More Lipophilic Dialkyldiamine-Based Diazeniumdiolates: Synthesis, Characterization, and Application in Preparing Thromboresistant Nitric Oxide Release Polymeric Coatings

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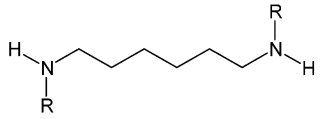
Received June 12, 2003

The synthesis, characterization, and biomedical application in preparing more thromboresistant polymeric coatings for a series of lipophilic dialkyldiamine-based diazeniumdiolates are described. Dialkylhexamethylenediamine diazeniumdiolates of the form $RN[N(O)NO]^-$-$(CH_2)_6NH_2^+R$, where R = CH_3, CH_2CH_3, $(CH_2)_2CH_3$, $(CH_2)_3CH_3$, $(CH_2)_4CH_3$, $(CH_2)_5CH_3$, and $(CH_2)_{11}CH_3$, are prepared via reaction of the corresponding diamine with NO. The more lipophilic diazeniumdiolates [e.g., R = $(CH_2)_3CH_3$] can be incorporated into hydrophobic polymeric films (e.g., plasticized PVC), and the resulting materials release NO for extended periods of time upon exposure to PBS buffer. The mechanism of NO release from these films is examined in detail. More stable initial NO release can be achieved by adding lipophilic anionic species (e.g., tetraphenylborate derivative) to the polymeric material to buffer the activity of protons within the organic phase. It is shown that the use of these new lipophilic NO-donors in polymers provides the ability to tailor NO release rates for a variety of medical applications. As an example, polymers doped with *N*,*N*′-dibutylhexamethylenediamine diazeniumdiolate and a tetraphenylborate derivative are employed as coatings for vascular grafts in sheep. The NO release grafts exhibited enhanced performance and had an average 95% thrombus-free surface area compared to 42% for the corresponding control grafts when examined after 21d of implantation.

Introduction

Nitric oxide (NO) has been shown to have several important physiological functions, including its unique vasodiolating properties,[1] cancer-fighting potency,[2−4] and antiplatelet activity.[5−7] Although NO is a stable radical, it is highly reactive with hemoglobin and oxygen; therefore, the ability to deliver NO to the site of need is challenging. Stable water-soluble as well as hydrophobic NO donors are desirable to best take advantage of the potency of NO for a wide range of biomedical applications.[8,9] These include NO-releasing pharmaceuticals[4,10−14] and the preparation of thromboresistive hydrophobic polymeric coatings for medical devices such as intravascular sensors and extracorporeal circuits[15,16] (based on NO's antiplatelet activity[17]). Indeed, many advances have been achieved using water-soluble diazeniumdiolates as NO delivery agents. For example, the diazeniumdiolate of proline (PROLI/NO), when infused into blood, has been shown to relieve muscle spasms.[18] In addition, it has been reported that the diazeniumdiolate of diethylene triamine (DETA/NO) completely suppresses overproliferation of cells after vascular injury,[19] and glycosylated diazeniumdiolates possess antitumor activity.[20]



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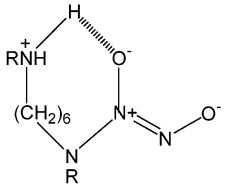

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Figure 1. Structures of parent dialkylhexamethylenediamine (**1**) and corresponding *N*-diazeniumdiolate (**2**) where R = CH_3, CH_2CH_3, $(CH_2)_2CH_3$, $(CH_2)_3CH_3$, $(CH_2)_4CH_3$, $(CH_2)_5CH_3$, and $(CH_2)_{11}CH_3$.

The use of such water-soluble diazeniumdiolates with hydrophobic matrixes to create more biocompatible coatings has, however, proven more problematic. For example, (*Z*)-1-[*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)amino]]diazen-1-ium-1,2-diolate (MAHMA/NO) (Figure 1 (R=CH_3)), when dispersed in a silicone rubber matrix, was shown to prevent thrombus formation on the surface of intravascular sensors[21] and greatly reduced platelet activity when employed within a polymer coating on the inner walls of PVC extracorporeal circuits.[22] However, MAHMA/NO and its corresponding diamine precursor were found to leach from the surface of the polymer matrix and back-react with an oxidative

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10.1021/jm030286t CCC: $25.00
Published on Web 10/29/2003

Table 1. Characteristics of Diazeniumdiolates (all measurements were $n > 3$)

compd	R	log P^a	k (s^{-1})	$t_{1/2}$ [b] (s)	ratio diamine:NO	ϵ ($M^{-1}\,cm^{-1}$)	$T_{NO\ loss}$ (°C)
2a	CH_3	0.97	0.010 ± 0.0001	67.2 ± 0.7	1:2.0 ± 0.1	7250[c]	104
2b	CH_2CH_3	2.03	0.0020 ± 0.0001	347 ± 26	1:2.10 ± 0.04	8640	104
2c	$(CH_2)_2CH_3$	3.09	0.0022 ± 0.0001	319 ± 23	1:2.00 ± 0.05	7868	104
2d	$(CH_2)_3CH_3$	4.15	0.0025 ± 0.0002	297 ± 34	1:2.0 ± 0.1	7818	104
2e	$(CH_2)_4CH_3$	5.21	0.0025 ± 0.0002	279 ± 31	1:1.9 ± 0.1	8045	104
2f	$(CH_2)_5CH_3$	6.26	d				
2g	$(CH_2)_{11}CH_3$	12.6	d	660[e]			

[a] Octanol/water partition coefficient calculated using ChemDraw. [b] "Apparent" $t_{1/2}$ and $t_{1/2}$ for diazeniumdiolates under investigation in PBS buffer at 37 °C and pH 7.4. [c] Reported in ref 24. [d] Unable to determine based on the lack of air stability. [e] Measured using an NO-selective electrochemical sensor in a nitrogen environment.

intermediate of NO to form potentially toxic nitrosamines.[23] In view of this, efforts to create useful NO-releasing polymeric films have now focused on synthesizing either more lipophilic diazeniumdiolates or diazeniumdiolates that are covalently anchored to a polymer backbone.[15,24,25] In this paper, the synthesis and characterization of a series of more lipophilic symmetrical diazeniumdiolates structurally similar to MAHMA/NO and their incorporation into thin polymer films are examined. The NO-release characteristics of these compounds alone as well as within a polymer matrix under physiological conditions are reported. Finally, the use of the resulting materials as thromboresistant coatings for vascular grafts is explored.

