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Enhancement of Blood Compatibility of Poly(urethane) Substrates by Mussel-Inspired Adhesive Heparin Coating

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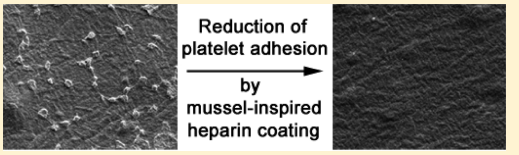
Supporting Information

ABSTRACT: Heparin immobilization on surfaces has drawn a great deal of attention because of its potential application in enhancing blood compatibility of various biomedical devices such as catheters, grafts, and stents. Existing methods for the heparin immobilization are based on covalent linkage formation and electrostatic interaction between substrates and heparin molecules. However, complicated multistep procedures and uncontrolled desorption of heparin are limitations of these methods. In this work, we report a new heparin derivative that exhibits robust adhesion on surfaces. The derivative, called hepamine, was prepared via conjugation of dopamine, a mussel-inspired adhesive moiety, onto a heparin backbone. Immersion of poly(urethane) substrates into an aqueous solution of hepamine resulted in robust heparin coating of the poly(urethane), the most widely used polymeric material for blood-contacting medical devices. The hepamine-coated poly(urethane) substrate showed significant inhibition of blood coagulation and platelet adhesion. The use of hepamine for surface modification is advantageous for several reasons: for example, no chemical pretreatment of the substrates is necessary, and surface functionalization is a simple, one-step procedure. Thus, the heparin immobilization method described herein is an excellent alternative approach for the introduction of heparin molecules onto surfaces.



INTRODUCTION

Heparin has been widely used in various blood-contacting materials because of its ability to inactivate blood coagulation pathways.[1,2] Therefore, stable immobilization of heparin molecules onto surfaces is of great importance in the development of blood-contacting medical devices, such as catheters, grafts, implants, and stents.[3−6] There are two common approaches for the surface immobilization of heparin: methods involving covalent linkage and those involving electrostatic interactions.[7−16] Approaches to the formation of covalent bonds require chemical derivatization of both the substrates and the heparin molecules—procedures that are often time-consuming and complicated. For example, heparin was chemically activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, and the substrate was modified to contain amine groups.[9] On the other hand, methods for utilizing an electrostatic interaction involve binding between anionic heparin molecules and cationic substrates. Examples in this category include a direct heparin deposition onto a cationic polyurethane surface.[10−12] Barbucci et al. prepared a cationic, poly(amidoamine)-immobilized polyurethane material to which heparin was immobilized by electrostatic interactions. Although the heparin immobilization was successful, uncontrolled desorption of immobilized heparin in media with a high ionic strength was a significant drawback. Furthermore, additional procedures to prepare cationic substrates are necessary for most substrates. Thus, the development of a simple and robust heparin immobilization method has remained an important goal.

Mussels are promiscuous underwater fouling organisms that attach to virtually any material, whether natural or synthetic. Studies of the fouling mechanisms have revealed that the unusual amino acid 3,4-dihydroxy-L-phenylalanine (DOPA) plays a critical role in mussel adhesion.[17−21] Single-molecule atomic force microscopy (AFM) studies showed that DOPA exhibits multifunctional, surface-dependent adhesion mechanisms in which it forms reversible coordination bonds to metal oxide surfaces and establishes irreversible covalent bonds to organic surfaces.[22,23] Thus, the introduction of DOPA or catechol (a side chain of DOPA) onto biopolymers has been a meaningful avenue for researchers seeking to enhance the adhesive properties of biopolymers.[24−28] For example, catechol-grafted poly(ethyleneimine) and poly(ethylene glycol) backbone showed successful adhesion onto various substrates including poly(tetrafluoroethylene) and poly(carbonate).[25,27] Recently, Park et al. reported the conjugation of heparin with dopamine, a small adhesive molecule inspired by mussel adhesive proteins.[29,30] They used dopamine-conjugated heparin as a stable platform for layer-by-layer coating of a mitotic inhibitor for cancer chemotherapy. Furthermore, the dopamine-conjugated heparin showed promise as a surface-modifying agent of gold nanoparticles (AuNPs) for computed tomography imaging. Despite the successful use of dopamine-conjugated heparin, the

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surfaces used in these studies were only metal surfaces: cobalt–chromium alloy and Au. We hypothesized that dopamine-conjugated heparin can modify other types of substrates, including synthetic polymers, because of dopamine's ability to adhere to versatile substrates.[17,22] Although the immobilization of heparin onto synthetic polymers used to manufacture blood-contacting medical devices is of great practical importance, studies for this purpose utilizing dopamine-conjugated heparin have not been completed.

