Microstructured scaffold coated with hydroxyapatite/collagen nanocomposite multilayer for enhanced osteogenic induction of human mesenchymal stem cells†

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Three dimensional microstructured scaffolds with precisely defined architectures have many promising features for tissue engineering applications, such as controllable porosity, optimal mechanical strength, and adjustable contour fitting to a tissue defect site. However, for enhanced cell adhesion and differentiation, surface modified scaffolds with bioactive agents are additionally required. In this study, we present an osteogenic nanocomposite coating strategy on the surface of a microstructured scaffold for applications in bone tissue engineering. A layer-by-layer multilayer assembly method was employed to coat the scaffold surface with hydroxyapatite and collagen. The amount of the two components in the multilayer could be easily controlled by adjusting the number of deposition layers, leading to improved adhesion, proliferation, and differentiation of seeded mesenchymal stem cells. The hydroxyapatite/collagen nanocomposite coated scaffold showed enhanced osteogenic activities compared to bare scaffold, allowing great potential for bone regeneration.

Introduction

Surface engineered biomaterials can present desirable physical, chemical, and biological functionalities, which favor cell adhesion, migration, and differentiation. In particular, tissue engineering scaffolds are frequently surface-modified with biologically active molecules to mimic the surface composition and topography of a natural extracellular matrix (ECM) structure. The consequent biofunctional cues on the surface play a critical role in regulating and accelerating tissue repair and regeneration processes. In the case of bone tissue engineering, hydroxyapatite (HAp), hyaluronic acid (HA), collagen, and several signaling molecules (e.g., bone morphogenetic protein and fibroblast growth factor) are utilized for surface modification for the enhancement of osteoconductivity or osteoinductivity.

Layer-by-layer (LbL) polyelectrolyte multilayer assembly is an attractive and facile surface modification approach, which generates a thin surface film from a few nano- to several micrometers in thickness with precise control. It comprises alternating deposition of polyanions and polycations on the basis of electrostatic force between two oppositely charged species, resulting in a self-assembled multilayer on the surface. Due to the versatility with regard to the introduction of any functional molecules into the multilayer, a wide variety of therapeutic components could be incorporated and released in a controlled manner. While the LbL assembly was often implemented on planar substrates due to the easy fabrication process, the challenge remains to assemble the multilayer on more complex three dimensional (3D) structures.

HAp is a naturally occurring crystalline calcium phosphate (Ca10(PO4)6(OH)2) and is clinically used as an excellent substitute for mineralized hard tissue due to the similar composition to the mineral components of human bone and enamel. HAp itself or in the form of a bioactive coating on biomedical implant devices has been proven to offer better osteoconductivity, leading to ultimate osteointegration. Collagen is a major organic component constituting natural bone, and has been also used to create favorable extracellular milieu for bone regeneration. Multicomponent scaffolds containing HAp and collagen would be highly desirable for bone tissue engineering due to the composite nature of bone matrix.

Here, we demonstrate a LbL nanocomposite multilayer coating composed of HAp and collagen on a microstructured scaffold fabricated by a rapid prototyping (RP)-based technique for bone tissue engineering applications. HAp nanoparticles (HApNPs) were surface-modified with dopamine-conjugated HA to produce stably dispersible HA-coated HApNPs in aqueous solution. HA-coated HApNPs and collagen were used to build up the nanocomposite multilayer on the surface of the biodegradable polymeric structure. The multilayer evolution was monitored using various surface analysis techniques, and the collagen content in the multilayer was determined. The cellular reactions of human mesenchymal stem cells including adhesion, proliferation, and osteogenic differentiation phenotype were

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examined with changing surface contents of HAp and collagen on the microstructured scaffold.

**Experimental**

**Surface modification of hydroxyapatite nanoparticles and characterization**

Hyaluronic acid (HA, Lifecore Biomedical, MN) was derivatized with dopamine (DA) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) chemistry according to the method previously reported.

