Nitric Oxide-Releasing Polyurethane–PEG Copolymer Containing the YIGSR Peptide Promotes Endothelialization With Decreased Platelet Adhesion

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Abstract: Thrombosis and intimal hyperplasia are the principal causes of small-diameter vascular graft failure. To improve the long-term patency of polyurethane vascular grafts, we have incorporated both poly(ethylene glycol) and a diazeniumdiolate nitric oxide (NO) donor into the backbone of polyurethane to improve thromboresistance. Additionally, we have incorporated the laminin-derived cell adhesive peptide sequence YIGSR to encourage endothelial cell adhesion and migration, while NO release encourages endothelial cell proliferation. NO production by polyurethane films under physiological conditions demonstrated biphasic release, in which an initial burst of 70% of the incorporated NO was released within 2 days, followed by sustained release over 2 months. Endothelial cell proliferation in the presence of the NO-releasing material was increased as compared to control polyurethane, and platelet adhesion to polyethylene glycol-containing polyurethane was decreased significantly with the addition of the NO donor. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 84B: 108–116, 2008

Keywords: vascular graft; endothelialization; polyurethane; nitric oxide; diazeniumdiolate; YIGSR; PEG

INTRODUCTION

The success of cardiovascular devices is highly dependant on their ability to resist thrombus formation and intimal hyperplasia. Synthetic vascular grafts are in demand largely due to the prevalence of coronary artery disease and preexisting disease in donor vessels. Grafts made of polyurethane have long been of interest due to their superior mechanical compliance and relative biocompatibility, however, platelet adhesion to the polymer surface has led to disappointing success rates as small-diameter (<6 mm) substitutes, such as coronary artery bypass grafts.[1–7] As platelets adhere and aggregate, they stimulate smooth muscle cell (SMC) proliferation and migration into the area of the injured tissue, forming an occlusive scar tissue and thereby inhibiting graft function. Polyethylene glycol (PEG) resists protein adsorption, platelet adhesion, and bacterial adhesion,[8–13] making it an attractive material for incorporation into vascular prostheses, and numerous methods have been developed to integrate PEG into biomaterials, including polyurethanes, through copolymerization and surface modification.[8,12,14–20]

The incorporation of cell adhesion moieties into vascular grafts is also employed as a means of enhancing graft function by encouraging endothelialization. Endothelial cell seeding has been extensively studied as a method to improve the long-term patency of synthetic grafts by providing the naturally nonthrombogenic luminal interface present in a normal blood vessel. However, upon implantation, there is usually a substantial loss of cells as a result of exposure to shear stress.[21–24] Biomaterials modified with adhesive peptide sequences such as the laminin-derived sequence tyrosine-isoleucine-glycine-serine-arginine (YIGSR) have demonstrated improved cell attachment, spreading and resistance to shear stress.[15,25–29] Retention of endothelial cells on the lumen of synthetic graft surfaces may prove essential to achieving a fully functioning vascular substitute.

One of the key biochemical functions of the endothelium is the production of nitric oxide (NO). NO has several

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critical functions in the vascular system, maintaining vascular homeostasis and acting as a potent vasodilator, regulator of vascular cell proliferation and migration, and inhibitor of thrombus formation.[30–36] NO donors, compounds that spontaneously decompose to release NO, are under investigation for a number of biomedical applications. For example, diazeniumdiolates, compound that contain the $[N(O)NO]^-$ functional group, are being investigated as NO-releasing pharmaceuticals[37–41] and thromboresistant coatings for blood-contacting medical devices.[40,42–49] NO-releasing materials have been shown to inhibit platelet adhesion and aggregation,[40,42–53] as well as decreasing the incidence of intimal hyperplasia in several animal models.[54–57] Our strategy for developing a nonthrombogenic candidate material for small-diameter synthetic vascular grafts therefore includes the incorporation of a diazeniumdiolate NO donor into the backbone of a polyurethane containing both PEG and the cell adhesive YIGSR peptide sequence in order to provide NO locally during the period of graft endothelialization. NO release from this polymer was studied, and the ability of PEG and NO to prompt a synergistic decrease in platelet adhesion was examined. The ability of NO to promote endothelialization on the surface of this material was also assessed.

