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Effect of Hyaluronic Acid Incorporation Method on the Stability and Biological Properties of Polyurethane-Hyaluronic Acid Biomaterials

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Abstract

The high failure rate of small diameter vascular grafts continues to drive the development of new materials and modification strategies that address this clinical problem, with biomolecule incorporation typically achieved via surface-based modification of various biomaterials. In this work, we examined whether the method of biomolecule incorporation (*i.e.*, bulk vs. surface modification) into a polyurethane (PU) polymer impacted biomaterial performance in the context of vascular applications. Specifically, hyaluronic acid (HA) was incorporated into a poly(ether urethane) via bulk copolymerization or covalent surface tethering, and the resulting PU-HA materials characterized with respect to both physical and biological properties. Modification of PU with HA by either surface or bulk methods yielded materials that, when tested under static conditions, possessed no significant differences in their ability to resist protein adsorption, platelet adhesion, and bacterial adhesion, while supporting endothelial cell culture. However, only bulk-modified PU-HA materials were able to fully retain these characteristics following material exposure to flow, demonstrating a superior ability to retain the incorporated HA and minimize enzymatic degradation, protein adsorption, platelet adhesion, and bacterial adhesion. Thus, despite bulk methods rarely being implemented in the context of biomolecule attachment, these results demonstrate improved performance of PU-HA upon bulk, rather than surface, incorporation of HA. Although explored only in the context of PU-HA, the findings revealed by these experiments have broader implications for the design and evaluation of vascular graft modification strategies.

Introduction

Due to the thrombogenic nature of many biomedical materials, modification of biomaterial or device surfaces with bioactive molecules has been widely employed as an approach to impart hemocompatibility upon blood-contacting devices [1-3]. While the use of highly hydrophilic polymers can limit device thrombogenicity [4-6], it is believed that direct anti-thrombotic bioactivity is necessary to reach appropriate levels of hemocompatibility for rigorous applications such as small diameter vascular grafts [7]. Such bioactive modifications of vascular materials (*e.g.*, ePTFE, Dacron, polyurethanes) have been achieved via immobilization of molecules such as anticoagulants, enzymes, antibodies, peptides, and native biomolecules [8-11]. These modifications are commonly accomplished by tethering the bioactive molecule to the material surface, either directly or indirectly, via covalent or non-covalent means [8,11]. One example is the covalent immobilization of

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heparin, which has been investigated extensively in the context of vascular grafts and other devices, with some clinical success [2,12]. However, despite many decades of implementing these modification strategies to improve the hemocompatibility to vascular devices, very few have proven to be clinically successful. Instead, the vast majority of vascular biomaterials remain passive in nature; intrinsic physical properties of the synthetic polymer surface (*e.g.*, hydrophobicity), rather than bioactivity, are responsible for limiting thrombogenic events on unmodified Dacron and ePTFE grafts [13,14], thereby restricting their usage to less demanding applications. However, these same surface properties are also responsible for limiting graft endothelialization, and may permit greater bacterial adhesion, which is another cause of graft failure [15]. Although microbial infection occurs less frequently than failure due to thrombosis, this phenomenon is associated with much higher mortality rates [16].

There exist many reasons why the modification of materials with bioactive molecules has primarily been focused on surface-based methods. Because this versatile approach involves modification of only the surface of existing materials, the bulk material structure is allowed to retain many of its existing physical properties (*e.g.*, mechanics, degradation) [17,18]. Moreover, numerous studies have documented excellent retention of the biological activity of molecules when tethered to a material surface [3,9,19-21], and surfaces may even be modified with more than one bioactive moiety to produce multi-functional materials [22-24]. However, as noted above, materials created by this approach frequently fail upon more rigorous testing. One potential reason for the failure of these surface-modified materials could be inadequate retention of bioactivity once exposed to dynamic or physiological conditions, but surprisingly few studies have explicitly investigated the stability of surface-grafted biomolecules in such conditions.

In theory, covalent incorporation of a biomolecule directly into the bulk (rather than surface) of a biomaterial structure could address some stability concerns by rendering the biomolecule less susceptible to enzymatic degradation or cellular internalization. However, the bulk modification approach is rarely explored in the context of biomolecules, largely due to concerns over retention of bioactivity once the molecule is crosslinked within the bulk polymer structure, as well as difficulties in the chemical processing of many biomolecules [25].