In this study, HA with molecular weight of 130 000 was used and the conjugation degree of DA was about 13.5% (determined by $^1$H NMR). For surface modification, spherical HApNPs (average size: 100.48 ± 66.96, Sigma, St. Louis, MO) were dispersed in pure deionized water (DW) at a concentration of 5 mg ml$^{-1}$, followed by addition of various amounts of DA-modified HA. Ultrasonication was then applied to the prepared solution for 10 min using a Branson sonifier 450 (20 kHz, output control 3, duty cycle 30%). After vigorously stirring at 60 °C for 3 h, the solution was centrifuged at 3000g for 10 min to collect nanoparticles, followed by rinsing with DW and centrifugation to completely remove unbound HA molecules. Surface charge and hydrodynamic size of HApNPs surface-modified with HA were measured using a dynamic light scattering instrument (DLS, Zeta-Plus, Brookhaven). For morphological investigation, HApNPs were subjected to scanning electron microscopy (SEM, S-4800, Hitachi) after sputter-coating with platinum with 10 nm thickness. To confirm the surface atomic composition, X-ray photoelectron spectroscopy (XPS, VG Microtech ESCA 2000 LAB MK-short parallel spectrometer) equipped with a MgKα X-ray ($h\nu = 1253.6$ eV) source was used. The XPS measurement was performed with an energy increment of 1 eV for survey scan and the adventitious carbon C1s peak with the binding energy of 284.8 eV was used as the reference for calibration.

**Fabrication of 3D microstructured scaffold**

The polymeric microstructured scaffold was fabricated using a direct polymer melt deposition (DPMD) process described previously. The process included firstly melting a biodegradable polymer (poly($\varepsilon$-caprolactone), PCL, average molecular weight of ca. 80 000, Aldrich) in a heated barrel, extruding the molten polymer through a micro-scale nozzle (inner diameter = 400 μm) by applying compressed air, and finally depositing a microfibrous array to produce a three dimensional structured scaffold in a layer-by-layer manner. The resultant structure was a 3D woodpile-like microfibrinous structure with periodical void spaces. The extruded microfiber with 200 μm diameter was obtained by adjusting the process parameters including applied air pressure (0.4 MPa) and moving velocity of the nozzle (2 mm s$^{-1}$). For convenient analysis of surface LbL coating, thin PCL films were also prepared by hot pressing under about 5 MPa at 80 °C for 10 min.

**Layer-by-layer multilayer assembly**

The PCL substrates (films or scaffolds) were firstly modified via aminolysis to promote LbL adsorption. The substrates were cleaned by sonication with DW and 2-propanol for 15 min each, followed by drying under a stream of N$_2$ gas. The cleaned substrates were immediately immersed in distilled 1,6-hexanedi-amine/2-propanol solution with a 10 wt% concentration and allowed to react at 37 °C for 10 min. The aminated substrates were washed with DW and treated with 0.01 N HCl solution for 30 min, followed by a brief rinse with DW. HApNPs solution was prepared by dispersion in 0.1 M 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (pH = 4) at a concentration of 10 wt%. Collagen (type I from calf skin, Sigma, St. Louis, MO) was dissolved in acetic acid at 10 mg ml$^{-1}$ and diluted 5 times with DW to obtain a final concentration of 2 mg ml$^{-1}$, followed by adjusting the pH to 4.0 with 1 N NaOH or HCl. For LbL multilayer construction on the PCL film, HApNPs were firstly absorbed for 15 min under gentle agitation. After rinsing with DW, the substrates containing HApNPs on their surface were dipped into the collagen solution for another 15 min and this alternate adsorption process was repeated to obtain the desired number of layers. For the nth bilayer deposition of HApNPs and collagen, the multilayer was denoted as (HApNPs/collagen)$_n$. In the case of the 3D microstructured scaffold, the flow of the polyelectrolyte solutions at a rate of 0.5 ml min$^{-1}$ was sequentially applied to the scaffold by using a peristaltic pump (Won Corp., Korea) for conformal LbL deposition. While the scaffold was affixed in a plastic cylindrical flow cell attached at both ends with a silicone tube, a fluid flow alternately passed through the front to back of the vertically standing scaffold for 10 min. The adsorption and rinsing sequences on the scaffolds were the same as those on the films.

