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Effect of Methylation of the Hydrophilic Domain of Tocopheryl Ammonium-Based Lipids on their Nucleic Acid Delivery Properties

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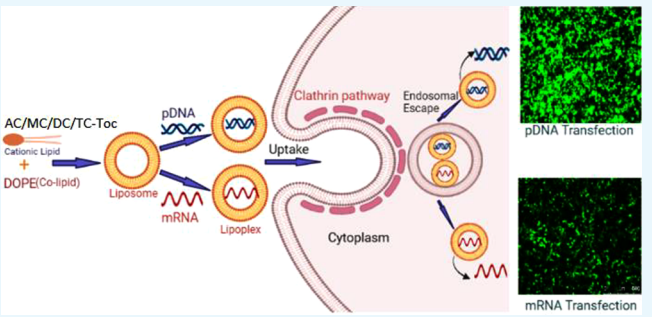
ABSTRACT: Lipid-enabled nucleic acid delivery has garnered tremendous attention in recent times. Tocopherol among the cationic lipids, 3b-[*N*-(*N*′,*N*′-dimethylamino-ethane)carbamoyl]-cholesterol hydrochloride (DC-Chol) with a headgroup of dimethylammonium, and cholesterol as a hydrophobic moiety are found to be some of the most successful lipids and are being used in clinical trials. However, limited efficacy is a major limitation for their broader therapeutic application. In our prior studies, we demonstrated tocopherol to be a potential alternative hydrophobic moiety having additional antioxidant properties to develop efficient and safer liposomal formulations. Inspired by DC-Chol applications and taking cues from our own prior findings, herein, we report the design and synthesis of four alpha-tocopherol-based cationic derivatives with varying degrees of methylation, AC-Toc (no methylation), MC-Toc (monomethylation derivative), DC-Toc (dimethylation derivative), and TC-Toc (trimethylation derivative) and the evaluation of their gene delivery properties. The transfection studies showed that AC-Toc liposomes exhibited superior transfection compared to MC-Toc, DC-Toc, TC-Toc, and control DC-Chol, indicating that methylation in the hydrophilic moiety of Toc-lipids reduced their transfection properties. Cellular internalization studies in the presence of different endocytosis blockers revealed that all four tocopherol lipids were internalized through clathrin-mediated endocytosis, whereas control DC-Chol was found to be internalized through both macropinocytosis and clathrin-mediated endocytosis. These novel Toc-lipids exhibited higher antioxidant properties than DC-Chol by generating less reactive oxygen species, indicating lower cytotoxicity. Our present findings suggest that AC-Toc may be considered as an alternative to DC-Chol in liposomal transfections.



INTRODUCTION

The commercially available cationic lipid 3b-[*N*-(*N*′,*N*′-dimethylamino-ethane)carbamoyl]-cholesterol hydrochloride (DC-Chol) was first synthesized in 1991 by Gao et al. This is one of the cationic lipids in combination with DOPE (1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine), which is widely used in liposomal preparations for gene delivery.[1,2] DC-Chol/DOPE has been used to treat cystic fibrosis through nasal application without altering ion transport, bacterial growth, or lung function.[3] DC-Chol also significantly enhances the effect of growth inhibition of antisense oligodeoxynucleotides on tumor cells to inhibit the tumor growth.[4] The important structural features of DC-Chol make it one of the most efficient cationic lipids in the delivery of biomolecules.[5]

In general, cationic lipids typically feature a hydrophilic headgroup, a hydrophobic backbone, and a linkage.[6−8] In these cationic lipids, systematic modifications of any or all of three domains can result in the variation of transfection efficiency. Several efforts have been made to generate novel amphiphiles by modifying these domains. However, the appropriate structural domains needed for stable transfection are still uncertain.[9−12] Therefore, investigations on the structure−activity of cationic lipids could reveal important insights into the correlation between structure, efficiency, and toxicity. Hence, the complications involved in the transfection pathway can be simplified. However, in many studies, superior cationic lipids have been additionally designed by understanding the structure−activity correlation and their mechanisms. This dictates the formation as well as the function of newly developed lipid formulations for clinical application.[13−15]

The development of novel gene carriers having less cytotoxicity and efficient gene transfection for effective and safe gene delivery is a major challenge. The efficiency of gene

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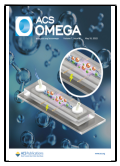

