Synthesis of Amine-Functionalized Diene-Based Polymers as Novel Gene Delivery Vectors

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Received June 5, 2006; Revised Manuscript Received September 25, 2006

ABSTRACT: A series of amine-functionalized diene-based polymers were synthesized via anionic and free radical polymerization techniques for evaluation of their potential as gene delivery vectors. Cytotoxicity, binding affinity, and transfection efficiency properties were examined. A systematic study of the structure−property relationships revealed that cytotoxicity decreased as the side group of the polymers changed from methyl to propyl group due to the decrease of the acidity after protonation. The binding affinity between the polymers and DNA also decreased as the size of the side group increased. Of the polymers studied, poly[2-(*N*,*N*-diethylaminomethyl)-1,3-butadiene], with lower cytotoxicity than poly(ethylene imine) and higher transfection efficiency at an N/P ratio of 2, is a promising, novel transfection vector.

Introduction

Gene therapy has received great interest in recent years because of its potential for the treatment of various diseases and genetic disorders. Nucleic acids cannot be delivered to a person's cells directly due to their large size and instability under physiological conditions. A carrier, or a gene delivery vector, is needed. The main limitation to the wide utilization of gene therapy is the lack of efficient, nontoxic vectors.[1−3] As a result, significant efforts have been made to develop improved materials. There are mainly two kinds of these delivery vectors: viral vectors and nonviral vectors. Although viral vectors are generally efficient at delivering genes, their application has been limited by their immunogenicity and potential oncongenicity.[1] As such, synthetic polycations have received increased attention as potential gene delivery vectors because of their lower safety risk, ability to incorporate large therapeutic DNA, ease of large-scale synthesis, and tunability.[1,4,5] Because of the electrostatic interactions between the positively charged groups in the polymers and the phosphate groups in the DNA, DNA binds with these polymers and forms polyplexes that cells can uptake. These polyplexes protect DNA from degradation by nucleases.

Currently the most widely used nonviral vector is poly(ethylene imine) (PEI). It is highly efficient but has moderate to high cytotoxicity. Understanding the structure−property relationship between gene delivery vectors and transfection efficiency plays an important role in designing improved materials.[3,6] In this article, the synthesis of a series of amine-functionalized homopolymers as well as block copolymers incorporating poly(ethylene glycol) (PEG) is described. The effect of the amine group on the mechanism of the anionic polymerization is studied. Because of the synthetic design of this series of materials, the effects of the size of the side group, molecular weight and polymer acidity on cytotoxicity, polyplex formation, and transfection efficiency are determined. The structures of the polymers studied are illustrated in Figure 1.

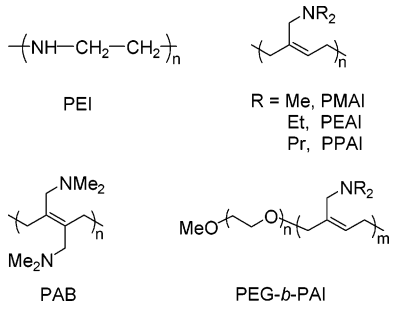

Figure 1. Structures of polymers as gene delivery vectors.

Experimental Section

Materials. *sec*-Butyllithium was purchased from FMC Corp. and used without further purification. Poly(ethylene glycol) monomethyl ether (mPEG-OH) was purchased from Polysciences, Inc. (Warrington, PA). Plasmid DNA (pCMV-Luc) was purchased from Elim Biopharmaceuticals, Inc. (Hayward, CA). Minimum essential medium (MEM), Hanks' balanced salt solution (HBSS), Dulbecco's phosphate buffered saline (PBS), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Invitrogen. CellTiter 96 AQ_{ueous} one solution cell proliferation assay and luciferase assay system were purchased from Promega Corp. (Madison, WI). BCA protein assay kit was purchased from Pierce (Rockford, IL). Toluene and benzene were degassed, dried over sodium, and freshly distilled before use. *N*,*N*-Dimethylformamide was distilled from calcium hydride and stored over molecular sieves. All other reagents were used without further purification.

Instrumentation. ^{1}H NMR spectra were acquired on a Bruker 400 AVANCE spectrometer using deuterated chloroform as the solvent. Molecular weights were measured using a Waters GPC system with polystyrene standards. The measurements were taken using THF as the solvent on three columns (Waters Styragel HR2, HR4, and HR5). Elemental analysis was performed by Atlantic Microlab, Inc.

Monomer Synthesis. Monomers 2-(*N*,*N*-dimethylaminomethyl)-1,3-butadiene (**I**), 2-(*N*,*N*-diethylaminomethyl)-1,3-butadiene (**II**), and 2-(*N*,*N*-dipropylaminomethyl)-1,3-butadiene (**III**) were synthesized and purified as described previously.[7]

*2-(N,N-Dimethylaminomethyl)-1,3-butadiene (**I**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.38 (dd, 1H, $J_1 = 17.6$ Hz, $J_2 = 11$ Hz), 5.41 (d, 1H, $J = 17.6$ Hz), 5.16 (s, 1H), 5.13 (s, 1H), 5.12 (d, 1H, $J =$

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10.1021/ma061249p CCC: $33.50
Published on Web 11/10/2006

11 Hz), 3.02 (s, 2H), 2.23 (s, 6H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 143.26 (CH_2=$C$$CH_2N$), 137.45 ($CH_2$=$CHCCH_2N$), 117.51 ($CH_2$=CHCC$H_2N$), 114.32 ($CH_2$=CC$H_2N$), 61.80 ($CH_2N$), 45.39 [N($CH_3$)$_2$]. Elem Anal. Calcd: C, 75.62%; H, 11.79%; N, 12.60%. Found: C, 75.77%; H, 11.97%; N, 12.25%.

