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Synthesis of lipopolyhydroxylalkyleneamines for gene delivery

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Abstract—Various bis(2-hydroxy-3-chloropropyl)alkylamines were synthesized by coupling primary amine with epichlorohydrin and utilized as a monomer to react with ethylenediamine (EDA), *N*,*N*′-dimethylethylenediamine (DMEDA), or tetramethylethylenediamine (TMEDA) to generate a series of lipopolyhydroxylalkyleneamines. The number- and weight-average molecular weight (M_n and M_w) and polydispersity index (M_w/M_n) of the lipopolyhydroxylalkyleneamines were dependent on reactant solvent and reaction temperature. The compounds with EDA as backbone have better transfection activity and lower toxicity than those with DMEDA and TMEDA as backbone.

Synthetic compounds such as cationic lipids[1–3] and polycations[4–7] have been considered as potential carriers for gene therapy due to their being easy to make and highly flexible in delivering transgene of various sizes. When mixing with plasmid DNA, these cationic compounds form stable complexes in which DNA is tightly condensed and protected from enzymatic degradation. Previous studies have shown that various cationic lipids and polycations are active in mediating gene transfer into cells in culture and in animals.[3] However, success in applying these synthetic compounds to human gene therapy has been limited primarily due to their poor delivery efficiency compared to that of virus-based delivery systems. In addition, recent studies have demonstrated that polyethyleneimine (PEI), the most studied polycation for gene delivery, is toxic to cells at higher doses due to its high charge density and lack of biodegradability.[8,9] Clearly, more efforts are needed to develop new synthetic gene carriers and to improve activity of existing compounds. Our ongoing research has focused on the development of new synthetic compounds. In the current study, we report the design, synthesis, and gene transfer activity of lipopolyhydroxylalkyleneamines, a novel class of compounds that bear the structural features of cationic lipid and polycation.

Lipopolyhydroxylalkyleneamine compounds were synthesized in two steps: preparation of various bis(2-hydroxy-3-chloropropyl)alkylamine monomers[10] and polymerization of monomers into lipopolyamines.[11] The procedures of Kim et al.[12] were modified to synthesize monomers **2a**–**2e** (Scheme 1). Bis(2-hydroxy-3-chloropropyl)dodecylamine **2a**, bis(2-hydroxy-3-chloropropyl)dodecylaniline **2b**, and bis(2-hydroxy-3-chloropropyl)octadecylamine **2d** were prepared by reaction of epichlorohydrin **1** with dodecylamine, dodecylaniline or octadecylamine, respectively. The same strategy was used for synthesis of **2c** and **2e** using hexylamine or methylamine to react with epichlorohydrin. Monomers **2c** and **2e** were not stable at room temperature and self-polymerization of **2e** was detected by ^{1}H NMR with a peak at 4.2–4.9 ppm. Thus, monomers **2c** and **2e** were immediately used without further purification. The yields of monomer synthesis are summarized in Table 1.

Assembly of various bis(2-hydroxy-3-chloropropyl) alkylamines into final product of lipopolyhydroxylalkyleneamines (**3a**–**3i**) is outlined in Scheme 2. Reaction of monomers of **2a**, **2b**, **2c**, **2d** or **2e** with ethylenediamine (EDA) afforded compounds **3a**, **3b**, **3c**, **3d**, and **3g**. Combination of **2e** with *N*,*N*′-dimethylethylenediamine (DMEDA) generated compound **3i**. Compounds **3e**, **3f**, and **3h** were produced by mixing *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) with **2a**, **2c** or **2e**, respectively. The reaction of monomers of **2a**, **2b**, **2c**, or **2d** with ethylenediamine in methanol/water at 40 °C

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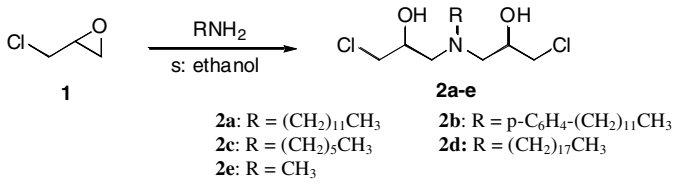


Scheme 1. Synthesis of monomers **2a**–**2e**.

