Synthesis of the First Poly(diaminosulfide)s and an Investigation of Their Applications as Drug Delivery Vehicles

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ABSTRACT: This paper reports the first examples of poly(diaminosulfide)s that were synthesized by the reaction of a sulfur transfer reagent and several secondary diamines. The diaminosulfide group has the general structure of R₂N⁻S⁻NR₂, and although it has been used in the synthesis of small molecules, it has never been utilized in the synthesis of macromolecules until this report. A series of poly(diaminosulfide)s were synthesized at elevated temperatures, and the molecular weights of the polymers were as high as 12 400 g mol⁻¹ with conversions for the polymerization reaction up to 99%. The rate constants for the transamination reactions that lead to the polymers were measured in several solvents to provide an understanding of the reaction conditions necessary to polymerize the monomers. The degradation of diaminosulfides was studied in D₂O, C₆D₆, C₃D₃OD, CDCl₃, and DMSO-d₆/D₂O to demonstrate that they were very stable in organic solvents but degraded within hours under aqueous conditions. These results clearly demonstrated that diaminosulfides are very stable in organic solvents under ambient conditions. Poly(diaminosulfide)s have sufficient stabilities to be useful for many applications. The ability of these polymers to function as drug delivery vehicles was studied by the fabrication of nanoparticles of a water-insoluble poly(diaminosulfide) with a dye. The microparticles were readily absorbed into human embryonic 293 cells and possessed no measurable toxicity toward these same cells.

INTRODUCTION

The integration of new functional groups into polymer chemistry opens new avenues for research and possible commercial applications. For instance, the development of well-defined carbene catalysts based on Mo, W, and Ru in the 1980s and 1990s increased the types and complexities of polymers that could be synthesized and the problems in macromolecular science that could be addressed.1−12 These catalysts led to the development of living ring-opening metathesis polymerization (ROMP) and acyclic diene metathesis (ADMET) polymerization, which were significant reasons the Nobel Prize was awarded to Schrock, Grubbs, and Chauvin in 2005.13−22 The use of “click” chemistry is another example, and its use has increased the complexity of the structure of macromolecules and has found widespread applications in polymer science.23−25 In a recent example by the Hawker group published in 2010, polymers were synthesized for the first time with a functional group that was a precursor to ketenes and provided a simple route to synthesize cross-linked polyethylene to systematically study its materials properties.26,27 From these examples and more, it is clear that when new functional groups are integrated into macromolecules, new applications are developed that take advantage of their unique reactivities.

In this article we report the first examples of polymers that utilize diaminosulfide functional groups along their backbones. The diaminosulfide functional group has the general structure of R₂N–S–NR₂ as shown in Figure 1. Small molecules with

Figure 1. (a) A polymerization to yield a poly(diaminosulfide). (b) Sulfur transfer reagents that are commonly used in small molecule synthesis. (c) A polymer of a benzo[1,2,5]thiadiazole.
Figure 2. Synthesis of two sets of sulfur transfer reagents. (a) The synthesis of a dithiosuccinimide. (b) The synthesis of a diaminosulfide in two steps. Molecules C and E were purified by distillation. (c) Molecule F was synthesized using the same procedure as molecule E.

one has used diaminosulfides to bond monomers together as shown in Figure 1a, and these polymers are the focus of this report.

An important characteristic of the diaminosulfide group is that it is based on inorganic atoms (one sulfur and two nitrogens). Most functional groups that are used to synthesize polymers are based on organic functional groups such as esters, amides, anhydrides, acetics, cyclic olefins, vinyl groups, carbonates, urethanes, and epoxides. Although many monomers are known to possess inorganic functional groups, it is uncommon that an inorganic functional group is transformed in the polymerization reaction and used to link monomers together as shown in Figure 1a. Most inorganic functional groups found in monomers or polymers are not transformed during the polymerization reaction. Three notable examples of inorganic functional groups that have been polymerized include the polymerization of thiols into poly(disulfides), the polymerization of cyclic phosphazenes into poly(phosphazenes), and the polymerization of cyclic siloxanes into poly(siloxanes).

Inorganic functional groups are interesting targets for polymer synthesis because they can be expected to have new reactivities that differ from those of organic functional groups and they have the potential to act as ligands for metals. The use of inorganic functional group transformations in the synthesis of polymers is understudied and represents a potentially rich source of functional group diversity in macromolecular science.

One part of our motivation to synthesize polymers through the polymerization of diaminosulfides was based on the chemical properties of this functional group in small molecule synthesis. These polymers are structurally related to polythiazyl (SN)_{x} which was first synthesized in 1953 from S_{2}N_{2}. This polymer is electrically conducting at room temperature and superconducting at low temperatures. Prior work by others, molecules with diaminosulfides were stable and readily isolated by traditional methods (distillation or chromatography). In addition, some examples of the synthesis of polymers containing diaminosulfides were found in 

To illustrate a possible application of poly(diaminosulfides), we completed initial experiments to investigate the application of a poly(diaminosulfide) as a delivery vehicle for drugs. Many drugs suffer from poor bioavailability, poor water solubility, short serum circulation lifetimes, and inadequate mechanisms to enter cells or have serious side effects that limit the amount of drug that can be administered. To overcome these and more limitations, drugs are often condensed with synthetic, biodegradable polymers into nanoparticle delivery vehicles that are administered to patients. The polymer protects the drugs from degradation in the bloodstream and allows their delivery to tumors by the enhanced permeation and retention effect where they can be taken into cancer cells. The polymers used in this field degrade slowly in the bloodstream but have a rapid rate of degradation when taken into the acidic compartments of cells—the endosome and lysosome—where they release their cargo. It is critically important that the polymer be biodegradable such that it will not accumulate within the body and cause a toxic response. In this article, some of the characteristics of poly(diaminosulfides) as drug delivery vehicles were investigated, including the stabilities of diaminosulfides in water under basic, acidic, and neutral conditions, whether nanoparticles fabricated from these polymers were internalized by cells, and whether any in vitro toxicity was observed from the nanoparticles. These studies are meant to illustrate an interesting application of poly(diaminosulfide) in medicine.