Results and Discussion

Synthesis. Using diamines to synthesize diazeniumdiolates has been previously reported;[26] however, in all cases the products examined were highly water-soluble. In this work, we explored the effect of the side alkyl chain length on the addition of NO to more lipophilic diamine structures. Figure 1 shows the parent *N*,*N*′-dialkylhexamethylenediamine structures (**1a**−**g**) and the corresponding *N*-diazeniumdiolates (**2a**−**g**) formed upon NO addition. Systematic variation in the length of the R chain R═CH_3 to $(CH_2)_{11}CH_3$ has enabled an investigation of the effect of increasing lipophilicity on the formation of stable diazeniumdiolates and the NO release properties of these basic diamine structures.

Decomposition of Diazeniumdiolates. Diazeniumdiolates have been shown to decompose and release NO by two mechanisms, proton-driven[27] and thermal dissociation.[15] To date, the proton-driven decomposition is most prevalent for discrete amine-based diazeniumdiolates.[27] Ultraviolet spectroscopy and NO-selective chemiluminescence measurements were used to monitor the decomposition of the diazeniumdiolates with time at pH 7.4. The UV spectra of **1a**−**e** show an absorbance maximum at 247 nm in methanol (compounds **1d** and **1e**) or basic solution (compounds **1a**−**c**), which is characteristic of diazeniumdiolates. This absorbance decreases with time when **1a**−**e** are exposed to PBS buffer (pH 7.4) at 22 °C, while there is a corresponding increase in the nitrite absorbance band at 214 nm (see Supporting Information, Figure 1S). However, because of the limited solubility of the more lipophilic species (**1d** and **1e**) in aqueous solution, it was not possible to measure the kinetics of all the diazeniumdiolates via UV-absorbance, owing to the turbidity of such samples. Therefore, NO measurements by chemiluminescence were used to obtain kinetic data for decomposition of all the diazeniumdiolates under investigation.

As expected, all intramolecular diazeniumdiolates release 2 mols of NO for each mole of diamine (see Table 1). These values were determined using chemiluminescence, after adding a given amount of the diazeniumdiolate to PBS buffer purged with nitrogen. The NO released was detected and integrated over time, until no further release of NO was observed.

Interestingly, as indicated in Table 1, it was not possible to form air-stable intramolecular diazeniumdiolates for the most lipophilic species (those with the highest octanol/water partition coefficients (log *P*)), *N*,*N*′-dihexylhexamethylenediamine (**1f**) and *N*,*N*′-didodecylhexamethylenediamine (**1g**). The reaction of NO with **1f** yielded a diazeniumdiolate (as determined by UV) that was initially air-stable but decomposed after 12 h, even with storage at −20 °C. While the reaction with NO with **1g** does also yield a diazeniumdiolated species that can be observed if maintained under a nitrogen environment, the presence of oxygen during the workup procedure immediately decomposes the most lipophilic intramolecular diazeniumdiolates to the corresponding ammonium nitrite salts. Thus, the most air-stable intramolecular diazeniumdiolate that could be isolated was that of diamine **1e**. Air stable bis-diazeniumdiolates of **1f** and **1g** can, however, be prepared when an exogenous base such as sodium methoxide is present in the reaction mixture.[28]

Thermal Stability of Diazeniumdiolates. To investigate the temperature stability of the various intramolecular diazeniumdiolates, thermal gravimetric analysis was performed on the analogue series. Thermal stability is important for storage and processing conditions, particularly if such compounds are to be used to prepare polymeric coatings for medical devices. Under a nitrogen atmosphere, the diazeniumdiolates studied remain stable to 104 °C (see Table 1) before losing their diazeniumdiolate moiety, leaving only the parent diamine, as confirmed by proton NMR. There appears to be no difference in the thermal stability of the diazeniumdiolates as a function of side chain length under a nitrogen atmosphere. The decomposition at this temperature is most likely due to disruption of the hydrogen-bonding interaction between the oxygen of the diazeniumdiolate and the ammonium hydrogen. On the basis of the percent weight change, the loss of only the diazeniumdiolate moeity is observed at a single temperature.

Kinetics. The decomposition of intramolecular zwitterionic diazeniumdiolates has been shown to follow pseudo-first-order kinetics.[1,24−25] As the pH of the environment becomes more basic, the rate of decomposition to liberate NO decreases. The decomposition of the

diazeniumdiolates prepared in this work, under physiological conditions (pH 7.4 and 37 °C), is also summarized in Table 1. On the basis of monitoring the amount of NO liberated with time in PBS buffer using chemiluminescence, the residual amount of diazeniumdiolate species at any given time can be determined. Plots of the natural logarithm of this diazeniumdiolate concentration vs time yielded a linear relationship, with r^2 values typically ≥0.99 for all diazeniumdiolates examined (suggesting first-order kinetics in all cases). There is an increase in the "apparent" half-lives as R is increased from CH_3 to CH_2CH_3. The term "apparent" half-life is used to refer to the half-lives of those compounds that have limited solubility in PBS buffer (heterogeneous suspensions) and therefore may not exhibit classical homogeneous kinetic behavior. As R is further increased from CH_2CH_3 to $(CH_2)_2CH_3$ to $(CH_2)_4CH_3$, the half-lives decrease slightly from that measured for R = CH_2CH_3. However, the differences observed in the "apparent" half-lives of **2c**−**e** are all within the standard deviation of the measurement, indicating that no clear trend with lipophilicity can be gleaned.

Use in Hydrophobic Systems. To date, the use of NO donors in polymeric systems has been limited despite the well-documented benefits of NO. Because NO can both prevent platelet activation and aggregation, polymeric materials doped with lipophilic NO donors are attractive with respect to preparing more blood-compatible polymer materials. However, to effectively use NO donors in these systems, the NO donor must remain stable through the preparation process of embedding the donor within the polymer matrix and must further be capable of spontaneously releasing NO when the polymer is exposed to solutions or blood under physiological conditions. Unlike discrete diazeniumdiolates, whose NO release is dependent on pH and temperature, the release of NO from molecules embedded within a polymer matrix has additional variables that could potentially govern NO-release profiles from within these materials. Under physiological conditions, the processes that are occurring between the polymer, the embedded NO donor, and the aqueous environment include, but are not limited to, the diffusion and ionization of water into/within the organic polymer film, ion-exchange between the buffer ions and ions within the polymer, protonation of the amine nitrogen bearing the diazeniumdiolate to yield NO, and deprotonation of water by secondary amine sites to yield organic ammonium hydroxides.