In order to create a suitable material for vascular applications, it is necessary to find a balance between the material modification strategy that yields optimal vascular-appropriate performance with the ability of these properties to be retained when exposed to physiological conditions. In this study, we modify a poly(ether urethane) with hyaluronic acid (HA) to investigate whether: 1) the method of biomolecule incorporation (*i.e.*, bulk vs. surface modification) impacts material bioactivity in the context of vascular applications, and 2) the stability of material bioactivity is impacted by the method of biomolecule incorporation. Our group has previously documented favorable vascular-appropriate properties of PU-HA materials [9,26], with PU being selected as the core material due to its versatile physical properties and extensive use in biomedical applications [27,28], while HA was chosen as the biomolecule because it possesses not only intrinsic anti-thrombotic [29] and angiogenic [30] properties but also numerous different reactive groups [31]. Although focused on the specific case of PU-HA, this investigation has implications for biomolecule modification strategies in general, as it explores phenomena that are broadly relevant to other polymer platforms and biomolecules.

Materials and Methods

Unless otherwise stated, all products are from Sigma-Aldrich, St. Louis, MO. Data were compared using one-way analysis of variance (ANOVA) with Tukey's HSD post-test for which statistical significance was defined as $p < 0.05$. Data are presented as mean $\pm$ standard deviation.

Preparation of DMF-Soluble HA Salts

To synthesize a copolymer of hyaluronic acid with polyether-based polyurethane, the HA (4.7 kDa sodium salt; Lifecore Biomedical, Inc., Chaska, MN) must be rendered soluble in an organic solvent. Thus, a simple ion exchange reaction was performed to replace the sodium salt of HA with a dodecyltrimethylammonium salt (DTA), which renders HA soluble in DMF. A 7.0% (w/v) solution of dodecyltrimethylammonium chloride in 1.6 ml of deionized water was added dropwise to 1.6 ml of HA 10% (w/v). This reaction formed a white precipitate. The precipitate was then separated from the supernatant by centrifugation. Once lyophilized overnight, the final product was obtained, HA-DTA powder.

Synthesis of Bulk-Modified PU-HA

Based upon previous investigations [32,33], a single HA concentration of 0.5 wt% was selected for synthesis of bulk PU-HA materials. According to previous work, this concentration of HA allows us to not only maintain desirable physical properties (*i.e.*, vascular-appropriate mechanics), but also possesses biological properties that are appropriate for vascular applications, such as good hemocompatibility and support of endothelial cell growth [32,33]. Bulk PU-HA synthesis was performed as previously described [26], starting with a 10% (w/v) solution of methylene di(p-phenyl isocyanate) (MDI; 2 mmol; MW=250 g/mol) in 5 ml of dimethylformamide (DMF) prepared in a 250 ml round-bottom flask and stirred at room temperature. A 10% (w/v) solution of poly(tetramethylene oxide) (PTMO; 1 mmol; MW=1000 g/mol) in 10 ml of anhydrous DMF was added, and the mixture was heated to 90°C and held there for three hours under argon. The reactor was cooled to room temperature before 1,4-butanediol (BD; 0.9 mmol; MW=90 g/mol) in 1 ml of anhydrous DMF was added as a chain extender. The DMF-soluble HA salt (HA-DTA, 0.025 g) was dissolved in 5 ml of DMF and added to the polymer solution. The polymer solution was then heated at 50°C for 3 hours. The reaction mixture was cooled on ice and precipitated in a methanol-ice mixture. The product (PU-HA) was dried at room temperature in a fume hood.

PU and bulk-modified PU-HA films were prepared by dissolving the polymer in an organic solvent mixture (1:1) composed of DMF:tetrahydrofuran (THF) at a concentration of 20 mg/ml and then spin-coating (Spin coater, Specialty Coating Systems, Inc., Model. P6700) on 40 mm diameter glass coverslips. Spin speeds between 100-3000 rpm and ramp accelerations between 5 and 3000 rpm/sec were used in 4 consecutive cycles. For all experiments that involved cells, the films were disinfected in 70% ethanol.

Synthesis of Surface-Modified PU-HA

Preparation of surface grafted PU-HA was also performed as previously described [9], with the HA concentration adjusted to match that displayed by bulk-modified PU-HA, as described below. Briefly, the urethane prepolymer was first synthesized via reaction of 1 g MDI (4 mmol; MW = 250) in 5 ml DMF with 1.3 g PTMO (2 mmol; MW = 650) in 11 ml DMF in a 100 ml round-bottom flask at room temperature. The mixture was heated to 90°C, held at this temperature for 3 hours under argon with constant stirring, and then cooled to room temperature before adding 0.18 ml of BD (2 mmol; MW = 90). The polymer solution was heated to 50°C for another 3 h and cooled to room temperature. A 1 wt% solution of

branched polyethylenimine (PEI; MW = 10,000; Polysciences, Inc., Warrington, PA) in 1 ml DMF was then added to the polymer solution, and this mixture was heated to 60°C for 1 hour under argon. The final polymer solution was cooled to room temperature, precipitated in methanol, and dried naturally in a fume hood. This product is referred to as PU-PEI.

