Surface Heparinization of Polyurethane Via Bromoalkylation of Hard Segment Nitrogens

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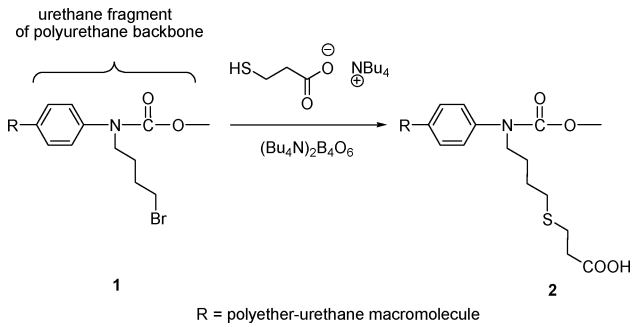
Previous research from our group has demonstrated that bromoalkylation of polyurethane elastomers via base mediated activation of the urethane-hard segment nitrogen groups can be used to either attach bisphosphonate groups to confer calcification resistance or append cholesterol to promote endothelial cell adhesion. In the present studies we further explore the potential of this chemical approach by investigating bulk carboxylation of polyurethanes via bromoalkylation to enable surface heparinization for thromboresistance. Thus, polyurethane (PU) was modified with pendant 7-carboxy-5-thiaheptyl groups using a polymer-analogous reaction of bromobutylated PU with tetrabutylammonium 3-mercaptopropionate in mild conditions. The grafting of polyallylamine (PAA) onto the surface of carboxylated PU via direct coupling of amino and carboxy groups resulted in high levels of PAA (up to 8 μg/cm^2). The surface-aminated PU was further covalently modified with unfractionated heparin as confirmed by FTIR. Fluorescence labeling of PAA hydrochloride and heparin with BODIPY−FL was used to quantify the extent of surface modifications. Heparin was covalently bound at a high level (1.11 ± 0.06 μg/cm^2) and was shown to be active, with demonstrable Factor Xa inhibition and platelet factor IV binding. It is concluded that surface amination of bulk-carboxylated PU represents a novel approach for heparinizing PU; carboxylation followed by surface amination represents another important dimension of bromoalkyl activation of polyurethane hard segments, thereby enabling heparinization.

1. Introduction

Postpolymerization derivatization of polyurethanes has been explored by our group as a means for therapeutic modifications of medical grade polyurethane elastomers. In particular, we reported bisphosphonate derivatized polyurethanes that resist pathologic calcification.[1,2] In these prior studies, bisphosphonates were covalently attached to polyurethanes via initial bromoalkylaton of the urethane nitrogens followed by specific conjugation chemistry reactions. Similarly, cholesterol derivatized polyurethane was also synthesized using the same bromoalkylation strategy, resulting in a polyurethane with endothelial cell adhesion properties significantly greater than nonmodified polyurethanes.[3] In the present studies, we have explored another important variant on this approach using bromoalkylation of the urethane nitrogens to enable heparinization of polyurethane surfaces.

Previously, we have reported that base-induced low-temperature bromoalkylation of PU with an excess of 1,6-dibromohexane[4] or 1,4-dibromobutane[5] provides PU with pendant bromoalkyl arms suitable for further derivatizations. As was shown using size exclusion chromatography, no degradation of the PU backbone takes place due to bromoalkylation.[4] Further reactions of the bromoalkyl side chains with various functionalized thiols can be then carried out in mild conditions, resulting in attachments of different functional groups to PU macromolecules.[1,4,5] In this paper, we report the synthesis of a novel PU with pendant 7-carboxy-5-thiaheptyl groups from a bromobutylated PU via a similar polymer-analogous reaction, using tetrabutylammonium 3-mercaptopropionate as a functionalized thiol (Scheme 1). Films of the bulk carboxylated PU were subjected to surface modifications employing the pendant carboxy groups. Thus, polyalylamine (PAA) was utilized to achieve surface amination followed by covalent attachment of heparin. The extent of heparinization and specific heparin activity were also assessed.

Scheme 1



2. Methods

A medical grade polyether-urethane (PU), Tecothane TT1074A, was obtained from Thermedics Inc. (Woburn, MA) and characterized as described previously.[5] All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. A Bruker Avance DMX 400 spectrometer was used for recording the NMR spectra. Fluorimetric measurements were carried out with a SpectraMax Gemini EM fluorimeter (Molecular Devices, Sunnyvale, CA).

2.1. *N*-Bromobutylated Polyether-Urethane (Scheme 1, 1). Polymer **1** was prepared from Tecothane TT1074A (15. 8 g, containing ca.

