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Synthesis and Characterization of Polymethacrylate-Based Nitric Oxide Donors

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Abstract: A synthetic path for the preparation of methacrylic homo- and copolymers containing secondary amine groups that can be converted into nitric oxide (NO) releasing N-diazeniumdiolates is described. The polymers are obtained by a multistep procedure involving synthesis of methacrylate monomers containing boc-protected secondary amine sites, free radical benzoyl peroxide initiated polymerization, deprotection of the amine sites, and subsequent reaction of the polymers with NO in the presence of sodium methoxide. Monomers with both linear and cyclic pendant secondary amines are examined as polymer building blocks. In most cases, polymers are obtained for both types with compositions that agree well with initial monomer ratios and with number average molecular weights (M_n) ranging from 1.69 to 2.58 $\times$ 10^6 Da. The final N-diazeniumdiolated methacrylic amine polymers are shown to release NO for extended periods of time with "apparent" $t_{1/2}$ values ranging from 30 to 60 min when suspended in phosphate buffer, pH 7.4. Total NO loading and release for these materials can reach 1.99 μmol per mg of polymer and is proportional to the amine content of the polymer. It is further shown that by using a dimethacrylate cross-linking agent in conjunction with the various methacrylate amines, suspension polymerization methods can be employed to create small (100−200 μm) polymeric methacrylate microbeads. Such microbeads that can be sequentially deprotected and converted to NO release particles via in-situ diazeniumdiolate formation as carried out for the non-crosslinked polymers.

Introduction

Ideally, the plastic components used in various biomedical procedures (e.g., open heart surgery, extracorporeal membrane oxygenation (ECMO), kidney dialysis, etc.) and in the design of intravascular devices (implantable chemical sensors, catheters, etc.) should be made of blood-contacting polymers that exhibit good hemocompatibility. Indeed, one of the great challenges in the area of biomaterials is the development of polymers that will not induce thrombus formation when in direct contact with blood (so-called "nonthrombogenic" surfaces). Since no such polymers currently exist, systemic or localized (e.g., heparin coatings) anticoagulation is usually required to reduce risk of clot formation during such clinical procedures.[1] Recent research in this laboratory[2−7] and elsewhere[8−12] has demonstrated that polymers that can release low levels of nitric oxide (NO) greatly reduce the degree of platelet activation on the surfaces of various polymeric films both in vitro and in vivo. Indeed, it is well known that NO is a potent inhibitor of platelet function,[13,14] and creating localized increases in the level of NO at a polymer/solution interface has been shown to greatly reduce the adhesion of radiolabeled platelets to polymers.[3,14] In this paper, we describe a synthetic approach to prepare new methacrylate homo- and copolymers that contain NO releasing N-diazeni-

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umdiolate functional groups and demonstrate that such materials release NO for extended time periods.

Diazeniumdiolates are widely known as useful NO donor molecules.[15−17] They are the product of the addition of two molecules of nitric oxide with an amine, usually a secondary amine structure. In the presence of water, diazeniumdiolates decompose releasing two moles of $NO_{(g)}$. There have been several efforts reported to date on the preparation of different NO release polymeric systems using N-diazeniumdiolate chemistry.[3,5−7,9,10,16,18,19] Some of these have consisted of polymeric films containing physically suspended small diazeniumdiolate molecules. For example, Kaul et al. incorporated spermine diazeniumdiolates within a biodegradable copolymer of polylactic and polyglycolic acid.[19] Mowery et al. as well as Annich et al. employed (*N*-methyl-N-[6-(*N*-methylammoniohexyl)amino]diazen)-1-ium-1,2-diolate (MAHMA-NO) doped within PVC and silicone rubber films to create more thombo-resistance NO release materials.[3,6] Others have developed methods to link the diazeniumdiolate NO donors covalently to the polymer backbone. Among these, diazeniumdiolated piperazine modified PVC and heparin, poly(ethyleneimine),[6] modified proteins,[20] poly(butanediol spermate),[12] dipropylenetriamine grafted on polysaccharide,[9] and amine modified silicone rubbers[7,21] have all been reported.

Acrylate-based polymers are potentially more attractive materials to prepare NO release coatings that can be covalently linked to metal surfaces or prepared in uniform microbead form and then incorporated, in controlled amounts, within other polymeric materials to create a wide variety of NO release films. The latter approach has been demonstrated recently by doping NO releasing fumed silica particles at varying wt % into silicone rubber and polyurethanes to create polymers with enhanced blood compatibility.[22] In principle, NO release acrylic coatings for metallic stents, and other blood-contacting metal surfaces (e.g., heat exchangers used in extracorporeal systems), could be achieved by first treating the metal surface with a silane agent possessing a pendant acrylate or methacrylate group, and then polymerizing appropriate monomers (containing secondary amines) on such surfaces to create thin films of covalently linked polymers that could then be reacted with NO, in situ, to form the desired NO releasing diazeniumdiolates.

Unfortunately, there is no straightforward route to the synthesis of acrylic polymers containing the required secondary amine sites necessary to generate N-diazeniumdiolate moieties. This is primarily because of the Michael addition reaction that takes place between amines and the unsaturated bonds of acrylates. Monomers with both amine and acrylic bonds would be unstable and could react with each other. Moreover, during polymerization, a competitive Michael reaction would occur leading to a reduction in the amount of secondary amine sites within the resulting polymeric material.

There are two possible solutions to this problem. One is the modification of existing acrylic polymers (e.g., poly(glicydyl methacrylate), poly(acrylic acid), etc.) by grafting residues that contain secondary amine sites to pendant side chains. A second approach involves the synthesis of modified monomers containing protected amine sites. It is this latter approach that is described herein, using boc-protected amine sites before introduction of methacrylic groups to create the new monomers and subsequent deprotection of these amine sites after polymerization. In this work, we present the synthesis of new methacrylic monomers possessing pendant secondary amine sites and their application for preparation of novel NO releasing polymethacrylates

Experimental Section

Materials. All reagents were purchased from Aldrich Chemical, Fisher, or Acros Organics. Methyl methacrylate was distilled before use and stored in the refrigerator. Tetrahydrofuran was distilled prior to use over potassium benzophenone ketyl. Compounds **1a**−**c**,[23] **2d**,[24] and **3d,f**[25] were synthesized according to previously described procedures.

Instrumentation. ^{1}H NMR spectra of the monomers and polymers were obtained on a Varian 300 or 400 MHz spectrometer in $CDCl_3$ or CD_3OD. Nitric oxide release was monitored with a Sievers NOA™ 280 nitric oxide analyzer. UV−vis spectra were recorded using a Beckman DU 640B spectrophotometer. Molecular weights were determined using size exclusion chromatography with Waters HT-4, HT-3, and HT-2 columns and a dichloromethane mobile phase. Glass-transition temperatures were measured with a Perkin-Elmer DSC 7.

Synthesis of Monomer Compounds. Synthesis of 2a,b,c,e Monomer Precursors. To a vigorously stirred solution of 0.1 mol of amine alcohol in 60 mL of dry THF, 0.105 mol of di-*tert*-butyl dicarbonate was added dropwise at 10 °C. The reaction mixture was stirred overnight. The solvent was removed in vacuo. The residue was redissolved in dichloromethane and washed with water and brine. The solution was dried with sodium sulfate and the dichloromethane was removed in vacuo. Liquid products where then kept on a vacuum pump overnight to remove any residual solvent, while the resulting solids were recrystallized and dried. The products and their purity were confirmed by ^{1}H NMR.