MATERIALS AND METHODS

Synthesis of PEG-Modified Cell Adhesive Polyurethane (PUBD-PEG-YIGSR)

A peptide containing the endothelial cell adhesive sequence YIGSR, glycine-glycine-tyrosine-isoleucine-glycine-serine-arginine-glycine-glycine-lysine (GGYIGSRGGK), was synthesized using standard fluorenylmethoxycarbonyl (Fmoc) chemistry on an Applied Biosystems 431A peptide synthesizer (Foster, CA). A polyurethane pre-polymer was synthesized by reacting methylene di(*p*-phenyl isocyanate) (MDI; Aldrich chemical, Milwaukee, WI) with a mixture of poly (tetramethylene oxide) (1.7 mmol; PTMO; Aldrich chemical, Milwaukee, WI) and poly(ethylene glycol) (0.13 mmol; PEG; Aldrich chemical, Milwaukee, WI) before chain extension using the GGYIGSRGGK peptide (0.1 mmol) and 1,4-butanediol (0.45 mmol; BD; Aldrich chemical, Milwaukee, WI). MDI was recrystallized in hexane, and a 10% (w/v) solution of MDI (4 mmol, MW: 250) in 10 mL anhydrous *N,N*-dimethylformamide (DMF; Aldrich Chemical, Milwaukee, WI) was prepared in a 100 mL three-neck round flask and stirred at room temperature. PTMO (1.7 mmol, MW: 2000) and PEG (0.3 mmol, MW: 4600) were dried for 48 h under vacuum and mixed at an 85:15 (PTMO: PEG) molar ratio in 20 mL anhydrous DMF. This was added to the MDI solution and the mixture heated to 75°C and held there for 3 h under argon. The reactor was cooled to room temperature before the peptide GGYIGSRGGK (0.1 mmol) and BD (0.65 mmol, MW: 90) in anhydrous DMF were added as chain extenders. The polymer solution was then stirred at 45°C for 3 h under argon. The polymer solution was cooled to room temperature, precipitated in ethyl acetate, and dried under vacuum.

Synthesis of NO-Releasing PEG-Modified Cell Adhesive Polyurethane (PUBD-PEG-YIGSR-NO)

A lysine-containing peptide sequence, serine-glycine-glycine-lysine-lysine-lysine-lysine-glycine-glycine-serine (SGG KKKKGGS), was synthesized using Fmoc chemistry on a peptide synthesizer. The peptide was purified, dissolved in DI 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 (Moore).[58] The diazeniumdiolate peptide, $SGG[K[N(O)NO]^-]_4GGS$, was freeze-dried and stored at −80°C.

The polyurethane pre-polymer was synthesized by reacting MDI with a PTMO/PEG mixture as described above and extended with a combination of $SGG[K[N(O)NO]^-]_4GGS$ peptide (0.2 mmol), GGYIGSRGGK peptide (0.1 mmol), and BD (0.45 mmol) in anhydrous DMF (Figure 1). The polymer mixture was stirred at 45°C for 3 h under argon, then cooled to room temperature, precipitated in ethyl acetate, and dried under vacuum.

Polymer Characterization

PUBD-PEG-YIGSR and PUBD-PEG-YIGSR-NO were characterized via ^1H NMR using a 400 MHz NMR spectrometer (Advance 400, Bruker, Germany) with *N,N*-dimethylformamide-d_7 (DMF-d_7; Aldrich Chemical, Milwaukee, WI) as the solvent. Molecular weight distributions were obtained by 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 (PS; Polymer Laboratories, Amherst, MA), ranging in molecular weight from 5,000–96,400 Da.

