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Dendrimers as a Scaffold for Nitric Oxide Release

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Abstract: The preparation and characterization of nitric oxide (NO)-releasing dendrimer conjugates are reported. Generation 3 and 5 polypropylenimine dendrimers (DAB-Am-16 and DAB-Am-64) were modified at the exterior to impart different amine functionalities. The ability to store NO on a dendritic scaffold using *N*-diazeniumdiolate NO donors was examined via the reaction of primary amine, secondary amine, and amide functionalities with high pressures of NO (5 atm). The secondary amine dendrimer conjugates exhibited a high storage capacity for NO (up to 5.6 μmol NO/mg), greatly increasing the "payload" of released NO over existing macromolecular NO donors. The mechanism of diazeniumdiolate decomposition was proton initiated, generating NO spontaneously under physiological conditions (pH 7.4, 37 °C). The NO release durations (>16 h) observed for the secondary amine dendrimers were significantly longer compared to small molecule alkyl secondary amine diazeniumdiolates, thus illustrating a dendritic effect on NO release kinetics. The multivalent exterior of dendrimers allows for the future combination of NO donors and other functionalities on a single molecular scaffold, enabling diverse utility as NO storage/delivery systems.

Introduction

Nitric oxide (NO), a diatomic free radical produced in the human body, regulates several biological functions in the cardiovascular, respiratory, and nervous systems.[1,2] Nitric oxide is also actively involved in the immune system response and mediates macrophage destruction of foreign pathogens.[3] The complex and wide ranging roles of NO in normal physiological function thus demand methods for chemically storing and releasing NO in a controlled manner. Metal complexes, nitrosothiols, nitrosamines, and diazeniumdiolates are all examples of molecular structures that have been developed as effective NO donors.[4] Such "NO donors" facilitate the improved understanding of NO's function in biological systems and may potentially serve as therapeutic agents for a number of disease states.[5,6]

Diazeniumdiolate NO donors are particularly attractive because they dissociate spontaneously under physiological conditions (i.e., 37 °C, pH 7.4) to yield two moles of NO per mole of NO donor.[7] *N*-bound diazeniumdiolates formed from the reaction of amines with high pressures of NO were initially reported by Drago and Paulik in the 1960s.[8,9] The rapid discovery of NO's role in biology has resulted in the resurgence of amine based diazeniumdiolate NO donors. Keefer and co-workers have reported the synthesis and characterization of multiple small molecule diazeniumdiolates, where NO release rates are governed by the chemical structure of the amine precursor.[7,10,11] For example, diazeniumdiolate derivatives of the polyamine diethylenetriamine (DETA/NO) resulted in lengthy durations of NO release with a half-life ($t_{1/2}$) of 20 h under physiological conditions while in contrast PROLI/NO has a $t_{1/2}$ of only 3 s.[7]

Larger molecular frameworks have also been modified with diazeniumdiolate NO donors to produce materials capable of storing large quantities of NO. Pulfer et al. synthesized diazeniumdiolated poly(ethylene imine) microspheres which were embedded into the pores of small-diameter prosthetic grafts to prevent thrombosis via the controlled release of NO.[12] Similarly, Meyerhoff and co-workers reported the synthesis of fumed silica particles with aminoalkoxysilanes grafted on the surface as substrates for diazeniumdiolate formation. The storage of NO was mediated by the type of aminosilane grafted and the silica particles prepared via this method released up to 0.56 μmol NO/mg.[13] These NO-releasing silica particles were initially employed as fillers for preparing silicone rubber coatings for extracorporeal circuits.[13] Hrabie et al. synthesized a water soluble macromolecular NO donor with a $t_{1/2}$ of 20 d via covalent attachment of a NO donor piperazine ligand to the solvent accessible lysine residues of bovine serum albumin.[14] Nitric oxide release was initiated via the slow hydrolytic

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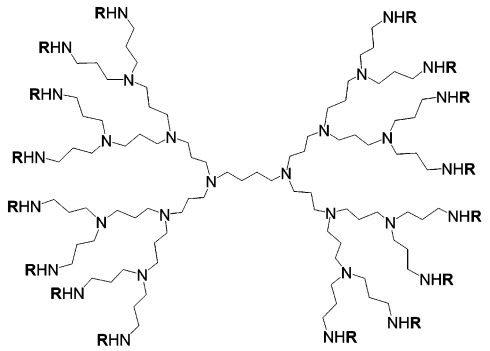


Figure 1. Generation 3 polypropylenimine dendrimer, DAB-Am-16, possessing a diaminobutane core and 16 primary amines where R = H.

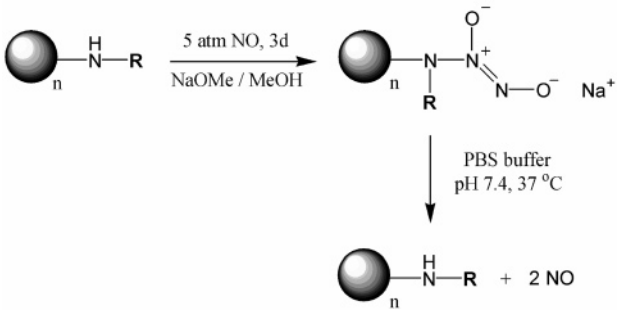
cleavage of the methoxymethyl protecting group. Proteins modified with diazeniumdiolate ligands demonstrate a strategy for increasing the stability and systemic half-life of NO donors in vivo.