The Effect of Organic Phase pH on NO Release from Polymeric Materials. As demonstrated previously, the rate constant for diazeniumdiolate decomposition is pH dependent.[27] Incorporation of 5 wt % of **2d** into a PVC film plasticized with dioctyl sebacate (DOS) (approximate thickness 150−200 μm) and then exposure to PBS buffer yielded an NO-release profile, as measured by chemiluminescence, that had an initial bolus of NO that decreases rapidly with time, never achieving theoretical total NO release (i.e., the amount of NO anticipated based on the mass of **2d** in the polymer film) (Figure 2). This is in sharp contrast to **2a**, which releases all of its NO in a short period of time (Figure 2). However, it was determined previously that the decomposition of **2a** occurs, partly, outside of the

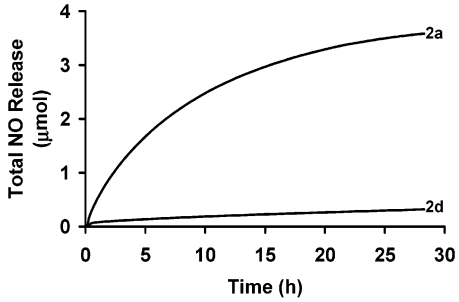

Figure 2. Total NO release curves for **2a** and **2d** (equal molar, ~5 wt %) dispersed in a 1:2 PVC/DOS polymer matrix (circular disks with a diameter of 8 mm and a thickness of ~150 μm) as a function of time in PBS buffer at 37 °C. NO was measured directly by chemiluminescence.

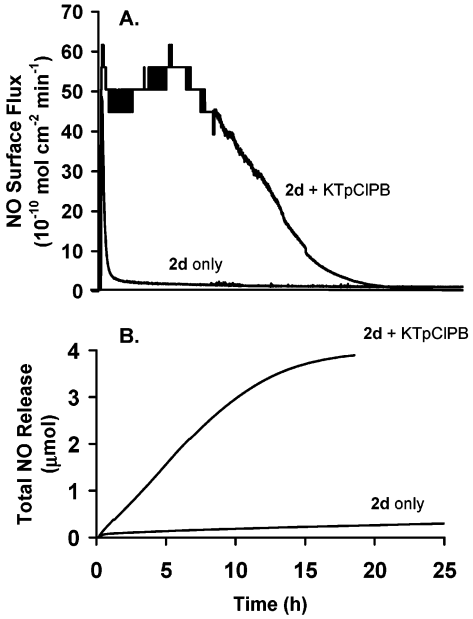

Figure 3. NO surface flux (A) and total NO release curves (B) for **2d** dispersed in a 1:2 PVC/DOS matrix (circular disks with a diameter of 8 mm and a thickness of ~150 μm) with and without KTpClPB (1:1 mol ratio KTpClPB: **2d**) soaked in PBS buffer (pH 7.4) at 37 °C. NO was measured directly by chemiluminescence.

polymer matrix;[23] that is, **2a** diffuses from the polymer matrix and then reacts with the protons within the soaking solution to release NO. For the more lipophilic NO donors, where the NO is released primarily from water-induced protonation within the polymer matrix, additional additives are required to prolong the release from polymeric films and achieve theoretical NO release (based on the total amount of diazeniumdiolate doped within the material).

The addition of a lipophilic tetraphenylborate salt [potassium tetrakis-4-chlorophenyl borate (KTpClPB)] into the polymer matrix both increases and prolongs the release of NO from plasticized PVC films containing **2d** (Figure 3). In the absence of the KTpClPB, NO release decreases dramatically after approximately 1 h. Similar NO release patterns are also observed for **2c** and **2e** dispersed within a plasticized PVC matrix (data not shown). The decrease in NO release observed is believed to result from an increase in the pH within the organic polymer film, which decreases the decomposition rate of lipophilic diazeniumdiolates. Indeed, as water diffuses into the film, initiating NO release, secondary amine

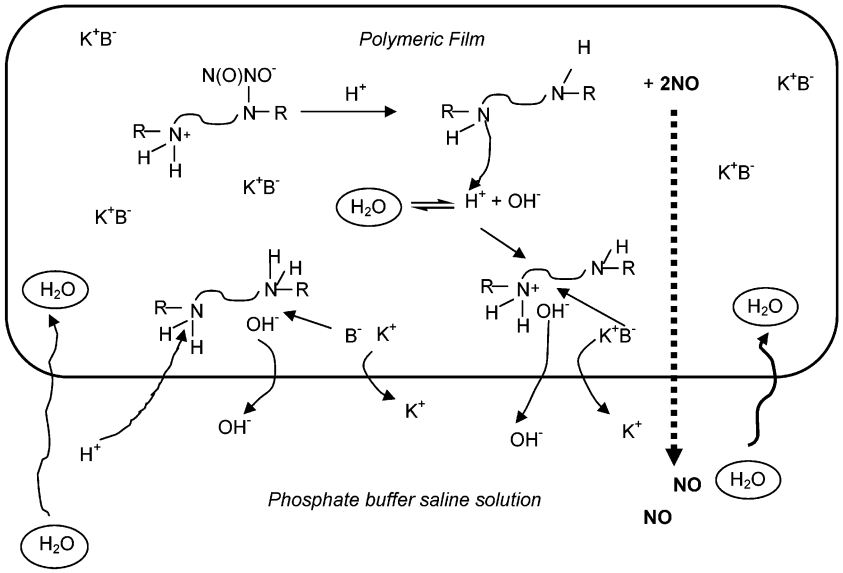

Figure 4. Schematic representing how the presence of potassium tetrakis(4-chlorophenylborate (depicted as K^+B^-) reduces pH changes within polymeric films containing *N*-diazeniumdiolates.

sites result. The secondary amine sites have a higher pK_a than water and can therefore deprotonate water to form hydroxide ions. The basic microdomain environment that results within the polymer, in turn, slows further decomposition of the remaining diazeniumdiolates that could generate NO. This retardation can occur as a result of the organic ammonium hydroxide microphases within the polymer, which, in turn, serves to stabilize the remaining diazeniumdiolates.

The incorporation of KTpClPB buffers the polymer phase by providing lipophilic anionic sites that can serve as counterions to the organic ammonium cations as depicted schematically in Figure 4. In this way, the potassium and hydroxide ions can diffuse from the polymer matrix to the surrounding aqueous phase and NO release is maintained at a more constant rate until the total concentration of diazeniumdiolate species decreases significantly.

