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Sustained Delivery of Nitric Oxide from Poly(ethylene glycol) Hydrogels Enhances Endothelialization in a Rat Carotid Balloon Injury Model

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Abstract—The continuing high incidence of vascular disease is leading to a greater need for interventional therapies and vascular prostheses. Nitric oxide (NO), which has been heavily investigated in recent years as an important biological mediator, is presented in this work as a sustained localized therapeutic for vascular disorders, specifically in the prevention of restenosis. NO-releasing PEG hydrogels were applied to the outer surfaces of carotid arteries following balloon denudation in a rat animal model. NO was allowed to diffuse into the vessel, and intimal thickening, as assessed after 2 and 28 days, was almost fully eliminated, showing an approximate 90% decrease. Meanwhile, endothelial cell migration and proliferation into the damaged vessel sections were observed. These results signify that these materials are suitable to prevent intimal hyperplasia and induce endothelialization *in vivo*, making these NO-releasing hydrogels an ideal candidate for incorporation into blood-contacting devices for the prevention of restenosis.

Keywords—Nitric oxide, Endothelialization, Hydrogel, Poly(ethylene glycol), Diazeniumdiolate.

INTRODUCTION

The incidence of restenosis, the reocclusion of arteries following percutaneous coronary intervention (PCI), remains a major clinical concern, affecting 20–50% of all patients within 6 months.[5] Initial interventional strategies largely focused on providing mechanical support to maintain the luminal diameter necessary for adequate blood flow; however, in recent years, the paradigm has shifted to reducing thrombus and scar tissue formation through local drug delivery from polymer-coated stent surfaces. This work focuses on the use of localized and sustained nitric oxide (NO) delivery from biocompatible polyethylene glycol (PEG) hydrogels to promote regeneration of the naturally thromboresistant arterial wall.

Since its introduction in the late 1970s, PCI, formerly known as percutaneous transluminal coronary angioplasty (PTCA) has been used as a treatment for coronary artery stenosis.[5] The procedure involves the inflation of a balloon attached to a catheter in the stenosed vessel to compress the occluding plaque. Restenosis is characterized as a healing response following the injury caused by PCI that occurs over a period of months. Elastic recoil of the artery causes fractional loss of the expansion instantly.[5] Beginning immediately after the initial injury, endothelial disruption, and the resulting lack of NO production by the endothelium, induces platelet adhesion and aggregation.[3] This stimulates the release of mitogenic substances including platelet derived growth factor (PDGF), which has been implicated in the migration and proliferation of smooth muscle cells into the lumen, leading to formation of a neointima.[3,18] Other mechanisms associated with the restenotic cascade include the activation of mitogens, including basic fibroblast growth factor (bFGF), through direct injury to subendothelial smooth muscle, which also leads to smooth muscle activation.[5,18,29] As smooth muscle cells proliferate and migrate into the injured area, they deposit matrix proteins that form an occlusive scar tissue, in a process termed neointimal hyperplasia.[3]

Though there is no effective therapy to prevent restenosis, there are a number of widely used treatments. Coronary stenting has seen considerable use since being introduced in 1986, accounting for more than 60% of PCI procedures.[7] Though useful in preventing elastic recoil of the artery, the implantation of coronary stents is not without complications.

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Wall injury at the site of stent deployment can lead to platelet activation and result in thrombus formation, and bare metal stent surfaces are also thrombogenic, leading to a high risk of thrombosis early after PCI.[10] There is also the potential for in-stent restenosis, in which smooth muscle cells migrate into the implant and form scar tissue, which develops in approximately 40% of patients.[7]

Drug-eluting stents, stents coated with drug-eluting polymers, and stents made of biodegradable polymers loaded with drugs or genetic material have both been shown to suppress neointimal formation[1,2,5,8,15,24,29,31]; however, the antiproliferative or antimigratory drugs released from these stents also inhibit desirable endothelial cell growth and migration, and are therefore unable to stimulate the healing response necessary to return the vessel to its original structure and function.

NO has several vital functions in the vascular system, one of the most important being the maintenance of homeostasis through the regulation of cellular growth and migration. NO donors, compounds that spontaneously decompose to release NO in solution, are extensively under investigation for a number of biomedical applications, including use as thromboresistant coating for medical devices. Diazeniumdiolate donors, which contain the $[N(O)NO]^-$ functional group, are increasingly investigated as NO-releasing pharmaceuticals, and have been shown to inhibit platelet adhesion and smooth muscle cell proliferation, while encouraging endothelial cell growth.[4,13,14,21,23,27] However, an important concern in the use of diazeniumdiolates in biological systems is that upon release of NO the by-product of dissociation, which bears an amine at the site where the NO groups were originally bound, may be toxic to the surrounding tissue,[16,25] and it has been suggested that tailoring donors with starting materials that are innately biocompatible and can only form nontoxic nitrosation products, such as amino acids, proteins, and polysaccharides, is the most promising route for development of novel NO-generating drugs.[16]

