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Poly(ethylene glycol)-lysine dendrimers for targeted delivery of nitric oxide

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Abstract—We have synthesized dendrimers of the amino-acid lysine bound to a central poly(ethylene glycol) (PEG) core, and then formed multiple diazeniumdiolate nitric oxide (NO) donors on the lysine residues. NO release from these materials occurred for up to 60 days under physiological conditions. These materials display the ability to regulate vascular cell proliferation and inhibit platelet adhesion to thrombogenic surfaces. When modified with a targeting ligand specific for inflamed endothelium (Sialyl Lewis X), we were able to demonstrate binding of fluorescently-labeled dendrimers to endothelial cells activated by interleukin 1β (IL-1β).

Key words: Nitric oxide; dendrimer; targeted drug delivery; diazeniumdiolate; Sialyl Lewis X.

INTRODUCTION

Atherosclerosis accounts for nearly 75% of deaths from cardiovascular disease and is a leading cause of death from stroke and heart attack [1]. Dysfunctional vascular endothelium is central to the onset of the complex pathology of atherosclerosis, displaying characteristics of inflammation and leading to leukocyte adhesion [2, 3]. During inflammation, endothelium is in an activated state and exhibits increased cell surface expression of adhesion molecules, including E-selectin, vascular cell adhesion molecule (VCAM) and intracellular cell adhesion molecule (ICAM) [4–6]. E-selectin plays a crucial role in the initial rolling interaction of immune cells with inflamed endothelium, transiently binding the carbohydrate Sialyl Lewis X (SLe^X) found on inflammatory cells [6–11] and has been previously been proposed as a target for site-specific delivery of therapeutic agents to regions of inflammation within the cardiovascular system [6, 12, 13]. This work reports the targeting of NO-

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releasing dendrimers to E-selectin on activated endothelial cell monolayers for the prevention of atherogenesis.

NO has a number of vital 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 [14–20]. NO donors, compounds that spontaneously decompose to release NO in solution, are extensively under investigation for a number of biomedical applications. Diazeniumdiolate NO donors, which contain the $(N(O)NO)^-$ functional group, are being investigated as NO-releasing pharmaceuticals [21–25] and thromboresistant coatings for blood-contacting medical devices [25–33]. These compounds contain anionic portions that spontaneously decompose in solution at rates dependent on chemical structure, temperature and pH.

NO releasing molecules have also been suggested as preventive therapeutics for the formation of atherosclerotic lesions [34–36]. Luminal release of NO from healthy endothelium inhibits the expression of cell adhesion molecules and chemoattractants [34–41], thus inhibiting the inflammatory phase of atherogenesis. The subsequent thrombotic phase, characterized by platelet adhesion to the surfaces of atherosclerotic lesions leading to thrombus formation, is also a major contributor to luminal narrowing [14, 16, 17, 42, 43] and can also be inhibited through localized NO release.

Dendrimers are synthetic, highly branched macromolecules of nanometer dimensions, in which bonds radiate from a central core in a regular branching pattern [44]. These molecules have several characteristics that make them attractive for biological and drug delivery applications, including uniform size, water-solubility, internal cavities and variable surface functionality [45–47]. Studies suggest that dendritic structures allow for loading and controlled release of drugs [47–49] and antibody–dendrimer conjugates have been shown to retain their immunoreactivity while displaying high binding specificity [47, 50], signifying that these nanoscale materials are ideal carriers for therapeutics targeted to localized pathologies. Dendrimers based on the amino-acid lysine have been tested for their ability to bind both high- and low-molecular-weight drugs while bearing targeting ligands to vascular extracellular matrix proteins [51, 52] and show promise of retention in the vascular wall under shear. Though *in vivo* applications of highly charged polylysines have, to date, been limited because the positive charges are associated with toxicity [52–54], various dendrimer formations based on lysine cores or branching units have shown efficacy in numerous applications, including proteolytically stable antimicrobials [55] and water-soluble complexes with DNA, capable of increasing plasmid stability in the presence of nucleases [56]. Lysine dendrimers have high numbers of surface amines capable of forming diazeniumdiolate NO donors; this would shield the potentially toxic positive charge while loading a high concentration of drug. The studies detailed here investigate the potential of NO-releasing lysine dendrimers as targeted drug-delivery agents to inflamed endothelium, a marker for active vascular disease. The ability to functionalize surface groups and encapsulate guest molecules