Preparation and Characterization of NO-Releasing PUBD Films

Polymers were dissolved in DMF (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 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 3342 (Instron Corp., Norwood, MA) at a crosshead speed of 25 mm/min with a 0.5 kN load cell. Polymers were dissolved in DMF

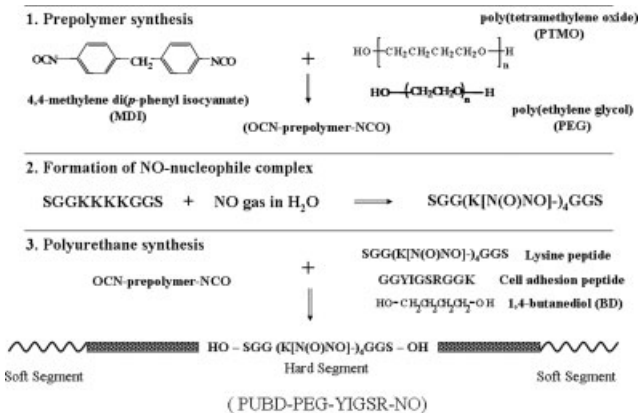

Figure 1. Synthesis of NO-releasing polyurethane-PEG copolymer containing the cell adhesive peptide YIGSR (PUBD-PEG-YIGSR-NO). The prepolymer was synthesized with MDI and PTMO and extended with BD and diazeniumdiolate-containing peptide sequences.

(Aldrich Chemical, 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 vacuum for 48 h. Test specimens were prepared according to ASTM D 638.[59] Tensile strength was calculated from the maximum load at break and the initial cross sectional area of the specimen. 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 smooth muscle cells (SMCs; Cell Applications, San Diego, CA), passages 2–5, were used in this study. Dulbecco's Modified Eagle's Medium (DMEM; Sigma Chemical, St. Louis, MO) was prepared with 10% fetal bovine serum (FBS; BioWhitaker, Walkersville, MD), 2 m*M* L-glutamine, 1 unit/mL penicillin, and 100 mg/L streptomycin (GPS; Sigma Chemical, St. Louis, MO) and endothelial basal medium (EBM; Sigma Chemical, St. Louis, MO) was prepared with 10% endothelial medium supplement (Sigma Chemical, St. Louis, MO) containing fetal bovine serum, basic fibroblast growth factor, heparin, epithermal growth factor, and hydrocortisone. BAECs were maintained on 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-PEG-YIGSR and PUBD-PEG-YIGSR-NO films were reacted with NO gas under argon at room temperature overnight. After rinsing the films with HEPES 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. In brief, samples were added to a combination of 1% sulfanilamide and 0.1% naphthylethylenediamine in 2% phosphoric acid and mixed to stimulate a colorimetric change; intensity was measured at 540 nm and compared to known sodium nitrite standards. Small amounts of released NO were not accounted for, as the conversion of nitrites to nitrates was not quantified; however, nitrite concentration has long been used as an accurate estimate for local NO production.[60,61]

Adhesion and Proliferation of BAEC and SDSMC

PUBD-PEG-YIGSR and PUBD-PEG-YIGSR-NO films were prepared and sterilized as described earlier. Films were anchored in 6-well polystyrene plates (Corning, Corning, NY) using hollow stainless steel molds having an inner diameter of 12 mm. To evaluate BAEC and SDSMC adhesion and proliferation, cells were seeded at a concentration of 17,000 cells/cm^2 on sterilized films and incubated for 48 h at 37°C in a 5% CO_2 environment. Cell adhesion was monitored at 6, 24, and 48 h following cell seeding using phase contrast microscopy (200×, Zeiss Axiovert 135, Thornwood, NY). After 48 h, films were rinsed thoroughly with PBS, trypsinized, and counted using a Coulter Counter (Coulter Counter ZM, Beckman Coulter, Miami, FL).

