Construction of extracellular microenvironment to improve surface endothelialization of NiTi alloy substrate

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A B S T R A C T
To mimic extracellular microenvironment of endothelial cell, a bioactive multilayered structure of gelatin/chitosan pair, embedding with vascular endothelial growth factor (VEGF), was constructed onto NiTi alloy substrate surface via a layer-by-layer assembly technique. The successful fabrication of the multilayered structure was demonstrated by scanning electron microscopy, atomic force microscopy, contact angle measurement, attenuated total reflection-fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy, respectively. The growth behaviors of endothelial cells on various NiTi alloy substrates were investigated in vitro. Cytoskeleton observation, MTT assay, and wound healing assay proved that the VEGF-embedded multilayer structure positively stimulated adhesion, proliferation and motogenic responses of endothelial cells. More importantly, the present system promoted the nitric oxide production of endothelial cells. The approach affords an alternative to construct extracellular microenvironment for improving surface endothelialization of a cardiovascular implant.

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1. Introduction
Cardiovascular disease is one of the most common causes of death worldwide, especially in low- and middle-income countries. Percutaneous transluminal coronary angioplasty, basing on stent implantation, is a common alternative for treating diseased vessels so far [1]. However, the high in-stent restenosis rate remains as a clinical challenge. Many approaches have been explored to develop stents with reduced in-stent restenosis. Among them, drug-eluting stent (DES) presents itself as a good candidate for intravascular treatment [2–4]. Among the high in-stent restenosis rate remains as a clinical challenge. Many approaches have been explored to develop stents with reduced in-stent restenosis. Among them, drug-eluting stent (DES) presents itself as a good candidate for intravascular treatment [2–4]. Despite the good performance of DES in reducing restenosis, the risk of late local endothelium regeneration and stent surface induced thrombosis still remains as one of the key challenges in long-term clinic applications.

Previous studies demonstrated that a rapid surface endothelialization of a cardiovascular stent was an efficient alternative for preventing thromboembolic complications with reduced restenosis [5–9]. The endothelium, composing of endothelial cells and extracellular matrix components, maintains a non-adhesive luminal surface, which has brinolytic and antithrombotic properties. As we have known, the growth behaviors of endothelial cells in vivo are highly dependent on their extracellular microenvironments. The natural extracellular microenvironment is a highly complicated network, which is composed of soluble signal (growth factors, cytokines, chemokines, etc.), physically bound signal (fibronectin, vitronectin, laminin, collagen, etc.) and signal arising from cell/cell interactions [10–12]. To reveal the interactions of those signals is particularly important to understand how the cellular microenvironment regulates cell survival, proliferation, and differentiation in vivo [13–16].

In recent years, many efforts have been devoted to constructing a desired extracellular microenvironment for inducing surface endothelialization of a stent. Extracellular matrix components, synthetic cell-adhesive peptides and growth factors have been introduced onto the surfaces of biomaterials via different techniques to mimic the extracellular microenvironment [17–22]. Taking advantage of the construction of appropriate extracellular microenvironment, these approaches could elicit specific biological responses of endothelial cells, such as adhesion, migration, proliferation, and differentiation. Considering the complexity of extracellular microenvironment, to immobilize only one kind of signal on stent surface would not mimic the complicated extracellular microenvironment for stimulating rapid surface endothelialization. Thus, it is urgent to induce more signals on material surface to construct artificial extracellular microenvironment.

In this work, multilayer films of chitosan (CHI), gelatin (GEL) and vascular endothelial growth factor (VEGF) were fabricated onto surfaces of NiTi alloy substrates to mimic extracellular microenvironment of endothelial cells. The rationale to select CHI is that it is the analogue of glycosaminoglycan (GAG), which presents in extracellular matrix [23]. Since GAG could specifically interact with growth factors, receptors