*2-(N,N-Diethylaminomethyl)-1,3-butadiene (**II**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.38 (dd, 1H, J_1 = 17.6 Hz, J_2 = 11 Hz), 5.44 (d, 1H, J = 17.6 Hz), 5.20 (s, 1H), 5.13 (s, 1H), 5.08 (d, 1H, J = 11 Hz), 3.15 (s, 2H), 2.50 (q, 4H, J = 7 Hz), 1.00 (t, 6H, J = 7 Hz). ^{13}C NMR (400 MHz, $CDCl_3$): δ 143.69 (CH_2=$C$$CH_2N$), 137.51 ($CH_2$=$CHCCH_2N$), 116.53 ($CH_2$=CHCC$H_2N$), 113.53 ($CH_2$=CC$H_2N$), 54.93 [$CH_2$N($CH_2CH_3$)$_2$], 46.63 [N($CH_2CH_3$)$_2$], 11.28 [N($CH_2CH_3$)$_2$]. Elem Anal. Calcd: C, 77.63%; H, 12.31%; N, 10.06%. Found: C, 77.63%; H, 12.30%; N, 9.89%.

*2-(N,N-Dipropylaminomethyl)-1,3-butadiene (**III**).* ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.38 (dd, 1H, J_1 = 17.6 Hz, J_2 = 11 Hz), 5.41 (d, 1H, J = 17.6), 5.18 (s, 1H), 5.09 (s, 1H), 5.02 (d, 1H, J = 11 Hz), 3.11 (s, 2H), 2.31 (t, 4H, J = 7 Hz), 1.42 (m, 4H, J = 7 Hz), 0.83 (t, 6H, J = 7 Hz). ^{13}C NMR (400 MHz, $CDCl_3$): δ 144.26 (CH_2=$C$$CH_2N$), 137.94 ($CH_2$=$CHCCH_2N$), 116.88 ($CH_2$=CHCC$H_2N$), 113.85 ($CH_2$=CC$H_2N$), 56.71 [$CH_2$N($CH_2CH_2CH_3$)$_2$], 56.22 [N($CH_2CH_2CH_3$)$_2$], 20.19 [N($CH_2CH_2CH_3$)$_2$], 11.89 [N($CH_2CH_2CH_3$)$_2$]. Elem Anal. Calcd: C, 78.97%; H, 12.65%; N, 8.37%. Found: C, 79.02%; H, 12.72%; N, 8.38%.

Synthesis of 2-Bromo-3-(*N,N*-dimethylamino)propene (IV). To a solution of dimethylamine (45 g, 1 mol) in ether was added 2,3-dibromopropene (100 g, 0.5 mol) dropwise at 0 °C. The reaction mixture was allowed to slowly warm to room temperature and stirred for 16 h. The solution was extracted twice with diethyl ether, washed once with brine, and dried over anhydrous sodium sulfate. After evaporation of the solvent, the residue was distilled to give 2-bromo-3-(*N,N*-dimethylamino)propene in quantitative yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.79 (s, 1H), 5.56 (s, 1H), 3.09 (s, 2H), 2.26 (s, 6H). ^{13}C NMR (300 MHz, $CDCl_3$): δ 131.60 (CH_2=CBrCH_2), 119.12 (CH_2=CBrCH_2), 68.20 (CH_2N), 45.14 [N(CH_3)$_2$].

Synthesis of 2,3-Bis(dimethylaminomethyl)-1,3-butadiene (V).[8] A Grignard reagent, readily prepared from **IV** (32.8 g, 0.20 mol) and magnesium (5.76 g, 0.24 mol), was added dropwise to **IV** (32.8 g, 0.20 mol) in the presence of dichloro[1,3-bis(diphenylphosphino)-propane]nickel (4.3 g, 0.008 mol) in THF at 0 °C. The reaction mixture was allowed to slowly warm to room temperature. After refluxing for 4 h, the reaction mixture was quenched with saturated sodium chloride, extracted with diethyl ether three times, washed once with brine, and dried over anhydrous sodium sulfate. After evaporation of the solvent, the residue was distilled to give 2,3-bis(dimethylaminomethyl)-1,3-butadiene in 30% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.29 (s, 2H), 5.06 (s, 2H), 3.03 (s, 4H), 2.18 (s, 12H). ^{13}C NMR (300 MHz, $CDCl_3$): δ 143.54 (CH_2=$C$$CH_2$), 113.35 ($CH_2$=CC$H_2$), 62.21 ($CH_2N$), 43.27 [N($CH_3$)$_2$]. Elem Anal. Calcd: C, 71.37%; H, 11.98%; N, 16.65%. Found: C, 71.46%; H, 12.22%; N, 16.35%.

Anionic Polymerization of 2-(*N,N*-Dialkylaminomethyl)-1,3-butadiene. Monomer was stirred over dibutylmagnesium for 20 min before vacuum distillation. Monomer (0.5 mL), solvent (1 mL), and *sec*-butyllithium (amount varies according to the target molecular weight) were added to a flame-dried round-bottom flask and stirred at the desired temperature for the prescribed amount of time depending on the monomer structure. The reaction was terminated with degassed methanol. ^{1}H NMR of the reaction mixture was taken before the polymer was precipitated. The conversion was calculated on the bassi of the integration ratio of the characteristic vinylic peak from the polymer at 5.24−5.29 ppm to the remaining vinylic peak from the monomer at 6.38 ppm. Poly-[2-(*N,N*-dialkylaminomethyl)-1,3-butadiene] (PAI) was purified by precipitation into acetone at −78 °C and dried under vacuum at room temperature for 24 h.