Previous work has shown that NO-releasing hydrogels can prevent neointimal thickening using donors capable of releasing NO for approximately 24 h after hydrogel formation.[19,21] Currently, the most popular treatment for restenosis is the deployment of either bare or drug-eluting stents. Bare stents are thrombogenic, and platelet adhesion to the surface stimulates the development. Drugs released from coated stents are generally antiproliferative or antimigratory, such as sirolimus, released from Johnson and Johnson's Cypher®, and paclitaxel, released from Boston Scientific's Taxus®. These drugs inhibit the growth of a restorative endothelium, and the coatings themselves may elicit an increased inflammatory response.[9,30] However, the implementation of drug-eluting stents in recent years has revealed the need for sustained therapies due to the onset of in-stent restenosis, in which the formation of an occlusive neointima occurs within the metallic mesh stent in 3–20% of patients.[6,11,24,26,28] This work addresses the potential for the prevention of restenosis through sustained localized NO therapy that results in the formation of a restorative endothelium following PCI. Herein, we report the formation of NO-donating PEG hydrogels that are formed through reaction of NO gas with PEGylated lysine chains to form diazeniumdiolate NO donors capable of releasing NO for several months. Upon exposure to cells *in vitro*, these materials inhibit smooth muscle proliferation and platelet adhesion, while stimulating the growth of endothelial cells. *In vivo* application of the materials in a rat carotid injury model demonstrates their effectiveness in preventing neointima formation after PCI.

METHODS AND MATERIALS

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Synthesis of Polyethylene Glycol Diacrylate

Polyethylene glycol diacrylate (PEG-DA) was synthesized by dissolving 12 g dry PEG (MW: 6000; Fluka, Milwaukee, WI) in 16 mL anhydrous dichloromethane (DCM) with an equimolar amount of triethylamine and 0.72 g acryloyl chloride (Lancaster Synthesis, Windham, NH) added dropwise. The mixture was stirred under argon for 24 h, washed with 2 μ K_2CO_3, and separated into aqueous and DCM phases to remove HCl. The DCM phase was dried with anhydrous $MgSO_4$ (Fisher Scientific, Pittsburgh, PA), and the PEG diacrylate was then precipitated in diethyl ether, filtered, and dried under vacuum at room temperature overnight. The resulting polymer was dissolved in *N,N*-dimethylformamide-d_7 and characterized via proton NMR (Avance 400 MHz; Bruker, Billerica, MA) to determine the extent of acrylation.

Synthesis of PEG-polylysine Derivatives

Polylysine peptide sequences (Lys_3 and Lys_5) were conjugated to PEG monoacrylate by reaction with acryloyl- PEG-*N*-hydroxysuccinimide (PEG-NHS; MW 3400; Nektar Therapeutics, Huntsville, AL) in 50 mM sodium bicarbonate (pH 8.5) at a 1:1 molar ratio for 2 h (Fig. 1). The mixture then was dialyzed (MWCO 1000), lyophilized, and stored at −20 °C. Gel permeation chromatography with UV and evaporative light scattering detectors (Polymer Laboratories,

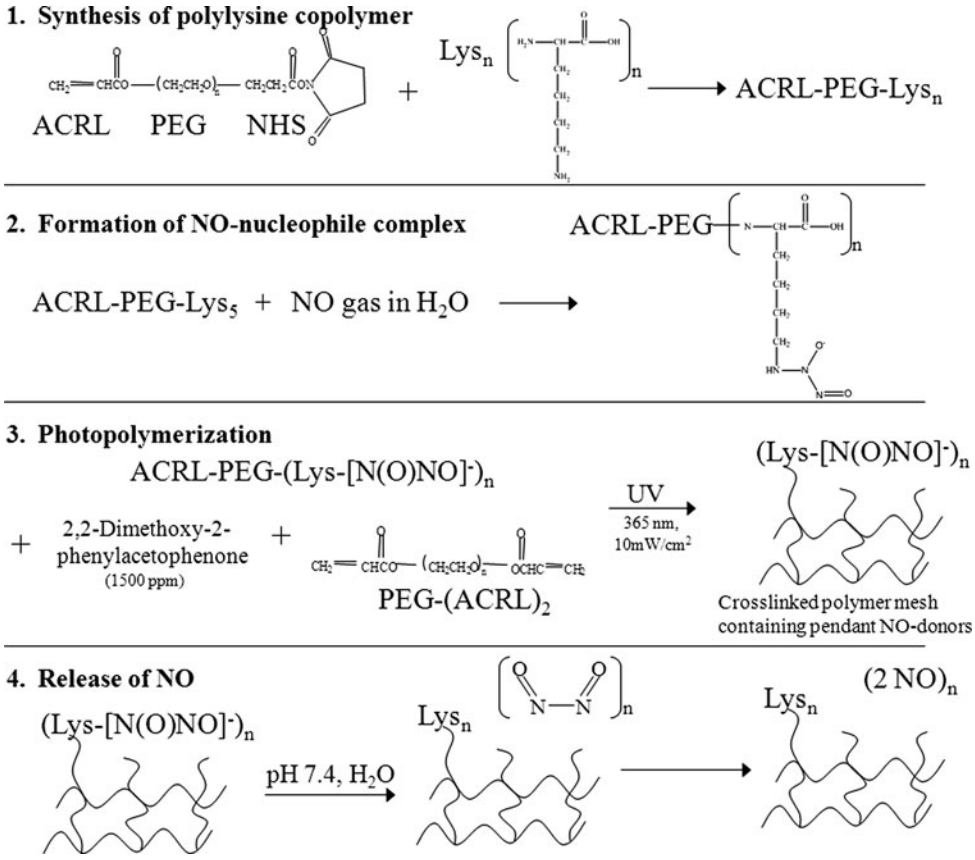

FIGURE 1. Synthesis of PEG–Lys–NO hydrogels begins with the synthesis of PEG–Lys from PEG–NHS and polylysine. NO donors are formed through reaction with NO gas in water, and this polymer is then incorporated in the crosslinked matrix of PEG-DA hydrogels.