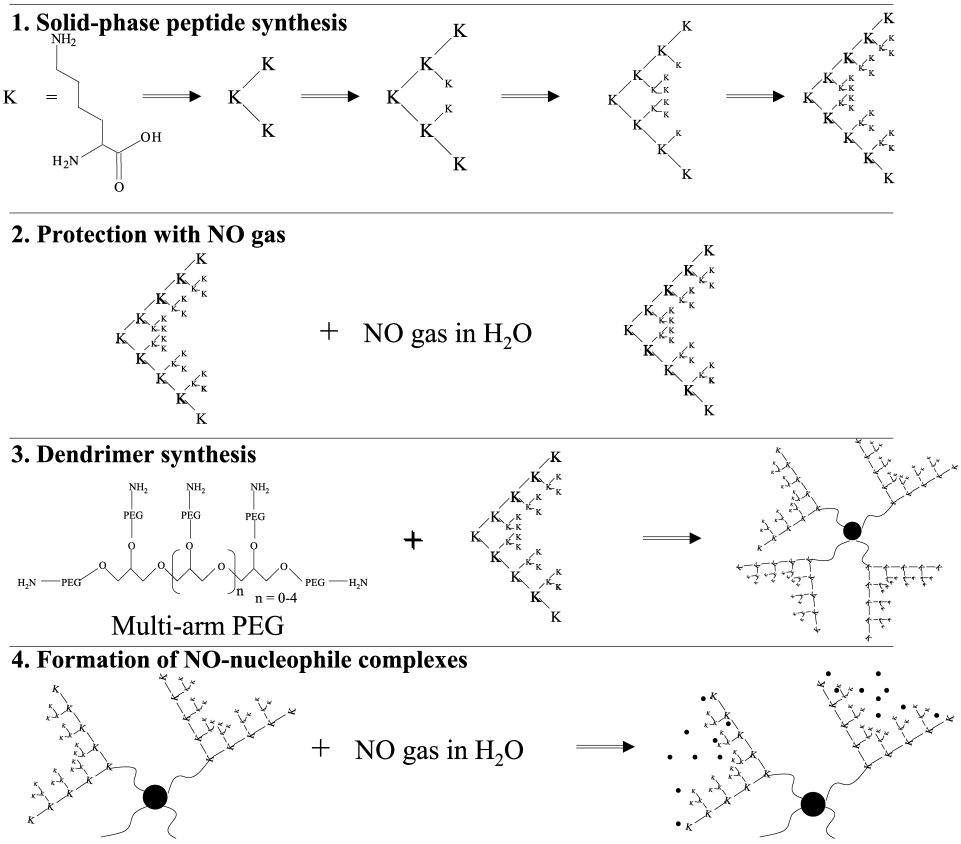

Figure 1. The amino-acid lysine was used as the basis for the branching unit, and solid-phase peptide synthesis was used to iteratively build a branched dendron. These were then reacted with NO gas in water and tethered to a multifunctional PEG to form a highly branched network with a PEG core. Each branching unit has many terminal amines, which were again reacted with NO gas in water.

makes these molecules ideal systems for drug delivery and offers the opportunity to design cell-targeted therapeutics for treatment of arterial disease. Our approach to prevent localized atherogenesis is the use of a targeted, diazeniumdiolate-modified dendrimer delivery system. The ability of these nanoscale materials to bind and release NO was assessed, as well as the effect of these materials on vascular cell viability and proliferation. Platelet adhesion in the presence of these materials was studied, and the efficacy of targeting these dendrimers using the ligand Sialyl Le^X was examined.