Table 1. Synthetic yield of monomers

Monomer	R	Yield[a] (%)
2a	Dodecyl	86.5
2b	4-Dodecylphenyl	91.5
2c	Hexyl	92
2d	Octadecyl	77
2e	Methyl	96

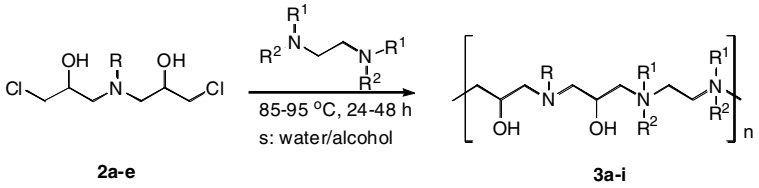
[a] Yield after column chromatography and based on the starting primary amine.

for 3 days afforded a mixture that contains the desired product, some oligomers (MW 600–750, 3–4 units), unreacted monomers, and partially decomposed compounds.

For compounds **3a**–**3f**, copolymerization in *n*-butanol/water at 90–95 °C for 1–2 days resulted in compounds with an average molecular weight of approximately 2000 as the major product, which was easily dissolved in water with the exception of compound **3d**. Methanol/water mixture served as an ideal solvent for synthesis of compounds **3g**–**3i**. When butanol was used as solvent for the reaction carried out with stirring at 85–95 °C for 1 h, a lot of solid was seen, which may be either chloride salt of product with a low MW, or ethylenediamine. If a mixture of methanol/water or butanol/water (2:1 or 1:1) was used as reaction solvent, pale yellow and viscous slurry was always seen during polymerization. The molecular weight of new compounds was around 2000 at an initial monomer concentration $[M]_0$ of 6 M. Polydispersity index (M_w/M_n) was 1.31–1.61 (Table 2), implying a well-controlled reaction. Structural features of compounds **3a**–**3i** are summarized in Table 2.

The chemical structures of the synthesized compounds were confirmed by ^1H and ^{13}C NMR and data are summarized in Refs. 10 and 11. Figure 1 is a representative ^1H NMR spectrum of lipopolyhydroxylalkyleneamine using compound **3a** as an example. It shows resonance signal at 3.75–4.22 ppm due to the protons of C*H*–OH. The single peak at 3.25 ppm corresponds to the protons of hydroxyl group O*H* of polymers. A broad multiple peak at 3.20–2.30 ppm corresponds to N–CH_2 of the structural unit. The peak at 1.72–1.41 ppm is assigned to the N–CH_2CH_2 of dodecyl chain unit. Resonance signals corresponding to the remaining protons on methylene groups of the dodecyl chain appear at 1.29 ppm. The signal at 0.871 ppm is assigned to the methyl group of the dodecyl chain unit. The ^1H NMR results indicate that the compounds with NH_2 as an end group are the overwhelming product.

Biological activities of the new compounds were evaluated using murine melanoma BL-6 cells according to an established procedure.[13] In brief, 5×10^5 cells/well in 48-well plates were seeded 24 h prior to transfection. DNA/lipopolyamine complexes were prepared by mixing various amounts of lipopolyhydroxylalkyleneamine in serum-free medium with a reporter plasmid



Scheme 2. Synthesis of lipopolyhydroxylalkyleneamines.

