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Nitric Oxide Releasing Polyurethanes with Covalently Linked Diazeniumdiolated Secondary Amines

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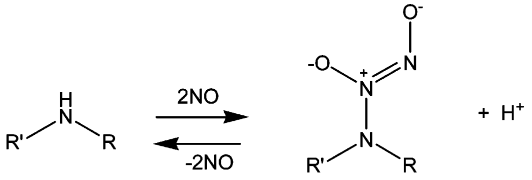
Two novel strategies for synthesizing stable polyurethanes (PUs) capable of generating bioactive nitric oxide (NO) are described. The methods rely on covalently attaching diazeniumdiolate ($N_2O_2^-$) groups onto secondary amine nitrogens at various positions within the polymer chain such that, when in contact with water or physiological fluids, only the two molecules of NO available from each diazeniumdiolate moiety are released into the surrounding medium, with potential byproducts remaining covalently bound to the matrix. Extensive analysis of the NO_x products released from the polymers was employed to develop appropriate strategies to better stabilize the diazeniumdiolate-based polymer structures. In one approach, diazeniumdiolate groups are attached to secondary amino nitrogens of alkane diamines inserted within the diol chain extender of a PU material. Oxidative loss of NO was minimized by blending the polymer with a biocompatible, relatively nonnucleophilic salt before exposing solutions of the polymer to NO during the diazeniumdiolation step. Fluxes of molecular NO from such materials during immersion in physiological buffer reached levels as high as 19 $\text{pmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ with a total recovery of 21 nmol of NO/mg of PU. A second general synthetic strategy involved ω-haloalkylating the urethane nitrogens and then displacing the halide from the resulting polymer with a nucleophilic polyamine to form a PU with pendent amino groups suitable for diazeniumdiolation. Commercially available Pellethane 2363-80AE that was bromobutylated and then reacted with diethylenetriamine and further exposed to gaseous NO proved stable in solid form for several months, but released NO with a total recovery of 17 nmol/mg upon immersion in physiological buffer. This material showed an initial NO flux of 14 $\text{pmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ when immersed in pH 7.4 buffer at 37 °C, with gradually decreasing but still observable fluxes for up to 6 days.

Introduction

For a polymer to be successfully used in biomedical applications, it must have good compatibility with the surrounding physiological environment. A significant problem encountered with current synthetic materials, such as polyurethane (PU), silicone rubber, and poly(vinyl chloride) (PVC), is thrombus formation. Typically, when a foreign substance comes in contact with blood, platelets adhere to its surface and activate, leading to the formation of a fibrin network.[1] This thrombus formation can cause device failure.[2,3] Hence, the focus of many researchers has been to create nonthrombogenic polymers.

Because polyurethanes have good biocompatibility, good mechanical properties, and are inherently more thromboresistant than other polymers, they are widely used in a variety of biomedical applications including catheters,[4] components of hemodialysis units,[5] and vascular grafts.[6] However, the thromboresistivity of polyurethanes still needs to be improved, as platelet activation, adhesion, and thrombus formation can still occur on existing biomedical grade polyurethanes. Fortunately, the versatile nature of PU synthesis allows for the tailoring of the specific properties known to decrease platelet adhesion, such as hydrophilicity and hydrogen bonding interactions.[7] For example, researchers have tried optimizing the hard-to-soft segment ratio,[8] changing the nature of the soft segment[9,10] and immobilizing heparin onto the polymer backbone[11] as means to further improve the biocompatibility of such materials.

Another approach to improve the thromboresistivity of polymers is to incorporate additives known to prevent thrombus formation into polymer coatings. For example, Espadas-Torre et al. in 1997 and Mowery et al. in 1999 demonstrated that blending small amounts of the nitric oxide (NO) releasing diazeniumdiolates into polymer films, including polyurethanes, greatly decreases the platelet adhesion and thrombus formation on the surface of various polymeric coatings.[12−14] The diazeniumdiolate decomposes spontaneously when exposed to water to release NO and a free amine (see eq 1 below). Although the results were very promising, in some instances, the diazeniumdiolate as well as the amine byproduct and an *N*-nitroso derivative thereof have been found to leach from the polymer film and could cause health risks.[14] In an effort to minimize



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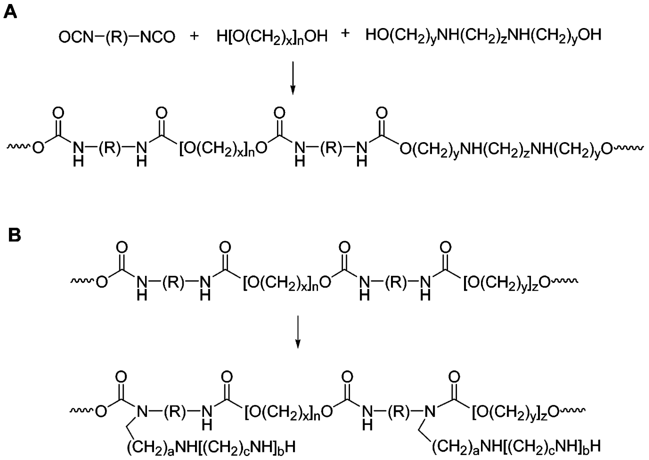
Scheme 1. Two General Methods of Preparing Polyurethanes Suitable for Diazeniumdiolation[a]



[a] The methods produce polymers that, upon diazeniumdiolation, are capable of releasing bioactive NO at physiological pH while keeping the carrier amines and other potentially toxic components covalently bound to the polymer matrix by (A) incorporating the carrier amine into the diol chain extender or (B) attaching the amine via a linker to the urethane nitrogen.

leaching, Batchelor et al. reported on efforts to covalently attach diazeniumdiolates onto polyurethane backbones and the potential instability of such diazeniumdiolate moieties even on secondary diamine structures.[15] Jun et al. recently reported that polyurethanes containing primary amine diazeniumdiolates could be prepared by incorporating a lysine-based polypeptide as the chain extender within the polymer backbone,[16] although questions exist as to whether the polyurethanes prepared liberate NO since primary amine diazeniumdiolates are known to be quite unstable[17] and no direct NO measurement technique was employed to quantitate the released NO_x species.