Amherst, MA) was used to determine the coupling efficiency. The polymer was dissolved in deionized (DI) water and reacted with NO at room temperature under argon gas in a 100 mL round bottom flask overnight to convert the amine resides of lysine to diazeniumdiolate NO donors. The extent of conversion of amine groups to diazeniumdiolates was measured using the Ninhydrin assay.[22] The diazeniumdiolate polymer was freeze-dried and stored at −80 °C.

Synthesis of NO-releasing PEG Copolymer Hydrogels

Hydrogels were formed by first dissolving 0.2 g/mL PEG-DA in 10 mM HEPES buffered saline (HBS, pH 7.4), and the polymer solution was sterilized by filtration using a 0.22 μm filter (Gelman Sciences, Ann Arbor, MI). The NO-releasing PEG-peptide was also dissolved in water and filtered sterilized in the same manner, then mixed with PEG-DA resulting in a total content of 1.25 μmol NO. The photoinitiator 2,2-dimethoxy-2-phenyl acetophenone in *N*-vinyl-pyrrolidinone (300 mg/mL) was added at 10 μL/mL polymer solution. This mixture was polymerized under UV light (365 nm, 10 mW/cm^2) for 1 min.

Cell Maintenance

Bovine aortic endothelial cells (BAECs; Clonetics, San Diego, CA) and Sprague–Dawley rat aortic smooth muscle cells (SDSMCs; Cell Applications, San Diego, CA), passages 2–5, were used in this study. Dulbecco's Modified Eagle's Medium (DMEM) 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). Endothelial basal medium (EBM; Cambrex, East Rutherford, NJ) was prepared with 10% endothelial medium supplement (Cambrex, East Rutherford, NJ). BAECs were maintained on mixture of EBM and DMEM (50/50%) at 37 °C in a 5% CO_2 environment. SDSMCs were sustained on DMEM at 37 °C in a 5% CO_2 environment.

NO Release

PEG–Lys–NO hydrogels 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 in a 96-well polystyrene plate 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 is an accurate estimate for local NO production.[17] Samples were taken every 4 h for the first 48 h, then at least once per week for over 2 months.

Proliferation of BAECs and SDSMCs

To evaluate BAEC and SDSMC proliferation, cells were seeded at 10,000 cells/cm^2 in 24-well polystyrene plates (Corning Inc., Corning, NY) and incubated for 24 h at 37 °C in a 5% CO_2 environment. PEG–Lys_5–NO hydrogels (20 mm diameter, 0.5 mm thickness) were polymerized after sterile filtration of the precursor solution, placed into sterile transwell inserts (0.4 μm pore polycarbonate membrane: Corning Inc., Corning, NY) and added to the cell culture plates containing 1 mL of media; 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 and permeabilized in methanol. A 3% hydrogen peroxide solution was used to block endogenous peroxidases, and cells were incubated with mouse IgG anti-PCNA antibody (Dako Corporation, Carpinteria, CA) diluted 1:100 in PBS with 3% FBS. After rinsing, cells were incubated with anti-mouse IgG HRP (Dako Corporation, Carpinteria, CA) diluted 1:100 in PBS with 3% FBS followed by aminoethylcarbazole chromogen (AEC; Dako Corporation, Carpinteria, CA), which generates a red precipitate. Cells were counterstained with Mayer's hemotoxylin (Dako Corporation, Carpinteria, CA). The percentage of proliferating cells per field of view (200×) was determined under light microscopy (Zeiss Axiovert 135, Thornwood, NY) by averaging 5 fields per sample.

Platelet Adhesion

PEG–Lys_5–NO hydrogels and PEG-DA control hydrogels (20 mm diameter, 0.5 mm thickness) were used in this study. A 2.5 mg/mL collagen I 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 surface. Heparin (10 U/mL) and mepacrine (10 μM), which fluorescently labels platelets, were both added to whole blood obtained from a healthy volunteer. Blood (3 mL per condition) was exposed to hydrogels for 20 min at 37 °C, and, as a negative control, a sample of labeled blood was not exposed to either type of hydrogel. Collagen I films were incubated with the mepacrine-labeled blood at 37 °C for 20 min to allow binding of platelets, then gently rinsed with PBS. The number of adherent platelets per square millimeter was determined using a fluorescent microscope (Zeiss Axiovert 135, Thornwood, NY) by assessing 5 fields per sample.

Perivascular Application of PEG–Lys–NO Hydrogels in Rat Carotid Model

These materials were examined *in vivo* using an experimental rat model of balloon angioplasty. Animal experiments were performed with the approval of the Rice University Institutional Animal Care and Use Committee (IACUC). Male Sprague-Dawley rats (425–450 g; Harlan, Indianapolis, IN) were anesthetized with isoflurane (Henry Schein, Melville, NY) and their left carotid arteries isolated. The external carotid artery was ligated and the common and internal carotids clamped to halt blood flow. An incision was made in the external carotid into which a Fogarty 2F embolectomy catheter (Edwards Lifesciences, Irvine, CA) was inserted to access the common carotid artery. In the common carotid, the balloon was inflated and withdrawn to denude the endothelium and induce a stretch injury. The balloon inflation was done a total of three times and the external carotid was then ligated again, above the incision, and blood flow was restored through the common and internal carotids.

