ELSEVIER

Biomaterials 21 (2000) 2273–2278

Biomaterials

Nitric oxide-generating polymers reduce platelet adhesion and smooth muscle cell proliferation

Kristyn S. Bohl[a], Jennifer L. West[a,b,*]

[a]*Department of Chemical Engineering, Rice University, PO Box 1892, MS-142, Houston, TX 77251-1892, USA*
[b]*Department of Bioengineering, Rice University, PO Box 1892, MS-142, Houston, TX 77251-1892, USA*

Abstract

We have developed polymeric biomaterials capable of providing localized and sustained production of nitric oxide (NO) for the prevention of thrombosis and restenosis. In the current study, we have characterized the kinetics of NO production by these materials and investigated their efficacy in reducing platelet adhesion and smooth muscle cell proliferation in vitro. Three nitric oxide donors with different half-lives were covalently incorporated into photopolymerized polyethylene glycol hydrogels. Under physiological conditions, NO was produced by these hydrogels over periods ranging from hours to months, depending upon the polymer formulation. NO production was inhibited at acidic pH, which may be useful for storage of the materials. The NO-releasing materials successfully inhibited smooth muscle cell growth in culture. Platelet adhesion to collagen-coated surfaces was also inhibited following exposure of whole blood to NO-producing hydrogels. The effects of NO production by these hydrogels on platelet adhesion and the proliferation of smooth muscle cells suggest that these materials could reduce thrombosis and restenosis following procedures such as balloon angioplasty.

Keywords: Restenosis; Thrombosis; Hydrogel; Photopolymerization

1. Introduction

The introduction of percutaneous transluminal coronary angioplasty (PTCA) for treatment of coronary artery disease has greatly reduced the mortality rate in patients with occluded coronary arteries. Approximately 500 000 coronary interventions were performed in the US in 1995 [1]. Unfortunately, 30–40% of patients treated with PTCA will experience restenosis, reocclusion of the diseased blood vessel [2].

Balloon inflation during angioplasty frequently damages the arterial wall, removing the non-thrombogenic lining of endothelial cells and injuring the underlying smooth muscle cells. This can result in rapid reocclusion of the vessel due to thrombosis and elastic recoil, or reocclusion months later due to restenosis, a type of wound-healing response [3]. Restenosis results, at least in part, from the migration and proliferation of smooth muscle cells and their secretion of matrix proteins to form an occlusive neointimal layer within the vessel [2,4,5].

Nitric oxide (NO) is a molecule produced by uninjured endothelial cells of the blood vessel intima. Nitric oxide synthase produced by endothelial cells converts L-arginine to L-citrulline and nitric oxide [6]. NO has been shown to reduce platelet adhesion and smooth muscle cell proliferation, while stimulating endothelial cell proliferation [7,8]. Thus, the delivery of NO to the injured blood vessel segment may significantly reduce thrombosis and restenosis. Several NO donors, low molecular weight compounds that hydrolyze to produce NO, have previously been identified and evaluated both in vitro and in vivo. The materials that we have developed are intended for use as tissue coatings to provide local and sustained NO therapy following vascular injury. In this study, we have investigated the release kinetics of three different NO-producing hydrogels and their effects on smooth muscle cell proliferation and platelet adhesion.

2. Materials and methods

2.1. PEG-Lys_5-NO hydrogels

All chemicals were obtained from Sigma Chemical Corp. (St. Louis, MO), unless otherwise specified.

* Corresponding author. Tel.: + 1-348-285-5955; fax: + 1-348-737-5877.
E-mail address: jwest@rice.edu (J.L. West).