In order to identify the concentration of surface-grafted HA that best matched the HA surface density presented by 0.5% bulk-modified PU-HA, a series of HA concentrations were grafted to the PU-PEI polymer. PU-PEI films were prepared on 40 mm diameter glass coverslips by spin coating a 20 wt% PU-PEI solution in a (1:1) solvent mixture composed of DMF:THF, and HA oligosaccharides (4.7 kDa; Lifecore Biomedical) were tethered onto the PU-PEI films using carbodiimide-based linking chemistry [34]. Briefly, a solution of 0.001, 0.01, 0.1, or 1 mg/ml HA in 0.05 M MES buffer (2-(N-morpholino)ethanesulfonic acid; pH 5.4) was combined with 100 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) hydrochloride and 50 mM N-hydroxysuccinimide. The amine groups of PU-PEI films were then reacted with the carboxyl groups present on HA by adding 5 ml of the HA solution to each 60 mm dish containing a PU-PEI film and allowed to react at room temperature for 16 hours. After removing the HA reaction solution, the HA-grafted PU-PEI films were rinsed with deionized water five times to remove unbound HA and then air-dried under argon gas. A quantitative HA test kit (described in following section) was used to identify the HA grafting concentration that resulted in a similar HA surface density as that presented by the 0.5% bulk-modified PU-HA.

Hyaluronic Acid Quantification

The surface density of HA presented by both bulk- and surface-modified PU-HA materials was quantified using a commercially available quantitative ELISA kit (HA-ELISA; Echelon Biosciences Inc., Salt Lake City, UT). The manufacturer's procedure was adapted to enable measurement of surface-bound HA on our samples by applying removable flexiPERM chambers (flexiPERM Micro 12, 0.2 cm² growth area/well) to PU-HA films (n=8 per condition) prepared on 40 mm diameter circular coverslips. The HA-grafted microwells were reacted with 100 µl hyaluronic acid binding protein (HABP) conjugated to alkaline phosphatase for 30 min at 37°C. After extensive washes with the 1X wash concentrate, 100 µl of the substrate solution p-nitrophenyl phosphate (pNPP) was added to the microwells, and the reaction was stopped after 30 min via the addition of 50 µl of stop solution to each well, with measurement of the colorimetric response at 450 nm.

PU-HA Mechanical Testing

Polymer solutions with a concentration of 40 mg/ml were homogeneously dispersed in Teflon dishes, and the solvent allowed to evaporate for 3 days. Using a razor blade and steel template, test samples were cut in a dogbone geometry according to ASTM D638-03 (4 mm gage length, 1.7 mm gage width, and 1 mm fillet radii; 3 specimens per condition). The sample thickness was measured using an optical microscope. Mechanical testing of the PU and PU-HA samples was carried out using an Instron 5648 Micro Tester equipped with a 10 N load cell. Crosshead displacement was used to determine axial strain in the gage section. All samples for all sections were loaded to rupture. A constant crosshead displacement rate of 25 mm/min was used for all tests as suggested by ASTM D638-03. Elastic modulus was obtained by calculating the slope of the initial linear region of the stress-strain curve for each sample.

Surface vs. Bulk PU-HA Enzymatic Degradation

To analyze the susceptibility of polymer-bound HA to enzymatic degradation, PU and PU-HA films (n=4 per condition) were prepared on 40 mm diameter glass coverslips by spin-coating and incubated in a solution of 100 U hyaluronidase (Hyase) per ml of PBS at 37°C

on an orbital shaker for 24 hours. Following enzymatic treatment, samples were extensively washed with PBS to remove unreacted enzyme and then air-dried. The amount of HA lost from the polymer film surface after enzymatic treatment was measured using the aforementioned quantitative HA ELISA kit. The results were compared against PBS-treated controls.

Dynamic Shear Experiments

Flow treatment of PU-HA materials was performed using a circular parallel plate flow chamber (Glycotech Corporation, Rockville, Maryland) connected to a recirculating system which provided a steady and uniform laminar flow. The chamber was placed on top of PU or PU-HA films and vacuum sealed to the surface using a portable vacuum pump (Fisher Scientific, Pittsburgh, PA). Phosphate buffered saline (PBS) was used as the shear medium and drawn through the flow channel (path width=10 mm, length=20 mm, height = 0.25 mm) using a programmable peristaltic pump (73-2949 Reglo Pump, IDEX Health & Science LLC, Rohnert Park, CA). Flow rates were ramped until reaching a shear stress of 12 dynes/cm², which is relevant to the physiological range in a human artery [35]. After 4 hours of exposure to shear, polymer samples were used in subsequent experiments.