Hydrogels were applied perivascularly to negate the effects of shear and NO scavenging by blood components. PEG–Lys_5–NO hydrogel precursor solutions were previously prepared with a total NO content of 3 μmol per 200 μL of polymer solution and stored at −20 °C. These hydrogels were designed based on our previous observations of NO release kinetics from PEG–Lys_5–NO hydrogels to release 1.25 μmol of NO over 2 weeks and 2 μmol over 4 weeks. Aliquots were thawed during each surgical procedure and 1 μL of the photoinitiator 2,2-dimethoxy-2-phenyl acetophenone in *N*-vinylpyrrolidinone (300 mg/mL) was added. Hydrogel precursors (201 μL of total solution) were then applied to the outer vessel wall and photopolymerized perivascularly upon exposure to UV light (365 nm, 10 mW/cm^2) for 2 min to coat the exterior of the vessel with a layer of hydrogel along the damaged vessel length. Hydrogels of identical volumes were made at the time of implantation and incubated under physiological conditions *in vitro* to monitor release rates for the duration of the study. After 4, 14, or 28 days the tissue was explanted, cryosectioned and

histologically stained. Upon excision, vessels were gently rinsed in PBS, and the tissue from the 4 day studies was immediately frozen at −80 °C. For the 14- and 28-day studies, the vessels were divided into five segments. The center, distal, and proximal segments were immediately frozen at −80 °C. The remaining two middle segments were fixed in formalin and sectioned to 10 μm using a MICROM cyrostat (Richard-Allan Scientific, Kalamazoo, MI). To examine intimal thickening after injury, slides were stained with van Gieson's elastin stain and imaged digitally for analysis. Each of the vessel layers was measured using Scion Image software to determine the intimal and medial area and the ratio of intimal to medial area, a normalized measure of intimal thickening.

Immunohistochemical Staining for Cellular Proliferation in the Medial Layer

Proliferation of cells in the medial layer of the injured vessels was assessed through immunohistochemical staining for PCNA. The left and right carotid arteries were excised after 4 days, rinsed in PBS, and stored at −80 °C. Vessels were cryosectioned to 10 μm radial sections and fixed in acetone for 5 min at −20 °C before staining. Samples were incubated in 3% H_2O_2 in methanol for 12 min, washed three times with PBS, then blocked for 20 min with 5% FBS in PBS and incubated overnight at 4 °C with mouse anti-PCNA (Zymed Laboratories Inc., Carlsbad, CA) diluted 1:100 in PBS containing 5% FBS. Sections were then washed three times with PBS and incubated for 1 h at room temperature with peroxidase-conjugated rabbit anti-mouse IgG2a as the secondary antibody (Zymed Laboratories Inc., Carlsbad, CA) diluted 1:50 in PBS containing 5% FBS. Samples were then washed three times with PBS, stained with the HRP substrate 3,3′diaminobenzidine (DAB; Vector Laboratories, Inc., Burlingame, CA) for 7 min, and counter-stained with Mayer's hematoxylin (Dako, Carpinteria, CA). Negative controls were exposed to the secondary antibody only. The number of proliferating cells and the total number of cells in the medial layer were quantified by counting three sections for each animal.

Immunohistochemical Staining to Assess Re-endothelialization

To assess re-endothelialization of the injured vessels at 14 and 28 days, frozen vessel segments were cryosectioned longitudinally to 10 μm and immunostained (with the previously described method) for the endothelial cell marker CD-31 using a mouse anti-rat CD-31 primary antibody (diluted 1:100 in PBS containing 5% FBS; Serotec, Raleigh, NC), peroxidase-conjugated goat anti-mouse as the secondary antibody (diluted 1:50 in PBS containing 5% FBS), and DAB as the peroxidase substrate. Sections were counterstained with Mayer's hematoxylin (Dako, Carpinteria, CA). Samples were scored based on the percentage of the lumen endothelialized by an observer blinded to the treatment groups. Control injuries were performed on four rats to determine whether the endothelial lining of the vessel had been completely removed during the procedure, and these vessels were explanted after 24 h, cryosectioned lengthwise, and immunostained for CD-31 using the procedure previously described for PCNA staining. No positive staining for CD-31 was observed along the control vessels, indicating that the vessels were completely de-endothelialized. Right carotid arteries (uninjured) were used as positive controls.

Statistical Analysis

All experiments were performed minimally in triplicate. Error bars reflect standard deviations, and *p*-values were assessed using two-tailed, unpaired *t*-tests; *p*-values less than 0.05 were considered significant. Animal studies were performed in groups of 8 animals per condition, and re-endothelialization was scored by an observer blind to the experimental groups. Statistical analysis was performed using nonparametric tests to determine if the treatment groups were statistically different.

RESULTS

NO Release

Reactions of polylysine-modified hydrogel with NO gas resulted in greater than 90% conversion of amines to diazeniumdiolate NO donors. NO release from diazeniumdiolate-modified hydrogels occurred over different time frames depending on the length of the lysine chain incorporated into the polymer. PEG–Lys_3–NO hydrogels were observed to release 95.89 ± 3.27% of the total NO loaded into the gel over approximately 30 days (Fig. 2a); however, PEG–Lys_5–NO hydrogels released 89.48 ± 0.31% of NO over roughly 60 days (Fig. 2b), indicating that the incorporation of longer chains of lysine into the polymer matrix induces more sustained rates of NO release and that these rates can be easily tailored. Subsequent studies were performed with PEG–Lys_5–NO hydrogels, since longer term release was achieved. Stability testing was performed to determine the effects of freeze-drying diazeniumdiolates, and no variations in NO donor activity were observed.