We report the synthesis of a small molecule that is a highly successful sulfur transfer reagent and how this molecule can be used to synthesize the first poly(diaminosulfide) reported in the literature. Some of the key, initial studies of a diaminosulfide in numerous solvents are reported to demonstrate their stabilities and, by extension, the stabilities of poly(diaminosulfide). Finally, one example of a poly(diaminosulfide) was fabricated into nanoparticles and studied for their ability to be internalized by human embryonic kidney-293 (HEK-293) cells and whether they showed any toxicity toward these cells.

### RESULTS AND DISCUSSION

#### Synthesis and Reactions of Sulfur Transfer Reagents.

We hypothesized that poly(diaminosulfide) could be synthesized by reacting secondary diamines with a sulfur transfer reagent as shown in Figure 1a. Many secondary diamines were commercially available or easily synthesized, so the challenge in the polymerization was to develop a useful sulfur transfer
reagent. Although SCl₂ is used in the synthesis of small molecules with diaminosulfides, its use has several drawbacks.80–85 This molecule has a low boiling point (59 °C), must be handled under inert atmospheres, is challenging to purify, reacts with multiple functional groups such as alcohols and alkenes, and releases HCl. Because of these limitations, we have not pursued the synthesis or use of SCl₂.

Two different sulfur transfer reagents were studied (Figure 2). Molecule B was initially explored as a sulfur transfer reagent based on the rapid reactions of thiosuccinimides with amines.86,87 Although the synthesis of B was straightforward and did not require any chromatography, its purification was challenging because of its poor solubility in many solvents. Molecule B was mostly insoluble in benzene, chloroform, DMSO, and methylene chloride. Molecule B was cleaned by washing the crude product with hexanes, and an isolated yield of 69% was obtained. To increase the purity of molecule B, it was recrystallized from methanol. Replacement of N-chlorosuccinimide with N-chlorophthalimide in the second step yielded a diphthalimide sulfur transfer reagent that also possessed limited solubility in organic solvents.

Although B was partially soluble in DMSO, it was not used to synthesize polymers for several reasons. First, the synthesis of B had poor atom efficiency. The addition of one sulfur (atomic weight: 32 g mol⁻¹) to yield a dianionsulfide functional group along the backbone of a polymer would require the use of 2 equiv of tributyltin chloride (MW: 326 g mol⁻¹) and 2 equiv of N-chlorosuccinimide (MW: 134 g mol⁻¹). Thus, significant amounts of waste were produced in the synthesis of molecule B. Second, the poor solubility of molecule B made it challenging to use in solvents that dissolve many polymers. Furthermore, it decomposed when heated in CDCl₃, and did not require any chromatography, its purification was challenging because of its poor solubility in many solvents.

A second sulfur transfer reagent was synthesized (molecule E in Figure 2) based on a literature procedure. In the first step, an excess of ethylmethyamine was reacted with sulfur chloride at −78 °C. Reactions run at 0 °C had unidentified side products, but the reaction at −78 °C yielded molecule C in high purity. Molecule C could be carried onto the next step without purification, or it could be purified by distillation. In the second step, C reacted with SO₂Cl₂ to yield D that was not isolated. Rather, D was slowly added to ethylmethyamine to yield the sulfur transfer reagent E. This procedure was followed to synthesize F using dimethyamine in both steps. Both E and F were readily purified by distillation and yielded clean products as shown by ¹H and ¹³C NMR spectroscopy and high-resolution mass spectrometry. Because no chromatography was necessary for the synthesis of E or F, these reactions could be scaled up to yield large amounts of product in a short period of time.

Kinetics of Transamination Reactions. To synthesize polymers via transamination reactions between molecule E and secondary diamines, the second-order kinetics of the reaction between molecule E and benzylmethyamine was studied in four solvents (Figure 3). Benzylmethyamine was chosen for these reactions because of the easily identified benzylic CH₂ group that shifted downfield in the ¹H NMR spectra when proceeding from benzylmethyamine to H to I.

The reactions between molecule E and 2 mol equiv of benzylmethyamine were studied, and the rate constants were measured in CD₂Cl₂ (7.81 × 10⁻⁵ M⁻¹ s⁻¹), DMSO-d₆ (4.89 × 10⁻⁵ M⁻¹ s⁻¹), CDCl₃ (2.79 × 10⁻⁵ M⁻¹ s⁻¹), and C₆D₆ (5.47 × 10⁻⁶ M⁻¹ s⁻¹). The rate constants were found using the data points for conversions of less than 10% using the assumption that the reaction was irreversible. Although the reaction was reversible, this assumption has been commonly used to find rate constants for reversible reactions at low conversions.88 It is important to note that the ethylmethyamine (boiling point = 36 °C) remained in the sealed NMR tube.