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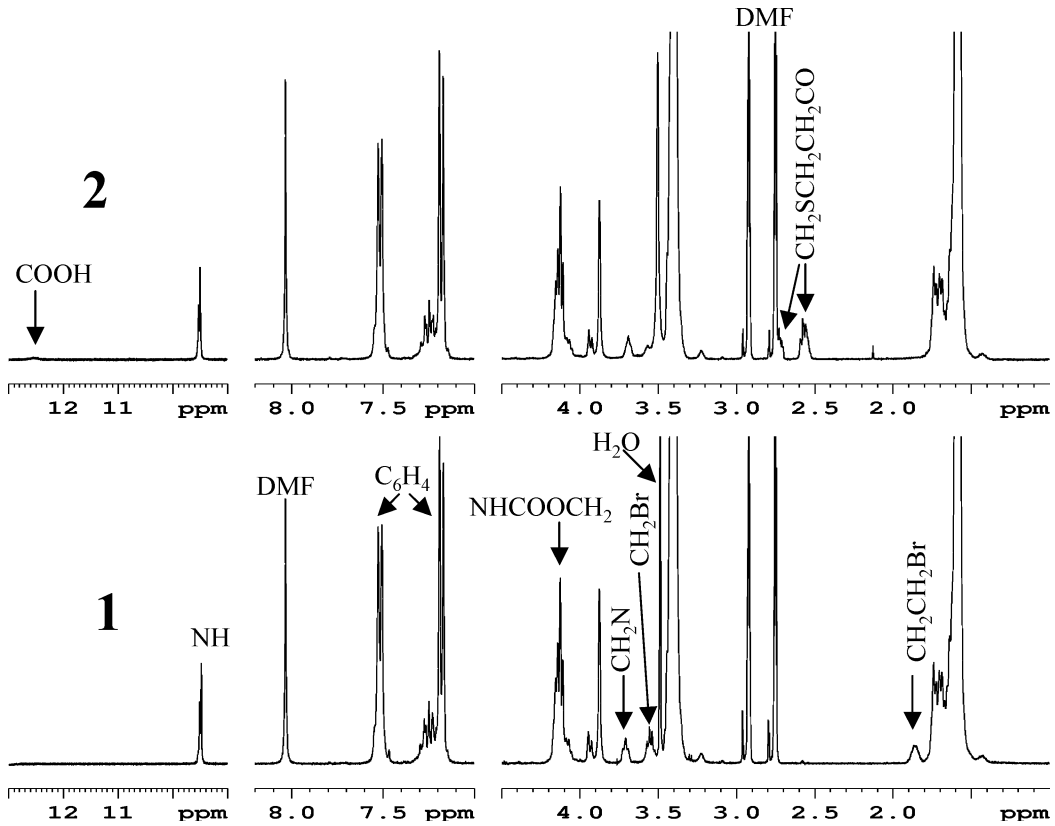
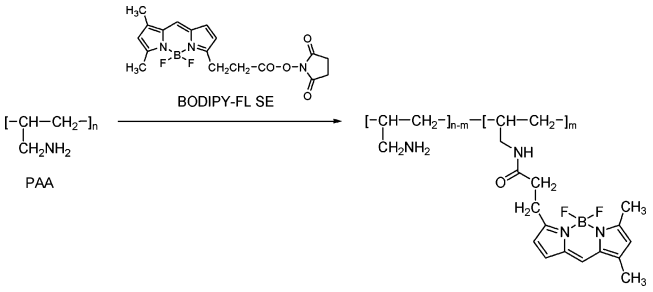


Figure 1. ^{1}H NMR (400 MHz, DMF-d_7) of bromobutylated (**1**) and carboxylated (**2**) polyurethanes. Signals at 3.56 ppm (CH_2Br) and 1.86 ppm (next CH_2 in pendant 4-bromobutyl) characteristic for the bromobutylated PU (**1**) disappear in the spectrum of **2**, whereas the fragments CH_2-SCH_2CH_2CO of 7-carboxy-5-thiaheptyl groups exhibit new signals at 2.57 ppm ($2CH_2$) and 2.74 ppm ($1CH_2$, on the shoulder of DMF signal). The signal of COOH appears as a broad peak near 12.5 ppm.

36 mmol of urethane NH groups) following a described[5] procedure, but with the amount of lithium *tert*-butoxide decreased to 4.8 mmol, yielding 15.28 g. ^{1}H NMR spectral analysis of polymer **1** (Figure 1) found ca. 13% of the urethane sites modified with 4-bromobutyl groups, corresponding to ca. 0.3 mmol/g of the thiol-reactive residues.

