Nitric Oxide-Producing Polyurethanes

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Thrombus formation and eventual intimal hyperplasia are the leading causes of small-diameter synthetic vascular graft failure. To combat these issues, we have incorporated a diazeniumdiolate-modified nitric oxide (NO)-producing peptide into a polyurethane to improve the thromboresistance of this biocompatible polymer. NO production by polyurethane films occurred for approximately 2 months under physiological conditions, and mechanical properties of the material were suitable for vascular graft applications. Platelet adhesion to NO-releasing polyurethane was dramatically decreased compared to control polyurethane. Furthermore, endothelial cell growth was stimulated in the presence of the NO-releasing polyurethane, while smooth muscle cell growth was greatly inhibited. The ability of this bioactive material to inhibit platelet adhesion and smooth muscle cell proliferation while encouraging endothelialization suggests that this NO-generating polyurethane may be suitable as a candidate material for small-diameter vascular grafts.

Introduction

Synthetic materials such as Dacron [poly(ethylene teraphthalate)], expanded poly(tetrafluoroethylene) (PTFE), and microporous polyurethanes have been successfully used as large-diameter (>6 mm) vascular substitutes.[1−3] However, synthetic materials have shown disheartening failures in small-diameter applications such as coronary artery bypass grafting because of rapid occlusion caused by thrombosis and intimal hyperplasia.[3−6] Numerous strategies have been investigated to improve the performance of small-diamater vascular grafts, but to date, clinical success has not been achieved.

Nitric oxide (NO), a natural mediator of vascular homeostasis, is an excellent candidate for improving the thromboresistance of cardiovascular biomaterials. NO, in recent years, has become one of the most widely investigated biological molecules, and its roles as a potent vasodilator, regulator of vascular cell proliferation and migration, and inhibitor of thrombus formation have made NO attractive for incorporation into blood-contacting devices.[7−11] As a result, compounds that spontaneously decompose to release NO are also increasingly under investigation. Notably, diazeniumdiolates, which contain the $[N(O)NO]^-$ functional group, are extensively studied for biological and clinical applications.[12−17] The anionic portions of these compounds spontaneously decompose in solution to release NO, leaving the amine group as a byproduct.[13] Rates of dissociation depend on several factors, such as structure, temperature, and pH of solution.[12] The formation of intimal lesions, due to platelet adhesion and aggregation and smooth muscle cell (SMC) proliferation, at sites of vascular graft placement is a major concern in the development of suitable engineered synthetic grafts to replace native blood vessels. NO donors have been shown to decrease the incidence of intimal hyperplasia in several animal models,[18,19] and the inhibition of platelet adhesion and aggregation by NO and several NO-releasing materials has also been widely reported.[7,14,16,20−24] We have, therefore, incorporated a diazeniumdiolate NO donor into a biocompatible polyurethane to assess the ability of this novel material to promote graft endothelialization while preventing thrombus formation and intimal hyperplasia. The NO release kinetics from this material were studied, and its effects on platelet adhesion as well as vascular endothelial and SMC proliferation were also evaluated.

Materials and Methods

Synthesis of Polyurethane. A polyurethane pre-polymer was synthesized by reacting methylene di(*p*-phenyl isocyanate) (MDI; Aldrich Chemical Co., Milwaukee, WI) with poly(tetramethylene oxide) (PTMO; Aldrich Chemical Co., Milwaukee, WI) and then was extended with 1,4-butanediol (BD; Aldrich Chemical Co., Milwaukee, WI), as shown in Figure 1.[25,26] A 10% (w/v) solution of MDI (4 mmol; MW, 250) in 10 mL of anhydrous *N*,*N*-dimethylformamide (DMF; Aldrich Chemical Co., Milwaukee, WI) was prepared in a 100-mL three-neck round flask and stirred at room temperature. A 10% (w/v) solution of PTMO (2 mmol; MW, 2000) in 20 mL of anhydrous DMF was added, and the mixture was heated to 75 °C and held there for 3 h under argon. The reactor was cooled to room temperature before BD (2 mmol; MW, 90) in 2 mL of anhydrous DMF was added as a chain extender. The polymer solution was then incubated at 45 °C for 3 h under argon. The polymer solution was cooled to room temperature, precipitated in methanol, and dried under a vacuum. This product is referred to as PUBD.

