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α-Tocopherol-ascorbic acid hybrid antioxidant based cationic amphiphile for gene delivery: Design, synthesis and transfection

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ABSTRACT

Natural antioxidants and vitamins have potential to protect biological systems from peroxidative damage induced by peroxyl radicals, α-tocopherol (Vitamin E, lipid soluble) and ascorbic acid (vitamin C, water soluble), well known natural antioxidant molecules. In the present study we described the synthesis and biological evaluation of hybrid of these two natural antioxidants with each other *via* ammonium di-ethylether linker, **Toc-As** in gene delivery. Two control cationic lipids **N14-As** and **Toc-NOH** are designed in such a way that one is with ascorbic acid moiety and no tocopherol moiety; another is with tocopherol moiety and no ascorbic acid moiety respectively. All the three cationic lipids can form self-assembled aggregates. The antioxidant efficiencies of the three lipids were compared with free ascorbic acid. The cationic lipids (**Toc-As**, **N14-As** and **Toc-NOH**) were formulated individually with a well-known fusogenic co-lipid DOPE and characterization studies such as DNA binding, heparin displacement, size, charge, circular dichroism were performed. The biological characterization studies such as cell viability assay and *in vitro* transfection studies were carried out with the above formulations in HepG2, Neuro-2a, CHO andHEK-293T cell lines. The three formulations showed their transfection efficiencies with highest in **Toc-As**, moderate in**N14-As** and least in **Toc-NOH.** Interestingly, the transfection efficiency observed with the antioxidant based conjugated lipid **Toc-As** is found to be approximately two and half fold higher than the commercially available lipofectamine 2000 at 4:1 charge ratio in Hep G2 cell lines. In the other cell lines studied the efficiency of **Toc-As** is found to be either higher or similarly active compared to lipofectamine 2000. The physicochemical characterization results show that **Toc-As** lipid is showing maximum antioxidant potency, strong binding with pDNA, least size and optimal zeta potential. It is also found to be least toxic in all the cell lines studied especially in Neuro-2a cell lines when compared to other two lipids. In summary, the designed antioxidant lipid can be exploited as a delivering system for treating ROS related diseases such as malignancy, brain stroke, etc.

1. Introduction

Basically, gene therapy is a potential strategy to deliver foreign DNA into a target cell nucleus, resulting in the modulation of hosts malfunctioning gene thereby helps in disease treatment [1]. Gene therapy plays an important role in the prevention of acquired diseases, such as cardiovascular diseases, cancer, and AIDS [2–4]. But, the ideal gene-carriers are needed to deliver and protect the genetic material that has to be entered into the host cell, against to the enzymatic degradation inside the cell milieu [5–7]. Though the viral vectors are one of the widely using DNA-carriers, they are proven to be imperfect in the past decades due to their less efficiency in gene transfer due to the existence of maximum immunogenicity, mutagenicity, and sometimes fatal toxicity [8,9]. However, synthetic DNA-delivering vehicles (non-viral vectors) were emerged as promising alternatives, because of their extensive characterization, structural and synthetic simplicity, as well as synthetic ease of modification of their size, functionality [10]. Synthetic cationic lipids were having been in use since 1987 for gene delivery. In general, non-viral vectors are categorized into cationic lipids, polymers, and peptides. The cationic lipids are considered to be one of the most efficient non-viral over viral approaches and it already has been using for *in vitro* transfection of a mammalian cell

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[11]. The cationic lipid-DNA complexes (lipoplexes) are one of the highly efficient systems for easy trafficking of gene of interest into host nucleus *via* an endocytosis pathway [12]. The transfection efficacy of liposomes depends up on the formulation of cationic lipids and the architecture of the gene-expression system [13–15]. However, toxicity is one of the barrier limiting the clinical applications of cationic liposomes [16,17]. Hence, regulating this cytotoxicity should facilitate the development of safe cationic liposomes for use as non-viral vectors and there by clarifying the mechanism of cytotoxicity of cationic liposomes [18]. Recently it is demonstrated that the cytotoxicity of cationic liposomes is a result of apoptosis [19,20], and that cationic liposome-induced apoptosis exhibited due to the following features: generation of reactive oxygen species (ROS) [21,22]; the activation of p38 mitogen-activated protein kinase (MAPK) [23,24]; the activation of caspase-8 [20–23,24] the cleavage of Bid and its translocation to the mitochondria and the release of cytochrome *c* [23,24]; and the activation of caspase-3 [25]. However, it remains unclear how cationic liposomes lead to ROS generation. Particularly neurons are vulnerable to increases in ROS levels [22,26], because these cells have a reduced capacity to detoxify ROS [24,27]. Hence, the delivery systems possessing radical scavenging ability along with transfection potency, may be helpful in treating ROS (reactive oxygen species) [28] related diseases such as brain stroke/ischemia and malignancy. Antioxidant cationic lipids display their obvious role as transporters of foreign DNA into the nucleolus of host cell, with-out showing any toxicity [29,30,31,32,33]. Herein, we designed and synthesized a new cationic lipid hybrid (**Toc-As**) by conjugating the α-tocopherol and ascorbic acid together by ammonium di-ether linker and allowed it to form the self-assembled amphiphilic molecule. The rational for a synthesis of this conjugated lipid is that these two vitamins (E and C) are potent natural antioxidants and can form an amphiphilic surfactant when conjugated. The main role of α-tocopherol as antioxidant is quenching of peroxyl radicals and per hydroxyl radicals and produce "tocopheroxyl radicals". Ascorbic acid (vitamin C) scavenges oxygen radicals in the aqueous phase and it converts tocopheroxyl radical into α- tocopherol, thereby permitting α-tocopherol to act as a free radical chain-breaking antioxidant and maintain biological systems. It is now apparent that ascorbic acid and tocopherol function together can protect membrane lipids from oxidative damage. To observe the individual role of each antioxidant in transfection, we have developed two control lipids one is with ascorbic acid moiety without tocopherol moiety (**N14-As**) and another is with tocopherol moiety without ascorbic acid moiety (**Toc-NOH**).