0142-9612/00/$ - see front matter
PII: S0142-9612(00)00153-8

A PEG-Lys_5-NO polymer was formed by first reacting polyethylene glycol N-hydroxysuccinimide monoacrylate (ACRL-PEG-NHS, MW 3400, Shearwater Polymers, Huntington, AL) with poly-L-lysine (DP = 5) at an equimolar ratio in 50 mM sodium bicarbonate buffer (pH 8.5) for 2 h; the product was then lyophilized. Analysis of the PEG-Lys_5 copolymer was performed using gel-permeation chromatography (GPC) with an evaporative light-scattering detector and a UV detector at 260 nm (Polymer Laboratories, Amherst, MA). Successful synthesis of PEG-Lys_5 was determined by a shift in the position of the peak from the evaporative light-scattering detector. The resultant copolymer was dissolved in water and reacted with NO gas in an evacuated vessel, thus forming NO-nucleophile complexes with the amine groups on the lysine side groups. The extent of conversion of amine groups to NO-nucleophile complexes was measured using the ninhydrin assay [9]. Following lyophilization, the PEG-Lys_5-NO polymer was incorporated into photopolymerizable hydrogels by mixing with PEG-diacrylate (MW 3400) at a 1 : 10 molar ratio in aqueous solution with 1500 ppm 2,2-dimethoxy-2-phenyl acetophenone as a long wavelength ultraviolet initiator. 0.15% *N*-vinylpyrrolidone was present in this mixture as it was used as a solvent for the photoinitiator. Exposure to UV light (365 nm, 10 mW/cm^2) was used to crosslink the polymer, resulting in conversion to a hydrogel [10]. Hydrogels were stored in HEPES buffered saline (HBS) at 37°C at pH 7.4 or 3. Production of NO by the hydrogels was quantified using the Griess assay [11].

2.2. PEG-DETA-NO hydrogels

Diethylenetriamine (DETA, Aldrich, Milwaukee, WI) was reacted with ACRL-PEG-NHS (MW 3400, Shearwater Polymers) in 50 mM sodium bicarbonate buffer (pH 8.5) at an equimolar ratio, lyophilized, and analyzed using GPC as described earlier. The copolymer was then dissolved in water and exposed to NO gas to form NO-nucleophile complexes as described for PEG-Lys_5-NO and assayed for amine content using the ninhydrin assay. The PEG-DETA-NO was lyophilized and then photopolymerized as described above to form hydrogels. The hydrogels were stored in HBS at 37°C, pH 7.4 or 2, and assayed for NO production using the Griess assay.

2.3. PEG-S-nitrosocysteine hydrogels

For *S*-nitrosocysteine (Cys-NO) hydrogels, a copolymer of ACRL-PEG-NHS (MW 3400, Shearwater Polymers) and L-cysteine was synthesized by reacting at a 1 : 2 molar ratio in 50 mM sodium bicarbonate (pH 8.5). The solution was dialyzed in a cellulose ester membrane (MWCO 500, Spectrum Labs, Laguna Hills, CA) in diH_2O, lyophilized, and analyzed using GPC as described above. The copolymer was then reacted with an equimolar amount of $NaNO_2$ at pH 2 and 37°C for 20 min to form *S*-nitrosocysteine. Conversion of thiol groups to *S*-nitrosothiols was measured using the Ellman's assay [12]. The *S*-nitrosothiol solution was adjusted to pH 7.4, and photopolymerized hydrogels were formed and stored at 37°C in HBS, pH 7.4 or 2.

2.4. Smooth muscle cell proliferation

Smooth muscle cells isolated from Wistar-Kyoto rats (passage 11–15, provided by T. Scott-Burden) were cultured in minimum essential medium supplemented with 10% FBS, 2 mM L-glutamine, 500 units penicillin, and 100 mg/l streptomycin, at 37°C in a 5% CO_2 environment. The cells were seeded into 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) at a density of 10 000 cells/cm^2. NO donors in either soluble or hydrogel form were added to the media in the wells one day after seeding. At 4 days culture, cell numbers were determined by preparing single cell suspensions with trypsin and counting three samples from each group using a Coulter counter (Multisizer #0646, Coulter Electronics, Hialeah, FL).