Table 2. Structural features of lipopolyhydroxylalkyleneamine

Compound	Monomer used	R	R^1	R^2	M_n	M_w	M_w/M_n	*n*
3a	**2a**	Dodecyl	H	H	1831	2536	1.39	7
3b	**2b**	4-Dodecylphenyl	H	H	1630	2168	1.33	5
3c	**2c**	Hexyl	H	H	2072	2736	1.32	10
3d	**2d**	Octadecyl	H	H	1690	2265	1.34	5
3e	**2a**	Dodecyl	CH_3	CH_3	1943	2736	1.41	6
3f	**2c**	Hexyl	CH_3	CH_3	1419	2404	1.69	7
3g	**2e**	Methyl	H	H	1500	2158	1.44	10
3h	**2e**	Methyl	CH_3	CH_3	1247	1783	1.43	6
3i	**2e**	Methyl	CH_3	H	1396	1829	1.31	7

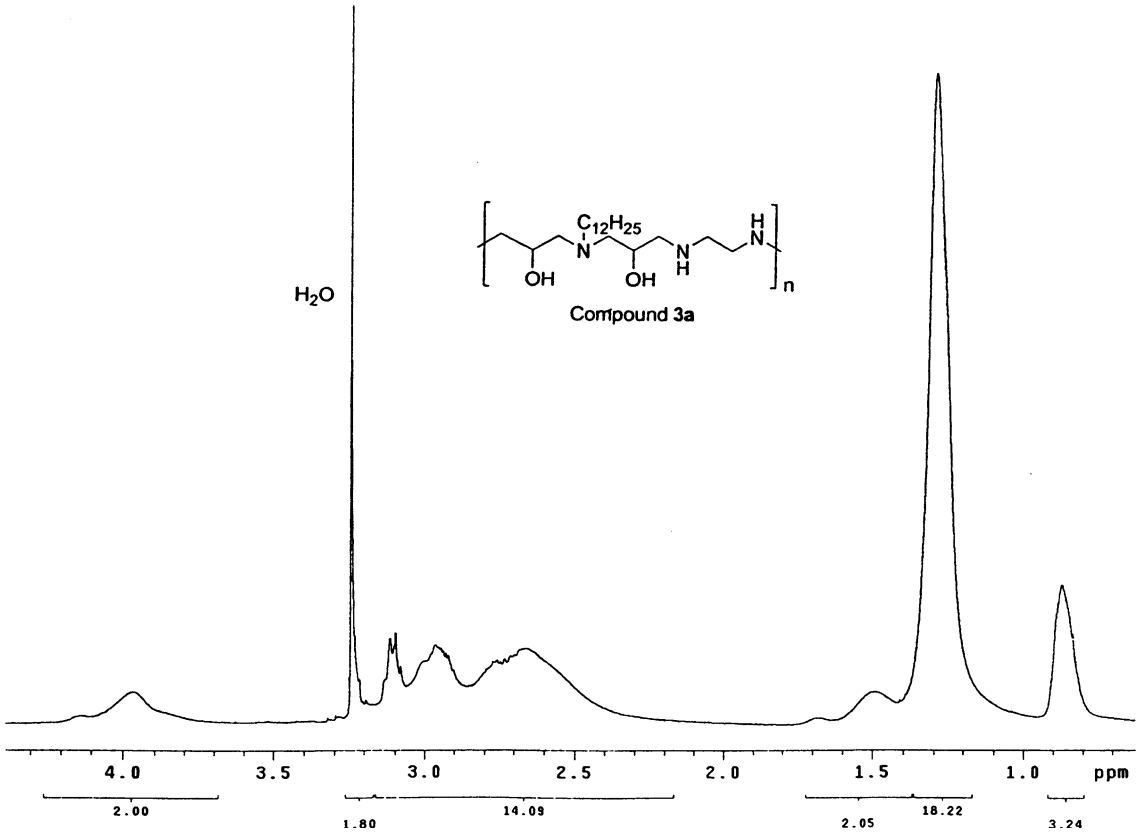


Figure 1. ^{1}H NMR spectrum in (D_2O) of lipopolyhydroxylalkyleneamine **3a**.

