Nitric Oxide-Releasing Polymers Containing the [N(O)NO]$^-$ Group

Daniel J. Smith,*[,†] Debashish Chakravarthy,[†] Sharon Pulfer,[†] Maia L. Simmons,[†] Joseph A. Hrabie,[‡] Michael L. Citro,[‡] Joseph E. Saavedra,[‡] Keith M. Davies,[§] Thomas C. Hutsell,[▽] Daniel L. Mooradian,[∥] Stephen R. Hanson,[⊥] and Larry K. Keefer*[,#]

Department of Chemistry, University of Akron, Akron, Ohio 44325, Biological Carcinogenesis and Development Program, SAIC Frederick, and Chemistry Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702, Department of Chemistry, George Mason University, Fairfax, Virginia 22030, Comedicus Incorporated, Columbia Heights, Minnesota 55421, Department of Laboratory Medicine and Pathology/Biomedical Engineering Center, University of Minnesota, Minneapolis, Minnesota 55455, and Division of Hematology and Yerkes Regional Primate Research Center, Emory University, Atlanta, Georgia 30322

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Ions of structure X[N(O)NO]$^-$ display broad-spectrum pharmacological activity that correlates with the rate and extent of their spontaneous, first-order decomposition to nitric oxide when dissolved. We report incorporation of this functional group into polymeric matrices that can be used for altering the time course of nitric oxide release and/or targeting it to tissues with which the polymers are in physical contact. Structural types prepared include those in which the [N(O)NO]$^-$ group is attached to heteroatoms in low molecular weight species that are noncovalently distributed throughout the polymeric matrix, in groupings pendant to the polymer backbone, and in the polymer backbone itself. They range in physical form from films that can be coated onto other surfaces to microspheres, gels, powders, and moldable resins. Chemiluminescence measurements confirm that polymers to which the [N(O)NO]$^-$ group is attached can serve as localized sources of nitric oxide, with one prototype providing sustained NO release for 5 weeks in pH 7.4 buffer at 37 °C. The latter composition, a cross-linked poly(ethylenimine) that had been exposed to NO, inhibited the *in vitro* proliferation of rat aorta smooth muscle cells when added as a powder to the culture medium and showed potent antiplatelet activity when coated on a normally thrombogenic vascular graft situated in an arteriovenous shunt in a baboon's circulatory system. The results suggest that polymers containing the [N(O)NO]$^-$ functional group may hold considerable promise for a variety of biomedical applications in which local delivery of NO is desired.

Introduction

Anions of structure **1** display vasorelaxant,[1−4] antithrombotic,[5,6] cytostatic,[6−8] and genotoxic[9,10] activity correlating with their ability to release the multifaceted bioregulatory agent nitric oxide (NO) in physiological fluids or culture media. The spontaneous generation of NO by these diazeniumdiolate anions has proven advantageous for research applications in which controlled, quantifiable exposure to NO is desired, but spontaneity of release can be a liability for therapeutic or medicinal chemistry research applications in which only one tissue is to be targeted among the many that could be affected by systemic administration. Additionally, it would be beneficial to have some general means of programming alterations into the rate of NO generation from a given compound.

X[N(O)NO]$^-$ (**1**)

With the aim of modulating the time course of NO release in a controllable way as well as limiting NO exposure to selected sites within the body, we have incorporated the [N(O)NO]$^-$ functional group into a variety of polymeric matrices. In the present report, we describe the synthesis and physicochemical characteristics of three representative structural types (structures **2**−**4** of Figure 1), provide data on their rates of decomposition and NO release, and illustrate their pharmacological potential using both an *in vitro* cytostasis model and a method for studying platelet-dependent thrombus formation *in vivo*.

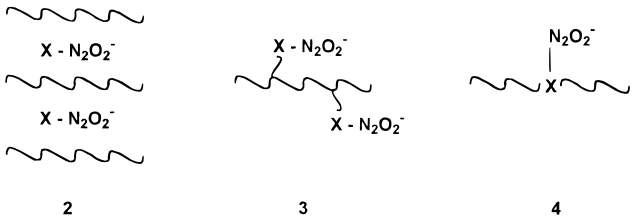

Figure 1. Generalized polymer types considered in the present investigation: X = nucleophile residue, $N_2O_2^-$ = NO-releasing unit, ∿ = polymer backbone.

Synthesis of Polymers of Structure Type 2

Many examples of low molecular weight ions containing the [N(O)NO]$^-$ group have been prepared by reacting nucleophiles with NO, as in eq 1.[2,11−15] Many of these products have been shown to regenerate NO when dissolved in aqueous media (eq 2).

$$X^- + 2NO \rightarrow \mathbf{1} \quad (1)$$

$$\mathbf{1} \xrightarrow{H^+} 2NO + X^- \quad (2)$$

[†] University of Akron.
[‡] SAIC Frederick.
[§] George Mason University.
[▽] Comedicus Inc.
[∥] University of Minnesota.
[⊥] Emory University.
[#] Laboratory of Comparative Carcinogenesis.
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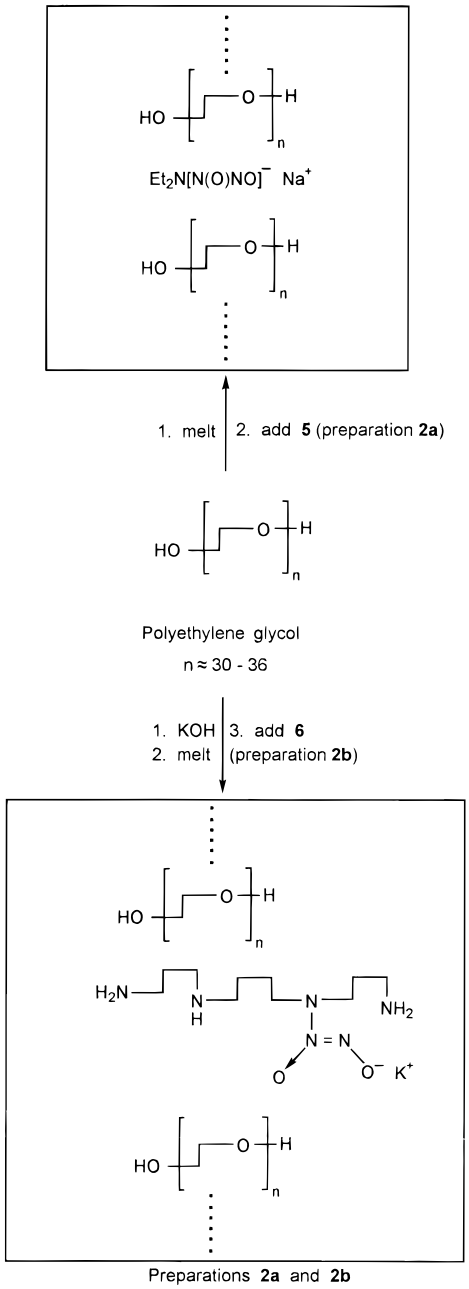
Scheme 1



