International Journal of Pharmaceutics 384 (2010) 181–188

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Controlled gene-eluting metal stent fabricated by bio-inspired surface modification with hyaluronic acid and deposition of DNA/PEI polyplexes

Taek Gyoung Kim, Yuhan Lee, Tae Gwan Park*

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, South Korea

ARTICLE INFO

Article history:
Received 31 July 2009
Accepted 22 September 2009
Available online 30 September 2009

Keywords:
Drug eluting stent
Dopamine
Hyaluronic acid
Gene delivery
Transfection

ABSTRACT

A metal stent that could elute plasmid DNA (pDNA) in a controlled manner for substrate-mediated gene transfection was fabricated by first coating with hyaluronic acid (HA) and subsequent deposition of pDNA. To create robust HA coating layer on stainless steel (SS316L) surface, HA was derivatized with dopamine which is a well-known adsorptive molecule involving mussel adhesion process. The HA-coated surface was verified by various analytical techniques and proved to be very hydrophilic and stable, also showing superior biocompatibility in terms of suppressed plasma protein adsorption. For surface loading of pDNA, cationic pDNA/polyethylenimine (PEI) polyplexes were prepared and ionically adsorbed onto the HA-coated SS316L surface. The adsorbed surface exhibited evenly distributed nano-granular topography while the polyplexes maintained the nano-particular morphology. The pDNA was released out in a controlled manner for a period of 10 days with maintaining structural integrity. The dual coated substrate with HA and pDNA/PEI polyplexes exhibited greatly enhanced gene transfection efficiency, when compared to both bare substrate adsorbed with the polyplexes and PEI/pDNA polyelectrolyte multilayers. Dually functionalized stent with HA and pDNA exhibited effective biocompatibility and gene transfection.

1. Introduction

During percutaneous coronary intervention, an expandable metallic stent has been served as a scaffolding material for preventing the re-occlusion of coronary artery (Acharya and Park, 2006; Gaffney et al., 2007; Venkatraman and Boey, 2007). However, during the deployment of the stent, a target blood vessel can be often injured with denudation of contacting endothelial cells. The bare metal stents are also known to elicit undesirable inflammatory and thrombogenic responses upon contact with the inner surface of blood vessel (Tamai et al., 2000). These induce abnormal hyper-proliferation of vascular smooth muscle cells (VSMCs), resulting in arterial re-obstruction, that is, in-stent re-stenosis (Regar et al., 2001). Drug-eluting stent (DES) has been recently utilized as an advanced approach to minimize the possibility of re-stenosis. With the DES angioplasty, the incidence of various stent related complications has been outstandingly reduced by the loco-regional surface delivery of a variety of small molecular weight anti-proliferative or anti-inflammatory agents such as paclitaxel and rapamycin to the neointimal area (Venkatraman and Boey, 2007). Therapeutic macromolecular drugs (e.g., proteins, DNA/virus, polysaccharides) have been also utilized as an active therapeutic agent for DES (Takahashi et al., 2007). In particular, intravascular gene delivery has been considered as a promising therapeutic approach in peripheral vascular disease and coronary artery disease (Simons et al., 1992). For example, a wide range of therapeutic DNA and viruses were delivered from the metallic stent surface to reduce the extent of re-stenosis (Klugherz et al., 2000; Perlstein et al., 2003; Fishbein et al., 2006). The stent-mediated DNA delivery enables effective gene transfection to the adjacent cells in a localized fashion (Klugherz et al., 2000). This is simply because surface immobilized DNA complexes have more chance to be transported within adhered cells. Intravascular stent coated with multilayered polyelectrolytes DNA films also showed the controlled release of DNA from the surface and effective cell transfection (Jewell et al., 2006).

For robust surface modification of a metal surface with synthetic and natural polymers for drug loading and elution, the metal surface should be first functionalized with various physical and chemical techniques such as gold deposition, plasma treatment, silanization, and pre-deposition of adhesive polycations (Nanci et al., 1998; Pitt et al., 2004; Charlot et al., 2007; Huang and Yang, 2008). For instance, biocompatible hyaluronic acid (HA) was immobilized on the stainless steel surface by pretreatment of reactive silane groups and subsequent covalent immobilization of hydrazide-derivatized HA (Pitt et al., 2004). Highly charged and

* Corresponding author. Tel.: +82 42 350 2621; fax: +82 42 350 2610.
E-mail address: tgpark@kaist.ac.kr (T.G. Park).

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doi:10.1016/j.ijpharm.2009.09.042

cationic polyethylenimine (PEI) was also directly adsorbed on the stainless steel surface for subsequent deposition of anionic heparin (Tan et al., 2003). A sequentially assembled PEI/heparin multilayer showed prolonged blood clotting time with reduced platelet adhesion. However, the PEI coated metallic surfaces exhibit undesirable cell toxicity, and the PEI surface layer was often delaminated when incubated under long-term physiological conditions. A facile and robust polymer coating strategy is highly needed for surface modification of metallic substrates with biocompatible polymers.

L-3,4-Dihydroxyphenylalanine (DOPA), an unusual amino acid highly present in mussel adhesive proteins, has been known to play a major role in strong adhesion of a mussel onto a variety of substrates in tidal environment (Deming, 1999; Yu et al., 1999). It has been established that a catechol group of DOPA is critically responsible for underwater adhesion to various inorganic and organic surfaces such as noble metals, oxides, ceramics, and plastics by combined, yet unknown mechanisms of metal complexation, hydrogen bonding, and π–π and π–cation interactions (Lee et al., 2006, 2007; Even et al., 2008). Dopamine (3,4-dihydroxyphenylethylamine, DA) is an analogue of DOPA, and has been recently used for facile modification of various surfaces by forming a melanin-like thin layer (polydopamine film), which could be used as a platform layer for additional deposition of metal nanoparticles and biomacromolecules (Lee et al., 2007). DA-derivatized polymers such as anionic HA and cationic PEI have also been used for modifying many different surfaces including magnetic nanoparticles (Lee et al., 2008). HA, a major glycosaminoglycan component of extracellular matrix, was previously employed for biocompatible and non-thrombogenic stent coating, demonstrating that the thrombus accumulation on the HA-coated stent could be effectively reduced (Verheye et al., 2000).