Although the reaction was most rapid in CD₂Cl₂ and reached equilibrium in 14 h, small amounts of unidentified side products were visible. The presence of side products made methylene chloride a poor choice for the polymerization. The reaction in CDCl₃ took 8 days to reach equilibrium, and the reaction in C₆D₆ did not reach equilibrium after 8 days. Despite the slow rates for reactions in these solvents, the reactions were clean and no side products were observed. The reaction in DMSO-d₆ also did not show any side products after 3 days, but this reaction reached 37% conversion and did not proceed any further. The final conversion was less than 50% because molecule I had limited solubility in DMSO-d₆ due to the apolar structure of molecule I and the polar structure of DMSO-d₆.

Figure 3. Kinetics of transamination reactions. (a) The reaction that was studied in a sealed NMR tube. (b) The conversion of the transamination reactions as a function of time. The conversion was defined as the sum of the S–N(CH₃)Bn bonds divided by the sum of all of the S–N bonds for molecules E, H, and I. (c) The plot of the initial data points used to find the rate constants for the reaction in each solvent. More data points were used to find the rate constant for the experiment in C₆D₆, but they are not shown here.
The $^1$H NMR spectra of this reaction in DMSO-$_d_6$ showed a lower than expected concentration of molecule 1 even after 3 days.

The reaction between molecule E and benzylmethylamine only reached 51% conversion in 17 h when completed at 40 °C in an uncapped NMR tube, despite the low boiling point of ethylmethylamine. Prolonged reaction times resulted in a slow increase in conversion, but this reaction was judged to be too slow. Molecule F was synthesized for the polymerization reactions because of the low boiling point of dimethylamine (boiling point 7 °C) which would make it simple to remove from a reaction.

Reactions between molecule F and benzylmethylamine were studied in CDCl$_3$, DMSO-$d_6$, and C$_6$D$_6$ in vented reaction vessels to allow dimethylamine to boil off (Figure 4 and Table 1).

1). Each of the reactions in Table 1 did not show any impurities by $^1$H NMR spectroscopy even when heated to 85 °C for extended periods of time. The conversions for the reactions were high for each solvent for reactions at 50 °C but went to quantitative conversions for reactions in C$_6$D$_6$ at 85 °C.

**Synthesis of Poly(diaminosulfide).** Poly-(diaminosulfide)s were synthesized by reaction of secondary diamines and molecule F at elevated temperatures (Scheme 1).

<table>
<thead>
<tr>
<th>entry</th>
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<th>temp (°C)</th>
<th>reaction time (h)</th>
<th>conv$^a$ (%)</th>
</tr>
</thead>
<tbody>
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<td>CDCl$_3$</td>
<td>50</td>
<td>24</td>
<td>39</td>
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<tr>
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<td>C$_6$D$_6$</td>
<td>50</td>
<td>24</td>
<td>41</td>
</tr>
<tr>
<td>5</td>
<td>C$_6$D$_6$</td>
<td>50</td>
<td>72</td>
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</tr>
<tr>
<td>6</td>
<td>C$_6$D$_6$</td>
<td>85</td>
<td>24</td>
<td>&gt;97</td>
</tr>
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</table>

$^a$The conversion was defined as the sum of the S−N(CH$_3$)$_2$Bn bonds divided by all of the S−N bonds in molecules F, J, and I.

**Scheme 1. Polymerization of Diamines with the Sulfur Transfer Reagent**

and Table 2). These polymerizations were run for 24 to 96 h, and the resulting polymers were characterized by GPC against polystyrene standards, $^1$H NMR spectroscopy, and $^{13}$C NMR spectroscopy.

The polymers in entries 1, 2, 5, and 6 had high molecular weights and degrees of polymerization. The degrees of polymerization were determined by two methods using the molecular weight measured by GPC and by end-group analysis in the $^1$H NMR spectra of the polymers. These values for the degree of polymerization agreed with each other and demonstrated that these reactions cleanly proceeded to high conversions. The polymerization with piperazine (entry 7) yielded an insoluble polymer in all solvents.

The polymer synthesized in entries 3 and 4 had limited stability. When this polymer was precipitated into methanol and water, it rapidly degraded as shown by the presence of numerous, unidentified peaks in the $^1$H NMR spectra. To isolate the polymer with minimal degradation, benzene was removed under vacuum after the polymerization was complete, and the polymer was characterized without further purification. The GPC and $^1$H NMR spectra were consistent with the indicated polymer. We believe that the internal, tertiary amine reacts with the diaminosulfide through an intramolecular reaction and was the source of the instability of this polymer.

The polymer shown in entry 6 was characterized by elemental analysis to provide further evidence that it possessed the indicated composition. The calculated weight composition of the repeat unit was carbon (64.95%), hydrogen (10.06%), nitrogen (11.65%), and sulfur (13.34%). The measured weight composition of the polymer was carbon (64.70%), hydrogen (9.97%), nitrogen (11.76%), and sulfur (13.44%). The agreement between the calculated and measured elemental compositions provided strong evidence that there was only one sulfur atom bridging between the nitrogens.