In the present work, we report two synthetic routes to prepare polyurethanes capable of being diazeniumdiolated (Scheme 1) using secondary amines linked to the polymer material. Each method produces polymers containing covalently bound NO-releasing segments that cannot leach undesired byproducts. The efficacy of forming stable diazeniumdiolate moieties on polymer backbones and the rate of NO release from the resulting structures are compared. Both methods produce materials that combine the innate biocompatibility of polyurethanes with the antiplatelet effects of NO release. Further, the strategies described, involving covalently binding the NO donor to the PU polymer, also serve to localize the effects of the released NO to the area in close proximity to the polymer, since no small molecule diazeniumdiolates (which could leach) are employed.

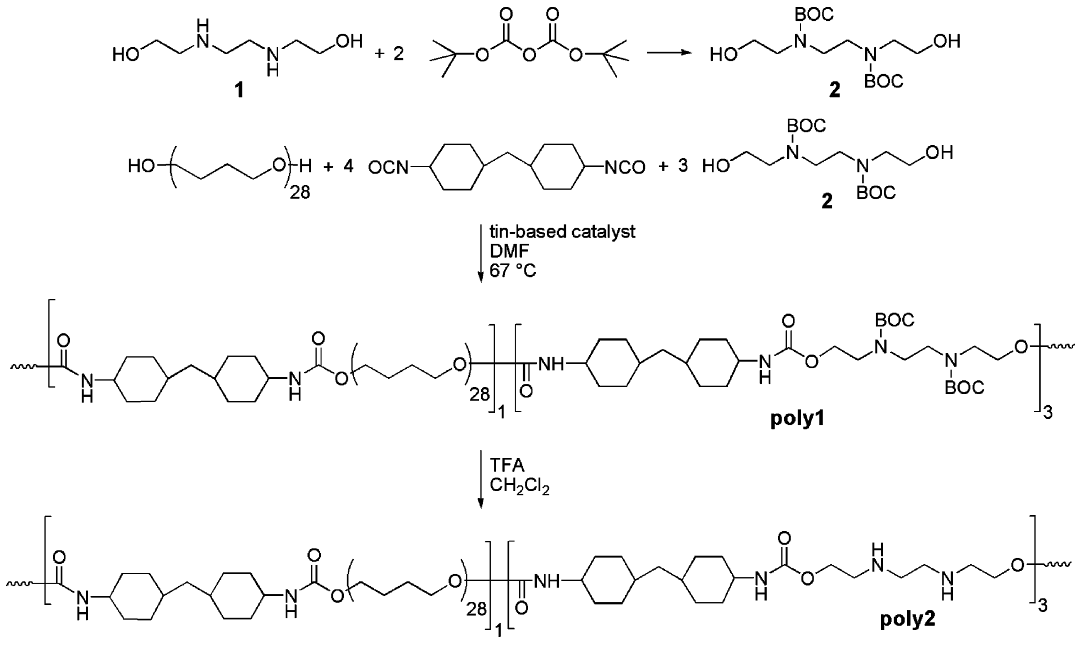
Experimental Section

Materials. *N*,*N*′-Bis(2-hydroxyethyl)ethylenediamine (**1**, see Scheme 2), di-*tert*-butyl dicarbonate, dibutyltin dilaurate, and terathane (MW = 2000) were purchased from Aldrich and used as received. Methylene bis(4-cyclohexyl isocyanate) (HMDI) was received from Bayer and used without further purification. Pellethane 2363-80AE was purchased from Dow Plastics. *N*,*N*-Dimethylformamide (DMF) was dried by distillation from CaH_2 under reduced pressure. Acetonitrile was stored over 3-Å molecular sieves. Tetrahydrofuran (THF) was distilled from benzophenone ketyl. Other reagents were purchased and used as received.