The nano structure of individual **Toc-As, Toc-NOH** and **N14-As** cationic lipids characterized in terms of size, antioxidant activity, stability, and morphology. The lipoplexes of **Toc-As**, **N14-As** and **Toc-NOH** were evaluated for theirs *in vitro* activities in multiple cell lines. The cytotoxicity of the lipids is also studied using MTT assay in multiple cell lines.

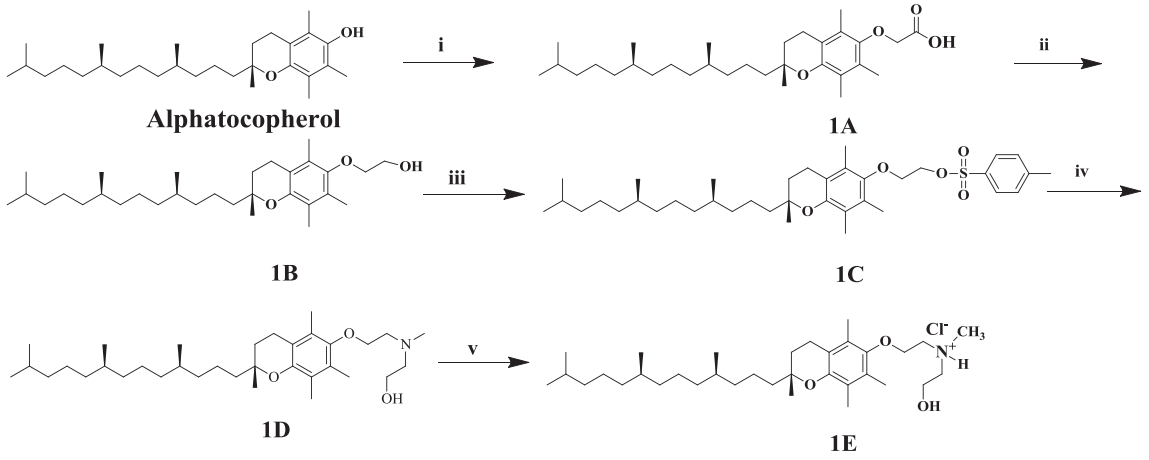
2. Results and discussion

2.1. Chemistry

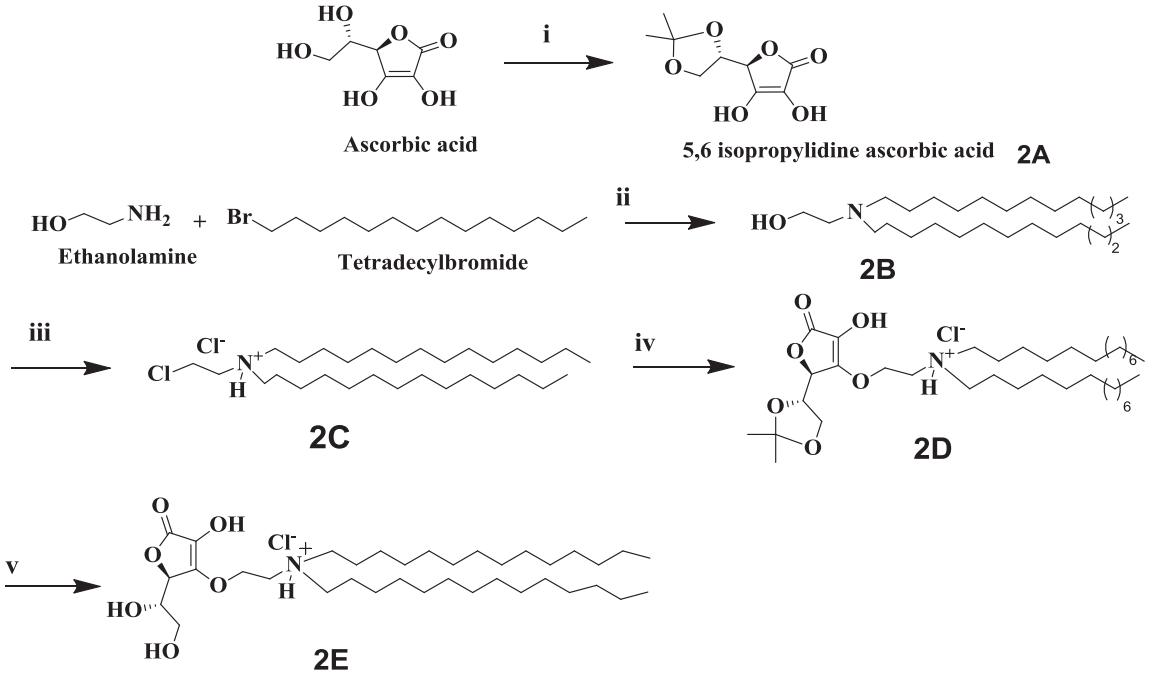
The titled cationic lipids **Toc-As, N14-As and Toc-NOH** were synthesized as described in the Schemes 1–3. The cationic lipid **Toc-As** possess α-tocopherol as the hydrophobic group and ascorbic acid as hydrophilic group linked through *N,N*-diehtylether ammonium linker, whereas **N14-As** lipid contains *N,N* dialkyl chain as hydrophobic group and ascorbic acid as hydrophilic group linked through ethyl ether linker. The lipids **Toc-NOH** possess α-tocopherol as the hydrophobic and hydroxyethyl group as hydrophilic moiety linked via ethyl ether linker. The precursor intermediate *O*-aminoethyl-[*N*-hydroxy ethyl, *N*-methyl]-α-tocopherol (**1D**, Scheme 1)in the synthesis of both the final lipids **Toc-NOH** and **Toc-As** is prepared conventionally as shown in Scheme 1. Briefly, *O*-alkylation of α-tocopherol using ethylbromo acetate in presence of 50% potassium hydroxide gave *O*-acetic acid-α-tocopherol, **1A** which upon reduction with Lithium aluminium hydride gave intermediate *O*-hydroxyethyl-α -tocopherol**1B** (Scheme 1). The intermediate **1D** was obtained by *O*-tosylation of intermediate **1B** followed by *N*-alkylation of intermediate **1C.** Subsequently the lipid **Toc-NOH** is obtained by the quaternization of **1D** using 1 N HCl in methanol. **Toc-As** is obtained by treating the intermediate **1D** with 5,6 isopropylidine ascorbic acid in DCM in presence of *N,N* dimethylamine followed by deprotection of acetonide group using dry hydrochloric acid in methanol (Scheme 3). The intermediate *N*-(2-chloroethyl)-*N*-tetradecyl tetradecane-1-amine, **2C** for synthesis of **N14-As**lipid is prepared conventionally as mentioned in the Scheme 2. The intermediate **2C** upon treatment with 5,6 isopropyledine ascorbic acid as described above for **Toc-As,** followed by the deprotection using dry hydrochloric acid gave lipid **N14-As** (Scheme 2). Chemical structures of all the synthetic intermediates lipids were confirmed by ^{1}H NMR, ESI-MASS and final lipids shown in Schemes 1–3 are confirmed by ^{1}H NMR, ^{13}C NMR and molecular ion peaks in their ESI–HRMS mass spectra. The purity of final lipids was characterized by RP-HPLC, as described in Section 4 (see Fig. 1).