Rothrock et al. recently reported the synthesis of NO-releasing gold monolayer protected clusters (MPCs) through diamine functionalization of bromine thiol ligands, followed by subsequent amine conversion to diazeniumdiolates.[15] Although capable of releasing NO, the NO donor-modified nanoparticles were characterized by poor amine to diazeniumdiolate conversion efficiencies, lack of aqueous solubility, and limited surface functionality. Such factors warrant the development of more efficient, multifunctional nanoparticle NO donor scaffolds.

In contrast to gold and silica particles, dendrimers are hyperbranched nanostructures possessing a multivalent surface with well-defined polymeric structure.[16,17] Dendrimers are assembled by one of two general strategies: (1) the divergent method, where repeat monomers are branched outward from a central core; or, (2) the convergent method developed by Frechet et al.,[18] where the synthesis begins at the periphery and grows inward. The resulting dendrimer products are monodisperse compared to bulk polymers, and their size (2−20 nm) varies with dendrimer type and exterior functionality.[19] The structure of a generation 3 polypropylenimine dendrimer (DAB−Am-16) is shown in Figure 1. Produced via divergent synthesis, this framework has become a widely utilized construct due to its easily modified, primary amine exterior.[17,20] The multivalent surface and unique structural properties of dendrimers have resulted in their widespread utility as drug delivery agents and medical diagnostic tools in the pharmaceutical and nanotechnology industries.[19,21−23]

Herein, we report on the development of nanoparticle NO donors that store and release large quantities of NO from dendritic scaffolds (Scheme 1). The primary advantage of NO releasing dendrimers over other NO donor systems is the ability

Scheme 1. Formation of Sodium Stabilized Diazeniumdiolates Followed by Decomposition under Physiological Conditions to Yield 2 mol of NO and Initial Dendrimer Conjugate (*n*=16, 64)



to store high concentrations of NO on a single molecular framework. The exterior of NO releasing dendrimers can also be manipulated to enable specific functionality (e.g., solubility) producing macromolecular NO donors tailored for a desired application. For example, dendrimer-based NO donors possessing lipophilic exteriors may be incorporated into hydrophobic polymers to impart thromboresistivity. Water soluble, hydrophilic dendrimers may allow for the development of therapeutic agents capable of delivering NO in vivo. The synthesis and NO storage capacity of several polypropylenimine dendrimer conjugates are presented. Furthermore, dendritic NO release kinetics are explored as a function of dendrimer size, structure of dendrimer bound amine precursor, and lipophilicity of the dendritic exterior.

Results and Discussion

Nitric Oxide Releasing Primary Amine Dendrimers. Dendrimers provide an attractive scaffold for storing NO because of their multivalent exterior. A dendrimer fully modified with diazeniumdiolates has the potential to store large quantities of NO on a single framework, thereby increasing the "payload" of NO per gram of substrate. Previously, polyamines have been shown to convert readily to diazeniumdiolates.[11] Secondary amines in the polyamine backbone react with NO to form a diazeniumdiolate NO donor that is stabilized via neighboring cationic amines. The diazeniumdiolate adduct of diethylenetriamine, a short polyamine, is depicted in Figure 2A. In addition to increasing the stability of the zwitterionic species, remote amines enhance the duration of NO release by providing alternate sites of protonation, thus slowing the proton driven dissociation of diazeniumdiolates to NO.[10] Polypropylenimine dendrimers are large polyamines grown in a divergent fashion, terminated by a multitude of primary amines based on the dendrimer generation. As such, the exterior of a polypropylenimine dendrimer has both a large number of nucleophilic amines to react with NO, and a multiple of neighboring amine sites for diazeniumdiolate NO donor stabilization (Figure 2B).

Primary amines typically form unstable NO donors and decompose rapidly to NO under ambient or aqueous conditions.[9]

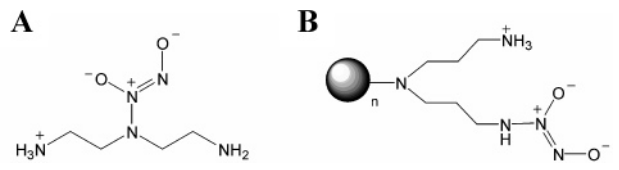


Figure 2. Ammonium cation stabilized diazeniumdiolates: (A) diazeniumdiolate of diethylenetriamine, DETA/NO;[11] and, (B) dendrimer bound diazeniumdiolate where *n* = 8 or 32 (DAB-Am-16 or DAB-Am-64).