In the present study, we used dopamine-conjugated heparin, termed hepamine, to coat the surface of a poly(urethane) (PU) substrate. PU has been extensively investigated for use in biomedical devices because of its unique mechanical property and biocompatibility.[31,32] However, PU's level of thromboresistance has been reported to be insufficient for long-term use in medical settings.[33,34] Thus, we chose to use hepamine-coated PU to evaluate thromboresistance at the interface. The hepamine-coated PU exhibited a significant reduction in platelet adhesion and blood coagulation, demonstrating enhancement of blood compatibility.

EXPERIMENTAL SECTION

Materials. Heparin sodium salt (unfractured heparin, avg. MW 12 000 Da) was purchased from Nanjing King-Friend Biochemical Pharmaceutical Co., Ltd. 2-Morpholinoethanesulfonic acid (MES), *N*-hydroxysuccinimide (NHS), 3-hydroxytyramine hydrochloride (dopamine hydrochloride), formaldehyde (37 wt % solution in water), and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich. Dialysis membranes (MWCO = 3500 Da) were obtained from Spectrum Laboratories (Canada) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was bought from Tokyo Chemical Industry. The gold (Au) and titanium (Ti) substrates were prepared by thermal evaporation onto silicon wafers (thickness = 100 nm). Poly(urethane) (PU), poly(carbonate) (PC), and silicon rubber (Si rubber) sheets were purchased from Hanmi Inc. (South Korea). For ellipsometric analysis, PU-coated Ti substrates were prepared by spin-casting of PU onto Ti substrates. PU (15 mg/mL in DMF) was spin-casted onto Ti substrates at 3000 rpm, and PU substrates were dried at 70 °C for 3 h. A concentrated platelet solution (1.4×10^6 cells/μL) was obtained from Redcross blood center (Daejeon, Korea), and the platelet was used to all assays as received.

Preparation of Dopamine-Conjugated Heparin: Hepamine. Heparin (200 mg) was dissolved in 0.05 M MES buffer (pH 5.5, 20 mL). NHS (0.05 M, 0.115 g) and EDC (0.1 M, 0.383 g) were subsequently added to the heparin solution to activate the carboxylic acid groups of heparin molecules. The solution was stirred at room temperature for 2 h, and dopamine (0.015 M, 0.06 g) was added to the solution. The resulting mixture was stirred at room temperature for additional 8 h. The solution was transferred to a dialysis membrane (MWCO = 3500 Da) and dialyzed for 12 h to remove unreacted coupling reagents and dopamine. Acidified water (1 mL addition of 5 M HCl per 1 L of deionized distilled water) was used for a dialysis procedure. The final product was obtained by lyophilization of the solution.

The number of conjugated dopamine per heparin was obtained from the intensity of dopamine peak in the UV–vis spectrum. From the molecular weight of heparin (12 kDa), the authors assume that each disaccharide repeating unit (M_w = 624 Da) has one carboxylic acid. The concentration of carboxylic groups of heparin can therefore be calculated by dividing the concentration of heparin (mg/mL) by 625 (mg/mmol). On the basis of the A_{280} intensity of hepamine, the catechol concentration of hepamine can be calculated, and the degree of dopamine conjugation can be determined by A280 intensity from a dopamine standard curve.

Hepamine Coating onto PU Substrates. Hepamine was dissolved in a 10 mM of Tris buffer solution (5 mg/mL, pH 8.5), and PU substrates were immersed into the hepamine solution. The PU substrate was cleaned by ultrasonication in an ethanol solvent for 10 min before use. After 10 h, the substrates were taken out, rinsed with water, and dried with N_2 gas. The hepamine-coated substrates were characterized by contact angle goniometer and X-ray photoelectron spectroscopy (XPS).

Platelet Adhesion and Serum Protein Adsorption onto Hepamine-Coated PU Substrates. Unmodified and hepamine-coated PU substrates were immersed in a PBS solution (pH 7.4) for 24 h, and then, each substrate was immersed into a 5 mL of platelet media (1.4×10^6 cells/μL). After 4 h incubation at 37 °C, the substrates were rinsed by PBS and immediately immersed into a formaldehyde solution (2%) for 24 h to fix the adhered platelets and were dehydrated by utilizing a series of ethanol solutions (50%, 60%, 70%, 80%, 90%, and 100%). Finally, the platelet adhesion onto the substrates was analyzed by scanning electron microscopy (SEM).