In this study, we present a new strategy for fabricating gene-eluting metallic stents by producing robust HA surface layer and subsequently depositing plasmid DNA on the surface for enhanced surface-mediated gene delivery. First, HA was derivatized with DA (HA-DA) by using carbodiimide chemistry. Austenitic stainless steel 316L (SS316L), which has been practically used for stent fabrication due to its great strength and corrosion resistance, was directly coated with HA-DA by a mussel-inspired adhesion mechanism. After the coating, the HA-coated SS surface was characterized to ensure its long-term stability in aqueous solution and the resistance of nonspecific protein adsorption upon contact with platelet-poor plasma. Cationic GFP plasmid DNA/polyethylenimine (PEI) polyplexes were then adsorbed on the HA-coated SS substrate. The dually functionalized metal substrate with HA and pDNA was characterized by assessments of the release rate of the pDNA and the gene transfection efficiency.

2. Materials and methods

2.1. Materials

Austenitic stainless steel (SS) 316L sheets (10 mm × 10 mm, 3 mm thick) and coronary SS stents (18.2 mm length × 1.6 mm i.d.) were kindly donated by Severance hospital (Seoul, Korea). Sodium hyaluronate (HA) (MW 130K) was purchased from Lifecore Biomedical (Chaska, MN). Dopamine hydrochloride (DA), 1-ethyl-3-(3-dimethylaminopropyl), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and polyethylenimine (PEI, branched, MW 25K) were obtained from Sigma–Aldrich (St. Louis, MO). RBS-35 detergent was purchased from Pierce (Rockford, IL). Plasmid DNA (pEGFP-C1) with a cytomegalovirus promoter was extracted from transformed *Escherichia coli* by using a DNA-purification column, and stored in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH = 7.5) at −20 °C until use. Plasmid DNA purity was analyzed by agarose gel electrophoresis and the concentration was determined by measuring a relative absorbance value at 260 and 280 nm. PicoGreen reagent was acquired from Molecular Probes (Eugene, OR).

2.2. Synthesis of HA-DA conjugate

HA was derivatized with DA using EDC as a coupling agent. Briefly, HA was dissolved in 250 ml of pure deionized water (DW) to a concentration of 2 mg ml^{-1}. To the solution, 237 mg of DA and 174 mg of EDC were added and the reaction mixture was stirred for 2 h at room temperature while maintaining pH value at 4.7 by addition of 0.1 N HCl. After reaction, HA-DA conjugate was precipitated in ethanol, re-dissolved in DW, and then purified by dialysis against 100 mM NaCl solution for 1 day and against DW for 2 days. The degree of DA substitution was determined from ^{1}H NMR spectroscopy (DRX 300, Brucker) in D_2O. Approximately 17 mol% of DA was substituted to initial carboxylic acid groups of HA.

2.3. Metal surface coating with HA-DA conjugate

For removing surface contaminants, SS316L sheets and stents were thoroughly cleaned by sonication with 5% RBS-35 detergent solution, acetone, and 2-propanol for 15 min each, followed by rinsing with DW. For HA coating, the cleaned metal samples were vertically immersed in an aqueous neutral (10 mM PBS, pH = 7.4) or slightly basic (10 mM Tris–HCl, pH = 8.0) buffer solution containing HA-DA conjugate at a concentration of 3 mg ml^{-1} and further incubated at room temperature or 50 °C for different periods up to 48 h for optimizing HA coating conditions. After coating, each sample was rinsed with excess amount of DW and dried under a stream of N_2 gas.

2.4. Surface characterization

The coating thickness of HA on the silicon wafer surface was evaluated by using a Gaertner L116 s ellipsometer (Gaertner Scientific Corporation, IL) equipped with a He–Ne laser (632.8 nm) at a 70° angle of incidence. Static water contact angles for bare and HA-coated substrates were measured using a contact angle measurement system (SEO 300A, SEO Co., Korea) mounted with a CCD camera at an ambient condition. A 10 mm × 10 mm sample was placed on a sample stage and a sessile drop of Milli-Q water was carefully applied to the metal surface. The surface elemental composition was analyzed by X-ray photoelectron spectroscopy (XPS, VG Microtech ESCA 2000 LAB MK-short parallel spectrometer) equipped with a Mg Kα X-ray ($h\nu$ = 1253.6 eV) source. The XPS measurement was performed with an energy increment of 1 eV for broad scans and 0.1 eV for high resolution scans. The morphological characterization of the sample surfaces was performed with scanning electron microscopy (SEM, S-4800, Hitachi) after sputter-coating with platinum with 10 nm thicknesses. Atomic force microscopy (AFM) image was obtained under ambient conditions using PSIA XE-100 AFM system (Santa Clara, CA) operating in a tapping mode.

2.5. Plasma protein adsorption on HA-coated surface

Human whole blood drawn from a healthy donor was collected in a Vacutainer® Plus Plastic Citrate Tube (BD Diagnostics, Franklin Lakes, NJ) and centrifuged at 3000 × g for 10 min at 22 °C. The platelet-poor plasma (PPP) freshly obtained was diluted to 50% with Dulbecco's phosphate-buffered saline (PBS, pH 7.4) solution. The PPP solution (0.5 ml) was each added onto the surface of the bare or HA-coated SS substrate, and incubated at 37 °C for 3 h. After gently retrieving the samples from the PPP solution, the substrates

were rinsed 5 times with PBS solution, followed by drying with filtered air. The adsorption extent of plasma proteins on the surface was evaluated by attenuated total reflection infrared spectroscopy (ATR-FTIR, Hyperion 3000, Bruker Optiks). For each measurement, 128 interferograms at a resolution of 4 cm^{-1} were collected and averaged over a range of 2000–1200 cm^{-1}. All spectra taken were processed by using an OPUS software package (Bruker Optiks). Due to the inherent amide signal of HA-DA conjugate, intensities of two characteristic amide peaks from the proteins adsorbed on the coated surface were adjusted by considering the presence of HA-DA conjugate on the surface.