2.2. Size and charge of liposomes and lipoplexes

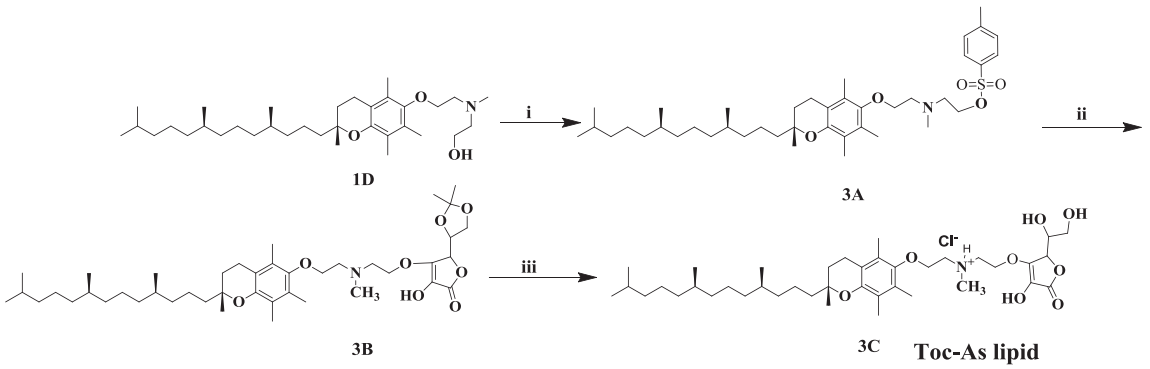
Physicochemical characteristics viz., particle size and surface potential of liposomes and lipoplexes of cationic lipids (**Toc-As**, **N14-As** and **Toc-NOH**) and DOPE as co-lipid were examined using DLS technique (Fig. 2). These measurements were made across the lipid:DNA charge ratios 1:1 to 8:1 in presence of Dulbecco's Modified Eagle's medium (DMEM). It is observed that the particle size of liposomes of **Toc-As** and **N14-As** are found to be 350 nm 398 nm respectively and slightly increased upon complexation with pDNA at 1:1. As we increase the charge ratio up 4:1 the particle size observed to be decreased up to 180 nm and 220 nm respectively and further increase in the charge ratio i.e. at 8:1 the particle size is increased i.e. 380 nm and 350 nm respectively. The surface potential of liposomes **Toc-As** and **N14-As** (+12 mV and +9.5 mV respectively) also observed to be decreasing with increasing charge ratio up to 2:1 (+6 mV and +3.6 mV respectively) and started increasing at higher charge ratio's 4:1 and 8:1 up to +16 mV and +20 mV respectively. The least size and optimal zeta potential of lipoplexes of **Toc-As** and **N14-As** at 4:1 charge ratio may be responsible for their greater transfection potentials at this charge ratio. It clearly indicate that when the resulting DNA-liposome complexes exhibit more charge positive or negative charge, electrostatic repulsive forces prevent extensive aggregation and/or fusion, thus leading to the formation of smaller complexes. It might also be the size of the nano-particle can be seen as a vital factor in evaluating lipoplexes suitability for gene delivery, given the size plays a role in lipoplexes endocytosis and transfection activity [34,35,36]. Moreover, the minimal global charge of lipoplexes benefit reduces the cytotoxicity and serum stability [37]. Whereas the sizes of lipoplexes of **Toc-NOH** observed to be increasing with increase in charge ratio 1:1 to 8:1 from 250 nm to 490 nm and similarly the surface charge also increasing with increase in charge ratios 1:1–8:1from +7.2 mV to 13.7 mV (Fig. 2). This may be because of the aggregation of lipoplexes in presence of DMEM. Such increase in the sizes of the lipoplexes in presence of DMEM is reported previously [38]. The same reason may be considered for the increase in the sizes of lipoplexes of **Toc-As** and **N14-As** at 8:1 charge ratio.