2.2. Polyether-Urethane Modified with Pendant 7-Carboxy-5-thiaheptyl Groups (Scheme 1, 2). Polymer **1** (15.1 g, containing ca. 4.7 mmol of 4-bromobutyl groups) was dissolved in dry *N,N*-dimethylacetamide (DMAc, 270 mL) under protection with a flow of argon, and the solution was cooled to −10 °C. 3-Mercaptopropionic acid (1.96 mL, 22.3 mmol) in a minimum of water was neutralized with aqueous tetrabutylammonium hydroxide to pH ca. 6. The mixture was dried and coevaporated with DMAc in vacuo to remove water. The resulting syrup of tetrabutylammonium 3-mercaptopropionate (11.7 g) was diluted with DMAc (25 mL) and added to the polymer solution simultaneously with a freshly prepared 0.25 M DMAc-solution of tetrabutylammonium tetraborate $(Bu_4N)_2B_4O_7$ (50 mL, 12.5 mmol) under vigorous stirring, while the temperature was not allowed to exceed −4 °C. The mixture was stirred at −1 to +1 °C for 1 h with continued Ar protection and acidified with acetic acid (20 mL). The reaction solution was filtered and poured into a large volume (1400 mL) of cold (−60 °C) methanol. The coagulate of polymer was separated, washed with copious amounts of methanol then water, and dried in vacuo. To remove residual amounts of Bu_4N^+ cation, the crude polymer **2** was redissolved in DMF (280 mL), acidified with acetic acid (30 mL), filtered, precipitated with cold methanol, washed with large volumes of methanol and water, stirred for 3 days with a large amount of water at 4 °C, and dried at 0.04 mmHg, yielding 13.4 g of polymer **2**. ^{1}H NMR spectral analysis of the polymer **2** (Figure 1) demonstrated ca. 13% of urethane sites bearing 7-carboxy-5-thiaheptyl groups (ca. 0.3 mmol/g), and no unreacted 4-bromobutyl groups could be detected. The films of polymer **2** were cast following the procedure described for acetylthio-modified PU,[5] thoroughly washed with large amounts of 0.1% aqueous $NaHCO_3$ then water, and air-dried.

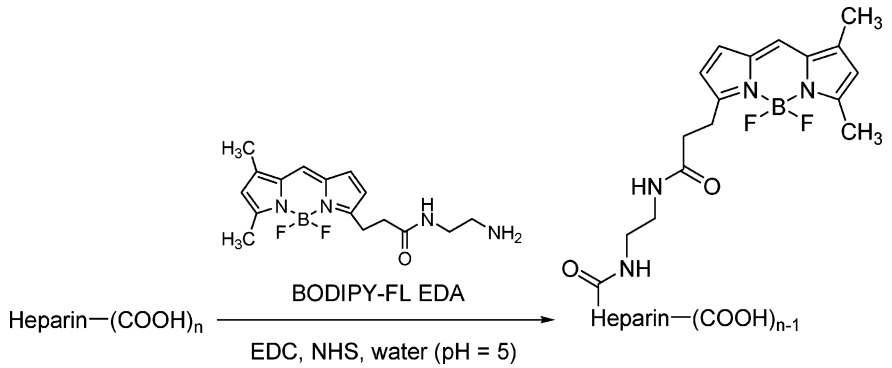
Scheme 2



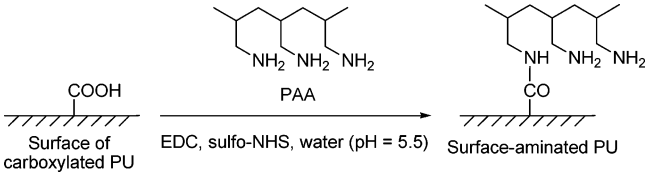
2.3. Labeling of Polyallylamine with BODIPY−FL (Scheme 2). Polyallylamine hydrochloride (PAA·HCl, M_w ca. 15 kDa, 0.45 g, ca. 4.8 mmol of NH_2) was dissolved in water (35 mL) and passed through a column filled with a strongly basic anionite Dowex G-55 in OH-form. The column was washed with water until the pH was neutral, and the eluate was dried in vacuo. The residue was repeatedly coevaporated with 2-propanol, dissolved in a mixture of 2-propanol (4 mL) and CH_2Cl_2 (10 mL), and cooled in ice. A solution of BODIPY−FL SE (Molecular Probes Inc., Eugene, OR, 5 mg, 12 μmol) in CH_2Cl_2 (5 mL) was added dropwise in ca. 1 min. The mixture was stirred in ice for 15 min and acidified with 12.1 M HCl (0.4 mL, 4.85 mmol). The solvents were removed in vacuo, and the residue was repeatedly coevaporated with $CHCl_3$−MeOH−2-propanol (equal volumes) until solidification. The solid was suspended in 2-propanol (11 mL) and left overnight at 4 °C. The precipitate was filtered off, thoroughly washed with 2-propanol, EtOAc, and pentane, and dried in vacuo. The yield of labeled PAA·HCl was 0.45 g. TLC (silica gel, $CHCl_3$−MeOH, 95:5) failed to detect any mobile fluorescent impurities.