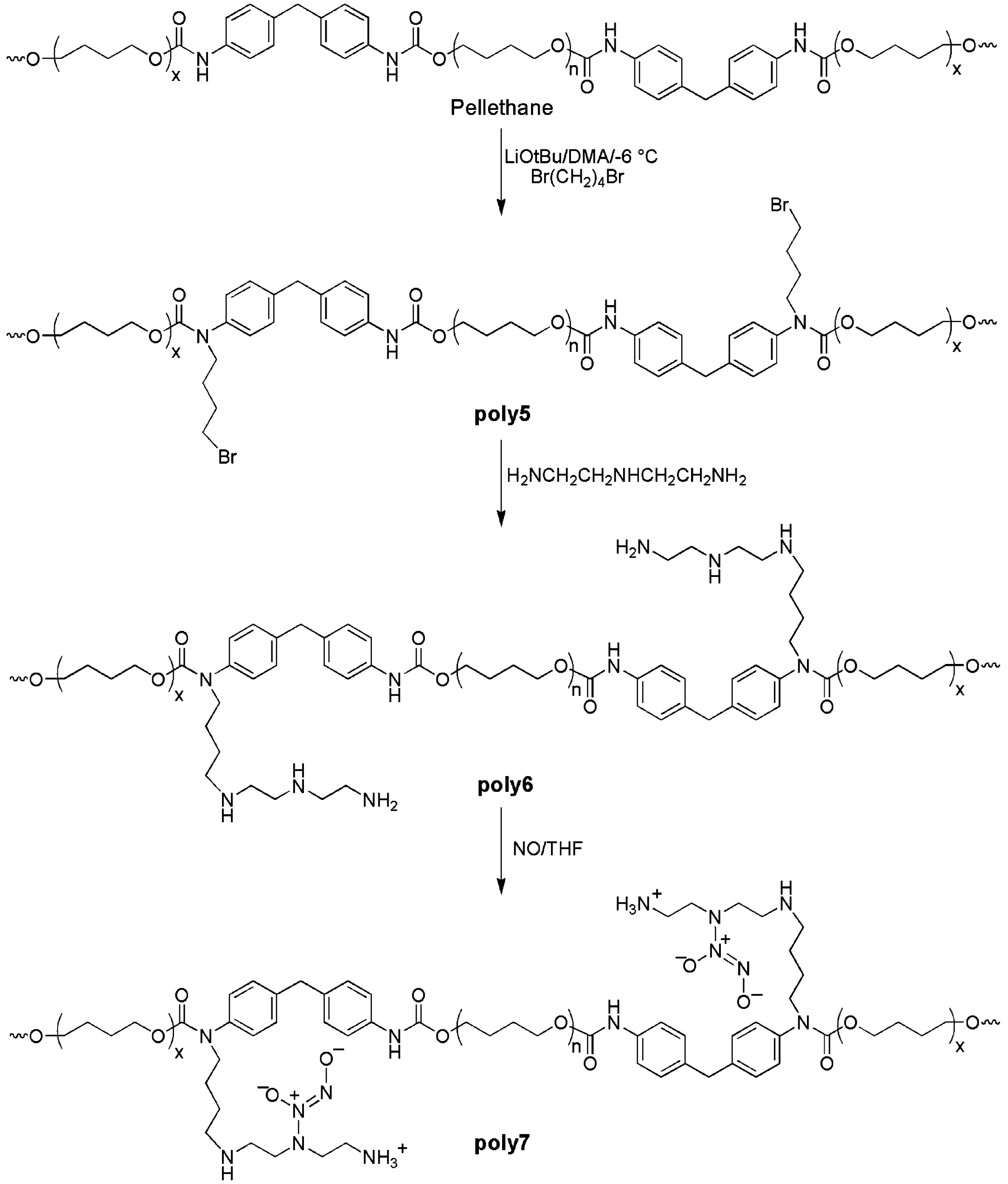
General Methods. UV−visible spectra were collected on a Beckman DU 640B spectrophotometer using samples prepared by dipping a quartz slide into a polymer solution to create a thin film on the surface. Fourier transform infrared (FT-IR) spectra were collected on a Perkin-Elmer SpectrumBX. Polymer films were cast onto a NaCl plate from a CH_2Cl_2 solution or immobilized between two NaCl plates. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were performed on a Perkin-Elmer DSC/TGA 7 under nitrogen. The DSCs were heated at 15 °C/min, and T_g's were obtained from the second heat. The TGAs were heated at 30 °C/min, and decomposition temperatures are reported at a 5% weight loss. Elemental analyses were performed by the University of Michigan Microanalysis Laboratory. Gel permeation chromatography was performed using a UV and RI detector, polystyrene calibration standards, and CH_2Cl_2 as the eluent. The columns were the Waters Styrogel Models HT 2, HT 3, and HT 4.

Synthesis of *N*,*N*′-Bis(2-hydroxyethyl)-*N*,*N*′-bis(*tert*-butoxycarbonyl)ethylenediamine (2). A 250-mL Schlenk flask equipped with a stir bar and N_2 inlet/outlet was charged with *N*,*N*′-bis(2-hydroxyethyl)-ethylenediamine (**1**, 2.03 g, 13.7 mmol) suspended in THF (100 mL). Di-*tert*-butyl dicarbonate (6.28 g, 28.8 mmol) was added with a syringe.

Scheme 2. Synthetic Scheme To Prepare Diamine-Containing Polyurethane **poly2**



Scheme 3. Synthetic Scheme To Prepare **poly5**−**poly7**

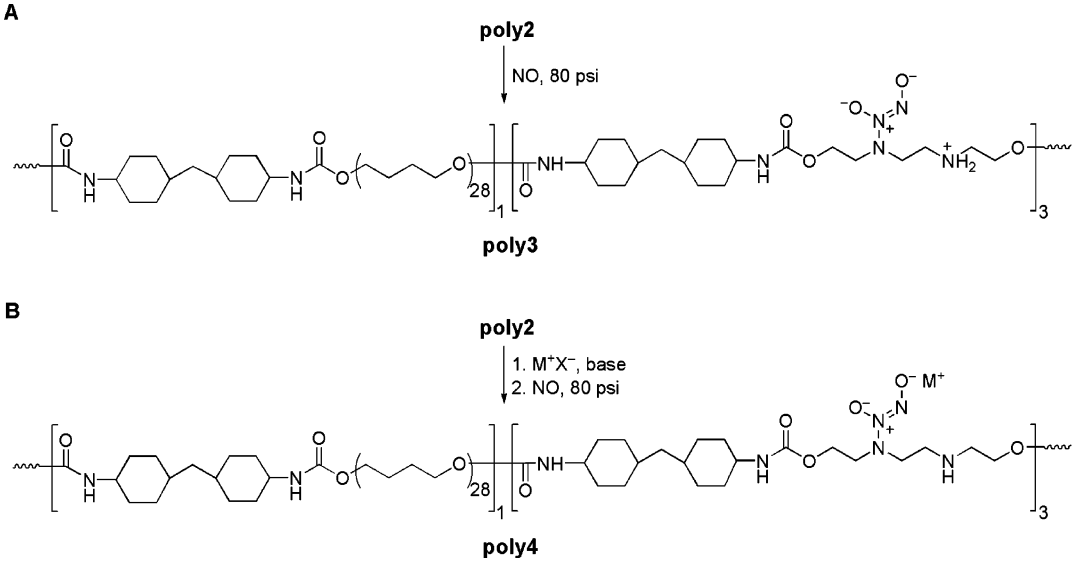


A condenser was placed onto the flask, and the reaction mixture was heated to reflux for 14 h. The solution was allowed to cool and was washed with saturated $NaHCO_3$ (2 × 100 mL). The organic layer was separated and dried over $MgSO_4$. The solvent was removed to give a white solid which was washed with hexanes, collected, and dried under vacuum. Yield = 4.27 g (89%): ^{1}H NMR ($CDCl_3$) δ 1.44 (18 H, s, $-C(CH_3)_3$), 3.25−3.58 (8 H, m, $-H_2CNCH_2-$), 3.70−3.80 (4 H, m, CH_2OH); FT-IR (KBr) $\nu_{C=O}$ = 1673 cm^{-1}, ν_{O-H} = 3466 cm^{-1}. Anal. Calcd for $C_{16}H_{32}N_2O_6$: C, 55.14; H, 9.27; N, 8.04. Found: C, 55.07; H, 9.39; N, 8.11.