The effects of NO donors in solution on the proliferation of SMCs were first investigated by performing a NO dose response curve, whereupon cells were cultured with a range of NO donor concentrations (1 μM–10 mM) in order to identify appropriate dosages for hydrogel studies. NO-nucleophile complexes (Lys-NO and DETA-NO) were formed by reacting either L-lysine or DETA with NO gas in water for 24 h. Soluble Cys-NO was synthesized by reacting an equimolar amount of L-cysteine with $NaNO_2$ at pH 2 and 37°C for 20 min. All NO donor solutions were adjusted to pH 7.4 prior to addition to cell cultures.

Smooth muscle cell proliferation in the presence of NO-producing and control hydrogels was then investigated using the optimal NO dose determined above. Hydrogels containing Lys-NO, DETA-NO, and Cys-NO were formed as described above, except that the gel solutions were sterile filtered through 0.2 μm syringe filters (Gelman Sciences, Ann Arbor, MI) prior to adding 2,2-dimethoxy-2-phenyl acetophenone. PEG-diacrylate hydrogels containing no NO donors were used as a control. The hydrogels were photopolymerized in cell culture inserts (8 μm pore size, Becton Dickinson, Franklin Lakes, NJ) and placed in the media over the cultured cells.

2.5. Platelet adhesion

Blood was obtained from a healthy volunteer by venipuncture and anticoagulated with 10 U/ml heparin. Platelets and white blood cells were fluorescently labeled

with mepacrine at a concentration of 10 μM. A solution of 2.5 mg/ml collagen I in 3% glacial acetic acid in diH_2O was prepared and applied to glass slides for 45 min in a humidified environment at room temperature. Cys-NO and PEG-diacrylate hydrogels were prepared as described above and incubated with the labeled whole blood at 37°C for 30 min. The hydrogels were removed and the blood was then incubated with the collagen-coated glass slides (two per group) for 20 min at 37°C and then rinsed with HBS. Platelet counts per field of view at 40 × were counted under a fluorescent microscope (Zeiss Axiovert 135, Thornwood, NY) in four randomly chosen areas per slide.

2.6. Statistical analysis

Data sets were compared using two-tailed, unpaired *t*-tests. *P*-values less than 0.05 were considered to be significant.

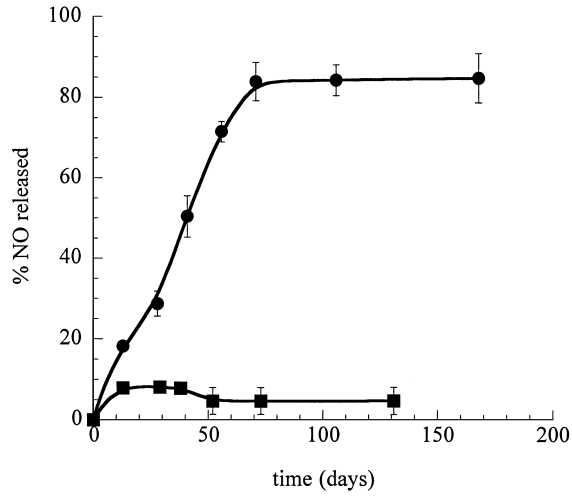

Fig. 1. Production of NO from PEG-Lys_5-NO hydrogels at pH 7.4 (circle) and pH 3 (square) was assessed using the Griess assay. Each data point is the mean of three samples and error bars are standard deviations.

3. Results

3.1. Polymer synthesis

Analysis of the polymers using gel permeation chromatography confirmed that NO donors were covalently bound to ACRL-PEG-NHS. As determined by the ninhydrin assay, the conversion of amine groups in the PEG-Lys_5 polymer to NO-nucleophile complexes ranged from 60–80%, and over 85% of the amine groups in PEG-DETA were converted to NO-nucleophile complexes. Approximately 90% of the thiol groups in PEG-Cys were converted to *S*-nitrosothiols, as measured by Ellman's assay.