Synthesis of Diazeniumdiolate-Modified Polyurethane. A lysine-containing peptide sequence, Ser-Gly-Gly-Lys-Lys-Lys-Lys-Gly-Gly-Ser (SGGKKKKGGS), was synthesized by using standard fluorenylmethoxycarbonyl chemistry on an

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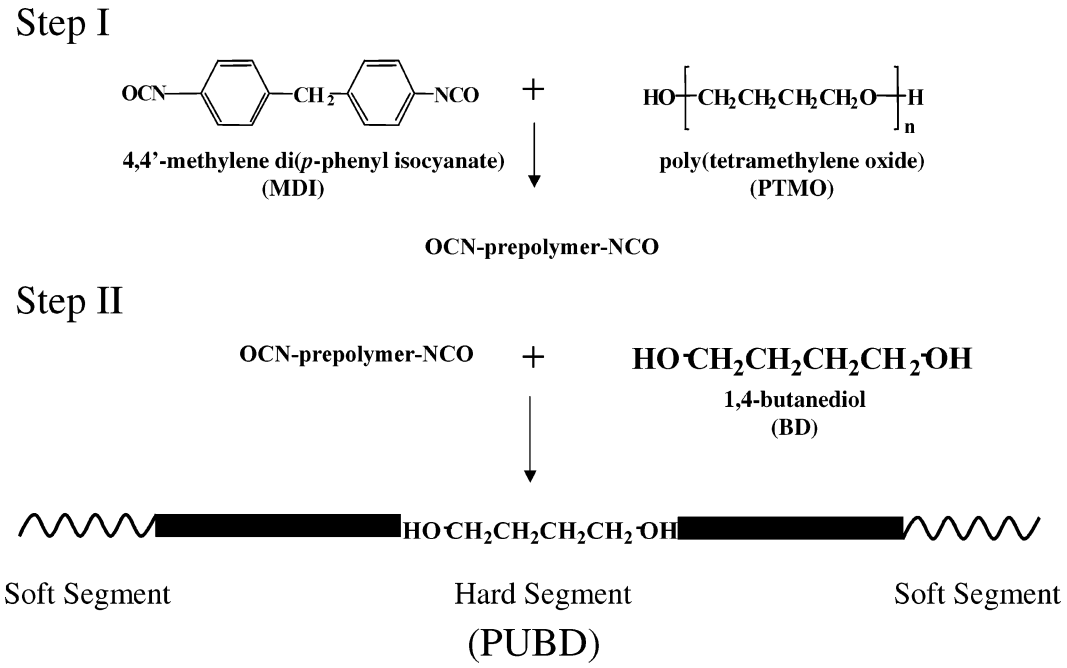

Figure 1. Synthesis of polyureathane (PUBD). The prepolymer was synthesized from MDI and PTMO and then extended with BD.