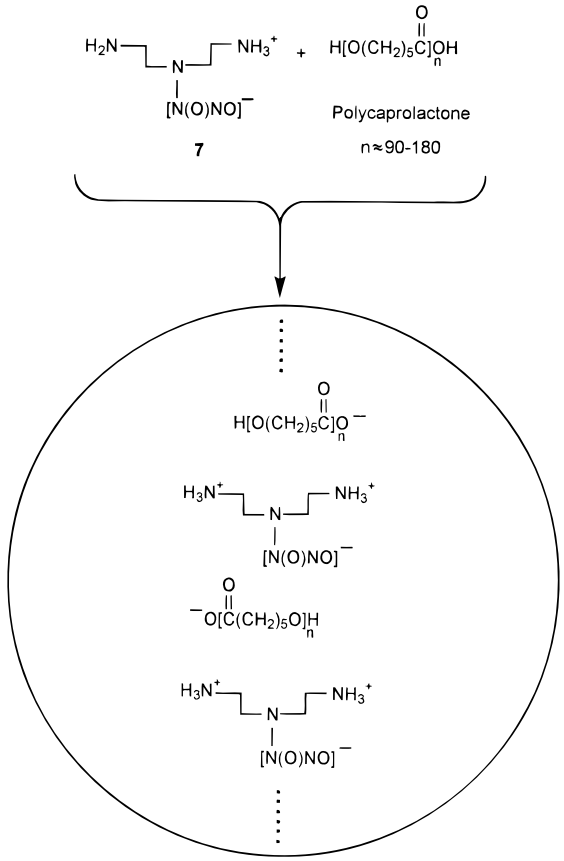
Preparations **2a** and **2b**

To test our hypothesis that incorporation into a solid matrix might drastically alter the time course of this NO release, we prepared a blend of compound **5** in poly(ethylene glycol). As indicated in Scheme 1, the polymer was melted and **5**, a salt with a 2-min half-life at pH 7.4 and 37 °C,[2] was dissolved in the resulting liquid. The matrix gradually solidified on cooling, producing preparation **2a**. The sample was stored in a clear glass vial under ordinary laboratory illumination and at ambient temperature. The strong chromophore at 250 nm seen when aliquots were dissolved in dilute base was followed in an effort to detect decomposition of **5**, whose extinction coefficient at this wavelength[2] is 6.5 mM^{-1} cm^{-1}. No loss of the NO-releasing functional group could be detected through the entire 7-week observation period.

$Et_2N[N(O)NO]^-Na^+$ (**5**)

$H_2N(CH_2)_3NH_2^+(CH_2)_4N[N(O)NO]^-(CH_2)_3NH_2$ (**6**)

Scheme 2



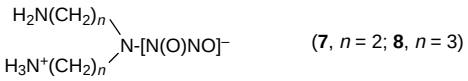
Preparation **2c**

A poly(ethylene glycol) solution of **6** was similarly prepared, but despite the order of magnitude longer half-life of this compound relative to **5** in physiological buffer,[2] the resulting blend suffered significant decomposition during the first 2 weeks of storage. This problem could be overcome by basifying the polymer before adding zwitterionic **6**. When this was done, only a small decrease (8%) in the 250-nm peak intensity was seen in aliquots of the basified polymer (sample **2b**) taken over 5 weeks of storage as the solid under ambient conditions. We speculate that the ammonium centers present in zwitterionic **6** retain enough protonating ability to promote acid-catalyzed decomposition of the $[N(O)NO]^-$ group in the blend, an effect that can be reversed by forming the potassium salt as in Scheme 1.

These examples illustrate the stabilization that can be achieved by incorporating the $[N(O)NO]^-$ group into solids of low hydrogen ion activity. Thus they might be useful as convenient storage forms for compounds of structure **1**, providing prolonged shelf life even in the absence of the low-temperature, low-humidity conditions normally recommended for such materials. Because they dissolve instantly in physiological fluids, however, preparations **2a**,**b** offer no obvious benefit in terms of targeted NO delivery.

For this reason, we prepared another example of structure **2** in which a second zwitterion containing the $[N(O)NO]^-$ group was noncovalently distributed in a biodegradable polymer that erodes only slowly in aqueous media. Polymer **2c** was prepared by melting poly(caprolactone) and mixing it thoroughly with **7** (Scheme 2). The material was cast into a disk-shaped mold as it solidified. Since the heating used in this preparation

could have induced decomposition of the temperature-sensitive starting material, we estimated the amount of intact **7** present in the resulting disks by taking up an aliquot in dichloromethane and extracting it with 1 M sodium hydroxide. The ultraviolet spectrum of the aqueous phase showed that ca. 50% of the **7** that was originally added had survived the blending process. To determine the rate of NO generation from the solid polymer, an analytical method that did not depend on dissolution thereof had to be devised.



(**7**, $n = 2$; **8**, $n = 3$)

Estimation of NO Release Rates from an Insoluble Solid under Physiological Conditions. Since one of the goals of this research is to provide localized sources of NO having medicinal value, it is necessary to verify that insoluble solids such as **2c** are capable of releasing NO under physiological conditions and, if so, to establish the time course for this action. The acid treatment/chemiluminescence protocol employed elsewhere[2] could be useful as a method of chemically characterizing freshly synthesized polymers, but it is not an adequate predictor of the materials' pharmacological behavior. Acidic conditions not only greatly accelerate fragmentation of ions **1** to NO, giving an inappropriately rapid time profile, but they also catalyze disproportionation of any nitrite ion present as an autoxidation product of NO, falsely exaggerating the amount of usable NO when NO_2^- (the autoxidation product of NO in aqueous media[16]) is present.

To overcome these drawbacks, the following analytical method was developed for appropriate characterization of the NO emanating as such from a solid. Polymer aliquots were immersed in pH 7.4 buffer at 37 °C in a vial open to the air. To determine the initial NO generation rate, the sample was removed from the vial, rinsed with fresh buffer to dispel nitrite, unreacted **1**, and other unwanted contaminants, and then placed into yet another aliquot of fresh buffer contained in a reactor vessel that could be continuously purged with an inert gas. By sweeping effluent gases with argon into a chemiluminescence detector, a continuous reading of the amount of NO produced as a function of time was obtained. Normally, the NO signal fluctuated widely when the purging was first begun for a given reading, but the recorder trace would eventually become relatively steady, producing a reasonably straight line for the long-lived NO sources. Integration of the resulting trace over several minutes provided an indication of the NO release rate during that particular interval. The process was repeated at various times thereafter to establish the time course of NO release from the insoluble solids prepared in this study.