Scheme 1. Synthesis of Toc-NOH lipid. Reagentsand condition: (i) Ethyl bromo acetate, Dry DMF, 50% KOH solution, 12 h RT. (ii) Dry THF, $LiAlH_4$, 6 h RT, 0–5 °C. (iii) p-Toulene sulfonyl chloride, DMAP, Dry DCM. (iv) *N*-methyl ethanol amine, Methanol. (v) 1 N HCl in Methanol, 12 h RT.



Scheme 2. Synthesis of N14-As lipid. Reagents and conditions: (i) Dry acetone, 2,2 Dimethoxy propane, $Sncl_2$, (ii) Anhydrous K_2CO_3, ethyl acetate, 48 h reflux (iii) $SOCl_2$, dry chloroform, 6 h reflux (iv) 5,6 isopropylidine ascorbic acid, dry DMSO, anhydrous K_2CO_3, refluxed for 18 h (v) 6 N HCl in Methanol, 6 h RT.



Scheme 3. Synthesis of Toc-As lipid. Reagents and conditions: (i) p-Toluene sulfonylchloride, Triethyl amine, DMAP, dry DCM, (ii) Diethylisopropylethylamine, 5,6 isopropylidine ascorbic acid, DMAP, dry DCM (iii) 1 N HCl in methanol, 12 h RT.

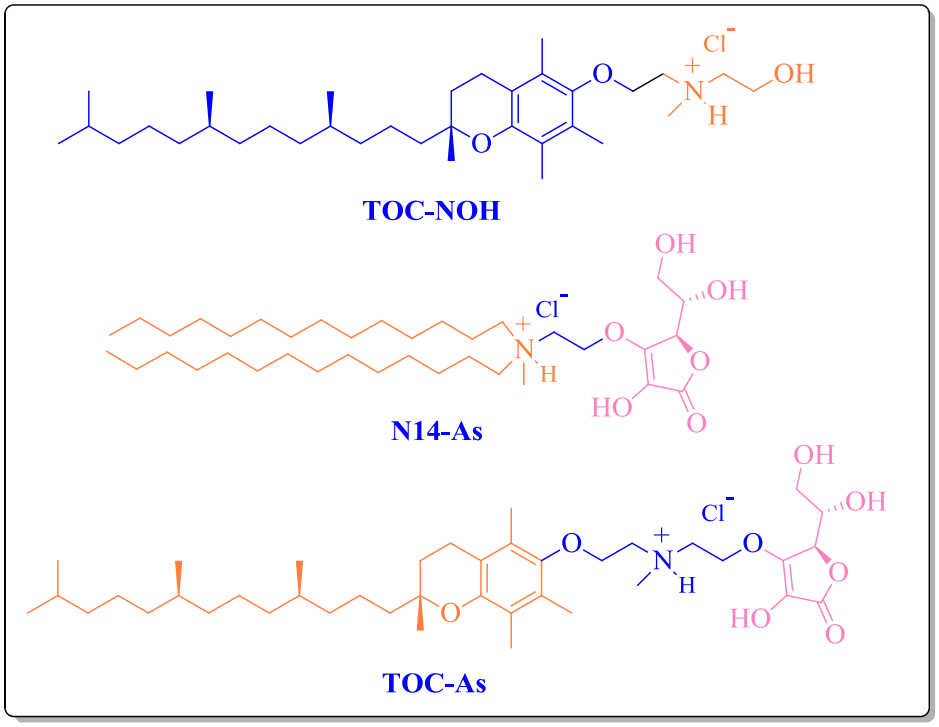


Fig. 1. Chemical structures of cationic lipids.

