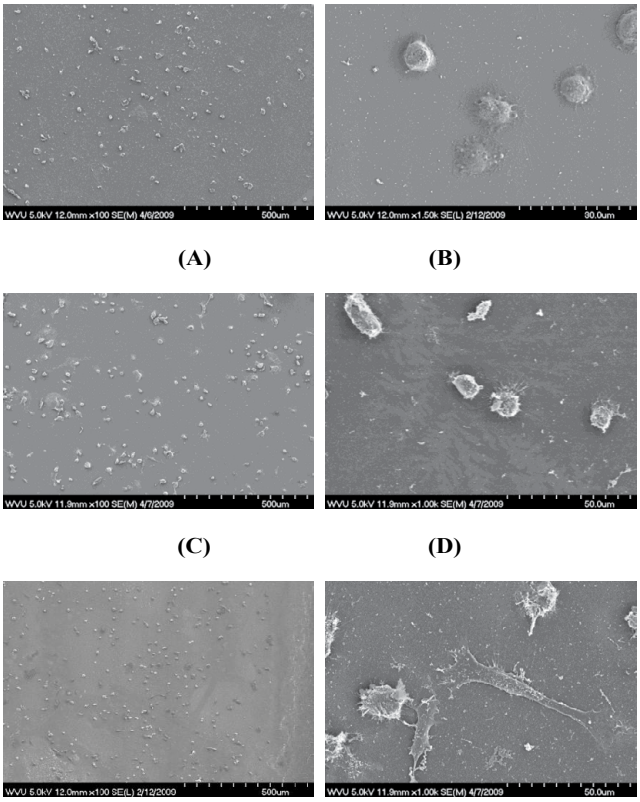


Figure 3: SEM images of osteoblast cells attaching to q non-coating quartz slide (A and B), $(PLL/PLGA)_{10}$ nanofilms (C and D) and $(PLL/RGD/PLGA)_{10}$ nanofilms (E and F). B, D and F are the magnified images of A, C and E, respectively.

Moreover, the effect of RGD incorporation on cell adhesion was confirmed as less osteoblast adhesion was observed on $(PLL/RGD/PLGA)_{10}$ nanofilms as the amount of RGD decreased within the nanofilms with increasing RGD elution time (Figure 4B). After incubating in PBS for 24 h, the adhesion of osteoblasts on $(PLL/RGD/PLGA)_{10}$ nanofilms (i.e. Film+RGD) was approximately the same as the control (i.e. $(PLL/PLGA)_{10}$ or Film).

After culturing for 1, 3, and 5 days (Figure 4C), the incorporation of RGD alone significantly enhanced osteoblast cell proliferation from day 1 to days 3 and 5. The loading of gentamicin decreased osteoblast cell proliferation at day 1 but such an effect waned at day 5. Compared to the cell proliferation of the $(PLL/RGD/PLGA)_{10}$ samples (i.e. Film+RGD), the loading of TGF-β1 to $(PLL/RGD/PLGA)_{10}$ nanofilms (i.e. Film+RGD+TGF) reduced the cell proliferation at days 1 and 3 but enhanced the cell proliferation at day 5 (Figure 4C).

The effects of incorporation of multiple drugs within polypeptide multilayer films on osteoblast cell viability were also determined.