Poly[2-(N,N-dimethylaminomethyl)-1,3-butadiene] (PMAI). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.26 (s, 1H), 2.80 and 2.70 (s, 2H from trans and cis CH_2N), 2.13−2.10 [broad, 10 H, 6 from 2(CH_3) and 4 from the polymer backbone]. ^{13}C NMR (400 MHz, $CDCl_3$): δ 136.75 (CH_2CH=$C$$CH_2$), 128.62 ($CH_2$$C$H=CC$H_2$), 66.61 [$CH_2$N-($CH_3$)$_2$, cis], 58.31 [$CH_2$N($CH_3$)$_2$, trans], 45.46 [N($CH_3$)$_2$], 35.90 ($CH_2$CH=C$C$$H_2$, trans), 28.83 ($CH_2$CH=C$C$$H_2$, cis), 26.38 ($CH_2$-CH=CC$H_2$). Elem Anal. Calcd: C, 75.62%; H, 11.79%; N, 12.60%. Found: C, 75.83%; H, 11.36%; N, 11.72%.

Poly[2-(N,N-diethylaminomethyl)-1,3-butadiene] (PEAI). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.29 (broad, 1H), 2.93 and 2.84 (s, 2 H from trans and cis CH_2N), 2.40 (q, 4H), 2.03−2.15 (broad, 4H), 0.98 (t, 6H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 137.80 (CH_2CH=$C$$CH_2$), 127.90 ($CH_2$$C$H=CC$H_2$), 60.30 [$CH_2$N($CH_2CH_3$)$_2$, cis], 52.23 [$CH_2$N($CH_2CH_3$)$_2$, trans], 46.97 [N($CH_2CH_3$)$_2$], 36.20 ($CH_2$-CH=C$C$$H_2$, trans), 29.40 ($CH_2$CH=C$C$$H_2$, cis), 26.90 ($CH_2$CH=CC$H_2$), 12.20 [N($CH_2CH_3$)$_2$]. Elem Anal. Calcd: C, 77.63%; H, 12.31%; N, 10.06%. Found: C, 77.37%; H, 12.41%; N, 9.94%.

Poly[2-(N,N-dipropylaminomethyl)-1,3-butadiene] (PPAI). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.24 (s, 1H), 2.89 and 2.80 (s, 2 H from trans and cis CH_2N), 2.23 (t, 4H), 2.09 (broad, 4H), 1.39 (m, 4H), 0.82 (t, 6H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 137.16 (CH_2-CH=$C$$CH_2$), 127.96 ($CH_2$$C$H=CC$H_2$), 61.05 [$CH_2$N($CH_2CH_2CH_3$)$_2$, cis], 55.64 [N($CH_2CH_2CH_3$)$_2$], 52.79 [$CH_2$N($CH_2CH_2CH_3$)$_2$, trans], 35.93 ($CH_2$CH=C$C$$H_2$, trans), 28.92 ($CH_2$CH=C$C$$H_2$, cis), 26.41 ($CH_2$CH=CC$H_2$), 20.21 [N($CH_2CH_2CH_3$)$_2$], 11.87 [N($CH_2$-$CH_2CH_3$)$_2$]. Elem Anal. Calcd: C, 78.97%; H, 12.65%; N, 8.37%. Found: C, 78.48%; H, 12.58%; N, 8.24%.

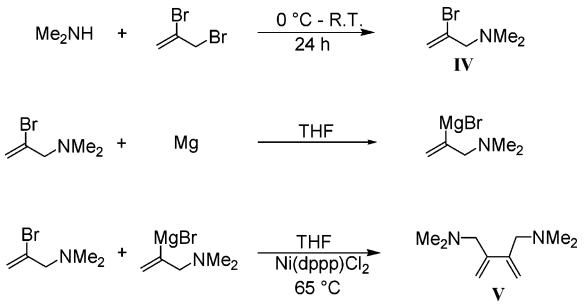
Synthesis of Poly[2,3-bis(dimethylaminomethyl)-1,3-butadiene] (PAB) by Free Radical Polymerization. Monomer **V** (0.48 g, 2.86 mmol) and *tert*-butyl peracetate (5.7 μL, 2.86 × 10^{-2} mmol) were added to an ampule with a magnetic stir bar. After three cycles of freeze−pump−thaw, the ampule was sealed under vacuum and placed in an oil bath preheated to 70 °C. After 48 h, the reaction was stopped by adding 1 mg of the inhibitor 2,6-di-*tert*-butyl-4-methylphenol. Polymer was precipitated twice in acetone and dried under vacuum for 48 h. Polymers with a molecular weight of 10 × 10^3 g/mol were obtained. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.92 (s, 4H), 2.05−2.30 [b, 16H, 12 from N(CH_3)$_2$, 4 from polymer backbone]. ^{13}C NMR (300 MHz, $CDCl_3$): δ 135.56 ($CH_2$$C$=$C$$CH_2$), 60.01 and 59.03 ($CH_2N$), 45.53 [N($CH_3$)$_2$], 30.08 ($CH_2CCH_2N$). Elem Anal. Calcd: C, 71.37%; H, 11.98%; N, 16.65%. Found: C, 70.85%; H, 12.08%; N, 16.47%.

Synthesis of the Diblock Copolymers by Anionic Polymerization. Monomer 2-(*N,N*-dimethylaminomethyl)-1,3-butadiene was stirred over dibutylmagnesium for 20 min and vacuum-distilled before use. Monomer (0.4 g, 3.6 mmol, 0.5 mL), benzene (1.0 mL), and *sec*-butyllithium (8.2 × 10^{-2} mmol, 50 μL) were added to a flame-dried round-bottom flask at 10 °C. The color of the reaction mixture became yellow immediately after the addition of *sec*-butyllithium to the aminoisoprene monomer. The propagation reaction was completed within 10 min, at which time freshly distilled ethylene oxide (0.4 g, 9.1 mmol) was introduced into the reaction flask. After the addition of ethylene oxide, the yellow color gradually faded and the reaction mixture became colorless within 50 min, indicating all carbanions had reacted with ethylene oxide. Phosphazene base *t*-BuP_4 solution (9.0 × 10^{-2} mmol, 90 μL) was introduced to the reaction system, and the temperature was increased to 55 °C. After stirring for 16 h, acidic methanol was added to the reaction mixture to terminate the polymerization. The polymer was purified by precipitation in diethyl ether.