Indroduction of Polymerizable Group: Synthesis of 3a,b,c,e. A solution containing 0.2 mol of the protected amine alcohol and 0.22 mol of triethylamine was prepared in dry THF at −10 °C under nitrogen. The solution was then stirred vigorously and 0.22 mol of (meth)acryloil chloride was added dropwise maintaining the temperature

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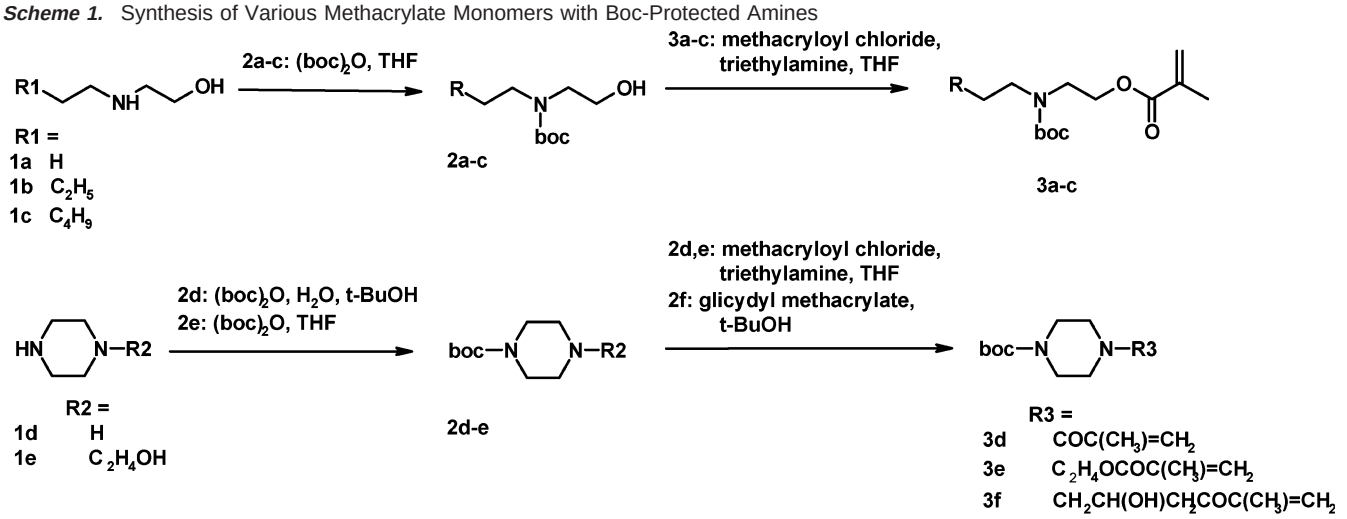
Scheme 1. Synthesis of Various Methacrylate Monomers with Boc-Protected Amines



below 0 °C. The reaction mixture was then stirred at room temperature overnight. A white precipitate of triethylammonium chloride was filtered off and washed with THF. The combined organic phases were evaporated; the resulting residue was dissolved in dichloromethane, washed with water and then brine, and then dried with sodium sulfate. After removal of the solvent, the product was purified by column chromatography on neutral alumina with a hexane/ethyl acetate mixture. Product confirmation and purity was confirmed by ^{1}H NMR. (see Supporting Information file, section EXP1s).

Polymerization and Copolymerization. The appropriate amount of methacrylic monomer or monomers (methyl methacrylate used to create copolymers) (total of 0.01 mol monomers) was dissolved in 3 mL of dry THF and placed in small reactor equipped with magnetic stirrer and Teflon seal. The initiator benzoyl peroxide (4.8 10^{-5} mol) was added. The solution was flushed with nitrogen for 5 min and the reactor was closed and placed in an oil bath at 75−80 °C. The reaction mixture was stirred at this temperature for 48 h. The polymer was precipitated with water or hexane and purified by reprecipitation. Typical ^{1}H NMR data for representative polymers resulting from this process are provided in the Supporting Information file (section EXP2s). Integration data was used to confirm the methyl methacrylate versus boc-protected amine methacrylate content of the final polymers.

General Procedure for Deprotecting Polymers. One gram of each polymer was dissolved in 7.5 mL of dichloromethane and then 2.5 mL of trifluoroacetic acid was added dropwise. The resulting mixture was stirred for 3 h at room temperature. The organic phase was then washed with water, sodium bicarbonate and brine, and finally dried with sodium sulfate. The dry solution was evaporated and any residual solvent was removed under vacuum overnight. ^{1}H NMR data for some representative deprotected polymers are provided in the Supporting Information file (section EXP3s). Deprotected polymers **poly[3a]$_{100\%}$**, **poly[3a]$_{40\%}$**, **poly-[3a]$_{20\%}$**, **poly[3b]$_{100\%}$**, **poly[3b]$_{40\%}$**, and **poly[3b]$_{20\%}$** exhibit analogous spectral data to those presented in the Supporting Information file. The only difference is the length of aliphatic chain within the amine monomer **3**.

General Procedure for NO Addition. Polymer solutions (25−55 mg/mL) in THF were placed in a high-pressure reactor and stirred under argon for 10 min. Then, 100% excess (with respect to free amine sites) of sodium methoxide (using 25% solution in methanol) was added and the reactor closed. The reactor was flushed with argon several times and charged with NO at 80 psi. The reaction mixture was stirred for 72 h at room temperature. At the end of this time, the resulting polymers were precipitated from dry hexanes. The precipitates were filtered, washed with dry solvent, dried under vacuum, and stored in the refrigerator. Representative ^{1}H NMR data for some of the diazenium-diolated polymers are provided in the Supporting Information file (section EXP4s).

Preparation of NO Releasing Methacylate Microbeads. Twenty grams of a mixture of monomers (methyl methacrylate and protected amine methacrylic monomer) as well as 0.5% mol of the cross-linking agent 1,6-hexanedioldimethacrylate and 0.5% mol of benzoyl peroxide were placed in a reactor equipped with mechanical stirrer, nitrogen inlet, and a condenser. A solution of 0.25 g of poly(vinyl alcohol) in 120 mL of distilled water was added. The reaction mixture was stirred at 2000 rpm and heated on an oil bath at 80 °C for 6 h. The reaction was cooled to room temperature. The resulting polymeric particles were separated, washed with water, and then dried under vacuum. The size of the resulting microbeads was determined by scanning electron microscopy.

Deprotection of Microbeads. Two grams of polymer particles were slowly added to a stirred solution of 30 mL of dichloromethane and 10 mL of trifluoroacetic acid. Stirring was continued for 10 h at room temperature. The particles were then filtered and washed thoroughly with dichloromethane. They were then placed in a beaker containing 30 mL of dichloromethane and excess of concentrated $NaHCO_3$. The mixture was stirred vigorously until all the dichloromethane evaporated. The particles were then filtered, washed with water, and dried under vacuum for 2 days.