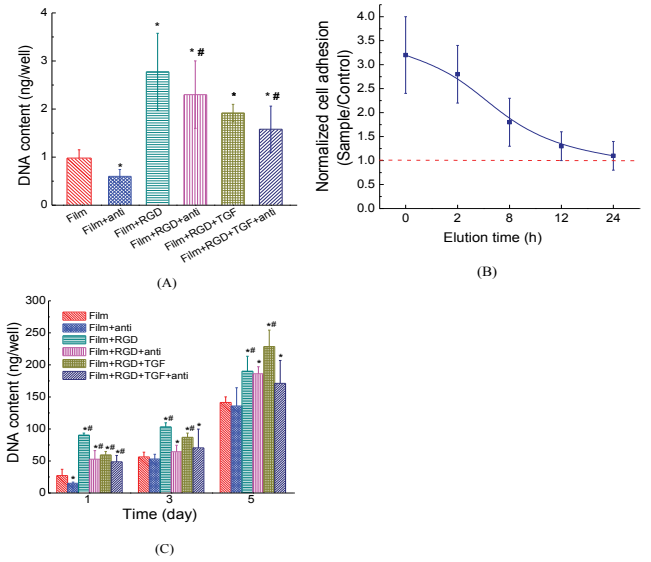


Figure 4: Effects of multidrug-loaded polypeptide nanofilms on cell adhesion and proliferation. Cells were cultured for 2 h at a cell density of 1 × 10^5 cells/well. (B) After various time elution in PBS, cells were cultured on $(PLL/RGD/PLGA)_{10}$ films (i.e. Film+RGD) for 2 h at a cell density of 1 × 10^5 cells/well; $(PLL/PLGA)_{10}$ nanofilms (i.e. Film) was used as control. (C) Cells were cultured for 1, 3, and 5 days at an initial cell density of 2 × 10^5 cells/well. *p<0.05 compared to $(PLL/PLGA)_{10}$ films (i.e. Film). #p<0.05 compared to samples of gentamicin loaded $(PLL/PLGA)_{10}$ nanofilms (i.e. Film+anti).

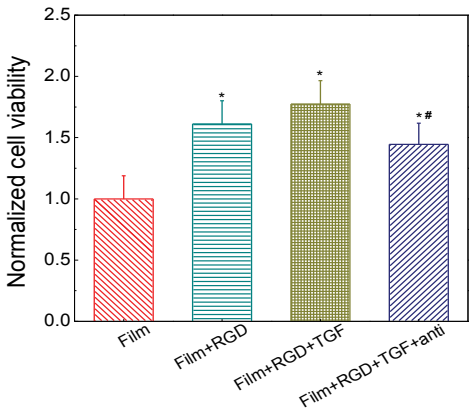


Figure 5: Cell viability, determined using MTT assay, of osteoblasts after culturing for 24 h on multidrug-loaded polypeptide nanofilms. *p<0.05 compared to $(PLL/PLGA)_{10}$ films (i.e. Film); #p<0.05 compared to TFG-β1 loaded (PLL/RGD/PLGA)10 samples (i.e. Film+RGD+TGF).

The cell viability of osteoblasts adhered to drug loaded polypeptide multilayer nanofilms, e.g. $(PLL/RGD/PLGA)_{10}$ (i.e. Film+RGD) and $(PLL/RGD/PLGA)_{10}$ loaded with TGF-β1 or a combination of TGF-β1 and gentamicin (i.e. Film+RGD+TGF and Film+RGD+TGF+anti), was significantly higher than that of osteoblasts adhered to $(PLL/PLGA)_{10}$ films (i.e. Film) without drugs (Figure 5). The incorporation of RGD and TGF-β1 enhanced the cell viability while the loading of gentamicin had resulted in a significant decrease in cell viability (Figure 5).

Antibacterial property against *S. aureus*

The ZOI of gentamicin incorporated polypeptide multilayer nanofilms increased with increasing number of PLL/RGD/PLGA layers (Figure 6A). The ZOI diameter increased from 18.8 ± 0.8 mm of $(PLL/RGD/PLGA)_5$ nanofilm to 27.0 ± 0.2 mm of $(PLL/RGD/PLGA)_{20}$ nanofilm (Figure 6A). The bacterial killing curves showed that polypeptide multilayer nanofilms incorporated with gentamicin (i.e. Film+anti, Film+RGD+anti,a nd Film+RGD+TGF+anti), with or without RGD and TGF-β1, had similar antimicrobial property and their percentage killing was more than 60% within the first two hours and reached 100% at 9 days (Figure 6B). By contrast, nanofilms without gentamicin (i.e. Film, Film+RGD, and Film+RGD+TGF) did not show any antimicrobial effects, and instead, more bacterial growth was seen with the Film+RGD and Film+RGD+TGF samples after 2 days (Figure 6B).