Synthesis of poly2 (see Scheme 2). A dry 100-mL three-neck flask equipped with a condenser, mechanical stirrer, and an N_2 inlet/outlet was charged with terathane 2000 (5.75 g, 2.88 mmol), HMDI (3.02 g, 3.0 mL, 11.5 mmol), and dibutyltin dilaurate (0.11 g, 0.17 mmol) in dry DMF (10 mL). The reaction mixture was heated to 67 °C. After 1.5 h, *N*,*N*′-bis(2-hydroxethyl)-*N*,*N*′-bis(*tert*-butoxycarbonyl)ethylenediamine (**2**, 3.01 g, 8.63 mmol) was added with an additional 7 mL of dry DMF. The solution was allowed to stir overnight at 67 °C, during which time the viscosity increased. The solution was poured into H_2O, and the solid was collected and dried under vacuum. The resulting BOC-protected polymer (**poly1**) was dissolved in 25% trifluoroacetic acid (TFA)/CH_2Cl_2 (133 mL) and allowed to stir for 10 h. The solvent was removed to give a viscous oil. The oil was dissolved in DMF, and the polymer was neutralized and precipitated by pouring into saturated $NaHCO_3$ (500 mL). The solid was collected, washed with H_2O, and dried under vacuum. Any residual water was removed by redissolving the polymer in THF (300 mL) and drying it over $MgSO_4$. The mixture was filtered through a 0.25-in. plug of Celite, and the polymer (**poly2**) was precipitated by pouring the filtrate into hexanes (600 mL). The polymer was filtered and dried under vacuum. Yield = 8.26 g (82%): TGA, 5% weight loss = 271 °C; DSC, T_g = 23 °C; FT-IR (KBr) $\nu_{C=O}$ = 1718 cm^{-1}, 1701 cm^{-1}, ν_{C-O} = 1113 cm^{-1}, ν_{N-H} = 3324 cm^{-1}. Anal. Calcd for $C_{190}H_{362}N_{14}O_{43}$: C, 64.61; H, 10.35; N, 5.55. Found: C, 64.34; H, 10.15; N, 5.44.

Procedure for Adding NO to poly2 to Produce poly3 (See Scheme 4A). A dry Parr bottle was charged with the amine-containing polyurethane **poly2** suspended/dissolved in CH_3CN or THF with or without the addition of a salt. The mixture was purged with argon to remove oxygen and then charged with NO up to 75 psig. The mixture was allowed to stir for 5 days, during which time NO was added as

Scheme 4. Method To Prepare Diazeniumdiolated Polyurethanes Using Amine-Containing Polymer Prepared by Method 1



necessary to maintain the pressure. The NO was released, and the mixture was bubbled with argon for 10 min and filtered. The polymer, **poly3**, was washed with diethyl ether. The polymer was dried under vacuum. TGA, 5% weight loss = 262 °C; DSC, $T_g = 32$ °C; FT-IR (KBr) $\nu_{C=O} = 1718$ cm^{-1}, 1701 cm^{-1}, $\nu_{N-H} = 3324$ cm^{-1}, $\nu_{C-O} = 1113$ cm^{-1}. Anal. Calcd for $C_{190}H_{362}N_{17}O_{46}$: C, 63.28; H, 10.14; N, 6.61. Found: C, 63.31; H, 10.49; N, 6.45.

Procedure for Adding NO to poly2 to Produce poly4 (See Scheme 4B). A dry Parr bottle was charged with the amine-containing polyurethane **poly2** dissolved in THF with the addition of a salt. The mixture was purged with argon to remove oxygen and then charged with NO up to 75 psig. The mixture was allowed to stir for 1−2 days, during which time NO was added as necessary to maintain the pressure. The NO was released, and the mixture was bubbled with argon for 10 min and precipitated into dry hexanes. The polymer, **poly4**, was washed with methanol and then diethyl ether; then it was dried under vacuum. TGA, 5% weight loss = 220 °C; FT-IR (NaCl) $\nu_{C=O} = 1717$ cm^{-1}, $\nu_{N-H} = 3329$ cm^{-1}, $\nu_{C-O} = 1111$ cm^{-1}.

Synthesis of 4-Bromobutylpellethane (poly5) (See Scheme 3). A solution of 14.28 g Pellethane 2363-80AE (this quantity is said to contain 38.0 mmol of NH groups[18] in 300 mL of *N*,*N*-dimethylacetamide) was prepared and cooled to −6 °C in a dry ice/carbon tetrachloride bath. To this was added 15 mL of 1,4-dibromobutane (126 mmol) followed by the dropwise addition of a solution of 7.6 mL of 1.0 M lithium *tert*-butoxide in hexane (7.6 mmol) dissolved in 20 mL of *N*,*N*-dimethylacetamide over 10 min. The bath was replaced by a simple ice bath, and the reaction was allowed to stir 1 h and was quenched by the addition of 6.5 mL of acetic acid. The dark yellow solution was then poured into cold (dry ice cooled) methanol (1.2 L), and the resulting slurry was allowed to stand 2 h as it warmed to room temperature. The large, fluffy clump of white rubbery product was removed using tongs, washed with three 500-mL portions of methanol, and then dried by squeezing between paper towels followed by drying in a vacuum overnight. The yield was 18.4 g of white rubbery solid. Elemental analysis: C, 64.86; H, 8.47; N, 3.70; Br, 3.98.