The validity of using this method to establish the NO generation rate profile for a slow-release preparation was confirmed by applying it to **7**, a zwitterion with a half-life of 20 h at pH 7.4 and 37 °C, as measured by following the first-order loss of its chromophore at 250 nm under these conditions.[8] The compound was subjected to the same procedure as described above except that it immediately dissolved when placed in the buffer, forming a homogeneous solution that was swept periodically with inert gas conducted into the chemiluminescence apparatus. Integration of the resulting traces

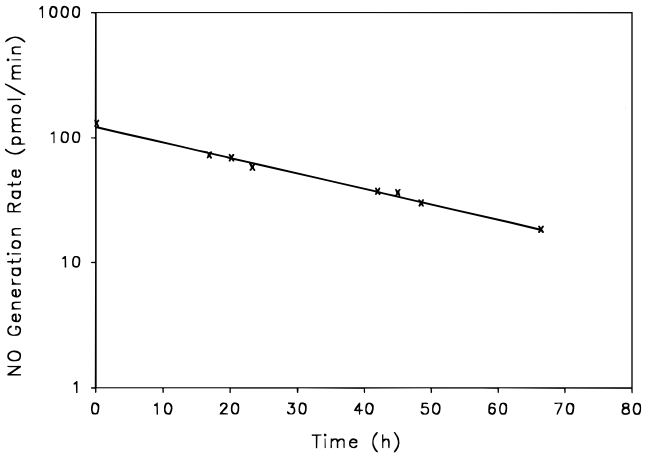


Figure 2. Time course of nitric oxide generation from 0.118 μmol of **7** dissolved in 2 mL of 0.1 M phosphate buffer at pH 7.4 and 37 °C. Linear regression analysis yielded a slope ($-k$) of 4.8×10^{-4} min^{-1} and a y-intercept of log(123 pmol/min), $r = 0.994$. Integration as in ref 17 provided an observed value of 2.2 mol of NO released/mol of **7** that dissociates.

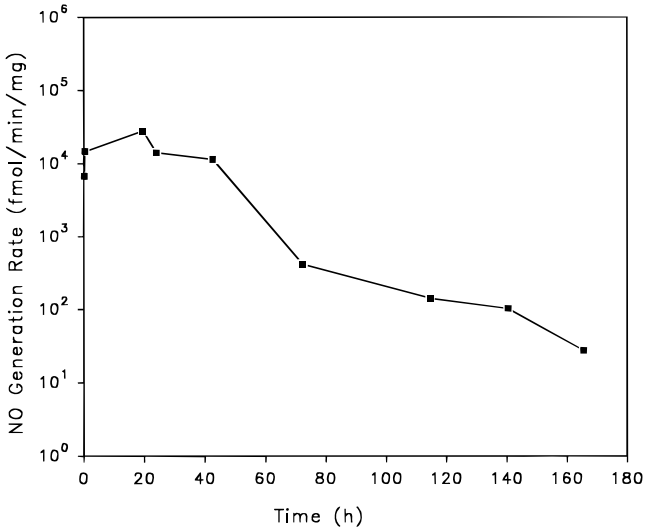
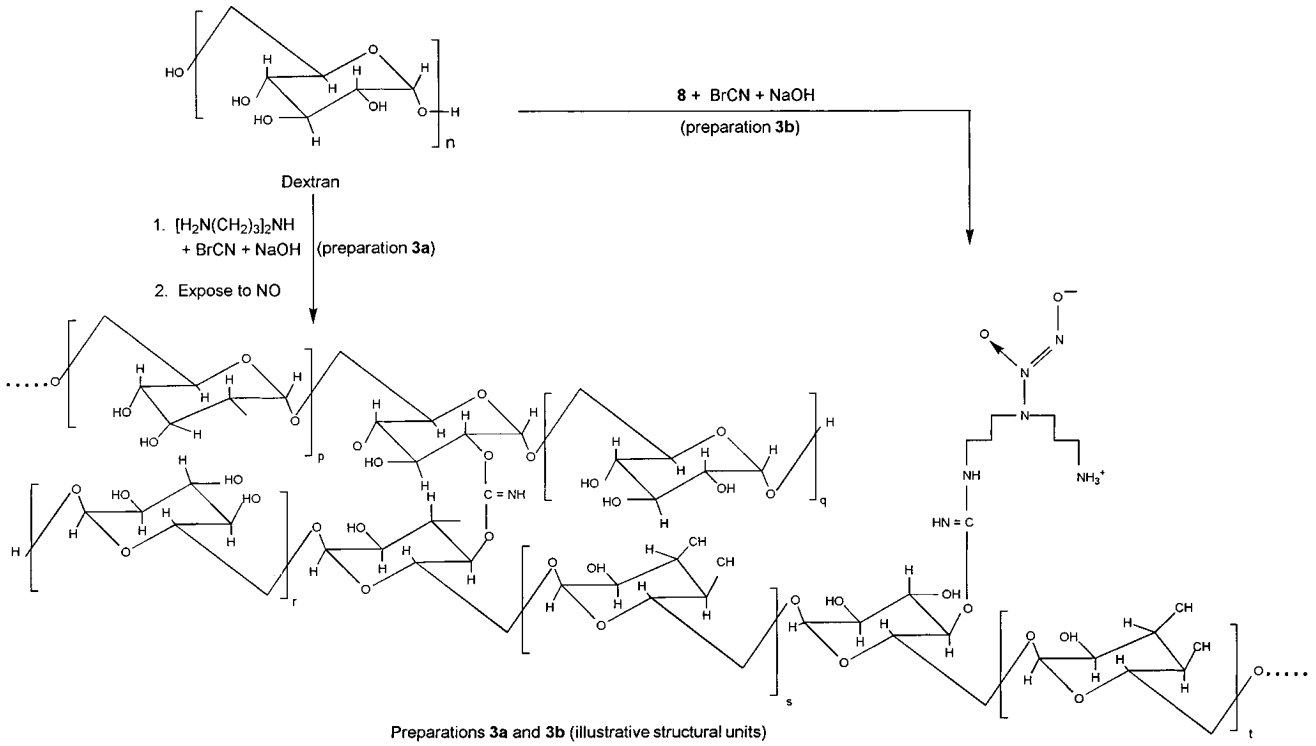


Figure 3. Time course of nitric oxide generation from **7** dispersed noncovalently in solid poly(caprolactone) (preparation **2c**) when immersed continuously in 0.1 M phosphate buffer (pH 7.4) at 37 °C as quantified by chemiluminescence detection.

as above led to the graph shown in Figure 2. The half-life obtained from this plot (23 h) closely matched the spectrophotometrically determined value, and integration[17] indicated that the full theoretical complement of NO was released, 2 mol/mol of **7** that dissociates, in agreement with the value obtained for this compound using the acid pulse technique.[15]

To determine the NO release profile of the poly(caprolactone)/**7** polymer described above, the method of the two previous paragraphs was applied to sample **2c**. Figure 3 shows the rate values thus derived as a function of the total elapsed time following its immersion in buffer. NO was seen to emerge from this sample for 1 full week before the chemiluminescence signal became undetectable. Graphical integration of the data in Figure 3 indicated that ca. 56 nmol of NO/mg of solid **2c** was released during this time. Since 30 nmol of intact **7** could be recovered unchanged from each milligram of **2c** and each mole of **7** could theoretically have released 2 mol of NO, we conclude that $56/(2 \times 30) = 93\%$ of the available nitric oxide was recovered as such during the week of immersion in buffer.