3.2. Release kinetics

There was evidence of production of NO by PEG-Lys_5-NO hydrogels over a period of several months at pH 7.4 (Fig. 1). The release of NO from these hydrogels at different pH levels was also examined in order to identify conditions where NO production can be inhibited for storage of the material. NO release was found to be significantly inhibited at acidic pH values (Fig. 1). PEG-DETA-NO hydrogels were synthesized to obtain a material with faster release kinetics. As shown in Fig. 2, these hydrogels produced NO over a period of approximately two days at pH 7.4, and NO release was inhibited at pH 2. PEG-Cys-NO hydrogels were designed to provide very rapid NO production, suitable for use in short-term applications. These hydrogels displayed rapid release kinetics, with the majority of NO being produced in the first 4 h (Fig. 3). There was significantly inhibited release of NO from the *S*-nitrosocysteine hydrogels at acidic pH.

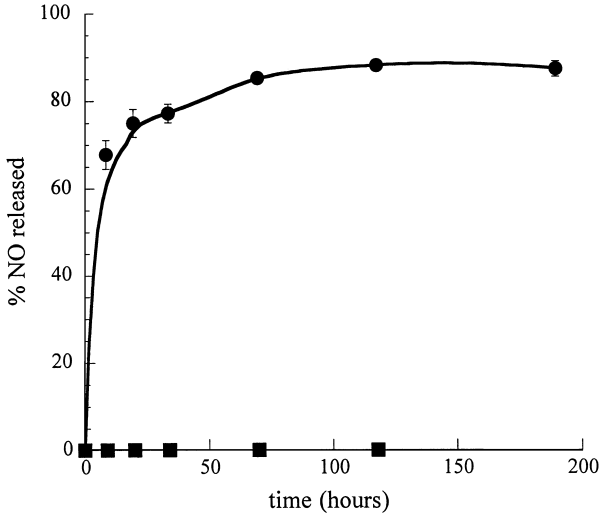

Fig. 2. Production of NO from PEG-DETA-NO hydrogels at pH 7.4 (circle) and pH 2 (square) was assessed using the Griess assay. Each data point is the mean of three samples and error bars are standard deviations.

3.3. Smooth muscle cell proliferation

Inhibition of smooth muscle cell (SMC) proliferation was investigated to probe possible efficacy of these materials in the prevention of restenosis. A concentration of 2 mM NO released over the course of the experiment was chosen as the optimal NO concentration from studies performed with a range of soluble NO donor concentrations; this concentration resulted in maintenance of cell number near the initial seeding density. At lower concentrations of NO (50 μM–1 mM), SMC proliferation was still

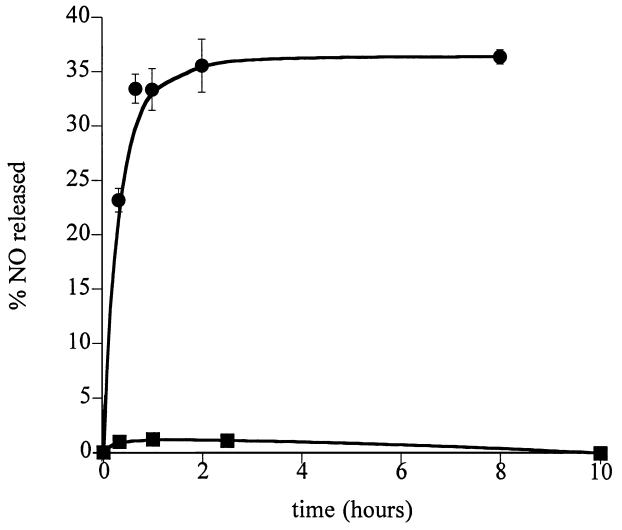

Fig. 3. Production of NO from PEG-Cys-NO hydrogels at pH 7.4 (circle) and pH 2 (square) was assessed using the Griess assay. Each data point is the mean of three samples and error bars are standard deviations.