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and proteins. CHI with analogous structure may share some similar biological properties to that of GAG. For GEL, as we know, it is a derivative of collagen, which is the main component of extracellular matrix. Vascular endothelial growth factor (VEGF) plays a key role in guiding endothelial cell in surrounding tissues to migrate, proliferate and form tubular structures. Previous studies demonstrated that the immobilization of VEGF on substrates could stimulate the growth of endothelial cells and the formation of new vessels [21,22,24–26]. It is well known that the growth factors (e.g. VEGF) are susceptible to denaturation and degradation when exposing to biological fluids, which consist of various chemicals and enzymes, leading to the loss of their bioactivities. Here, layer-by-layer (LBL) assembly technique was employed to construct multilayer films containing VEGF on NiTi alloy substrate. LBL assembly technique has emerged as a versatile, inexpensive yet efficient technique to build biologically active surfaces, which is based on the consecutive adsorption of polyanions and polycations via electrostatic interactions [27–29]. Previous studies demonstrated that multilayer film via LBL could retain the bioactivities of drugs/proteins in mild aqueous conditions and present a desired platform for controlled drug delivery [30,31].

We intended to simultaneously introduce physically bound signal (CHI and GEL) and soluble signal (VEGF) on NiTi alloy substrates to construct desirable extracellular microenvironment for endothelial cells. Thus, the NiTi alloy substrate functionalized with multilayer films containing VEGF could potentially regulate adhesion, proliferation and other biological responses of endothelial cells.

2. Experimental section

2.1. Materials

NiTi alloy substrates (Ti49.2Ni50.8 at.%) were kindly supplied by Northwest Institute for Non-ferrous Metal Research (China). Fluorescent rhodamine-phalloidin was supplied by Invitrogen Co (USA). CHI, GEL, VEGF, Hoechst 33342, 3, 4-dihydroxyphenylalanine (dopamine), dimethyl sulfoxide (DMSO) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co, (MO, USA).

2.2. Fabrication of polyelectrolyte multilayer films

CHI solution (5 mg/ml) was prepared with 0.3% (v/v) acetic acid. GEL (5 mg/ml) and VEGF (200 ng/ml) solution were prepared with phosphate buffered saline (PBS, pH 7.4). Firstly, NiTi alloy substrates were immersed into dopamine solution (2 mg/ml) in Tris buffer solution (10 mM, pH 8.5) overnight in the dark. Then, the treated substrates were rinsed with double distilled water 6 times to remove the unreacted dopamine. Next, the treated substrates were immersed into CHI solution overnight at ambient temperature. Subsequently, the substrates were immersed into CHI, GEL, VEGF and GEL solution each for 10 min respectively to induce LBL assembly. The samples were rinsed twice with double distilled water for 1 min after each step. The process was repeated until the desired layers of (GEL/CHI/GEL/VEGF)5 were achieved onto the substrates. Finally, the substrates were kept in a sterile environment before use. The (GEL/CHI/GEL/VEGF)5 film modified NiTi alloy substrates were denoted as NiTi-LBL-VEGF substrates. In addition, (GEL/CHI)5 multilayer films without VEGF were also fabricated on NiTi alloy substrates, which were denoted as NiTi-LBL substrates.

2.3. Surface characterization

Field-emission scanning electron microscopy (FE-SEM) (Quanta 200, Philips–FEI Corporation, Netherlands) and atomic force microscopy (AFM) (WET-SPM-9500J3, Shimadzu Co., Japan) were employed to characterize surface morphology of the samples. Contact angle (CA) measurement was employed to test the wettability of different substrates, which was performed with a Model 200 video based optical system (Future Scientific Ltd Co, Taiwan, China). Six values of contact angles were collected per group of samples. Attenuated total reflection-Fourier transform infrared (ATR-FTIR, Equinox 55, Bruker Co., Germany) and X-ray photoelectron spectroscopy (XPS, AxisUltra, Kratos Analytical Ltd, England) were used to analyze functional groups and chemical composition of the treated samples. For ATR-FTIR and XPS measurements, (GEL/CHI/GEL/VEGF)70 films were fabricated on NiTi alloy substrates.

2.4. Ni ion release test

Inductively coupled plasma mass spectrometry (ICP-MS) (Elan DRC-e, Perkin Elmer USA) was used to determine the amount of Ni ion leached from the samples. The samples were immersed into 2 ml of simulated body fluid (SBF) solution and incubated in a thermostatic chamber at 37 °C for 1 and 3 days. At each time point for each group, the SBF solution was taken out and analyzed by ICP-MS.