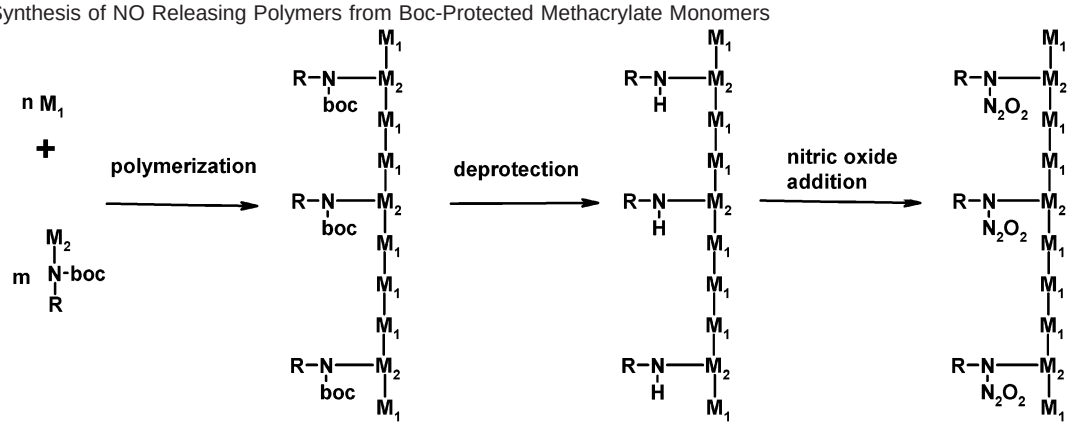
NO Addition to Deprotected Mircobeads. One-half gram of the polymer particles were suspended in 20 mL of dry THF containing 100% excess (with respect to free amine sites) of sodium methoxide (25% solution in methanol). The solution was placed in a high-pressure reactor. The reactor was charged with argon several times to remove all the remaining air and then filled with NO at 80 psi. The reaction mixture was stirred at room temperature for 72 h. Particles were separated, washed twice with dry solvent, and dried under vacuum.

Results and Discussion

Synthesis of Monomers. A relatively simple synthetic route can be applied for the synthesis of several methacylic monomers, which can then be utilized for the preparation of the NO releasing methacrylate polymers. The synthetic approach to obtain the desired monomers is presented in Scheme 1.

The precursor secondary amines (**1a**−**c**) were synthesized via two routes. One utilized 2-ethanoloamine (in excess) and an alkyl halide; the other utilized 2-halogenethanol and an excess of primary alkylamine. In both cases, products were purified by vacuum distillation. The best yields were achieved for **3c** when synthesized from hexylamine and 2-chloroethanol. Reactants used in excess were recovered from the reaction mixture by distillation and recycled. Compounds **1d,e** were purchased from Aldrich Chemical Co.

Scheme 2. Synthesis of NO Releasing Polymers from Boc-Protected Methacrylate Monomers



Most compound **1** species have both hydroxyl and secondary amine groups. The more reactive amines were protected by reaction with di-*tert*-butyl dicarbonate. The reaction is selective and provides good yields (56−99%). The products (**2**) needed only simple workup.

The final step, to introduce the polymerizable methacrylate group, also provides good yields (64−95%), as long as certain precautions are followed. First, all methacrylic anhydrides cannot be used in reaction with alcohols **2**. The reaction byproduct (methacrylic acid) deprotects the amine group, which can then further react with final methacrylate and starting anhydride leading to a mixture of products. Methacryloyl chloride with triethylamine as the hydrogen chloride acceptor was used successfully in this case. After the usual workup, the product was 90% pure and ^{1}H NMR spectra showed small peaks of other species having unsaturated acrylic bonds (e.g., sodium methacrylate). These species can be further removed by filtration through neutral alumina (silica gel is not effective in this case) from hexane or hexane/ethyl acetete mixtures.

The monomers **3d,f** containing piperazine to provide the secondary amine sites were synthesized using a different approach. Piperazine **1d** was first monoprotected with boc. The remaining amino group was then either reacted with methacryloyl chloride leading to the final product **3d** or with glicydyl methacrylate yielding **3f**. The yield in the latter case was a little lower (42%) than in other cases, but the product could be easily purified by recrystallization.

Characterization of the Monomers. All the monomers **3a**−**c** and **3e** are colorless liquids with the characteristic methacrylate odor. Compounds **3d** and **3f** are white crystalline solids. They are stable when stored in the refrigerator, except for **3e** which eventually forms a polymeric, nonsoluble material even at low temperatures.

All the new monomers were characterized by ^{1}H NMR spectroscopy. For example, in the spectrum for the linear amine monomer **3c** (see Figure 1s in the Supporting Information file), the methacrylic group (A) appears as a set of three peaks (CH_2 = two singlets between 5.5 and 6.5 ppm, CH_3 singlet around 2 ppm). The most downfield shifted signal (above 4 ppm) for a methylene group is the one neighboring the oxygen atom (B). The signals for the methylene groups neighboring the nitrogen atom (C) are between 3 and 4 ppm. For the remainder of the molecule, the aliphatic chain signals (D) are below 2 ppm. The boc-protecting group singlet (E) is always at 1.48 ± 2 ppm. These bands are characteristic for all the **3a**−**c** compounds. The piperazine-based monomers **3d**−**f** yielded analogous NMR spectra (spectra not shown).

Synthesis of NO Releasing Polymers. To obtain NO releasing polymeric materials from the previously synthesized monomers, there were three additional synthetic steps required (Scheme 2). First, the monomers were homo- or copolymerized with methyl methacrylate. Then, the amine groups were selectively deprotected. Finally, the free secondary amine sites within the polymers were reacted with NO under high pressure in the presence of sodium methoxide.

Compounds with methacrylate groups can be copolymerized with a variety of different monomers to yield polymers that differ significantly in their chemical and physical properties. For this research, aimed at demonstrating the feasibility of preparing NO releasing polymethacylates, only methyl methacrylate was used as a copolymerization monomer with the various new methacrylate molecules containing protected secondary amine sites. Indeed, to better understand changes in the structure by NMR measurements after polymerization, it was important not to introduce too many additional proton signals into the methylene proton region of the spectra. Methyl methacrylate, when polymerized, exhibits only a singlet for the CH_3 group protons in this region.

Homo- and copolymers were synthesized in THF solution by free-radical polymerization. The solution of monomer or mixture of monomers containing 0.5 mol % of benzoyl peroxide (relative to total moles of monomer) was deoxygenated with nitrogen, sealed in a reactor equipped with magnetic stirrer, and kept at 75 °C for 48 h. The resulting polymers were precipitated from water (**3a**−**e** polymers) or hexanes (**3f** polymers).

Scheme 3 shows the structures of monomers and final polymers prepared in this study. Monomers **3** were homo- and copolymerized. In the copolymerization reactions, 20 and 40 mol % of nitrogen containing monomers **3** were used in combination with methyl methacrylate. The yields for all of the polymers are tabulated in Table 1.