**Stability of Diaminosulfides in Organic Solvents and in Water.** Although numerous small molecules possessing diaminosulfide functional groups have been synthesized, no report on their long-term stabilities in organic solvents or water have been published. The stability of this functional group was investigated to estimate the stabilities of poly(diaminosulfide)s for future work. Molecule E and an internal standard of diethylene glycol dimethyl ether were added to CDCl$_3$, DMSO-$d_6$/D$_2$O (10/1 by volume), and C$_6$D$_6$ and allowed to sit at room temperature in capped NMR tubes (Figure 5). Periodic $^1$H NMR spectra were collected to determine the percent decomposition by the mole ratio of molecule E to the ether. After 32 days the amount of decomposition ranged from no detectable decomposition in C$_6$D$_6$ to 38% decomposition in DMSO-$d_6$/D$_2$O. Because molecule E was not soluble in methanol, the stability of molecule K was studied in CD$_3$OD. After 32 days, 15% of molecule K degraded.

These results demonstrated that the diaminosulfide functional group was stable in apolar, aprotic solvents but that it very slowly degraded in polar, protic solvents. The rate of degradation was slow enough that polymers with diaminosulfide functional groups are expected to have reasonable stabilities in these solvents, and this stability was observed for the prepared poly(diaminosulfide)s. The polymers were synthesized in benzene and chloroform at elevated temperatures and isolated by precipitation into methanol. Despite these conditions, the polymers possessed high degrees of polymerization.

To further explore the stability of the diaminosulfide functional group, molecule L was synthesized and studied in water (Figure 6). Molecule L and an internal standard of tert-butanol were added to D$_2$O with 9 mol equiv of acetic acid (acidic conditions), 9 mol equiv of KOH (basic conditions), or no additional acid or base (neutral conditions). The rate constants for the decomposition of this molecule were 1.29 × 10$^{-8}$ s$^{-1}$ under neutral pH conditions and 9.88 × 10$^{-5}$ s$^{-1}$ under basic conditions. Under acidic conditions, molecule L completely degraded by the time the first $^1$H NMR spectrum was obtained so only a lower limit of the rate constant was calculated (1.70 × 10$^{-2}$ s$^{-1}$).
The only product of degradation determined by \(^1\)H NMR spectroscopy was the secondary diamine used in the synthesis of molecule L. From prior work by others, it was known that diaminosulfides react in water to form sulfur monoxide, which possessed a half-life of seconds and decomposed to release SO\(_2\) and elemental sulfur.\(^8\)\(^9\),\(^9\)

Fabrication of Microparticles from a Poly(diaminosulfide) and Their Uptake into Cells. Synthetic polymers are widely used in drug delivery. In this field a polymer and drug are fabricated into nano- to micrometer sized particles and delivered to the body. Most of the polymers used in this field are based on polyesters\(^\) although other polymers are under investigation—because of the need to have the polymer degrade \textit{in vivo} before it accumulates in the body and provokes a toxic response. Polysters are widely used because they degrade in the body under neutral or acidic conditions without the need for enzymes. This observation of the role of polysters in drug delivery and the degradation of diaminosulfides in water led us to speculate that poly(diaminosulfide)s may be useful as drug delivery vehicles. The diaminosulfide functional group degrades several orders of magnitude faster than ester bonds under acidic conditions, and they possess reasonable stabilities in water under neutral conditions.\(^9\)\(^1\)

Some of the first key experiments to demonstrate the ability of poly(diaminosulfide)s to function as drug delivery vehicles are described here, and more results will be published in subsequent articles.

A polymer with the structure of entry 6 in Table 2 was used to fabricate microparticles that were studied as potential drug delivery vehicles (Figure 7). The microparticles were prepared according to a water/oil/water double emulsion-solvent evaporation method using poly(vinyl alcohol) as a surfactant. Briefly, the poly(diaminosulfide) was insoluble in water, and it was added to dichloromethane with a dye (FITC-dextran). A surfactant solution of water with 1 wt % poly(vinyl alcohol) was added to the dichloromethane and sonicated to produce the particles. This solution was diluted with more water and poly(vinyl alcohol) and further sonicated. After removal of the dichloromethane by evaporation, the microparticles were

<table>
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<th>Entry</th>
<th>Diamine</th>
<th>Solvent</th>
<th>Temperature (°C)</th>
<th>Reaction time (h)</th>
<th>(M_n) (g mol(^{-1}))</th>
<th>PDI</th>
<th>Yield (%)</th>
<th>DP(^b) (%)</th>
<th>DP(^c) (%)</th>
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</thead>
<tbody>
<tr>
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<td>24</td>
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<td>5,200</td>
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<td>97</td>
<td>98</td>
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</tr>
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<td>(\text{C}_2\text{H}_6)</td>
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\(^{a}\) The \(M_n\) and PDI were measured using size exclusion chromatography versus polystyrene standards. \(^{b}\) The degree of polymerization were based on the values for \(M_n\) measured by GPC. \(^{c}\) The degree of polymerization were based on \(^1\)H NMR spectra. \(^{d}\) The polymer was insoluble.

\(N\)-S-N

\(\text{CDCl}_3\) 8% degradation after 32 days

\(\text{DMSO-d}_6/D_2\text{O}\) 38% degradation after 32 days

\(N\)-S-N

\(\text{CDOD}\) 15% degradation after 32 days

Figure 5. Stability of two molecules were studied at room temperature in organic solvents including DMSO-\(d_6/D_2\)O (10/1 v/v).