transfection and cytotoxicity mainly depends on the molecular structure of genetic vectors. Hence, selection of suitable molecular domains and their linkers is very important in gene therapy to construct highly efficient and low toxicity gene vectors.[5,16,17] Vitamin E used as an antioxidant has been widely reported, and it has therapeutic use for treating multiple disorders. Moreover, because of their solvent capacity and biocompatibility, vitamin E-based nanomedicines in cancer therapy have been developed. Furthermore, the serum stability and low toxicity of vitamin E, and the presence of physiological pathways of tocopherol transport to various tissues including brain from blood have led to several investigations on systemic delivery applications.[22,23] In recent times, the efficiency of α-tocopherol as a delivery system *in vivo* for the delivery of nucleic acids has been reported.[15,24] It is reported from previous studies that α-tocopherol performed similarly as cholesterol in changing liposomes, i.e., making the liposomes highly protein-induced disruption-resistant, and it as found that this inhibition of protein-induced disruption is more effective with tocopherol compared to cholesterol. Therefore, this made tocopherol-based liposomes more efficient vectors for *in vivo* gene delivery.[13,25,26] Together, impressed by DC-Chol applications and the biological importance of tocopherol, we were inspired to design, synthesize, and evaluate four tocopherol-based cationic derivatives, with varying degrees of methylation, AC-Toc (3β-[*N*-(aminoethane)carbamoyl]-tocopherol) hydrochloride), MC-Toc (3β-[*N*-(*N*′-methylaminoethane)carbamoyl]tocopherol hydrochloride), DC-Toc (3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]-tocopherol hydrochloride), and TC-Toc (3β-[*N*-(*N*′,*N*′,*N*′-trimethylaminoethane)carbamoyl]tocopherol hydrochloride). We synthesize the derivatives with high purities synthesized and compared their transfection potentials with commercially available DC-Chol.

RESULTS AND DISCUSSION

In the present study, the four tocopherol-based cationic lipids AC-Toc, MC-Toc, DC-Toc, TC-Toc were synthesized, and physicochemical characteristics of these lipids, viz., size, surface charge, and DNA binding abilities, were illustrated. Herein, we also report the results of *in vitro* transfection experiments of lipoplexes of lipids AC-Toc, MC-Toc, DC-Toc, TC-Toc, and DC-Chol performed on four different cell lines, viz., hepatic (Hep-G2), neural (NEURO-2a), kidney (HEK-293), and ovary (CHO).

Synthesis of Lipids. The synthesized cationic lipids with their molecular structures (AC-Toc, MC-Toc, DC-Toc, TC-Toc) are given in Figure 1. The detailed synthesis process is shown in Scheme 1. As shown in Scheme 1, alpha-tocopheryl chloroformate was synthesized as previously reported from our laboratory.[14] Alpha-tocopheryl chloroformate was coupled with ethylenediamine followed by protonation to yield AC-Toc. Alpha-tocopheryl chloroformate was coupled with *N*-methyl ethylenediamine followed by protonation to yield MC-Toc. Alpha-tocopheryl chloroformate was coupled with *N*,*N*-dimethyl ethylenediamine to form an amide derivative. This amide derivative was further treated with HCl to yield DC-Toc and treated with methyl iodide to yield TC-Toc iodide salt. The iodide salts of final lipids were converted to chloride salts by passing them repeatedly onto a column containinga chloride ion exchange resin, which yielded the title lipid TC-Toc having chloride as a counterion. The structure of the intermediate (**I**) (Scheme 1) was confirmed by ^{1}H NMR and

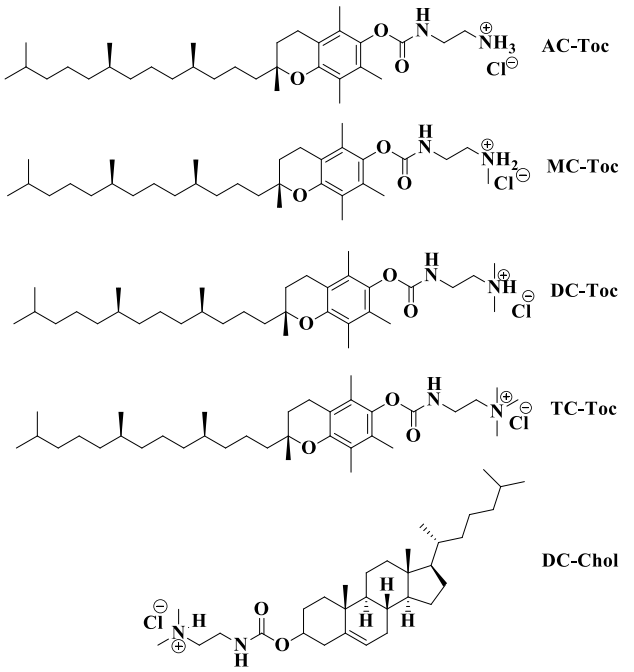

Figure 1. Structures of the synthesized and control cationic lipids.

mass spectra, and the final target lipids (Scheme 1) were confirmed by ^{1}H NMR, ^{13}C NMR, and ESI-HRMS spectra (Supplementary Data).

Reagents and Conditions. (A) Dry DCM, room temperature, 24 h; (B) dry methanol/conc. HCl (1:1), 0 °C (1 h), 12 h room temp; (C) methyl iodide, reflex 12 h; (D) amberlite chloride ion exchange resin.

Preparation of Liposomal Aggregates. As mentioned in the Experimental Procedures, liposomes can be efficiently made by mixing lipid with DOPE as a colipid in a 1:1 molar ratio. DOPE assists in the release of pDNA from the lipoplex by providing the necessary characteristics following endosomal fusion with membranes at low pH.[27] All the liposomes (AC-Toc, MC-Toc, DC-Toc, TC-Toc, and DC-Chol) produced were optically transparent and stable water suspensions with no apparent turbidity even after a month of storage at 4 °C.