Synthesis of Butyl-Linked Diethylenetriamine/Pellethane (poly6) (See Scheme 3). A solution of 5.13 g of 4-bromobutyl/pellethane **poly5** in 100 mL of *N*,*N*-dimethylacetamide was prepared, and to this was added 13 mL of diethylenetriamine (120 mmol). The solution was stirred at room temperature for 6 h, poured slowly into 1.7 L of cold methanol, and stored in a refrigerator overnight. The resulting large off-white spongy clump of polymer was removed using tongs, washed with three 500-mL portions of methanol, squeezed dry between layers of paper towels, and dried in a vacuum for 1 day. The yield was 4.73 g of a single large, rubbery clump of off-white polymer that was cut into thin strips using scissors. Elemental analysis: C, 65.12; H, 8.44; N, 4.94; Br, 0.78.

Preparation of Pellethane/DETA/NO (poly7) (See Scheme 3). A solution of 4.73 g of butyl-linked diethylenetriamine/Pellethane **(poly6)** in 250 mL of THF was prepared and placed in a large Parr bottle. The solution was purged with argon and placed under NO at an initial pressure of 78 psig. The reaction was stirred at room temperature for 2 days as the pressure slowly fell to 75 psig; then the NO was vented and the bottle was flushed with argon. The resulting mixture (some polymer had separated as a gel) was poured into a large excess of ethyl ether, the solvent was removed by decantation, and the off-white fluffy polymer was rinsed with two additional portions of ether. After drying in vacuo, the yield was 3.44 g of a dense yellow rubbery polymer. TGA, 5% weight loss = 210 °C; FT-IR (NaCl) $\nu_{C=O} = 1716$ cm^{-1}, 1699 cm^{-1}, $\nu_{N-H} = 3317$ cm^{-1}, $\nu_{C-O} = 1119$ cm^{-1}.

Nitrite-Release Measurements via Griess Assay. Polymer films were prepared by casting a solution containing 200 mg of the appropriate polymer dissolved in 3 mL of THF into a 2.5 cm i.d. Teflon ring. The rings were covered to allow slow evaporation of the solvent. After 24 h, 1-cm disks were cut and soaked in phosphate buffered saline (PBS) solution (composition 0.138 M NaCl, 0.0027 M KCl, 0.01 M phosphate; pH 7.4) maintained at 37 °C. At selected time intervals, the films were transferred to fresh PBS soaking solutions. Nitric oxide was measured indirectly via the Griess assay fitted to a microtiter plate format. The Griess assay is a two-step process: diazotization followed by azo coupling. Briefly, 200-μL samples were pipetted into a 96-well microtiter plate and chilled to 4 °C. Forty microliters of a 1:1 mixture of 6 M HCl and 12.5 mM sulfanilic acid were then added to each well, and the samples were chilled for an additional 10 min at 4 °C. Twenty microliters of 12.5 mM *N*-(naphthyl)ethylenediamine dihydrochloride (NEDA) was then added to make an azo compound that has an absorbance maximum at 540 nm. The samples were analyzed with a Labsystems Multiskan RC 96-well microplate reader. The absorbance at 540 nm was directly proportional to the concentration of nitrite in the sample.

NO-Release Measurements via Chemiluminescence. NO gas measurements were performed using a Sievers nitric oxide analyzer (NOA), Model 280 or 280i. The instruments were calibrated before each experiment with house nitrogen as the zero gas. The flow rate was set to 200 mL/min with a cell pressure of 5.4 Torr and an oxygen pressure of 6.0 psig. The measurement was performed by inserting the diazeniumdiolated PUs into a clean, dry, NOA measurement cell, sealing the cell with a rubber septum, and collecting a baseline level of NO. Deoxygenated PBS was then injected via syringe through a septum into the NOA measurement cell. The NO generated from the sample was removed from the solution and passed into the NOA via a constant nitrogen purge. At the end of the experiment, the NO-reacted material was removed from the cell under a positive pressure of nitrogen. The soaking solution was then purged with nitrogen until the baseline level of NO was obtained. The soaking solution was saved for subsequent measurement of nitrite. The data as recorded on an internal RAM card were processed using Excel.

Results and Discussion

The first step in preparing diazeniumdiolated polyurethanes was the synthesis of a polyurethane that contains secondary amine sites. It has been shown that secondary amines react with NO to yield diazeniumdiolates that, when exposed to water, release NO and the starting amine (see eq 1).[14,19,20] Additionally, the presence of the amines should increase the hydrophilicity and hydrogen-bonding capability of the polymers, both of which are known to improve biocompatibility.[7] At the same time, however, the amine sites can become protonated, causing a positively charged surface. Charged surfaces can cause increased protein adhesion. However, such effects can be reduced by using a non-amine-containing polyurethane overcoat layer.

Amine sites were incorporated into the polymer backbone using two synthetic methods as outlined generally in Scheme 1. In the first method (Scheme 1A), polyurethanes are synthesized by adding an amine-containing chain extender during the polymerization reaction. In method 2 (Scheme 1B), commercially available polyurethanes are derivatized to yield polyurethanes with pendent secondary amine sites.

Preparation of Amine-Containing Polyurethanes via Method 1. The synthesis of polyurethanes is well developed[21,22] and provides methods of synthesizing polymers containing NO-releasing diazeniumdiolate moieties. Polyurethanes are typically synthesized from three components: (1) a polyether- or polyester-based macroglycol; (2) a diisocyanate linker; (3) a chain extender. The most important component in polyurethane synthesis is the diol chain extender since it contains chemical functionalities that give the polymer its desired characteristics. For example, sulfonated polyurethanes are synthesized by incorporating chain extenders that contain sulfur groups,[23] and phospholipid polyurethanes have been prepared from chain extenders with pendent phospholipid side chains.[24,25] NO-releasing polymers can thus be prepared, in principle, by using chain extenders that allow the incorporation of diazeniumdiolate moieties into the polymer as suggested by Batchelor et al.[15] and Jun et al.[16] As an exemplary chain extender containing secondary amine sites, *N*,*N*′-bis(2-hydroxyethyl)ethylenediamine was chosen (see Scheme 2, compound **1**). This diamine-based chain extender was used because it is structurally similar to diamines that had been reported previously to form intramolecular zwitterionic diazeniumdiolates when reacted with NO.[19]