Platelet Adhesion

PUBD-PEG-YIGSR and PUBD-PEG-YIGSR-NO films were prepared and sterilized as previously described. A solution of 2.5 mg/mL collagen I (Sigma Chemical, 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 thrombogenic reference material. Whole blood was obtained from a healthy volunteer and 10 U/mL heparin (Sigma Chemical, St. Louis, MO) and 10 μM mepacrine (Sigma Chemical, St. Louis, MO), which fluorescently labels platelets, were both added. Collagen I (positive control), PUBD-PEG-YIGSR, and PUBD-PEG-YIGSR-NO films were incubated with the mepacrine-labeled blood at 37°C for 20 min, then rinsed with PBS. The number of adherent platelets per square millimeter (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

Polymer Synthesis and Characterization

The Ninhydrin assay was used to assess the conversion of free amine groups on the side chains of the SGGKKKKGGS peptide to diazeniumdiolates, and (97.6 ±

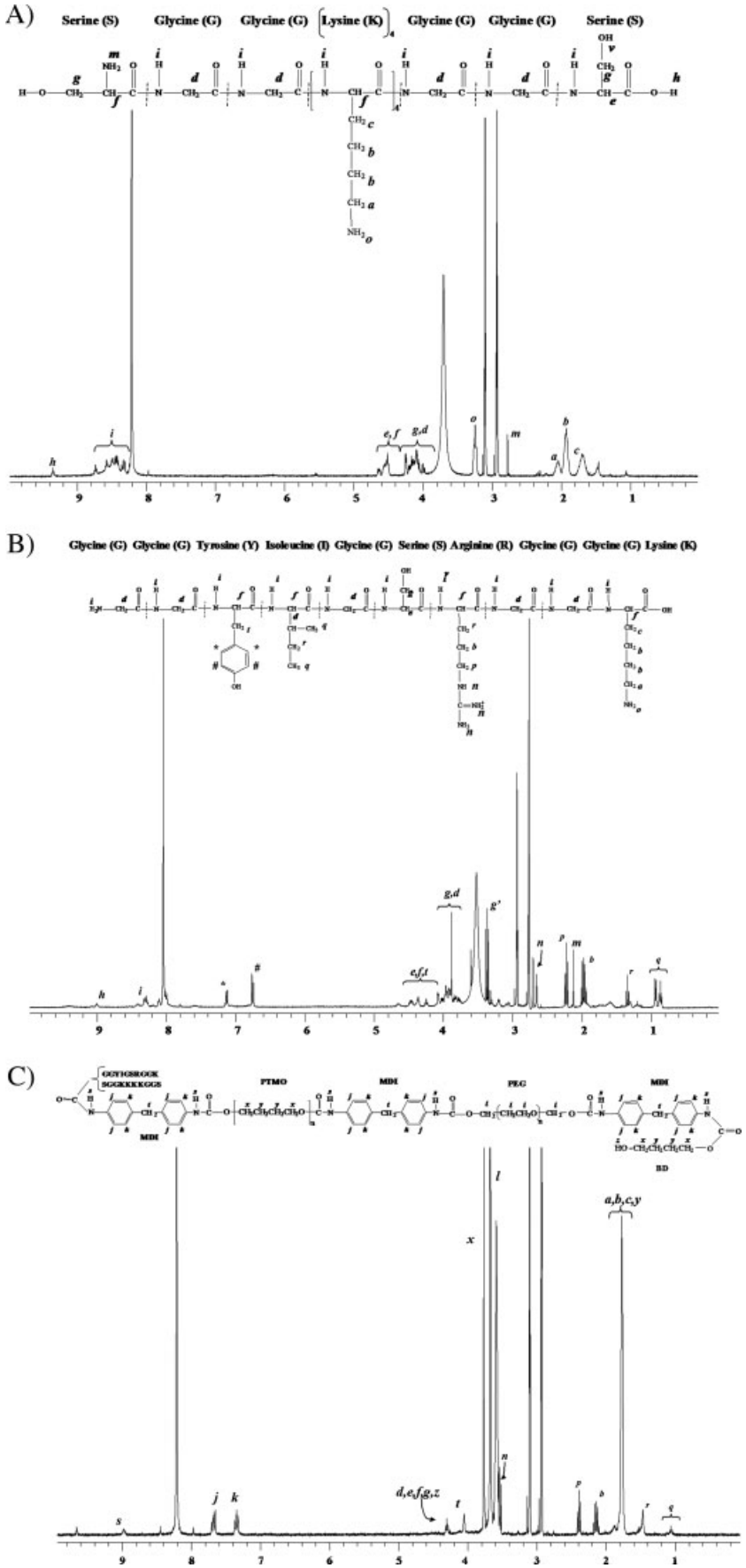

Figure 2. [1]H NMR spectra of (A) GGYIGSRGGK, (B) SGGKKKKGGS, and (C) PUDB-PEG-YIGSR-NO. Characteristic peaks of each peptide are evident in the spectra of PUDB-PEG-YIGSR-NO.