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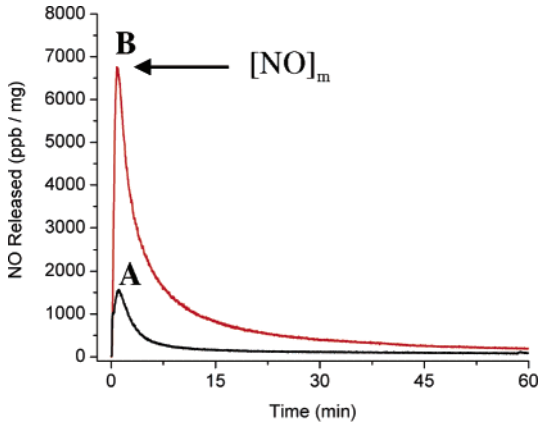


Figure 3. Real time NO release profiles for **1** (DAB-Am-16) charged in (A) MeOH and (B) NaOMe/MeOH.

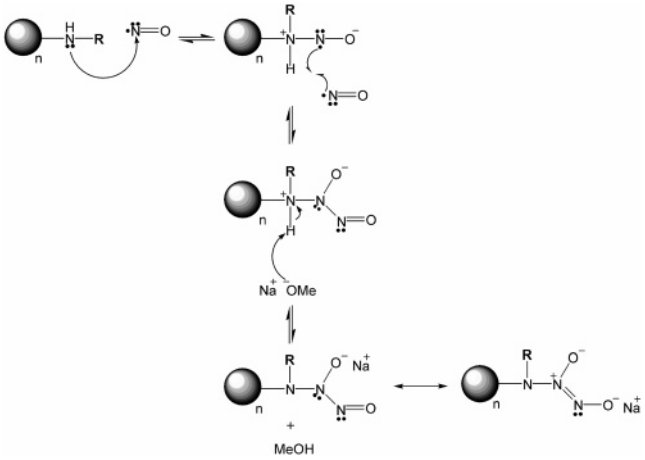
However, the density of primary amines at the exterior and the conformational freedom of the dendritic architecture may lead to enhanced diazeniumdiolate stability over small molecule primary amine substrates. To test the ability of primary amine terminated dendrimers to store NO via diazeniumdiolate NO donors, generation 3 polypropylenimine dendrimer (DAB-Am-16) was selected as an initial substrate for diazeniumdiolate conversion. DAB-Am-16 (**1**) was dissolved in MeOH and exposed to high pressures (5 atm) of NO for 3 d. Herein, this process will be referred to as "charging," or the introduction of the multiply charged, zwitterionic diazeniumdiolate moiety (Scheme 1). An aliquot of the charged MeOH solution containing **1** (200 μM) was added to a reaction flask containing degassed phosphate buffered saline (PBS; 0.01 M, pH 7.4) and the corresponding NO release was monitored at physiological temperature (37 °C) via chemiluminescence.[24] Despite an initial burst of NO (Figure 3A), the polyamine dendrimer failed to yield the lengthy NO release durations of the polyamine diazeniumdiolate DETA/NO.

The conversion efficiency of amine precursor to diazeniumdiolate NO donor was calculated to examine the effect of the dendritic structure and amine functionality on NO storage capacity. Assuming the maximum theoretical yield of two moles of NO per one mole of amine precursor, the conversion efficiency is defined as the total moles of NO released divided by twice the number of dendrimer bound substrate amines (eq 1).

$$\%\ \text{conversion} = \frac{\text{moles NO released}}{2\times\ \text{moles amine}} \qquad (1)$$

The conversion efficiency for **1** exposed to NO under these conditions was poor (<1%). Zhang et al. previously reported that the addition of methoxide base during NO exposure shifts the reaction equilibrium toward diazeniumdiolate formation by deprotonating the amine and stabilizing the diazeniumdiolate via a countercation (e.g., sodium), thereby increasing the NO addition efficiency on a macromolecular scaffold.[13] Primary amine dendrimer **1** exposed to NO in 0.5 M NaOMe/MeOH released greater amounts of NO over preparation in MeOH alone (Figure 3B), with a maximum flux of NO release ($[NO]_m$) of 6760 ppb/mg. Control NaOMe/MeOH solutions did not release

Scheme 2. Mechanism of Diazeniumdiolate Formation on Dendrimer Bound Amine Functionalities under Basic Conditions (Adapted from Drago et al.)[9]



NO. Hereafter, all NO releasing dendrimer conjugates were prepared under basic conditions as shown in Scheme 1.

A similar NO release profile was observed for the sodium stabilized primary amine diazeniumdiolates of **2** (DAB-Am-64) charged under identical conditions. As shown in Table 1, the total NO released (t[NO]) and conversion efficiency were slightly greater for DAB-Am-64/NO, suggesting diazeniumdiolate formation in methanolic solution favors the larger, more compact globular structure. Also, the abundance of neighboring amine sites on the larger dendrimer slowed diazeniumdiolate decomposition, as evidenced by a $t_{1/2}$ of 29 min for DAB-Am-64/NO compared to 12 min for the smaller DAB-Am-16/NO. Although DAB-Am-16 and DAB-Am-64 primary amine dendrimers released NO at levels similar to previously reported macromolecular NO donors,[12−14] the conversion efficiencies of the NO donor-modified dendrimers were low, failing to harness the anticipated storage capacity offered by a dendritic scaffold.