**Characterization of multilayer**

The HApNPs/collagen multilayer build up was analyzed by tapping-mode atomic force microscopy (AFM) using a Dimension 3100 AFM (Digital Instruments/Veeco, Woodbury, NY). For the thickness measurement, the multilayer was freeze-fractured by a razor blade, and scans of 5 μm × 5 μm on the fractured boundary were taken at a scanning rate of ~0.5 Hz ($n = 10$). Multilayered collagen content was determined using a colorimetric ninhydrin method. In brief, various HApNPs/collagen multilayers constructed on the PCL film were put into a mixture of 1 ml of 6 N HCl and 4 ml of phosphate buffered saline (PBS) solution and heated at 120 °C under a nitrogen atmosphere for 6 h to hydrolyze the collagen to amino acids. After centrifugation of the resultant solution, the supernatant (1 ml) was neutralized with 1 N NaOH and then the ninhydrin reagent solution was added. The solution was boiled for 15 min to ensure complete color development and then cooled to room temperature. After adding 5 ml of 50% ethanol, the absorbance at 570 nm was measured by using a UV spectrophotometer (UV-1601, Shimadzu). Collagen content was determined using a standard calibration curve from known amounts of collagen while subtracting the contribution from the aminated PCL film. The structure of the LbL multilayer was confirmed with attenuated total reflection infrared spectroscopy (ATR-FTIR, Hyperion 3000, Bruker Optiks). The spectra in absorbance mode were obtained with a resolution of 4 cm$^{-1}$ and an average of 128 scans for each sample in the range 700-4000 cm$^{-1}$. All spectra acquired were analyzed by using an OPUS software package (Bruker Optiks). Scanning electron microscopy (SEM)–energy dispersive X-ray...
spectrometry (EDX) micro-analysis (INCA Energy 350, Oxford Instruments, Witney, UK) was performed on the HApNPs/collagen multilayer to investigate the elemental composition.

hMSCs culture and seeding on scaffolds

Human mesenchymal stem cells (hMSCs, Lonza, MD) were grown in α-modified eagle’s medium (α-MEM, Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Gibco, Gaithersburg, MD) and antibiotics (100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin) and maintained in a 75 cm² tissue culture flask in a 5% CO₂ incubator at 37 °C. hMSCs were passaged at 80% confluence and used at a passage number of 4–5 for this study. Prior to cell seeding, the scaffolds were sterilized by ultraviolet irradiation, soaked in 75% ethanol, and then rinsed with sterile DW and PBS solution. The cultured cells were harvested with 0.05/0.02% trypsin/EDTA, centrifuged at 1000 rpm for 3 min and resuspended in the culture medium, followed by cell-counting using a haemocytometer. The hMSCs suspension (0.2 ml, 2 × 10⁵ cells) was inoculated drop-wise on both sides of the scaffolds in 24-well cell culture plates. After 30 min incubation, culture medium was added to a final volume of 2 ml and the cell/scaffold constructs were incubated for a predetermined period. Four hours after seeding, cellular adhesion was investigated using a CCK-8 assay kit (CCK-8, Dojindo laboratories, Kumamoto, Japan) containing a fluorescent dye for staining double-stranded DNA. For each time point, the cell/scaffold constructs were treated with 0.05% trypsin/EDTA for 30 min with occasional gentle vortexing. The detached cells from scaffolds were harvested and lysed with a lysis buffer (0.5% Triton X-100, 20 mM Tris-HCl, 1 mM EDTA, 0.02% trypsin/EDTA) for 30 min with occasional gentle vortexing. This journal is