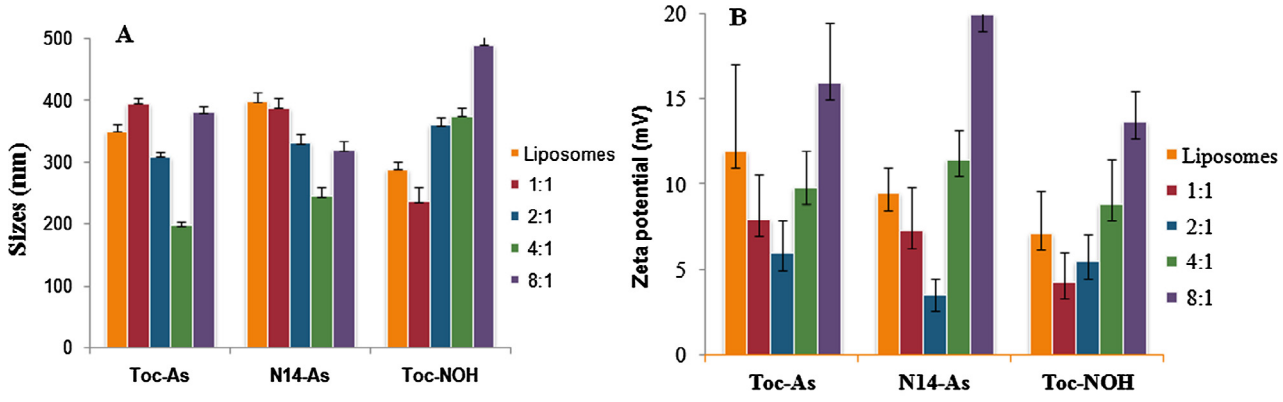


Fig. 2. Physicochemical characterization of liposomes and lipoplexes: Particle sizes (A) and zeta-potentials (B) of liposome of lipids Toc-As, N14-As and Toc-NOH and pDNA complexes at various N/P ratios (DLS at room temperature). Data represent mean ± SD (n = 3).

2.3. Liposome-DNA binding and heparin displacement

The electrostatic binding interactions between the cationic liposome and pDNA were studied using conventional agarose gel retardation assay and heparin displacement assay across 1:1–8:1 charge ratios. The corresponding gel images reveal that) all the three cationic lipids have similar capabilities in inhibiting the mobility of pDNA (Fig. 3A). All the three lipids is capable of inhibiting 90% of pDNA mobility even at lower charge ratio's such that 2:1 (Fig. 3A). Further, heparin displacement assay is carried out to observe the DNA binding strengths of these liposomal formulations. The results of heparin displacement assay showed significant

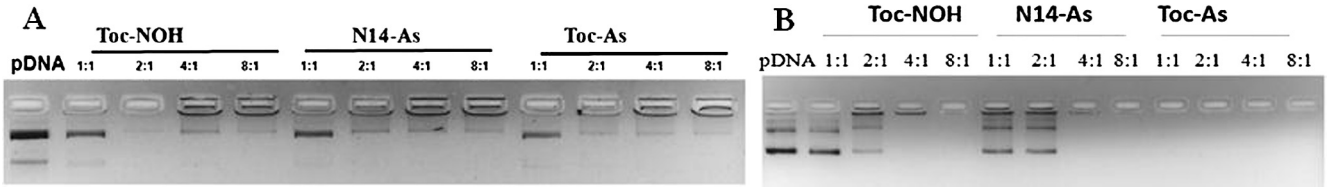


Fig. 3. (A) Electrophoretic gel patterns for lipoplex-DNA in gel retardation assay, (B) Electrophoretic gel patterns for lipid-DNA complexes in Heparin displacement assay for lipids Toc-NOH, N14-As and Toc-As. The lipid: DNA charge ratios are indicated at the top of each lane. The details of the treatment are as described in the text.

difference in the binding strengths of the three liposomal formulations. Liposomal formulation with **Toc-As** liposomal formulation completely resisted displacement of DNA with heparin across the lipid: DNA charge ratio 8:1–1:1 (Fig. 3B). While, **N14-As** liposomal formulation could survive DNA displacement by heparin only at higher charge ratios i.e. 4:1 and above. (Fig. 3B). Whereas, liposomal formulations of **Toc-NOH** could not resist much even at charge ratio 4:1 and could survive heparin displace only at higher charge ratio 8:1. It therefore appears that **Toc-As** and **N14-As** lipids having ascorbic acid moiety in the head group region are capable of binding pDNA strongly and it may show any crucial role in the transfection biology. These observations are well in agreement with the size and surface potential data. The CD spectrum data also supports the binding abilities of the lipids having ascorbic acid moiety with pDNA.

2.4. CD spectra of pDNA in lipoplexes

To observe the change in the morphology of pDNA when complexed with the liposomes of the present lipids, circular dichroism (CD) measurements were carried out. Surprisingly, the formulations of **Toc-As** and **N14-As** lipids perturbed the double helix structure of DNA (Fig. 4). Such transformed mode of compaction of pDNA by **Toc-As** and **N14-As** might be responsible for their relatively higher transfection activity, though this may be further corroborated by other physical methods to provide a comprehensive model and to prove the role of ascorbic acid derivative of the lipids in gene transfection activity. As ascorbic acid is generally negatively charged, it cannot neutralize the charges on the negative phosphate groups of DNA. **Toc-As** and **N14-As** lipids having ascorbic acid head group, may interact with the bases inside the double-helix structure of pDNA and causes distortion in the double-helix structure. Neault et al. [39]. carried out an infrared and Raman spectroscopic studies on the effect of ascorbic acid on DNA [40]. They found that the OH and C—O groups of ascorbic acid interact directly with DNA bases [39,41,42,43,44]. Fig. 4 demonstrate that, **Toc-As** and **N14-As** lipoplexes showed linear conformation changes when compared to **Toc-NOH** lipoplex and this indicates that morphology change of lipoplexes induced by ascorbic acid head group contained lipids. These results suggest that torsional stress is generated along the double-helix DNA in ascorbic acid based lipids and this may be useful for the gene delivery. To the best of our knowledge, gene delivery efficiency of ascorbic acid head group (**Toc-As** and **N14-As**) lipids has not previously reported. In this study, it has become clear that ascorbic acid head group lipids have a dramatic effect on the conformation of giant DNA molecules.