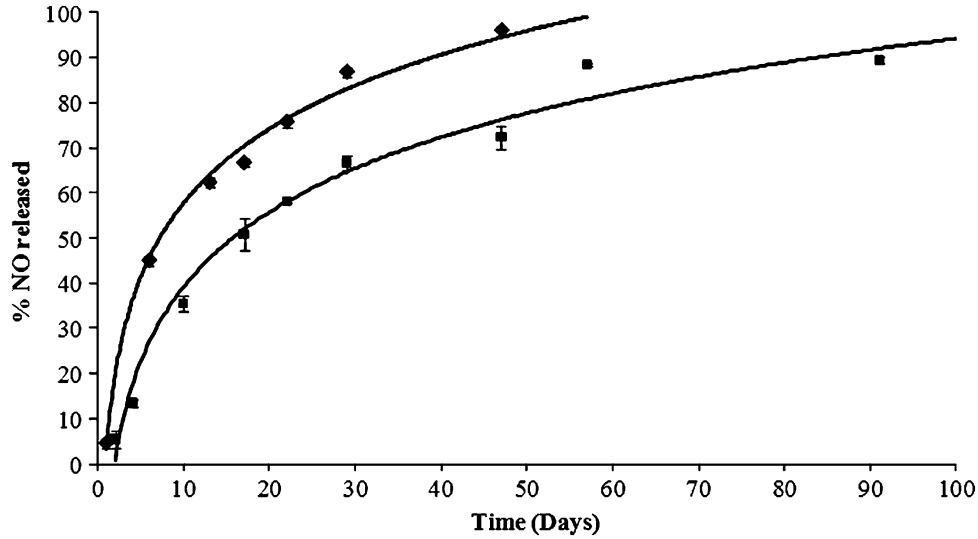

FIGURE 2. Release of NO from PEG–Lys_3–NO hydrogels occurred for roughly 30 days under physiological conditions ($n = 5$), while release of NO from PEG–Lys_5–NO hydrogels occurred for twice as long, approximately 60 days ($n = 5$).

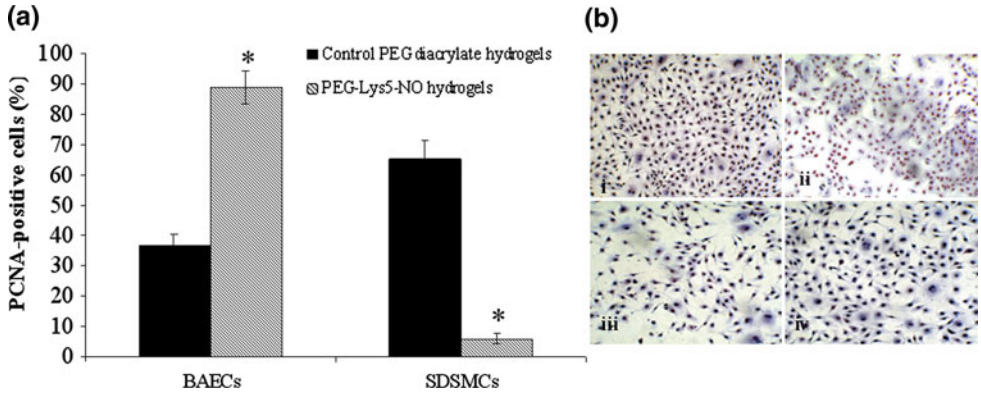

FIGURE 3. (a) BAEC proliferation is greater in the presence of PEG–Lys_5–NO hydrogels, and SMC proliferation is inhibited when cells were exposed to NO-releasing hydrogels. (b) (i) BAECs exposed to PEG-DA, (ii) BAECs exposed to PEG–Lys_5–NO, (iii) SMCs exposed to PEG-DA, (iv) SMCs exposed to PEG–Lys_5–NO. Data represent the mean of four samples (*, # $p < 0.01$).

Proliferation of BAECs and SDSMCs

The effect of NO release from PEG–Lys_5–NO hydrogels on BAEC and SDSMC proliferation was examined using immunohistochemical staining for PCNA. The percentage of PCNA-positive BAECs exposed to PEG–Lys_5–NO ($88.91 \pm 5.22\%$) was significantly greater than those exposed to PEG-DA ($36.47 \pm 4.12\%$) after 48 h of culture (Fig. 3). However, the percent of PCNA-positive SMCs cultured with NO-releasing hydrogels ($5.84 \pm 1.50\%$) was significantly lower than those cultured with PEG-DA gels ($65.12 \pm 6.19\%$).

Platelet Adhesion

The ability of diazeniumdiolate-modified hydrogels to decrease thrombus formation was assessed, and platelet adhesion to collagen I was approximately 80% less when blood was exposed to PEG–Lys_5–NO (729.3 ± 274.5platelets/mm^2) compared to blood exposed to PEG-DA (3447.9 ± 717.1 platelets/mm^2), as shown in Fig. 4. These results suggest that NO-releasing hydrogels can be used in the formation of blood-contacting surfaces such as stent coatings, reducing thrombus formation and thereby increasing the effectiveness of the device. Previous work has shown that thrombogenic materials engineered to contain PEG have decreased platelet adhesion, and that the inclusion of NO even further reduces platelet adhesion.[27] The system described herein is composed entirely of hydrophilic PEG with the capability to release NO over a prolonged period, greatly reducing the potential for thrombus formation.