2.6. pDNA/PEI complexation and electrostatic deposition onto the surface of HA-coated SS substrate

For preparing pDNA/PEI polyplexes, pDNA (100 μg) dissolved in 100 μl of DW was mixed with PEI (133 μg) dissolved in 100 μl of DW at a nitrogen/phosphate ratio (N/P) of 10, followed by vortexing and incubation for 15 min. After the formation of complexes, the solution was diluted to a desired final volume at an appropriate pDNA concentration with 10 mM NaCl solution (pH = 6.5). Surface charge and hydrodynamic size of the resultant polyplexes were measured using dynamic light scattering instrument (DLS, Zeta-Plus, Brookhaven). For depositing polyplexes on the surface, bare and HA-coated SS substrates were immersed into 0.5 ml of pDNA/PEI polyplex solution at various pDNA concentrations for 1 h under gentle agitation at room temperature, rinsed with DW three times, and dried under a laminar flow of filtered air. The amount of loaded pDNA on the surface was measured by PicoGreen assay. Briefly, the coated substrates deposited with pDNA polyplexes were treated with 3 ml of heparin solution (20 mg ml^{-1}) to extract pDNA in a solution phase. The procedure was repeated until pDNA elution was not detected. The PicoGreen reagent was added to the pDNA solution to measure fluorescence intensity at an excitation of 480 nm and an emission of 520 nm. A calibration curve was constructed from known quantities of DNA. For control experiments, PEI/pDNA multilayer was also assembled via a layer-by-layer fashion on the surface of HA-coated SS316L as described previously (Sukhorukov et al., 1996; Pei et al., 2001; Ren et al., 2005).

2.7. In vitro pDNA release

In vitro DNA release was performed by incubating pDNA/PEI polyplex loaded bare, HA-coated SS sheet and coronary stent in 1 ml of PBS solution at 37 °C in a humidified 5% CO_2 environmental incubator under gentle shear condition. At predetermined time points, 100 μl of release medium was removed and replaced with an equal volume of fresh buffer medium. The released DNA amount was quantified by PicoGreen assay as described above. Experiments were all performed in triplicate. The integrity of collected DNA was confirmed by agarose gel electrophoresis with ethidium bromide staining.

2.8. Cell culture and viability test

COS-7 cell line was used for evaluating cell viability and GFP gene transfection efficiency. COS-7 cells were routinely maintained in the culture medium consisting of Dulbecco's modified Eagle's medium (DMEM, Grand Island, NY) supplemented with 10% FBS, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin under a humidified atmosphere of 5% CO_2 at 37 °C. COS-7 cells harvested from a tissue culture flask using trypsin/EDTA were inoculated onto various substrates at a seeding density of 5×10^4 cells cm^{-2}. The culture medium was replenished every day. After 2-day culture, cell viability was evaluated by CCK-8 assay kit (CCK-8, Dojindo laboratories, Kumamoto, Japan) that generated an orange formazan product by cellular dehydrogenases. Briefly, each substrate was removed from the incubation medium, washed with PBS solution three times, and placed into 1 ml of fresh culture medium containing the CCK-8 solution. After 2 h incubation, 100 μl of each medium was transferred to a 96-well plate for measuring absorbance value at 450 nm using a microplate reader (Bio-Rad, Model 550).

2.9. Transfection in vitro

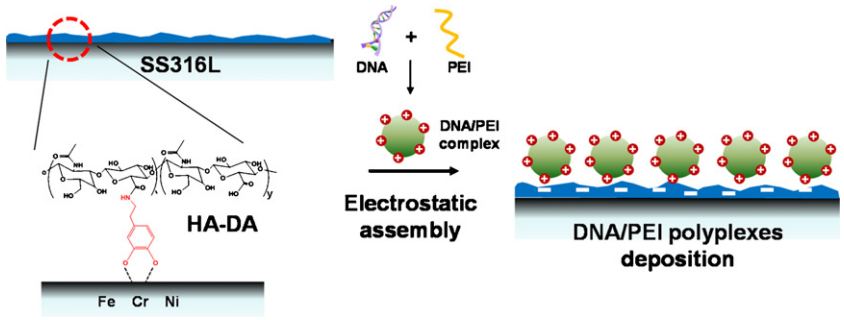
COS-7 cells in the culture medium were plated onto various substrates deposited with pDNA/PEI polyplexes at a seeding density of 10^5 cells cm^{-2} and incubated for 3 days with replenishing the medium daily in a humidified 5% CO_2 incubator at 37 °C. As a control, pDNA/PEI polyplexes (N/P ratio of 10, an equivalent amount of pDNA depositing onto the surface of HA-coated SS substrate) were added to pre-plated cells in a 24-well tissue culture plate. Following 5 h incubation, the culture medium was replenished every day. For gene transfection for the HA-coated coronary stent deposited with pDNA/PEI polyplexes, the stent were placed into a siliconized cylindrical tube, and 1.5 ml of cell suspension (1×10^5 cells ml^{-1}) was added. After incubating for a total of 4 h with intermittent agitation for 3 min an every half hour, the resultant cell/stent constructs were washed with DMEM and transferred to a siliconized 12-well tissue plate containing the culture medium for additional incubation. After 3 days of incubation, GFP gene transfected cells were directly visualized using laser scanning confocal microscopy (LSCM, LSM5100, Carl Zeiss). To determine GFP gene expression level, transfected cells were lysed with 1% Triton X-100 and the green fluorescence intensity was quantified by a spectrofluorometer with an excitation of 488 nm and an emission of 509 nm. The measured fluorescence intensity was normalized by the total cellular protein amount that was determined by BCA assay (Pierce, Rockford, IL).