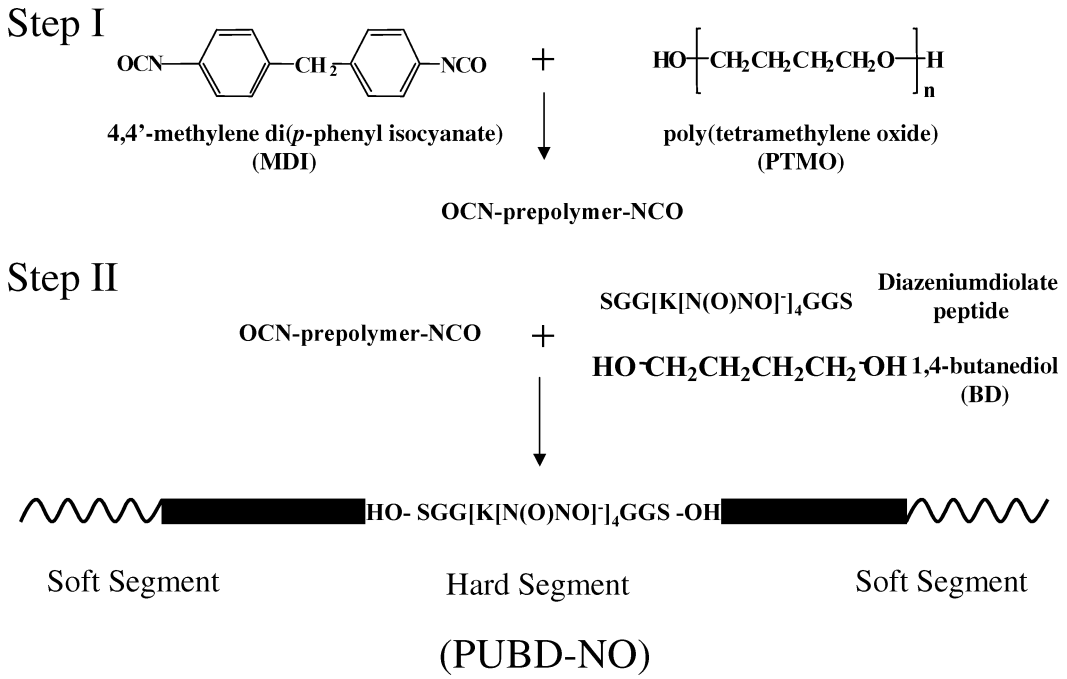

Figure 2. Synthesis of NO releasing polyurethane (PUBD-NO). The prepolymer was synthesized from MDI and PTMO and then extended with BD and peptide sequences containing diazeniumdiolates.

Applied Biosystems 431A peptide synthesizer (Foster, CA). The lysine residues have pendant amine groups that can be converted to diazeniumdiolates, while serine residues contain pendant hydroxyl groups to allow incorporation of the peptide into the polyurethane chain. The peptide was dissolved in deionized water and reacted with NO at room temperature under argon gas in a 100-mL round-bottom flask overnight. The extent of conversion of amine groups to diazeniumdiolates was measured using the Ninhydrin assay.[27] The diazeniumdiolate peptide, SGG[K[N(O)NO]$^-$]$_4$GGS, was freeze-dried and stored at −80 °C.

The polyurethane pre-polymer was synthesized by reacting MDI with PTMO as described above and was extended with a combination of SGG[K[N(O)NO]$^-$]$_4$GGS peptide (0.22 mmol) and BD (0.54 mmol) in 5 mL of anhydrous DMF (Figure 2).[25,26] The polymer mixture was incubated at 45 °C for 3 h under argon, then cooled to room temperature, precipitated in ethyl acetate, and dried under a vacuum. This product is referred to as PUBD-NO.

Polymer Characterization. PUBD and PUBD-NO were characterized via ^{1}H NMR using a 400-MHz NMR spectrometer (Advance 400, Bruker, Germany) with *N*,*N*-dimethylformamide-d_7 (Aldrich Chemical Co., Milwaukee, WI) as the solvent. Molecular weight distributions were obtained by gel permeation chromatography (GPC) with UV and evaporative light scattering detectors (Polymer Laboratories,

Amherst, MA). Samples for GPC analysis were dissolved in HPLC-grade DMF at a concentration of 1 mg/mL and run at 70 °C through Plgel 5-μm Mixed-C columns (Polymer Laboratories, Amherst, MA) at a flow rate of 1 mL/min. Calibration was performed using polystyrene standards (Polymer Laboratories, Amherst, MA), ranging in molecular weight from 5000 to 96 400 Da.

Preparation and Characterization of NO-Releasing PUBD Films. Polymers were dissolved in tetrahydrofuran (0.3 wt %) and filtered using 0.2-μm-pore-size PTFE syringe filters (Whatman, NJ). Polymer films were prepared on glass coverslips (18 mm; Fisher Scientific, PA) by solvent casting at room temperature. Polymer films were held under a vacuum for 48 h to ensure removal of the solvent and then sterilized under UV light (254 nm) overnight.

Uniaxial mechanical testing of polymer films was performed using an Instron model 5565 at a crosshead speed of 25 mm/min with a 5-kN load cell. Polymers were dissolved in DMF (Aldrich Chemical Co., Milwaukee, WI) at 10 wt % and filtered using 0.2-μm-pore-size PTFE syringe filters (Whatman, NJ). Polymer films were prepared in Teflon molds by solvent casting at 60 °C under a vacuum for 48 h. Test specimens were prepared according to ASTMD-638-VI. Tensile strength was calculated from the maximum load at break and the initial cross-sectional area of the specimen. The sample thickness was measured using a digital caliper (Mitutoyo, Aurora, IL).

Cell Maintenance. Bovine aortic endothelial cells (BAECs; Clonetics, San Diego, CA) and Sprague−Dawley rat aortic SMCs (Cell Applications, San Diego, CA), passages 2−5, were used in this study. Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) was prepared with 10% fetal bovine serum (FBS; BioWhitaker, Walkersville, MD), 2 mM L-glutamine, 1 unit/mL penicillin, and 100 mg/L streptomycin (GPS; Sigma Chemical Co., St. Louis, MO), and endothelial basal medium (EBM; Sigma Chemical Co., St. Louis, MO) was prepared with 10% endothelial medium supplement (Sigma Chemical Co., St. Louis, MO) containing FBS, basic fibroblast growth factor (bFGF), heparin, epithermal growth factor, and hydrocortisone. BAECs were maintained on a mixture of EBM and DMEM (50/50%) at 37 °C in a 5% CO_2 environment. SMCs were sustained on DMEM at 37 °C in a 5% CO_2 environment.