To determine, experimentally, if the pH within the film does change with time, a liphophilic pH chromoionophore was incorporated into PVC films containing 5 wt % of **2d** both in the presence and absence of added KTpClPB. The intensity of the UV−vis absorbances corresponding to the protonated ($\lambda_{max} = 650$ nm) and deprotonated ($\lambda_{max} = 514$ nm) peaks of the chromoionophore was monitored with time spectrophotometrically. It was found that films without KTpClPB showed an increase in the absorbance of the deprotonated peak and a decrease in the protonated peak as a function of exposure time to the PBS buffer (Figure 5A). In contrast, once equilibrium is established, no change is observed in the ratio of the protonated and deprotonated peaks and primarily only a protonated species is observed for films containing KTpClPB, where the intensity of the protonated peak remains constant (Figure 5B). This further supports the notion that as NO is being released from within the polymer phase, the unreacted diazeniumdiolate groups require a buffering system to prevent the film from becoming too basic, which slows further diazeniumdiolate decomposition.

The Effect of Polymer Matrix on NO Release. Diazeniumdiolates of similar structure to those investigated herein decompose to generate NO by a primarily proton-driven mechanism.[27] Thus, polymer matrixes that favor water or proton partitioning and diffusion within the matrix should provide the fastest NO release profiles. By formulating different polymer compositions, thereby yielding different water uptake properties and diffusion coefficients of reacting species, it is possible that the NO release profiles of a diazeniumdiolate in a particular polymer matrix could be controlled.

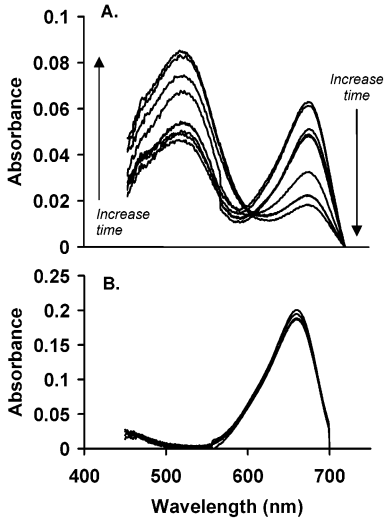

Figure 5. Visible spectra of 9-(dimethylamino)-5-[4-(16-butyl-2,14-dioxo-3,15-dioxaeicosyl)pneynylimino]benzo[*a*]-penoxazine (chromoionophore II) incorporated into a 1:2 PVC/DOS matrix containing **2d** soaked in PBS buffer pH 7.4 as a function of time with (A) and without (B) KTpClPB. The protonated absorption peak of Chromoionphore II is at 650 nm and the deprotonated band is at 514 nm.

It has been previously reported that the water uptake values for plasticized PVC films are dependent on the matrix composition, and as the polymer-to-plasticizer ratio is increased, the water uptake values decrease.[29] This decrease in the water uptake may lead to a lower proton activity within the polymer matrix as the polymer-to-plasticizer ratio is increased. In addition, the diffusion coefficients of species in a plasticized

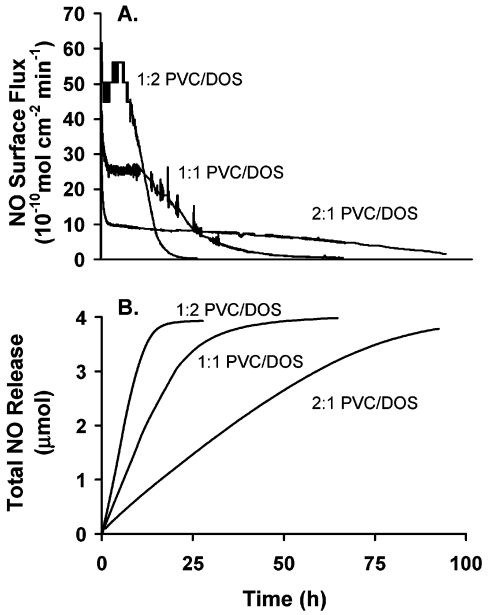

Figure 6. NO surface flux (A) and total NO release curves (B) for **2d** dispersed in plasticized PVC films (ratios 1:2, 1:1 and 2:1 PVC:DOS by mass) containing KTpClPB in PBS buffer (pH 7.4) at 37 °C. Circular films of 8 mm in diameter and ~150 μm thickness were used. NO was measured directly by chemiluminescence.

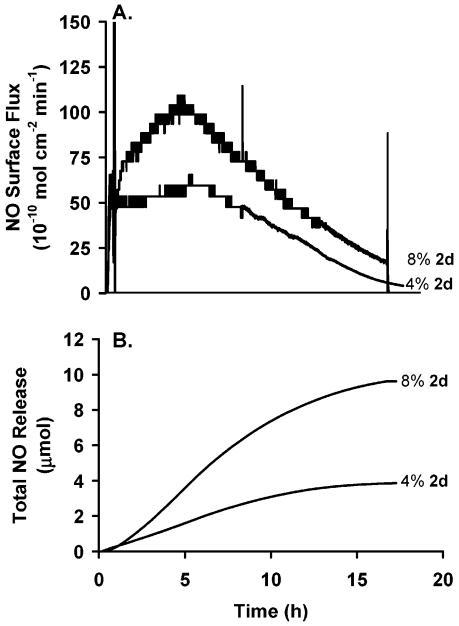

Figure 7. NO surface flux (A) and total NO release curves (B) for 4 and 8 wt % **2d** dispersed in a 1:2 PVC/DOS matrix containing KTpClPB (1:1 mol ratio with **2d**) in PBS buffer (pH 7.4) at 37 °C. Circular disks of diameter 8 mm and ~150 μm thickness were used. NO was measured directly by chemiluminescence.

polymer decreases with lower plasticizer content. The coupling of lower proton activity with decreased diffusion coefficients as the polymer-to-plasticizer ratio increases could lead to a dramatic decrease in the rate constant for the decomposition of the diazeniumdiolate due to a decrease in the probability of the reacting species (the diazeniumdiolated N and the proton) to collide and liberate NO. Thus, a lower flux of NO from films with higher polymer-to-plasticizer ratios would be predicted.

Indeed, as shown in Figure 6, as the polymer-to-plasticizer ratio is increased from 1:2 to 2:1 for films containing **2d** and KTpClPB, the NO-release rate decreases as hypothesized, and the release is prolonged. For each variation in the polymer-to-plasticizer ratio, there exists a linear region of NO release. As the polymer-to-plasticizer ratio is increased, the time over which the NO release is steadily lengthened. This is especially advantageous for applications that require constant NO release for longer periods of time. Indeed, it can be envisioned that blends with even higher polymer-to-plasticizer ratios will have even more prolonged release that may be very useful for certain medical applications that require continued NO generation over several days (i.e., implantable sensors, extracorporeal membrane oxygenation (ECMO), vascular grafts, etc.).