Synthesis of Macroinitiator. A PEG block macroinitiator was synthesized according to the reported procedure.[9] A solution of 4,4′-azobis(4-cyanopentanoic acid) (0.280 g, 1 mmol) and 4-(dimethylamino)pyridinium 4-toluenesulfonate (DPTS) (0.882 g, 3 mmol) in a mixture of methylene chloride and dry *N,N*′-dimethylformamide was added to mPEG-OH (10 g, 2 mmol). A solution of *N,N*′-dicyclohexylcarbodiimide (1.236 g, 6 mmol) in methylene chloride was then added dropwise to the mixture. The reaction was stirred at room temperature for 36 h and poured into 300 mL of 2-propanol. The precipitate was collected and dried under vacuum before use.

Synthesis of Diblock Copolymer by Free Radical Polymerization. Monomer (0.48 g, 2.86 mmol) and macroinitiator (7.15 × 10^{-3} mmol) were added to a 20 mL ampule. After three cycles of freeze−pump−thaw, the reaction ampule was sealed under argon and placed in an oil bath preheated to 80 °C. The polymer was

Scheme 1. Synthesis of 2,3-Bis(dimethylaminomethyl)-1,3-butadiene by Nickel Coupling Chemistry



purified by first precipitated in diethyl ether, then redissolved in 2 mL of THF, precipitated in 20 mL of acetone, and centrifuged at 0 °C.

Cytotoxicity Assay. HeLa cells were grown in 96-well plates at a density of 1×10^4 cells per well in 150 μL of growth medium (89% MEM, 10% FBS, and 1% penicillin/streptomycin). Cells were grown for 24 h, after which time the growth medium was removed. Cells were washed once with Dulbecco's phosphate buffered saline. To 1 mg of polymer was added 40 μL of acetic acid. A 150 μL aliquot of the polymer solution was added to each well in triplicate. Cells were incubated at 37 °C, 5% CO_2. After 4 h of incubation, growth medium was removed and replaced with 100 μL of medium and 20 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS, from CellTiter 96 AQ_{ueous} one solution cell proliferation assay kit). The samples were incubated for 1 h at 37 °C, 5% CO_2. Optical absorbance was measured at 492 nm using a microplate reader (model 3550, Bio-Rad).

Agarose Gel Electrophoresis Assay. Polymer solutions were made by dissolving 1 mg of polymer in 10 mL of Opti-MEM solution containing 4 μL of acetic acid. DNA/polymer complexes were formed by mixing the desired amount of polymer solution (0.1 μg/μL) with 10 μL of DNA solution (0.1 μg/μL). The resulting mixture was vortexed for 10 s. The electrophoresis assay was carried out on 0.75% agarose gel in Tris-acetate-EDTA buffer for 30 min at 92 V. DNA bands were visualized using ethidium bromide staining.

Transfection Efficiency Test. For calculation of the N/P ratio, a molecular weight of 650 g/mol was used for the base pairs in DNA. Polymer/DNA polyplexes were formed at N/P (amine/phosphate) ratios of 4 and 2. HeLa cells grown in 24-well plates were treated with 100 μL of polyplex solution containing 0.8 μg of plasmid DNA. The growth medium was removed after the cells were incubated at 37 °C, 5% CO_2 for 4 h. Cells were rinsed once with Hanks' balanced salt solution. Minimum essential medium containing 10% fetal bovine serum and 1% penicillin/streptomycin were added to the cells. Cells were reincubated for 48 h and treated with 150 μL of lysis buffer. After centrifugation, supernatant was collected. Luciferase activity was quantified by measuring the light generated by the mixture of 100 μL of luciferase assay reagent and 20 μL of cell lysate. The total protein content was calculated using the BCA protein assay.

Results and Discussion

Monomer Synthesis. The monomers chosen for this gene delivery study were amine-functionalized 1,3-butadienes. Specifically, dimethyl-, diethyl-, and dipropylamine derivatives were synthesized in order to systematically study the effect of the size of the side groups on cytotoxicity, DNA binding affinity, and transfection efficiency. Monomer 2,3-bis(dimethylaminomethyl)-1,3-butadiene (**V**) was studied to investigate the effect of doubling the number of amine groups within each repeat unit (Scheme 1). The synthetic approach to prepare monomer **V** is similar to that of monomers **I**, **II,** and **III**.[7] However, the reaction to synthesize **V** utilizes an amine-functionalized Grignard reagent, 10 times more nickel coupling catalyst, and increased temperature.

Anionic Polymerization of 2-(*N*,*N*-Dialkylaminomethyl)-1,3-butadiene. Having synthesized and purified the desired monomers, the anionic polymerization studies were carried out. While there are several examples in the literature describing the anionic polymerization of similar monomers, one common theme is that the conversion from monomer to polymer is not quantitative.[10−16] The reason is not well understood. A better understanding of the reaction mechanism is critical for preparing block copolymers by sequential monomer addition. Therefore, we undertook a detailed study to further investigate the nature of the polymerization. The effects of side groups, solvent, and temperature were studied. Table 1 summarizes the experimental conditions and results of the anionic polymerization of monomers **I**, **II**, and **III**.

For the polymerization of monomer **I** in toluene, although the conversion was always greater than 96%, quantitative conversion could not be achieved. This is likely due to a chain transfer reaction to the solvent. By changing the reaction solvent from toluene to benzene, 100% conversion was achieved. When the polymerization of monomer **II** was carried out in toluene with a target molecular weight of 10×10^3 g/mol, the conversion was increased from 82% to 98% as the reaction temperature was decreased from 10 to −40 °C. It is likely that both chain transfer and propagation reactions were suppressed at lower temperatures, but the chain transfer was suppressed more, leading to an increase in conversion. When benzene was used as the solvent, conversion was increased to 98% at a reaction temperature of 10 °C. The freezing point of benzene at 5.5 °C prevents any further decrease in temperature, leaving open the question of whether quantitative conversion can be achieved at lower temperatures in benzene. Lower conversion was observed in the polymerizations of monomer **III** in benzene compared to that of monomers **I** and **II**. The conversion was 93% when the target molecular weight was 5×10^3 g/mol and only 68% for a target molecular weight of 10×10^3 g/mol.