Scheme 3



Synthesis of Polymers of Structure Type 3

To demonstrate that the $[N(O)NO]^-$ function could be covalently attached to the polymer, as in structure **3** of Figure 1, dipropylenetriamine was grafted onto a polysaccharide (dextran) using cyanogen bromide as the coupling agent (see Scheme 3). The dextran was cross-linked during the process through formation of the potentially biodegradable imidocarbonate linkage in an oil-in-water microsphere preparative technique. The polymeric microspheres were exposed to NO to give **3a** as a free-flowing white powder.

NO was also found to emanate from this preparation when it was wrapped in filter paper and subjected to the same kinetic procedure as was used for the poly(caprolactone)/**7** disk described above, but the effect was very short-lived. In fact, NO was undetectable after only 6 h in contact with 37 °C buffer. The results are shown in Figure 4.

Reasoning that a conceptually different procedure for arriving at such a structure might yield different and hopefully better results, we reacted dipropylenetriamine first with NO as in eq 1 to produce the known[8] NO prodrug **8** and then grafted the product to the dextran using the same cyanogen bromide procedure as was employed for the preparation of **3a**. Despite the fact that the $[N(O)NO]^-$ group's terminal oxygen is nucleophilically reactive[18,19] and might have been partially bound covalently during the grafting and cross-linking process in a form incapable of regenerating NO, this sample (**3b**, see Scheme 3) released more NO for a longer period when in contact with pH 7.4 buffer than did **3a**.

These results, also shown in Figure 4, show that covalent attachment of the $[N(O)NO]^-$ group to a polymer can provide a relatively steady source of NO; after dropping by 2 orders of magnitude over the first 20 h, the rate of NO generation was essentially constant for the next 5 days before becoming undetectable.

Because the imidocarbonate linkage of preparations

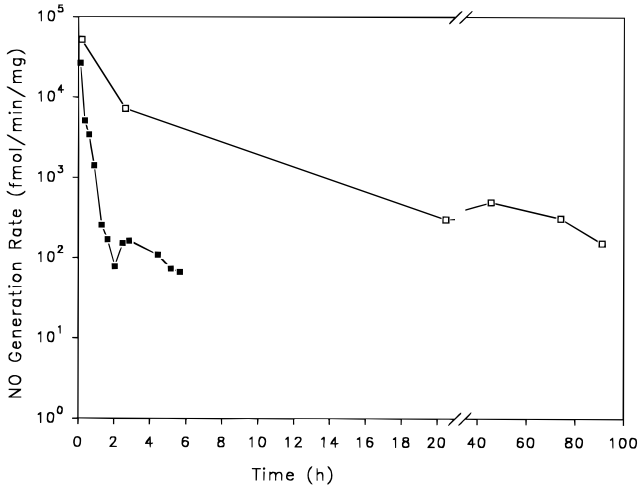


Figure 4. Time course of NO release from dextran covalently bound via a linker moiety to the $[N(O)NO]^-$ group prepared by (■) grafting dipropylenetriamine to the dextran and then exposing it to NO (sample **3a**) and (□) grafting preformed **8** directly to the dextran (sample **3b**).

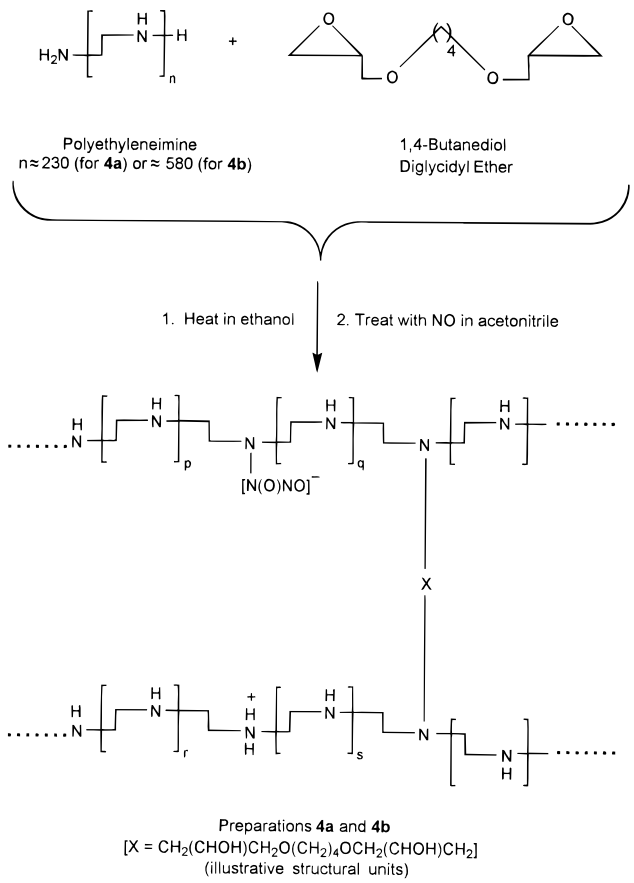
3 is hydrolytically scissile, these materials might be expected to release free **8** as well as molecular NO *in vivo*; such a circumstance could be of benefit if it is desired to deliver both NO and an NO donor into the tissue contacting the solid polymer to provide greater penetration than the notoriously short *in vivo* lifetime of free NO will allow.

Synthesis of Polymers of Structure Type 4

When a localized source of NO alone is desired, the $[N(O)NO]^-$ function can be attached directly to nucleophilic centers in the backbone of a hydrolytically stable, insoluble polymer from which only free NO could be released into solution.

To accomplish this, we chose poly(ethylenimine)[20,21] as the nucleophile, cross-linked it by reaction with 1,4-butanediol diglycidyl ether to render it insoluble, and

Scheme 4



exposed the product to 5 atm of NO (see Scheme 4). The resulting polymer had a rather rubbery texture, suggesting that it might be useful as a coating for other materials. To test this possible application, we coated poly(tetrafluoroethylene) vascular grafts with this material by dipping them into the cross-linking solution of poly(ethylenimine) and 1,4-butanediol diglycidyl ether, allowed them to dry, and exposed them to NO. When one of the resulting coated grafts (preparation **4a**) was immersed in buffer, NO was found to be generated at a relatively constant rate for 5 weeks before exhaustion, as shown in Figure 5. Since the initial rate of NO release from this graft was only about 20 pmol/min/mg, a heavier loading was applied when preparing a second, larger batch of **4a** for biological testing.