Size and Zeta Potential of the Liposomes. In the approach of physicochemical characterization, a DLS experiment was performed to examine the size and zeta potential of these nanoparticles suspended in water. This provides the information about their stability as well as their self-assembly pattern which is used for assessment of the transfection potential. The particle diameters (nm) given in Figure 2A demonstrate the liposomal formulations AC-Toc, MC-Toc, DC-Toc, TC-Toc, and DC-Chol, and it was observed that the particle sizes of the liposomal formulations were found to be 189, 171.9, 161, 189, and 174 nm, respectively. Hence, all the liposomes had a constant particle size of <200 nm, which aids in the increased entry of lipoplexes into cells through the endocytotic pathway.[38−40] The zeta potential values of AC-Toc, MC-Toc, DC-Toc, TC-Toc, and DC-Chol liposomal formulations showed moderate to high surface charges, 34.4 mV, 36.1 mV, 39.3 mV, 44.6 mV, and 49.3 mV respectively (Figure 2B).

DNA Binding Assay and Heparin Displacement Assay. The effectiveness of gene transfection depends on the electrostatic interactions between liposomes and pDNA.[28]

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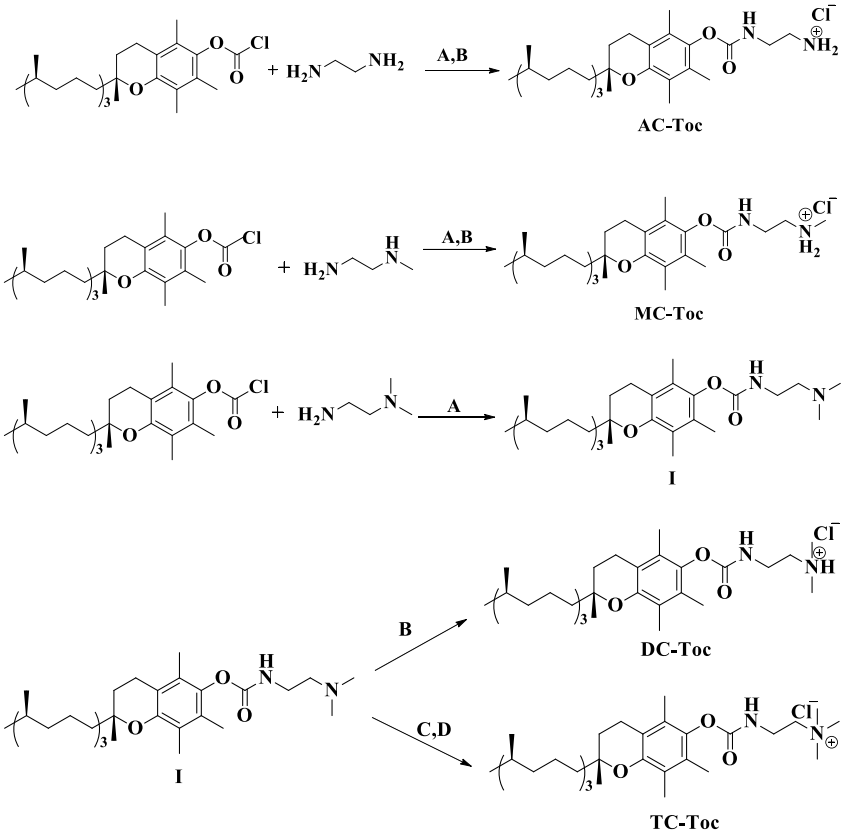
Scheme 1. Synthesis Pathway of Tocopherol-Based Cationic Lipids



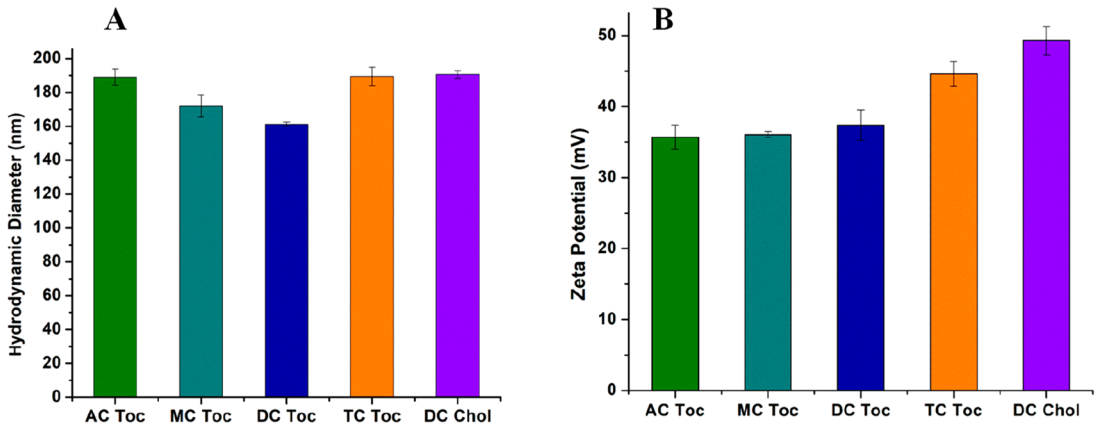


Figure 2. Particle sizes (A) and zeta potential values (B) of different synthesized liposomes.