MATERIALS AND METHODS

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

Synthesis and characterization of PEG-core polylysine dendrimers

Branched lysine dendrons were synthesized by standard fluorenylmethoxycarbonyl (Fmoc) chemistry on an 431A peptide synthesizer (Applied Biosystems, Foster City, CA, USA) using N_α-N_ε-di-Fmoc-L-lysine (Peninsula Laboratories, San Carlos, CA, USA). In brief, 4-molar equivalents of di-Fmoc-lysine was bound to Fmoc-Lysine(Boc) resin (0.25 mmol; Applied Biosystems), deprotected and then reacted with another 4-fold excess of di-Fmoc-lysine, iteratively building a branching structure (Fig. 1). The procedure was repeated a total of 4 times to build generation-4 dendrons. Dendrimers were synthesized after reacting dendrons with NO gas in water to protect the terminal amines from reaction, then mixing 4 molar equivalents of lysine dendrons with a 4-arm PEG-amine (25 mg; 10 kDa; Nektar Therapeutics, San Carlos, CA, USA) for 4 h in anhydrous N-N-dimethylformamide (DMF; Aldrich, Milwaukee, WI, USA) in the presence of 4 molar equivalents each of N-hydroxybenzotriazole (HOBT), O-benzotriazol-1-yl-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) and N,N-diisopropylethylamine (DIPEA) to facilitate formation of a peptide bond between PEG-amine and the carboxyl terminus of the dendrons. Dendrimers were precipitated in ethyl ether and filtered using a 0.45 μm Durapore filter (Millipore, Billerica, MA, USA).

Fluorescent dendrimers were synthesized by adding fluorescein isothyocyanate (FITC) to dendrons prior to reaction with PEG-amine. Dendrons, at a concentration of 10 mg/ml, were incubated with FITC at a final concentration of 250 μM in anhydrous dimethyl sulfoxide (DMSO) in the dark for 4 hr. Dendrons were then reacted with PEG-amine and filtered as described above.

Targeted dendrimers were formulated using an avidin-biotin linkage between the dendrimer and the targeting agent. A 10 molar excess of biotin-N-hydroxysuccinimide was first reacted with 10 mg/ml of lysine dendrons in DMF to biotinylate each terminal amine. The product was then precipitated in ethyl ether and filtered using a 0.45 μm Durapore filter (Millipore), while 500 μg SLe^X-biotin (Glycotech, Gaithersburg, MD, USA) was separately reacted with 2 molar equivalents of avidin in HEPES-buffered saline (HBS). These products were then combined to facilitate the conjugation of the targeting ligand. Control materials were modified with Lewis X-biotin (Le^X-biotin; Glycotech), which is structurally similar to SLe^X, but does not bind E-selectin. Fluorescent, targeted, NO-releasing dendrimers are synthesized with the addition of previously functionalized dendrons to PEG-amine, each at a 1:4 molar ratio with the 4-arm PEG-amine.

Lysine dendrons and PEG-lysine dendrimers were characterized *via* ^{1}H-NMR using a 400 MHz NMR spectrometer (Advance 400, Bruker, Rheinstetten, Germany) with *N,N*-dimethylformamide-d_7 (DMF-d_7) as the solvent. Molecular weight distributions were obtained by GPC with UV and evaporative light scattering detectors (Polymer Laboratories, Amherst, MA, USA). 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) at a flow rate of 1 ml/min.

Calibration was performed using PEG standards (Polymer Laboratories), ranging in molecular mass from 0.4 to 20 kDa.

Cell maintenance

Bovine aortic endothelial cells (BAECs; Clonetics, San Diego, CA, USA), Sprague–Dawley rat aortic smooth muscle cells (SMCs; Cell Applications, San Diego, CA, USA) and human umbilical vein endothelial cells (HUVECs; Clonetics) 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, USA), 2 mM l-glutamine, 1 unit/ml penicillin and 100 mg/l streptomycin (GPS). Endothelial basal medium (EBM; Cambrex, East Rutherford, NJ, USA) was prepared with endothelial microvascular medium supplement (Cambrex). Endothelial basal medium-2 (EBM-2; Cambrex) was prepared with endothelial medium-2 supplement (Cambrex). 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. HUVECs were maintained on EBM-2 at 37°C in a 5% CO_2 environment.