One polymer blend in which this prolonged high steady surface flux of NO has been observed is silicone rubber (SR). The NO released from silicone rubber tubes coated with a solution of plasticized SR with 4.2 wt % **2d** and 8.1 wt % KTpClPB is steady over a 40 h period, with only 15% of the total estimated NO released from the surface of the tube after this time upon exposure to PBS buffer at 37 °C (see Figure 2S in the Supporting Information). This suggests that release may continue for weeks. This prolonged NO release is believed to be a result of the relatively low water uptake of the SR matrix[30] coupled with the buffering effect of having the lipophilic borate salt present as well.

Another approach that can be used to alter the NO release profile of diazeniumdiolates from a polymer matrix is to change the plasticizer. Plasticizers are often blended with polymers to make the matrix more flexible and promote diffusion of species within the material (e.g., ion selective electrodes[31]). To examine the effect of plasticizer on NO release from PVC films, two plasticizers with different dielectric constants were used: *o*-nitrophenyloctyl ether (NPOE) and dioctyl sebacate (DOS). The formulation of the polymer matrix (i.e., ratio of polymer to plasticizer, incorporation of KTpClPB) remained constant. By using a more polar plasticizer such as NPOE [dielectric constant (ϵ) of 21], not only is water partitioning into the polymer more favorable, leading to an increased source of protons, and therefore a faster rate of NO release, but the intrinsic pK_a of the amine that possesses the diazeniumdiolate group may increase (become more basic) in matrixes prepared with the more polar plasticizer. This would lead to faster NO release from polymer matrixes containing a more polar plasticizer. Indeed, changing the plasticizer type does alter the NO release profile (see Figure 3S in the Supporting Information), where higher initial NO release is observed for **2d** incorporated into a PVC/NPOE matrix compared with a PVC/DOS matrix.

Effect of NO Donor Amount on NO Release. In addition to matrix modifications to alter NO surface flux of materials, changing the amount of lipophilic NO donor that is incorporated within the polymer matrix can also be employed to alter the rate and total amount of NO released. As shown in Figure 7, doubling the NO donor (**2d**) in a PVC film nearly doubles the surface flux. This readily allows the preparation of materials with a

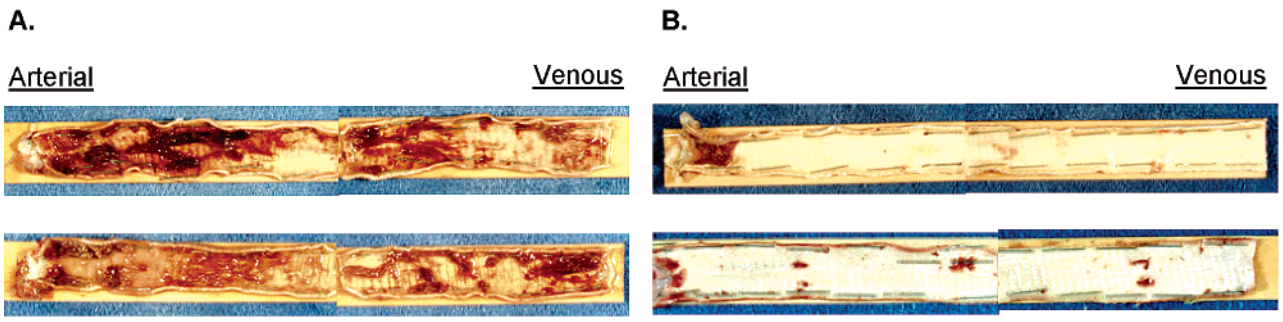

Figure 8. Images of polymer (plasticized PVC) coated Vectra vascular access grafts after removal from 21 d implantation in sheep: control (A) and with NO release (containing **2d**) (B).

wide range of NO surface fluxes from the same polymer matrix.

Stability. One concern with using polymeric materials that contain diazeniumdiolates for medical applications is the stability of the diazeniumdiolates with respect to air and temperature. For example, it has been shown that diazeniumdiolated diaminoalkyltrimethoxylsilane cross-linked poly(dimethylsiloxane) polymer (DACA/N_2O_2) continuously releases NO at room temperature before even being exposed to water, necessitating cold storage (freezer) for optimal stability.[15] For an NO-donor material to be used practically, the shelf-life and storage conditions must be suitable to preserve the diazeniumdiolate moiety. It is preferred that these conditions be close to ambient.

As stated previously, the diazeniumdiolate species under investigation herein remain thermally stable up to 104 °C under a nitrogen environment. To determine if polymer films containing such diazeniumdiolates would also remain stable, NO-release measurements were conducted for films containing 29 wt % PVC, 60 wt % plasticizer (DOS), 4.4 wt % **2d**, and 6.6 wt % KTpClPB under two storage conditions: (a) room temperature under nitrogen and (b) ambient conditions. Initial NO-release measurements were performed for freshly prepared films. Additional NO-release measurements were conducted for both storage conditions after 1, 2, and 4 weeks. Films stored under ambient conditions showed the greatest loss of NO after a 4 week period, achieving only 62% of the theoretical total NO release. This loss of NO could be due to the slow decomposition of the diazeniumdiolate within the polymer matrix owing to permeation of water vapor into the polymer film. Although this loss represents a significant percentage of the total NO-releasing capability of the film, such films still yield NO surface fluxes higher than that of stimulated endothelial cells (i.e., 4×10^{-10} mol cm^{-2} min^{-1}).[32] On the other hand, films stored under a dry nitrogen environment at room temperature maintained 99% of their NO release capability after a 4 week period.

Given the high thermal stability of **2**(**a**−**e**) in a nitrogen environment and the stability of these NO donors embedded into polymer films under appropriate storage conditions, polymer materials prepared with these small molecule diazeniumdiolates and stored properly have the potential to remain shelf stable for extended periods of time.

Preliminary Application to Vascular Grafts. Thrombus formation is a major factor limiting the long-term patency of synthetic vascular grafts used for arterial reconstruction and hemodialysis access.[33] It is hypothesized that NO-releasing biopolymers may prolong graft patency by reducing platelet adhesion and hence thrombus formation on the surface of synthetic vascular grafts.