3. Results and discussion

3.1. Bio-inspired HA coating on SS316L substrate

The ultimate goal of surface modified biomedical devices is to accomplish bio-mimicking adaptation over diverse body responses with retaining sufficient biocompatibility at the implant site (Chen et al., 1997, 2008; Holland et al., 1998). A variety of surface modification methods with vastly different polymers have been attempted to minimize or eliminate adverse cell-biomaterial interactions, such as inflammation, thrombosis, and hypersensitive reaction, occurring at the interfaces in immediate contact with human tissues and organs (Dalsin et al., 2005; Zurcher et al., 2006; Cho et al., 2007; Yang et al., 2008; Yuan et al., 2008). In this regard, surface modification techniques bio-inspired from nature would be valuable to create bio-mimicking and biocompatible devices. Recently, it was reported that a catechol group, abundantly present in mussel adhesive proteins, offers a unique adhesive moiety for facile surface modification. In this study, dopamine containing a catechol moiety was conjugated to the backbone of HA. HA-DA conjugate was used for robustly coating the SS316L substrates mainly composed of Fe, Cr, and Ni, and then pDNA/PEI polyplexes were electrostatically adsorbed on the HA-coated surface for enhanced substrate-mediated gene delivery, as shown in Scheme 1.

To optimize HA coating condition, SS316L substrates were incubated in neutral (pH 7.4) or slightly basic (pH 8) buffer solution containing HA-DA at 37 °C. After 24 h, the substrate incubated under basic solution evidently showed the formation of a thick HA layer in contrast to that formed under neutral pH condition (determined from XPS and AFM, data not shown). Surface color for the HA-coated SS substrate was changed to light brown, indicative of



Scheme 1. Schematic representation of the surface coating with HA and subsequent electrostatic deposition of DNA/PEI polyplexes on SS316L substrate.

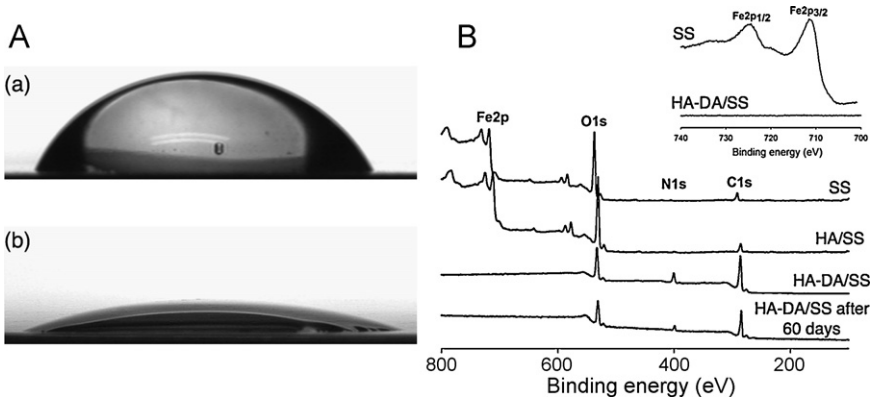


Fig. 1. (A) Static water contact angles of the bare (a) and HA-coated SS substrates (b). (B) XPS survey scan of bare SS, pristine HA-treated SS, HA-coated SS, and HA-coated SS after 60 days incubation in PBS at 37 °C. (Inset) The multiplex scan of Fe_{2p} of bare and HA-coated SS substrates.