NO release

PEG-Lys dendrimers were reacted with NO gas in water under argon at room temperature overnight. A portion of dendrimers not reacted with NO were reserved for quantification of terminal amines using the ninhydrin assay [57], and the reaction efficiency was determined by comparison of amines still detected after reaction with NO gas. Dendrimers were then incubated at 37°C in HBS, and NO release was measured using the Griess assay [58]. Samples were taken every 4 h for the first 48 h, then at least once per week for over 2 months.

Viability of BAECs and SDSMCs

NO-releasing dendrimers were dissolved in deionized water and sterilized by filtration using a 0.22-μm syringe filter (Whatman, Clifton, NJ, USA). Viability was assessed by first seeding BAEC and SDSMCs at 1×10^4 cells/cm^2 in 6-well polystyrene plates (Corning, Corning, NY, USA) and incubating them for 24 h at 37°C in a 5% CO_2 environment. Dendrimers were added to the cell culture media, and after another 48 h of culture at 37°C in a 5% CO_2 environment, cells were stained with a calcein AM and ethidium homodimer Live/Dead viability stain kit (Molecular Probes, Eugene, OR, USA) to visibly quantify cell viability. In brief, a solution of 4 mM ethidium bromide and 2 mM calcein AM was incubated with cells for 45 min at room temperature. Ethidium bromide enters cells with damaged membranes and binds nucleic acids, causing dead cells to fluoresce red [59]. Calcein AM is cell-permeable and is converted to a fluorescent calcein dye by intracellular esterase activity; thus, live cells fluoresce green [59]. Fluorescence

was assessed using a fluorescence microscope (Zeiss Axiovert 135, Thornwood, NY, USA) by counting red and green cells and determining the percentage of the cells that fluoresced green, averaging 5 fields per sample.

Proliferation of BAECs and SDSMCs

PEG-Lys-NO dendrimers were dissolved in deionized water and sterilized by filtration using a 0.22-μm syringe filter (Whatman). To evaluate BAEC and SDSMC proliferation, cells were seeded at a concentration of 1×10^4 cells/cm^2 in 6-well polystyrene plates (Corning) and incubated for 24 h at 37°C in a 5% CO_2 environment. Dendrimers were added to the cell culture media, and 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, Carpinteria, CA, USA) diluted 1:100 in PBS with 3% FBS. After rinsing, cells were incubated with anti-mouse IgG horseradish peroxidase (HRP, Dako) diluted 1:100 in PBS with 3% FBS followed by aminoethylcarbazole chromogen (AEC, Dako), which generates a red precipitate. Cells were counterstained with Mayer's hematoxylin (Dako). The percentage of proliferating cells per field of view (200×) was determined under light microscopy (Zeiss Axiovert 135) by averaging 5 fields per sample.

Static platelet adhesion

NO-releasing dendrimers were dissolved in deionized water and sterilized by filtration using a 0.22-μm syringe filter (Whatman). A solution of 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 reference material. Heparin (10 U/ml) and mepacrine (10 μM), which fluorescently labels platelets, were both added to whole blood obtained from a healthy volunteer. Blood was exposed to dendrimers that released 1.25 μmol NO over 20 min at 37°C. 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 (200×) was determined using a fluorescent microscope (Zeiss Axiovert 135) by assessing 5 fields per sample.

Targeting studies

Fluorescent, targeted dendrimers were dissolved in deionized water and then sterilized by filtration using a 0.22-μm syringe filter (Whatman). HUVECs were seeded in flat-bottomed 24-well tissue culture plates at a density of 1×10^4 cells/cm^2, and allowed to adhere for 24 h. Cells were then stimulated with 1 ng/ml interleukin-1β (IL-1β) for 4 h to stimulate the endothelial cells and encourage the presentation

of cell adhesion molecules. Next, cells were exposed to either fluorescent SLe^X-conjugated dendrimers or to fluorescent dendrimers that had no SLe^X for 30 min. As a control, a portion of the cells exposed to the SLe^X-conjugated dendrimers were pre-incubated with an antibody to human E-selectin (10 μg/well; anti-CD62E) for 30 min before exposure to the dendrimers in order to block E-selectin availability. Cells were rinsed thoroughly three times with sterile PBS. Fresh media was added to the cell cultures, and dendrimers bound to the surface of the HUVECs were visualized using a fluorescent microscope (Zeiss Axiovert 135).