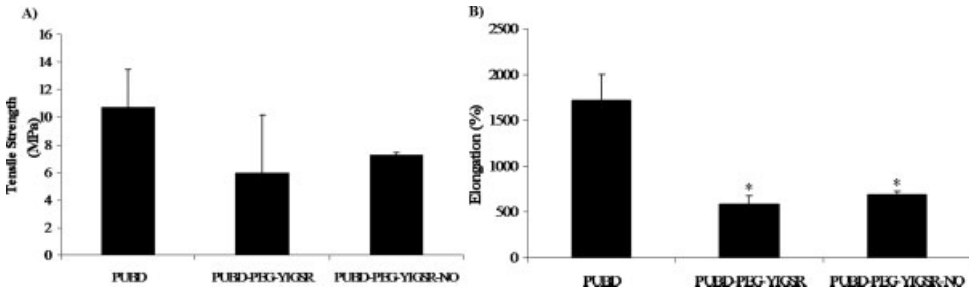

Figure 3. The tensile strengths and elongations of PUBD-PEG-YIGSR and PUBD-PEG-YIGSR-NO as compared to a control polyurethane that did not contain PEG or either peptide (PUBD). Data represent the mean of three samples (*$p < 0.02$ as compared to PUBD). Properties are comparable to commercial polyurethane vascular grafts.

4.2)% ($n = 6$) of amines were converted to these NO-nucloephile complexes. PUBD-PEG-YIGSR-NO was synthesized by incorporating the SGG[K[N(O)NO]$^-$]$_4$GGS sequence into the polymer backbone with the GGY IGSRGGK peptide. The ^{1}H NMR spectra of the peptides and polyurethanes were obtained, and the characteristic proton peaks of the GGYIGSRGGK and SGGKKKKGGS sequences indicated the successful incorporation of the peptide sequences into the polymer (Figure 2). 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. The PUBD and PUBD-NO polymers had comparable molecular weights (PUBD-PEG-YIGSR: $M_n = 95{,}852$; $M_w = 1{,}267{,}524$; PDI = 1.32; and PUBD-PEG-YIGSR-NO: $M_n = 78{,}271$; $M_w = 94{,}708$; PDI = 1.21).

Mechanical Properties

The incorporation of PEG into polyurethanes had been shown to decrease mechanical properties.[28] However, the addition of the peptides into the polymer backbone does increase mechanical strength.[28,52] Both the elastic modulus and tensile strength of PUBD-PEG-YIGSR-NO were slightly greater than those of PUBD-PEG-YIGSR (Figure 3). The mechanical properties of these materials were comparable to commercial polyurethane vascular grafts.[62]

NO Release Kinetics

NO release from PUBD-PEG-YIGSR-NO films is shown in Figure 4, occurring over ~2 months. Rapid release occurred over the first 48 h, in which 70% ± 1.22% of the incorporated NO was detected, followed by much slower, sustained release for almost 60 days. After 60 days, 86% ± 0.53% of the total NO had been released, and no further release was detected. No release of NO was detected from PUBD-PEG-YIGSR films reacted with NO gas.