To understand the binding efficiencies of liposomes, a gel retardation assay was performed. Using gel retardation electrophoresis experiments, the N/P ratio (1:1, 2:1, 4:1, and 8:1) of lipid/pDNA complexes at which the effective binding between lipid and pDNA takes place was determined. It is observed from the Figure 3A that AC-Toc was found to be efficient in binding pDNA even at a 1:1 N/P ratio, and MC-Toc was found to be efficient in binding pDNA at an N/P ratio of 2:1. DC-Toc, TC-Toc, and DC-Chol liposomes were able to retard pDNA efficiently at a 2:1 N/P ratio, and finally complete binding was observed at 4:1 and 8:1 N/P ratios as illustrated in Figure 3A.

To evaluate the above-mentioned lipoplexes' stability in the presence of competing negatively charged molecules, we tested the susceptibility of these lipoplexes to heparin displacement. Heparin is one of the negatively charged polysaccharide glycosaminoglycans (GAGs) which are found in many tissues and also on the cell surface as major components of the extracellular matrix. We monitored the sensitivities of lipoplexes formed with lipids after treatment with heparin displacement, which indicated the highest stability of lipoplexes formed with lipids. It is observed from Figure 3B that the binding efficacies of all the lipoplexes coincided with the gel retardation assay (Figure 3B). In conclusion, all the lipids were capable of binding to pDNA more reliably.

pDNA Transfection. Qualitative and quantitative analyses of prepared liposomes (1:1, 2:1, 4:1, and 8:1 N/P ratios) with enhanced green fluorescent protein (eGFP) encoded plasmid

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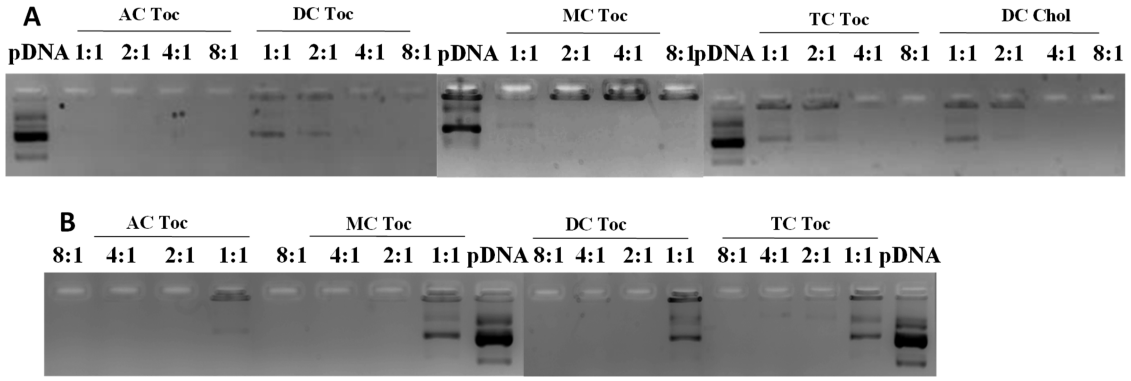

Figure 3. (A) DNA binding patterns of lipoplexes at different N/P ratios of liposome/pDNA on agarose gel electrophoresis. (B) Heparin-\ displacement assay of various liposomes associated with pDNA at different N/P ratios.