The polymers were characterized by ^{1}H NMR spectroscopy (see Figures 2s and 3s in Supporting Information file). The ^{1}H NMR spectra of **poly[3b]** polymers (Figure 2s) exhibit no resonances for unreacted monomers. Proton signals characteristic for **3b** can be observed. With an increase in the content of methyl methacrylate within the polymer, the singlet of the CH_3 group appears at 3.5 ppm. The signal integration ratio between 0.7 and 1.2 ppm is also changed because of the presence of the methyl methacrylate units. The amount of each monomer

Scheme 3. Polymers of Various Methacrylate Monomers Synthesized in This Work

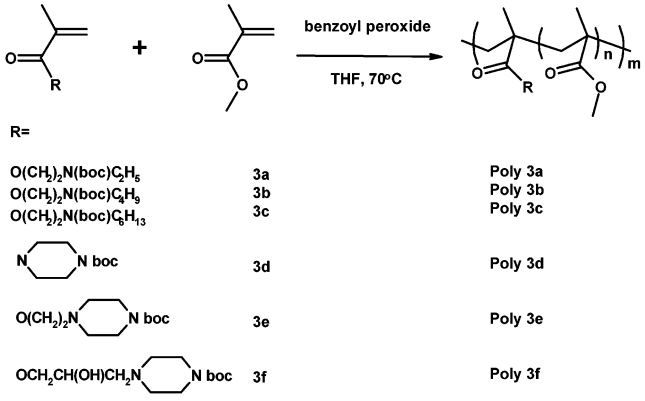


Table 1. Composition and Yields of the Various Homo- and Copolymers (with methyl methacrylate) Prepared Using Boc-Protected Amine Monomers

	theoretical composition [mol % of **3**]	actual composition [mol % of **3**]	yield [%]
poly3a	20	24	100
	40	41	100
	100	100	100
poly3b	20	21	100
	40	39	100
	100	100	97
poly3c	20	20	100
	40	41	100
	100	100	97
poly3d	20	3.8	60.2
	40		
	100		
poly3e	20	27	75
	40	48	85
	100	100	75
poly3f	20	28	72
	40	49	74
	100	100	70

incorporated into the polymer backbone can be determined from integration ratios, using the singlet at 3.5 ppm and any of the resonances for the CH_2 groups neighboring the N or O atoms (4, 3.4, 3.2 ppm). The large size of one of the monomers (methacrylate with protected amine) does not influence the polymer structure, which from the NMR spectrum appears to be heterotactic. This can be seen more clearly from the NMR spectrum of **poly[3e]** (Figure 3s), in which there are no CH_3 signals from the aliphatic side chain.

The yields of piperazine containing polymers (**poly[3e]** and **poly[3f])** were generally lower than that observed for the **poly-[3a−c]** species (see Table 1). They ranged from 70 to 85%. The NMR data revealed a slightly higher amount of piperazine monomer (**3e,f**) incorporated into the polymers than was used in the reaction mixtures. The ^{1}H NMR spectra show well-resolved proton resonances for the homopolymer and both of the respective copolymers. As shown in Figure 3s, there are clear signals for the piperazine ring methylene groups, the C_2H_4 spacer, protective groups, and CH_3O in the copolymers with methyl methacrylate. In the range between 1.2 and 0.7 ppm, there are two proton signals that can be assigned to the CH_3 groups next to pro-chiral carbon atoms. The signal at higher field value belongs to *racemic* sequences, while the other, at lower field, to *meso* dyads of isotactic sequences. The polymer is again heterotactic with an excess of racemic sequences.

Table 2. Characterization Data for **Poly[3b,b]** Polymers

	yield [%]	content of amine units [mol %]	molecular weight M_w	molecular weight M_n	polydispersity	T_g [°C]
poly3b$_{100\%}$	97	100	9258296	2504805	3.70	23
poly3b$_{40\%}$	100	39	8745710	2479545	3.53	
poly3b$_{20\%}$	100	21	6976657	1706468	4.09	118
poly3c$_{100\%}$	97	100	11181072	2575532	4.34	22
poly3c$_{40\%}$	100	41	8243963	2529218	3.26	95
poly3c$_{20\%}$	100	20	7084962	1689461	4.19	125

Monomer **3d** did not homopolymerize under the conditions used for all of the other polymerization reactions. However, small amounts could be incorporated into the polymer backbone when **3d** was copolymerized with methyl methacrylate. The composition of all the polymers is provided in Table 1. Polymers **poly[3a−c]** contained theoretical amounts of the monomer **3** species in the polymer backbone. Polymers **poly[3e,f]** contained slightly greater than theoretical amounts of **3** incorporated into the backbone: 48 and 27% for **poly[3e]$_{40\%}$** and **poly[3e]$_{20\%}$**; 49 and 28% for **poly[3f]$_{40\%}$** and **poly[3f]$_{20\%}$**, respectively. This indicates that some of the methyl methacrylate was not completely converted into polymer and explains the lower yields for **poly[3e,f]**.

Polymers **poly[3b,c]** were investigated in more detail. Molecular weights were determined by GPC and the results are shown in Table 2. The M_n of the polymers was between 1.6 and 2.6 $\times$ 10^6 Daltons. The highest values were obtained for homopolymers **poly[3b]$_{100\%}$** and **poly[3c]$_{100\%}$**. The copolymers with 60% of methyl methacrylate had almost the same M_n values. The copolymers containing 80% of methyl methacrylate were generally found to have a significantly lower M_n value and the widest molecular weight distribution.

Weight-average molecular weight (M_w) values are dependent on the molecular weight of the monomers (see Table 2). The highest values are found for homopolymer **poly[3c]** (molecular weight of the monomer **3c** is 313.44 g/mol). Polymers containing the **3b** monomer (M = 285.39 g/mol) had proportionally lower molecular weights (M_w). The M_w of the copolymers are again proportionally lower since the methyl methacrylate (M = 100 g/mol) molecule is much smaller than the nitrogen containing monomers **3**. The polydispersity of the polymers range between 3.2 and 4.4 which is typical for radical polymerizations performed in the solution.[26]

Glass-transition temperatures (T_g) for the polymers were determined by DSC. The T_g values of the polymers showed significant dependence on the amount of amine containing monomer within the polymer. Homopolymers **poly[3b,c]$_{100\%}$** exhibit T_g values close to room temperature. The data collected for the copolymers had glass-transition temperatures closer to the typical T_g values for poly(methyl methacrylate).[26]

Deprotection of the Amine Sites. Methacrylate polymers containing free secondary amine sites were obtained by selective removal of boc-protecting group. Reactions were performed in dichloromethane in the presence of trifluoroacetic acid. The reaction conditions were tested beforehand using poly(methyl methacrylate) as a model and were found not to cause chemical changes in the polymer structure. In all cases, after boc deprotection, the polymer solution was washed with water, concentrated sodium bicarbonate, and then carefully dried under

(26) Stevens, M. P. *Polymer Chemistry: an Introduction*; Oxford University Press: New York, 1990.