Figure 6. Degradation of molecule L was studied in D\(_2\)O under acidic (with acetic acid), neutral, and basic (with KOH) conditions. The amount of the diaminosulfide that degraded as a function of time was plotted.
The cell viability of HEK-293 cells was investigated to determine whether the microparticles derived from poly-(diaminosulfide)s were toxic. The toxicity of microparticles fabricated from the polymer with the structure shown in entry 6 of Table 2 was studied via a MTS assay that is widely accepted as one method to determine cell viability in the presence of foreign molecules.\textsuperscript{92,93} Briefly, the MTS assay measures the mitochondrial activity of the cells and is used as an indication of the cell growth and viability. In living cells the MTS reagent (a yellow, water-soluble tetrazolium salt) is cleaved by the mitochondrial enzyme dehydrogenase (NADH-dependent reduction of the tetrazolium ring in MTS) to generate a water-soluble purple product called formazan. The concentration of formazan can be measured, and in this way, the relationship between the cell number and the amount of formazan generated is established since the absorbance is directly proportional to the number of viable cells. Damaged or dead cells exhibit a reduced or diminished enzyme activity and therefore less or no formazan production. Here, the incubation period of 24 h ensured the exposure of the cells to the different treatments in their exponential growth phase. Figure 9 shows the cell viability as a function of the concentration of microparticles and demonstrates excellent biocompatibility of these novel polymeric microparticles in HEK-293 cells. Microparticles in the concentration range of 1–1000 \( \mu \text{g/mL} \) had no adverse effect on cell viability. Even high concentrations of the microparticles did not reduce cell viability with cell survival rates greater than 85\% for all the concentrations tested.

## CONCLUSIONS

This paper described the first synthesis of poly-(diaminosulfide)s from two simple starting materials. The sulfur transfer reagent used in the synthesis was readily synthesized in two steps, and because it was purified by distillation rather than column chromatography, large quantities could be synthesized in only a few days. These polymers have many of the right properties to be used as synthetic polymers for different applications. For instance, we investigated the stabilities of diaminosulfides in different solvents so that future applications of poly(diaminosulfide)s could be envisioned. This functional group was very stable in organic solvents and not prone to oxidation; in fact, no evidence of oxidation of the sulfur was observed in any sample. One exciting application of these polymers as drug delivery vehicles was explored, and the results were very promising. A poly(diaminosulfide) was readily fabricated into nanoparticles that were absorbed into cells. These nanoparticles were also nontoxic toward HEK-293 cells. These results were promising, but more work is needed to investigate the advantages poly(diaminosulfide)s may possess.
over polymers used in drug delivery. We propose a general label of poly(NSN) for any poly(diaminosulfide) to emphasize the functional group used in their synthesis and found in their backbones. Poly(NSN) can be used to describe a general family of polymers in the same way that the terms polysytyrenes and polyacrylates are used.

One significant characteristic of diaminosulfides is that they are based on an inorganic functional group. Their structures differentiate them from the numerous organic functional groups used in the synthesis of most polymers. We believe that by working with inorganic functional groups with reactivities that differ from those of organic functional groups, new opportunities in macromolecular science will be realized.

**EXPERIMENTAL SECTION**

**Materials.** Sodium sulfide nonahydrate (Na₂S·9H₂O), tributyltin chloride, N-chlorosuccinimide, sulfur monochloride, N-ethylmethylamine, N,N-dimethyl-1,6-hexanediame, N,N-bis[3-(methylamino)propyl]methyamine, 4,4′-trimethylenedi-piperidine, N,N-di-sc-butyryl-p-phenylenediamine, dimethylamine, p-toluenesulfonyl chloride, and 3-methoxypropylamine were purchased from Aldrich or Acros Organics at their highest purity and used as received. Piperazine (99%) was purchased from Fisher Scientific. 4,4′-Dithiobisdimethylamine.95 Bis(succinimide) Sulfide (Molecule B).94 N-Chlorosuccinimide (2.22 g, 16.6 mmol) was slowly added to a solution of molecule A (5.08 g, 8.30 mmol) in CHCl₃ (22 mL) at 0 °C and stirred. After 1.7 h, the ice bath was removed and the reaction was stirred for 8 h. A yellow solid stuck to the walls of the flask. The yellow organic phase was decanted. The yellow solid was washed with hexanes and dried under vacuum to give a crude yellow solid (1.30 g, 69% yield).1H NMR (DMSO-d₆): δ 2.57 (s, 8H).13C NMR (DMSO-d₆): δ 29.55, 179.47. N,N′-Dithiobis(bisethylmethyamine) (Molecule C).95 A solution of N-ethylmethyleneimine (10.5 g, 178 mmol) in petroleum ether (400 mL) was cooled to −78 °C for 30 min. To this solution, sulfur monochloride (6.00 g, 44.4 mmol) was added dropwise for 10 min. The reaction was stirred for 20 min at −78 °C and another 35 min at room temperature. The mixture was washed with a saturated NaCl solution in water. The organic layer was dried over anhydrous magnesium sulfate and evaporated to give a yellow–green oil (7.0 g). The product was isolated by vacuum distillation at 30–35 °C to yield a colorless oil (6.20 g, 78% yield).1H NMR (CDCl₃): δ 1.14 (t, 6H, J = 7.2 Hz), 2.64 (s, 6H), 2.69 (q, 4H, J = 7.1 Hz).13C NMR (CDCl₃): δ 13.81, 46.28, 53.54. HRMS calcd for C₆H₁₄N₂S: 180.0755. Found: 180.0759.

N-Ethylmethylsulfonyl Chloride (Molecule D).96 A solution of molecule A (4.81 g, 26.7 mmol) in CH₂Cl₂ (70 mL) was precooled to 0 °C for 40 min under N₂. Sulfuryl chloride (3.96 g, 29.4 mmol) was added dropwise to the solution for 17 min under N₂. The reaction was stirred for 30 min at 0 °C and another 50 min at room temperature to give a crude product (6.70 g, 53.4 mmol), which was used in situ for the preparation of molecule E.