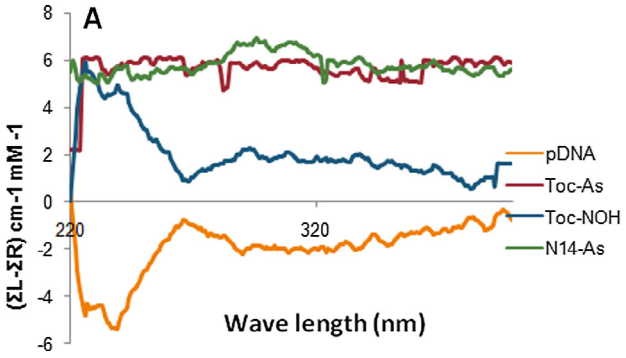


Fig. 4. Circular dichroism (CD) spectra of lipoplexes. Liposomes were prepared at 1/1 M ratio of Toc-As, N14-As and Toc-NOH as cationic lipids DOPE taken as a co-lipid. Complexes were prepared at 4:1 charge ratio of cationic liposome-double helix structure of DNA, and CD was recorded at a pDNA concentration of 50 μg/mL. The CD spectra have been compared to the profile for pDNA alone. Spectral profiles of liposomes with dextrose or of dextrose alone were subtracted as blank.

2.5. Antioxidant activity

As the cytotoxicity of cationic liposomes is majorly because of apoptosis exhibited due to the generation of reactive oxygen species (ROS) during transfection [45,16]. Hence, the cationic lipids having radical scavenging ability along with transfection potency may be helpful in treating ROS (reactive oxygen species) induced toxicity [16]. So, it is necessary to study the antioxidant efficiencies of the designed anti-oxidant cationic lipid **Toc-As** along with the control lipids **N14-As** and **Toc-NOH.** The radical scavenger ability of the synthesized lipids was evaluated calorimetrically by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. The DPPH radicals show a deep-violet color in an aqueous solution of ethanol giving a strong absorption in the visible region (460–560 nm). The increase in the intensity of DPPH solution is directly proportional to the concentration of radicals and hence used as a measure of the radical-scavenging ability of **Toc-As**, **N14-As** and **Toc-NOH.** The antioxidant efficiencies of the cationic lipids **Toc-As**, **N14-As** and **Toc-NOH** were calculated with respect to the control samples that contain only DPPH. The cationic lipids at different concentrations (0–50 μM) were incubated in aqueous ethanol solutions of DPPH (100 μM) at room temperature in the dark. The difference in the concentration of DPPH radicals upon treatment with different cationic lipids was calculated by measuring the colorimetric intensity of the samples at 490 nm using a microplate reader after 30, 60, 90, and 120 min of incubation (Fig. 5). The untreated DPPH sample at every time point used as controls and the anti-oxidant efficiencies observed were time and concentration dependant. After 30 min of incubation, low concentrations of the cationic lipids (<5 μM) did not show significant antioxidant activity (Fig. 5). However, the cationic lipids, **Toc-As, Toc-NOH** and **N14-As**showed antioxidant activity at concentrations that are above 10 μM. **Toc-As** showed higher radical scavenger abilities when compared to control lipids **Toc-NOH**, **N14-As**. Moreover, the antioxidant activity of **Toc-As** was found to be 2-fold higher than the natural antioxidant ascorbic acid which is used as a positive control (Fig. 2). The cationic lipid **Toc-NOH** did not show any appreciable antioxidant activity even at higher concentrations (Fig. 5).

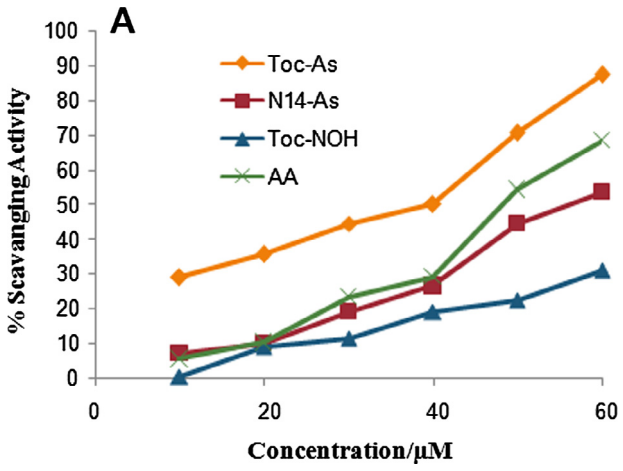


Fig. 5. (A) Antioxidant assay of cationic lipids. Concentration-dependent radical-scavenging ability of Toc-As, N14-As and Toc-NOH cationic lipids against DPPH free radicals after 30 min of incubation at room temperature in the dark. The absorbance was measured at 490 nm. The values shown are mean ± SEM of three independent experiments carried out in three to five replicates. DPPH = 2, 2-diphenyl-1-picrylhydrazyl radicals, AA = ascorbic acid.

However, **N14-As** lipids molecules having ascorbic acid moiety as head group showed some radical-scavenging effect after 30 min of incubation. The experimentally found superior radical-scavenging activity of **Toc-As is** attributed to the ability of the natural antioxidant properties and neutralization of the DPPH radicals.