Studies were also conducted to evaluate whether the primary amine NO donors release nitroxyl (HNO) and the effect of HNO release (in place of NO) on the NO donor conversion efficiencies. Nitroxyl has garnered recent attention due to its importance in the pharmacological treatment of heart failure.[25] Miranda et al. reported that the primary amine diazeniumdiolate adduct of isopropylamine (IPA/NO) represents an attractive HNO donor at physiological pH.[25] Further studies by Dutton et al. have confirmed the pH dependent production of HNO and NO from primary amine diazeniumdiolate adducts using computational methods.[26] At neutral pH (7.4), primary amine adducts are predicted to release HNO, whereas at lower pH (3), decomposition follows the traditional diazeniumdiolate pathway and generates 2 equiv. of NO.[26] Since nitroxyl primary amine adducts release NO at acidic pH, citric acid buffer (pH 3) was employed to assess whether HNO formation was responsible for the poor NO donor conversion efficiencies observed for DAB-Am-16/NO. A 67% increase in NO was detected at the lower pH, suggesting that HNO is indeed produced. Studies

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Scheme 3. Synthesis of Polypropylenimine Dendrimer Conjugates. (A) DAB-C7-16 and DAB-C7-64; (B) DAB-PO-64; and, (C) DAB-Ac-16 and DAB-Ac-64.

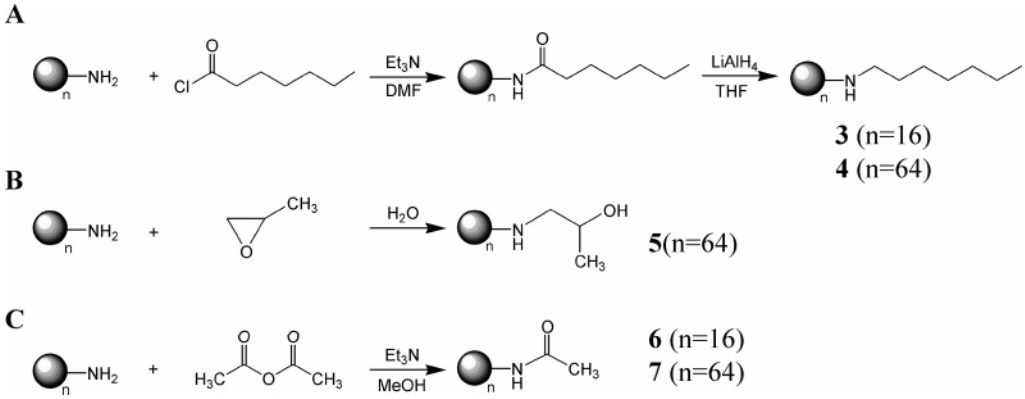
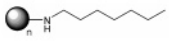
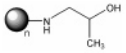
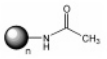


Table 1. Summary of NO-Release Properties for Dendritic NO Donors

Dendrimer	Functionality	t[NO] (mmol NO/mg)	$[NO]_m$ (ppb/mg)	$t_{1/2}$ (min)	% Conv
1 (DAB-Am-16)		0.44	6760	12	2.3
2 (DAB-Am-64)		0.69	7460	29	3.9
3 (DAB-C7-16)		3.5	11100	77	38
4 (DAB-C7-64)		3.2	15600	86	36
5 (DAB-PO-64)		5.6	53100	28	47
6 (DAB-Ac-16)		0.02	6630	1.4	<0.2
7 (DAB-Ac-64)		0.02	3720	2.5	<0.2

are underway to further quantitate the HNO release from primary amine dendrimer diazeniumdiolates and to explore the potential of dendrimers as novel HNO donors.

Nitric Oxide Releasing Secondary Amine Dendrimer Conjugates. Secondary amines convert to diazeniumdiolates more readily than primary amines since they form more stable adducts.[9] The addition of an electron donating substituent, **R**, serves to both enhance the acidity of the amino nitrogen and provide electron density to stabilize the zwitterionic diazeniumdiolate product (Scheme 2).[27] Several secondary amine dendrimer conjugates were synthesized to assess the potential for increasing the NO storage on a dendritic scaffold. Modifications to yield both hydrophobic and hydrophilic NO releasing dendrimers were explored.