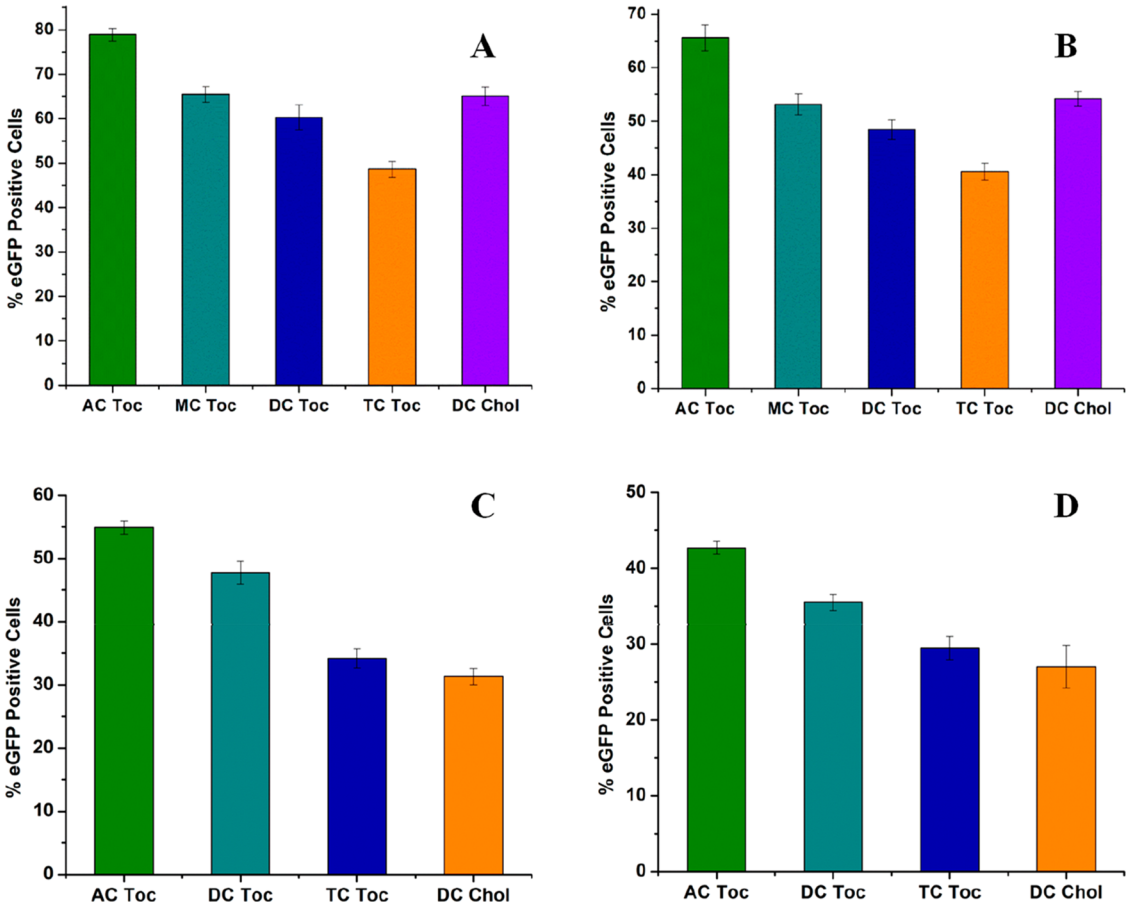

Figure 4. Comparative *in vitro* pDNA transfection efficiencies by flow cytometry in different cell lines at a lipid/pDNA 2:1 N/P ratio, HEK-293 (A), CHO (B), Neuro-2a (C), Hep-G2 (D); cationic lipid DC-Chol was used as a positive control.

DNA were performed in HEK-293, CHO, Neuro-2a, and Hep-G2 cell lines. It was observed from fluorescence microscopy as well as flow cytometry that a 2:1 N/P ratio shows high eGFP expression. The percentage of eGFP-positive cells in HEK-293, CHO, Neuro-2a, and Hep-G2 cell lines measured at a 2:1 N/P ratio using flow cytometry is depicted in Figure 4A–D, respectively. Herein, effective mediation of cell transfection was observed for all of the liposomes studied. Specifically, AC-Toc liposomes exhibited superior transfection compared to MC-Toc, DC-Toc, TC-Toc, and the control lipid DC-Chol in all the cell lines studied. In addition, DC-Toc showed better transfection than the control DC-Chol in Neuro-2a and Hep-G2 cell lines, whereas TC-Toc showed the least transfection in all cell lines. It is interesting to note that specifically in cancerous cell lines Neuro-2a and Hep-G2, lipid DC-Toc exhibited greater transfection, and TC-Toc showed almost similar transfection efficiency compared to DC-Chol. Thus, AC-Toc may be considered as an alternative to DC-Chol in liposomal transfections.

Determination of the Endocytic Pathway. Knowledge of intracellular pathway is important in gene transfection studies.[29−31] The internalization pathway of lipoplexes was studied by using various endocytosis inhibitors at optimized concentrations, e.g., a clathrin pathway inhibitor, chlorproma-

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zine (CPZ), a caveolae pathway inhibitor nystatin (NYS), a macropinocytosis pathway inhibitor, amiloride (AML), and a caveolae pathway inhibitor, filipin-III (Fil III). The results demonstrated that, for AC-Toc, MC-Toc, DC-Toc, and TC-Toc, the amiloride inhibitor had less effect on transfection, and the macropinocytosis internalization pathway may be ruled out. It is observed that when a CPZ inhibitor was used, maximum reduction (~70%) of gene expression of these lipoplexes was observed, whereas caveolae inhibitors filipin-III, and nystatin reduced activity ~20%, which confirmed that the maximum internalization of these lipoplexes happened with clathrin-mediated endocytosis (Figure 5). DC-Chol showed a

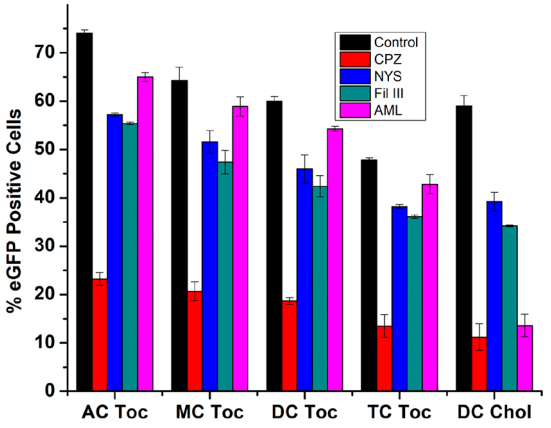

Figure 5. Effect of endocytosis inhibitors on eGFP expression in a HEK-293 cell line: % of GFP-positive cells was determined using flow cytometric analysis after normalization. Cells were pretreated with inhibitors (control: black), chlorpromazine (CPZ, red), nystatin (Nys, blue), filipin-III (Fil III, dark green), and amiloride (AML, pink) for about 30 min before complex addition.

maximum reduction in activity by using both chlorpromazine and amiloride, which confirms that the DC-Chol internalization pathway occurs through both macropinocytosis and clathrin-mediated endocytosis. Hence, changing the hydrophobic moiety can influence the specificity of the lipoplexes internalization pathway.