2.5. Cell culture and seeding

Human umbilical cord vein endothelial cells (HUVECs) were cultured according to previous reports [32,33]. HUVECs were grown in polystyrene flasks with media of RPMI1640 (Hyclone), 10% fetal bovine serum (PBS, Gibco), 100 units/ml of penicillin and 100 mg/ml of streptomycin at 37 °C under 5% CO2 atmosphere. Cell culture media was initially changed at the first day, and every two days thereafter. HUVECs were grown to near confluence and passaged when required. HUVECs at population of the 3rd passage were used in the following experiments. For cell seeding, all samples were placed in a 24-well plate.

2.6. Fluorescent staining

HUVECs were seeded in native NiTi alloy substrates, NiTi-LBL substrates and NiTi-LBL-VEGF substrates at an initial cell seeding density of 104 cells per disk. After culturing for 3 days, the cells layers were rinsed with PBS 3 times. The cells were fixed with 5% glutaric dialdehyde at 4 °C for 30 min followed by rinsing with PBS 3 times. The treated cells were then permeabilized with 0.2% Triton X-100 at 4 °C for 2 min followed by rinsing with PBS for 3 times. Then, the treated samples were incubated with 1% bovine serum albumin (BSA)/PBS at 37 °C for 1 h. The cells were stained with 5 U/ml of rhodamine-phalloidin at 4 °C overnight and counterstained with 10 µg/ml of Hoechst 33342 at room temperature for 5 min. The stained samples were finally observed with confocal laser scanning microscopy (CLSM, Leica DMI 6000, Germany).

2.7. Cell viability assay

HUVECs were seeded into tissue culture polystyrene (TCPs), native NiTi alloy substrates, NiTi-LBL substrates and NiTi-LBL-VEGF substrates at an initial cell seeding density of 105 cells per disk. After HUVECs were incubated for 1 and 3 days, the medium was removed and the samples were washed twice with PBS. Subsequently, 1 ml of fresh medium (without FBS) and 100 µl of MTT (5 mg/ml) were added and incubated at 37 °C for 4 h. Then the medium containing MTT was removed and 500 µl of DMSO was added to each well dissolving the blue formazan reaction product. The absorbance of the solution was measured by using a microplate reader (BIO-RAD 680) at wavelength 490 nm. The percentage difference of cell viability between the NiTi sample and the TCPs sample was calculated based on the average of six replicates.

2.8. Wound healing assay

For wound healing assay, HUVECs were seeded to pristine NiTi alloy substrates, NiTi-LBL substrates and NiTi-LBL-VEGF substrates at an
initial cell seeding density of $5 \times 10^4$ cells per disk. After HUVECs growing near 75% confluence, the cell monolayer was scratched with a plastic pipette as previously described [34]. Then cells were washed with PBS and incubated with complete medium for another 8 h. Finally, cell cytoskeleton and nuclei were stained and observed.

2.9. Total protein content assay

HUVECs were cultured on various substrates at an initial density of $10^4$ cells per disk for 1 and 3 days. Cells were lysed by 1% Triton X-100 with three freeze–thaw cycles. Total protein content in the cell lysate was determined using a commercially available kit (BCA, Sigma). Light absorbance of these samples was measured at 570 nm with a Bio-Rad 680 spectrophotometric microplate reader. Total intracellular protein (expressed as mg) synthesized by cells cultured on the sample was determined from a standard curve of absorbance versus known concentration of albumin run in parallel experiments.

2.10. NO assay

NO assay was performed according to instructions of the commercial NO Kit (Beyotime Biotechnology Co., Jiangsu China). HUVECs were seeded to samples at an initial cell seeding density of $10^4$ cells per disk and incubated for 1 and 3 days, respectively. Cells were lysed by 1% Triton X-100 with three freeze–thaw cycles. Then, the supernatant was harvested and centrifuged at 1500 g/min for 15 min to remove cellular debris. NO production in cells was measured by Griess method. The absorbance at 540 nm was measured using a spectrophotometric microplate reader (Bio-Rad 680). The NO concentration (expressed as $\mu$mol ml$^{-1}$) was determined from a standard absorbance curve versus known concentration of NO run in parallel experiments. The NO concentration (expressed as $\mu$mol ml$^{-1}$) was normalized by total intracellular protein production. The NO concentration was thus expressed as $\mu$mol ml$^{-1}$ mg protein$^{-1}$.