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 of PEG-core polylysine dendrimers

The ^{1}H-NMR spectra of PEG-lysine dendrimers contained characteristic proton peaks of both lysine dendrons and PEG chains, indicating the successful attachment of the dendrons to the PEG core (Fig. 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 PEG standards to further confirm dendrimer structure (lysine dendrons: $M_n = 3491.8$, $M_w = 3963.2$, PDI = 1.13 and PEG-Lys dendrimers: $M_n = 13\,566.8$, $M_w = 14\,385.1$, PDI = 1.06).

Approximately $83 \pm 9\%$ of amines was converted to diazeniumdiolate NO-donors as assessed by the ninhydrin assay. NO release from PEG-Lys-NO dendrimers occurred for over 60 days under physiological conditions (Fig. 3), with the majority of release occurring within the first 10 days. The subsequent sustained release of NO from these materials should provide extended inhibition of atherosclerotic plaque enlargement.

Vascular cell responses to NO-releasing dendrimers

No loss of cell viability was observed in cultures exposed to dendrimers that released NO; $100 \pm 1\%$ of BAECs and $99 \pm 3\%$ of SMCs were viable after 48-h exposure to NO-releasing dendrimers. $89 \pm 3\%$ of BAECs were viable after exposure to non-NO-releasing dendrimers, as were $87 \pm 6\%$ of SMCs, suggesting that the positively charged PEG-Lys dendrimers exhibit some cytotoxicity that is eliminated through reaction with NO. The effect of NO release on vascular cell proliferation was observed after 48-h exposure to PEG-Lys dendrimers, and the percentage of PCNA-positive BAECs was approximately 50% higher after incubation with NO-releasing dendrimers ($86 \pm 2\%$) as compared to cells exposed to dendrimers that were not reacted with NO ($43 \pm 4\%$). Conversely, SMC proliferation