2.4. Labeling of Heparin with BODIPY−FL (Scheme 3). Unfractionated heparin sodium USP, 158 units/mg (Pharmacia & Upjohn, Franklin, OH, 0.305 g, containing approximately 0.25 mmol of COOH groups) was dissolved in water (8.6 mL) and adjusted to pH = 5 with

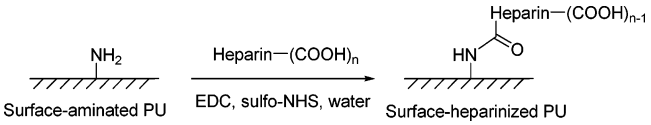
Scheme 3



Scheme 4



Scheme 5



HCl. BODIPY−FL EDA·HCl (Molecular Probes Inc., 5 mg, 13 μmol), *N*-hydroxysuccinimide (NHS, 0.103 g, 0.89 mmol), and 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 0.23 g, 1.2 mmol) were added, and the mixture was stirred for 19 h at room temperature. Water was removed in vacuo, and the residue was repeatedly coevaporated with 2-propanol until solidification. The solid was suspended in 2-propanol (15 mL), filtered off, washed with 2-propanol, $CHCl_3$, and pentane, and air-dried. Finally, the product was dissolved in water, exhaustively dialyzed (cell membrane cutoff: 3500 Da) at 4 °C against 0.2 M NaCl, then 0.2 M NaCl + 0.01 M $NaHCO_3$, and finally against water, filtered, and lyophilized. The yield of labeled heparin was 0.299 g.

2.5. Surface Amination of Carboxylated PU 2 Films (Scheme 4). Films cast from polymer **2** (with an average thickness of ca. 0.15 mm) were rinsed with water and incubated at pH = 5.5 (adjusted with $KHCO_3$) in an aqueous solution containing PAA·HCl (M_w ca. 15 kDa, 58 mg/mL), hexadecylpyridinium chloride (0.5 mg/mL), *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS, Fluka Chemicals, Milwaukee, WI, 3 mg/mL), and EDC (22 mg/mL, added immediately before application) for 24 h at room temperature. The films were rinsed with water and subjected to a second amination in a freshly prepared solution as above for 2 days. After exhaustive washing with 0.1 M HCl and water, the films were incubated in 0.3% aqueous K_2CO_3 for 4 h to hydrolyze labile esters, rinsed with water, and air-dried. Control samples, lacking PAA groups, were prepared from functionalized PU by reacting PAA·HCl (M_w ca. 15 kDa, 58 mg/mL) in the absence of the above coupling agents. The surface amination with BODIPY-labeled PAA·HCl was carried out analogously on aminated and control films, omitting the incubation in K_2CO_3 solution (which significantly degraded the fluorescent label).

2.6. Immobilization of Heparin onto the Surface-Aminated PU Films (Scheme 5). Heparin was bound to the surface-aminated PU-films by reaction with an aqueous solution of unfractionated heparin (Pharmacia & Upjohn) in the presence of 10 mg/mL sulfo-NHS and 5 mg/mL EDC at pH 7.0, with mild shaking at room temperature for 18−20 h, followed by exhaustive washing with water at pH 7.0. To ensure that heparin was chemically bound to the PU surface, and not merely adsorbed, control samples of PU were reacted with heparin in the absence of 10 mg/mL sulfo-NHS and 5 mg/mL EDC.

2.7. Fluorescence Measurements of PU Films Modified with BODIPY-Labeled Polymers. Circles (6 mm in diameter) of PU films (150 μM thick) modified with BODIPY-labeled polymers (PAA or heparin) were placed into wells of a 96-well polystyrene plate (Costar, Corning, NY) and fluorescence measured with excitation/emission set at 490/515 nm and the emission cutoff filter at 515 nm. Aqueous solutions (0.2 mL) of labeled polymers with different concentrations were used as standards. The weight amounts of bound heparin or PAA per 1 cm^2 were calculated from the calibration curves assuming that equal weights of polymers either in the solutions or on the films will produce equal intensities of fluorescence. Both sides of the PU film were taken into account.