NO Release. Sterilized PUBD-NO films were reacted with NO gas under argon at room temperature overnight. After rinsing the films with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffered saline (HBS, pH 7.4) three times, the films were incubated in HBS at 37 °C. Release of NO from the films was measured using the Griess assay, which quantifies nitrites, the primary degradation product of NO.[23,28]

Proliferation of BAEC and SMC. PUBD and PUBD-NO films were prepared and sterilized as described above. To evaluate BAEC and SMC proliferation, cells were seeded at a concentration of 17 000 cells/cm^2 in six-well polystyrene plates (Corning Inc., Corning, NY) and incubated for 24 h at 37 °C in a 5% CO_2 environment. PUBD and PUBD-NO films were then placed into transwell inserts (24-mm diameter, 0.4-μm pore polycarbonate membrane, Corning Inc., Corning, NY) in the six-well plates. After another 48 h of culture at 37 °C in a 5% CO_2 environment, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was employed to assess cellular proliferation. Cells were fixed in a 10% buffered formalin solution (Sigma Chemical Co., St. Louis, MO) and permeabilized in methanol (Sigma Chemical Co., St. Louis, MO). A 3% hydrogen peroxide solution was used to block endogenous peroxidases, and cells were incubated with mouse IgG anti-PCNA antibody (Dako Corp., Carpinteria, CA) diluted 1:100 in phosphate-buffered saline (PBS) with 3% FBS. After rinsing, cells were incubated with anti-mouse IgG HRP (Dako Corp., Carpinteria, CA) diluted 1:100 in PBS with 3% FBS followed by aminoethylcarbazole chromogen (Dako Corp., Carpinteria, CA), which generates a red precipitate. Cells were counterstained with Mayer's hemotoxylin (Dako Corp., Carpinteria, CA). The percentage of proliferating cells per field of view (200×) was determined using phase contrast microscopy (Zeiss Axiovert 135, Thornwood, NY) by averaging five fields per sample.

Platelet Adhesion. PUBD and PUBD-NO films were prepared and sterilized as previously described. A solution of 2.5 mg/mL collagen I (Sigma Chemical Co., St. Louis, MO) solution was prepared in 3% glacial acetic acid. Collagen I was adsorbed onto glass coverslips for 45 min at room temperature to provide a highly thrombogenic reference material. Whole blood was obtained from a healthy volunteer, and to this was added 10 U/mL heparin (Sigma Chemical Co., St. Louis, MO) and 10 μM mepacrine (Sigma Chemical Co., St. Louis, MO) to fluorescently label the platelets. Collagen I (positive control), PUBD, and PUBD-NO films were incubated with the mepacrine-labeled blood at 37 °C for 20 min and then rinsed with PBS. The number of adherent platelets per field of view (200×) was determined using a fluorescent microscope (Zeiss Axiovert 135, Thornwood, NY) by assessing five fields per sample.

Statistical Analysis. Data were compared with two-tailed, unpaired *t* tests. *p* values less than 0.05 were considered to be significant.

Results

Synthesis and Characterization. The conversion of free amine groups on the side chains of the SGGKKKKGGS peptide to diazeniumdiolates was measured using the Ninhydrin assay, and 92.7% (±9.1%, $n = 6$) of amines were converted to NO−nucleophile complexes. PUBD-NO was synthesized by incorporating the SGG[K[N(O)NO]$^-$]$_4$GGS sequence into the polymer backbone. The ^{1}H NMR spectra of the peptide and PUBD-NO were obtained, and the characteristic proton peaks of the SGGKKKKGGS sequence indicated the successful incorporation of the peptide sequence into the polymer (Figure 3). The peptide concentration of the polymer matrix was determined to be approximately 100 μmol/g.

The number-average molecular weight (M_n), the weight-average molecular weight (M_w), and the polydispersity index (PDI) were determined by GPC using polystyrene standards.