Measurement of Protein Adsorption to Surface vs. Bulk PU-HA

Removable flexiPERM chambers were applied to PU and PU-HA films that had either been held static or had been pre-treated with flow, and films were equilibrated with PBS for 3 hours at room temperature. After removing the PBS, 200 µl of a 5% solution of fetal bovine serum (FBS) in PBS was added to each well (n=4 wells per condition) and serum proteins were allowed to adsorb to the surfaces at 37°C overnight. Films were rinsed three times with PBS in order to remove unbound proteins. To recover the adsorbed proteins, 75 µl of a 1 wt % solution of sodium dodecyl sulfate (SDS) in PBS was added to each well, and the plate was shaken (150 rpm) on an orbital shaker overnight at room temperature. The solution was then collected for protein quantification via the Micro BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions and compared against bovine serum albumin standard solutions.

Platelet and Red Blood Cell Adhesion to Surface vs. Bulk PU-HA

PU and PU-HA films were spin-coated on glass coverslips and then held static or pre-treated with flow, as described above. Whole blood and platelets collected from healthy donors were purchased from Innovative Research (Novi, MI). The human platelets were then subjected to an additional centrifugation at 400×g for 15 minutes in order to separate and collect platelet-rich plasma (PRP). To create a thrombogenic surface as a positive control, glass coverslips were incubated overnight with 0.6 mg/ml Collagen I solution (Angiotech Pharmaceuticals, Vancouver, Canada). Coverslips coated with Collagen I, PU, or PU-HA (bulk- or surface-modified) were incubated on an orbital shaker plate with PRP or citrated whole blood for 30 minutes at 37°C followed by five rinses in PBS and fixation in 2.5% glutaraldehyde. The numbers of adherent platelets and red blood cells (RBCs) were determined by manually counting four randomly chosen fields of view for each of the four samples per condition (n=4), captured at a 200X magnification.

Bacterial Adhesion to Surface vs. Bulk PU-HA

Escherichia coli (BL21; Novagen, Madison, WI) colonies were cultured under standard conditions (37°C, 250 rpm) in LB Broth, Miller (Fisher Scientific, Fairlawn, NJ). The final bacteria concentration was adjusted to an OD 600nm ~0.3, using an optical spectrophotometer (Beckman, Model DU530). PU and PU-HA polymer films were cast upon coverslips, either held static or pre-treated with flow, and disinfected as described

earlier; removable 12-well silicone flexiPERM chambers were applied to the polymer-coated coverslips in order to create discrete wells upon the sample surfaces. In order to create a positive control surface for comparison, agar samples were prepared in a 96 well plate. A volume of 100 μ l bacterial suspension was added to each flexiPERM well. After incubation at 37°C for 24 hours, six samples from each condition were rinsed once with PBS before adding 50 μ l of B-PER buffer (Bacterial Protein Extraction Reagent, Pierce) to lyse the cells. The concentration of adherent cells in each sample was quantified via performance of a DNA assay (Quant-iT Pico Green Assay Kit, Invitrogen Corp., Carlsbad, CA) on the collected lysate according to manufacturer instructions.

Endothelial Cell Viability, Adhesion, and Growth on Surface vs. Bulk PU-HA

Bovine aortic endothelial cells (ECs; passage 2-11; Lonza, Switzerland) were cultured in DMEM supplemented with 5% FBS, 100 μ g/ml streptomycin and 100 U/ml penicillin. The cells were seeded at a concentration of 20,000 cells/cm² on the PU control and both PU-HA modifications. Prior to cell seeding, films were disinfected in 70% ethanol. At time points of five hours and five days post seeding, a Live/Dead assay (Invitrogen) was performed to evaluate EC viability upon the polymer films. Fluorescent photomicrographs of live and dead cells were captured at 100 $\times$ magnification (Olympus IX51 inverted microscope).

In order to assess cell number, PU and PU-HA films were first pre-treated with flow as described above. ECs were then seeded on the films (n=6 per condition) at a concentration of 60,000 cells/cm² and harvested after 2 days of culture for cell number quantification. At this time, non-adherent cells were removed by rinsing with PBS, and cells were lysed in M-PER solution (Mammalian Protein Extraction Reagent, Pierce). The number of adherent cells was quantified via a PicoGreen DNA assay (Invitrogen).

Results

Synthesis of Surface- vs. Bulk-Modified PU-HA Materials

One of the first steps in achieving comparable bulk- vs. surface-modified PU-HA materials was tailoring the HA modification chemistry to result in equivalent amounts of HA being presented on the polymer surface for both bulk and surface PU-HA. For both physical and biological reasons described above, the amount of HA presented by 0.5 wt% bulk-modified PU-HA was selected as the target. As shown in Figure 1A, application of a range of HA concentrations in the coupling solution used to generate surface-modified PU-HA enabled the identification of a grafting condition that matched the bulk PU-HA target. Namely, use of 0.001 mg/ml HA solution for surface grafting resulted in a statistically similar HA density when compared to 0.5 wt% bulk PUHA, with a final HA density of approximately 530 ng/cm². It is also important to note that this assay is based upon the biological recognition of HA by HABP, and the resulting measurements are thus indicative of the amount of bioactive HA present on the PU-HA surfaces.