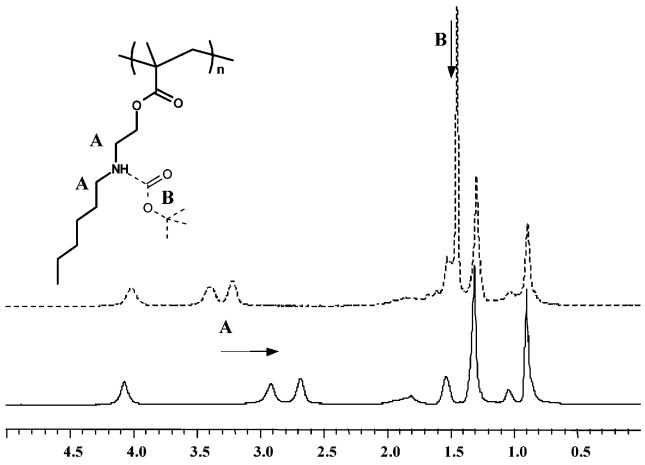


Figure 1. Changes in the ^{1}H NMRspectrum of **poly[3c]$_{100\%}$** after deprotection (−); before (---).

vacuum. An alternate method of deprotection ($HCl_{(g)}$, AcOEt) resulted in insoluble materials and a more complicated workup. The average yield of the deprotection procedure was 90% as determined from ^{1}H NMR. An example of the change in the NMR spectrum after deprotection is shown in Figure 1 for **poly[3c]$_{100\%}$**. The protons of the Bu^t group disappear (B, Figure 1) and protons of the two methylene groups adjacent to nitrogen atom (A, Figure 1) are shifted upfield ca. 0.5 ppm after deprotection. The number of signals and integration confirm complete deprotection of amine sites. Similar data were obtained for **3c** copolymers.

Nitric Oxide Addition. The deprotected polymers were soluble in common solvents. Solutions (usually in THF) of the polymers were placed in pressure reactors and stirred for 72 h under pure NO at 80 psi in the presence of 100% excess (in respect to the amine sites concentration) of sodium methoxide. The 72-h time period was optimal for NO loading. Longer periods for the reaction did not result in any further increase in diazeniumdiolate formation as determined by NMR and CL. The resulting diazeniumdiolated polymers usually partially precipitated from the solution during the reaction (approximately 15 wt %). After the reaction, the remainder of the polymer was precipitated from dry hexane. Typically, the material that precipitated spontaneously had only slightly greater diazeniumdiolate content than the material remaining in solution and then precipitated with hexane. The pooled diazeniumdiolated polymers were washed with dry solvent, dried under vacuum, and finally stored under nitrogen in the freezer.

Characterization of Polymethacrylate Diazeniumdiolates. The final diazeniumdiolated polymers were not soluble in most of the organic solvents and water. The only solvent found to dissolve the materials completely was methanol. Hence, these new materials were characterized by means of NMR and UV−vis spectroscopy in methanol or deuterated methanol. Further, NO release measurements, using a chemiluminescence NO analyzer, were carried out by suspension of the polymeric materials in PBS buffer.

The UV−vis absorption spectra of diazeniumdiolated **poly[3c]** polymers in methanol with the different amounts of methyl methacrylate as the copolymerization monomer (see Figure 4s in Supporting Information file) show the characteristic absorption band for diazeniumdiolates at 248 nm.[17] As expected, the intensity of absorption at this wavelength increases with the

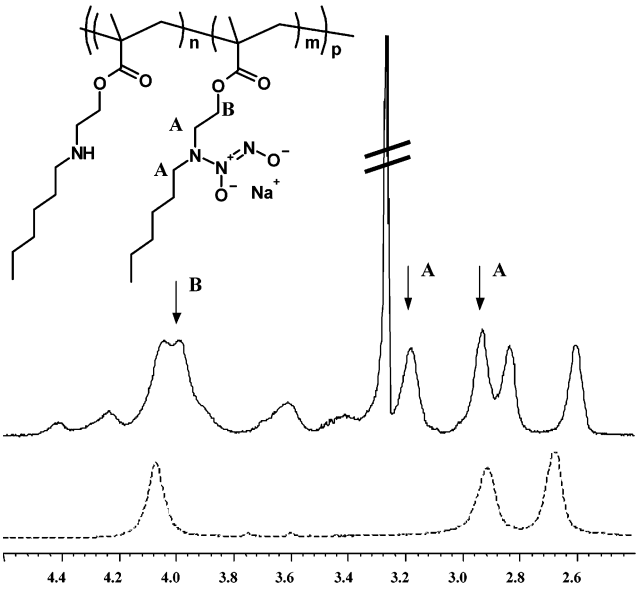


Figure 2. ^{1}H NMR spectrum (−) showing partially substituted diazeniumdiolated amine sites in **poly[3c]$_{100\%}$** (in CD_3OD); arrows indicate methylene groups of diazeniumdiolated polymer units. For reference purposes, ^{1}H NMR spectrum (---) of starting material is shown below (^{1}H NMR, $CDCl_3$).

greater amine content of the **poly[3c]** polymers. Similar UV absorption data were obtained for the other diazeniumdiolated polymethacrylate species.

Figure 2 shows the ^{1}H NMR spectrum of the diazeniumdiolated polymer **poly[3c]$_{100\%}$**. The resonances labeled A and B are assigned to methylene groups neighboring the diazeniumdiolated amine site. These signals are downfield shifted from the unreacted polymer (data not shown), which is typical for other diazeniumdiolates.[27] It can be seen, however, that the material still contains a significant amount of unreacted amine sites as evidenced by the resonances at 2.6 and 2.85 ppm. There are several explanations possible for the incomplete conversion of the amine sites to diazeniumdiolate moieties. One is partial precipitation of the polymer material during the NO addition reaction. Precipitated material, which still contains free amine groups, may not be able to react further, owing to the inability of sodium methoxide to penetrate the material. The methoxide serves as an exogenous base to deprotonate the amine site, enabling the reaction with NO.[28] The sodium ion can then serve as the stabilizing countercation for the anionic diazeniumdiolate species. Another explanation for incomplete diazeniumdiolate formation may be related to steric interference by the polymer backbone and neighboring polymer units. Indeed, such interference surely precludes nearby amine sites from serving as the required base and subsequent countercation to stabilize diazeniumdiolate formation on adjacent amine sites. This is confirmed by the inability to form appreciable amounts of diazeniumdiolated polymer in the absence of sodium methoxide in the NO addition reaction mixture (even for **poly[3a−c]$_{100\%}$**).

As shown in Figure 2, additional proton resonances can also be observed in the same methylene region of the NMR spectrum. These are likely from nitrosoamine formation. Such nitrosamines

(27) Parzuchowski, P. G.; Meyerhoff, M. E. Quantification of the formation and decomposition of diazeniumdiolates under various conditions, in preparation.
(28) Feelisch, M.; Stamler, S. *Methods in Nitric Oxide Research*; John Wiley & Sons: New York, 1996.

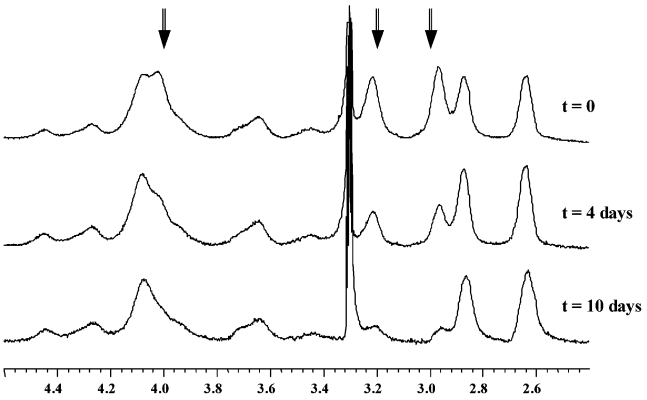


Figure 3. Decomposition of diazeniumdiolates within **poly[3c]$_{100\%}$** with time as determined by ^{1}H NMR (in CD_3OD).

result when the polymer is reacted with NO in the presence of trace levels of residual oxygen[27,29] that is inevitably present no matter how much care is taken to purge the reactor system.