BAEC and SMC Adhesion and Proliferation

The effect of NO release on BAEC and SMC adhesion to polyurethane films was examined at 6, 24, and 48 h by phase contrast microscopy [Figure 5(A)]. While SMCs did

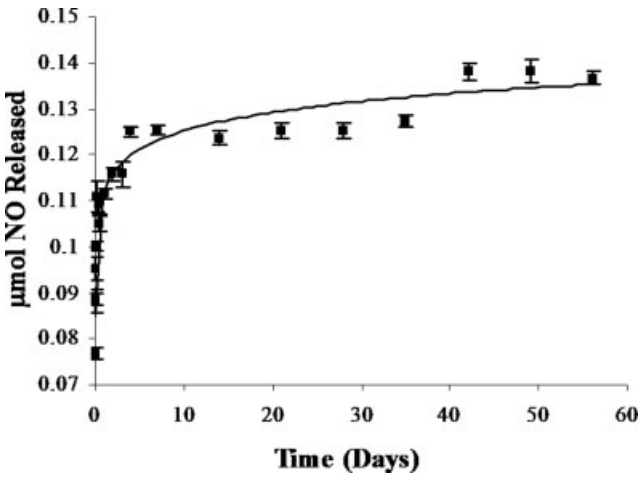

Figure 4. NO release from PUBD-PEG-YIGSR-NO films occurred in two stages over ~2 months as assessed by the Griess assay ($n = 6$). An initial burst of ~70% of the total NO loaded was released within 48 h, followed by sustained release for 60 days.

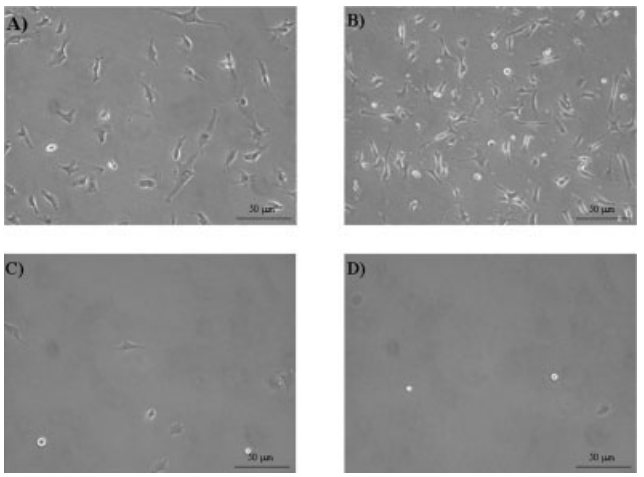

Figure 5. BAECs adhered and spread on (A) PUDB-PEG-YIGSR and (B) PUDB-PEG-YIGSR-NO, while SDSMCs did not adhere significantly to either (C) PUDB-PEG-YIGSR or (D) PUDB-PEG-YIGSR-NO. Photos were taken 24 h after cell seeding ($n = 3$).

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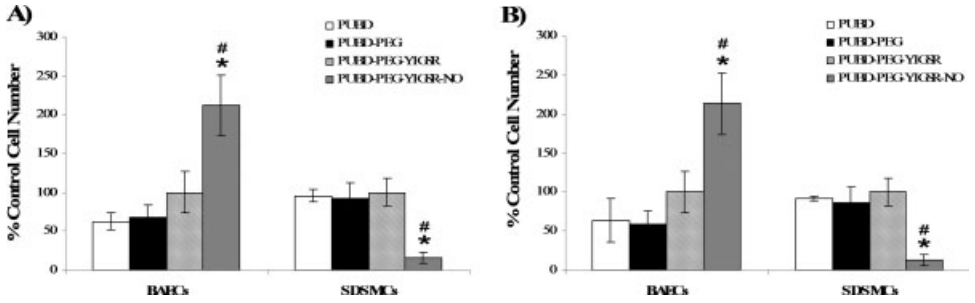

Figure 6. (A) Cellular proliferation on PUBD-PEG-YIGSR and PUBD-PEG-YIGSR-NO in rapid phase of NO release. (B) Proliferation of BAECs and SMCs on PUBD-PEG-YIGSR and PUBD-PEG-YIGSR-NO in the slower phase of NO release. Proliferation was assessed by cell counting after 48 h and was compared to control polymers that either contained no PEG and neither peptide (PUBD) or contained PEG and nor peptides (PUBD-PEG) (*, #$p < 0.002$; $n = 6$).

not adhere well to YIGSR-modified polyurethane, BAECs adhered and spread on the material within 6 h. No cells adhered to polyurethane that did not contain the YIGSR peptide. Adhesion was also assessed after films were allowed to release NO for 48 h prior to cell seeding, and SMCs did not adhere while BAECs adhered and spread within 6 h.