2.8. Fourier Transform Infrared Spectroscopy-Attenuated Total Reflectance. Fourier transform infrared spectra of triplicate hydrated samples were measured on nonfluorescent PU, aminated PU, and heparinized PU samples prepared as described above by attenuated total reflectance spectroscopy (FTIR-ATR) using a Nicolet 5-Protégé 460 spectrophotometer E.S.P. (Nicolet, Madison, WI). All spectra were obtained from 200 scans collected at a resolution of 2 cm^{-1} at a 45° angle of incidence. All spectra (4000−650 cm^{-1} regions) were recorded under identical conditions and adjusted for atmospheric water vapor and carbon dioxide transmittance by subtraction of the appropriate reference spectrum using the Omnic software package (Nicolet).

2.9. Quantitation of Heparin Binding Capacity. Total heparin bound to the polyurethane (PU) surface was determined by saturation analysis, using unfractionated heparin (Pharmacia & Upjohn) that had been labeled with the fluorescent probe BODIPY as detailed above. Increasing concentrations of BODIPY-heparin were reacted with 0.5 cm^2 circles of aminated or nonaminated PU, and the amount bound was determined by fluorimetry as above and compared to the original reaction concentrations. Data were reduced and maximum binding (Bmax) was calculated using Prism 4.03 (GraphPad, Software Inc., San Diego, CA) and normalized to 1 cm^2 surface area.

2.10. Quantitation of Bound Heparin Bioactivity. The anti factor Xa activity of unlabeled PU-bound heparin was determined by inhibition of factor Xa[6] activity in the presence of excess antithrombin III (AT3, generously provided by Dr. Sriram Krishnaswamy) using an established methodology[7] involving the cleavage of a chromogenic fXa substrate (Spectrozyme fXa, American Diagnostica, Greenwich, CT) and in comparison to a standard curve generated in solution using the same lot number of heparin used in all of the binding experiments (Pharmacia & Upjohn;158U/mg, 106% anti factor Xa activity). Absorbance of factor Xa-generated chromogen at 405 nm was measured using a SpectraMax 190 (Molecular Devices) spectrophotometer, after removal of 0.1 cm^2 circles of PU (previously reacted with unlabeled heparin under saturating conditions) from the reaction wells. Results were normalized to a 1 cm^2 surface area.

2.11. Quantitation of Platelet Factor 4(PF4) Binding Capacity. 1 cm^2 samples of heparinized PU or nonheparinized PU were incubated with excess recombinant human PF4 was prepared according to

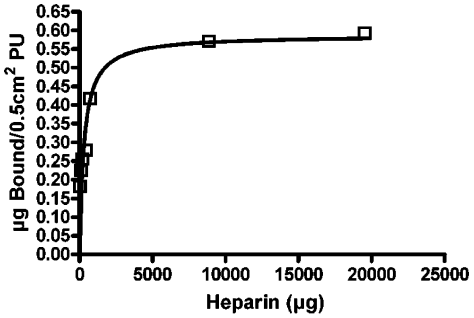


Figure 2. Saturation analysis of the heparin binding capacity of aminated PU. Representative results showing maximal binding capacity of 0.59 μg heparin per 0.5 cm^2 of aminated PU analyzed. Average results of repeated analyses showed the material was capable of binding 1.11 $\pm$ 0.06 μg of heparin per cm^2.

established procedures[8] and was dissolved in phosphate buffered saline at room temperature for 30 min with mild shaking, then washed extensively with 0.5 M NaCl to remove unbound PF4. Bound PF4 was eluted from the PU samples with 100μL of 2 M NaCl for 30 min at room temperature with vigorous shaking. The concentration of PF4 in this eluent was determined after dilution using an Asserachrom PF4 ELISA (Diagnostica Stago, Asnieres, France) according to manufacturer's directions. Results were calculated from a standard curve using PF4 dilutions and normalized to 1 cm^2 surface area.

3. Results

To quantify the amount of grafted PAA, we prepared BODIPY−FL labeled PAA·HCl, via a reaction of PAA base with the succinimide ester, BODIPY−FL SE (Scheme 2) in nonaqueous medium. The labeled PAA base was then immediately transformed into a hydrochloride, to prevent degradation of the fluorescent label under basic conditions. The labeled PAA·HCl exhibited properties similar to those of the nonlabeled compound, whereas the fluorescent label was sufficiently stable.

Fluorescence measurements demonstrated quantifiable levels of PAA grafting. After one amination, 4 μg/cm^2 of surface immobilized PAA·HCl were detected. After a repeated amination in a fresh aminating solution, the amount of grafted PAA·HCl continued to increase and reached 8 μg/cm^2. Thus, there were abundant surface amination sites for subsequent heparinization. Heparin was immobilized onto the surface of aminated PU films via the same direct coupling of NH_2 and COOH groups in the presence of sulfo-NHS and EDC in aqueous media (Scheme 5). BODIPY−FL labeled heparin was used to quantify the amounts of heparin bound to the surface of PU films (modified with nonlabeled PAA), analogously to the estimations of PAA grafting.