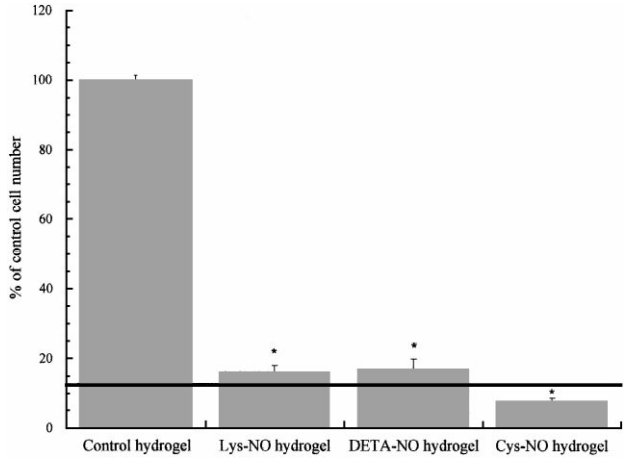

Fig. 4. Inhibition of smooth muscle cell growth by PEG-Lys-NO, PEG-DETA-NO, and PEG-Cys-NO hydrogels. PEG-diacrylate hydrogels were used as a control. The solid line represents the initial cell seeding density. $N = 3$ per group, data presented are mean $\pm$ SD. (*): $p < 0.0001$ compared to control.

significantly inhibited. All three hydrogel NO donors significantly inhibited SMC growth ($p < 0.0001$). The number of smooth muscle cells remained near that of the seeding density, which ranged from 10 to 15% of the final control cell number for all experiments (Fig. 4).

3.4. Platelet adhesion

The effect of NO release on platelet adhesion was investigated to assess the potential of these materials for prevention of thrombosis. Glass slides coated with collagen were used as a thrombogenic surface to which platelets would normally adhere. When the blood was incubated with control PEG-diacrylate hydrogels, 69.25 ± 4.46 (mean $\pm$ SD) adherent platelets were observed per field of view (40×). This number was reduced to 7.65 ± 6.16 when blood was pre-exposed to the PEG-Cys-NO hydrogels ($p < 0.0001$). Fluorescence micrographs of control and NO-exposed surfaces are shown in Fig. 5.