A powdered version of this material was prepared by extensively washing the poly(ethylenimine)/1,4-butanediol diglycidyl ether copolymer with ethanol before exposing it to NO. This preparation (**4b**) also released NO when immersed in buffer, as described below.

The results suggested that such polymers might be suitable as localized NO sources for two of the medicinal applications we had considered—improving the performance of vascular grafts and lowering the risk of restenosis after angioplasty.

Antithrombotic Activity. Intraluminal thrombus formation is an important cause of failure in prosthetic vascular grafts.[22−24] Protein absorption onto the graft surface begins as soon as circulation is established after surgical implantation, coating the lumen with adhesive glycoproteins.[24] Platelet deposition occurs rapidly within hours[24] and can thwart even short-term applications requiring only 1−3 weeks of graft patency.[25] For this reason, it is generally recommended that patients

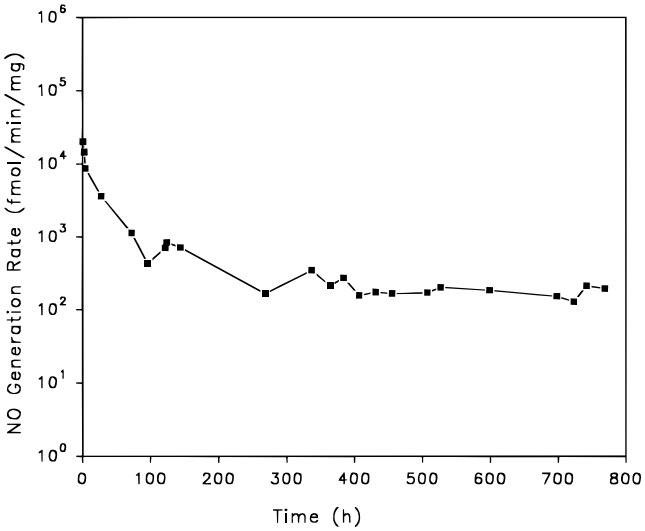


Figure 5. Time course of NO release from sample **4a**, prepared by coating a 1,4-butanediol diglycidyl ether/poly(ethylenimine) copolymer onto a poly(tetrafluoroethylene) vascular graft and then exposing it to NO. Rates of NO generation are plotted as a function of time after immersion in pH 7.4 phosphate buffer at 37 °C.

receiving such prostheses be given oral aspirin or other antiplatelet therapy to reduce the chances of potentially life-threatening graft occlusion.[24]

We speculated that coating the graft with a localized NO source might obviate the need for systemic administration of anticoagulants. Various NO prodrugs have been shown to be potent inhibitors of platelet adhesion and aggregation.[26,27] To determine the effect of our NO-releasing polymers on platelet function *in vivo*, we exposed the coated poly(tetrafluoroethylene) vascular grafts (preparation **4a** described above) to flowing blood by inserting them between segments of an artery-to-vein shunt in baboons.[28] The accumulation of blood platelets was quantified by scintillation camera imaging of platelets labeled with indium-111. The animals were not anticoagulated with heparin or given other agents known to affect platelet function, conditions that lead to rapid thrombus formation and graft failure in this model when untreated poly(tetrafluoroethylene) grafts are employed.[29] As shown in Figure 6, the NO-releasing grafts were substantially less thrombogenic than the controls, which were identical in construction except that they were not exposed to NO after being coated with cross-linked poly(ethylenimine). It would appear that localized NO generators such as **4a** may hold considerable promise for improving the performance of prosthetic vascular grafts in the absence of systemic antiplatelet therapy.

The results further suggest that such materials might be useful in other vascular surgery applications, e.g., for reducing the incidence of restenosis following angioplasty.[30] The reactions of blood platelets appear to promote the development of restenotic lesions.[31] By adhering in large numbers to the vessel wall, platelets may contribute directly to the lesion mass. In addition, platelet membrane phospholipids catalyze important coagulation reactions leading to the local generation of potent mitogenic as well as prothrombotic activities.[32] The ability of platelets to deliver growth factors to damaged tissues by adhering to them immediately after angioplasty may initiate and/or enhance the migration and proliferation of vascular smooth muscle cells as well

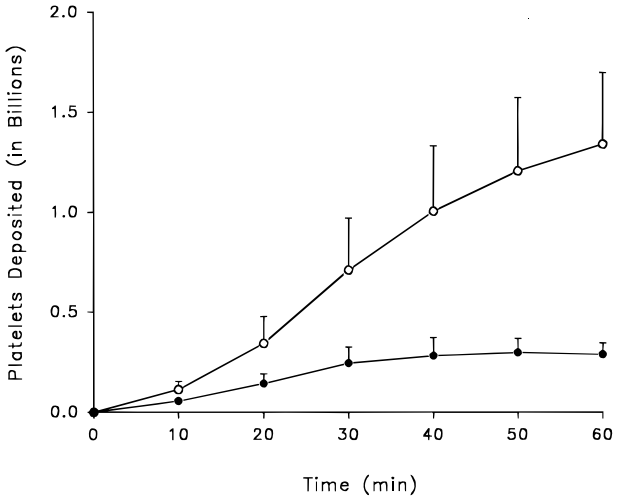


Figure 6. Inhibition of platelet deposition in poly(tetrafluoroethylene) vascular grafts coated with cross-linked poly(ethylenimine) that was (●) generating NO at a rate estimated to be 1−2 nmol/min/mg (preparation **4a**) and (○) generating no NO. Data were collected by quantifying accumulation of radioactivity in grafts placed in arteriovenous shunts in baboons whose platelets had been labeled with ^{111}In but who were not treated with heparin. Data are means ± SEM (n = 5). The difference between the two curves by repeated measures ANOVA was significant at the $p < 0.05$ level. The means for the 60-min time point were significantly different at the $p < 0.01$ level by the Mann−Whitney rank sum test.