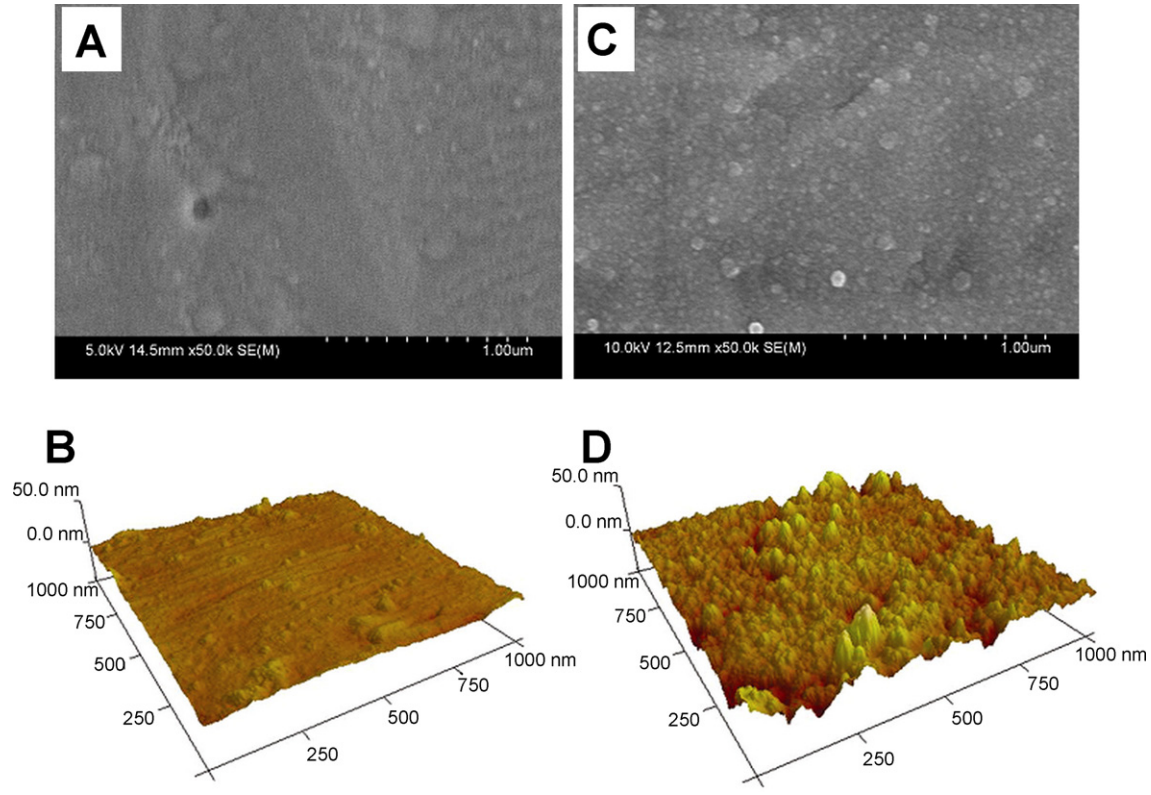


Fig. 2. Surface morphology acquired by SEM (A and C) and AFM (B and D) of bare (A and B) and HA-coated (C and D) SS substrates.

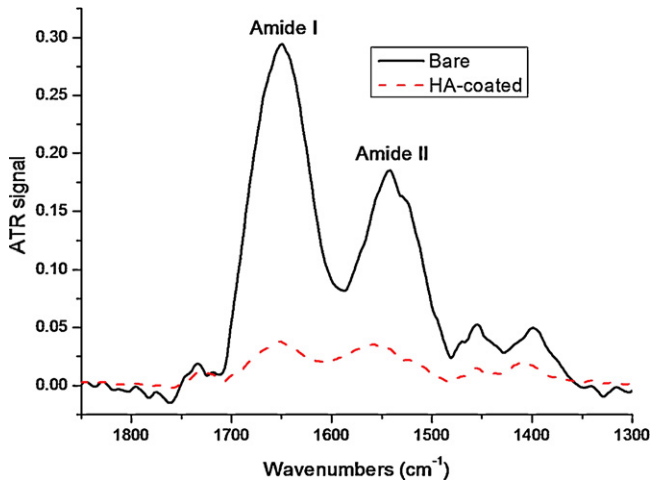


Fig. 3. Demonstration of the resistance to human plasma protein adsorption. ATR-FTIR spectra of bare and HA-coated SS substrates after exposed to human PPP at 37 °C for 3 h.

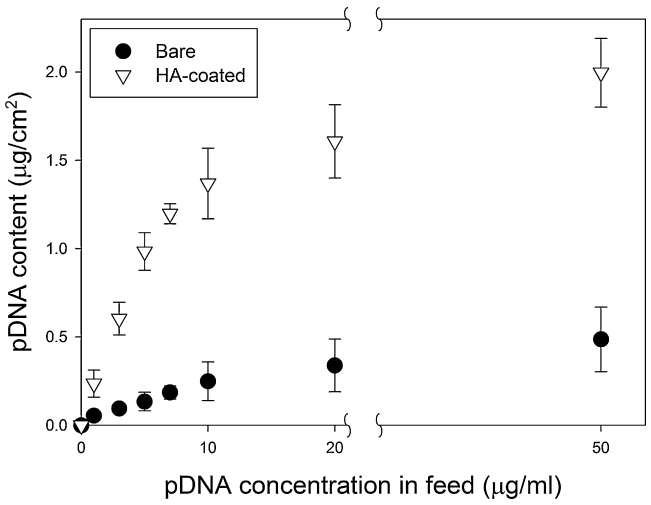


Fig. 4. Surface pDNA/PEI polyplexes adsorption. Increase of assembled DNA content on bare and HA-coated SS substrates as function of DNA concentration in the feed.

dopamine oxidation and crosslinking (Lee et al., 2007). An ellipsometry analysis was performed for measuring the coating thickness of HA layer under basic aqueous condition at different temperatures and incubation times. Since the SS surface was very rough with poor reflection, the thickness measurements were conducted on a silicon oxide substrate. The thickness of HA layer was negligibly changed at room temperature, but substantially increased at 50 °C up to approximately 12 nm after 24 h (data not shown). This