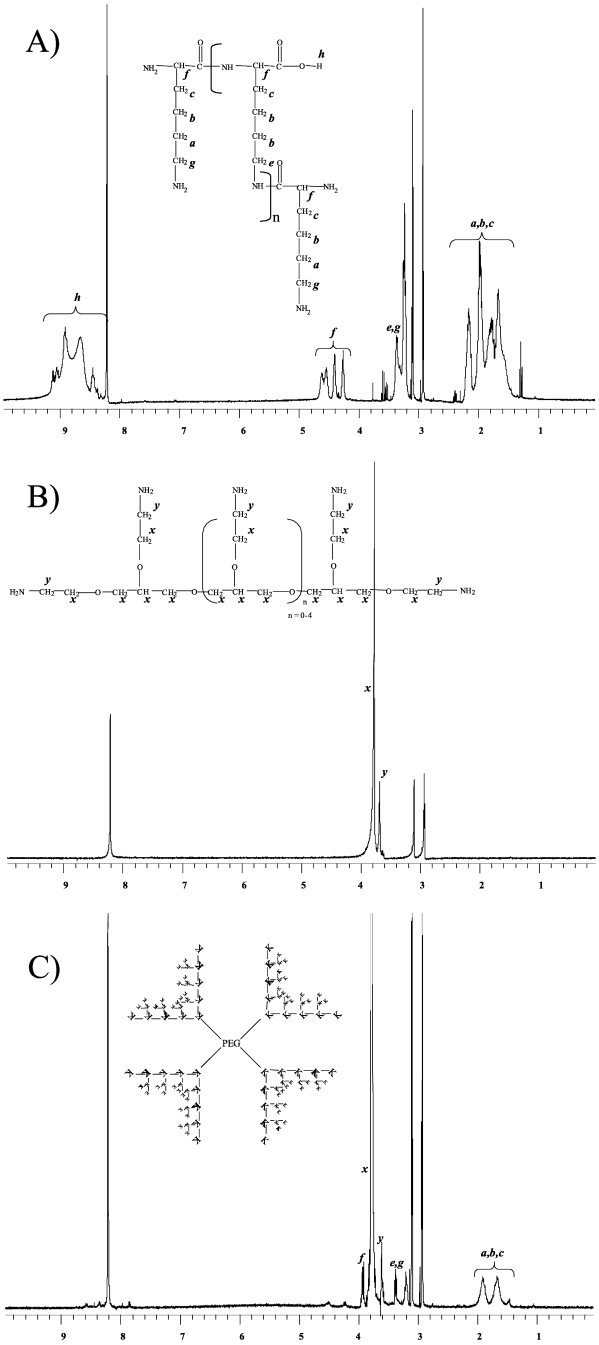

Figure 2. ^{1}H-NMR spectra of (A) branched lysine dendrons, (B) PEG-amine and (C) PEG-Lys dendrimers. Characteristic peaks associated with lysine and PEG-amine are clearly present in the spectra of the dendrimers, indicating the successful incorporation of both components in the resulting material.

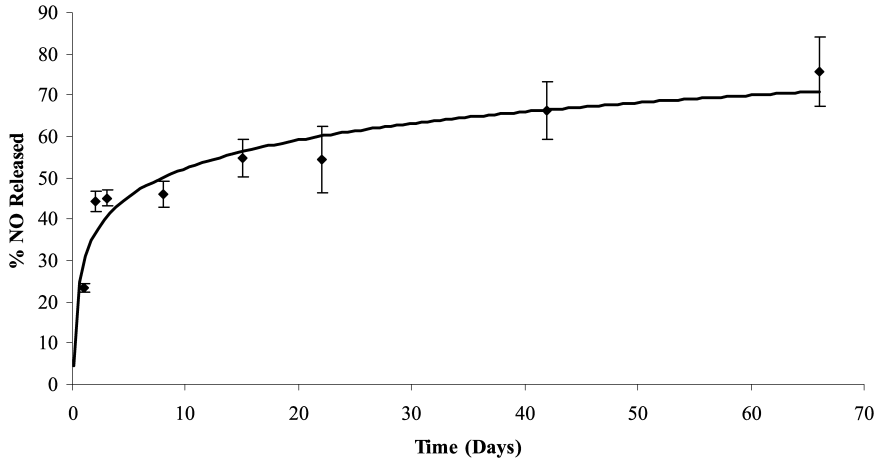

Figure 3. Release of NO from PEG-lysine dendrimers occurred for over 60 days at pH 7.4 and 37 °C ($n = 4$).

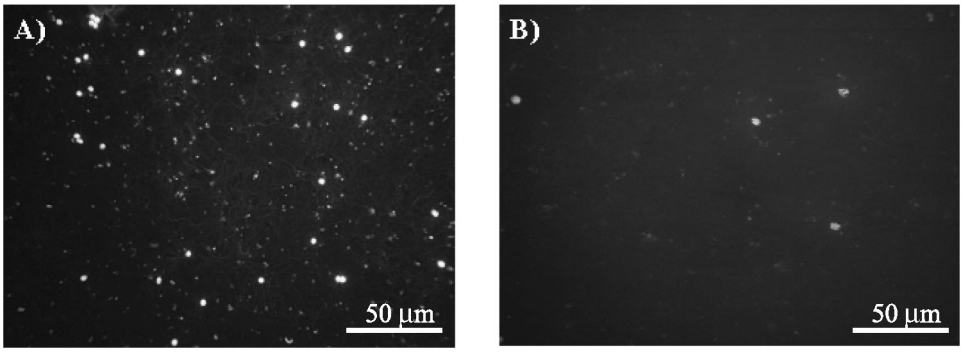

Figure 4. Platelet adhesion to collagen I was greater after exposure to (A) control dendrimers than to (B) NO-generating dendrimers ($P < 0.002$; $n = 6$).

was approximately 86% lower upon exposure to NO-releasing dendrimers ($8 \pm 1\%$ for NO-releasing dendrimers *vs.* $57 \pm 2\%$ for controls), confirming that NO-releasing dendrimers were able to influence vascular cell growth as expected.