For investigation of blood component adsorption, PU-coated Ti substrates were used. PU substrates were modified by hepamine in alkali solution (pH 8.5) overnight. Subsequently, unmodified and hepamine-coated substrates were immersed in 50% EtOH for 10 min and incubated in human blood for two and seven days. Resulting substrates were taken out and sufficiently rinsed with water. The thickness of adsorbed blood component on the surface was measured by ellipsometer.

Characterization. UV–vis spectra were obtained using an UV–vis spectrophotometer (8453, Hewlett-Packard). Fourier transform infrared (FT-IR) spectra were obtained in a single-reflection mode using a dry N_2-purged Thermo Nexus FT-IR spectrophotometer. We averaged 1000 scans to yield the spectra at a resolution of 4 cm^{-1}. The contact angle measurements were performed with a Phoenix 300 goniometer (Surface Electro Optics Co., Ltd., Korea). Static water contact angles were measured at three different locations on each sample, and the average values are reported. Platelet-adhered substrates were studied by Field Emission SEM (S-4800, Hitachi). XPS was performed with a ESCA 2000 (Thermo VG Scientific, England) with a monochromatic twin X-ray source (Mg/Al target). Emitted photoelectrons were detected by a multichannel detector at a takeoff angle of 45° relative to the surface. During the measurements, ultralow vacuum (10^{-9} to 10^{-10} Torr) at an analysis chamber pressure was maintained. The thickness of adsorbed serum protein was measured with a Gaertner L116s ellipsometer (Gaertner Scientific Corporation, IL) equipped with a He–Ne laser (632.8 nm) at a 70° angle of incidence. A refractive index of 1.46 was used for all samples. The bioactivity of heparin, hepamine, and surface-immobilized hepamine was analyzed using Coatest heparin kit (Chromogenix, USA) (1 IU of heparin corresponded to 7.05 μg).

RESULTS AND DISCUSSION

The hepamine was prepared using carbodiimide chemistry as described in the methods section (Figure 1). The reaction mixture was dialyzed (MWCO = 3500 Da) to remove unreacted reactants and then lyophilized. Acidified water was used during

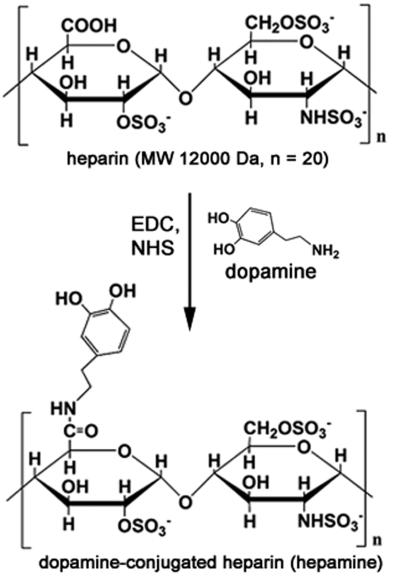

Figure 1. Reaction scheme to prepare hepamine.

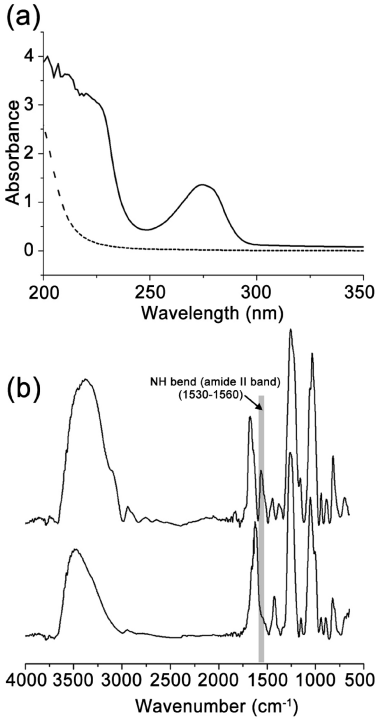

Figure 2. (a) UV−vis spectra of hepamine (1 mg/mL, solid) and heparin (1 mg/mL, dash). (b) FT-IR spectra of hepamine (upper) and heparin (lower).

dialysis to avoid catechol oxidation. UV−vis spectroscopy results showed that dopamine was successfully conjugated to heparin, as indicated by the presence of a peak at 280 nm (Figure 2a, solid line). The intensity at 280 nm was used to quantify the extent of dopamine conjugation to the carboxylic groups of heparin. Utilizing a dopamine standard curve, we found that 27 ± 8% of the carboxylic groups in heparin were conjugated with dopamine. Furthermore, FT-IR spectroscopy results confirmed the amide bond formation (1530−1560 cm^{-1}) (Figure 2b, upper line); this