Because mechanics alone can influence the biological properties and performance of a material, mechanical testing of bulk- vs. surface-modified PU-HA films was also performed in order to verify comparable mechanical properties of the two materials. As shown in Figure 1B, the elastic moduli of bulk- and surface-modified PU-HA were statistically identical (example stress-strain curves shown in Supplementary Figure S1). Moreover, both surface and bulk PUHA materials possessed an elastic modulus similar to that of native vessels (native vessel elastic modulus: ~3 MPa [36]). The percent elongation to break for the surface-modified PU-HA was significantly lower when compared to bulk-modified PU-HA (Supplementary Figure S1), although it is not known whether this property can affect biological outcomes, as most studies have related only elastic modulus to biomolecular and

cellular interactions with materials. Thus, upon confirming that these bulk- and surface-modified PU-HA materials possessed similar HA densities and elastic moduli, we proceeded to investigate whether the HA modification strategy impacted the performance of these materials with respect to HA degradation and HA stability upon exposure to shear stress.

Effect of Surface vs. Bulk Modification on PU-HA Degradation

To investigate whether HA modification strategy impacted the biological stability of the HA, the surface concentration of HA was assessed following exposure of bulk- vs. surface-modified PUHA materials to hyaluronidase for 24 hours. As shown in Figure 2, the surface concentration of HA in both bulk- and surface-modified materials decreased upon hyaluronidase treatment. However, surface-modified materials exhibited significantly greater susceptibility to enzymatic HA degradation than bulk-modified materials ($p < 0.02$) resulting in the surface-modified PU-HA materials displaying a significantly lower surface HA concentration than the bulk-modified PUHA following hyaluronidase treatment. PBS-treated control samples performed as expected, showing no change in HA surface density after 24 hours (not shown).

Retention of Surface vs. Bulk HA Upon Exposure to Flow

To investigate whether HA modification strategy impacted the physical stability of the HA, the density of HA on the different PU-HA materials was quantified after exposing the material surfaces to a shear stress of 12 dynes/cm² for 4 hours. As shown in Figure 3, despite the covalent attachment confirmed by XPS (Supporting Information Methods and Table S1), surface-grafted materials experienced a significant ($p < 0.005$) loss of immobilized HA upon exposure to physiological shear conditions when compared to the static control, with approximately 21% of surface-grafted HA not retained after flow. In comparison, for bulk-modified PU-HA exposed to the same shear levels, the density of HA remained at a level similar to that observed for the static control.

Protein Adsorption to Surface vs. Bulk Modified PU-HA

One motivation for modifying PU with HA is to decrease protein adsorption to these materials (as we have previously shown in [32]), as protein adsorption can mediate undesirable events such as platelet and bacterial adhesion [37,38]. Consistent with the increase in hydrophilicity observed for both PU-HA materials when compared to PU (confirmed by contact angle measurements, Supplementary Figure S2), protein adsorption to PU-HA materials was decreased with either the bulk or surface HA modification approach relative to unmodified PU (Figure 4), although only bulk PU-HA was statistically different from PU ($p < 0.01$). Similarly, only the bulk-modified PU-HA materials were able to retain the same resistance to protein adsorption following exposure to flow. For the surface-grafted PU-HA materials, the consequence of shear-induced instability of the surface-bound HA was an increase in protein adsorption when compared to the static control, which also matches the increase in hydrophobicity to a level similar to that found for PU (Supplementary Figure 3).

Hemocompatibility of Surface vs. Bulk Modified PU-HA

Under static conditions, both surface and bulk modified PU-HA were able to significantly attenuate the adhesion of platelets and red blood cells relative to unmodified PU (Figure 5). However, pre-treatment of PU-HA materials with flow significantly decreased the ability of the surface-modified PU-HA to resist platelet and RBC adhesion ($p < 0.03$). In fact, exposure to physiological shear stresses rendered the thrombogenicity of the surface-modified PU-HA equivalent to that obtained for unmodified PU. In contrast, exposure to flow did not alter the properties of the bulk-modified PU-HA with respect to either platelet or RBC adhesion.

Bacterial Adhesion to Surface vs. Bulk Modified PU-HA

Under static conditions, similar levels of *E. coli* had adhered to both the PU and PU-HA materials at 24 hours post-seeding (Figure 6). However, pre-treatment with flow affected the extent of bacterial adhesion to the surface-grafted PU-HA. Specifically, exposure of surface-modified PU-HA films to flow resulted in significantly higher bacterial adhesion when compared to the static surface-modified control ($p < 0.003$). The extent of *E. coli* adhesion to bulk-modified PU-HA was unaffected by exposure to shear stress.