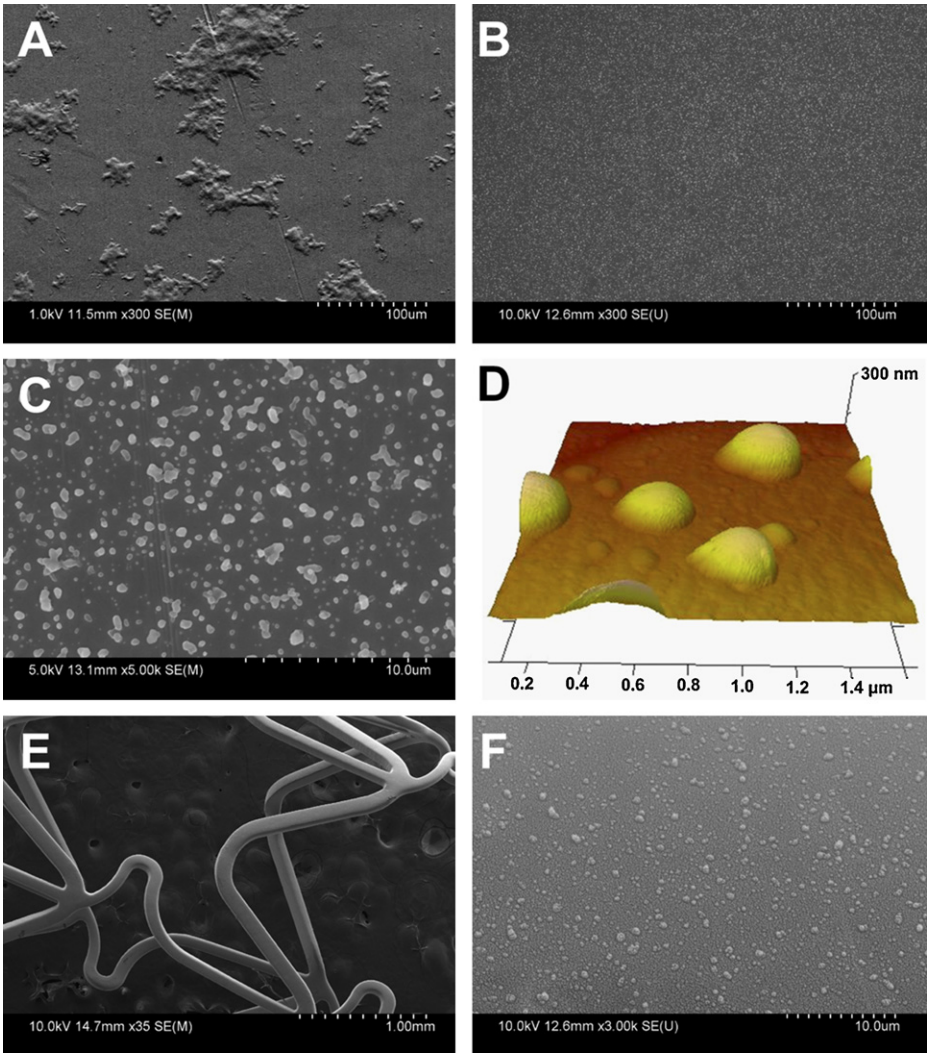


Fig. 5. Deposition with pDNA/PEI polyplexes on bare (A), and HA-coated (B–D) SS substrate. Expanded HA-coated SS coronary stent (E) and its nano-topographical surface (F) assembled by pDNA/PEI polyplexes.

layer growth under slightly basic condition in combination with elevated temperature can be correlated with the rapid oxidation of catechol groups, resulting in the formation of o-quinone groups with further self-crosslinking (Deming, 1999). It is known that an oxidized quinone form of dopamine self-polymerizes to produce a sticky polydopamine layer on the surface of various substrates under basic condition by inter- and intra-molecular crosslinking (Yu et al., 1999). Thus, it is reasonable to say that HA-DA was stably coated on the SS surface by direct catechol–metal coordination interactions as well as the quinone-induced HA-DA crosslinking, resulting in a HA-DA self-crosslinked thin hydrogel layer on the surface of SS substrate. Based on these observations, all subsequent experiments were performed with SS substrates coated under basic aqueous solution at 50 °C.

Static water contact angle and XPS analysis were undertaken to verify the surface modification with HA. As shown in Fig. 1A, the contact angle was greatly reduced from $70.2 \pm 2.9^{\circ}$ to $19.3 \pm 1.5^{\circ}$ upon HA coating on the SS substrate. The XPS analysis also provides the evidence of stable HA layer formation on the SS surface (Fig. 1B). In the survey scan spectra, it can be seen that a new N_{1s} peak appears and C_{1s} signal intensity increases for HA-coated SS substrates. XPS spectra of the SS substrate incubated with pristine HA were indistinguishable from those of bare surface, suggesting that HA was immobilized via a catechol-mediated adhesion. After HA coating, $Fe_{2p2/3}$ peak at 710.8 eV and $Fe_{2p1/2}$ peak at 724 eV from the bare SS surface were completely diminished (Fig. 1B inset), implying the superb surface coverage of HA on the SS substrate as a thin hydrogel layer of at least 10 nm (considering the penetration depth limit of X-ray beam onto the surface). More importantly, there was little change in chemical composition and the Fe_{2p} signal was undetected for 60 days incubation in PBS solution at 37 °C. HA coating layer was highly stable in aqueous solution, exhibiting no delamination of the HA layer from the SS surface. SEM and AFM were also used to visualize surface topography after HA coating (Fig. 2). The SEM picture of the coating layer shows a granular topography, possibly due to the formation of DA-mediated inhomogeneous crosslinking of HA molecules. AFM images show that the HA-coated SS substrate displayed substantially increased roughness (rms: 7.29 nm, measured on $1\,\mu m \times 1\,\mu m$ region), compared with that of bare surface (rms: 1.71 nm). It was likely that a thin, rough, but stably crosslinked HA hydrogel layer was formed on the SS surface.

3.2. Surface protein adsorption

Nonspecific protein adsorption on the surface of blood-contacting materials induces thrombosis and inflammatory responses (Chen et al., 2008). Therefore, a wide variety of non-fouling polymers like polyethylene glycol were chemically or physically adsorbed on the surface to suppress protein adsorption by combined mechanisms of steric repulsion and/or water-structured layer formation. HA has been known to effectively shield the underlying substrates by strong interaction with water molecules, enabling to endow non-fouling property to the surface (Morra and Cassineli, 1999). Bare and HA-coated SS substrates were exposed to fresh human platelet-poor plasma at 37 °C for 3 h to test the non-fouling effect. The extent of plasma protein adsorption was qualitatively analyzed by ATR-FTIR (Fig. 3). The amount of plasma proteins adsorbed on the bare surface were evidently high as observed from two intensive absorption bands around 1650 and 1550 cm^{-1}, which can be assigned to amide I and amide II, respectively. The amount of protein adsorption on the coated surface was greatly reduced as judged from the diminished two absorption peak intensities, revealing that the high water absorbing HA layer played a key role in repelling nonspecific protein adsorption.