NMR spectroscopy can also be used to follow the stability of the diazeniumdiolated polymethacrylates. Diazeniumdiolates, in the presence of oxygen, can exhibit accelerated decomposition rates.[23] Decomposition of **poly[3c]$_{100\%}$** was observed in methanol solution over a two-week period. Figure 3 illustrates that over this period, the proton signals due to the diazeniumdiolate species (at 2.96, 3.20, and 4.04 ppm) disappear. This experiment was performed in the presence of ambient oxygen at room temperature. Under these conditions, diazeniumdiolates decompose to free amines as well as significant amounts of nitrosoamine. Indeed, Figure 3 clearly shows the relative increase of amine (2.64, 2.88 ppm) and nitrosoamine (4.28, 4.44 ppm) content over the two-week period.

The ^{1}H NMR spectrum of the freshly prepared NO releasing polymer **poly[3c]$_{100\%}$** shows that approximately 45% of amine groups in the polymer were diazeniumdiolated. The same material was also investigated for nitric oxide release by monitoring the NO generated via a chemiluminescence (CL) NO analyzer. After 15 h of continuous release in PBS buffer (as suspension of polymeric particles in buffer), the total diazeniumdiolate load calculated with respect to total amount of amine groups was 21%. However, the sample was still releasing NO when the experiment was terminated. On the basis of these results, the NMR data suggest a higher yield of diazeniumdiolated sites on **poly[3c]$_{100\%}$** than observed by the NO release measurements. This difference is likely due to the solubility of NO gas in the PBS buffer and the incomplete decomposition of diazeniumdiolates during the measurement period owing to localized increases in pH within the polymeric particles suspended in PBS buffer. Indeed, when acid (HCl) was subsequently added to the suspensions of diazeniumdiolated polymers in PBS to dramatically lower the solution pH, the total integrated amount of NO evolved and detected by the CL analyzer typically matched (within 10%) the total diazeniumdiolate content as determined by NMR.

The typical NO release curve for **poly[3c]$_{40\%}$** suspended in PBS buffer as determined by CL is shown in Figure 4. As also shown in this figure, control experiments, in which poly(methyl methacrylate) was placed in the NO reactor for 3 days, yielded only trace levels of NO release over the same initial time period. In all the investigated methacrylic polymers, the maximum of release appeared always during the first half hour of the experiment.

The three diazeniumdiolated polymers, **poly[3c]$_{100\%}$**, **poly[3c]$_{40\%}$**, and **poly[3c]$_{20\%}$**, were investigated in detail. Three independent NO reactions for each of these polymers were performed and the NO release from the various materials was measured by CL by suspending particles of the polymers in PBS buffer at room temperature. Results are tabulated in Table 3. The "apparent" half-lives for different samples varied from 45 to 60 min. It is clear from this data that polymers containing more amine groups (**poly[3c]$_{100\%}$**) released proportionally more nitric oxide. The NO load with respect to the amount of amines in the starting polymer increased slightly with the decrease of

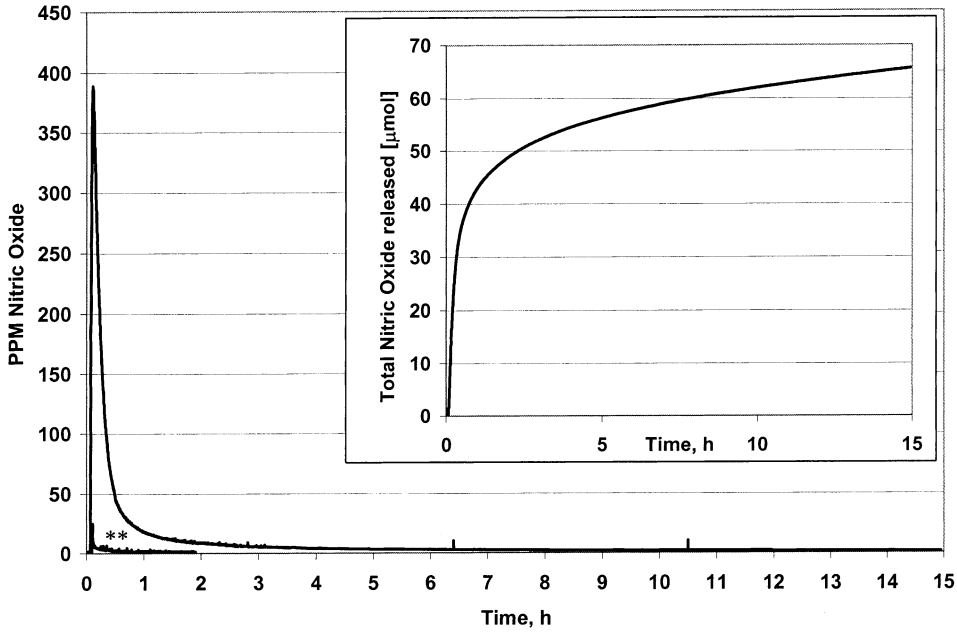


Figure 4. Typical NO release curve of 50 mg of **poly[3c]$_{40\%}$** in 4 mL of deoxygenated 0.1 M PBS buffer as detected by chemiluminescence NO analyzer. (**) Blank experiment, showing NO release for poly(methyl methacrylate) reacted with NO for 3 days.

Table 3. Chemiluminescence (CL) Characterization of NO Released from **Poly[3c]** Polymers in PBS Buffer

	max no. released[b]		average no. released[c]		
	μmol/mg	%[a]	μmol/mg	%[a]	"apparent" $t_{1/2}$[c] [min]
poly[3c]$_{100\%}$	1.99	23.0	1.84 ± 0.14	21.2 ± 1.7	56
poly[3c]$_{40\%}$	1.31	25.2	1.24 ± 0.07	23.7 ± 1.5	30
poly[3c]$_{20\%}$	0.90	28.8	0.64 ± 0.13	24.5 ± 4.3	40

[a] % of diazeniumdiolated amine sites. [b] Maximum released without acid addition. [c] Average from three independent NO addition reactions.

amine monomer within the copolymer. This may be due to the increased solubility of the material during NO addition. Again, the total amount of NO liberated by the polymers when suspended in the PBS buffer is generally 50% of that predicted on the basis of NMR data, unless the solution phase is further acidified while making the CL measurement (see above).

Kinetic studies of the NO release reaction for **poly[3c]$_{100\%}$** were also performed in a solution phase consisting of methanol/PBS buffer (1:2). The presence of the methanol helps solubilize the polymer so that kinetic data for a true homogeneous reaction could be obtained. The rate of NO release was determined by chemiluminescence, and the initial concentration of diazeniumdiolates in each reaction mixture was calculated according to NMR data. Since the reaction rate is pH dependent, pH was monitored during the experiment and remained constant. The reaction exhibited near-first-order behavior (plot of ln(rate) vs ln(diazeniumdiolate concentration) yielded a straight line with slope of 0.89 and a rate constant of 4.62×10^{-8} sec^{-1}) with a $t_{1/2}$ value of 100 min. Under such homogeneous conditions, nearly all of the diazeniumdiolates on the polymer eventually decompose to yield NO, without need to add strong acid to the reaction mixture (i.e., no local increase in pH at surface or within insoluble particles).