Bis(N-ethylmethyl) Sulfide (Molecule E).96 A solution of molecule D (6.70 g, 53.4 mmol) in CH₂Cl₂ (40 mL) was slowly added to a solution of N-ethylmethyleneimine (7.89 g, 13.3 mmol) in CH₂Cl₂ (60 mL) at 0 °C under N₂ and stirred for 1 h. The mixture was washed with a saturated NaCl solution in water. The organic phase was dried over anhydrous magnesium sulfate and evaporated to give a yellow–green oil (4.22 g). The product was purified by distillation under vacuum at room temperature to yield a colorless oil (2.53 g, 32% yield).1H NMR (CDCl₃): δ 1.14 (t, 6H, J = 7.2 Hz), 2.95 (s, 6H), 3.11 (q, 4H, J = 7.1 Hz).13C NMR (CDCl₃): δ 14.24, 46.29, 54.89. HRMS calcd for C₅H₁₁N₂S: 148.1034. Found: 148.1033.

N,N′-Dithiobisdimethylamine.98 A solution of dimethylamine (8.01 g, 178 mmol) in anhydrous ether (400 mL) was cooled to −78 °C for 44 min. Sulfur monochloride (6.00 g, 44.4 mmol) was added dropwise to the solution for 14 min. The solution was stirred for 30 min at −78 °C and another 30 min at room temperature. The mixture was washed with a saturated NaCl solution in water. The organic layer was dried over anhydrous magnesium sulfate and evaporated to give a colorless oil (6.66 g, 99% yield), which could be used directly for the preparation of molecule F without further purification.1H NMR (CDCl₃): δ 2.63 (s, 12H).13C NMR (CDCl₃): δ 48.31. HRMS calcd for C₁₂H₂₄N₄S: 152.0442. Found: 152.0444. N-Dimethylsulfonyl Chloride.96 A solution of N,N′-dithiobisdimethylamine (6.03 g, 39.6 mmol) in anhydrous Et₂O (50 mL) was cooled to 0 °C for 1 h under N₂. Sulfuryl chloride (5.88 g, 43.6 mmol) was added dropwise to the solution under N₂. The reaction was stirred for 36 min at 0 °C and another 50 min at room temperature to give a crude product (8.84 g, 79.2 mmol), which was used in situ for the preparation of molecule F.

Bis(N,N′-dimethyl) Sulfide (Molecule F).96 A solution of N-dimethylsulfonyl chloride (8.84 g, 79.2 mmol) in anhydrous Et₂O (50 mL) was slowly added to a solution of dimethylamine (17.9 g, 39.6 mmol) in anhydrous Et₂O (75 mL) at −5 °C under N₂ and stirred for 1.2 h. The reaction was washed with saturated aqueous NaCl. The organic phase was dried over anhydrous magnesium sulfate, and the solvent was removed after freezing the product at −5 °C to give yellow-green oil (7.0 g). Further purification was achieved by distillation under vacuum at 30 °C to yield a colorless oil (4.39 g, 46% yield).1H NMR (CDCl₃): δ 3.02 (s).13C NMR (CDCl₃): δ 49.69. HRMS calcd for C₆H₁₄N₂S: 120.0721. Found: 120.0719.

Bis(N,N′-3-methoxypropyl(triethylene glycol monomethyl ether) sulfide) (Molecule L). In a flask was added N-(3-
methoxypropyl-triethylene glycol monomethyl ether) (2.27 g, 9.64 mmol) and 3.6 mL of benzene. Next, molecule F (0.503 g, 4.18 mmol) was added, and the flask was connected to a reflux condenser and heated to 85 °C for 4 h. After the benzene was removed under vacuum. The product was cleaned by chromatography on basic alumina oxide using ethyl acetate. The product was a clear oil (1.54 g, 73% yield). 1H NMR (CDCl3): δ 1.70 (m, 4H), 3.29 (s, 6H), 3.34 (m, 4H). 13C NMR (CDCl3): δ 25.59, 42.43, 46.90, 55.38, 59.01.

Entry 1, Table 2. Molecule F (0.942 g, 7.83 mmol) was reacted with N,N′-dime-thyl-1,6-hexanediamine (1.13 g, 7.83 mmol) in refluxing benzene (1.4 mL) at 85 °C for 24 h. After evaporating the solvent, the polymer was precipitated into methanol (10 mL). The polymer was dried under vacuum to yield a brown oil (1.02 g, 75% yield). 1H NMR (CDCl3): δ 1.29 (m, 4H), 1.54 (m, 4H), 2.94 (s, 6H), 3.07 (t, 4H, J = 7.2 Hz). 13C NMR (CDCl3): δ 26.88, 28.66, 46.90, 55.38, 61.05.

Entry 3, Table 2. Molecule F (0.186 g, 1.55 mmol) was reacted with N,N-bis-[3-(methylamino)propyl]methyleneamine (0.268 g, 1.55 mmol) in refluxing benzene (1.4 mL) at 85 °C for 72 h. The benzene was removed under vacuum. When the polymer was redissolved in 4 mL of CH3OH and precipitated into 9 mL of water, the polymer decomposed to unknown products, and the 1H NMR spectrum became too complicated to assign the peaks. Therefore, after the polymerization was complete the polymer was dried under vacuum to yield a brown oil (0.310 g, 97% yield) that was used without further purification. 1H NMR (CDCl3): δ 1.71 (m, 4H), 2.21 (s, 3H), 2.32 (t, 4H, J = 7.5 Hz), 2.95 (s, 6H), 3.12 (t, 4H, J = 7.1 Hz). 13C NMR (CDCl3): δ 25.59, 42.43, 46.90, 55.38, 59.01.