2.11. Statistical analysis

All data were expressed as means ± standard deviation (SD). Single factor analysis of variance (ANOVA) technique was used to assess the statistical significance of results between groups. The statistical analysis was performed at confidence levels of 95% and 99%.

3. Results and discussion

3.1. Preparation and characterization of multilayer film

New generation of biomaterials are being developed for controlling over cell adhesion, proliferation, differentiation and stimulating tissue formation in vivo. The behaviors of individual cell and multi-cellular tissues are highly regulated by their surviving extracellular microenvironment. Materials modified with molecular cues mimicking certain functions of an extracellular microenvironment may ultimately stimulate cell functions and tissue formation.

In the present study, we constructed bioactive multilayer films onto surfaces of NiTi alloy substrates to mimic extracellular microenvironment of endothelial cells. By employing CHI and GEL as polycation and polyanion, the multilayered structures were constructed by LBL assembly technique. Meanwhile, VEGF was embedded into GEL/CHI multilayer. These multilayer films could provide physically bound signals and soluble signals to mimic extracellular microenvironment. Thus, the ultimate fate of endothelial cells, such as cell adhesion, proliferation, migration and apoptosis, could be directed by those cues in the extracellular microenvironment.

Fig. 1 shows SEM images of pristine and modified NiTi alloy substrates. Bare NiTi alloy substrates displayed visible scratches, which contributed to polishing abrasion (Fig. 1a). After deposition of GEL/CHI multilayer film, the NiTi-LBL substrates displayed relatively smooth surface morphology (Fig. 1b). After VEGF-infiltrating multilayer film was deposited onto NiTi alloy substrates, the NiTi-LBL-VEGF substrates presented granular structures, which were distributed into multilayer film (Fig. 1c). In addition, AFM images also demonstrated similar rough morphology of bare NiTi substrates (Fig. 2a). In contrast, the NiTi-LBL substrates displayed relatively smooth surface morphology (Fig. 2b). However, the NiTi-LBL-VEGF substrates displayed granular structures (Fig. 2c). The root mean square (rms) roughness of native NiTi alloy substrates was about 22.3 nm. The rms value of the GEL/CHI multilayer film coated NiTi alloy substrates was about 14.4 nm, which was lower than that of native NiTi substrates.

The buildup process of the multilayer on native NiTi alloy substrates was also monitored by contact angle (CA) measurement. The CA of bare NiTi alloy substrate was about 90° (Fig. 3). For the NiTi-LBL substrate with CHI as the outmost layer, the CA was around 60°. Furthermore, for the NiTi-LBL-VEGF substrate with VEGF as the outmost layer, the CA was about 43°.

Furthermore, ATR-FTIR analysis was used to characterize the surface of the NiTi-LBL-VEGF substrates. It indicates the presence of several relevant peaks of the CHI and GEL (Fig. 4). The wide absorption peak at about 3440 cm$^{-1}$ was attributed to overlapping of stretching vibration of –OH from CHI and –NH from CHI and GEL, respectively. The absorption peak at about 1640 cm$^{-1}$ was attributed to corresponding to C=O stretching vibration from CHI and GEL. Amide III peak (C–N stretch plus N–H in phase bending) at 1260 cm$^{-1}$ was the distinguishing feature of GEL molecules. In addition, the LBL coated NiTi surface showed the peak at 1054 cm$^{-1}$, which was assigned to the C–O–C from CHI.