Results and discussion

Hyaluronic acid-modified hydroxyapatite nanoparticles

Pure HApNPs have a relatively low surface charge and are easily aggregated to precipitate in aqueous solution, which has led to the development of surface modified HApNPs for stable dispersion. Recently, dopamine (3,4-dihydroxyphenylethylamine, DA) inspired by mussel adhesion protein and DA-derivatized polymers were proven to have an extraordinary adhesion property to a broad range of materials mostly by catechol-mediated interaction. Catechol groups can be stably bound onto various organic, inorganic, and metallic surfaces through noncovalent interactions including chelation with metal ions. In particular, catechol and its derivatives were shown to have strong affinity to hydroxyapatite. It was also demonstrated that glycosaminoglycans such as chondroitin sulfate, heparan sulfate, and HA could interact with the surface of HAp by ionic interactions with conformational matching. Dopamine-modified HA (HA-DA) was previously utilized to coat...
inorganic and metallic surfaces including iron oxide and stainless steel.\textsuperscript{22,23} In this regard, HA-DA was here exploited to modify HApNPs to enhance the stability of their colloidal dispersion by highly negative charge and steric repulsion effects of HA. HA is well known to play an important role in cellular signaling and proliferation.\textsuperscript{31} Thus, it is likely that HA immobilized onto the surface of HApNPs can synergistically induce an additional and beneficial effect on the adhesion, proliferation, and differentiation of hMSCs with other bioactive components. The alteration of surface charge was monitored with addition of various amounts of HA-DA to the HApNPs solution (Fig. 1A). With increasing the concentration of HA-DA, surface charge values changed from near zero to $-28$ mV, a saturation plateau value at a HA-DA concentration of 2 mg ml$^{-1}$. The average hydrodynamic size of the HA-DA-modified HApNPs was around 280 nm (Fig. 1A inset). A representative SEM image of HApNPs modified with HA-DA is shown in Fig. 1B. Partial agglomerates composed of spherical particles with diameter of approximately 100 nm were observed, which were formed possibly due to linear assembly of HApNPs along the HA chain. For dispersion stability test, three different samples of pure, native HA-bound, and HA-DA-modified HApNPs were dispersed in PBS solution (Fig. 1C). After 24 h, while pure HApNPs were mostly precipitated, HA-DA-modified HApNPs showed superior colloidal stability to native HA-bound HApNPs. These results demonstrate that HA-DA molecules containing multiple catechol groups can be securely anchored onto the HAp surface via catechol-HAp interactions. Thus, HA-DA-modified HApNPs can be used as building blocks for multilayer assembly on the basis of relatively high negative surface charge and excellent colloidal stability. To confirm the surface atomic composition, pure HAp and HA-DA-modified HApNPs were subjected to XPS analysis (Fig. 1D). The survey spectrum before coating confirms HApNPs elemental composition (peaks at binding energy of 533 eV, 349 eV, and 135 eV for O$_{1s}$, Ca$_{2p}$, and P$_{2p}$, respectively). After modification with HA-DA, HAp signals (Ca$_{2p}$ and P$_{2p}$ peak) were completely attenuated while a new N$_{1s}$ peak (399 eV) was developed and the intensity of the carbon C$_{1s}$ signal (285 eV) significantly increased. The oxygen-to-carbon atomic ratio (O/C) was calculated to be 0.68, which is similar to the theoretical value for HA (O/C $\approx$ 0.7).\textsuperscript{32} The XPS analysis results confirmed the existence of HA on the HApNPs surface with a thickness of at least 10 nm (judged from the limited escape depth of photoelectrons).