The number of BAECs exposed to PUBD-PEG-YIGSR-NO films releasing NO in a rapid burst was significantly greater than those exposed to PUBD-PEG-YIGSR after 48 h of culture. However, the number of SMCs cultured with fast-releasing PUBD-PEG-YIGSR -NO films was significantly lower than those cultured with PUBD-PEG-YIGSR films [Figure 6(A)].

We also examined the ability of our materials to stimulate cellular proliferation during the later, slower phase of NO release. PUBD-PEG-YIGSR-NO and PUBD-PEG-YIGSR films were incubated in HBS for 48 h, and then both BAECS and SMCs were exposed to these films as previously described. Again, the number of BAECs cultured with NO-releasing PUBD-PEG-YIGSR-NO films was higher than that of BAECs exposed to control PUBD-PEG-YIGSR films, and the count of SMCs cultured with PUBD-PEG-YIGSR-NO films was drastically lower than SMCs in the presence of PUBD-PEG-YIGSR films [Figure 6(B)].

Platelet Adhesion

Platelet adhesion to PUBD-PEG-YIGSR and PUBD-PEG-YIGSR-NO was examined using mepacrine-labeled whole blood. Platelet adhesion to PUBD-PEG-YIGSR was roughly 90% less than on the positive control, collagen I [Figure 7(A)]. However, platelet adhesion on PUBD-PEG-YIGSR-NO was dramatically lower, 95% percent less than those adhering to collagen I, and 50% less than those adhering to PUBD-PEG-YIGSR. A polyurethane that did not contain PEG or either peptide had 60% more adherent platelets than PUBD-PEG-YIGSR.

We also assessed whether 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 ear-

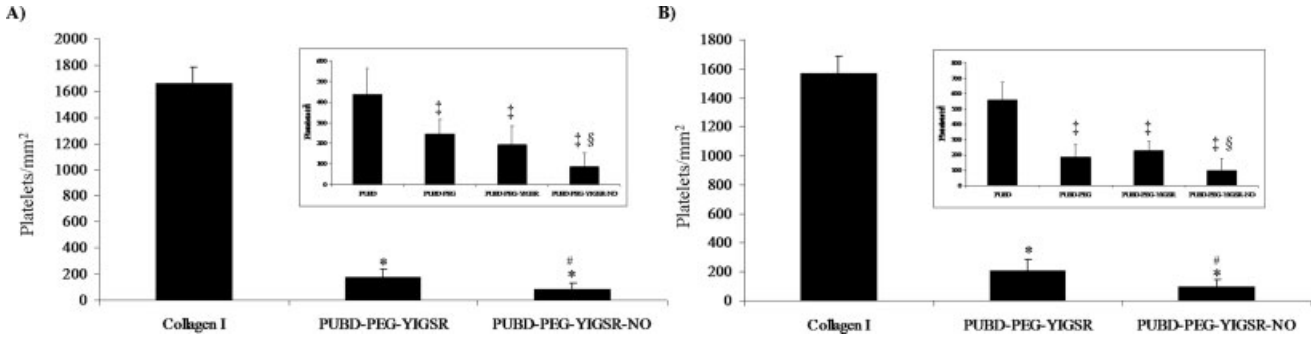

Figure 7. (A) Platelet adhesion to PUBD-PEG-YIGSR and PUBD-PEG-YIGSR-NO was significantly lower than to collagen I in the burst phase of NO release. (B) Platelet adhesion was also significantly decreased in the slower phase of NO release (*$p < 0.002$ compared to collagen I, #$p < 0.002$ compared to PUBD-PEG-YIGSR; $n = 4$). Inset figures show platelet adhesion as compared to a polyurethane that does not contain PEG or either peptide in both phases of NO release as well as a control that contains PEG but no peptides (‡$p < 0.002$ compared to PUBD, §$p < 0.002$ compared to PUBD-PEG-YIGSR; $n = 4$).