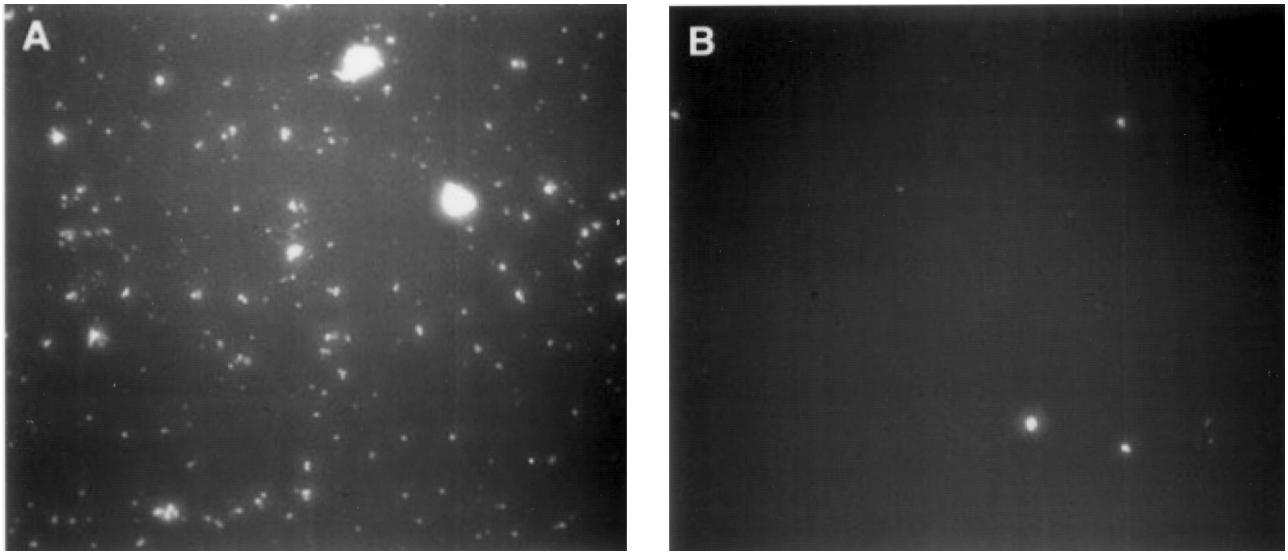

Fig. 5. Fluorescence micrographs of platelets adherent to collagen I-coated glass after exposure to PEG-diacrylate hydrogels (A) or PEG-Cys-NO hydrogels (B). Exposure to the NO-producing hydrogels for 30 min significantly reduced platelet adhesion to collagen I ($p < 0.0001$).

4. Discussion

The current study has described the synthesis and characterization of a new group of biomaterials that can be photopolymerized in situ to coat tissues and that produce NO for prolonged periods of time. We have previously reported the use of interfacially photopolymerized PEG hydrogels to prevent thrombosis and intimal thickening in rat and rabbit balloon injury models [13,14]. The materials reported here can be used to serve the same functions as non-thrombogenic barriers, but also provide sustained and localized treatment with NO. Based on the results of this study regarding reduction of smooth muscle cell proliferation and platelet adhesion, we expect these new materials to be substantially more effective for the prevention of thrombosis and restenosis than PEG hydrogels without pharmacological activity. These materials may have applications in other disease states affected by NO, such as asthma, mucosal injury, and dermal wound healing [15–19].

Intravenous administration of NO donors such as molsidomine and *S*-nitroso-*N*-acetylpenicillamine (SNAP) has been shown to reduce smooth muscle cell proliferation following animal models that resemble angioplasty [20,21]. However, their administration has not been concentrated at the site of injury, thereby introducing issues of systemic toxicity. Synthesis of NO-producing polymers has been previously reported [22]. These materials were fabricated into microspheres and then incorporated into vascular grafts to reduce thrombogenicity. The materials that we have developed combine both pharmacological and barrier functions to prevent thrombosis and restenosis following vascular injury. Our hydrogels may be polymerized via interfacial photopolymerization in direct contact with the damaged vessel to form a thin, conformal coating on the surface of the vessel wall [13,14]. In addition, we can easily alter the NO release kinetics from the hydrogels by using NO donors with different half-lives. There still exist many uncertainties in the process of restenosis, so varying NO donors may allow us to determine an optimal treatment protocol for the prevention of restenosis. In the current study, we have combined the monoacrylate NO-producing PEG derivatives with PEG-diacrylate to allow crosslinking into hydrogels. Biodegradable PEG-diacrylate derivatives, such as copolymers with α-hydroxy acids [10] or proteolytically degradable peptides [23] could be substituted to create biodegradable hydrogels. This allows separate determination of NO production kinetics and biodegradation characteristics. Flexibility in the duration of NO release may also prove useful in the extension of this therapy to applications other than thrombosis and restenosis.

Acknowledgements

The authors wish to thank Dr. Timothy Scott-Burden, Texas Heart Institute, for his advice and support and Nancy Turner for her technical assistance. Research funding was provided by an NSF CAREER Award (BES-9875607), the Theodore N. Law Foundation, the Whitaker Foundation, and an NIH Biotechnology Training Grant.