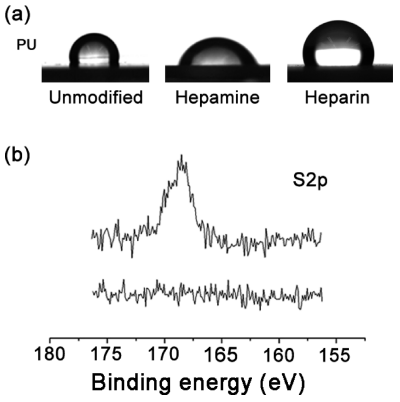

Figure 3. (a) Static water contact angle images for the unmodified PU surface (left), hepamine-coated PU surface (middle), and PU surface incubated in heparin solution for 10 h (right). (b) High-resolution S2p spectra of hepamine-coated (upper) and unmodified (lower) PU surfaces.

bond was absent in unconjugated heparin (lower line). The activity determined by Coatest heparin kit showed that there was little effect by dopamine conjugation under the same concentration (2 μg/mL): 0.38 IU/mL for heparin and 0.34 IU/mL for hepamine.

We hypothesized that hepamine can be immobilized onto various substrates as the conjugated catechol moiety demonstrated robust surface immobilization. PU was chosen as a model substrate because it has been a widely used material for manufacturing blood-contacting medical devices.[31,32] Also, the presence of phenyl groups in PU chains might enable the catechol moiety in hepamine to interact with PU substrates by $\pi-\pi$ interactions.[22] PU substrates were functionalized through immersion into the hepamine solution, and the functionalized PU surface was characterized by water contact angle measurement and XPS analysis (Figure 3). The unmodified PU substrate was hydrophobic, with a contact angle value of 103.3°, which was reduced to 62.0° after hepamine immobilization for 10 h. The decrease in the water contact angle originated from the hydrophilic nature of heparin,[35] and this result clearly indicated successful hepamine coating (Figure 3a, left and center). The presence of catechol moieties in hepamine was critical in surface functionalization; the PU substrate that was incubated in a solution of heparin alone showed a negligible change in the contact angle, indicating that unmodified heparin was not able to modify the PU substrate (Figure 3a, right). The hepamine-coated surface was further characterized by XPS. After hepamine coating onto the PU surface, a new peak for sulfur (S) 2p (168.5 eV) was observed (Figure 3b, upper curve), whereas no S 2p peak was detected for the unmodified PU surface (Figure 3b, lower curve). The S 2p peak originated from the sulfate groups of hepamine, and the presence of the peak clearly demonstrates successful hepamine coating. We also applied hepamine coating to other substrates to demonstrate the versatility of its coating ability (Supporting Information Figure S1). Significant changes of the contact angle values indicated successful hepamine coating: poly(carbonate) (PC) (85.4° → 48.1°), Au (81.9° → 45.4°), and elastic Si rubber (97.9° → 57.8°). This result indicates that the use of hepamine is an efficient approach to immobilize heparin molecules onto surfaces without the need for chemical pretreatment of substrates.

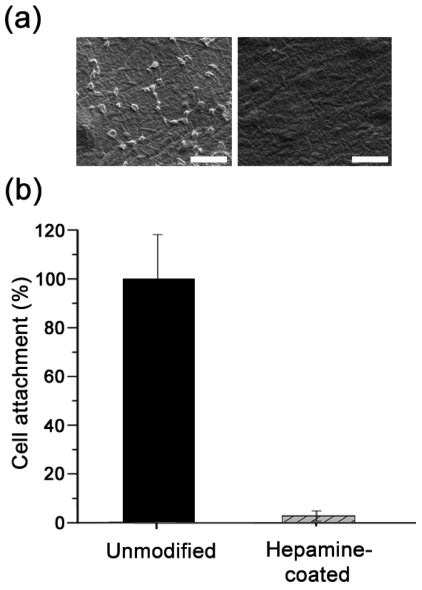

Figure 4. (a) SEM images of unmodified (left) and hepamine-coated (right) PU surfaces after 2 h platelet adhesion. (The scale bar is 10 μm.) (b) Quantitative analysis of platelet adhesion results shown above. Adhered platelets were counted at five different locations on each sample, and the average values are reported.