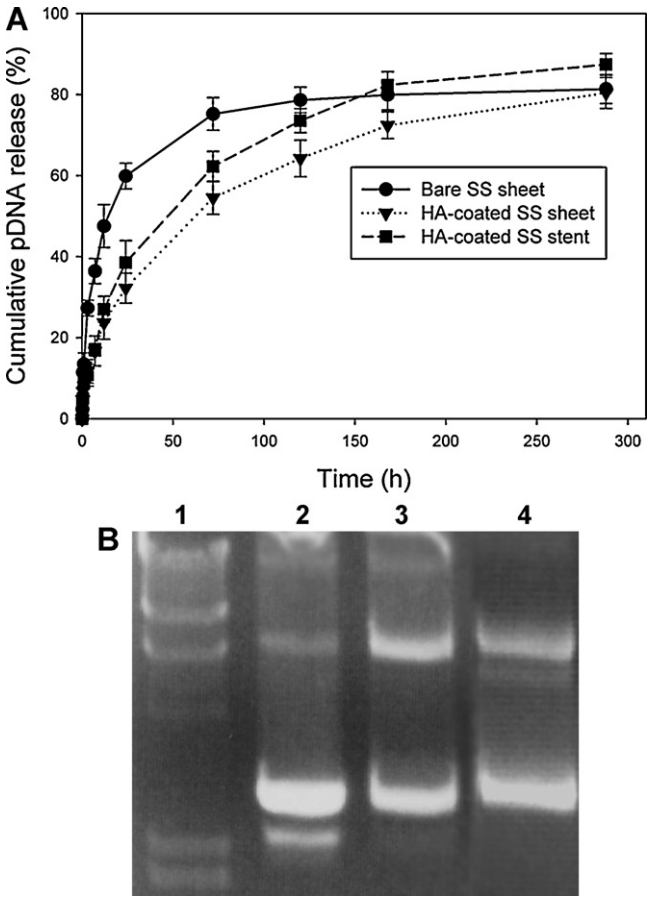


Fig. 6. (A) DNA release kinetics from pDNA/PEI polyplexes adsorbed on bare, HA-coated substrate and stent. (B) DNA structural integrity by agarose gel electrophoresis. Lane 1: molecular weight marker. Lane 2: original plasmid DNA. Lanes 3 and 4: released plasmid DNA from bare and HA-coated SS substrate, respectively after 3 days of incubation.

3.3. Deposition of pDNA/PEI polyplexes on the surface and in vitro pDNA release

Average size and surface charge values of pDNA/PEI polyplexes were determined to be 282.1 ± 89.3 nm and $+24.06 \pm 0.24$ mV, respectively. After pDNA/PEI polyplexes deposition, the loading amount of pDNA on the surface of bare and HA-coated SS substrate was assessed as a function of initial loading amount of pDNA in the feed (Fig. 4). The loading amount of pDNA increased with the amount of pDNA in the feed. The amount of pDNA on the coated surface was $2.0 \pm 0.2\,\mu g\,cm^{-2}$ at the feed concentration of $50\,\mu g\,ml^{-1}$, which was about fourfold higher than that on the bare surface ($0.49 \pm 0.2\,\mu g\,cm^{-2}$). In the case of HA-coated SS316L coronary stent, the pDNA loading amount was $1.1 \pm 0.1\,\mu g$/stent. The increased pDNA loading amount for the coated substrate was clearly due to strong electrostatic interactions between cationic pDNA/PEI polyplexes and anionic HA on the surface (Pannier et al., 2005). SEM and AFM images (Fig. 5) show deposition behaviors of pDNA/PEI polyplexes on the surface of bare, HA-coated SS sheet, and HA-coated SS coronary stent. It can be seen that pDNA/PEI polyplexes are irregularly aggregated on the bare surface, while they are uniformly deposited on the HA-coated SS surface and stent with preserving their spherical morphology and size. It was previously reported that pDNA/PEI polyplexes nonspecifically adsorbed on hydrophobic surfaces were highly aggregated, resulting in reduced gene transfection efficiency (Segura and Shea, 2002). The surface deposited pDNA/PEI polyplexes with less aggregation and better