Cross-Linked Microbeads. Monomers **3** can also be used for preparation of more advanced nonsoluble microbead materials. This approach involves use of cross-linking agents to form the microbeads in conjunction with suspension polymerization.[20] Such NO releasing microparticles (100−200 μm) could potentially be used as additives in other polymeric materials to create alternate NO releasing matrixes.[15,16] The general synthetic route for the preparation of such microparticles is schematically illustrated in Figure 5.

The particles were synthesized in suspension, with poly(vinyl alcohol) as a surface active agent and with 0.5% of 1,6-hexanedioldimethacrylate as the cross-linking agent. Two different lots of microbeads were prepared containing 20% (**Part[3c]$_{20\%}$**) and 40% (**Part[3c]$_{40\%}$**) of **3c** with the remainder being methyl methacrylate for copolymerization. The reulting protected microbeads were swollen in dichloromethane and the boc-protecting groups were removed with TFA. It is important to remove all the residual acid left after this deprotection step since decomposition of diazeniumdiolate species is accelerated by protons.[22] To accomplish the complete purge of acid from within the bead structure, the microparticles were maintained in a swollen state in dichloromethane while washing repeatedly with sodium bicarbonate.

The amine content within the insoluble microbeads was determined by elemental analysis, since only monomer **3c** contains nitrogen. The results of elemental analysis before deprotection of the polymers were 41.0% for **Part[3c]$_{40\%}$** and 20.6% for **Part[3c]$_{20\%}$** and 41% and 21.7%, respectively, after deprotection of the amine sites. This data suggests that deprotection of amine groups within the microbead structure was essentially complete. The size of the microbeads was estimated to be in the range of 100−200 μm by scanning electron microscopy (see Figure 6). The cross-linked microbeads were loaded with NO in THF in the presence of sodium methoxide by placement within reactor pressurized with NO for 3 days.

All diazeniumdiolated microbead materials were water, air, and temperature sensitive. They are, however, relatively stable when stored under nitrogen in the refrigerator. The typical NO release curves for 5 mg of **Part[3c]$_{40\%}$** suspended in 4 mL of PBS buffer is shown in Figure 7. The total amount of NO released (1.05 μmol/mg) was similar but lower than in the corresponding NO loaded soluble polymer **poly[3c]$_{40\%}$** (1.31μmol/mg, see Table 2). Both materials have almost the same composition; however, there is a small amount of cross-linking

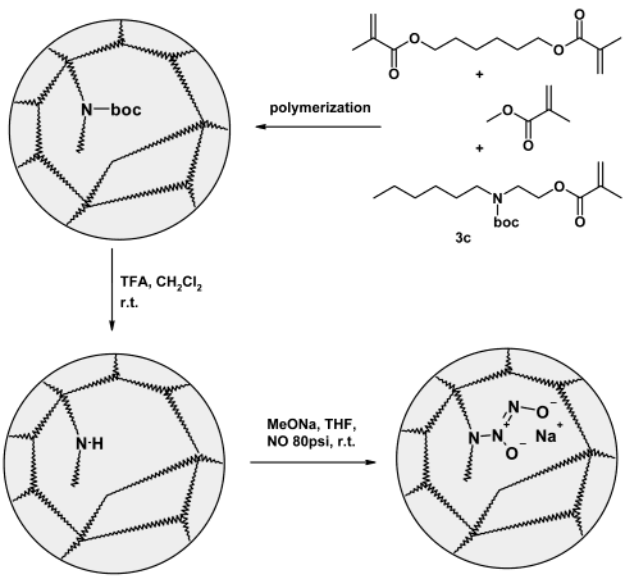


Figure 5. Synthetic approach for preparing cross-linked NO releasing poly(methacrylate) microbeads.

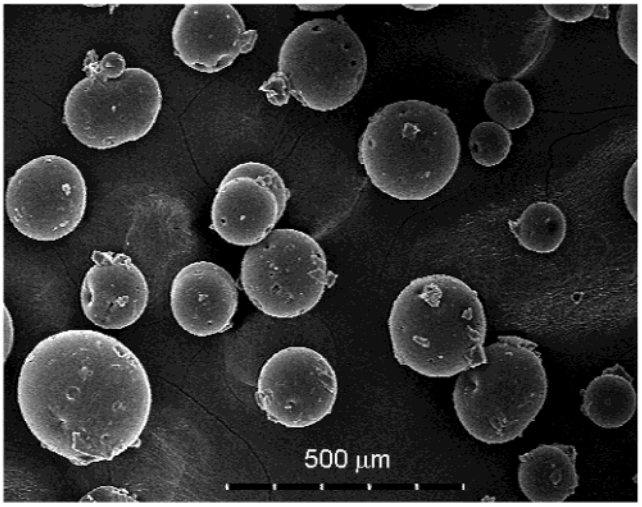


Figure 6. SEM of poly[3c]-based microbeads (**Part[3c]$_{40\%}$**).

(29) Challis, B. C.; Kyrtopoulos, S. A. *J. Chem. Soc., Perkin Trans. 1* **1979**, 299−304.

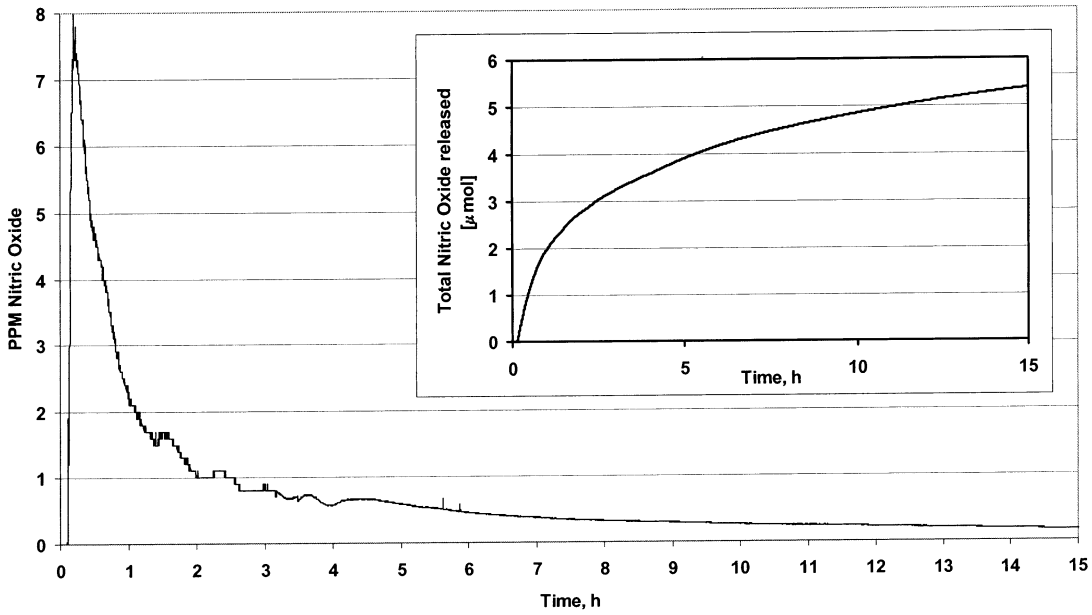


Figure 7. NO release curve as measured by CL for 5 mg of cross-linked microbeads **Part[3c]$_{40\%}$** in 4 mL of deoxygenated 0.1 M PBS buffer. Total NO released = 1.05 μmol/mg/15h.