Quantitative analysis of the surface-immobilized hepamine onto PU substrates (1 cm^2) was achieved by a chromogenic anti-FXa assay. The amount of immobilized hepamine was 22.25 $\pm$ 2.75 ng/cm^2, which corresponded to 3.15 $\times$ 10^{-3} IU/cm^2. To investigate the blood compatibility of hepamine-coated PU substrates in a more detailed manner, we carried out a platelet adhesion experiment on the hepamine-coated PU substrate. Heparin is a well-known polysaccharide that inhibits platelet adhesion onto surfaces, and thus, we expected that the hepamine-coated substrate could prevent blood coagulation on surfaces.[36,37] Platelets (1.4 $\times$ 10^6 cells/μL) were seeded onto unmodified and hepamine-coated PU surfaces, and after 4 h of incubation, the substrates were examined by scanning electron microscopy (SEM) (Figure 4a). The hepamine-coated PU surface showed excellent resistance against platelet adhesion, whereas the unmodified surface exhibited significant adhesion of platelets. The average platelet densities adhered onto the unmodified and functionalized PU surfaces were 32 000 and 650 cells/mm^2, respectively. Quantitatively, the adhered platelet density was reduced by 98% after hepamine coating (Figure 4b). The result indicates that the hepamine remains stably immobilized on PU surfaces, markedly enhancing blood compatibility of the most widely used catheter material, PU. Although the detailed mechanisms of platelet interactions with heparin molecules are unclear, there are a number of studies demonstrating a significant reduction of platelet adhesion onto heparin-immobilized substrates.[6,36,37] A proposed mechanism is probably due to electrostatic repulsion between heparins and platelets.[38] The anticoagulation effect of hepamine-coated substrates was also evaluated by measuring the thickness of the adsorbed blood component layers by ellipsometry. PU-coated Ti substrate was used, and hepamine functionalized the substrate. Hepamine coating thickness determined by an ellipsometer was 26.46 $\pm$ 4.1 Å. Subsequently, human blood (2 mL) taken from a human volunteer was applied directly to the substrate (Supporting Information Figure S2, inset). After 2 and 7 days of blood contact, the substrates were rinsed with water, and then, the amount of adsorbed blood components was measured. The hepamine-coated PU substrates exhibited a significant reduction in blood component adsorption; the thickness was 100 Å (2 days) for the unmodified PU substrate, which was greatly increased to 307 Å (7 days). However, the hepamine-coated substrates showed reduced adsorption 32 Å and 150 Å for 2- and 7-day exposure, respectively (Supporting Information Figure S2). These results suggest that hepamine-coated PU substrates showed improved resistance against adsorption of blood components.

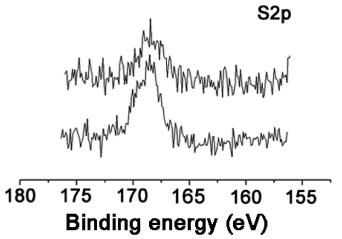

Figure 5. High-resolution S2p spectra of hepamine-coated PU (lower) and hepamine-coated PU immersed in 50% EtOH for 2 h (upper).

Stability of the hepamine layer upon exposure of a sterilizing solution is an important factor for practical utility of the hepamine coating approach. A popular sterilizing solution, 50% (v/v) ethanol, was prepared to which the hepamine-coated PU substrate was immersed for 2 h. XPS was used to monitor delamination of the coated hepamine layer (Figure 5). XPS surface analysis revealed that the hepamine-coated PU substrate exhibited a clear S2p signal, which still remained after incubation in 50% ethanol solution. This result demonstrated that the stability of hepamine coating was suitable in practical medical settings.

CONCLUSIONS

In this study, a new adhesive heparin derivative called hepamine was introduced. Hepamine was prepared by conjugation of dopamine, a mussel-inspired adhesive compound, and showed excellent surface coating capability on various substrates particularly on PU substrates. Unlike existing approaches that require multistep, complicated heparin immobilization, one-step immersion of a substrate into the hepamine solution was shown to be sufficient for the coating without chemical treatment of PU. The resulting hepamine immobilized surfaces showed significantly improved blood compatibility, preventing blood coagulation and platelet adhesion. The coating was stable in a typical disinfectant solution (50% ethanol), and the anticoagulant activity of the hepamine-coated surface remained for 7 days. These results suggest that the use of hepamine can be an effective alternative approach for stable surface immobilization of heparin. Thus, we believe that the strategy described herein can be generally applied in a variety of biomedical devices such as catheters, grafts, and stents to achieve a thromboresistance property.

ASSOCIATED CONTENT

Supporting Information. Supporting Figures S1, S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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