2.6. Cationic liposome preparation

Toc-As/DOPE, **N14-As** /DOPE and **Toc-NOH**/DOPE liposomes were prepared at 1:1 mol ratio with 1.4×10^{-2} M total lipid concentration, as stock solution. The mixture was dissolved in chloroform (1.5 mL) in an autoclaved glass vial. The solvent was evaporated and dried under a thin flow of moisture-free nitrogen gas. The resulting thin film is dried farther under high vacuum for 4 h. The dried film was hydrated by the addition of 1 mL of deionized water. The mixture was allowed to swell overnight. The liposomes were vortexed for 1–2 min to remove any adhering lipid film and then sonicated in a bath sonicator for 5 min at room temperature to obtained multilamellar vesicles. The vesicles were then sonicated in an ice bath using a probe sonifier to afford the cationic liposomes. All the liposomes formed were stable and uniform. Liposomes were stored at 4 °C and it was observed that no precipitation even after 3 months.

2.7. Transfection biology: In vitro transfection studies

The relative transfection potentials of lipoplexes of lipids **Toc-As**, **Toc-NOH** and **N14-As**wereestablished against multiple cell lines viz., HepG2, HEK-293T, CHO andNeuro-2a across charge ratio 1:1 to 8:1.*p*CMV-SPORT-β-gal plasmid DNA was used as a reporter gene and is complexed with cationic liposomes across1:1 to 8:1 lipid:DNA charge ratios. The transfection efficacies of **Toc-As**, **Toc-NOH** and **N14-As** lipids were compared with that of commercial formulation, lipofectamine 2000 and the results are summarized in Fig. 6. All the cationic lipids showed their maximum transfection efficacies at the charge ratio 4:1 irrespective of the cell lines. This may be due to the optimal particle sizes and zeta potentials of the lipoplexes at these charge ratios, which in turn affect the cellular uptake (Fig. 6). The lipoplexes of lipids **Toc-As** and **N14-As** showed similar transfection profile in HEK-293T and CHO cells at charge ratios 2:1 and 4:1 and nearly two folds higher efficient than Lipofectamine 2000 in HEK-293T cell lines at 4:1 charge ratio. Where as in Neuro-2a cell lines **Toc-As** is slightly better active than **N14-As**at 4:1 charge ratio and comparable to that of lipofectamine 2000. The lipoplexes of **Toc-As**, i.e. lipid having both tocopherol and ascorbic acid moieties, exhibited much superior transfection efficacy among three lipids and two and half fold higher transfection than Lipofactamine 2000 against HepG2 cell lines at 4:1 charge ratio (Fig. 6). It is apparently, due to the better uptake of tocopherol lipids through cellular transport pathways mechanism involving cell surface receptors (tocopherol-transfer protein) present on HepG2 cell lines [46]. At the same time the superior activity of **Toc-As** may be attributed because of the least cytotoxicity of the lipid due to its maximum anti-oxidant activity. In contrast the lipoplexes derived from **Toc-NOH** showed least transfection against all the tested cell lines, indicating that the ascorbic acid in the head group region in others played a crucial role in transfection. The higher transfection efficiencies of **Toc-As and N14-As** compared to **Toc-NOH** may also be due to the presence of more number of hydroxyl groups present in the head group region as reported earlier [38] which is also confirmed by CD spectral data. The superior activity of **Toc-As** cationic liposomes also can be attributed because of the diehtylether ammonium linker, which might have greatly influenced the process of liposomes formation and lipoplex-membrane fusion than **N14-As** liposomes. In detail, the lipid **Toc-As** possessed α-tocopherol and chiral ascorbic acid may show some degree of lateral freedoms to each other because of the diehtylether liker and it might contributed to the maximum release of DNA from lipoplexes into cytosol and also the flexibility in the lipid favours the multiple H-bonding interaction of ascorbic acid moiety of the lipid with negatively charged surface of double helix plasmid DNA to significantly enlarge the overall strength of interaction between the lipid and the DNA. In summary**, Toc-As** lipid is capable of transfecting multiple cell lines effectively along with its higher radical scavenging ability and low cytotoxicity may be helpful in treating ROS (reactive oxygen species) related diseases such as brain stroke/ischemia and malignancy.

2.8. EGFP expression

The transfection results revealed that the present lipids **Toc-As**, **N14-As** and **Toc-NOH** showed their maximum transfection ability at 2:1 & 4:1, which was further supported by the qualitative examination using eGFP plasmid under epifluorescence microscopy. Towards this end, lipoplexes were prepared using eGFP plasmid at transfection efficient charge ratios 2:1 and 4:1. The formulations were incubated with HEK-293T and HepG2 cell lines in DMEM + 10% FBS for 4 h. The green fluorescent images were visually observed under epi-fluorescent microscope and evaluated in terms of% GFP positive transfected cells (Figs. 7 and 8**)** and geometric mean fluorescence intensities (GMFI) (Figs. 7C and 8**C**). The representative fluorescent images (Figs. 7A and 8A) show that the relative fluorescent gene expression obtained from HepG2 was relatively higher than that obtained from HEK-293T cell lines. The results represent that maximum GFP expression was observed at 4:1 charge ratio irrespective of the cell lines for all the three lipid formulations. It is also observed that **Toc-As** lipoplex showed maximum GFP expression when compared to **N14-As** and **Toc-NOH** lipoplexes at both the charge ratios in both the cell lines studied (Figs. 7 and 8). These results were reliable with the transfection results and it reemphasize that tocopherol-ascorbic acid hydride cationic lipid has superior transfection profile. The lipoplexes of **Toc-NOH** showed least expression of eGFP among the three lipids studied (Figs. 7 and 8). The order of cationic lipids according to their intensity of fluorescent expression observed in Figs. 7 and 8 can be given as **Toc-As > N14-As > Toc-NOH.**