(pCMV-Luc) at the final DNA concentration of 4 μg/ml. The mixture was incubated at room temperature for 15 min and then added to each well (1 μg of DNA/well in 250 μl) after removal of the culture medium. Five hour after transfection, 27.9 μl of fetal bovine serum (FBS) was added to each well and incubation proceeded for additional 19 h. Transfection medium was then replaced with fresh medium and cells were lysed 24 h later with lysis buffer. Total proteins and luciferase activity in cell lysates were determined. Figure 2 (left panel) shows that transfection activity was much better for compounds **3a**, **3c**, **3g**, and **3h** than for the rest of compounds. Compounds with a secondary amine (compound **3g**), or quaternary amine (compound **3h**), exhibited similar transfection activity. Longer R groups in compounds **3b**, **3d**–**3f** appeared less active compared to that of compound **3g** with R = CH_3. The lowest activity was seen in compound **3f** with R = $(CH_2)_5$–CH_3 and a quaternary amine. Compounds **3a** and **3c** showed significant cytotoxicity, as reflected by decreased amount of proteins recovered from each well of transfected cells (Fig. 2, right panel). Overall, compound **3g** (R = CH_3, R_1 = R_2 = H) has the structure exhibiting the best transfection activity with relatively low toxicity.

In summary, a series of new lipopolyamines containing hydroxyl, different amine groups (secondary, tertiary or quaternary), and various alkyl groups were synthesized

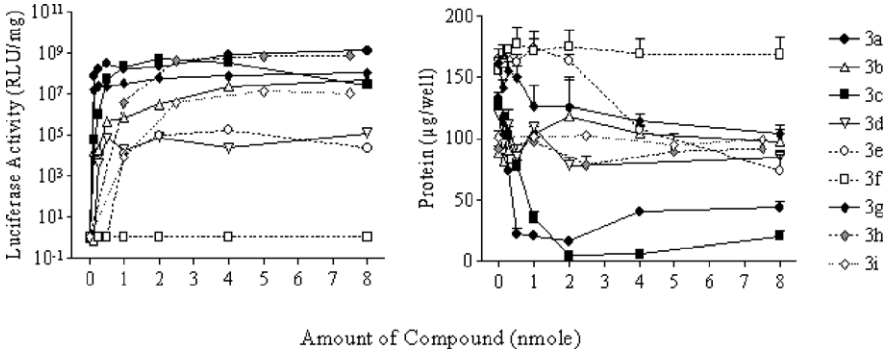


Figure 2. Transfection activity (left) and cytotoxicity (right) of lipopolyhydroxylalkyleneamine **3a**–**3i**. Cells (5×10^5 cells/well) were seeded in a 48-well plate 24 h before the addition of DNA/compound complexes (1 μg DNA and various amounts of compounds). The level of luciferase gene expression is expressed as mean of relative light units (RLU) per milligram of extracted protein. Cytotoxicity was indicated by the total amount of proteins recovered from each well. Data represent means + SD (n = 3).

and characterized by NMR. Under our experimental conditions, the number-average molecular weights (M_n) of the compounds ranged from 1396 to 2072, and the weight-average molecular weights (M_w) ranged from 1783 to 2736. Biological tests in BL6 cells revealed excellent but variable biological activities in gene delivery, depending on the backbone structure and the chain length of the hydrophobic moiety. The detailed investigations on structure–activity relation of these compounds in vivo are in progress.