Antioxidant Properties of Lipids. Reactive oxygen species (ROS) is generated by cells constantly. The generation of ROS leads to oxidative stress in cells, nonspecific protein damage, and consequently leads to cell death.[32,33] During transfections, lipid peroxidation and the membrane lipid bilayer get disrupted due to the generation of ROS, and it leads to cell damage.[34] Hence, to treat the toxicity induced by ROS, development of cationic lipids having antioxidant properties along with transfection potency may be helpful. Previous results demonstrated that α-tocopherol shows better antioxidant properties than cholesterol, and it prevents oxidation by two distinct mechanisms such as a free radical scavenging mechanism and a membrane structural mechanism.[35,36] Hence, it is essential to examine the antioxidant potentials of the designed cationic lipids AC-Toc, DC-Toc, and TC-Toc. To study the antioxidant potentials of the synthesized lipids through their radical scavenger ability, a fluorescence-based assay using DCF-DA was conducted which tested the ability of these lipids in ROS quenching along with DC-Chol as a control lipid. N-Acetylcysteine (NAC), a powerful antioxidant, was used as a negative control. Hydrogen peroxide (H_2O_2), a ROS inducer, was used as a positive control. Results demonstrate that the ROS generation by AC-Toc is lower than by DC-Toc, and ROS generation by DC-Toc is lower than by TC-Toc (Figure 6). However, these three lipids showed less ROS generation than control lipid DC-Chol did.

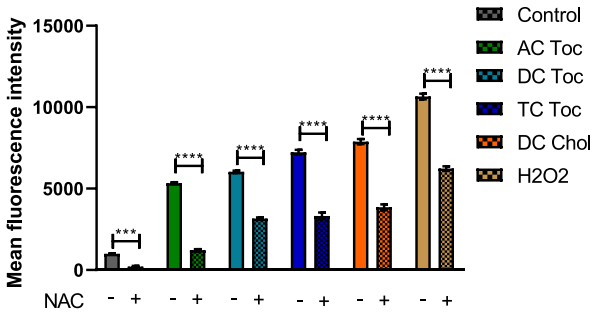

Figure 6. ROS measurement in HEK-293 cells treated with liposomal formulations. An in vitro assay on ROS generation was carried out using DCFDA fluorescence dye and measured by flow cytometry.

Cytotoxicity Study of Lipids. Another important feature that increases the probability of a synthetic gene delivery vector being used in medical gene therapy is its low cytotoxicity. To determine the safety of the synthetic cationic lipids under evaluation, the MTT assay was used.[37] The effect on cell viability of AC-Toc, MC-Toc, DC-Toc, TC-Toc, and DC-Chol lipid/pDNA complexes in HEK-293T and N2a cell lines was examined (Figure 7) at N/P ratios of 1:1−8:1. Cell viability of all the lipid complexes was observed to be maximum at 1:1 and 2:1 lipid/pDNA N/P ratios and slightly decreased at 4:1 and 8:1 lipid/pDNA N/P ratios (Figure 7). It was also observed that the impact on cell viability of all the lipids at the transfection efficient N/P ratio 2:1 was minimal. As a result, the mentioned cationic lipids are potential transfection reagents with minimal cytotoxicity, which is an important feature for *in vitro* applications.

CONCLUSIONS

The four cationic lipids with tocopherol moieties employed in this present investigation vary with increasing degrees of methylation on their headgroup. Hence, we observed remarkable differences in performances of these vectors in delivering genes. The results of this study emphasize that the cellular interactions of these four cationic lipids varied dramatically, though they have similarities in physicochemical properties and their interaction with pDNA. The AC-Toc liposome is a promising candidate for pDNA delivery compared to MC-Toc, DC-Toc, TC-Toc, and control DC-Chol in all cell lines. However, MC-Toc and DC-Toc showed better transfection in cancerous cell lines. The endocytic pathway inhibitor assay proved that the pDNA-derived complexes were involved in clathrin-mediated endocytosis during the internalization process. These novel Toc-lipids exhibited higher antioxidant properties than DC-Chol by generating less ROS, indicating less cytotoxicity. The results from the MTT assay proved that the estimated toxicity of these complexes is low. In summary, our findings collectively demonstrate that AC-Toc may be considered as an alternative to DC-Chol in liposomal transfections, and these cationic lipids could be more effective, economic, and safer for nucleic acid delivery.