Entry 5, Table 2. Molecule F (0.186 g, 1.55 mmol) was reacted with 4,4′-trimethylenebridiphenide (0.326 g, 1.55 mmol) in CHCl3 (1.6 mL) at 60 °C for 72 h. After evaporating the solvent and redissolving it in CHCl3 (4 mL), the polymer was precipitated into methanol (8 mL) to give a white-yellow powder (0.330 g, 88% yield). 1H NMR (CDCl3): δ 1.22 (m, 12H), 1.59 (m, 4H), 3.08 (t, 4H, J = 11.0 Hz), 3.44 (m, 4H). 13C NMR (CDCl3): δ 23.68, 34.02, 34.96, 36.72, 58.57.

Reactions of Molecule E and N-Benzylmethyleneamine (Figure 3). Molecule E (463 mg, 312 μmol) was dissolved in 1.35 mL of CDCl3, and 1 mL (34.4 mg, 232 μmol) of the solution was transferred to a NMR tube. After the addition of N-benzylmethyleneamine (56.3 mg, 464 μmol) and sealing the NMR tube with a rubber septum, 1H NMR spectra were continually recorded for 3 days. The reaction was monitored by conversion of the benzyl hydrogens in N-benzylmethyleneamine at 3.71 ppm to the benzyl hydrogens in molecule H at 4.31 ppm and in molecule I at 4.36 ppm.

The same procedure was also followed for the kinetics in CDCl3. The conversion of molecule E to molecules H and I was monitored by comparing the benzyl hydrogens at 3.71 ppm in molecule H and 4.34 ppm in molecule I at 4.36 ppm.

Transamination Reaction of Molecule F and N-Benzylmethyleneamine (Table 1). N-Benzylmethyleneamine (153 mg, 1.26 mmol) was added to a solution of molecule F (75.8 mg, 631 μmol) in 1.26 mL of CDCl3. After connecting a condenser to the flask, the mixture was reacted at 50 °C, and the reaction was monitored by 1H NMR spectroscopy every 24 h showing 9% conversion to J and 88% conversion to I after 72 h.

The same procedure was followed for the reaction of molecule F (88.3 mg, 735 μmol) and N-benzylmethyleneamine (178 mg, 1.47 mmol) in 1.47 mL of DMSO-d6 showing 13% conversion to J and 77% conversion to I after 72 h. The reaction of molecule F (82.3 mg, 685 μmol) and N-benzylmethyleneamine (166 mg, 1.37 mmol) in 1.37 mL of CD3OD showed 17% conversion to J and 75% conversion to I after 72 h.

Molecule F (89.9 mg, 748 μmol) and N-benzylmethyleneamine (181 mg, 1.50 mmol) were reacted in 1.5 mL of benzene at 85 °C showing 3% conversion to J and 97% conversion to I after 24 h.

Stability of Molecule E in Organic Solvents. The stability of molecule E was studied in CDCl3, DMSO-d6/D2O (10/1 v/v), and CD3OD following the same procedure. Molecule E (34.4 mg, 2.32 × 10−4 mol) was added to a NMR tube with 1 mL of solvent. Next, diethylene glycol dimethyl ether (31.2 mg, 2.32 × 10−4 mol) was added. The NMR tube was capped, and 1H NMR spectra were periodically collected. The amount of decomposition was determined by the difference in the ratio of the peaks due to molecule E and the ether measured on days 1 and 32.

Stability of Molecule L in D2O. Molecule L (31.4 mg, 6.27 × 10−5 mol) was added to an NMR tube. A 1 mL solution of D2O of tert-butanol (5.96 mg, 6.27 × 10−5 mol) and acetic acid (30.5 mg, 0.20 × 10−4 mol) was added to the NMR tube, and it was vigorously shaken. The first 1H NMR spectrum after 271 s showed no evidence of molecule L and showed the secondary amine as the only degradation product.

The same procedure was followed except that no acetic acid was added (the neutral conditions). The decomposition of molecule L was followed by 1H NMR spectroscopy. The same procedure was followed except that no acetic acid was added and KOH (9 mol equiv) was added (the basic conditions). The decomposition of molecule L was followed by 1H NMR spectroscopy.

Formulation of Microparticles. Microparticles were fabricated from the polymer in entry 6 of Table 2 using a double emulsion-solvent evaporation method that is widely used for the encapsulation of hydrophilic drugs. The surfactant solution (1 wt % PVA in water, internal water phase or W1) was added to the polymer solution (in dichloromethane, oil phase or O) under microtip probe sonication for 30 s (10 W energy output, Fisher Scientific sonic dismembrator Model 100) to form the first emulsion (W1/O). This was then immediately added to the second PVA solution (in water, external water phase or W2) and further sonicated at the same speed for another 30 s to form the second emulsion (W1/O/W2). These processes were carried out under an ice bath. The final emulsion was then added to aqueous PVA solution under magnetic agitation and stirred at room temperature and under atmospheric pressure until complete evaporation of dichloromethane. The microparticles were collected by centrifugation at 8500 rpm for 10 min (Fischer Scientific Accuspin 400), washed twice with deionized distilled water at a concentration of 1 mg/mL using folded capillary cells. Sample dilution is often necessary in order to avoid multiscattering events. The suspension is necessary in order to avoid multiscattering events. The FITC-dextran loaded microparticles were prepared in the same manner by dissolving FITC-dextran in the internal water phase used in making the primary emulsion.