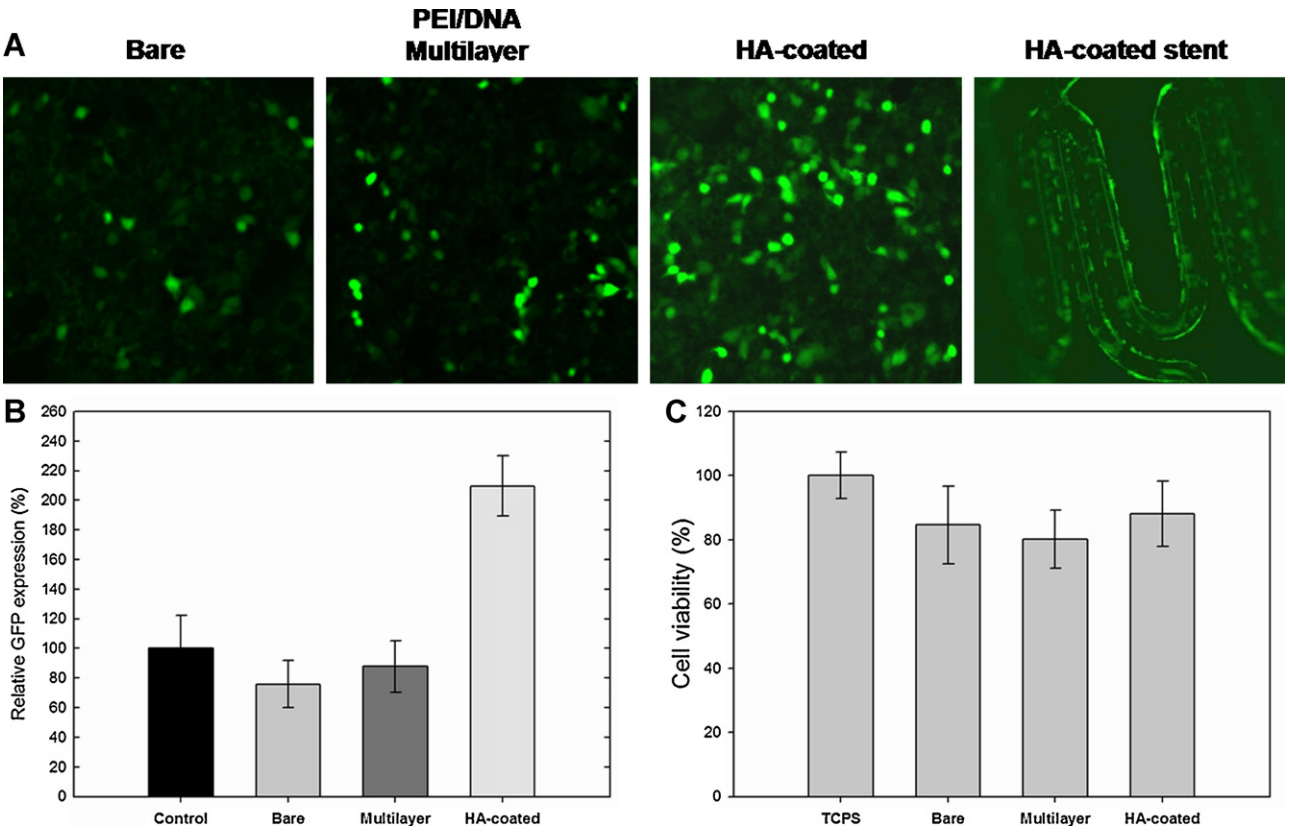


Fig. 7. GFP expression and viability in COS-7 cell on various substrates. (A) LSCM images of transfected cells with green fluorescence. (B) Relative GFP expression level of cells on control (solution-state transfection), pDNA/PEI polyplexes absorbed bare, PEI/pDNA multilayer, and pDNA/PEI polyplexes absorbed HA-coated SS substrate. (C) Cell viability on various surfaces.

distribution would have more chance to be transported within adhered cells through substrate-mediated gene transfection.

Fig. 6A shows release profiles of pDNA from bare, HA-coated SS substrate, and HA-coated SS stent. The bare SS substrate exhibited an approximately 45% initial burst within 1 day and a subsequent rapid release up to 80% during 5 days of incubation. In contrast, the coated substrate and stent exhibited less extents of initial burst (less than 30%) and sustained releases of pDNA up to approximately 80% over a period up to 12 days. It seems that pDNA/PEI polyplexes were more slowly desorbed from the coated surface than from the bare surface via an ion-exchange mechanism. The structural integrities of pDNA released from bare and the coated SS substrates after 3 days of incubation were examined by gel electrophoresis with comparison to that of naked plasmid DNA (Fig. 6B). The released pDNA from the two SS substrates showed slightly reduced band intensity in a supercoiled form as compared to that of naked pDNA, indicating that polyplexed pDNA was partially nicked during the release period, but sufficiently maintained its conformation (Shea et al., 1999).

3.4. Substrate-mediated gene transfection

The substrate-mediated DNA delivery could offer higher and prolonged gene transfection efficiency, compared to the solution-state DNA delivery, even though the same DNA amount was used for transfection (Luu et al., 2003; Shen et al., 2004; Liang et al., 2005; Jang et al., 2006). When the cells are seeded and contact with the DNA-coated surface, they could experience far more increase in concentration of DNA, as compared with DNA suspended in solution. The surface immobilized DNA in close proximity or in contact with cells can lead to high and long-lasting exogenous gene expression by immediate internalization and sustained release effects (Luo and Saltzman, 2000; Shen et al., 2004). In addition, the formation of nanoscale polyplexes can be crucial for cellular internalization of DNA and subsequent trafficking within cells (Jang et al., 2006). Prior to the gene transfection experiment, the adhesion extents of cells to different substrates were taken into account for normalizing gene expression efficiency per surface adhered cell number. As depicted in Fig. 7B, pDNA/PEI polyplexes deposited on the bare surface show $76.9 \pm 15.8\%$ gene transfection efficiency, compared with the solution-state pDNA/PEI polyplex transfection (100%). On the other hand, those on the HA-coated SS substrate exhibit $209.9 \pm 20.2\%$ gene transfection efficiency. The HA-coated coronary stent deposited with pDNA/PEI polyplexes also demonstrated significantly high green fluorescence on the surface after transfection (Fig. 7A, right panel). The higher level of gene transfection on the coated SS substrate relative to solution-state gene transfection can be attributed to the effect of surface-mediated gene delivery. In order to prove that the maintenance of intact spherical morphology and size of DNA polyplexes on the surface was crucial for substrate-mediated gene transfection, a PEI/pDNA multilayer was separately built on the surface of HA-coated substrate in a layer-by-layer fashion. At an approximately equivalent amount of pDNA, the assembled multilayer showed only about $87.7 \pm 17.4\%$ gene transfection efficiency (Fig. 7B). The result implicates that the adsorbed pDNA/PEI polyplexes with nano-granular morphology and dimension is probably important for improved cellular uptake. As shown in Fig. 7C, the viability of adhered cells also suggested no significant cytotoxic effect upon direct contact with the pDNA/PEI polyplexes, revealing that dually functionalized SS substrates with HA and DNA were biocompatible and could be used for stent-mediated gene delivery.

4. Conclusion

Through surface coating with HA and subsequent deposition of pDNA/PEI polyplexes, dually functionalized metal surface with HA and DNA was created for DES application. For producing robust HA layer on the SS surface, DA-derivatized HA was prepared and immobilized on the surface in a bio-inspired manner. HA-DA formed a thin hydrogel layer on the metal surface, offering superior biocompatibility in terms of higher resistance to human plasma protein adsorption. The HA-coated surface also allowed improved deposition of pDNA/PEI polyplexes, giving rise to enhanced gene expression mainly due to the surface-mediated DNA transfer into the adhered cells. The current dual functionalization strategy on the metal surface can be a promising approach for preventing re-occlusion of coronary artery after angioplasty surgery.

Acknowledgements

This study was supported by the grants from the National Research Laboratory program of the Ministry of Education, Science and Technology, and the Korea Research Foundation (KRF-2006-005-J04602), and the Polymer Technology Institute, Sungkyunkwan University, Republic of Korea.

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