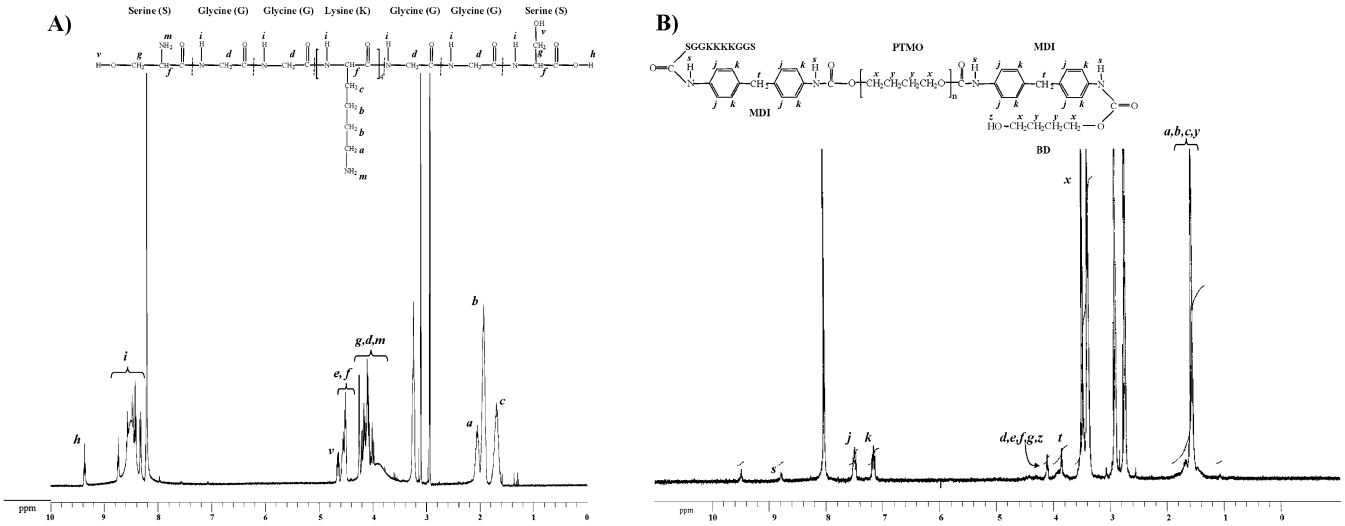

Figure 3. ^{1}H NMR spectra and peak assignments of (A) SGGKKKKGGS peptide and (B) PUBD-NO.

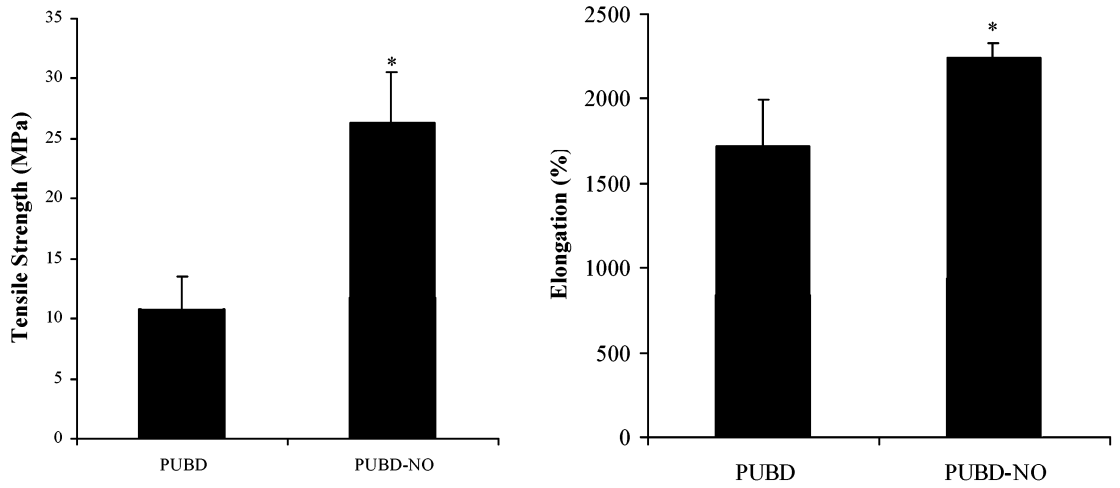

Figure 4. Tensile strengths and elongations of PUBD and PUBD-NO. Data represent the mean of three samples (*: $p < 0.01$).

The PUBD and PUBD-NO polymers had similar molecular weights (PUBD, $M_n = 54\,701$, $M_w = 75\,167$, PDI = 1.37; PUBD-NO, $M_n = 50\,323$, $M_w = 71\,006$, PDI = 1.41).