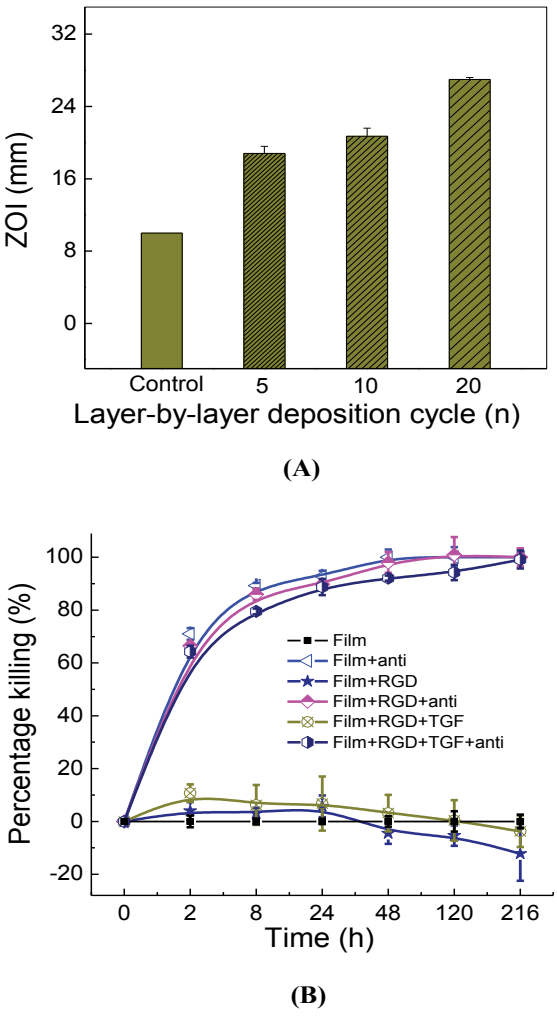


Figure 6: Antibacterial properties of multi-drug loaded polypeptide nanofilms against *S. aureus*. (A) ZOI tests of gentamicin loaded $(PLL/RGD/PLGA)_n$ nanofilms (n=5, 10, and 20). $(PLL/RGD/PLGA)_{20}$ without gentamicin was used as control. (B) Percentage of bacterial killing of multidrug-loaded polypeptide multilayer nanofilms.

Discussion

Cell adhesion is very crucial to biomedical and biotechnological applications [3]. For instance, cell adhesion to orthopedic implant surfaces may regulate cell growth, proliferation, differentiation, and apoptosis [25,26], and consequently impacts osseointegration; osseointegration requires secure and strong attachment and growth of bone cells on the surgically implanted devices [4,5]. In this study,

we successfully incorporated RGD, a cell-adhesive ligand, TGF-β1, a growth factor, and gentamicin, a common antibiotic, into one single nanofilm on orthopedic implant models, and we showed that the incorporation of the three drugs could be controlled by tuning the preparation variables including the assembly layers and incubation time (Figure 1B and 1C); the combination of these three drugs within a single nanofilm was intended to achieve improved cell adhesion and growth and meanwhile to inhibit implant-associated infection thereby achieving improved osseointegration and long-lasting implantation.

We first assembled RGD within polypeptide multilayer nanofilms using layer-by-layer nanoassembly. Immobilization of small peptides found in extracellular matrix is one of the most popular approaches to obtain bioactive surfaces that promote cell adhesion [27]. RGD, derived from fibronectin, is the most investigated peptide. The peptide can build a bridge between cell receptor and surface and subsequently improve cell adhesion, differentiation and proliferation as well as cell survival on, various substrate surfaces [28-31]. Our studies showed that the incorporation of RGD in multi-drug loaded polypeptide nanofilms indeed led to significantly improved cell adhesion (Figures 3, 4A and 4B), proliferation (Figure 4C), and viability (Figure 5).

We subsequently loaded commonly-used antibiotics (e.g. gentamicin) and growth factors (e.g. TGF-β1) into polypeptide nanofilms using a post-preparation approach. As we previously reported that positively-charged drug molecules could be loaded into polypeptide multilayer nanofilms by changing the solution pH and their loading and release could be finely tuned [21,32]. By changing the pH, net charges are generated within the polypeptide nanofilms and can interact with oppositely-charged drug molecules during drug loading [21]. In this study, polypeptide nanofilms were prepared at pH 4.0 and allowed successful loading of gentamicin and TGF-β1 at pH 7.0 where the drugs are positively charged. One advantage of nanofilm post-preparation drug loading is that the post-preparation approach relieves the concerns over drug stability during storage and opens the opportunity for loading appropriate drugs minutes before surgical implantation.