To introduce a fluorescent label into heparin macromolecules, carboxylic groups of heparin were partly reacted with the commercial fluorescent probe BODIPY−FL EDA containing a primary amino group in the presence of EDC (Scheme 4). The efficiency of coupling in this case was increased by addition of uncharged NHS instead of the anionic sulfo-NHS. The fluorescence measurements showed a maximal binding capacity of 1.11 $\pm$ 0.06 μg heparin/cm^2 aminated PU film, (Figure 2) and all further binding reactions were carried out with an excess of heparin, 10 000 μg/cm^2. Parallel analysis of non-aminated PU showed less than 0.05 μg/cm^2 heparin bound (data not shown).

FTIR was also used to confirm the surface carboxylation and heparinization, and was in agreement with the NMR (for bulk-carboxylated PU) and fluorescent studies described above. Examination of PU samples by FTIR after amination, and after subsequent heparinization, showed changes in absorbance characteristic of these modifications (Figure 3). The appearance of peaks at approximately 1670 and 1520 cm^{-1} are characteristic of C═O stretching of carboxylation with peak shoulders appearing at 1112 cm^{-1} after heparinization. Heparinization of PU by carboxylation and plasma glow discharge and by subsequent amination[9,10] has been previously described, and in

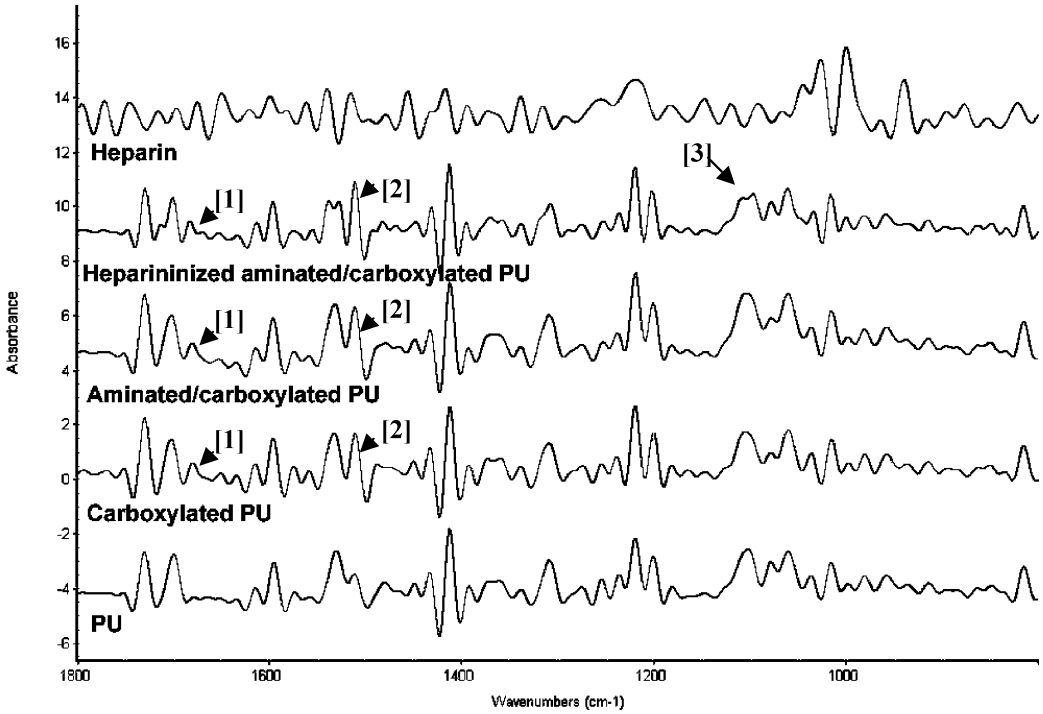


Figure 3. Absorbance peaks of representative FTIR. Five materials are shown: unmodified PU (Tecothane), carboxylated PU, PU which has been surface aminated after carboxylation, heparinized aminated PU, and a heparin solution. The presence of peaks at approximately 1670 (arrow 1)and 1520 (arrow 2) cm^{-1} are characteristic of C═O stretching of carboxylation, as are peak shoulders appearing at 1112 (arrow 3) cm^{-1} after heparinization (S═O of heparin overlapping PU related peaks).

both cases peak shoulders appearing at 1112 cm^{-1} have been indicative of immobilization of heparin on the PU (S═O of heparin overlapping strong PU absorbances).