DAB-C7-16 (3). Generation 3 polypropylenimine dendrimer **1** was acylated with heptanoyl chloride and the corresponding amide was reduced with $LiAlH_4$ to produce DAB-C7-16, **3**, an alkyl terminated dendrimer (Scheme 3A). Following purification on an Al_2O_3 column, confirmation of the seven carbon alkyl secondary amine functionalization was obtained via ^1H and ^{13}C NMR. The NMR chemical shifts matched those of previously reported octyl (C8) functionalizations of other generation polypropyelenimine dendrimers.[28] The alkyl secondary amine dendrimer was exposed to NO (5 atm, NaOMe/MeOH) to form DAB-C7-16/NO. This NO donor-modified dendrimer exhibited high yields of NO release upon addition to PBS (pH 7.4, 37 °C). Indeed, the t[NO] from DAB-C7-16/NO corresponded to nearly 40% conversion of secondary amines to diazeniumdiolate NO donors (Table 1). The 3.5 μmol NO/mg released for the alkyl secondary amine dendrimer was a sizable increase over the NO released from the primary amine substrates. Confirmation of the diazeniumdiolate functional group was obtained via UV−vis spectroscopy. As shown in Figure 4, DAB-C7-16/NO exhibits a λ_{max} of 252 nm, characteristic of the diazeniumdiolate absorption maximum.[11]

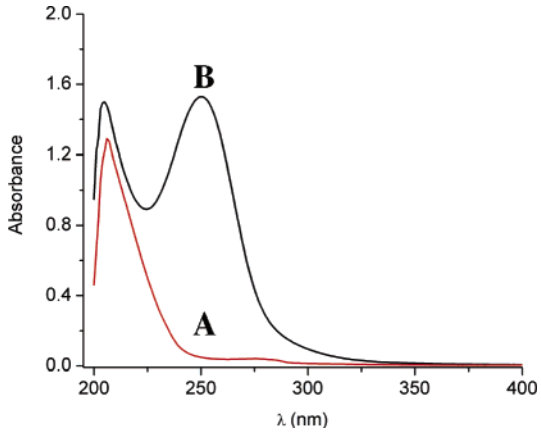


Figure 4. UV−vis spectra collected in MeOH for (A) DAB-C7-16 dendrimer conjugate and, (B) the diazeniumdiolate functionalized dendrimer DAB-C7-16/NO.

Similar to the primary amine dendrimers, the alkyl conjugate was characterized by an initial burst of NO release, reaching $[NO]_m$ in just minutes (Figure 5). Subsequently, significant levels of NO continued to be released for up to 14 h, albeit at a lesser rate. Previous work by Price et al. indicated that the diazeni-

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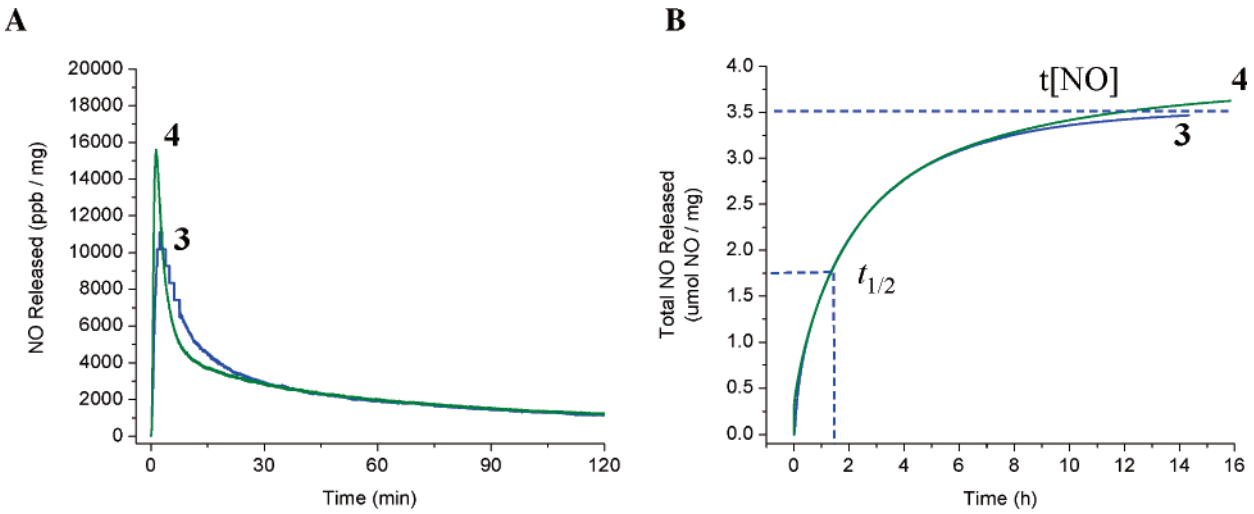

Figure 5. (A) Real time NO release profile for NO-releasing dendrimer conjugates of **3** and **4**; and, (B) plot of t[NO] vs time for conjugates of **3** and **4** depicting values for the $t_{1/2}$ for DAB-C7-16/NO and long duration of NO release.

umdiolate decomposition rate constant (k_{obs}) and half-life for the secondary alkylamine dipropylamine diazeniumdiolate (DPA/NO) were 4.45×10^{-3} s^{-1} and 2.6 min, respectively.[29] Since the diazeniumdiolate structures for both DPA/NO and the alkyl secondary amine dendrimer NO donors (DAB-C7-16/NO) are nearly equivalent (secondary amine diazeniumdiolates bracketed by three alkyl carbons on each side), similar NO release would be expected. However, the surrounding chemical environments of the two different diazeniumdiolate NO donors are drastically different. The dendritic effect on the duration of NO release is evident from the lengthy NO release duration of DAB-C7-16/NO ($t_{1/2}$ = 77 min). The enhanced duration of NO release is attributed to increased local pH upon regeneration of free secondary amines. The effective decrease in proton concentration at or nearby the diazeniumdiolate NO donors results in slower dissociation rates and longer overall durations of NO release.