Mechanical Properties. The incorporation of the peptides into the polymer backbone improved mechanical properties. Both the elastic modulus and the tensile strength of PUBD-NO were significantly greater than those of PUBD (Figure 4). Subsequent NO release from PUBD-NO should not alter the mechanical properties, as the original amine, in this case lysine, is the result. The mechanical properties of PUBD-NO were comparable to commercial polyurethane vascular grafts as well as to native tissue.[29−31]

NO Release Kinetics. NO release from PUBD-NO films is shown in Figure 5, occurring over 2 months. Rapid release occurred over the first 48 h followed by much slower, sustained release for almost 60 days. No release of NO was detected from PUBD films reacted with NO gas.

BAEC and SMC Proliferation. The effect of NO release on BAEC and SMC proliferation was examined using immunohistochemical staining for PCNA. The percentage of PCNA-positive BAECs exposed to PUBD-NO films releasing NO in a rapid burst was significantly greater than the percentage of those exposed to PUBD after 48 h of culture. However, the percent of PCNA-positive SMCs cultured with fast-releasing PUBD-NO films was significantly lower than the percent of those cultured with PUBD films (Figure 6).

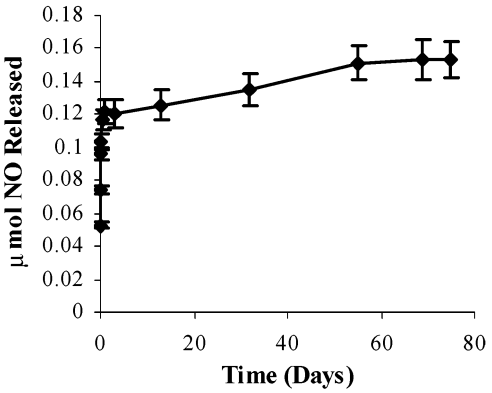

Figure 5. NO release from PUBD-NO films in HBS at pH 7.4, 37 °C. Data represent the mean of three samples.

We also sought to determine the efficacy of our materials during the slower phase of NO release. PUBD-NO and PUBD films were incubated in HBS for 48 h, and then both BAECS and SMCs were exposed to these films as previously described. Again, the percentage of PCNA-positive BAECs

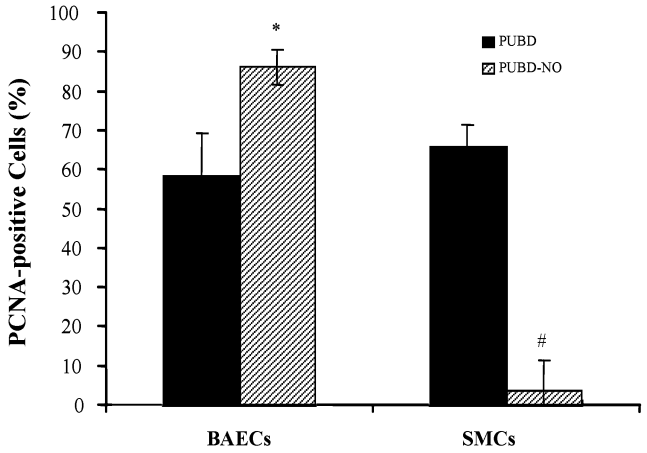

Figure 6. BAEC proliferation was greater and SMC proliferation was inhibited when cells were exposed to NO-releasing PUBD films in the burst phase of release as compared to regular PUBD films. Data represent the mean of four samples (*, #: $p < 0.001$).

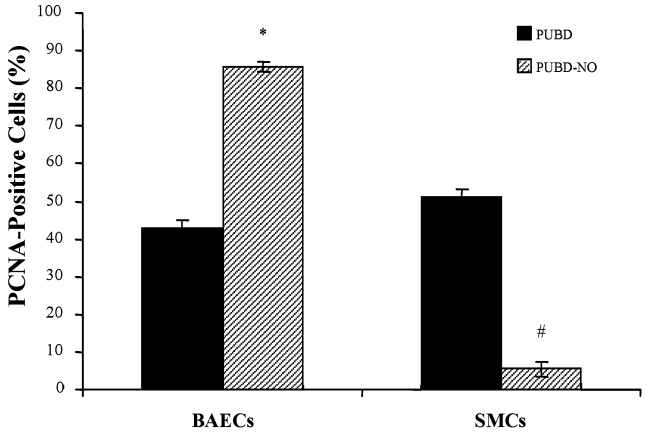

Figure 7. BAEC proliferation was greater and SMC proliferation was inhibited when cells were exposed to NO-releasing PUBD films in the slower phase of NO release as compared to regular PUBD films. Data represent the mean of four samples (*, #: $p < 0.002$).

cultured with NO-releasing PUBD films was higher than that of BAECs exposed to control PUBD films, and the number of positively stained SMCs cultured with PUBD-NO films was drastically lower than SMCs in the presence of PUBD films (Figure 7).