Synthetic vascular access grafts (Vectra), a proprietary blend of segmented polyetherurethane and siloxane (20 cm in length and 5 mm in diameter), were coated with **2d** dispersed within a PVC/DOS matrix with appropriate additives. A sheep model was used for in vivo testing. Arteriovenous grafts connecting the common carotid artery to the ipsilateral external jugular vein were surgically implanted in a subcutaneous tunnel in adult sheep. Over a three-week period, duplex ultrasound and clinical examination were performed to assess graft patency. Grafts were removed at 21 d and underwent gross and histological evaluation. Each control graft ($n = 2$) occluded prior to 21 d and was found to have a mean luminal thrombus-free surface area of 42%. In contrast, the NO-coated grafts ($n = 2$) were patent at 21 d and had a mean luminal thrombus-free surface area of 95%. Figure 8 clearly shows the gross thrombus formation on the control grafts and the greatly reduced degree of thrombus formation on the NO release grafts. In addition, histological studies, using appropriate stains to highlight different cell regions, confirm the thrombus adherent at the luminal surface and red blood cell infiltration into upper layers of the control grafts (Figure 9). In contrast, the NO release grafts showed minimal thrombus formation and red blood cell infiltration.

These preliminary results strongly suggest that NO-releasing polymers, prepared with more lipophilic diazeniumdiolates reported herein, may prove effective in reducing thrombus formation on prosthetic vascular grafts as well as other bioprosthetic medical devices. This work corroborates results reported by Smith et al. using poly(tetrafluoroethylene) vascular grafts coated with poly(ethylenimine) and prereacted with NO in a baboon model.[14] In that work, vascular grafts coated with the NO-releasing polymer showed greatly reduced platelet deposition onto their surfaces as measured using ^{111}In-labeled platelets.

Significance and Conclusion. The potential advantages of the new more lipophilic diazeniumdiolates described herein for preparing NO release polymeric coatings are numerous. First, the amount of NO donor incorporated into thin polymeric films can be easily controlled, thereby giving different release profiles (e.g.,

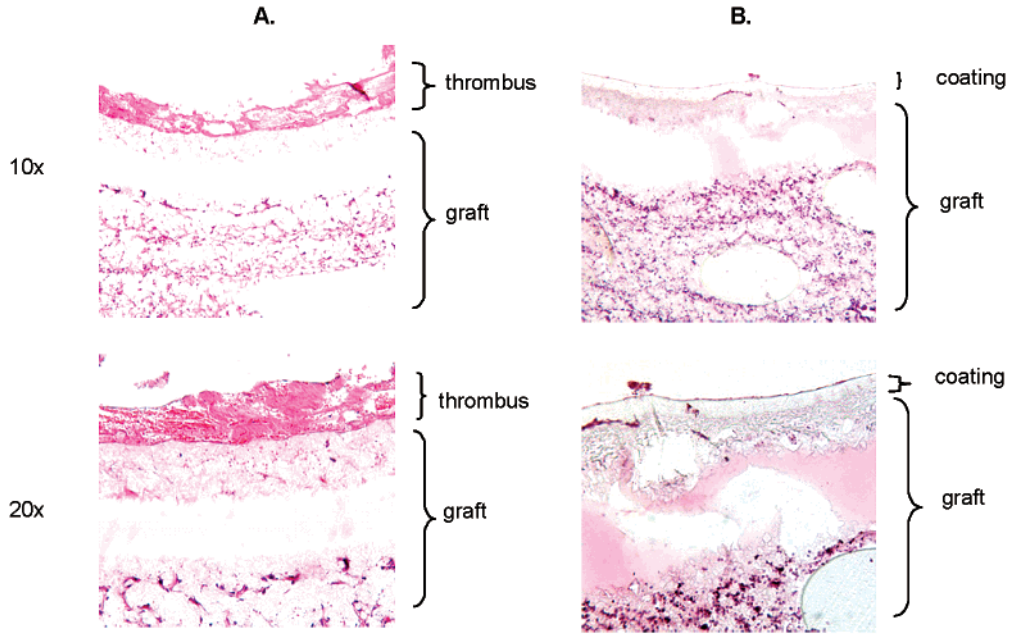

Figure 9. Representative histology images of Vectra vascular access grafts after removal from 21 d implantation in sheep at magnifications of 10× and 20×: control (A) and with NO release (containing **2d**) (B).

NO fluxes), for a given application. In addition, preparation of such coatings is less troublesome than the covalent attachment of NO donors to the polymer backbone. Indeed, with these new materials, thin coatings with high NO loading can be prepared for circumstances where polymer thickness is limited (e.g., coatings for catheters) and NO can be stored until needed and then delivered under physiological conditions. Finally, potential byproducts resulting from diazeniumdiolate decomposition are more confined to the polymer matrix, owing to the increased lipophilicity of these species, thereby reducing the toxicity threat to biological systems.

Many medical devices suffer from blood compatibility issues including platelet adhesion and activation on their surfaces. The ability to synthesize and incorporate NO donors into hydrophobic polymers to prevent such a response is desirable. Currently, systemic anticoagulant treatments are required to minimize the risk of thrombus formation, but this approach has associated with it the increased risk of uncontrolled bleeding elsewhere in the body. The use of the new lipophilic diazeniumdiolates described in this report could prove useful in developing polymeric coatings with greatly improved thromboresistivity, thereby minimizing the need for systemic anticoagulation. Indeed, preliminary studies reported here using NO-releasing arterial grafts in a sheep model strongly support the potential biomedical utility of these compounds.

Experimental Section

Instrumentation. ^{1}H NMR spectra were collected on a Varian Mercury 300 or Inova 400 and were referenced to the residual proton solvent resonance. UV−vis spectra were monitored on a Beckman DU 640B spectrophotometer. FT-IR were collected on Perkin-Elmer SpectrumVX. Thermogravimetric analysis (TGA) was performed on a Perkin-Elmer DSC/TGA 7 under nitrogen. Elemental analyses were performed by the University of Michigan Microanalysis Laboratory and by Atlantic Microlab, Inc.