Moreover, in addition to this physical characterization, we have also demonstrated significant retention of heparin bioactivity with several specific assays. By measuring anti-factor Xa activity, which is used clinically to monitor therapeutic heparin levels, we showed that the anti-factor Xa activity of 1.11 $\mu g/cm^2$ PU-bound heparin was found to be 0.13 ± 0.09 μg heparin/cm^2 (20.5 ± 13.6 mU/cm^2), or $11.7\% \pm 7.8\%$ of the same weight of unfractionated heparin in solution. Further evidence for retention of heparin structural integrity was provided by PF4 studies. PF4 binding to the immobilized heparin was demonstrated at a level of 0.14 ± 0.02 $\mu g/cm^2$, while less than 0.001 ng/cm^2 was detected on unheparinized PU (data not shown).

4. Discussion

The present studies illustrate the broad conjugation potential of the bromo-alkylation strategy that has been investigated by our group in prior studies[1−3] demonstrating successful covalent attachment of either bisphosphonates or cholesterol to urethane nitrogen groups using comparable initial reactions to those investigated herein. Attachment of mercapto-carboxylates to PU modified with pendant 6-bromohexyl residues in the presence of $(Bu_4N)_2B_4O_7$ as a base was described previously by our group[1] and is analogous to the PU modification with thiol-containing bisphosphonates.[4] Here we report a modification of the method, which employs bromobutylation instead of bromohexylation. Bromobutylation of the medical grade PU Tecothane TT 1074A in DMAc-solution with a large excess of 1,4-dibromobutane and lithium *tert*-butoxide as a base[5] permits reducing the exposure of PU to strongly basic conditions. At the same time, reactivity of the resulting PU (**1**) activated with pendant 4-bromobutyl groups toward tetrabutylammonium 3-mercaptopropionate was similar to that of the bromohexylated polymers and resulted in a PU with nonhindered 7-carboxy-5-thiaheptyl groups (**2**, Scheme 1) suitable for further surface modifications. We have found that for the Tecothane-based carboxylated PU this value is close to the upper limit that still allows solvent casting and processing the polymer into films.

Bulk-modifications of PU with pendant carboxylic groups were described in the literature for improving the biomedical properties of PU[11] or rendering the PU surface (normally lacking reactive functional groups) suitable for further covalent immobilizations with useful biomolecules.[12] Two different approaches were generally used to introduce carboxy groups into the PU macromolecules: base-induced alkylation of urethane segments in already formed polymers with lower haloalkyl carboxylates[11] or the use of carboxyl-containing chain extenders in the last step of polymerization.[12] We have found[1] that base-induced alkylation of PU with haloalkyl carboxylates even under optimal conditions results in a noticeable degradation. On the other hand, carboxylated chain extenders can provide the PU macromolecules only with carboxylic functions that are substantially sterically hindered and thus poorly reactive.

Thus, we also demonstrated a novel strategy for surface modification that builds on bromoalkylation chemistry pursued grafting of PAA (as a hydrochloride, M_w ca. 15 kDa and M_n ca. 10 kDa) in aqueous solutions onto the surface of carboxylated PU (**2**) via direct coupling of NH_2 and COOH groups in the presence of EDC (Scheme 4). As recommended in the literature,[13] we introduced sulfo-NHS into the reaction solution to increase the efficiency of coupling. Thus, each molecule of PAA contains on average ca. 100 NH_2 groups and is capable of the formation of multiple bonds with the carboxylated PU, which in turn, contains up to 30 COOH groups per molecule. Total cross-linking of the modified surface into a single interlocked layer seems to be very likely under the chosen conditions. Thus, we expected a sufficient stability of the modified surface layer to allow further modifications, even with extremely hydrophilic macromolecules such as heparin, and the demonstrable specific heparin activity present in our heparinized-PU illustrates the success of this approach.

Heparin modified polyurethanes have been investigated by others as a means to reduce the thrombogenecity of vascular devices fabricated from PU elastomers. Controlled release of heparin from PU was used previously for short-term delivery of bioactive heparin.[7] This strategy confers short-term thromboresistance to the vascular implant, but the heparin is eventually expended.[14] Others have investigated a variety of chemical approaches for immobilization of heparin to PU surfaces as a means to reduce the thrombogenecity of vascular devices fabricated from PU elastomers. For example, plasma glow discharge has been used to increase the concentration of immobilization sites for heparinization on the surface of PU films.[9,10] In these studies, the introduction of chemically reactive species (carboxylic acid, amine groups, or poly(ethylene oxide)) on the surface of PU allowed for the subsequent attachment of heparin at concentrations of 1.1−2.0 $\mu g/cm^2$.[9,10] Another heparinization strategy[15] engrafted a polycarbonate PU surface using an azobis compound followed by the subsequent grafting of an acrylamide monomer providing reactive sites for the attachment of carbodiimide linked heparin moieties. This study demonstrated retention of heparin, via X-ray photoelectron spectroscopy, on the PU surface and resistance to degradation, after exposure to physiological shear forces for 10 hours.[15] This formulation represents a very different approach to surface amination of PU than used in the present study but, nevertheless, enabled successful heparinization via amination as in our experiments. The present studies demonstrate the attachment of similar levels (1.1 $\mu g/cm^2$) of heparin to the PU as was reported by plasma glow discharge. However, it is not possible at this time to note any critical distinctions that distinguish any of these heparinization strategies above the others (including the present report).