Acknowledgments

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10. Preparation of monomers. Synthesis of monomer **2a**: epichlorohydrin (3.9 ml, 50 mmol) was added to a solution of dodecylamine (4.63 g, 25 mmol) in ethanol (13 ml) at room temperature. The mixture was stirred for overnight and the solvent was evaporated under vacuum. The crude product was purified by flash chromatography on a silica gel column with chloroform/methanol (14:1) as the eluent to yield **2a** as a white solid (8 g). ^{1}H NMR ($CDCl_3$) δ: 0.861 (t, 3 H), 1.26 (single, 18H), 1.37–1.52 (brand, 2H), 2.74–2.51 (m, 6H), 3.25–3.48 (br s, 2H), 3.50–3.60 (m, 4H), 3.85–3.90 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 69.30, 68.76, 58.87, 58.31, 56.12, 56.10, 47.64, 47.51, 32.13, 29.87, 29.85, 29.83, 29.76, 29.56, 27.51, 27.19, 27.13, 22.91, 14.34.
Synthesis of monomer **2b**: mixture of epichlorohydrin (1.16 g, 12.5 mmol), dodecylaniline (1.31 g, 5 mmol), and ethanol (l ml) was stirred at room temperature for 18 h. A white solid after removal of solvent under vacuum was obtained and purified by flash chromatography using a chloroform/isopropyl ether (10:0.7) eluent (R_f = 0.38), resulting in 2.03 g white solid. The NMR results indicate that this compound exists in two isomers. ^{1}H NMR ($CDCl_3$) δ: 7.08–7.05 (quat, 2H), 6.79–6.77 (d, 1H), 6.64 (d, 1H), 4.20–4.11 (m, 2H), 3.88 (d, 1H), 3.68–3.48 (m, 5H), 3.41–3.36 (m, 2H), 3.18–3.08 (m, 1H), 2.53–2.49 (m, 2H), 1.57 (m, 2H), 1.39–1.11 (m, 18H), 0.82–0.92 (t, 3H); ^{13}C NMR ($CDCl_3$) δ: 146.45, 145.76, 133.63, 132.72, 129.62, 129.54, 114.95, 113.16, 69.83, 69.12, 58.53, 56.43, 47.49, 47.05, 35.10, 35.04, 32.17, 32.03, 31.97, 29.93, 29.88, 29.80, 29.60, 22.94, 14.38.
Synthesis of **2c**: epichlorohydrin 8.33 g (7.1 ml, 90 mmol) was added dropwise to the solution of hexylamine 3 g (4 ml, 30 mmol) in ethanol (30 ml) at 5 °C over 15 min. The mixture was stirred at room temperature for 8 h and the solvent was removed by vacuum desiccation. A transparent and viscous liquid was obtained, which was further purified by flash chromatography using hexanes/ethyl acetate (3:1, R_f = 0.57) as the eluent to give the final product (12.5 g). ^{1}H NMR ($CDCl_3$) δ 0.845 (t, 3H), 1.215 (m, 6H), 1.416 (m, 2H), 2.493–2.72 (m, 6H), 2.95–3.41 (br s, 1H), 3.49–3.58 (m, 4H), 3.84–3.88 (m, 2H).
Synthesis of **2d**: mixture of epichlorohydrin (10 g, 108 mmol) and octadecylamine (7 g, 25 mmol) in 30 ml isopropyl ether and 60 ml ethanol was stirred at room temperature overnight and concentrated under reduced pressure. The crude product was isolated by chromatography on a silica gel column with chloroform/methanol (14:1 v/v, R_f = 0.65) as the eluent to afford a white solid of 9.1 g. ^{1}H NMR ($CDCl_3$) δ 3.91–3.86 (m, 2H), 3.61–3.50 (m, 4H), 3.49–3.18 (br s, 2H), 2.75–2.51 (m, 6H), 1.45 (br s, 2H), 1.24 (br s, 30H), 0.87 (t, 3H); ^{13}C NMR ($CDCl_3$) δ: 69.30, 68.75, 58.89, 58.34, 56.13, 56.10, 47.64, 47.50, 32.15, 29.92, 29.88, 29.84, 29.76, 29.59, 27.51, 27.16, 27.09, 22.92, 14.34.