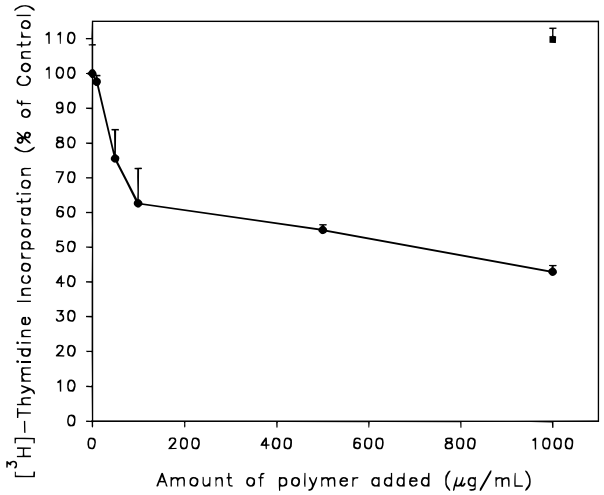


Figure 7. Inhibition of DNA synthesis in vascular smooth muscle cells exposed to a cross-linked poly(ethylenimine) preparation (sample **4b**) that was (●) generating NO at a rate estimated as 25 pmol/min/mg at the moment tritiated thymidine was added and (■) generating no NO. Data are means ± SD (n = 3) relative to growth rates in cells treated with neither polymer.

as provide a matrix for subsequent cellular ingrowth and thrombus reorganization. Polymeric films that shield freshly damaged vascular tissue from platelets and other blood components for <1 day after balloon injury have been shown to reduce both thrombosis and intimal thickening.[33]

Several groups have demonstrated that NO donor drugs can impede the growth of vascular smooth muscle cells in culture,[8,34−36] and it has been reported that both systemic administration of NO donor molecules[37] and transfection of the endothelial NO synthase gene into freshly damaged rat artery[38] had similar effects *in vivo*. We postulated that the polymeric NO sources described above might, if applied locally, inhibit the neointimal lesion formation that can result in restriction of blood flow.

Inhibition of Vascular Smooth Muscle Cell Proliferation *in Vitro*. To test this hypothesis, we cultured rat aorta smooth muscle cells and placed them into close proximity with (but not touching) the NO-releasing powder **4b**. As a control, a material that was identically prepared except that it was not exposed to NO was employed. DNA synthesis in these cultures was shown to be dose-dependently inhibited by **4b** but not by the control relative to that in cells treated with neither polymer. The results are shown in Figure 7.

To determine the degree to which powdered **4b** was acting as a localized source of molecular NO rather than of a solubilized, bound form thereof that would generate NO homogeneously throughout the medium, we performed the following experiment. Aliquots were wrapped in filter paper, immersed in pH 7.4 phosphate buffer, and incubated at 37 °C for 1 day to simulate the conditions that prevailed in the medium at the moment the tritiated thymidine was added in the cell culture experiments of Figure 7. Measurement of the NO generation rate at that point using the chemiluminescence method described above revealed that samples that had been rinsed three to five times with fresh buffer during the 1-day incubation were generating NO at the rate of 16.8 ± 6.0 (mean ± SD, n = 3) pmol/min/mg, while those that were soaked continuously in the same buffer solution for the entire observation period released 33.8 ± 11.1 pmol/min/mg at the 1-day time point. These findings indicate that solid particles unable to escape the filter paper crypt on rinsing were responsible for ca. one-half of the observed NO release.

The amount of NO required to induce significant cytostasis in this experiment was comparable to that produced by effective concentrations of a soluble NO donor. The quantity of **4b** that inhibited DNA synthesis by 50% 22 h after immersion was estimated from Figure 7 to be 0.7 mg/mL. Assuming that this amount was producing 25 pmol/min/mg (the average for all the measurements of the previous paragraph), the NO generation rate responsible for 50% inhibition was 0.7 × 25 ≈ 18 pmol/min/mL. When a virtually identical experiment was performed with **7**, a soluble compound containing the $[N(O)NO]^-$ group having a half-life in pH 7.4 phosphate buffer of 20 h at 37 °C, an IC_{50} of 40 μM was observed.[8] Since the latter value was the *initial* concentration giving rise to 50% inhibition at 22 h, ca. 1 half-life later, the *actual* concentration of **7** at the time of measurement in the previous work must have been about 20 μM. Multiplying the rate constant ($=0.69/t_{1/2}$) by 20 μM and then by 2 (since each mole of **7** that dissociates produces 2 mol of NO), a 50% effective NO generation rate of 23 pmol/min/mL for soluble **7** in the earlier study (compared with 18 pmol/min/mL calculated above for powdered **4b**) is obtained. The fact that two such physically dissimilar carriers of the $[N(O)NO]^-$ group were so similar in this respect is consistent with the view that NO release rate is one of the most important determinants of pharmacological activity in compounds containing this functional unit.

Significance

The seven preparations introduced here illustrate the diversity of structural type, chemical behavior, and physical form that one can achieve by incorporating the

$[N(O)NO]^-$ group into polymeric matrices. Preparation **2a** demonstrates that formulation of an $X[N(O)NO]^-$ ion as a water-soluble blend in poly(ethylene glycol) can greatly increase its shelf life under conditions of ambient temperature and humidity that are otherwise inimical to its longevity, and **2b** emphasizes the importance of lowering the hydrogen ion activity in prolonging the useful life of such materials. Preparation **2c** shows that a water-insoluble blend of an $X[N(O)NO]^-$ ion in moldable poly(caprolactone) can serve as a reasonably efficient source of NO for an extended period, roughly one-half of the theoretical maximum amount of NO being recovered as such during 1 week of immersion in pH 7.4 phosphate buffer at 37 °C. Preparations **3a**,**b** show that the $X[N(O)NO]^-$ unit can be covalently attached to a polysaccharide by either of two approaches: grafting a polyfunctional nucleophile, X, to the polymer and then reacting it with NO to form the $[N(O)NO]^-$ group or grafting the $X[N(O)NO]^-$ moiety directly to the polymer via a nucleophile residue remote from the $[N(O)NO]^-$ group in X. Preparation of **4a** illustrates how covalent attachment of the $[N(O)NO]^-$ function to a nucleophilic center in the backbone of poly(ethylenimine) can provide a flexible coating for a clinical poly(tetrafluoroethylene) vascular graft that proved substantially less thrombogenic when inserted into a baboon's circulatory system than control grafts that were identically prepared except that they were never exposed to (and hence could not release) nitric oxide. Preparation **4b** is structurally similar to **4a** but served as a localized source of NO that was capable of dose–responsively inhibiting the proliferation of vascular smooth muscle cells in culture.

Given the remarkable number of physiological processes that are mediated by NO, such NO-releasing polymers would appear to constitute important new tools in medicinal chemistry research as well as potentially offer improved solutions to a variety of pressing clinical problems. Of particular interest to us in this connection is the goal of reducing the risk of restenosis following angioplasty and other interventional procedures for vascular repair. Our results offer preliminary evidence that such polymeric sources of NO are capable of inhibiting two of the most important risk factors in the development of this debilitating disorder—excessive proliferation of vascular smooth muscle cells and pathogenic thrombus formation. Additionally, the data also suggest that improvements in vascular graft function may be possible using this technology. Work aimed at demonstrating the clinical promise of these materials in *in vivo* model systems is currently in progress.

Experimental Section

Compound **5** was prepared as previously described,[2] as were **6**, **7**, and **8**.[15] 1,4-Butanediol diglycidyl ether, dipropylenetriamine, and cyanogen bromide were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dextran (520 kDa) was purchased from Sigma Chemical Co., St. Louis, MO. Poly(ethylenimine) (25 kDa) was generously provided by BASF Corp. (Polymin, water-free; Holland, MI). Poly(ethylenimine) (10 kDa) and poly(caprolactone) (10–20 kDa) were obtained from Polysciences (Warrington, PA). Poly(ethylene glycol) (1.3–1.6 kDa) was obtained from Union Carbide Chemicals (Sentry PEG-1450; New York, NY). Ethanol was purchased from Quantum Chemical Co. (Cincinnati, OH). Acetonitrile was anhydrous grade (Aldrich), but all other solvents were reagent grade. NO was purchased from Matheson Gas Products (Twinsburg, OH) and used as received. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA).