Layer-by-layer multilayer assembly

As illustrated in Fig. 2A, alternating LbL adsorption of two oppositely charged species (anionic HA-DA-modified HApNPs and cationic collagen at pH = 4) produced a nanocomposite multilayer coated on the PCL surface. The HApNPs/collagen multilayer was constructed on the aminolysed PCL film for characterization of the buildup process. The surface topographical evolution during the LbL process was monitored by AFM images. After the build-up of (HApNPs/collagen)$_3$, depleted voids on the surface were often observed with a multilayer coverage of 60–70% (Fig. 2B). At the end of 5 deposition cycles, the surface coverage percent increased to more than 80% with minor blank spaces (Fig. 2C). As shown in Fig. 2D and E, after 10 deposition cycles, the multilayer completely covered the substrate area, and the root-mean-square roughness value also gradually increased from 35.8 nm for (HApNPs/collagen)$_{10}$ to 53.6 nm for (HApNPs/collagen)$_{20}$. After depositing 20 HApNPs/collagen layers, surface morphology was investigated by SEM.
The HApNPs/collagen multilayer thickness was estimated by AFM height profiling along the two-point straight line (Fig. 3A and inset). Freeze-fractured edge of the multilayer showed an abrupt increase in height from the flat surface of PCL substrate film, indicating that the height difference across the fractured edge represents the multilayer thickness. As shown in Fig. 3B, the linear relationship between the number of layers and thickness was observed, showing the increase in thickness from 281.3 ± 51.4 nm for 3 bilayers to 1627.9 ± 109.3 nm for 20 bilayers. ATR-FTIR analysis was carried out in order to confirm the composition of the multilayer. From the collagen IR spectrum, three distinctive peaks such as N–H stretching at ~3300 cm⁻¹ for amide A, C=O stretching at ~1635 cm⁻¹ for amide I, and N–H deformation at ~1550 cm⁻¹ for amide II can be observed. The HApNPs spectrum revealed one prominent absorption peak at ~1030 cm⁻¹ corresponding to the PO₄³⁻ v3 mode. After LbL multilayer assembly, combined absorption peaks characteristic of both collagen and HApNPs were detected on the coated surface, indicative of a successful formation of the nanocomposite multilayer. With increasing number of deposited layers, the deposited amount of collagen grew exponentially (Fig. 3C). As mentioned above, the surface roughness increased with the layer deposition cycle, meaning that the surface area available for the adsorption of the next layer also increased. Thus, increased surface area provided more chance for more collagen adsorption, causing an exponential growth of collagen content.

**Nanocomposite coating of 3D microstructured scaffold**

The biodegradable polymeric scaffold, 3D woodpile-like microstructure composed of PCL microfibers, was fabricated using a DPMD process (fiber diameter = 200 µm, fiber spacing = 400 µm) (Fig. 4A). 3D coating by LbL is generally considered to be more difficult than 2D planar coating due to inhomogeneous deposition, originating from gravity, surface curvature, and uncontrollable convective flow in a coating medium.³³ To realize the conformal coating of 3D complex microstructure, the flow of coating medium was needed to be precisely controlled along the surface of the microstructure. In this study, the upper and lower sides of the scaffold were sequentially exposed to continuous flow of HApNPs and collagen solution at a constant flow rate. As a result, this coating strategy allowed more uniform multilayer deposition of charged species compared to passive coating under static conditions (judged from SEM analysis, data not shown). As shown in Fig. 4B–E, the nanocomposite multilayer was evenly deposited throughout the surface of the microfibers. The surface topography showed the HApNPs-embedded collagen network structure with open porosity consistent with the planar substrate. By EDX analysis, the atomic ratio of calcium-to-carbon (Ca/C) at different locations was monitored throughout the scaffold. The Ca/C value of ~0.73 was relatively invariable with less fluctuation, resulting in homogeneous nanocomposite coating on the 3D microstructured scaffold.

**In vitro hMSCs growth and osteogenic differentiation**

For an ideal tissue engineering scaffold, the scaffold should provide a cell-favorable microenvironment for supporting cellular adhesion and proliferation. The seeded hMSCs were attached evenly along with the defined architecture of the scaffold and were grown on the...
skeletal structure without occupying the inner macropore space. As shown in Fig. 5A, the adhesion of hMSCs onto the scaffold coated with nanocomposite multilayer was greatly enhanced as compared to bare scaffold (~230% increase). In addition, cellular adhesion was significantly enhanced with the number of deposited layers. For assessment of hMSCs proliferation, DNA amount was quantified as a function of incubation period (Fig. 5B). As expected, the presence of surface multilayer played an important role in favoring the cellular growth in the scaffold. In addition, the multilayer coverage with appropriate thickness seems to have prominent effects on cell proliferation. Among the multilayer components, collagen can be a major component responsible for the cellular adhesion and proliferation. Collagen is known to have a variety of cell adhesive peptide moieties for anchoring the cells and acts as a structural cue for stimulating the cell growth signal. As a consequence, surface chemical and topographical cues created by HApNPs/collagen LbL multilayer deposition mimicked the natural ECM to some extent.