To investigate the successful formation of LBL multilayer onto NiTi substrates, the chemical compositions of the surfaces of NiTi-LBL-VEGF substrates were determined by XPS. Fig. 5 shows the representative XPS spectra of the LBL coated NiTi substrates. The NiTi-LBL-VEGF substrates demonstrated three elements (C, O, and N), which were derived from CHI, GEL and VEGF multilayer films. In addition, after being coated with multilayer film, the peaks of Ni and Ti from NiTi substrates disappeared. The result suggests that NiTi substrates were completely

![Fig. 1. SEM images of (a) native NiTi, (b) NiTi-LBL, and (c) NiTi-LBL-VEGF substrates.](image-url)
covered by LBL multilayers. In short, all results of SEM, AFM, CA, ATR-FTIR, and XPS provided direct evidence for the successful formation of LBL film onto the surfaces of NiTi substrates.

The Ni ion release from native and modified NiTi alloys in SBF solution was measured by ICP-MS. After 1 and 8 days of immersion in SBF solution, Ni ion concentrations of NiTi substrates were 1.13, and 6.71 μg/ml, respectively. Furthermore, Ni ion concentrations of NiTi-LBL-VEGF substrates were 0.65 and 4.54 μg/ml. The results indicate that LBL composite coating could inhibit the release of Ni ion from NiTi substrates.

3.2. Assessment of HUVECs growth behaviors on NiTi substrates

The cellular behavior on a biomaterial is an important factor for evaluation of the biocompatibility of the biomaterial. Cell adhesion on a substrate is the first sequential response when cells come into contacting with a material surface, which is crucial for regulation of successive cell functions including proliferation, migration and differentiation [35]. To investigate HUVEC adhesion on the native and treated NiTi alloy surfaces, cells were visualized with a double staining of actin (cytoskeleton) and nucleus. HUVECs displayed characteristic features, such as a large and clear nuclei, polygonal or spindle, and typical cobblestone-like arrangement (Fig. 6). However, there were only limited cells on the native NiTi surface after culture for 3 days (Fig. 6a). The cell numbers on GEL/CHI multilayers modified NiTi surface (NiTi-LBL) were similar to those of native NiTi surface (Fig. 6b). However, HUVECs grown on NiTi-LBL-VEGF substrates displayed significantly higher cell numbers with spreading cell morphology, almost covering the entire surface of the sample (Fig. 6c). The results suggest that VEGF functionalized nanocoating on NiTi substrates was beneficial for cell adhesion.

To evaluate cell viability of HUVECs cultured on both native and modified NiTi substrates, the MTT assay was employed in the present study. The mechanism of the MTT assay is based on the mitochondrial succinodehydrogenase within viable cells forming blue formazan crystals. It thus indirectly indicates the viability of cells. Fig. 7 shows relative cell proliferation of HUVECs adhered to different substrates after culture for 1 and 3 days, respectively. After culture for 1 day, although the average cell viability of HUVECs adhered to NiTi-LBL-VEGF substrates was higher than those of the native NiTi and NiTi-LBL substrates, no statistically significant difference was observed (p > 0.05). However, after culture for 3 days, HUVECs grown on NiTi-LBL-VEGF substrate displayed statistically higher cell viability than those grown on NiTi-LBL (p < 0.05) and native NiTi substrates (p < 0.01).

The increase in cell adhesion and viability suggests that GEL/CHI multilayers with VEGF could improve the affinity for endothelial cells on the NiTi substrate surfaces, which might affect further cellular functions, such as migration.

The migration of vascular endothelial cells is central to many vascular physiological and pathological processes, including wound healing, embryonic development, angiogenesis, reendothelialization, and vascular remodeling. In postoperative (i.e., balloon angioplasty or bypass surgery) wound progress, the migration of nearby endothelial cells into the injured area is crucial for wound closure and it can prevent the development of restenosis.