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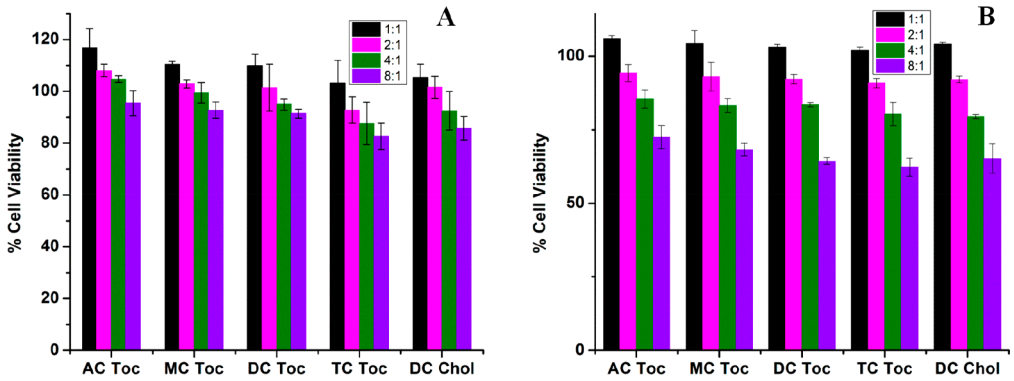

Figure 7. % Cell viability of AC-Toc, DC-Toc, TC-Toc, and DC-Chol lipoplexes at different N/P ratios in HEK-293 (A) and Neuro-2A (B) cell lines.

EXPERIMENTAL PROCEDURES

Synthesis of AC-Toc. A solution of 0.49 g (1 mmol) of alpha-tocopheryl chloroformate which was previously prepared by our laboratory in dry DCM (10 mL) was added to ethylenediamine (0.6 g, 10 mmol) in 15 mL of DCM dropwise and stirred for 24 h at room temperature. After observation of the starting materials' consumption using TLC, the resulting mixture was transferred to a separating funnel using excess DCM and washed with water (2 × 50 mL) and then 50 mL of brine. The pooled organic layer was dried by adding sodium sulfate, and the solvent was evaporated on a rotary evaporator. The crude product was further purified by column chromatography using 100−200 mesh silica gel, eluting with 3% (v/v) $CH_3OH/CHCl_3$. Subsequently, the obtained compound was dissolved in 1 mL of dry methanol and kept in an ice bath with stirring. At 0 °C, 1 mL of concentrated HCl was progressively added drop by drop and stirred for 12 h. After the starting components were consumed, the solvent was removed by washing three to four times with ethyl acetate and kept under a high vacuum for 3−4 h. Yield: 0.44 g (85%), (R_f = 0.3, 5% $CH_3OH/CHCl_3$). ^{1}H NMR [δ/ppm] (400 MHz, $CDCl_3$) δ 3.48 (s, 2H), 3.10 (s, 2H), 2.50 (s, 2H), 2.13−1.97 (m, 9H), 1.71−1.65 (m, 2H), 1.55−1.06 (m, 24H), 0.86−0.83 (m, 12H). ^{13}C NMR [δ/ppm] (126 MHz, $CDCl_3$) 156.21, 149.26, 140.48, 127.76, 126.07, 122.87, 117.45, 75.10, 45.57, 40.06, 39.51, 38.88, 37.81, 37.62, 37.60, 37.55, 37.43, 32.93, 32.89, 31.27, 31.20, 29.83, 28.11, 24.95, 24.64, 24.61, 23.44, 22.86, 22.77, 21.18, 20.58, 19.88, 19.81, 19.75, 19.71, 19.70, 19.66, 13.04, 12.21, 11.84. ESI-HRMS m/z: calculated: 517.4364, found: 517.4358 $[M]^+$.

Synthesis of MC-Toc. A solution of 0.49 g (1 mmol) of alpha-tocopheryl chloroformate which was previously prepared by our laboratory in dry DCM (10 mL) was added to 0.74 g (10 mmol) of *N*-methyl ethylenediamine in 15 mL of DCM dropwise and was stirred at room temperature for 24 h. After observation of the starting material consumption using TLC, the resulting mixture was transferred to a separating funnel using excess DCM and washed with water (2 × 50 mL) and then 50 mL of brine. The collected organic layer was dried on sodium sulfate, and the solvent was evaporated on a rotary evaporator. Column chromatography (100−200 mesh silica gel) was used to purify the crude product by eluting with 2% (v/v) $CH_3OH/CHCl_3$. Subsequently, obtained compound was dissolved in 1 mL of dry methanol and the mixture was stirred at 0 °C by keeping it in an ice bath. At 0 °C, 1 mL of concentrated HCl was added drop by drop and stirred for 12 h. After the starting components were consumed, the solvent was removed by washing it three to four times with ethyl acetate and keeping it under a high vacuum for 3−4 h. Yield: 0.41 g (83%). (R_f = 0.4, 5% $CH_3OH/CHCl_3$) ^{1}H NMR [δ/ppm] (400 MHz, $CDCl_3$) δ 3.79 (d, J = 12.0 Hz, 2H), 3.37 (s, 5H), 2.54 (t, J = 4.0 Hz, 2H), 2.0 (m, 9H), 1.73 (m, 2H), 1.55−1.04 (m, 24H), 0.87−0.83 (m, 12H). ^{13}C NMR [δ/ppm] (101 MHz, $CDCl_3$) δ 155.58, 147.89, 147.01, 127.47, 125.62, 122.67, 117.43, 74.55, 46.16, 39.38, 37.50, 37.43, 37.31, 32.80, 29.70, 27.97, 24.81, 24.49, 22.73, 22.64, 21.05, 19.75, 19.69, 19.59, 13.89, 11.77, 11.59. ESI-HRMS m/z: calculated: 531.4526, found: 531.4553 $[M]^+$.