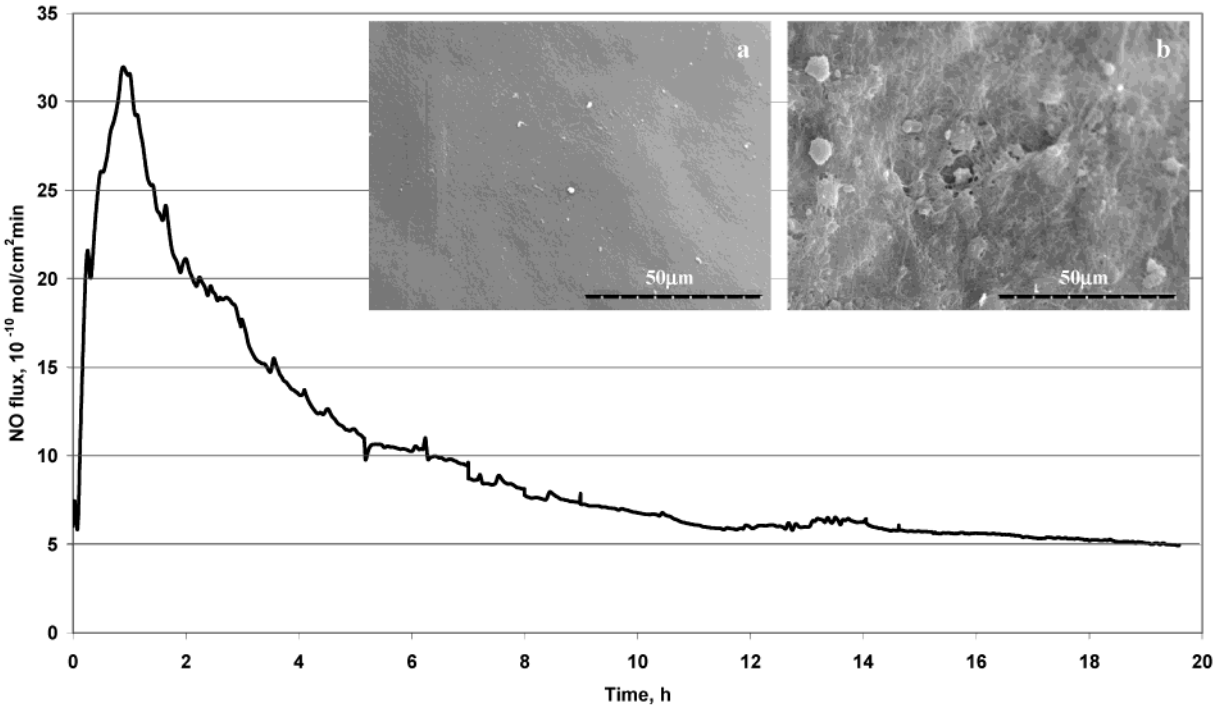


Figure 8. NO surface flux, as measured by CL, for catheter style electrochemical oxygen sensor probes coated with the poly[3c]$_{20\%}$-SR and the typical SEM images of **poly[3c]$_{20\%}$**-SR coated probes after being implanted for 16 h in porcine arteries; (a) NO releasing, (b) control.

agent in **Part[3c]$_{40\%}$** that is not present in **poly[3c]$_{40\%}$** and this may be partly responsible for the difference in NO load.

Although in these preliminary studies regarding the preparation of NO release microbeads relatively large particles have resulted, it is well known that optimized suspension or emulsion polymerization can be employed to prepare much smaller particles. Indeed, Kopelman and co-workers prepared polymethacrylate beads in the range of 20−200 nm, small enough to be inserted within single cells.[30] It is likely, therefore, that much smaller NO-release microbeads can be formulated by further optimization of the conditions used for polymerization and cross-linking of the boc-protected aminomethacrylate monomers reported in this paper.

Preliminary in vivo Studies. To determine the potential biomedical utility of the new NO releasing polymethacrylate polymers, preliminary in vivo experiments were performed. Because of the short apparent half-lives of the polymers, to decrease NO release rates they were incorporated into a silicone rubber coating. For example, **poly[3c]$_{20\%}$** was incorporated at 10 wt % into a silicone rubber matrix that was then used to

(30) (a) Clark, H. A.; Hoyer, M.; Philbert, M. A.; Kopelman, R. *Anal. Chem.* **1999**, *71*, 4831−4836. (b) Clark, H. A.; Kopelman, R.; Tjalkens, R.; Philbert, M. A. *Anal. Chem.* **1999**, *71*, 4837−4843.

coat the surface catheter style electrochemical oxygen sensors.[4] As shown in Figure 8, substantial levels of NO are generated for an extended time period from the surface of the sensor. When these sensors were then implanted into the arteries of a pig for 16 h (no systemic anticoagulation), the probes coated with the **poly[3c]$_{20\%}$**-SR material exhibit far less platelet adhesion and activation compared to control sensors implanted in the same animal and prepared with the same amount **poly[3c]$_{20\%}$** that was not diazeniumdiolated (see Figure 8 inset). This result is consistent with previous in vivo experimental data obtained with other polymers that release NO and that have been coated on similar intravascular oxygen sensors.[4,7]

Conclusions

The main goal of this work was to develop a convenient synthetic route for the preparation of NO releasing methacrylate-based polymers. This was achieved by first synthesizing a series of methacrylic monomers possessing protected secondary amine sites. These monomers can vary in lipophilicities and can be easily copolymerized with most of the commercially available acrylic/methacrylic monomers. The results for copolymerization with methyl methacrylate gave theoretical yields of polymers and copolymers. After boc deprotection, these polymers could be loaded with NO to form correponding diazeniumdiolated materials. The apparent half-lives for NO release when the polymers were suspended in PBS buffer were in the range of 30−60 min. Similar NO release rates were observed for small microbeads of the same polymers prepared by suspension polymerization in the presence of a cross-linking agent.

The ability to create acrylic materials with pendant NO releasing diazeniumdioate groups could provide an attractive means to enhance the biocompatibility of a variety of biomedical substrates. In addition, recent studies have suggested that NO release from surfaces also greatly reduces bacterial adhesion.[11] The fact that polymethacrylates and polyacrylates can be covalently bonded to a variety of metal and silica surfaces using methacrylate silanes suggests that the chemistry reported here can potentially be used to create tightly bound NO releasing coatings for such materials. While the apparent half-lives for NO release for the present polymer formulations is relatively short for biomedical applications, it is likely that the fundamental kinetics of NO release could be varied substantially by incorporating the current diazeniumdiolated polymethacrylates into other polymer matrixes, as described above for preliminary studies with implantable oxygen sensors (see Figure 8).

Beyond incorporating the current diazeniumdiolated polymethacrylates into other polymer matrixes to control NO release rates, it should be possible to synthesize methacrylate monomers with pendant alkyldiamine units that can be converted to diazeniumdiolates without the need for sodium methoxide. This could result in zwitterionic diazeniumdiolate structures that may be more stable, thus increasing half-lives for NO release. Efforts to prepare such diamine monomers and the corresponding series of diazeniumdiolated homo- and copolymers of these species are currently in progress in this laboratory.

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Supporting Information Available: Experimental details and spectra (PDF). This material is available free of charge via the Internet at http: //pubs.acs.org.

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