Static platelet adhesion

When platelet adhesion to collagen I was assessed after exposure to PEG-lysine-NO dendrimers, platelet adhesion was reduced by approximately 81% (636 ± 207 platelets/mm^2) than when blood was exposed to dendrimers that did not release NO (3272 ± 419 platelets/mm^2), as shown in Fig. 4. These results suggest that these materials should inhibit platelet adhesion to damaged endothelium.

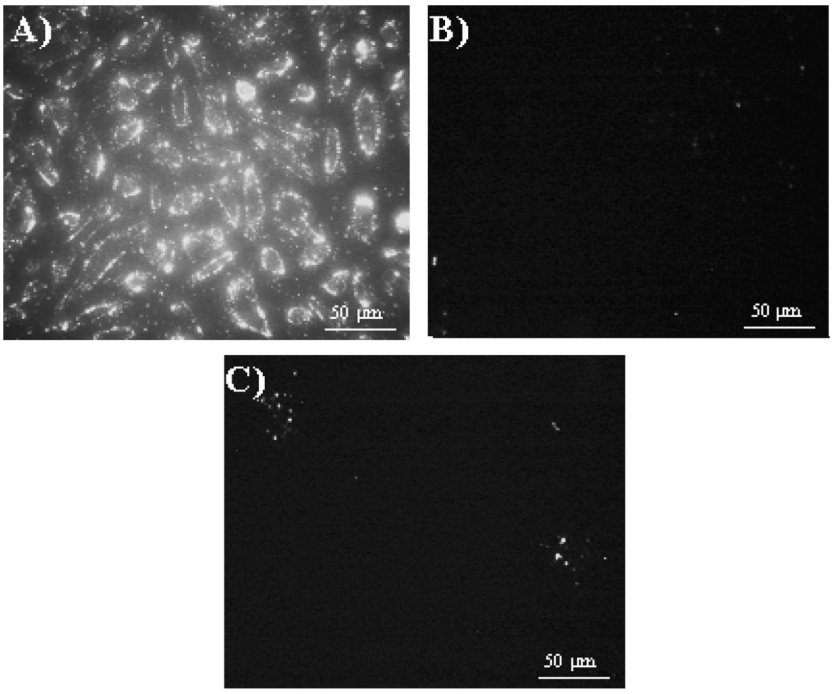

Figure 5. Binding of (A) targeted dendrimers to activated HUVECs, (B) non-targeted dendrimers to activated HUVECs, and (C) targeted dendrimers to unstimulated HUVECs.

Targeting to activated endothelium

SLe^X-conjugated dendrimers preferentially bound HUVECs stimulated by IL-1β (Fig. 5A). Dendrimers bearing Le^X exposed to activated HUVECs did not significantly bind to cells (Fig. 5B). Cells that were not stimulated were also exposed to targeted dendrimers, and low levels of binding were observed, likely due to basal levels of E-selectin on the cell surface (Fig. 5C), suggesting that NO delivery can be localized to sites of inflammation with little or no binding to healthy tissue.

DISCUSSION

Atherosclerosis, the occlusive vascular disease characterized by the accumulation of cells, platelets, lipids and extracellular matrix at the vascular wall, is a slow disease that can start in childhood and progresses with age. Initial endothelial activation leads to an inflammatory phase in which leukocytes adhere to the vascular wall, contributing to plaque formation and encouraging lipid accumulation. An ensuing thrombotic phase, caused by platelet adhesion and aggregation at the plaque surface, often lead to heart attacks and strokes. Current treatment for atherosclerosis can cause further damage to the endothelial lining of the vessel and often lead to further occlusion in the form of restensosis. This work introduces a novel preventive

strategy for atherosclerosis through using NO-releasing dendrimers as injectible, targeted therapeutics.