Introduction of RGD in nanofilm demonstrates dramatically improved cell adhesion and cell viability on the surface (Figure 2). Cell numbers on film of Film+RGD had up to 150% increases than that of sample Film. Comparing to the sample Film+anti, cell number on Film+RGD+anti showed a threefold increase. Gentamicin embedded in nanofilm had a negative effect on cell adhesion and cell viability, which is probably due to the high antibiotic concentration nearby the interface between adhesive cells and film surface. The exposure of osteoblast cells to high concentration of gentamicin had reported a negative effect on cell attached number, viability and cell function, such as alkaline phosphatase (ALP) activity [17,18]. It has reported that TGF-beta 1 treatment of primary human osteoblast (HOBs) could enhance cell-substrate adhesion [19,33]. However, the effect of introduction of TGF-β1 on cell adhesion in our experiments seemed negligible, and no significant different was illustrated in the comparative groups of Film+RGD vs. Film+RGD+TGF and Film+RGD+anti vs. Film+RGD+TGF+anti.

RGD and TGF-β1 did have impact on cell proliferation. At day 1, sample Film+RGD demonstrated largest cell numbers. At day 3 and day 5, nanofilms embedded with RGD and TGF-β1 presented a similar cell proliferation even with gentamicin incorporation.

For long-time contact with cells, RGD mostly is covalently bonded to the substrates surface or conjugated to biomacromolecules as a coating component [19,34]. However, in considering of bacterial prevention, RGD existence may not be preferred. Only a few papers reported the effects of the adhesive ligand on bacterial adhesion to surface, and it seemed that these RGD-immobilized coating surfaces had no apparent impact on bacterial adhesion [35-38]. But the intervention of antibacterial/anti-adhesive constituent, such as antimicrobial chitosan [36], and poly(L-lysine)-grafted poly(ethylene glycol) (PLL-g-PEG) [37,38], in the surfaces made these results controversial. For instance, a lower decrease of the adhesion of *S. aureus* demonstrated on PLL-g-PEG/PEG-RGD surfaces by 69% than that on PLL-g-PEG surface by 89-93%. It sounded the PEG-lated surface without RGD more effective for inhibiting bacterial adhesion. Therefore, function of RGD to improve cell adhesion is preferred in "decisive time" for implantation (normally 0-6 h) in order to avoid of negative effect for bacterial killing or non-adhesion [14,15]. Here, RGD is physically immobilized in nanofilm according to the electrostatic interaction between RGD and PLGA and can be released in a desirable time. *In vitro* release showed that most of RGD (up to 60%) was released into the medium at 4 h. Most RGD could be released and didn't exist after 24 h. At meantime, gentamicin at a concentration upto 150 μg/ml was released, which is much higher than MIC of gentamicin against *S. aureus*, 50 μg/ml [24]. All gentamicin-containing films had a high bacterial killing percentage up to 85% (Figure 6B). Effect of RGD on bacterial growth was negligible.

The effect of TGF-β1 on bacterial adhesion and growth is still unclear. Lahouassa et al. reported that TGF-β1 release from bMEC did not show an anti-inflammatory potential [39]. However, Wang et al. considered that impaired TGF-β1 could enhance *E. coli*-induced inflammatory [20]. From bacterial killing assay test, Film+RGD+anti showed a little higher killing percentage at day 3 and day 5 than Film+RGD+TGF+anti. This might be due to the competitive loading of TGF-beta 1 with gentamicin during drug loading procedure and subsequently led to a decreased antibiotic loading content.

Conclusion

We developed and engineered multi-drug loaded polypeptide nanofilms using layer-by-layer nanoassembly. An adhesive ligand RGD and a growth factor TGF-β1 were incorporated within polypeptide nanofilms to enable the nanofilms with enhanced cell adhesion and proliferation. An antibiotic of gentamicin was incorporated within polypeptide nanofilms to endow the surfaces with antibacterial properties. The combined incorporation of a cell-adhesive peptide (i.e. RGD), growth factor (TGF-β1), and a bacterial-killing agent (i.e. gentamicin) on an orthopedic implant had led to enhanced osteoblast adhesion and proliferation as well as effective bacterial killing. The developed multi-drug loaded polypeptide nanofilms on implants could potentially be used to improve cell adhesion while simultaneously prevent bacterial infection.

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