Platelet Adhesion. Platelet adhesion to PUBD and PUBD-NO was examined using mepacrine-labeled whole blood. Platelet adhesion to PUBD was approximately 40% less than on the positive control, collagen I (Figure 8). However, platelet adhesion on PUBD-NO was dramatically lower, with almost no adherent platelets observed.

We also sought to ascertain if these materials would retain their thromboresistance during the slower stages of NO release. Films were allowed to release in HBS for 48 h before they were exposed to whole blood as described above. Once more, we see a 40% reduction in the number of platelets adhering to PUBD when compared to collagen I (Figure 9). Platelet adhesion to PUDB was again significantly lower.

Discussion

Synthetic vascular grafts have not demonstrated success when used in small-diameter applications because of complications caused by thrombosis and intimal hyperplasia. In this study, NO generating polyurethane (PUBD-NO) was successfully synthesized by incorporating diazeniumdiolated-modified lysine peptide sequences into polyurethane, and this material's active resistance to platelet adhesion and intimal hyperplasia enhances its performance as a candidate material for small-diameter vascular grafts while retaining the mechanical properties of commercially available synthetic grafts.

NO release from polyurethane films showed two-phase kinetics: an initial burst within 48 h and a much slower, sustained release over 2 months. The initial rapid NO release is likely caused by dissociation of diazeniumdiolate complexes on the surface of the films, while the prolonged release is the escape of NO embedded in the matrix of the material. The rate of NO release is crucial because though too high a dose of NO may cause a cytotoxic response, too little may not stimulate endothelial cell proliferation or inhibit platelet adhesion and SMC growth.[32,33] The multiphasic release observed here is ideally suited to stimulate the desired cellular responses while functioning as a thromboresistant scaffold

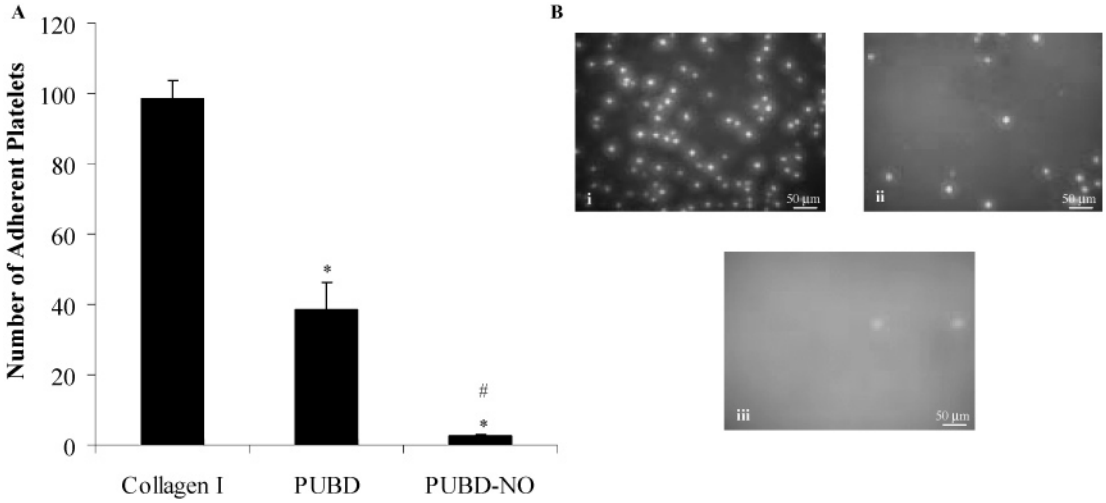

Figure 8. (A) Platelet adhesion is significantly less to PUBD-NO films in the rapid phase of NO release than to PUBD or collagen I-coated films. Data represent the mean of three samples (*: $p < 0.001$ compared to collagen I; #: $p < 0.001$ compared to PUBD). (B) Digital images of mepacrine-labeled platelets adhering to (i) collagen I, (ii) PUBD, and (iii) PUBD-NO.