In the above reactions, even with benzene as the solvent, living anionic polymerization was not achieved. The hypothesis is that side reactions other than chain transfer to the solvent exist in the system to compete with propagation and thus terminate the chains. The amine-functionalized monomers have side chains that are similar in structure to *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA). As a result, they should have similar effects on polymerization.

It has been reported that the presence of TMEDA promotes a side reaction that leads to a cyclic structure.[17] In the polymerization of our monomers, when the penultimate unit of the chain has a vinyl microstructure, the carbanion active center can attack the vinyl carbon in the penultimate unit to form a five-membered ring structure. This compound will then undergo a lithium hydride elimination reaction which leads to chain termination (Scheme 2). The driving force for this elimination reaction vs reinitiation is the formation of the stable lithium hydride molecule and p−π conjugation. Moreover, the dialkylaminomethyl group on the new active center is sufficiently bulky to prevent the addition of monomer to this site. ^{1}H NMR verified the formation of the cyclic side product. The peaks at 5.03 and 4.79 ppm come from the vinyl protons in the 4,3-microstructure, and the peak at 4.97 ppm is the result of the vinyl protons in the cyclic structure. This peak was not observed in the ^{1}H NMR of PMAI when the conversion to polymer was quantitative. It was observed in the ^{1}H NMR of PEAI and PPAI

Table 1. Results of Anionic Polymerization of Monomers I, II, and III

polymer	monomer	solvent	temp (°C)	conv[a] (%)	$\langle M_n \rangle \times 10^{-3}$ (g/mol) target	$\langle M_n \rangle \times 10^{-3}$ (g/mol) expl[b]	PDI[b]
P1	I	toluene	10	99	5.5	6.3	1.1
P2	I	toluene	10	96	11.1	9.3	1.2
P3	I	benzene	10	100	5.5	7.0	1.1
P4	I	benzene	10	100	11.1	13.4	1.2
P5	II	toluene	10	97	5.0	6.7	1.3
P6	II	toluene	10	82	10.0	10.4	1.5
P7	II	toluene	−5	97	5.0	8.8	1.2
P8	II	toluene	−5	92	10.0	15.3	1.4
P9	II	toluene	−40	98	10.0	11.5	1.3
P10	II	benzene	10	98	5.0	5.5	1.3
P11	II	benzene	10	98	10.0	9.9	1.2
P12	III	benzene	10	93	5.0	8.2	1.4
P13	III	benzene	10	68	10.0	16.6	1.4

[a] Conversion, determined by ^{1}H NMR. [b] Determined by GPC.

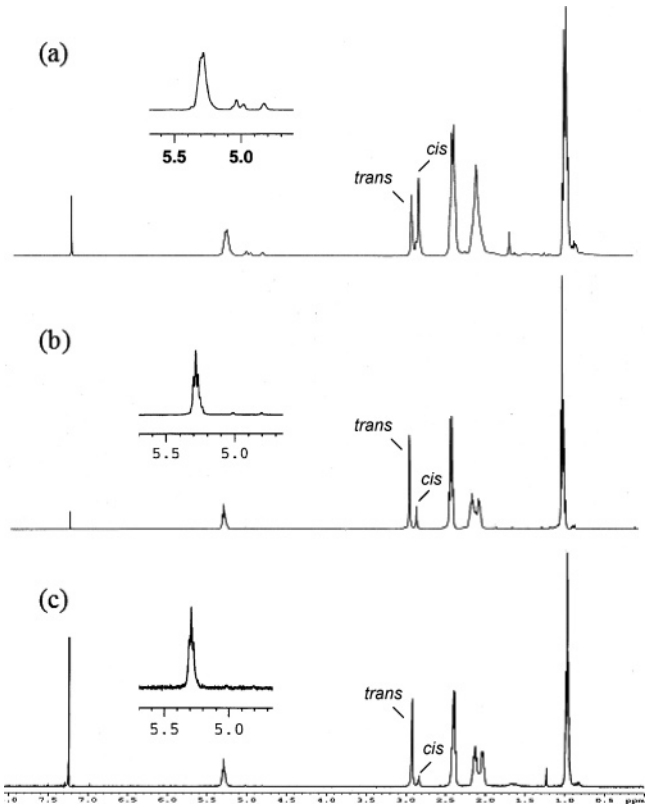
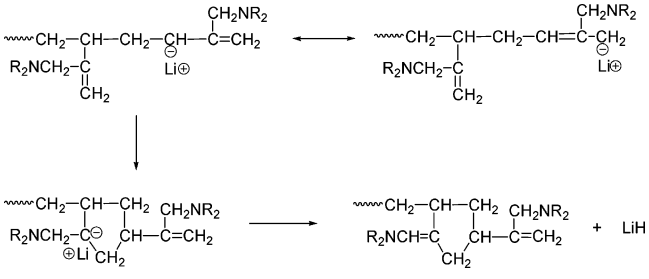


Figure 2. ^{1}H NMR of poly[2-(*N*,*N*-diethylaminomethyl)-1,3-butadiene] prepared at (a) 10, (b) −5, and (c) −40 °C.

Scheme 2. Possible Side Reactions To Terminate the Chain Propagation



when 100% conversion was not achieved. The peak decreases in the ^{1}H NMR of PEAI as the temperature decreases (Figure 2), which corresponds to increasing conversions.