DAB-C7-64 (4). The structure of DAB-C7-16 in solution and the hydrophobic packing of the alkyl tails may affect both the extent of diazeniumdiolate formation and rates of NO release. The primary amine dendrimer with 64 exterior functionalities, **2**, exhibited slightly greater NO release properties than **1**, the smaller dendrimer. To probe the size dependence and effects of alkyl secondary amine density on NO storage, **4** (DAB-C7-64) was synthesized using generation 5 polypropylenimine dendrimer as the substrate. Changing the dendrimer size from generation 3 to 5 showed little effect on the conversion efficiency and t[NO] for DAB-C7-16/NO and DAB-C7-64/NO (Table 1). The maximum of near 40% conversion achieved for both dendrimer sizes is attributed to the Coulombic repulsion of multiple anionic functional groups upon diazeniumdiolate formation. Additionally, the formation of a zwitterionic species in a primarily hydrophobic environment may also contribute to the observed limit of conversion efficiency.

As shown in Figure 5, the NO release profiles appeared remarkably similar for both alkyl conjugates. However, DAB-C7-64/NO was characterized by a greater $[NO]_m$ and longer $t_{1/2}$. The dissimilarity of the two release profiles at early times (<30 min) indicates a slight size dependent effect on the initial rates of NO release. At longer times, DAB-C7-16/NO and DAB-C7-64/NO adopted similar rates of diazeniumdiolate decomposition.

Synthesis of more lipophilic diazeniumdiolate NO donors has been the goal of other studies aimed at developing NO-releasing hydrophobic polymer coatings.[30] Such coatings have been used to improve the thromboresistivity of intravascular sensors. Dendrimers possessing alkyl secondary amines represent novel NO releasing macromolecules with increased lipophilicity over traditional NO donors, and may elicit similar potential as polymer dopants for preparing NO-releasing polymers. The larger payload of NO and increased duration of NO release from a dendritic scaffold may prove advantageous in formulating NO release coatings with extended NO release properties.

Macromolecular NO Release Kinetics. The extended duration of NO release observed for DAB-C7-16/NO and DAB-C7-64/NO indicates a likely deviation from pseudo first-order kinetics reported for the dissociation of small molecule alkyl secondary amine diazeniumdiolates. Zhang et al. previously reported a method for determining an "apparent" reaction order (n) of diazeniumdiolate dissociation from silica-based NO-releasing macromolecules.[13] To determine the reaction order for dendrimer-bound diazeniumdiolate decomposition, ln υ vs ln C_d graphs were plotted, where υ represents the dissociation rate of the *N*-diazeniumdiolate moiety ($-dC_d/dt$) and C_d is the *N*-diazeniumdiolate content (μmol/mg) of the dendritic NO donors. The reaction order (n) is given by the slope of this plot.[13] After the initial burst of NO release, both DAB-C7-16/NO and DAB-C7-64/NO shared the same order of reaction (n = 1.89) for diazeniumdiolate decomposition. The relatively high reaction order observed for the dendrimer bound alkyl secondary amine diazeniumdiolates (n = 1.89) is comparable with the value reported for the alkyl secondary amine diazeniumdiolates on the surface of fumed silica (n = 1.75 for 1N−C1).[13] These data illustrate the complexity of macromolecular NO release kinetics and the dependence on the pH, lipophilicity of the local chemical environment, and nanoparticle solution structure.

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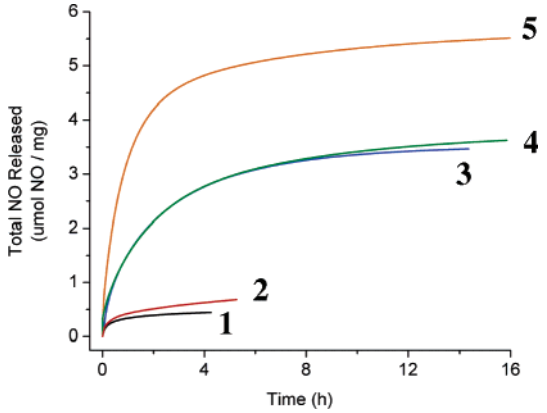

Figure 6. Total NO release profiles for NO releasing dendrimers 1−5.