Endothelial Cell Culture on Surface vs. Bulk Modified PU-HA

EC adhesion and growth studies were performed after 5 hours and 5 days of culture to determine the effect of the method of HA incorporation on the ability of the two PU-HA materials to support EC culture. Live/Dead images (Figure 7A) indicate that both bulk and surface modifications supported similar levels of viable ECs with excellent morphology at both time points. However, the HA modification strategy did affect the ability of the PU-HA materials to support EC culture following exposure of the PU-HA material to shear stress (Figure 7B). Following flow treatment of the PU and PU-HA materials, the number of ECs present on the surface-modified PU-HA was significantly decreased relative to the static control ($p < 0.03$). Meanwhile, both PU and bulk-modified PU-HA retained similar cell numbers after exposure to physiological shear stress when compared to their respective static controls.

Discussion

Instability of material or device modifications can yield serious consequences for device performance and patient health; in the case of vascular grafts, premature loss of anti-thrombotic properties can result in graft occlusion and failure. Yet, despite the development of many surface-based approaches for improving graft hemocompatibility [9,20-22,39-41], very few have yielded successful results *in vivo*, with one potential reason being lack of persistence of the surface modification. Although bulk incorporation of bioactive molecules directly into the polymer backbone could theoretically address stability issues, this approach has generally not been pursued, likely due to a combination of synthetic challenges and concern over loss of biomolecule availability or functionality upon copolymerization. In this work, we first demonstrate that, under static conditions, comparable physical and biological outcomes can be achieved via modification of PU with HA via either surface or bulk methods, thereby indicating that bulk-modified PU-HA can be as efficacious as surface-modified PU-HA in many aspects of vascular performance. Given the assumption that surface-tethered molecules should exhibit greater mobility and flexibility, this result is not intuitive, and is promising with respect to the potential for using bulk-modified materials. Moreover, we go on to show that surface-modified PU-HA was significantly more susceptible to enzymatic degradation and detachment via shear stress than the HA in bulk-modified materials, resulting in a significantly deteriorated ability to sustain an endothelial cell layer and resist protein adsorption, platelet adhesion, and bacterial adhesion. Although explored only in the context of PU-HA, the findings revealed by these experiments have broader relevance in the evaluation of vascular graft modification strategies. The results obtained from these experiments are expected to provide insight into optimal incorporation techniques that will enable the improvement of small diameter vascular graft patency and blood compatibility.

The success of many material modifications has been hindered by the fact that achieving a stable covalent attachment of the desired biomolecule is frequently complicated by loss of bioactivity during the modification process. For instance, while heparin modification remains the most common approach to improving hemocompatibility of most synthetic graft

materials [1,2,42], covalent tethering of heparin can compromise its ability to bind antithrombin III, resulting in loss of bioactivity [43]. In our case, successful modification with HA by both surface- and bulk-methods was accomplished, as determined by a quantitative assay based on the binding of labeled HA-binding protein (HABP) to the surface-available HA. The specific recognition of the covalently attached HA by HABP is promising, as it indicates that there is bioavailable HA bound to our PU-HA materials despite the material modifications implemented. Subsequent experiments examined both the biological and physical stability of this incorporated HA via treatment with hyaluronidase or physiological shear stress, respectively.

One potential disadvantage facing the use of biomolecules in graft-modification approaches is their susceptibility to enzymatic degradation. Thus, it is desirable to develop material modification strategies that minimize premature degradation of the bioactive moiety while retaining its beneficial characteristics. After exposing the different PU-HA materials to hyaluronidase, a significant loss of surface-detectable HA was identified in both types of PU-HA materials. However, significantly more HA was lost from the surface-modified PU-HA than from the bulk-modified material. While surface-grafting can theoretically achieve better chain mobility than bulk incorporation, this may come at the cost of increased susceptibility to enzymatic degradation. In the case of bulk modification, incorporation of HA into the PU backbone may allow the PU chains to serve as a protective matrix that hinders the ability of HA-targeting enzymes to degrade the modified substrate. Bulk vs. surface-modified PU-HA were also found to differ in their physical stability, as indicated by measurements of HA surface density following exposure to physiological shear stresses. Specifically, despite the covalent attachment confirmed by XPS (Supporting Information Methods and Table S1), surface-grafted materials were less stable upon exposure to shear, experiencing a significant loss of HA. In contrast, the HA density on bulk-modified materials remained unaltered after exposure to physiological conditions. In a physiological environment, this HA detachment may leave parts of the bare PU surface directly exposed to proteins, enzymes, bacteria, and platelets, which may negatively affect the material stability and overall hemocompatibility, as examined in this work.