The synthesis of the diamine-containing polyurethane is shown in Scheme 2. The first step was the reaction of the chain extender, *N*,*N*′-bis-(2-hydroxyethyl)ethylenediamine (compound **1**), with di-*tert*-butyl dicarbonate to form the BOC-protected amine, compound **2**. This was done to prevent the undesirable side reaction of the secondary amine sites with the diisocyanate linker to produce urea groups. The BOC group was chosen because it is a selective amine-protecting group that is easily removed. The BOC-protected polymer (**poly1**) was prepared using a one-step polymerization procedure by the reaction of 4,4′-methylene-bis-cyclohexyl diisocyanate (HMDI), polytetramethylene glycol (terathane), and the chain extender in the presence of dibutyltin dilaurate at 67 °C for 12 h. A 4:3:1 ratio of diisocyanate to chain extender to terathane was used for synthesizing **poly1**. Deprotection of the BOC protecting groups was achieved by treating the polymer with 25% v/v trifluoroacetic acid (TFA) solution to give the resulting diamine-containing polyurethane (**poly2**). Excess acid was neutralized by washing the polymers with $NaHCO_3$ solution.

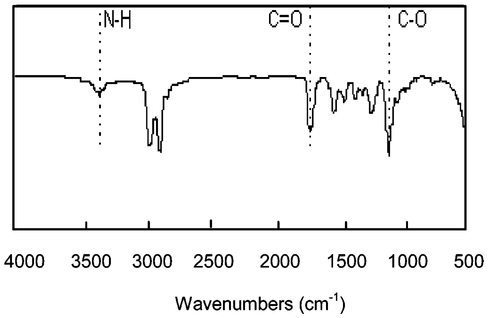


Figure 1. FT-IR spectrum of **poly2**.

Preparation of Amine-Containing Polyurethanes via Method 2. A second method to introduce potentially NO-reactive amine sites into a polyurethane backbone is shown in Scheme 1B. A procedure has been described previously[26] that allows the bromoalkylation of the urethane hard segments in various commercially important polyurethanes via low-temperature deprotonation with lithium *tert*-butoxide. We elected to apply this method to Pellethane 2363-80AE, a thermoplastic polyurethane elastomer prepared from methylenediphenyl diisocyanate (MDI), 1,4-butanediol, and polytetramethylene glycol (terathane) by Dow Plastics. This polyurethane is known to contain 2.66×10^{-3} mol of urethane NH sites per gram.[18] Pellethane 2363-80AE was reacted with 1,4-dibromobutane to prepare a PU derivative containing pendent 4-bromobutyl groups as shown in Scheme 3. This material was then treated with various polyamines to substitute amine-containing chains for the bromine. Diethylenetriamine proved to be the best polyamine to use since it produced a PU **(poly6)** containing minimal cross-linking.

Characterization of Polyurethanes Prepared by Method 1. The polyurethanes were characterized using elemental analysis, IR spectroscopy, DSC, and TGA. The molecular formula of a repeat unit was determined for **poly2** using the ratio of the reactants, and elemental analysis confirmed the calculated chemical composition.

Measurements using IR spectroscopy were performed and showed absorbances indicative of amine, alcohol. and carbonyl functionalities. Figure 1 shows the FT-IR spectrum for **poly2**. The defining features come from the N−H (3324 cm^{-1}), carbonyl (1701 cm^{-1}, 1718 cm^{-1}), and ether (1113 cm^{-1}) linkages in the polymer backbone.

There was no observable UV absorption present, between 200 and 400 nm, for **poly2**. The molecular weight of the polymer was determined by gel permeation chromatography (GPC). The M_n was 2.03×10^6 with a polydispersity index (PDI) of 2.33 for **poly2**. Molecular weight was also determined for the protected polyurethane. **Poly1** had an M_n of 5.45×10^5 and a PDI of 1.15.

Characterization of Polyurethanes Prepared by Method 2. Since Pellethane is a well-characterized polymer of commerce, no further effort was made to study its basic polyurethane structure. The satisfactory addition of the bromobutyl side chain to Pellethane was verified by obtaining elemental analysis data that clearly showed the intermediate polymer to contain 3.98% halogen. Better than 80% of the bromine was removed by

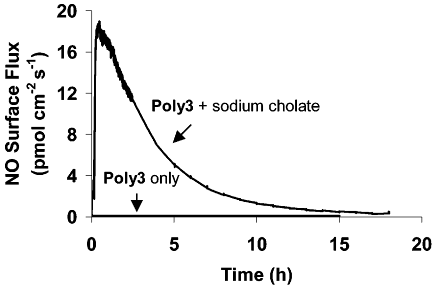


Figure 2. NO release curves for **poly3** in the presence and absence of sodium cholate. NO release was measured via chemiluminescence at 37 °C in PBS (pH 7.4).

substitution with diethylenetriamine, and this was verified by the decrease in the halogen content as measured by elemental analysis.

Nitric Oxide Addition to Polyurethanes Prepared by Method 1. Polyurethanes, prepared using Scheme 2, were successfully diazeniumdiolated only when exogenous salts were incorporated during the NO addition step (Scheme 4). Representative NO release curves (based on direct detection of NO using chemiluminescence) are shown in Figure 2 for NO-reacted **poly2** (synthesized using a 1:3:4 ratio of polyether to diamine chain extender to diisocyanate) in the presence and absence of sodium cholate. This material released 2.1×10^{-8} mol of NO/mg of PU over a 24 h period, corresponding to an initial surface flux of 19 pmol·cm^{-2}·s^{-1}. Approximately 0.91% of the diamine sites were diazeniumdiolated. Without the addition of the exogenous salt, the diazeniumdiolate groups decomposed readily under ambient conditions to yield predominantly nitrite-releasing polymers (Figure 3C) (based on direct detection of nitrite using the Griess assay compared to direct detection of NO using an electrochemical NO sensor—see Supporting Information). We believe this finding is a direct result of the decomposition of diazeniumdiolates to form nitrite under ambient conditions.