Perivascular Application of PEG–Lys–NO Hydrogels in Rat Carotid Model

The intimal thickening was decreased 88.27 ± 2.04% (Fig. 5a) and the ratio of intimal are to medial area was lessened 83.76 ± 4.59% (Fig. 5b) when PEG–Lys_5–NO hydrogels were applied perivascularly for 14 days following balloon injury. Intimal thickening was decreased by 87.5 ± 4.7% (Fig. 5c) and the ratio of intimal to medial area was decreased by 92.2 ± 1.7% (Fig. 5d) when applied for 28 days, demonstrating that these NO-generating materials are effective when applied peri-adventitially.

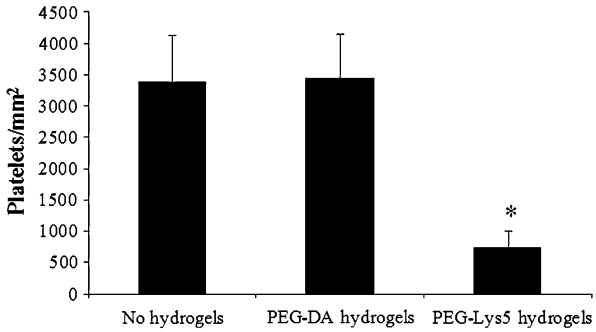

FIGURE 4. Platelet adhesion to collagen I was greater after exposure to nohydrogels and PEG-DA than to NO-generating hydrogels ($p < 0.02$; $n = 6$).

Intimal thickening was examined by measuring the size of the vessel layers after staining with van Gieson's elastin stain, which stains the elastic lamina separating the layers of the vessel. Representative vessels from the 28-day treatment are shown in Fig. 6. The internal elastic lamina, highlighted by the arrows in Fig. 6, separates the media from the intima. In an undisturbed vessel, one would see only one thin layer of cells above the internal elastic lamina, and while the animal treated with the NO-releasing hydrogel shows very little intimal growth into the lumen (Fig. 6a), the tissue treated with the control PEG-DA gels shows a distinct neointima (Fig. 6b). Previous studies have demonstrated that neither PEG-DA hydrogels nor the in situ photopolymerization modifies the vascular response when applied perivascularly.[32] There were no observations of any toxic or adverse effects caused by any hydrogel formulations upon examination of the surrounding tissues, and no effects of the surgical procedure or hydrogel application were observed when compared to uninjured vessels.

Cellular Proliferation in the Medial Layer

After 4-day exposure to NO-releasing hydrogels, proliferation in the medial layer was significantly inhibited (Fig. 7; 38.42 ± 4.08%) as compared to vessels exposed to PEG-DA control hydrogels

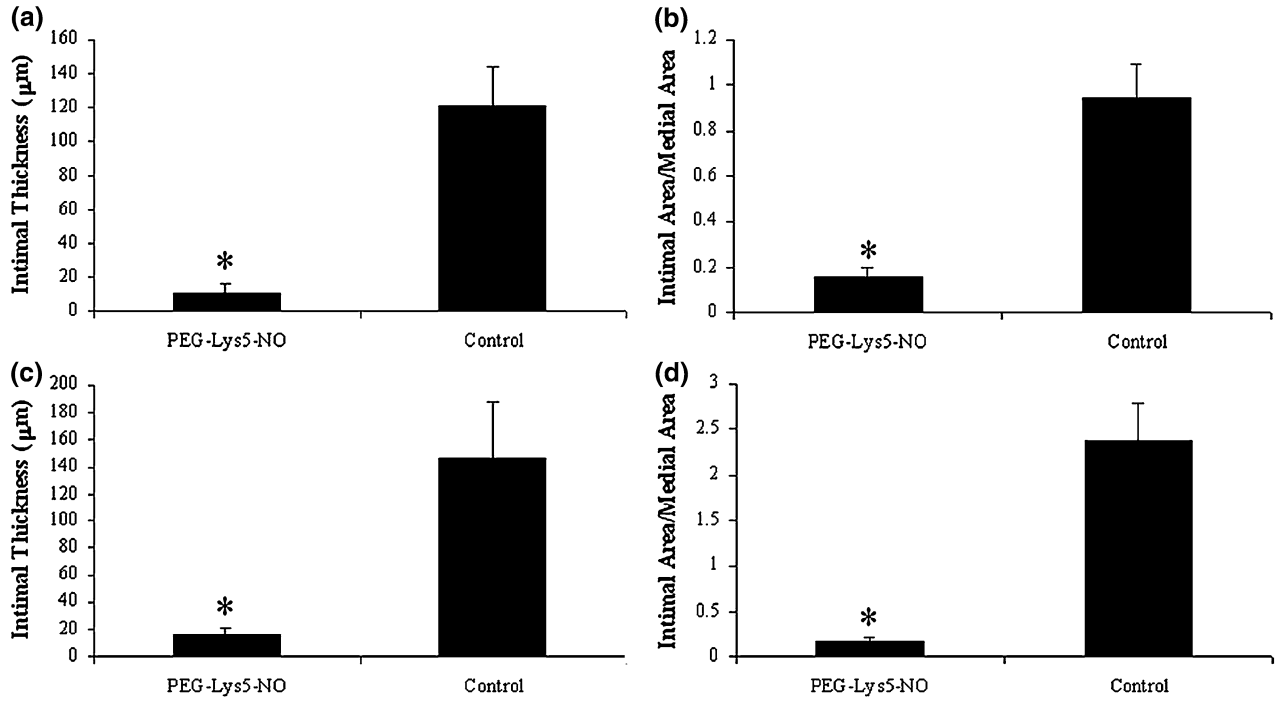

FIGURE 5. (a) Intimal thickness was significantly decreased in animals exposed to PEG–Lys_5–NO hydrogels for 14 days and (b) normalized measurements of intimal thickness were also decreased after both 14 days of exposure. After 28 days of exposure to NO-releasing hydrogels, (c) intimal thickness was further decreased, as were (d) normalized measurements of intimal thickness ($n = 8$; * $p < 0.02$).