References

[1] Feuerstein GZ. Restenosis: basic resesarch and clinical perspective. In: Feuerstein GZ, editor. Coronary restenosis: from genetics to therapeutics. New York: Marcel Dekker, 1997. p. 1–4.
[2] Schwartz RS, Holmes DRJ, Topol EJ. The restenosis paradigm revisited: an alternative proposal for cellular mechanisms. J Am Coll Cardiol 1992;20:1284–93.
[3] Ip JH, Fuster V, Israel D, Badimon L, Badimon J, Chesebro JH. The role of platelets, thrombin and hyperplasia in restenosis after coronary angioplasty. J Am Coll Cardiol 1991;17:77B–88B.
[4] Haudenschild CC. Pathobiology of restenosis after angioplasty. Am J Med 1993;94(Suppl. 4A):S40–4.
[5] Bauters C, Isner JM. The biology of restenosis. Prog Card Dis 1997;40:107–16.
[6] Feldman PL, Griffith OW, Stuehr DJ. The surprising life of nitric oxide. Chem Eng News 1993;71:26–38.
[7] Loscalzo J, Welch G. Nitric oxide and its role in the cardiovascular system. Prog Card Dis 1995;38:87–104.
[8] Loscalzo J. Nitric oxide and restenosis. Clin Appl Thromb/Hemostas 1996;2:7–10.
[9] Moore S. Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. J Biol Chem 1968;243:6281–3.
[10] Sawhney AS, Pathak CP, Hubbell JA. Bioerodible hydrogels based on photopolymerized poly(ethylene glycol)-co-poly(α-hydroxy acid) diacrylate macromers. Macromol 1993;26:581–7.
[11] Scott-Burden T, Schini VB, Elizondo E, Junquero DC, Vanhouette PM. Platelet-derived growth factor suppresses and fibroblast growth factor enhances cytokine-induced production of nitric oxide by cultured smooth muscle cells: effects on cell proliferation. Circ Res 1992;71:1088–100.
[12] Hermanson GT. Bioconjugate techniques. San Diego, CA: Academic Press, 1995. p. 88–90.
[13] Hill-West JL, Chowdhury SM, Slepian MJ, Hubbell JA. Inhibition of thrombosis and intimal thickening by in situ photopolymerization of thin hydrogel barriers. Proc Natl Acad Sci USA 1994;91:5967–71.
[14] West JL, Hubbell JA. Separation of the arterial wall from blood contact using hydrogel barriers reduces intimal thickening after balloon injury in the rat: the roles of medial and luminal factors in arterial healing. Proc Natl Acad Sci USA 1996;93:13188–93.
[15] Kuo PC, Schroeder RA. The emerging multifaceted roles of nitric oxide. Ann Surg 1995;221:220–30.
[16] Wang R, Ghahary A, Shen YJ, Scott PG, Tredget EE. Human dermal fibroblasts produce nitric oxide and express both constitutive and inducible nitric oxide synthase isoforms. J Invest Dermatol 1996;106:419–27.
[17] Weller R. Nitric oxide—a newly discovered chemical transmitter in human skin. Br J Dermatol 1997;137:665–72.

[18] Elliott SN, Wallace JL. Nitric oxide: a regulator of mucosal defense and injury. J Gastroenterol 1998;33:792–803.
[19] Sharara AM, Hijazi M, Tarawneh M, Ind PW. Nebulized glyceryl trinitrate exerts acute bronchodilator effects in patients with acute bronchial asthma. Pulm Pharmacol Ther 1998; 11:65–70.
[20] Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J Clin Invest 1989;83:1774–7.
[21] Groves PH, Banning AP, Penny WJ, Newby AC, Cheadle HA, Lewis MJ. The effects of exogenous nitric oxide on smooth muscle cell proliferation following porcine coronary angioplasty. Card Res 1995;30:87–96.
[22] Pulfer SK, Ott D, Smith DJ. Incorporation of nitric oxide-releasing crosslinked polyethyleneimine microspheres into vascular grafts. J Biomed Mater Res 1997;37:182–9.
[23] West JL, Hubbell JA. Polymeric biomaterials with degradation sites for proteases involved in cell migration. Macromol 1999; 32:241–4.