The requirement of adding exogenous salts was surprising given that this same synthetic methodology had been used previously to prepare diazeniumdiolated silicone rubber materials[27] and poly(ethyleneimine) polymers.[28] Originally, it was believed that ammonium ions from other diamine nitrogens within the NO-reacted **poly2** structure could serve as the counterions to the diazeniumdiolates through either intra- or intermolecular interactions (see idealized structures in Figure 3A,B). However, this interaction apparently does not occur or is too weak to yield stable diazeniumdiolated structures. The rapid decomposition of diazeniumdiolates in air has been demonstrated previously for more lipophilic diamine-based zwitterionic diazeniumdiolates.[29] Although the exact cause of the destabilization is not yet known, it could be reasoned that the long monomer units and the rigidity of the hard segments of the polyurethane prevent the formation of strong intramolecular hydrogen bonds required to stabilize the diazeniumdiolate. Therefore, a counterion (from added salts) that is not attached covalently to the polymer is required to form a stable diazeniumdiolate as shown in Scheme 4B and Figure 3D,E.

Incorporation of External Countercations to Increase the Stability of the Diazeniumdiolate Moiety. The incorporation of exogenous cationic sites via the addition of base during the reaction of NO with the diamine-based polyurethane increased the NO-to-nitrite ratio. This increase in the number of stable diazeniumdiolate groups formed is presumably due to the enhanced stability of the diazeniumdiolate moiety via electrostatic interaction of the exogenously supplied cation with the negative charge of the diazeniumdiolate group. In addition, because the hydrogen bonding interaction from the neighboring ammonium ion is no longer needed to ensure stability of the diazeniumdiolate, the possibility of diazeniumdiolating twice as many amine sites exists and could offer even greater NO loading for a given mass of PU (see Figure 3E).

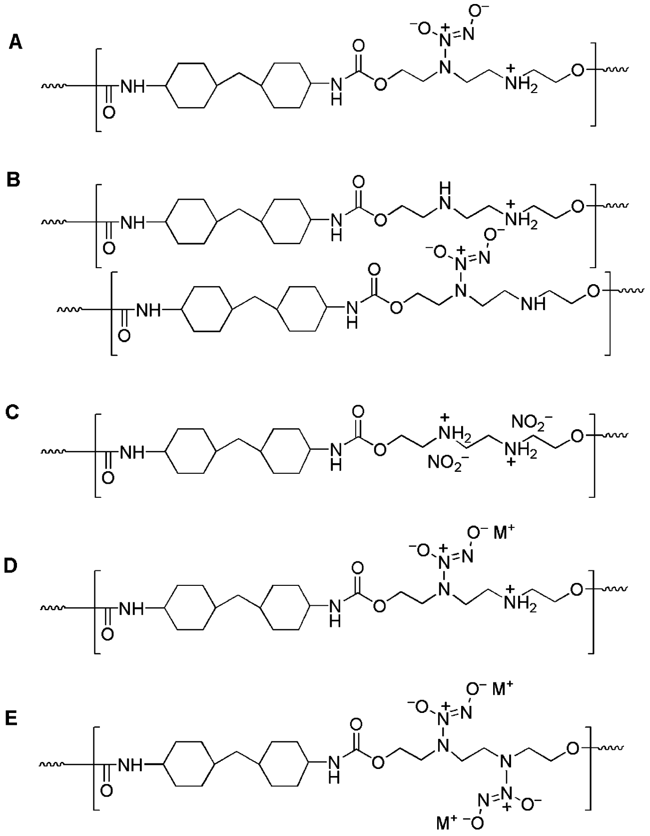


Figure 3. Possible structures of **poly3** and **poly4** after **poly2** is reacted with NO under various conditions: (A) intramolecularly hydrogen bonded diazeniumdiolate from the same monomer unit; (B) self-stabilized diazeniumdiolate through hydrogen bond interactions from another polymer chain; (C) ammonium nitrite salt; (D) single anionic diazeniumdiolate form; (E) bis-anionic diazeniumdiolates for each chain-extender unit.

Ideal attributes of the added base salt would include an alkali metal cation, a nonnucleophilic or sterically hindered anion, and solubility in the reaction solvent (THF). Bases such as the trimethylsilanolates and methoxides have been used extensively to create anionic diazeniumdiolates by removing a proton from secondary amine nitrogens.[30,31] However, since urethane linkages are susceptible to cleavage by base, an efficient and easy screening method was needed to assess whether bases of interest can decompose urethane linkages. A method was developed that employed a rapid and simple analysis using a model urethane that contained no other functionality (see Supporting Information for method). In addition, the proposed additives were evaluated for their possible reaction with NO alone using spectrophotometric methods (see Supporting Information). Using the screening methods, several salts, bases, and combinations thereof were investigated for their reactivity to NO and their ability to hydrolyze urethane linkages. These additives and their reactivity with urethane linkages and NO are summarized in Table 1. Only salt and base combinations that emerged from the screening process were used as candidates for reaction with **poly2**. These candidates were 2,6-di-*tert*-butyl-4-methylpyridine (2,6-DTB-4-MP), sodium hexafluorophosphate (NaPF$_6$), sodium thiocyanate (NaSCN), lithium phosphate (Li$_3$PO$_4$), lithium tetrafluoroborate (LiBF$_4$), and sodium cholate (NaC$_{24}$H$_{39}$O$_5$).