During osteogenic induction of hMSCs seeded on various types of scaffolds, the activity of ALP, which is a conventional marker of osteoblastic activity, was monitored at 3, 7, 12, and 21 days to confirm the effect of the multilayer coated scaffold on osteogenic differentiation. As shown in Fig. 6A, ALP production by hMSCs increased overall with osteogenic induction time, whereas the ALP activity in monolayer-cultured hMSCs dropped at day 21. ALP activity was 1.83-fold greater for hMSCs cultured in the scaffold having 20 bilayers of HApNPs/collagen than for those cultured in the bare scaffold. In consistent with hMSCs adhesion and proliferation, ALP activity increased with number of deposited layers, indicating that the multilayer components, HApNPs and collagen, could facilitate matrix-mediated intracellular signaling related to osteoblastic activity.

To further prove osteogenic differentiation, quantitative real-time RT-PCR analysis was performed for typically expressed osteoblast-related genes including bone sialo protein-II (BSP), bone morphogenetic protein-2 (BMP2), osteopontin (OP), and osteocalcin (OC). For hMSCs cultured on the 20 bilayer-coated scaffold, expression levels of the investigated genes except for OC were unregulated at 1.64, 1.85, and 4.45-fold for BSP, BMP2, and OP, respectively, when compared to hMSCs cultured on the bare scaffold. On the other hand, hMSCs cultured on the 5 bilayer-coated scaffold showed no significant differences in BSP, BMP2, and OP gene expression, as compared to the bare scaffold. The OC gene from hMSCs cultured on the 20 bilayer-coated scaffold was slightly down-regulated as compared to other cases. However, the OC gene level was statistically not significant compared to those of bare and 5-bilayer scaffolds. The increase in BMP2 expression is known to increase mRNA levels and protein synthesis of BSP and OP as a result of the autocrine effect of BMP2. The simultaneous up-regulation of three marker genes (BSP, BMP2, and OP) observed in this study can be attributed to a biomimicking osteogenic surface created by HApNPs/collagen multilayer deposition. The results indicated that the osteoblast-related gene expression can be readily regulated in an ECM-mediated manner. Cell contact with ECM proteins is of great importance for regulating osteogenic differentiation of hMSC. The gradual increment in multilayer thickness, coverage, and roughness with increasing number of deposition cycles could result in increasing the number of cellular recognition sites on the scaffold surface. Thus, the improved cellular responses such as adhesion, proliferation, and differentiation can be attributed to the enhanced contact of hMSCs to the bioactive multilayered surface of the scaffold.
Conclusions
In this study, an LbL multilayer coating strategy was successfully applied to a RP-based microstructured scaffold for the first time. We first surface-modified HApNPs with HA containing catechol groups, resulting in highly negatively charged HA-coated HApNPs with stable dispersibility in aqueous solution. The modified HApNPs and collagen were used as building blocks for LbL multilayer assembly, which produced a nanocomposite coating with an open porous network structure on the surface of a biodegradable PCL microstructured scaffold. The amounts of HAp and collagen on the surface were easily controlled by adjusting the number of deposited layers. In the growth conditions of hMSCs, the nanocomposite multilayer-coated scaffold induced significantly enhanced cell adhesion and proliferation. When the hMSCs/scaffold constructs were placed in osteogenic conditions, ALP activity and osteoblast-related gene expressions increased in a matrix-dependent manner. The main advantage of this multilayered coating strategy is that the composition and density of hydroxyapatite and collagen can be readily controlled on the scaffold surface. The introduction of other charged bioactive ingredients such as bone growth factors onto the multilayer assembly can further facilitate bone tissue regeneration.

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Notes and references