DAB-PO-64 (5). The therapeutic implications of NO are well understood,[4,31,32] but failure to deliver NO to a specific cell or tissue type of interest has limited the use of NO donors as therapeutic entities. Dendrimers offer a solution to traditional drug delivery problems because of their tailored solubility, globular solution behavior, and highly functionalizable exterior. The biocompatibility of several dendritic structures, including polyester dendrimers synthesized by Frechet and co-workers possessing possess primary alcohols at the exterior, have been reported as acceptable for drug delivery applications.[23]

A water-soluble, hydrophilic dendrimer with terminal hydroxyl groups and secondary amines required for efficient storage of NO was synthesized to demonstrate a potential delivery vehicle for the controlled release of NO in vivo. Generation 5 polypropylenimine dendrimer **2** was reacted with propylene oxide under aqueous conditions to yield **5** (DAB-PO-64) (Scheme 3B).[33] Functionalization was confirmed via ^{1}H, ^{13}C NMR, and ESI−MS. Notably, the NO releasing conjugate DAB-PO-64/NO displayed the largest t[NO] of the dendrimer conjugates synthesized to date (5.6 μmol NO/mg, 46% conversion) (Figure 6). The enhanced storage capacity of the hydrophilic dendrimer is likely attributed to a fully extended solution structure of the dendrimer in the polar methanol solvent used during NO exposure. Indeed, the structure of polypropylenimine dendrimers are known to be responsive to solvent,[34] thereby affecting changes in globular solution behavior. Compared to the more lipophilic dendrimer of similar size (DAB-C7-64/NO), DAB-PO-64/NO was characterized by a greater $[NO]_m$ (53 100 versus 15 600 ppb/mg). DAB-PO-64/NO also exhibited prolonged NO release for up to 16 h. As was the case with the alkyl secondary amine dendrimers, the dendrimer bound diazeniumdiolates seem to adopt a slower rate of dissociation at longer times. The reaction order for DAB-PO-64/NO decomposition was calculated to be 1.71. The slight shift toward first-order kinetics is attributed to the hydrophilic nature of DAB-PO-64/NO and NO donor dissociation closer to that of small molecule diazeniumdiolates.

DAB-Ac-16 and DAB-Ac-64 (6 and 7). To demonstrate control over the NO storage potential of multivalent dendrimers, acetamide functionalities were introduced via the reaction of primary amines with acetic anhydride in the presence of triethylamine (Scheme 3C). The functionalization was confirmed by ^{1}H, ^{13}C NMR, and ESI−MS analysis. The absence of a nucleophilic amine required for diazeniumdiolate formation makes amides an unfavorable moiety for storing NO. Following NO charging, both acetamide dendrimers (**6** and **7**) exhibited low NO release and a short half-life ($t_{1/2}$ < 3 min) (Table 1). The trace amount of NO observed (<0.2% conversion) is attributed to NO donor formation at residual primary amine sites (from incomplete acetamide functionalization) and/or loosely associated NO within the tertiary amine backbone structure. The lack of amide reactivity toward NO suggests that the acetamide functionality may prove useful in future applications for stoichiometrically limiting the number of NO donor functional groups. Acetamide modifications have been used previously to control functionality for other dendrimer constructs.[35,36] In addition to tuning the storage capacity of NO, the amide exterior may enable increased water solubility and eliminate the toxicity of primary amine dendrimer constructs.[35,37]

Proton Initiated Diazeniumdiolate Dissociation. The sustained duration of NO release observed from the secondary amine dendrimer conjugates at extended periods (>12 h) indicates a likely deviation from the first-order exponential decay reported for the kinetics of small molecule alkyl secondary amine diazeniumdiolates.[10] Consistent with the mechanism of decomposition for small molecule diazeniumdiolates,[10,27] the buffer pH influenced the kinetics of NO release from the dendrimer conjugates. As expected, NO release from DAB-C7-64/NO was more rapid under acidic conditions (pH 3) and slowed drastically at pH > 11 (Supporting Information). To test the contribution of thermal degradation on NO release, decomposition was measured under an inert nitrogen atmosphere as a function of temperature. At 37 °C, negligible NO was released from the NO donor-modified dendrimer conjugates. Upon increasing the temperature to 70 °C, NO release was initiated albeit at an extremely low rate (∼0.5 ppb/s). After 60 min, degassed PBS buffer was added to the sample, resulting in a rapid release of NO (Supporting Information). Such behavior, combined with the pH dependent dissociation confirms that the dominate mechanism of NO release for dendrimer conjugates was proton initiated. Dendrimers stored under basic conditions at −20 °C retained 96% (DAB-C7-16/NO) of the stored NO as diazeniumdiolate after 3 wks. Future studies aim to elucidate how diazeniumdiolate structure, solvent behavior, and remote amine sites alter the local dendritic environment and influence the rate of diazeniumdiolate dissociation. These parameters are essential for predicting NO release kinetics from macromolecular NO donors. Efforts to develop a model for evaluating the structure−activity relationships of macromolecules and NO release rates will be of importance for designing NO releasing materials bearing therapeutic significance.

Conclusions

A new class of NO-releasing macromolecules was prepared from primary and secondary amine functionalized dendrimers.