Determination of Particle Size (Hydrodynamic Diameter) and Size Distribution. Particle size and particle size distribution of microparticles were analyzed at a concentration of approximately 1 mg particles/1 mL of deionized water. Appropriate dilution of the particle suspension is necessary in order to avoid multiscattering events. The measurements were carried out on microparticle suspensions using a Zetasizer Nano-ZS (Malvern Instruments). The particle size and size distribution by intensity were measured by dynamic light scattering (He–Ne laser with a fixed wavelength of 633 nm, 173° backscatter at 25 °C) in 10 mm diameter cells.

Measurement of Surface Charge. The zeta potential of microparticles was analyzed by dispersing the microparticles in deionized distilled water at a concentration of 1 mg/mL using folded capillary cells. Sample dilution is often necessary in order to eliminate particle interactions. Zeta potential is an indicator of the charge on the
surface of the microparticles. The surface charge measurements of the blank microparticles were performed using the electrophoretic laser scattering method (Laser Doppler Microelectrophoresis, He–Ne laser 633 nm at 25 °C).

Scanning Electron Microscopy (SEM). The shape and the surface morphology of the microparticles were studied using a scanning electron microscope. The particles were mounted on silicon wafers which were placed on aluminum specimen stubs using adhesive carbon tape. The mount was then coated by ion sputtering (K550 Emitech sputter coater, set at 10 mA for 2.5 min) with conductive gold and examined using a Hitachi Model S-4800 SEM, operated at 4 kV accelerating voltage.

Cell Culture. The cells were maintained in DMEM supplemented with 10 vol % FBS and gentamycin at a concentration 50 μg/mL in a humidified incubator (Sanyo Scientific Autiflow, IR direct heat CO2 incubator) at 37 °C containing 95% air and 5% CO2. The cells were plated and grown as a monolayer in 75 cm² polystyrene cell culture flasks (Corning Inc., Corning, NY) and subcultured (subcultivation ratio of 1:4) after 80–90% confluence was achieved. Cell lines were started from frozen stocks, and the medium was changed every 2–3 days. The passages used for the experiment were between 4 and 15.

Investigation of FITC-Dextran Loaded Microparticle Uptake by HEK-293 Cells Using Confocal Microscopy. To determine the qualitative in vitro intracellular uptake of microparticles, cells were plated at a density of 50 000 cells/well in a clear, flat-bottom, 8-chambered glass slide with cover (Lab-Tek, Nunc, NY) that were previously coated with 0.1 wt % poly(l-lysine). The cells were allowed to attach overnight, and the next day the cell culture medium was removed and the cells were treated with an aliquot of a suspension of FITC-dextran loaded microparticles in medium and further incubated at 37 °C for 24 h. The experiment was terminated by removing the particulate suspension and washing the cell monolayer two times with PBS in order to remove particles not internalized by the cells. The cells were then fixed with 4 vol % paraformaldehyde, followed by permeabilization of cells with 0.2 wt % Triton X-100 (Sigma, Sigma-Aldrich, St. Louis, MO). The cells were later treated with phalloidin permeabilization of cells with 0.2 wt % Triton X-100 (Sigma, Sigma-Aldrich, St. Louis, MO). The cells were washed with PBS during every step in the process. Cellular uptake of FITC-dextran loaded microparticles and their intracellular distribution was visualized by confocal microscopy (Carl Zeiss LSM 710, 60× oil objective lens) by using DAPI, FITC, and phallolidin filters equipped with Zen 2009 imaging software.

Evaluation of the Cytotoxicity of Microparticles Incubated in HEK-293 Cells. The in vitro cytotoxicity of blank nanoparticles was examined by a colorimetric MTS assay. A stock suspension of microparticles was prepared by dispersing freeze-dried particles in an appropriate volume of cell culture medium. To obtain different test concentrations (1–1000 μg/mL), serial dilutions from the stock microparticle suspension were prepared with the medium. The first day of the experiment, confluent cells were seeded in clear polystyrene, flat bottom, 96-well plates (Costar, Corning Inc., Corning, NY) at a density of 10 000 cells/well and allowed to attach overnight in the incubator. Next day, the cells were exposed to the polymer by replacing the culture medium with different dilutions of stock suspensions and further incubating for 24 h. On the last day of the experiment, the treatments were removed and fresh medium was added along with 20 μL of MTS reagent. The plate was incubated at 37 °C in a humidified, 5% CO2 atmosphere for 4 h. To measure the amount of soluble formazan produced by the reduction of MTS reagent by viable cells, the plate was read by Spectramax 384 Plus (Softmax Pro, Molecular Devices, Sunnyvale, CA) at a wavelength of 490 nm. The absorbance readings were recorded and quantitated for the colorimetric assay and the cell viability was expressed by the following equation:

\[
\text{cell viability(\%)} = \frac{\text{[absorbance intensity of cells treated with MPs]}}{\text{[absorbance intensity of cells without any treatment (control)]}} \times 100
\]

The cytotoxic effect of different treatments was calculated as a percentage of cell growth with respect to the control. Values are expressed as mean ± SEM for each microparticle concentration (n = 6).

**REFERENCES**