The ratio of 4,1-microstructure to vinyl microstructure in Figure 2 is 1.000:0.070, 1.000:0.038, and 1.000:0.003 for 10, −5, and −40 °C, respectively. As the temperature decreases, the degree of association between chain end anion and lithium counterion increases. Thus, the negative charge is more localized on the α-carbon (Figure 3a), resulting in more 4,1-microstructure. Since the five-membered cyclic ring side product would only form when the penultimate unit of the chain has vinyl microstructure, the side reaction is less likely to occur at lower temperature. This results in less side product and higher conversion to polymer. The side reaction also explains why a PDI higher than the typical PDI for living anionic polymerization reactions was obtained. Additionally, the side reaction competes with the chain propagation reaction. The longer the side chain, the slower the chain propagation reaction due to steric reasons and thus the more pronounced the effect of the side reaction and the lower the conversion from monomer to polymer.

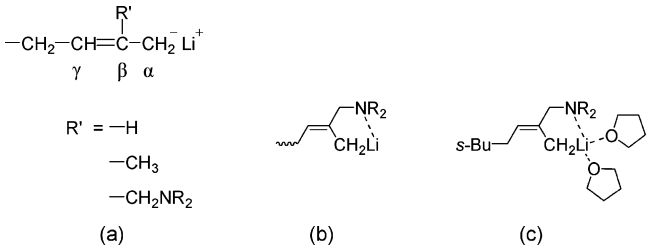
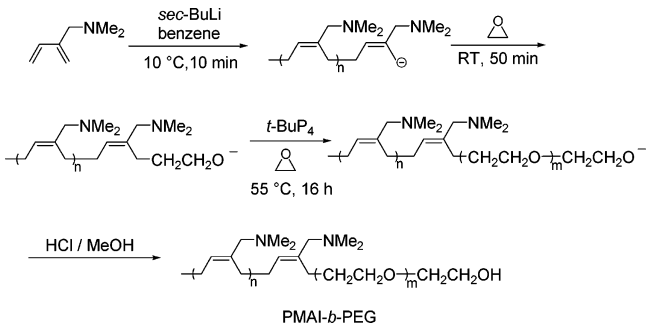


Figure 3. (a) Structure of polydiene chain end; (b) structure of PAI chain end; (c) structure of PAI chain end n THF.

Scheme 3. Synthesis of PMAI-*b*-PEG Copolymer by Anionic Polymerization



The ^{1}H NMR of PEAI (Figure 2) shows peaks at 2.93 and 2.84 ppm from the proton in the trans and cis microstructure, respectively. Peaks were assigned according to a previous 2D NMR study (^{1}H-ROESY).[11] The integrated ratio of trans proton and cis proton was 1.00:1.33, 1.00:0.27, and 1.00:0.14 for 10, −5, and −40 °C, respectively. In the propagation step, the monomer is always added to the polymer chain end in a cis fashion. As the reaction temperature decreases, the rate of

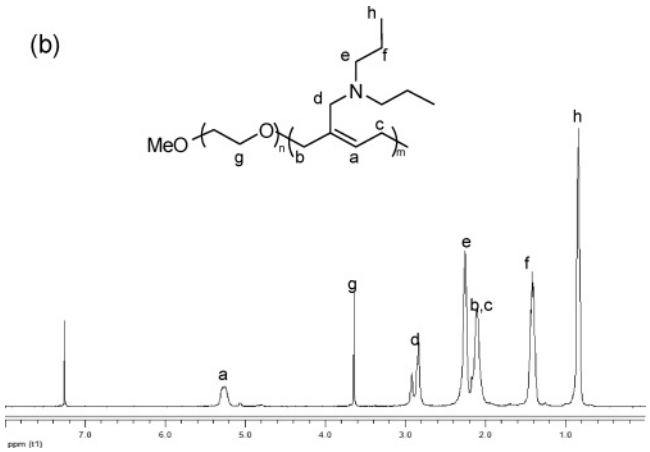


Figure 4. ^{1}H NMR of (a) PEAI-*b*-PEG and (b) PPAI-*b*-PEG.

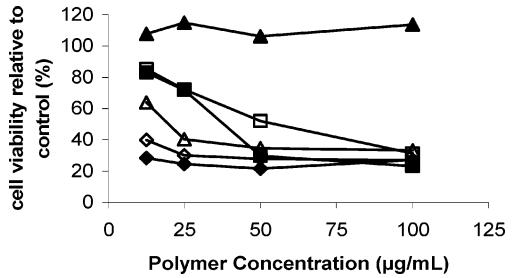


Figure 5. Cytotoxicity of the polymers in vitro: PMAI (◆), PEAI (■), PPAI (▲), PAB (◇), PEAI−PEG (□), and PEI (△).

propagation also decreases, leaving more time for the chain end to isomerize to the more stable trans microstructure.

An interesting observation is that all of the 2-(*N*,*N*-dialkylaminomethyl)-1,3-butadiene polymers obtained have almost exclusively 4,1-microstructure although there were many polar groups in the reaction system. No polymer was obtained when the reaction was carried out in THF, while butadiene and isoprene form polymers with high vinyl microstructure content when the polymerization reactions are carried out in polar solvents such as THF or with TMEDA added to the reaction. This can be explained by the chelation of the lithium to the amino group on the active chain end.[10] Unlike polybutadiene and polyisoprene in polar solvents, where the negative charge is mostly localized on the γ-carbon (Figure 3a),[18] for the amine-functionalized polymers the negative charge is mainly localized on the α-carbon due to the chelation (Figure 3b). Because of this chelation, a high 4,1-microstructure was obtained. When the reaction was carried out in THF, the chain end was surrounded by the polar THF molecules, preventing the addition of monomers (Figure 3c). The chelation of lithium to the amino group on the active chain end also explains why no 1,2-microstructure was obtained.