Table 1. Summary of Additives' Reactivity with NO and Urethane Linkages

reagent	react with NO?	hydrolyze urethane linkage?
$NaPF_6$	no	no
NaSCN	no	no
Li_3PO_4	no	no
$NaC_{24}H_{39}O_5$	no	no
$LiBF_4$	no	yes (concentration dependent)
NaOMe	no	yes
$NaOSiMe_3$	no	yes
2,6-DTB-4-MP	no	no
proton sponge	yes	no

Table 2. Summary of Improvement in the NO Loading of **poly2** with Additives

salt	base	mole ratio diamine:salt:base	% NO[a]
			0.7
$LiBF_4$		1:1:0	0.3
	2,6-DTB-4-MP	1:0:2.1	0.3
$LiBF_4$	2,6-DTB-4-MP	1:1:1.1	22
$LiBF_4$	2,6-DTB-4-MP	1:1:2	18
$LiBF_4$	2,6-DTB-4-MP	1:2:2.1	31
Li_3PO_4		1:2:0	0.3
Li_3PO_4	2,6-DTB-4-MP	1:1.4:1.5	6
Li_3PO_4	2,6-DTB-4-MP	1:1:2	18
$NaPF_6$		1:2.1:0	0.3
$NaPF_6$	2,6-DTB-4-MP	0.5:2:2.1	13
$NaPF_6$	2,6-DTB-4-MP	0.5:1:1	41
NaSCN		1:2.1:0	0.5
NaSCN	2,6-DTB-4-MP	1:2:2.1	4
$NaC_{24}H_{39}O_5$		1:2.1	*b*

[a] % NO is defined as the moles of NO gas generated as measured by soaking samples in PBS buffer (pH 7.4) after various time periods at 37 °C divided by the nitrite (from the soaking solution) + NO detected by chemiluminescence measurements. [b] Not able to determine nitrite quantity due to interference of sodium cholate with the Griess assay; however, significant amounts of NO were generated.

Table 2 summarizes the NO reaction conditions employed and the percentage of NO (based on total NO and nitrite) released from polymers prepared using these additives once exposed to PBS solution. Notable is that all of the salt/base combinations produced polyurethanes with higher NO-to-nitrite ratios than reactions in the absence of additives. Second, the highest ratio of NO to nitrite is also achieved with sodium as a countercation compared to lithium. Finally, in terms of the NO release profiles, similar profiles are generated from all sodium salts and 2,6-DTB-4-MP regardless of the anion.

Although all the sodium salts produced polyurethanes with similar NO release profiles, sodium cholate was considered to be the most ideal salt studied for synthesizing NO-releasing polyurethanes using method 1. This is because both sodium ion and cholate ion are biologically inert agents. Any leaching of these species from the polymer would not pose health risks to the patient. In addition, it is hypothesized that cholate could serve a function similar to that of borate for these films containing free amine sites by buffering the pH of the polymer phase as demonstrated by Batchelor et al.[29] Taken together, these results prove that the diazeniumdiolate moiety can indeed be stabilized by the addition of countercations under NO reaction conditions.

Nitric Oxide Addition to poly6 Prepared by Method 2. Direct reaction of amine-containing Pellethane **poly6** with NO afforded polyurethanes containing diazeniumdiolate functionality presumably because the amine-containing side chains are more flexible, when compared to the materials described above for method 1. Formation of these NO-releasing polyurethanes did not require the addition of exogenous base or cations. This is presumably due to having pendent amine sites on the polymer that are not constrained by the rigid polymer backbone. This flexibility would be expected to allow the zwitterionic diazeniumdiolate to form more easily. In fact, in the presence of added salts, less diazeniumdiolation was found to occur.

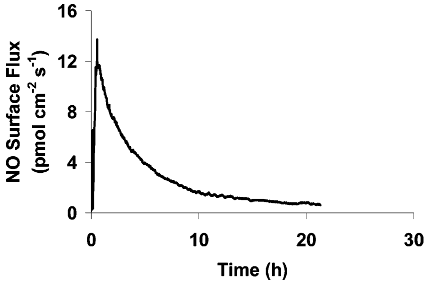


Figure 4. NO release curve for Pellethane/DETA/NO (poly 7) for the first 21 h of soaking in PBS (pH 7.4) at 37 °C. Detectable NO release continued for a total of 6 days.

The best NO release was obtained from a Pellethane derivative containing diethylenetriamine linked to the urethane nitrogens via a butyl group. This material showed an initial NO release of 14 pmol$\cdot$cm$^{-2}\cdot$s^{-1} which decreased in an approximately exponential fashion over a period of 6 days. During this 6-day period, the polymer released a total of 1.7×10^{-8} mol of NO/mg of PU. Figure 4 shows the NO release curve from this material for 20 h. During this time, NO release reached fluxes well above those of the normal endothelium, ~7 pmol$\cdot$cm$^{-2}\cdot$s^{-1}. In addition, the polymer was also evaluated to determine how much nitrite was released as a measure of decomposition of the diazeniumdiolate. It was found that, of the total NO_x ($NO + NO_2^-$) released from the polymer, 12% was due to NO gas. The remaining 88% was determined to be nitrite as measured by the Griess assay. This is the highest ratio of NO to NO_x release for any PU system reported to date using this methodology without external additives.

Conclusions

As shown herein, it is possible to prepare nitric oxide releasing polyurethanes via two methods. The NO may be added to the polymer backbone by the reaction of NO with a series of secondary amines that were incorporated into the main chain of the polymer as well as by derivatization of polyurethanes to contain pendent polyamine sites. FT-IR spectroscopy and elemental analysis were used to confirm the polymer compositions, and GPC, DSC, and TGA were used to characterize various polymer properties. Stable diazeniumdiolates can be formed on a nitrogen incorporated into the backbone only in the presence of additional counterions and bases, preferably sodium and cholate, during the NO addition reactions. The new polyurethanes are thus unlike a previously reported silicone rubber polymer system[27] containing diamines that were successfully reacted with NO to form diazeniumdiolates in the absence of such additives. However, by using polyurethanes containing polyamine side chains, air-stable diazeniumdiolates can be formed readily on the same polymer backbones without need for reaction additives. The amount of NO release provided should improve the thromboresistivity of such polyurethanes when they are used as coatings for blood-contacting biomedical devices. Indeed, it has recently been suggested that NO-releasing

PU materials may be the materials of the future for vascular grafts.[32] Hence, the in vivo biocompatibility of these novel PU materials described herein will be examined soon to determine whether they are candidates for this and other biomedical applications.

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Supporting Information Available. Details of the conduct and analysis of NO-generating reactions; method for screening for PU decomposition. This material is available free of charge via the Internet at http://pubs.acs.org.

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