The poor outcomes associated with current synthetic grafts (*e.g.*, Teflon, Dacron) used in arterial reconstruction are primarily due to early thrombosis [44,45]. Protein adsorption is one of the initial events that occur when a material is exposed to blood and can significantly impact numerous subsequent biological processes, such as cell or platelet adhesion. Thus, prevention of protein adsorption is one important strategy for enhancing material hemocompatibility. Although both bulk- and surface-modified PU-HA materials demonstrated similar resistance to protein adsorption, only bulk-modified materials were able to retain this resistance following exposure to flow. Protein adsorption experiments performed using non-covalent PU-HA modifications confirmed that the differences in the protein adsorption behavior observed between bulk- and surface-modified PU-HA were a consequence of flow exposure and not a resulting from incomplete covalent bonding between PU and HA (Supporting Information Methods and Figure S3).

In terms of hemocompatibility, exposure of PU-HA materials to shear resulted in retention of thromboresistant properties by bulk-modified PU-HA, but over a two-fold increase in platelet and RBC adhesion to surface-modified PU-HA, rendering them indistinguishable from the unmodified PU. Other modifications to PU, such as incorporation of poly(ethylene glycol) [4], phospholipids [46,47], heparin [48,49], and combinations thereof [50,51] have yielded similar (or better) improvements to PU hemocompatibility compared to what we have illustrated using PUHA. However, the stability and function of the majority of these alternate modification strategies have not been examined following exposure to flow. Also, unlike PU-HA, polyurethanes modified via these aforementioned alternative techniques

have not demonstrated the ability to support robust endothelialization without further modification. Finally, it is important to note that the PU-HA formulations described herein were specifically designed to perform a stability study of surface vs. bulk HA incorporation, and do not represent the optimal hemocompatibility that could be achieved with these materials. As noted earlier, we elected to use a fairly low HA content in order to easily fabricate bulk and surface PU-HA with comparable HA densities and mechanics, but our previous studies that examined ranges of HA content in PU-HA indicate that we may further improve upon the thromboresistant properties of these materials by raising the HA concentration in PU-HA [52].

For vascular applications, the ability to support a healthy endothelial layer is crucial in creating an anti-thrombogenic surface that can interact with the circulating blood and prevent the development of anastomotic or intimal hyperplasia. As noted earlier, most current approaches to improving graft hemocompatibility (*i.e.*, modification with heparin or PEG) generally require additional modification to enable EC adhesion and growth [53]. For instance, polyurethanes that incorporate a thromboresistant functionality have also been modified with specific cell adhesion peptides [54-56] in order to render the material more favorable for EC adhesion and growth. However, these dual modifications greatly increase the complexity of the material fabrication, and are also complicated by the non-thrombogenic functionality competing for available surface space with the pro-EC adhesion sequence. In contrast, the PU-HA materials presented herein contain just a single functionality to simultaneously discourage platelet adhesion while supporting endothelialization. PU-HA enabled the formation of a morphologically healthy, confluent monolayer of ECs in five days, independent of the modification strategy implemented. However, the bulk- vs. surface-modified PU-HA did not exhibit equivalent performance with respect to support of EC culture following exposure to shear. Flow treatment of surface-modified PU-HA rendered this material less supportive of EC culture than the bulk-modified PU-HA, which did not experience any change in its ability to support ECs.

Finally, in addition to thrombosis, infection is a potential life-threatening complication that hinders the long term success of many implanted vascular grafts [38,45,57]. Between 1 and 5% of patients develop vascular graft infection, with a mortality rate of up to 50%, depending on the graft site [16,58]. Other groups have modified polyurethanes to impart bactericidal properties via a range of techniques, with inclusion of quaternary ammonium salts [59,60] or silver [61-63] being the predominant approaches. While these polyurethanes have been shown to possess excellent antimicrobial properties, in most cases it is not known how well these properties are maintained following exposure to flow. Also, they generally have not exhibited (or been tested for) hemocompatibility or the ability to support endothelialization, and most have not been fabricated to possess vascular-appropriate mechanics. Although modifications of PU with HA slightly attenuated *E. coli* adhesion prior to flow exposure, the differences were not significant. Our previous work demonstrated that the bactericidal properties of PU-HA were dependent upon the HA density [32]; thus, although we decided to pursue modifications with low HA concentrations in this specific study to better analyze outcomes related to bulk vs. surface grafting methods, these HA modifications can be easily tailored in order to produce a significant bacteriostatic effect. However, differences in bacterial adhesion between the two HA modification methods were apparent after flow exposure, where a significant increase in *E. coli* adhesion occurred on surface-modified PU-HA, but not on bulk-modified PU-HA. The ability of the bulk PU-HA materials to maintain the same bacterial adhesion levels after flow exposure is also advantageous with respect to hemocompatibility, since there is evidence to suggest that an increase in adherent bacteria may activate clotting and platelet recruitment in their vicinity to facilitate the process of colonization, resulting in potential graft failure [64].