Estimation of Physiological NO Regeneration Rates. Weighed samples of polymer were placed in 0.1 M phosphate buffer (pH 7.4), and the resulting mixture was allowed to stand open to the air in a 37 °C water bath, with NO measurements being conducted intermittently. Polymers that were insoluble powders, microspheres, etc., were enveloped in a filter paper crypt before immersion. An individual measurement was obtained by rinsing the sample with buffer, adding 2 mL of fresh buffer, closing the system, and purging the buffer with argon via a fritted glass tube placed at the bottom of the reactor vessel such that the gaseous effluent was passed into a Thermal Energy Analyzer Model 502A instrument (Thermedics, Inc., Woburn, MA) set to monitor NO content by chemiluminescence. Bubbling was continued until a steady, relatively horizontal NO trace was achieved, whereupon the signal was integrated over a span of several minutes. The number of integral units was converted to a value for moles of NO by comparison with integrals obtained for gaseous standards containing 55.3 ppm of NO in helium (certified standard obtained from MG Industries, Morrisville, PA). The rate of NO release over that time increment (calculated by dividing the integrated signal by the number of minutes the integration was conducted) was plotted versus the total elapsed time since the sample was first placed in the buffer bath to provide the time profile for NO generation from that sample.

Synthesis of Polymers in which Small Molecules Containing $[N(O)NO]^-$ Groups Are Noncovalently Dispersed (2a–c). Poly(ethylene glycol) was the base polymer for preparations **2a**,**b**. In the former case, 2.5 g of poly(ethylene glycol) was heated to 46 °C until completely melted, whereupon 36 mg of **5** was added. The container was placed on a vortex mixer until a homogeneous solution was obtained that gradually solidified upon cooling to room temperature to produce **2a**. No change was seen when the absorbance of aliquots dissolved in 10 mM sodium hydroxide was measured periodically over a 7-week observation period on storage at room temperature in air.

When **6** was similarly mixed with molten poly(ethylene glycol), substantial decomposition occurred; <40% of **6** remained after 5 weeks. To overcome this decomposition, an aqueous solution containing 0.11 g of potassium hydroxide and 8.9 g of poly(ethylene glycol) was evaporated to dryness. The amber residue was melted to ensure homogeneity, and a 1.1-g portion was mixed with 12 mg of **6**. After blending to a uniform mass, the mixture was allowed to solidify, producing **2b**. A decrease of only 8% could be seen in the absorbance at 250 nm of aliquots dissolved in dilute alkali over 5 weeks of storage at 22 °C in a clear glass vial in contact with air.

Disks of **2c** were prepared by heating 10 g of 10–20 kDa poly(caprolactone) at 60 °C until molten and then mixing it with 0.1 g of **7**. The resulting viscous liquid was poured into the wells of a 24-well polystyrene plate to a depth of ca. 1 mm. The wells were broken carefully after cooling to release the solid disks of **2c**. One of the disks was taken up in a 1:1 (v:v) mixture of dichloromethane with 0.01 M sodium hydroxide. The clear aqueous layer was saved, and the organic phase was again extracted with an equal volume of 0.01 M sodium hydroxide. The absorbance of the combined aqueous extracts at 250 nm indicated that the disk contained 30 nmol of **7**/mg of solid. Thus, 50% of the **7** added to the polymer in preparing the disk could be recovered as such from the resulting solid. Anal. Calcd for poly(caprolactone) containing 1% of **7** by weight: C, 62; H, 9; N, 0.4. Found: C, 62.57; H, 8.65; N, 0.37.

Synthesis of Polymers Containing Pendant $[N(O)NO]^-$ Groups (3a,b). Dipropylenetriamine was grafted onto dextran using cyanogen bromide to begin the synthesis of **3a** as follows. Nine grams of dextran was dissolved in 0.8 L of water and basified with 8 mL of 10 M sodium hydroxide. Cyanogen bromide (8 g) freshly dissolved in 0.1 L of water was added followed quickly by 31.8 g of dipropylenetriamine. The pH was adjusted to 10, and the stirring was continued overnight. The solution was extracted with two 150-mL volumes of dichloromethane and then dialyzed overnight against cold running water. After adjusting the pH to 7, the solution was lyophi-

lized to yield 9.2 g of a translucent yellow solid. The success of grafting was affirmed by titrating the product against 1 M sodium hydroxide. A 3.6-g aliquot of the grafted dextran was placed in 15.6 mL of doubly distilled water and treated with 2 mL of 10 M sodium hydroxide. The resulting solution was poured into 0.3 L of light mineral oil in a Waring blender and stirred for 2 min. Two grams of cyanogen bromide freshly dissolved in 16 mL of water was added to the blender. After stirring for an additional 2 min, the mineral oil was removed by stirring three times for 30 s each with 300-mL volumes of petroleum ether and decanting the organic phase after each addition. The resulting microsphere/water suspension was dehydrated by adding two 0.5-L volumes of 95% ethanol and filtering each time. Final dehydration was achieved by washing the microspheres with 0.5 L of absolute ethanol and filtering. The microspheres were air-dried under vacuum for 6 h to yield 0.8 g. This material was suspended in 50 mL of dry acetonitrile and stirred under 5 atm of NO for 48 h, whereupon the slurry was filtered, washed with 0.2 L of acetonitrile, and dried to yield a white, free-flowing powder (sample **3a**).

Preparation **3b** was begun by dissolving 3.6 g of dextran in 15.6 mL of water and adding 5 mL of 10 M sodium hydroxide. To this was added 1.5 g of **8**. The mixture was poured into 300 mL of light mineral oil and stirred in a Waring blender for 2 min. Five grams of cyanogen bromide freshly dissolved in 35 mL of distilled water was added while stirring, which was continued for 2 min more. The mineral oil was removed by stirring three times for 30 s each with 300-mL volumes of petroleum ether and carefully decanting the organic phase each time. The microsphere/water suspension was mixed with 1 L of 35% ethanol and centrifuged; the collected microspheres were similarly extracted three more times with 35% ethanol (1 L each time). When no ultraviolet peak corresponding to unreacted **8** could be detected in the supernatant, the microspheres were washed with 1 L of absolute ethanol, filtered, and air-dried on the filter paper under vacuum for 6 h to give 2.7 g of **3b**.