Synthesis of Compound I. To a solution of *N*,*N*-dimethyl ethylenediamine (0.88 g, 10 mmol) in dry DCM (15 mL) was added dropwise a solution of 1.0 g (2.03 mmol) of alpha-tocopheryl chloroformate in dry DCM (10 mL) and the mixture was stirred at room temperature for 24 h. After the starting material disappeared on TLC, the reaction mixture was taken into a separating funnel with excess DCM, and washed with water (2 × 50 mL) and 50 mL brine. The organic solvent was dried with sodium sulfate and evaporated with a rotary evaporator. Column chromatography (100−200 mesh silica gel) was used to purify the crude product by eluting with 2% (v/v) $CH_3OH/CHCl_3$. Yield: 0.91 g (91%). (R_f = 0.4, 5% $CH_3OH/CHCl_3$). ^{1}H NMR [δ/ppm] (400 MHz, $CDCl_3$) 5.68 (s, 1H), 3.30 (dd, J = 12.0, 8.0 Hz, 2H), 2.50 (t, J = 8.0 Hz, 2H), 2.45 (t, J = 8.0 Hz, 2H), 2.23 (s, 6H), 2.00−1.90 (m, 9H), 1.73−1.66 (m, 2H), 1.46−0.97 (m, 24H), 0.80−0.76 (m, 12H). ESI-HRMS m/z: calculated: 544.4604, found: 545.4706 $[M + H]^+$.

Synthesis of DC-Toc. A solution of 0.45 g of compound **I** (0.82 mmol) in methanol (1 mL) was taken in an RB flask and kept for stirring in an ice bath. At 0 °C, 1 mL of concentrated HCl was added slowly drop by drop, and stirring was continued for 12 h. After consumption of substrate, the solvent was removed by giving 3−4 ethyl acetate washings and keeping it in a high vacuum for about 3−4 h. Yield: 0.39 g (86%). ^{1}H NMR [δ/ppm] (400 MHz, $CDCl_3$) 3.71 (t, J = 4.0 Hz, 2H), 3.33 (s, 2H), 2.91 (s, 6H), 2.56 (s, 2H), 2.06 (s, 9H), 1.74 (s, 2H), 1.53−1.07 (m, 24H), 0.85 (t, J = 4.0 Hz, 12H). ^{13}C NMR [δ/ppm] (101 MHz, $CDCl_3$) 155.60, 149.28, 140.31, 127.41, 125.69, 122.87, 117.31, 75.04, 58.30, 57.98, 44.34, 40.20, 40.16, 39.37, 37.57, 37.45, 37.40, 37.28, 36.84, 32.78, 32.72, 31.11, 29.69, 27.97, 24.81, 24.45, 23.83, 22.74, 22.64, 21.04, 20.58, 19.76, 19.69, 19.65, 19.59, 13.11, 12.29,

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11.80. ESI-HRMS m/z: calculated: 545.4677, found: 545.4682 $[M]^+$.

Synthesis of TC-Toc. 0.45 g (0.82 mmol) of compound (**I**) taken in methyl iodide was (1 mL) in a sealed tube and stirred for 12 h on reflex conditions. TLC was montored to show the consumed starting materials, and the obtained final TC-Toc iodide salt was exposed to repeated chloride ion exchange chromatography using chloride ion exchange resin (Amberlite A-26) and by eluting with chloroform (approximately 100 mL) as the eluent to yield the final product (TC-Toc). Yield: 0.38 g (84%). ^{1}H NMR [δ/ppm] (400 MHz, $CDCl_3$) δ 4.00−3.92 (m, 4H), 3.48 (s, 9H), 2.59 (t, J = 8.0 Hz, 2H), 2.09−2.01 (m, 9H), 1.82−1.73 (m, 2H), 1.58−1.10 (m, 24H), 0.89−0.86 (m, 12H). ^{13}C NMR [δ/ppm] (126 MHz, $CDCl_3$) δ 156.27, 149.46, 140.03, 127.39, 122.92, 118.17, 75.23, 65.66, 54.79, 39.57, 37.54, 37.24, 36.26, 32.86, 31.26, 29.74, 28.07, 24.86, 24.50, 24.01, 22.70, 21.17, 20.69, 19.82, 13.10, 12.23, 11.94. ESI-HRMS m/z: calculated: 559.4833, found: 559.4834 $[M]^+$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c06889.

Proton NMR, ^{13}C NMR, and ESI-HRMS spectra of the final lipids. Fluorescent microscopic images of liposomes with the best N/P ratio 2:1 in the HEK-293 cell line given as Figure S1 (PDF)

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Notes

The authors declare no competing financial interest.

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