The dendrimers introduced in this work show promise as localized therapeutics for the prevention of arterial disease through long-term release of NO, regulation of vascular cell growth, inhibition of platelet adhesion, and the preferential binding of cell adhesion molecules displayed during an inflammatory response. These studies detail the synthesis of dendrimers of the amino-acid lysine bound to a central PEG core, which can form multiple diazeniumdiolate NO donors through the reaction of dendrimers with NO gas in water. NO release from these materials occurred for over 60 days under physiological conditions, offering the potential for extended inhibition of the inflammatory and thrombotic phases of atherogenesis, leaving the surrounding healthy tissue undamaged. Though highly charged molecules have seen limited successes in biomedical application due to cytotoxicity [52, 53], the therapeutic in this work also acts as a protecting agent. The positive charge is shielded by reaction with NO, and no cell death is observed in the presence of NO-releasing dendrimers, indicating an added benefit of sustained release of the chosen therapeutic. Since NO-release occurs long-term, depletion of NO will not likely occur until dendrimers have been fragmented or cleared from the bloodstream; thus, no cytotoxicity should be observed *in vivo*. Proliferation of vascular endothelial cells in the presence of PEG-Lys-NO dendrimers was enhanced, while proliferation of SMCs was decreased. The capacity to influence vascular cell proliferation demonstrates that these materials can mimic the function of undamaged endothelium in regulating vascular homeostasis, as well as in maintaining their naturally antithrombogenic properties.

The NO-releasing dendrimers developed here show efficacy in preventing platelet adhesion to thrombogenic surfaces under static conditions, and the inhibition of leukocyte and platelet adhesion to sites of vascular inflammation has previously been suggested as a means to inhibit atherogenesis [41, 60, 61]. Although results may vary in different flow regimes, NO is a known potent antithrombotic in the vasculature, where it is released from healthy endothelium under shear [14, 62] and has also been implicated in preventing leukocyte adhesion [34, 35, 37]. Our strategy for the initial prevention of leukocyte adhesion is to have dendrimers bound to available E-selectin on the cell surface, followed by sustained NO release to diminish levels of cell adhesion molecules in the target area. When modified with a targeting ligand specific for inflamed endothelium, fluorescently labeled dendrimers were able to preferentially bind endothelial cells activated by IL-1β. SLe^X-modified dendrimers were able to bind stimulated endothelium, while dendrimers bearing Le^X or no ligand were unable to bind the cell surface. Cells exposed to antibodies to E-selectin did not show bound fluorescent dendrimers. The blocking of E-selectin by the antibody inhibits dendrimer binding to the surface, therefore targeting is taking place by the interactions of SLe^X with E-selectin at the cell surface. Low levels of NO released form PEG-Lys-NO dendrimers did not greatly decrease E-selectin expression, suggesting that dendrimers will be retained at the site of

inflammation while delivering NO. NO has been implicated in decreasing immune cell adhesion, and studies have shown that inhibition of cell adhesion molecules by NO causes this decrease in adhesion [34–41]. However, the low levels of NO released in this study do not significantly lessen the levels of available E-selectin over the short term, suggesting that dendrimers can remain bound at the targeted site, hindering the ability of leukocytes to adhere *via* E-selectin. The ability of these materials to prevent immune cell adhesion to activated endothelium is achieved both by their attachment to available E-selectin and possibly through decreasing adhesion molecule expression after sustained release of NO. These bioactive polymers may prove beneficial as NO-delivery systems in numerous applications, offering the ability to design injectable, targeted therapeutics.

CONCLUSIONS

The NO-releasing dendrimers developed in this work release NO for approximately 2 months and can be selectively targeted to stimulated vascular cells with the selectin ligand SLe^X. The capacity of these dendrimers to decrease platelet and immune cell adhesion at sites of inflammation can lessen the onset of atherosclerotic plaque formation. These nanoscale materials show promise as localized therapeutics for the prevention of arterial disease through long-term release of NO, regulation of vascular cell growth, inhibition of platelet adhesion and the preferential binding of cell adhesion molecules displayed during an inflammatory response.

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