Reagents. High molecular weight poly(vinyl chloride) (PVC), dioctyl sebacate (DOS), 2-nitrophenyl ether (NPOE), 9-(dimethylamino)-5-[4-(16-butyl-2,14-dioxo-3,15-dioxaeicosyl)-pneynylimino]benzo[*a*]penoxazine (Chromoionophore II), and potassium tetrakis(4-chlorophenylborate) (KTpClPB) were purchased from Fluka (Ronkonkoma, NY). Phosphate-buffered saline (PBS), pH 7.4, containing 138 mM NaCl and 2.7 mM KCl, was obtained from Sigma (St. Louis, MO). *N*-Dodecylamine, *N*-hexylamine, *N*-pentylamine, *N*,*N*′-dibutyl-1,6-hexanediamine (**1d**), *N*,*N*′-dimethyl-1,6-hexanediamine (**1a**), adipoyl chloride, triethylamine (NEt_3), and lithium aluminum hydride (LiAlH) were purchased from Aldrich (Milwaukee, WI). *N*,*N*′-Diethyl-1,6-hexanediamine (**1b**) and *N*,*N*′-dipropyl-1,6-hexanediamine (**1c**) were purchased from Pfaltz and Bauer (Waterbury, CT). Tetrahydrofuran (THF), ethyl acetate, hexane, dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), and acetonitrile (CH_3CN) were products of Fisher (Fair Lawn, NJ). NO was purchased from Matheson Gases. Didodecylhexamethylenediamine (**1g**), dihexylhexamethylenediamine (**1f**), and dipentylhexamethylenediamine (**1e**) were synthesized as described below. All other reagents were analytical reagent grade or better and were used without further modification.

General NO Addition Procedure. The NO-addition process was carried out as described by Hrabie et al.[26] In brief, a dry Parr bottle, equipped with a magnetic stir bar, was charged with the diamine compound dissolved in an appropriate solvent (either CH_3CN or diethyl ether). The reaction vessel was attached to the NO reactor (a modified hydrogenation system) and the headspace purged with argon, up to 1 atm six times, to remove air from the connector lines, and then up to 80 psi argon 25 times over a 1 h period. The solution was then charged with NO up to 80 psi. The solution was allowed to stir between 15 and 24 h during which time a white precipitate formed. The NO was then released and the headspace purged thoroughly with argon. The NO adducts were obtained by filtration and washed three times with either CH_3CN or diethyl ether. The products were finally collected and dried under vacuum.

Molar Extinction Coefficients. Ultraviolet spectra were collected on a Beckman DU 640B spectrophotometer. The samples were prepared by dissolving the NO adduct in 25 mL of 0.0375 M NaOH to make a 0.125 mM diazeniumdiolate solution. The sample was placed into a quartz cuvette and the spectrum was obtained by scanning between 200 and 500 nm. The maximum wavelength and absorbance were collected. The

molar extinction coefficient was then calculated using Beer's Law.

Kinetic Studies. The half-lives and "apparent" half-lives for the diazeniumdiolates under investigation were determined using chemiluminescence. To 3 mL of 100 mM phosphate buffer (pH 7.4) containing 137 mM NaCl and 2.7 mM KCl heated to 37 °C was injected into the reaction cell between 25 and 100 μL of the respective diazeniumdiolate solution in 10 mM NaOH and the NO released was monitored at 0.25 s intervals until baseline NO levels were achieved. From the total NO release, the time at which half of the diazeniumdiolate groups had decomposed was determined to be the half-life. The "apparent" half-lives of the non-water-soluble diazeniumdiolate compounds were determined by dispersing/dissolving the diazeniumdiolate in THF or a mixture of NaOH and MeOH then injecting between 25 and 100 μL of the diazeniumdiolate solution into 3 mL of deoxygenated PBS buffer. The NO released was measured using chemiluminescence. The "apparent" half-lives were calculated as the time taken to release half of the total NO possible based on the total diazeniumdiolate species present.

Preparation of Polymer Films Containing Diazeniumdiolates. Polymer membranes containing the dialkylhexanediamine diazeniumdiolates were prepared by dissolving poly(vinyl chloride) (PVC) and dioctyl sebacate (DOS) (totaling 200 mg) in 1.5 mL of freshly distilled tetrahydrofuran. The diazeniumdiolates were dispersed within the polymer cocktail via sonication for 10 min to obtain a slightly cloudy dispersion of the diazeniumdiolate. The polymer cocktail was then cast into a 2.5 cm diameter Teflon ring on a Teflon base. The membranes were allowed to cure overnight covered. Polymer films containing additives were prepared in a similar manner. Smaller disks were cut from the parent films the next morning and evaluated for their NO release via chemiluminescence.

NO Release Measurements by Chemiluminescence. Nitric oxide measurements were performed using a Sievers Nitric Oxide Analyzer (NOA), model 280. The instrument was calibrated before each experiment using an internal two-point calibration (zero gas and 45 ppm). The measurement was performed by inserting the NO adducts or polymeric films into a clean, dry NOA measurement cell, sealing the cell with a rubber septum, and collecting a baseline level of nitric oxide. Nitrogen-purged PBS buffer was then injected via a syringe through a septum into the NOA measurement cell. The NO generated from the sample was removed from the solution via a constant nitrogen purge. The data were recorded on-line using a Dell computer. The data were processed using Excel. See Supporting Information for details on how the amount of NO was quantified.

pH Experiments. Two polymer solutions containing 1-hydroxy-2-oxo-3-(*N*-butyl-6-aminohexyl)-3-butyl-1-triazene (**2d**) were prepared by dissolving 66 mg of PVC and 134 mg of DOS in 1.5 mL of THF. To one sample was added 15.7 mg of KTpClPB (cocktail 1). (*Z*)-1-{*N*-Butyl-*N*-[6-(*N*-butylammoniohexyl)-amino]}diazen-1-ium-1,2-diolate (10 mg) was added and dispersed within each of the polymer solutions via sonication. To a 25 μL aliquot (5.8×10^{-7} mol of diazeniumdiolate) of the cocktail without KTpClPB were added 75 μL of a 1:2 PVC/DOS solution and 1.25 μL (4.0×10^{-9} mol) of 2 mM KTpClPB along with 6.81 μL (1.8×10^{-8} mol) of a 1.25 mM Chromoionophore II solution. The 2 mM KTpClPB solution was prepared by dissolving 4.95 mg of KTpClPB in 5 mL of THF, and the 1 mM Chromoionphore II solution was prepared by dissolving 1.47 g of Chromoionphore II in 2 mL of THF. The aliquot was vortexed and cast onto quartz slides. To a 100 μL (2.3×10^{-6} mol) aliquot of the cocktail with KTpClPB was added 14.5 μL (1.8×10^{-8} mol) of Chromoionophore II. The solution was vortexed thoroughly and cast onto a quartz slide. The slide was immobilized within a cuvette, 2 mL of PBS added, and the spectrum recorded from 400 to 800 nm. Additional spectra were recorded with time.