Synthesis of Polymers Containing $[N(O)NO]^-$ Groups Attached to Heteroatoms in the Backbone (4a,b). Vascular grafts (preparations **4a**) containing the $[N(O)NO]^-$ group were prepared as follows. A 250-mL flask equipped with a magnetic stirring bar and a reflux condenser was charged with poly(ethylenimine) (10 kDa, 4 g) and 1,4-butanediol diglycidyl ether (0.4 g, 2.0 mmol) in 40 mL of ethanol and maintained at 80 °C. After 20 min, the viscosity of the solution increased, and tubular segments of expanded poly(tetrafluoroethylene) (Gore-Tex, 2.5 cm, 4-mm i.d.; W. L. Gore & Assoc., Flagstaff, AZ), presoaked in absolute ethanol, were dipped into the solution for 60 s. After four repetitions of this dipping procedure, the grafts were placed in an oven at 55 °C for 3 h to complete the cross-linking. The devices were soaked in absolute ethanol overnight to remove low molecular weight leachables, dried *in vacuo* for 3 h, suspended in acetonitrile, and exposed to NO for 3 days. As a result of this procedure, an 80-mg graft segment increased in weight to 97.4 mg; this piece was used to accumulate the data of Figure 5.

To obtain a sample of **4a** providing more rapid initial rates of NO release for the biological studies, the procedure was repeated as in the previous paragraph except that the grafts were dipped only once into a more concentrated solution of the cross-linking polymer (7 g of poly(ethylenimine) and 0.7 mL of 1,4-butanediol diglycidyl ether in 40 mL of ethanol) and exposed to NO for 5 days instead of 3 days. In this case, both ribbed and nonribbed graft segments were used. For the former, the mean weight of the replicate pieces before coating was 273 ± 13 (SD) mg; dipping increased this value to 307 ± 17 mg, and the mean graft weight was 314 ± 17 mg after exposure to NO. For the nonribbed graft segments, the corresponding weights were 137 ± 1, 183 ± 8, and 188 ± 10 mg, respectively. These **4a** samples were used to collect the data for Figure 6.

4b was prepared similarly, except that poly(ethylenimine) of molecular weight 25 kDa was used and the clear, rubbery material produced on cross-linking was washed with hot ethanol (50 °C) for 24 h and then with acetonitrile for 24 h to remove leachable material. The white, tacky polymer thus produced was filtered and dried *in vacuo* for 4 h yielding 3.1 g of cross-linked poly(ethylenimine). Sample **4b** was prepared by suspending the polymer in acetonitrile and treating it with 5 atm of NO three times over 6 days. The product was isolated by filtration, washed with acetonitrile and then ether, and dried *in vacuo* yielding 1.1 g of yellow, powdery **4b**. This sample was used in the cytostasis experiment of Figure 7.

Antiplatet Activity in the Baboon Circulatory System. Measurements of platelet thrombus accumulation onto vascular grafts were performed as described previously.[28] Briefly, these studies employed five normal male baboons (*Papio cynocephalus*) weighing 9−12 kg that had been quarantined and observed to be disease-free for at least 3 months. All studies were approved by the Institutional Animal Care and Use Committee and were in accordance with federal guidelines (*Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 86-23). Each baboon had a chronic exteriorized silicone rubber shunt placed between the femoral artery and vein.[28] These shunts do not shorten platelet survival detectably or produce measurable platelet activation.[39] To assess the capacity of the polymer-coated vascular grafts (**4a**) to induce thrombus formation, they were inserted into the shunt system and exposed to non-anticoagulated blood for 1 h. Blood flow was maintained at 100 mL/min by a clamp placed distal to the test section and measured continuously using an ultrasonic flowmeter (Model 201; Transonics Systems, Ithaca, NY).

Autologous baboon platelets were labeled with 1 mCi of indium-111-oxine as previously described.[28] Labeling efficiencies averaged >90%. After allowing at least 1 h for the reinfused cells to distribute within the vasculature, the grafts were incorporated into the shunt system and exposed to native blood for 1 h. One control graft and one NO-releasing graft were evaluated individually in each study animal. The accumulation of ^{111}In-labeled platelets within each graft was measured continuously using a gamma scintillation camera (General Electric 400T; MaxiCamera, Milwaukee, WI). Data were stored at 5-min intervals and analyzed using a computer-assisted image processing system interfaced with the camera (medical data systems A^3; Medtronic Inc., Minneapolis, MN). Since the amount of injected isotope was not a limiting factor, optimal resolution was achieved by acquiring the low-energy indium-111 peak (172 keV) with a high-sensitivity collimator.[28] Images of the vascular graft and distal expansion segment were taken in 128 × 128 byte mode using a 15% energy window and analyzed using 4-cm long (20 pixel) regions of interest for each graft. The total number of deposited platelets in each region was calculated by dividing the deposited platelet radioactivity (cpm) by the whole blood ^{111}In platelet activity (counts/min/mL) and multiplying by the circulating platelet count (platelets/mL), as described.[28,39]

Measurement of *in Vitro* Cytostatic Activity. Inhibition of DNA synthesis was investigated using a procedure similar to the one previously described.[8] Briefly, rat aorta smooth muscle cells (passage 9) were seeded at a concentration of 4×10^4 cells/well in 24-well trays containing MCDB medium (MCDB 131 medium complete with trace elements; Sigma Chemical Co., St. Louis, MO) + 10% fetal bovine serum (FBS) and allowed to attach at 37 °C in an atmosphere of 5% CO_2/95% O_2 for 5 h. Serum-containing medium was then removed and replaced with serum-free medium. Forty-eight hours later, the serum-free medium was removed and replaced with MCDB + 10% FBS. Powdered **4b** (5.7 mg) was washed with 4 mL of MCDB + 10% FBS, recovered by centrifugation, and suspended in 0.82 mL of fresh MCDB + 10% FBS. Dilutions of this stock suspension were prepared in MCDB + 10% FBS and placed in a Millipore tissue culture insert (Bedford, MA). The insert, the bottom of which consists of a polycarbonate filter with 0.45-μm pores, was suspended 1 mm above the base of the tissue culture well; this prohibited direct contact between the polymer and the cells but presumably permitted free diffusion of NO from the polymer compartment into the cell compartment. [^{3}H]Thymidine (1 μCi) was added 22 h later. Following a 2-h incubation, the cells were washed twice with Hank's balanced salt solution to remove unincorporated [^{3}H]-thymidine. Cells were then washed four times with ice-cold 10% trichloroacetic acid and lysed with 2% sodium dodecyl

sulfate in 1 M NaOH. Trichloroacetic acid-precipitable material was transferred to scintillation vials, EcoLite cocktail (ICN, Irvine, CA) was added, and radioactivity was counted in a Beckman LS-6500 scintillation counter. Controls consisted of cells receiving either no treatment or a cross-linked poly(ethylenimine) powder identical with **4a** except that it had never been exposed to NO. Data are expressed as percent [^{3}H]-thymidine incorporation relative to untreated controls.

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