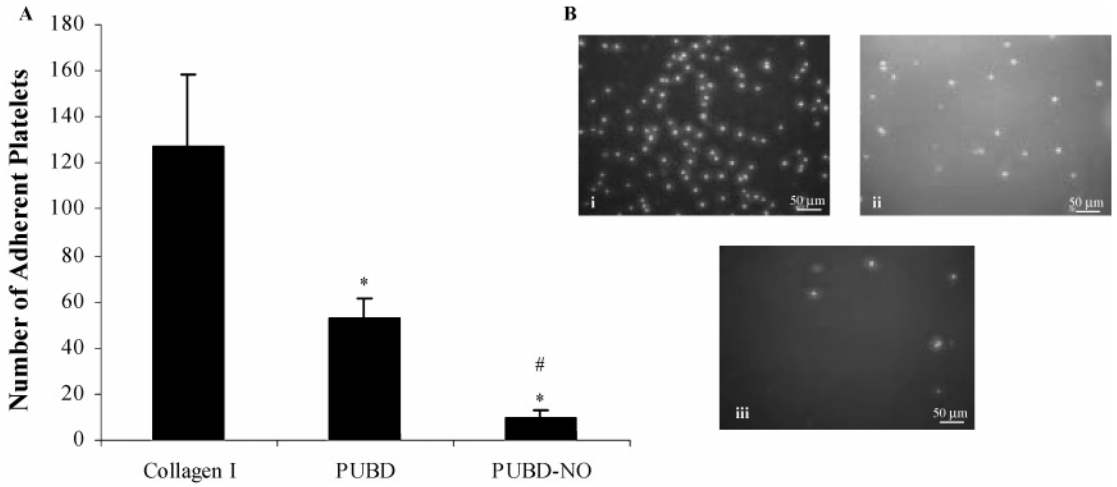

Figure 9. (A) Platelet adhesion is significantly less to PUBD-NO films in the slow phase of release than to PUBD or collagen I-coated films. Data represent the mean of three samples (*: $p < 0.001$ compared to collagen I; #: $p < 0.001$ compared to PUBD). (B) Digital images of mepacrine-labeled platelets adhering to (i) collagen I, (ii) PUBD, and (iii) PUBD-NO.

for endothelialization. However, both phases of NO release from peptide-modified polyurethane films were able to stimulate vascular cell response. BAECs showed an increased proliferative response when exposed to films in both the rapid stage of NO release and the slower period of release, signifying that our material should show enhanced endotheliazation due to an initial burst of NO release that should continue through the sustained release phase. This should aid in the formation of an endothelial lining similar to that of native vessels. Rat aortic SMCs displayed decreased proliferation upon exposure to NO-releasing PUBD, indicating that small amounts released after an initial surge of NO are sufficient to halt smooth muscle growth. Platelet adhesion to PUBD-NO was drastically reduced in both phases of NO release as well, suggesting greatly improved thromboresistance of our modified polyurethane over the long term. In addition, intimal formation is inhibited by both a lack of platelet adhesion to the material and the lack of SMC growth.

The inhibition of platelet adhesion and intimal growth is critical for the success of engineered materials in the cardiovascular system. The lack of an endothelial lining in synthetic grafts, as well as the disruption of native endothelium upon graft placement, presents a surface that is highly susceptible to thrombus formation. Platelet adhesion and aggregation initiates the cascade of vascular SMC migration and proliferation due to the local release of mitogens, including platelet-derived growth factor.[34,35] Another contributing mechanism includes smooth muscle activation through direct injury to subendothelial smooth muscle, which also leads to the activation of mitogens, including bFGF.[36] As SMCs proliferate and migrate into the injured area, they deposit matrix proteins that form an occlusive scar tissue, or neointimal hyperplasia.[34,37] NO, which is released in low levels from the endothelial linings of healthy blood vessels, inhibits platelet adhesion and SMC proliferation, thereby effectively cutting off potential pathways for the failure of an engineered vascular substitute. The material developed in this study has demonstrated success in preventing the major events in the formation of an occlusive intimal thickening in blood vessels.

Conclusions

NO-generating polyurethane (PUBD-NO) has been successfully synthesized by incorporating a diazeniumdiolate−peptide sequence into the polymer backbone. NO was successfully generated from the polymer and reduced platelet adhesion and SMC proliferation while improving endothelial cell proliferation. This material also has mechanical properties similar to those of commercially available synthetic vascular grafts and may be useful for a wide variety of cardiovascular applications. Future modifications to this material could include the incorporation of cell-adhesive peptide sequences, poly(ethylene glycol), and NO donors and might result in synergistic effects leading to faster levels of high endothelialization without intimal formation.

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