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Table 2. Summary of NO-Releasing Macromolecules Reported in the Literature.

diazeniumdiolated species	NO released (μmol NO/mg)	$t_{1/2}$ (min)	ref
gold nanoparticles (C6−HD)	0.09	68	15
PEIX/NO microspheres	0.19	3.96×10^3	12
proteins (BSA)	0.54	1.72×10^5	14
fumed silica (2N[6]-N_2O_2)	0.56	43	13
DAB-Ac-16/NO	0.02	1.4	
DAB-Ac-64/NO	0.02	2.5	
DAB-Am-16/NO	0.44	12	
DAB-Am-64/NO	0.69	29	
DAB-C7-16/NO	3.4	77	
DAB-C7-64/NO	3.2	86	
DAB-PO-64/NO	5.6	28	

The total NO released from secondary amine conjugates was significantly greater than from the primary amine parent dendrimers due to the enhanced stability of secondary amine diazeniumdiolates. The level of NO released (3−6 μmol NO/mg) for the secondary amine dendrimers represents the greatest "payload" of an NO-releasing macromolecule to date (Table 2). Kinetic data confirmed that the mechanism of diazeniumdiolate dissociation was proton initiated. In addition to the increased storage capacity of the dendritic architecture, the dendrimer bound NO donor half-lives greatly exceed those for small molecule equivalents, thereby illustrating a "dendritic effect" on diazeniumdiolate decomposition.

Expansion of the current work to introduce multiple functionalities on the dendritic exterior may enable the use of NO-releasing dendrimers as a vehicle for further understanding NO's role in physiological systems of interest. For example, dendrimers equipped with targeting ligands (e.g., folic acid) to deliver NO selectively to cancer cells may potentially help elucidate the mechanistic effects of NO on tumor cell biology. Fluorescent probes and/or poly(ethylene glycol) chains may also be added to produce fully biocompatible nanoparticles capable of harnessing the therapeutic potential of NO. Such studies are currently underway.

Experimental Section

General. All reagents including generation 3 and generation 5 polypropylenimine dendrimers (DAB-Am-16 and DAB-Am-64) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Methanol was distilled over magnesium prior to use. Water was purified with a Millipore Milli-Q gradient A-10 purification system (Bedford, MA). Spectra/Por Float-A-Lyzers were purchased from Spectrum Laboratories Inc. (Rancho Dominguez, CA). Absorption spectra were recorded on a Perkin-Elmer Lambda 40 UV−vis spectrophotometer (Norwalk, CT). Nuclear magnetic resonance (NMR) spectra were collected in $CDCl_3$, CD_3OD, or D_2O using a 400-MHz Bruker Nuclear Magnetic Resonance spectrometer. Mass spectra were acquired in positive ion mode using a Micromass Quattro II triple quadrupole mass spectrometer equipped with a nano-electrospray ionization source. Nitric oxide release was measured using a Sievers 280i Chemiluminesce Nitric Oxide Analyzer (Boulder, CO). Details on the synthesis and characterization of polypropylenimine dendrimer conjugates are provided in Supporting Information.

Formation of the Diazeniumdiolate NO Donor. Parent primary amine dendrimers and the dendrimer conjugates were dissolved in 0.5 M NaOMe/MeOH and placed in 5 mL glass vials equipped with a stir bar prior to exposure to NO gas. The vials were placed in a Parr bottle, connected to the NO-reactor, and flushed six times with Ar, followed by a series of six longer charge/discharge cycles with Ar (6×10 min) to remove oxygen from the stirring solutions. The Parr bottle was then filled with 5 atm of NO (purified over KOH pellets for 30 min to remove trace NO degradation products) and sealed. After 3 d, the NO was expunged using the same Ar procedure described above to remove unbound NO from the dendrimer-diazeniumdiolate product solutions.

Characterization of NO−Releasing Dendrimers. Two methods were employed to characterize the formation of the *N*-bound diazeniumdiolate NO donor species. First, absorption spectroscopy was used to confirm diazeniumdiolate formation. UV−vis spectra recorded on a Perkin−Elmer spectrophotometer in dilute MeOH solutions displayed the characteristic diazeniumdiolate absorption maximum (λ_{max}) between 250 and 260 nm for diazeniumdiolates in all cases.[9] Second, NO released from the polypropylenimine dendrimer conjugates was measured upon donor decomposition via chemiluminescence. Aliquots (10 μL) of the NO exposed dendrimer solution were added to 0.01 M phosphate buffered saline (PBS, pH = 7.4) at 37 °C to initiate NO release under physiological conditions.[38] Dendrimer concentrations ranged from 0.2 to 1.0 mM. The chemiluminescence analyzer was calibrated with NO gas (24.1 ppm). A parameter for converting the instrument response (ppb) to moles of NO was obtained via the conversion of nitrite standards to NO in a 0.1 M KOH/H_2SO_4 solution (1.19×10^{-13} moles NO/ppb). Chemiluminescence data for the NO-releasing dendrimers were represented in two graphical forms or plots: (1) chemiluminescence response in ppb NO/mg dendrimer vs time; and, (2) the total amount of NO-release (t[NO]) vs time. The maximum flux of NO release ($[NO]_m$) and the time required to reach that maxima (t_m) were obtained from plot 1. The half-life ($t_{1/2}$) of NO release as well as the t[NO] (μmol NO/mg) can be determined from plot 2.

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Supporting Information Available: Details on the synthesis and characterization of polypropylenimine dendrimer conjugates and NO release kinetics (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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