In other investigations, activation of the PU surface, via urea or urethane residues, with isocyanate groups allowed for the subsequent attachment of heparin.[16] PU surface amination via diisocyanate reactions followed by heparinization was also reported as part of a combined NO-donor/heparinized PU formulation with anti-fXa activity comparable to the results of the present investigations;[17] PF4 binding studies were carried out in these investigations. In other studies, plasma glow discharge was used to couple heparin to a polyethyleneimine surface layer on a polyurethane C1E3 blood pump surface.[7] Heparin bioactivity on this surface, determined via antifactor Xa assay, was initially 88.5 mIU/cm^2, which declined to 31.4 mIU/cm^2 after washing, indicating the predictable instability of charge-based bindings. Furthermore, other investigations showed, via Factor Xa binding assays, that hydrophillic poly(ethylene oxide) spacers enhanced bioactivity of heparin[18] to levels approaching 19% of activity of free heparin. In the studies reported herein, our formulations demonstrated via a factor Xa assay, 20 mIU/cm^2 or 11.7% of activity of free heparin. These data suggest that the covalent binding of heparin to surface amino groups reduced heparin activity. However, in these experiments, the levels of heparin activity as a function of

surface amino group concentrations were not examined, and it is possible that increasing the amount of appended PAA groups would in turn immobilize more heparin but reduce heparin activity by binding more carboxylic groups on the heparin chain.

In addition, we further determined bioactivity, as a function of PF-4 binding. PF-4 is a heparin binding protein, secreted from platelets, which reduces the anticoagulation properties of heparin.[19] In fact, recombinant PF-4 has been investigated as a novel alternative for neutralization of heparin to terminate systemic heparinization.[20] Thus, we assessed the PF-4 binding activity of heparin immobilized with our bromoalkylation chemistry. These data demonstrated that the heparin immobilized on our surface aminated PU films would very likely be neutralized by the cumulative binding of PF-4. Taken together, these present results, the Factor Xa and PF-4 data, indicate that there would be anticoagulant activity of these surfaces that would eventually be neutralized by the PF-4 effects.

Several limitations of the present studies need to be mentioned, especially since they actually represent the focus of future investigations. Although specific clotting factor (Xa) and platelet interactions (PF4) were demonstrated for the heparinized PU reported in this paper, no in vitro or in vivo coagulation experiments were carried out. However, in vitro clot assays have not predicted the success of heparinized surfaces in the past,[21−23] and thus, we chose not to pursue this direction. Long-term circulatory implants in experimental animals represent the most definitive and aggressive model system for assessing heparinized biomaterials, and these investigations will provide definitive insights about the thromboresistance of the heparinized-PU formulation reported in the present studies. In addition, although we studied a single novel formulation of PU involving bromoalkylation to enable heparinization, prior studies from our group using a comparable approach to append either cholesterol to promote successful endothelial cell binding[3,24] or bisphosphonates, to prevent ectopic calcification,[1,2] suggest a combinatorial approach to create a multifunctional, therapeutically modified PU. While this was beyond the scope of the present studies, the possible synergy resulting from combining therapeutic modifications holds great promise. Our preliminary experiments (data not shown) showed that it is possible to modify up to 44% of urethane groups in PU Tecothane TT-1074A with pendant 4-bromobutyl residues without sacrificing mechanical properties or ability to process the configured polyurethane. It is estimated that only 0.3 mmol/g or 13% of urethane nitrogens were utilized in the carboxylation reactions in the PU modifications reported herein, and thus there is a considerable reserve for additional urethane-nitrogen reactivity that could be used to create combinatorial PU formulations that could hypothetically provide synergistic therapeutic modifications of PU.

5. Conclusion

It is concluded that bromoalkylation of prepolymerized polyurethane elastomers via activation of the hard segment nitrogen can be used to heparinize polyurethane. A novel strategy has been presented involving using bromoalkylation to first achieve bulk-carboxylation of polyurethane followed by surface amination with subsequent heparinization. Specific heparin-related activity (Factor Xa conversion and PF-4 binding) demonstrates the success of this approach. These results taken together with prior successful therapeutic derivatizations by our group of polyurethane via initial bromoalkylation suggest that a combinatiorial is possible to create therapeutic biomaterials with controllable synergy.

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