Thermal Gravametric Analysis. TGAs were obtained by increasing the temperature in an nitrogen environment slowly.The data were recorded on Perkin-Elmer DSC/TGA 7.

(Z)-1-{N-Ethyl-N-[6-(N-ethylammoniohexyl)amino]}-diazen-1-ium-1,2-diolate (2b) was synthesized by treating *N,N′*-diethyl-1,6-hexanediamine (2 mL, 9.54 mmol) in 250 mL of CH_3CN with NO for 22 h: yield 0.152 g (6.8%); mp 118 °C; ^{1}H NMR (0.01 M NaOD) δ 0.67 (6 H, tt); 1.11 (6 H, s); 1.51 (2 H, s); 2.29 (4 H, t), 2.65 (4 H, t); ^{13}C NMR δ 11.1, 13.9, 25.9, 26.0, 26.2, 28.2, 42.8, 48.3, 49.0, 54.1. Anal. $C_{10}H_{24}N_4O_2$ (C, H, N).

(Z)-1-{N-Propyl-N-[6-(N-propylammoniohexyl)amino]}-diazen-1-ium-1,2-diolate (2c) was synthesized by treating *N,N′*-dipropyl-1,6-hexanediamine (2 mL, 8.20 mmol) in 200 mL of CH_3CN with NO for 17 h: yield 0.336 g (15.7%); mp 120 °C; ^{1}H NMR (0.01 M NaOD) δ 0.68 (6 H, t); 0.1.12 (6 H, s); 1.56 (4 H, s); 2.29 (4 H, m), 2.71 (4 H, m); ^{13}C NMR δ 11.1, 11.3, 19.8, 22.0, 25.8, 26.0, 26.2, 28.4, 48.7, 50.8, 54.2, 56.0. Anal. $C_{12}H_{28}N_4O_2$ (C, H, N).

(Z)-1-{N-Butyl-N-[6-(N-butylammoniohexyl)amino]}-diazen-1-ium-1,2-diolate (2d) was synthesized by treating *N,N′*-dibutyl-1,6-hexanediamine (1.5 mL, 5.40 mmol) in 250 mL of CH_3CN with NO for 16.5 h: yield 0.504 g (32.7%); mp 120 °C; ^{1}H NMR (0.01 M NaOD) δ 0.72 (6 H, m), 1.15 (12 H, m), 1.26 (6, m), 2.34 (4 H, m), 2.72 (4H, t); ^{13}C NMR δ 15.78, 15.86, 22.20, 22.50, 28.37, 28.65, 28.93, 30.65, 30.94, 33.39, 50.87, 51.09, 56.46, 56.63. Anal. $C_{14}H_{32}N_4O_2$ (C, H, N).

(Z)-1-{N-Pentyl-N-[6-(N-pentylammoniohexyl)amino]}-diazen-1-ium-1,2-diolate (2e) was synthesized by treating *N,N′*-dipentyl-1,6-hexanediamine **(1e)** (0.7321, 2.86 mmol) in 20 mL of CH_3CN with NO for 22 h: yield 0.420 g (46.4%); mp 113 °C; ^{1}H NMR (CD_3OD) δ 0.92 (6 H, t); 1.33 (16 H, m); 1.52 (4 H, m); 2.53 (4 H, m), 2.84 (4 H, m). Anal. $C_{18}H_{36}N_4O_2$ (C, H, N).

Hexanedioic acid bis-pentylamide (3e) was synthesized by equipping a dry 250 mL three-neck flask with a condenser, addition funnel, stir bar, and N_2 inlet/outlet and charging it with *N*-pentylamine (15 mL, 0.129 mol) and triethylamine (30 mL, 0.215 mol) in $CHCl_3$ (125 mL). Adipoyl chloride (8.8 mL, 0.0605 mol) in $CHCl_3$ was added dropwise over 20 min, during which time a white precipitate formed. After 3 h, the solvent was removed under vacuum to give a white solid. The solid was stirred in hot water for 30 min and filtered. The solid was washed with additional water followed by acetonitrile. The white solid was collected and dried under vacuum: yield 12.33 g (71%); mp 152 °C; ^{1}H NMR δ 0.89 (6 H, t), 1.3 (8 H, m), 1.45−1.50 (4 H, m), 1.66 (4 H, m), 2.1−2.3 (4 H, m), 3.2 (4 H, m), 5.7 (2 H, s); ^{13}C NMR δ 14.1, 22.0, 25.3, 29.2, 37.3, 40.0, 172. MS (CI) = $[M + H]^+$ = 285.2532. Anal. $C_{16}H_{32}N_2O_2$ (C, H, N).

***N,N′*-Dipentylhexane-1,6-diamine (1e)** was synthesized by charging a dry 250 mL three-neck flask equipped with a condenser, stir bar, and N_2 inlet/outlet with lithium aluminum hydride (LiAlH) (2.82 g, 74.3 mmol) in dry THF (150 mL). Hexanedioic acid bis-pentylamide **(3e)** (4.32 g, 15.1 mmol) was carefully added as solid portions over 30 min. The reaction was heated to reflux for 16 h. The reaction flask was placed in an ice bath, and the LiAlH was carefully quenched with 100 mL of 1 M sodium potassium tartrate. The mixture was filtered, and the solid residue was washed with ethyl acetate (100 mL). The aqueous phase was extracted with ethyl acetate several times. The organic portions were combined and dried over $MgSO_4$. The solvent was removed to give an oil, which was purified via vacuum distillation (0.3 mmHg at 120 °C). The colorless liquid solidified upon standing. The white solid was dried under vacuum to give 2.26 g (60%) of **1e**: ^{1}H NMR δ 0.88 (6 H, t), 1.27−1.43 (12 H, m), 1.49 (8 H, m), 2.58 (8 H, tt). ^{13}C NMR δ 14.1 (2), 22.6 (2), 27.4 (2), 39.9 (2), 30.5 (2), 30.9 (2), 50.5 (4). MS (CI with ammonia) = $[M + H]^+$ = 257.3. Anal. $C_{16}H_{36}N_2$ (C, H, N).

Acknowledgment. The authors are grateful for funding from the National Institutes of Health through grant NIH EB-00783, and Michigan Critical Care Consultants, Inc.

Supporting Information Available: UV spectra for **2d**, NO surface flux and release curves for **2d**, and details of NO release measurements by chemiluminescence. This material is available free of charge via the Internet at http://pubs.acs.org.

Note Added after ASAP Posting. This manuscript was released ASAP on 10/29/2003 with a missing Supporting Information paragraph. The correct version was posted on 10/31/2003.

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JM030286T