Synthesis of the Diblock Copolymers. When the polyplexes formed by interaction between DNA and polymers are neutral charge, they usually have sizes that are too large (greater than 150 nm) for endocytosis. To form polyplexes small enough for endocytosis, a large polymer to DNA ratio generally is preferred. While smaller polyplexes can be prepared this way, the result is a high net positive charge, leading to cytotoxicity. To address this problem, block copolymers composed of polycations and PEG have been employed as gene delivery vectors.[15,19−21]

Two different approaches were used to prepare the diblock copolymers. The first was the anionic polymerization of the amine-functionalized dienes and ethylene oxide by sequential monomer addition (Scheme 3). To break the strong lithium alkoxide aggregate and facilitate the propagation reaction, phosphazene base *t*-BuP_4 was introduced to the system to afford the block copolymer. In addition to the desired diblock copolymer, homopolymers PMAI and PEG were also recovered as side products. PMAI homopolymer can be removed from the block copolymer mixtures using diethyl ether. However, because of the similar solubility properties of the block copolymers and PEG, it was very difficult to separate the two by conventional methods.

Given the difficulty encountered in the synthesis of PMAI-*b*-PEG using anionic polymerization techniques, a free radical macroinitiator approach was investigated to prepare the block copolymers (Scheme 4). Macroinitiator **B** was synthesized by reacting mPEG-OH with 4,4′-azobis(4-cyanopentanoic acid) (**A**). This was followed by free radical polymerization of the aminoisoprene monomers using macroinitiator **B**. Because of the similarity of the properties of the resulting block copolymers and PEG, it was very difficult to separate the two polymers. Nonetheless, some block copolymers PEAI-*b*-PEG and PPAI-*b*-PEG were isolated by precipitation in acetone, followed by centrifugation at 0 °C. GPC showed monomodel peaks, indicating the copolymers were purified. The PEAI *b*-PEG obtained has a molecular weight of 31 $\times$ 10^3 g/mol with a PDI of 1.2, and the PPAI-*b*-PEG obtained has a molecular weight of 103 $\times$ 10^3 g/mol with a PDI of 1.7. Figure 4 illustrates the ^{1}H NMR of PEAI-*b*-PEG and PPAI-*b*-PEG.

Cytotoxicity. Given the potential biological applications of these materials, their cytotoxicity properties were evaluated using the MTS assay. In this test, the MTS tetrazolium compound was bioreduced by living cells to provide a formazan product that has an absorbance at 490 nm. The absorbance at 490 nm is directly proportional to the quantity of formazan product, which is directly proportional to the number of living cells. Figure 5 illustrates the results of the cytotoxicity test. The cytotoxicity decreased from PMAI to PEAI and dramatically decreased for PPAI. In fact, PPAI showed the lowest cytotoxicity among all the polymers tested. Diblock copolymer PEAI-*b*-PEG showed similar cytotoxicity to PEAI homopolymer at lower concentrations (less than 25 μg/mL), while at higher concentrations the diblock copolymer showed much lower cytotoxicity than the homopolymer. Most notably, all materials, except PMAI and PAB, demonstrated lower cytotoxicity than poly(ethylene imine), the most widely used transfection agent. The order of cytotoxicity was PMAI > PAB > PEI > PEAI > PEAI−PEG > PPAI. Cytotoxicity is possibly due to the acidity of the polymer solution. As the alkyl group changes from the methyl to the propyl group, the basicity of the amine increases, thus reducing the overall acidity.

Self-Assembly of Polycations and DNA. Following cytotoxicity evaluation, the ability of the materials to complex to DNA was investigated by gel electrophoresis experiments (Figure 6). DNA has a negative charge due to the phosphate groups, whereas our polymers have a positive charge due to the amino groups. As a result, the positively charged polymer will bind with negatively charged DNA, causing the DNA to condense into a compact particle. Pure DNA or incompletely neutralized DNA migrates toward the anode on the agarose gel, while neutral or positively charged complexes do not migrate.

The result showed that more PEAI (0.6 μg) was needed than PMAI (0.4 μg) to bind with the same amount of DNA (1.0 μg), while PPAI did not demonstrate the ability to form charge neutral polyplexes up to 3.0 μg. Overall, binding affinity decreased as the alkyl group was changed from methyl to ethyl

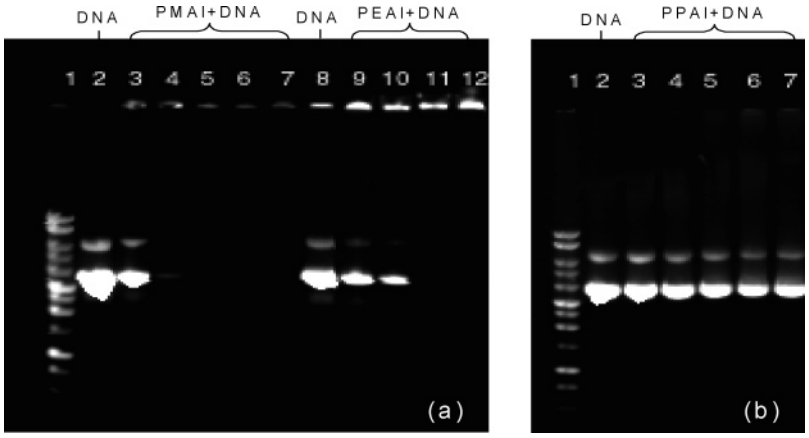
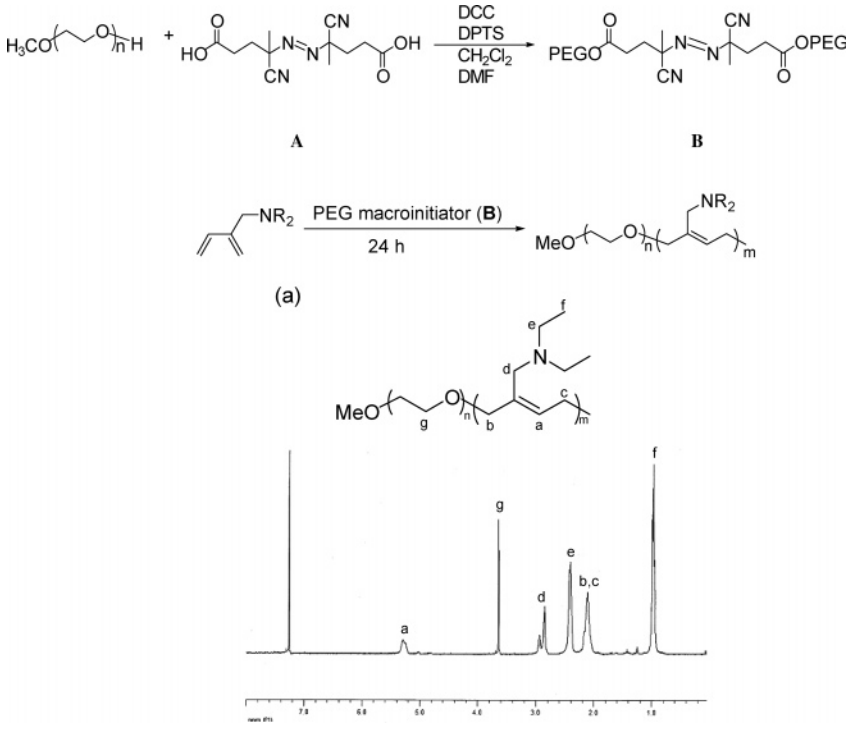