Synthesis of **2e**: to 13 ml of ethanol epichlorohydrine (13.1 g, 0.141 mmol) was added dropwise methylamine (33% wt solution in absolute ethanol, 0.645 mmol) at −5 °C. The mixture was stirred at rt for 6 h and then at 4 °C overnight. The reaction mixture was then concentrated under vacuum to afford the product as a colorless and viscous liquid (96%). ^{1}H NMR (CD_3CN) δ 2.28 (d, 3H), 2.41–2.56 (m, 4H), 3.51–3.57 (m, 2H), 3.61–3.66 (2H), 3.82–3.89 (m, 2H).
11. Preparation of lipopolyhydroxylalkyleneamines: compounds **3a**–**3f** were synthesized by condensation of bis(2-hydroxy-3-chloropropyl)alkylamine and ethylenediamine (EDA) in 1-butanol/water (2:1) at around 95 °C for 24–48 h (compounds **3a**–**3d);** or bis(2-hydroxy-3-chloropropyl)-alkylamine and tetramethylenediamine in butanol/water (1:1) with reflux overnight (compounds **3e**, **3f**). The reaction mixture was concentrated under reduced pressure. After washing with chloroform, acetone, and cooled methanol to remove unreacted monomers and low molecular weight oligomers, a crystalline amorphous solid was obtained.
Compound **3a**: ^{1}H NMR (CD_3OD) δ 0.80 (t, 3H), 1.15–1.40 (br s 18H), 1.40–1.70 (br s 2H), 2.45–3.16 (br s, 14H), 3.82–4.17 (br s, 2H).
Compound **3b**: (D_2O + DMSO) ^{1}H NMR (D_2O) δ 0.81 (br s, 3H), 0.98–1.28 (br s, 18H), 1.30–1.45 (br s, 2H), 2.20–2.49 (m, 2H), 2.65–3.88 (br s, 12H), 4.20–4.11 (m, 2H), 6.71 (br s, 2H), 7.08–7.05 (br s, 2H).
Compound **3c**: ^{1}H NMR (D_2O) δ 0.87 (br s, 3H), 0.95–1.42 (br s, 8H), 2.1–3.15 (14H), 3.81–4.10 (br s, 2H).
Compound **3d**: ^{1}H NMR (D_2O) δ 0.82 (br s, 3H), 0.95–1.52 (br s, 36H), 2.1–3.15 (14H), 3.51–4.15 (br s, 2H).
Compound **3e**: ^{1}H NMR (D_2O) δ 0.75–0.90 (br s, 3H), 1.15–1.35 (br s, 18H), 1.38–1.52 (br s, 2H), 2.25–2.50 (br s, 6H), 2.60–2.8 (br s, 2H), 2.95–3.52 (m, 16H), 4.2–4.4 (br s, 2H).
Compound **3f**: ^{1}H NMR (D_2O) δ 0.85 (t, 3H), 1.24 (br s, 6H), 1.35–1.5 (br s, 2H), 2.48–2.65 (m, 6H), 2.74–2.95 (m, 2H), 3.10–3.65 (m, 18H), 3.95–4.35 (m, 2H).
Addition of monomer **2e** to EDA/TMEDA/DMEDA was accomplished using a 1:1 mixture of water/methanol

as solvent at 85–95 °C (Table 2) for 48 h to give compounds **3g**, **3h**, and **3i** which are hygroscopic white solid. The compounds formed were purified as described above.

Compound **3g**: ^{1}H NMR (H_2O) δ 4.18–3.96 (m, 2H), 3.10–2.45 (m, 17H).

Compound **3h**: ^{1}H NMR (H_2O) δ 4.48–4.31 (m, 1H), 4.26–4.14 (m, 0.5H), 4.10–3.91 (m, 1H), 3.68–3.14 (m, 15H), 3.10–2.65 (m, 2H), 2.65–2.42 (m, 4H), 2.40–2.30 (m, 3H), 2.28 (s, 1H).

Compound **3i**: ^{1}H NMR (H_2O) δ 4.24–4.00 (m, 2H), 3.06–2.62 (m, 15H), 2.60–2.40 (m, 8H), 2.29 (s, 3H).

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