To investigate migration of HUVECs on different substrates, a “wound healing” assay was performed. The “wound healing” assay is generally initiated by removing a section of a cell monolayer which
results in two wound edges. Cells around the wound will extend their filopodia or lamellipodia and migrate to form a new cell monolayer and “heal the wound”. Thus, the more cells move to the field of wound, the higher the motogenic responses of those cells are. Fig. 8 shows the motogenic responses of HUVECs adhered to different NiTi substrates. As shown in Fig. 8a and b, few HUVECs adhered to native NiTi and NiTi-LBL substrates migrated to the center of the wound field. It is worthwhile noting that a few HUVECs that adhered to NiTi-LBL-VEGF substrates migrated to the center of the wound field (Fig. 8c). The result suggests that NiTi-LBL-VEGF substrates were beneficial for the migration of HUVECs. The migration process is directionally regulated by chemotactic, haptotactic, and mechanotactic stimuli and further involves degradation of the extracellular matrix to cytoskeletal remodeling. Previous studies have demonstrated that a number of important bio-molecules regulate the migration of endothelial cells, such as peptide, growth factors, bioactive lipids, ephrins, angiopoietins, netrins, chemokines, and their receptors. Among them, VEGF was considered to be the most important stimulant for the migration of endothelial cells and angiogenesis. Nevertheless, the short lifespan of VEGF hinders its potential application under physiological environment in a host. In this work, GEL/CHI multilayers played an important role in preventing VEGF from denaturation. Thus, VEGF in a controlled-release manner would continuously stimulate the migration of HUVECs.

Endothelial cells in vivo secrete various substances and directly affect the balance of hemostasis and thrombosis in the cardiovascular system. Nitric oxide (NO) is an endogenous gas molecule secreted by endothelial cells and plays some key physiological roles such as prevention of platelet adhesion and activation, inhibiting bacterial adhesion and proliferation, enhancing vasodilation, promoting angiogenesis, and aiding in wound healing [36–39]. Fig. 9 shows the content of NO secreted by HUVECs grown onto pristine NiTi, NiTi-LBL and NiTi-LBL-VEGF substrates after culture for 1 and 3 days. After culture for 1 day, HUVECs grown on NiTi-LBL-VEGF substrates displayed significantly higher content of NO release than that of corresponding native NiTi and NiTi-LBL (p < 0.01). The high level of NO release indicates that the embedding of VEGF into LBL multilayer film was advantageous to stimulate HUVECs to release NO, which is potentially important for preventing the adhesion/activation of platelets on normal blood vessel walls. After culture for 3 days, HUVECs adhered to NiTi-LBL and NiTi-LBL-VEGF substrates both displayed significantly higher (p < 0.01) NO release than that of native NiTi substrates. The phenomenon could be interpreted that the extracellular matrix analogues of GEL/CHI multilayer coating also contributed to the improved secretion of NO from HUVECs.

In this work, LBL multilayer film embedding with VEGF was beneficial for promoting endothelial cell biological responses, which could be attributed to two factors. First, CHI and GEL could afford physical bound signals of extracellular microenvironment to mediate cell adhesion and proliferation; second, except for physical bound signal, soluble VEGF signal was another important factor that contributed to extracellular microenvironment, which mediated cell migration and biological functions.

4. Conclusion

To summarize, we developed a multilayer film composed of GEL/CHI pair and VEGF for mimicking extracellular microenvironment of
HUCVEs via LBL method. Combined techniques of SEM, AFM, CA, ATR-FTIR and XPS confirmed that the multilayered structure was successfully constructed onto the surface of NiTi alloy substrate. Furthermore, taking advantage of the construction of appropriate extracellular microenvironment, the resultant multilayer film presented here was used to elicit specific biological responses of endothelial cells. Cell morphology observation and MTT assay demonstrated that the multilayered structure were beneficial for HUCVE adhesion and proliferation. Wound healing assay revealed that this structure stimulated the motogenic response of HUCVEs. More importantly, such surface functionalization promoted the production of NO of HUCVEs. Overall, the methodology reported in this paper is very helpful for promoting endothelial cell biological responses.

This study has great implication for the development of high quality of cardiovascular implants for potential clinical applications. The methodology presented here is not limited to the demonstrated NiTi alloy substrate. It might be extended to generate other functional coatings on other metal and its alloys to meet the requirements of (including but not limited to) biomaterial applications.

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References


Fig. 8. Motogenic responses of HUCVEs cultured onto (a) native NiTi, (b) NiTi-LBL, and (c) NiTi-LBL-VEGF substrates after culture for 8 h.

Fig. 9. NO releasing content of HUCVEs cultured onto native NiTi, NiTi-LBL, and NiTi-LBL-VEGF substrates after culture for 1 and 3 days. Error bars represent means ± SD for n = 4, *p < 0.01.