Figure 6. Agarose gel electrophoresis assay. Lanes correspond to various DNA/polymer weight ratios. (a) Lane 1: DNA molecular ladder; lanes 2 and 8: naked DNA; lane 3: DNA/PMAI = 10:1; lane 4: DNA/PMAI = 10:2; lane 5: DNA/PMAI = 10:3; lane 6: DNA/PMAI = 10:4; lane 7: DNA/PMAI = 10:5; lane 9: DNA/PEAI = 10:1; lane 10: DNA/PEAI = 10:2; lane 11: DNA/PEAI = 10:3; lane 12: DNA/PEAI = 10:4. (b) Lane 1: DNA molecular ladder; lane 2: naked DNA; lane 3: DNA/PPAI = 10:1; lane 4: DNA/PPAI = 10:2; lane 5: DNA/PPAI = 10:3; lane 6: DNA/PPAI = 10:4; lane 7: DNA/PPAI = 10:5.

Scheme 4. Synthesis of PAI-*b*-PEG Copolymer by Free Radical Polymerization



and dramatically decreased in the case of the propyl group. This is likely due to the increased steric hindrance.

Transfection Efficiency Test. Having demonstrated the ability to bind with DNA and to form condensed polyplexes, the transfection efficiency of the amine-functionalized polymers was investigated. Cells were treated with DNA containing information to make luciferase, a catalyst for a bioluminescent reaction to generate light. The light intensity is directly proportional to the amount of luciferase present. The transfection efficiency was evaluated by comparing the light intensity generated. Figure 7 illustrates the experimental results. PMAI, PAB, and PPAI showed similar transfection efficiency to naked DNA. This is presumably because PPAI does not bind with DNA sufficiently, while PMAI and PAB bind with DNA so tightly that the release of DNA from the polyplexes was not efficient. As a result, they do not work as well as PEAI, which showed higher transfection efficiency. PEAI, PEI, and PEAI-*b*-PEG showed higher transfection efficiency at an N/P ratio of 4 than 2. The transfection efficiency of the diblock copolymer was lower than that of the PEAI homopolymer. One possible explanation is that while the PEG block can help reduce undesired blood clearance in vivo, it may also reduce the interaction of the cell membrane and the polyplexes, leading to a less effective endocytosis. Even though transfection efficiency of PEAI was 2.6 times lower than PEI at an N/P ratio of 4 (optimum condition for PEI transfection), it was 75 times higher than PEI at an N/P ratio of 2.

Although some of the polymers that have been synthesized previously demonstrated higher transfection efficiency than PEI under certain conditions, they usually require a high N/P ratio (at least greater than 10), which is not desirable.[4,22,23] PEAI, which had lower cytotoxicity than PEI and showed reasonable transfection efficiency at an N/P ratio of 2, is a promising novel transfection vector.

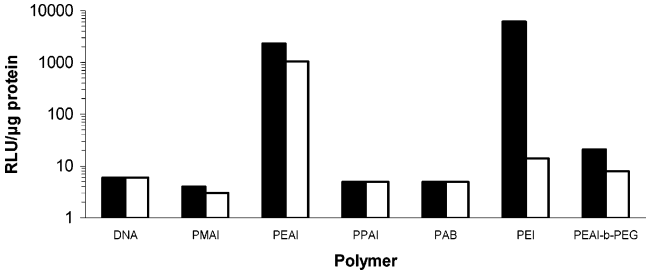


Figure 7. Transfection efficiency of various polymers in Hela cells at N/P = 4 (filled bars) and N/P = 2 (open bars). Molecular weights of PMAI, PEAI, PPAI, PAB, and PEI are 13, 15, 16, 10, and 10×10^3 g/mol, respectively.

Conclusion

Anionic polymerization of amine-functionalized diene-based monomers was studied in detail. The potential of the prepared polymers as gene delivery vectors was investigated. The results showed that in order to design an efficient gene delivery vector the synthetic polymers must bind with DNA sufficiently to protect it. On the other hand, they cannot bind with DNA so tightly as to prevent the release of the DNA from the polyplexes. The amine-functionalized polymer works most efficiently as a gene delivery vector when the alkyl group in the tertiary amine is an ethyl group. An efficient, nontoxic gene delivery vector was successfully synthesized.

Acknowledgment. The authors thank financial support from the National Science Foundation (Department of Materials Research) under Grant 0418499.

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MA061249P