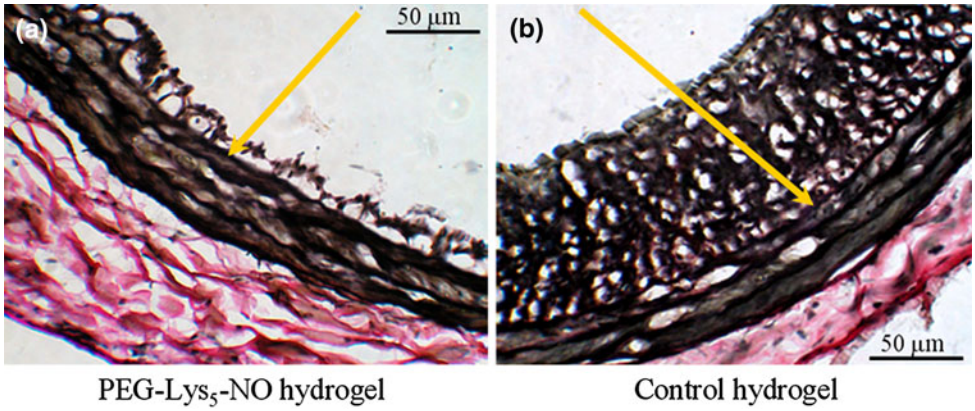

FIGURE 6. Arterial sections stained with van Gieson's elastin stain after (a) 28-day exposure to PEG–Lys_5–NO and (b) 28-day exposure to PEG-DA. Arrows indicate the internal elastic lamina, above which lie the intima and the lumen. The tip of the arrow identifies the elastic lamina which separates the intimal and medial layers.

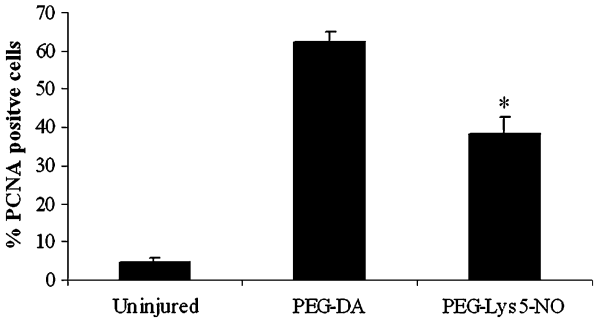

FIGURE 7. Smooth muscle cell proliferation in the medial layer was inhibited by NO-releasing hydrogels after 4 days as compared to controls treated with PEG-DA ($n = 4$; * $p < 0.02$).

(62.31 ± 2.64%). An uninjured control vessel had only 4.6 ± 1.28% cells stain positive for PCNA, suggesting that the injury stimulates smooth muscle cell proliferation in the medial layer, and the local delivery of NO reduces cellular growth into the intima. Though we did not study the phenotypic characteristics of the cells within the neointima, it has been observed that SMCs cultured from the neointima of injured rat carotid arteries have a different shape and organization *in vitro* than SMCs from the uninjured media.[20]

Re-endothelialization Following Injury

Endothelialization was enhanced in animals treated with NO-releasing hydrogels when compared with animals treated with PEG-DA controls at both 14 and 28 days. Vessel sections were scored according to the percentage of lumen endothelialized by an observer blinded to the treatment groups (Fig. 8). A significant majority of both the middle and proximal sections were found to have higher percentages of endothelium when treated with PEG–Lys_5–NO hydrogels, and the morphology of these sections was comparable to those taken from uninjured vessels.

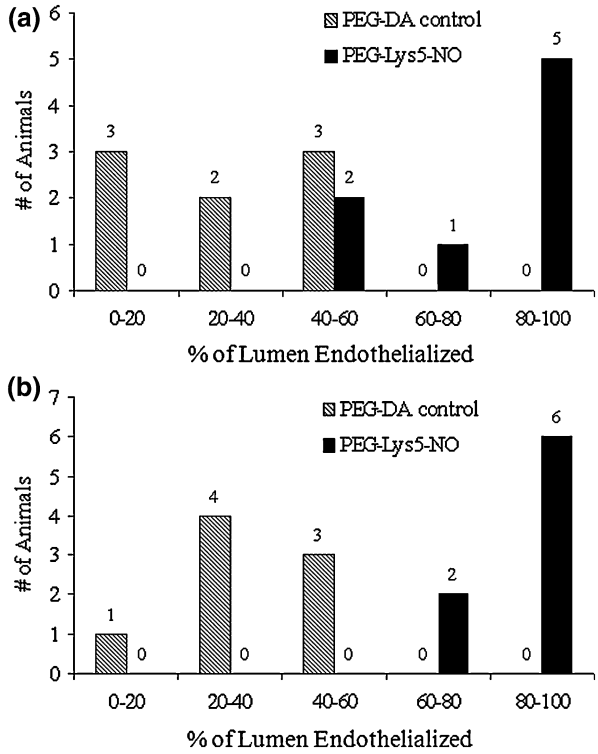

FIGURE 8. Percent of vessel re-endothelialization after (a) 14 days and (b) 28 days of treatment with either PEG-DA or PEG–Lys_5–NO hydrogels. The majority of sections were scored with higher percentages of endothelium when treated with PEG–Lys_5–NO. Analysis by the Mann–Whitney *U*-test found the treated group scored significantly higher than the untreated control during each time period.