Study Limitations

The current study was limited to studying material performance *in vitro*; although the materials were exposed to physiological levels of shear stress, *in vivo* studies will be needed to fully characterize material performance and evaluate both the stability and efficacy of the HA modification strategies. Moreover, this study included measurement of the loss of surface HA upon short-term treatment with a high concentration of hyaluronidase, but does not characterize long-term PU-HA degradation behavior with respect to persistence of HA in or on the PU-HA material. However, because the presence of HA is needed only at the early stage after material implantation in order to resist initial thrombotic events and achieve establishment of a functional endothelium, its longer-term stability may not be an issue with respect to graft performance. Similarly, the experiments herein involved only short-term exposure of materials to flow conditions. Although examination following longer-term flow exposure will be needed prior to device implantation, the short duration used for the current work was sufficient to address the research question driving this investigation, namely whether bulk vs. surface HA modification strategies yielded differences in PU-HA material performance under static or dynamic conditions. Thus, although much work remains to be done to characterize the long-term performance of these materials in the presence of other vascular cells, or in an *in vivo* setting, the initial findings presented in this communication indicate promise for use of these materials (specifically, bulk-modified PU-HA) in vascular applications.

Conclusions

This work examined whether the method of HA incorporation (*i.e.*, bulk vs. surface modification) into a PU polymer impacted PU-HA performance in the context of vascular applications, both before and after exposure to flow. Under static conditions, modification of PU with HA (by either surface or bulk methods) yielded materials that possessed no significant differences in their ability to resist protein adsorption, platelet adhesion, and bacterial adhesion, while supporting endothelial cell culture. However, only bulk-modified PU-HA materials were able to fully retain these characteristics following material exposure to flow. The finding that bulk incorporation of HA into PU could not only yield equivalent biological performance as surface HA modification, but also that this bulk modification strategy might provide a more stable biomaterial, is important to vascular graft design, where surface tethering of biomolecules remains the predominant approach in the modification of vascular graft materials. These findings motivate further exploration of the bulk modification approach to produce materials with more stable biomolecule presentation and improved vascular performance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) A range of HA grafting concentrations was tested to achieve surface-modified PUHA that possessed a comparable HA surface density to that found in the target polymer, bulk-modified 0.5% PU-HA. * $p < 0.0001$ vs. 0.5% bulk PU-HA; $n=8$ samples per condition. B) Elastic moduli of PU and bulk- and surface-modified PU-HA polymer films. # $p < 0.002$ vs. unmodified PU; $n=3$ samples per condition.

Figure 2. Percent of starting HA surface density after incubation of PU-HA materials with 100 U/ml hyaluronidase for 24 hours. No HA was detected on unmodified PU. * $p < 0.02$ vs. bulk PUHA; $n=4$ samples per condition.

Figure 3. Surface density of hyaluronic acid after exposing bulk- vs. surface-modified PU-HA to 12 dynes/cm² shear stress for 4 hours. *p< 0.005 vs. static control from the same modification; n=4 samples per condition.

Figure 4.

Amount of protein adsorbed to the bulk and surface HA modifications after exposure to shear for 4 hours. * $p < 0.01$ vs. unmodified PU, # $p < 0.05$ vs. static control from the same modification. $n=4$ samples per condition.

Figure 5.

A) Platelet adhesion to PU and PU-HA materials modified via either bulk or surface methods following exposure to platelet rich plasma. B) Red blood cell (RBC) adhesion to PU and PU-HA materials after exposure to whole blood. * $p < 0.02$ vs. unmodified PU; # $p < 0.03$ vs. static control from the same modification; all bulk and surface PU-HA materials were statistically different from the collagen positive control ($p < 0.02$); $n=4$ samples per condition.

Figure 6.

E. coli adhesion to PU, bulk-modified PU-HA, and surface-modified PU-HA at 24 hours, as measured via DNA quantification. * $p < 0.001$ vs. agar (positive control); # $p < 0.003$ vs. static control from the same modification; $n=6$ samples per condition.

Figure 7.

A) Endothelial cell adhesion and growth upon bulk- and surface-modified PU-HA. Live cells are stained green, while dead cells are stained red. Scale bar =100 μ m. B) Quantification of endothelial cell adhesion after exposure of PU, bulk-modified PU-HA, and surface-modified PU-HA to shear stress for 4 hours. * $p < 0.03$ vs. static control from the same modification; $n=3$ samples per condition.