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lier. Once more, we see an approximate 90% reduction in the number of platelets adhering to PUBD-PEG-YIGSR when compared with collagen I [Figure 7(B)]. Platelet adhesion to PUBD-PEG-YIGSR-NO was again significantly lower.

DISCUSSION

Small diameter synthetic vascular grafts have, to date, shown poor success rates because of the formation of thrombi and intimal hyperplaysia. While several studies have observed decreases in platelet adhesion and thrombus formation in the presence of NO-releasing polymeric coatings *in vitro*[40,43,45] and *in vivo*,[63] the leaching of potentially harmful amine byproducts from these coatings has also been observed.[43] The NO-producing materials developed in our group contain covalently bound NO donors, derivatives of natural amino acids, and have been shown to decrease platelet adhesion and cellular proliferation leading to intimal hyperplaysia both *in vitro*[50,53] and *in vivo*.[55,56] We have previously shown that the addition of an NO-releasing peptide into the main chain of a polyurethane can improve thromboresistance while increasing endothelial cell proliferation, while retaining the mechanical properties of commercially available vascular graft materials.[52] Prior studies have also shown that the addition of a cell adhesion peptide and PEG into a biocompatible polyurethaneurea encourages endothelial cell adhesion to the polymer surface while decreasing platelet adhesion.[15,28] In this study, we have successfully combined the effects of NO, PEG, and the cell-adhesive peptide YIGSR to further reduce platelet adhesion and encourage endothelialization of polyurethane, enhancing its performance as a candidate material for small-diameter vascular substitutes.

An initial burst of NO from the polymer films was observed within 48 h, followed by slow sustained release over $\sim$60 days. This multiphasic release is likely due to initial release of NO from the surface of the polymer films, followed by slower release of NO bound within the bulk of the polyurethane. These two phases of release provide an early stimulation of cellular response and thromboresistance, discouraging platelet adhesion and intimal formation over a time range that may allow sufficient endothelialization to mimic the function of a normal blood vessel. An appropriate dose of NO is vital to encourage endothelial cell growth and the inhibition of thrombus formation and SMC proliferation; however, too high a dosage could be cytotoxic.[40,63] In both phases of NO release, we saw increased proliferation of BAECs adhered to the surface of PUBD-PEG-YIGSR-NO, suggesting that endothelial cell growth will continue throughout the sustained release of low levels of NO. The growth of an endothelial lining on the luminal surface of a graft will then function as a normal intima, releasing NO and maintaining thromboresistance.

Thrombus formation on blood contacting devices is a major concern for the success or failure of engineered materials. Recently, several animal models have been employed to examine the ability of NO releasing coatings on PTFE[40] and polyurethane[42,45,64] vascular grafts to reduce the formation of thrombi on the graft surface. However, these materials do not encourage endothelial cell adhesion, proliferation, or migration, which are all essential to a fully functional graft.

SMC proliferation and migration into the area of vascular injury leads to the formation of an occlusive scar tissue, or neointimal hyperplasia. Rat aortic SMCs did not significantly adhere to YIGSR-containing polyurethane, and the proliferation of SMCs was drastically decreased in the presence of our NO-releasing peptide. The inhibition of SMC adhesion and growth indicates that our material should resist intimal hyperplasia, thus improving the patency of a small-diameter vascular prosthetic.

CONCLUSIONS

An NO-generating, cell adhesive polyurethane-PEG copolymer (PUBD-PEG-YIGSR-NO) has been synthesized by incorporating a diazeniumdiolate-modified peptide as well as the cell adhesive YIGSR peptide sequence into the polymer backbone. NO release was observed for $\sim$2 months and reduced platelet adhesion and SMC growth while stimulating endothelial cell proliferation. This material also encouraged endothelial cell adhesion while SMCs did not adhere to the polymer. The ability to modify polyurethane with PEG and peptide sequences may afford us the ability to design an optimal scaffold for tissue engineered blood vessels.

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