DISCUSSION

The NO-generating hydrogels described in this study are able to release NO over a period of months, depending on design, and are able to cause changes in vascular cell viability and proliferation, platelet

adhesion to thrombogenic surfaces, intimal thickening, and the re-endothelialization of arteries after denudation. Stents, both bare and drug-eluting, are still unable to fully prevent restenosis and promote vessel recovery after angioplasty. Though further study is necessary to examine the effect of blood flow on these materials and their effect on the inflammatory response *in vivo*, the materials presented here show promise in improving vascular healing after PCI and are excellent candidate materials for stent coatings and as endoluminal barriers. Ideally, the polymeric system could be coated onto a stent and freeze-dried to inhibit hydrolytic NO release. Upon deployment of the stent inside the body, the hydrogel would absorb water, triggering NO release.

The rates of NO release in this work were dependent on the size of the lysine chained covalently bound to the hydrogel matrix. An increase of only two amino acids practically doubles the time course of release. Previous research has shown that small changes in the NO-releasing species can lead to relatively large increases in donor half-life,[12] and the results presented here indicate that altering NO release kinetics from PEG hydrogels is possible through material design. Tailoring treatments to particular disorders or patients can be easily accomplished by choosing the proper polylysine-based NO donor. In treating restenosis, which is the normal healing response following coronary intervention and occurs over a period of several months, sustained release of NO will guide enhanced regeneration of the vessel by encouraging endothelial cell proliferation and migration into the damaged area.

The lack of a functioning endothelium following vascular interventional procedures starts a cascade of events that leads to intimal thickening. Endothelium is destroyed during denudation and cells are unable to proliferate and migrate into the region. The NO-producing hydrogels in this study promote endothelial cell growth and, at the same time, inhibit smooth muscle cell proliferation. Since these are two of the key contributors to the onset of intimal hyperplasia, the materials presented here should prove highly effective in the prevention of restenosis. The major contributor to the cascade leading to vessel re-occlusion is platelet adhesion. Thrombus formation at the injury site leads to degranulation of platelets which release of their granular contents, including growth factors such as PDGF, enhancing smooth muscle proliferation and migration. Thus, PEG–Lys–NO hydrogels have several preventive roles, decreasing the onset of intimal thickening while returning the vessel to its native structure and function.

To further validate the efficacy of NO-releasing hydrogels in preventing intimal hyperplasia, PEG–Lys_5–NO hydrogels were applied to the outer surface of the carotid artery following balloon denudation in a rat animal model. NO was allowed to diffuse into the vessel, and no toxicity was observed in any of the surrounding tissue as indicated by the absence of necrotic tissue or abnormal morphology. After 4 days, cellular proliferation in the medial layer was examined, and found to be decreased in the animals treated with NO-producing hydrogels. At the 4-day time point for these results, very little of the NO has been released and thus the response of the SMCs in the medial layer, while inhibited, is not totally restricted and there is some cellular proliferation. As the NO levels increase during the following days, the inhibition is likely increased and the proliferation greatly decreased. It is expected that the small amount of intimal thickening that we observe is occurring early within the experiment (i.e. within the first 4–7 days). Intimal thickening was assessed after 2 and 4 weeks, and was almost fully eliminated, showing an approximate 90% decrease. Meanwhile, endothelial cell migration and proliferation into the damaged vessel sections were observed. These results signify that these materials are suitable to prevent intimal hyperplasia and induce endothelialization *in vivo*. Though similar results in decreasing neointima formation have been observed using biocompatible materials that release NO rapidly,[14,19,21,23] it is important to note that the hydrogels used in this study have only released approximately 67% of the loaded NO over the 4-week time course and have the potential to continue encouraging endothelialization for several weeks. The potential for this extensive NO release greatly increases the chances for restoration of a continuous lumen that that may not be otherwise achieved with fast-acting donors.

The materials used here prevent the cascade leading to the onset of restenosis *in vivo* through release of NO, a natural vascular mediator, from non-immunogenic PEG hydrogels, and these NO-releasing hydrogels should be considered as localized barriers for the prevention of neointimal hyperplasia. Clinical applications of these NO-producing hydrogels may lead to even further decreases in the onset of restenosis, enhancing the effects of mechanical barriers (stents and endoluminal coatings) against the migration of smooth muscle cells across the elastic lamina. In addition, the incorporation of NO-releasing moieties into a PEG-DA hydrogel matrix causes a significant decrease in platelet adhesion. The inhibition of smooth muscle cell migration and proliferation, as well as the potential for the elimination of platelet adhesion, make these NO-releasing hydrogels an ideal candidate for incorporation into blood-contacting devices for the prevention of restenosis.

CONCLUSIONS

Modified polylysine copolymer hydrogels released NO over extended time periods, on the order of months, under physiological conditions. The materials synthesized in the present work demonstrate that NO release kinetics vary depending on material design, and may be tailored to release specific amounts of NO over defined time periods. The capacity of these materials to inhibit thrombus formation and initmal thickening while encouraging endothelialization implies that these hydrogels can not only prevent restenosis after angioplasty, but can also return the vessel to its original structure and function, promoting superior healing through tissue regeneration.

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CONFLICTS OF INTEREST

No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this manuscript.

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