Representative fluorescence images of eGFP expression at the indicated charge ratios. Liposomes of all cationic lipids formulated with colipid DOPE (1:1) lipid and were complexes with negatively charged GFP in the optimized charge ratios 2:1 and 4:1 were used. The liposome-DNA complexes were incubated for 4 h in presence of 10% serum. Images were acquired 48 h post transfection. (B) Representative graph depicts the transfection efficiencies in terms of percentage GFP positive cells. (C) The graph indicating geometric mean fluorescent intensity (GMFI) of transfected cells using eGFP as the reporter gene. The data shown is the mean and standard deviation of three different experiments.

2.9. Effect of serum on transfection efficiency

Generally the gene transfer efficacies of cationic amphiphiles are evaluated either in absolute absence of added serum or in the presence of only 10% (v/v) serum as reported in many prior investigations [38,47,48]. Still the serum-incompatibility is one of the major setbacks retarding the clinical success of cationic transfection lipids. The high *in vitro* transfection of many cationic amphiphiles are often found to be adversely affected in the presence of serum. The cationic lipids show their serum incompatibility due to adsorption of negatively charged serum proteins onto the positively charged cationic liposome surfaces which in turn prevents their efficient interaction with cell surface and/or internal-

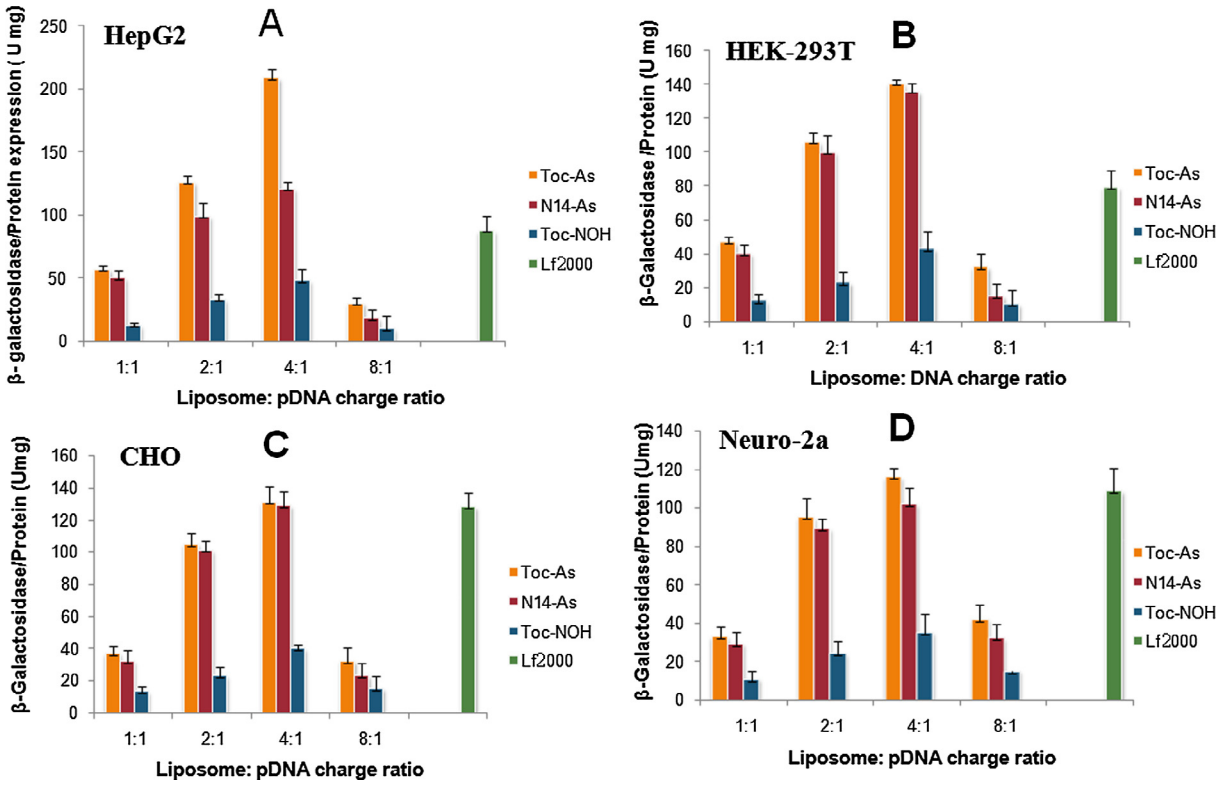


Fig. 6. (A–D) Transfection efficiencies of lipids Toc-As, N14-As and Toc-NOH in (A) HepG2 cells, (B) HEK-293T cells, (C) CHO cells and (D) Neuro-2a cells with DOPE as co-lipid (each lipid formulated with co-lipid in 1:1 M ratio respectively). The transfection efficiencies of the lipids were compared to that of commercial formulation Lf2000. Transfection experiments were performed as described in the text. All the cationic lipids were tested on the same day, and the data presented are the average of three experiments performed on three different days. The error bar represents the standard error. The difference in the data obtained is statistically significant in all charge ratios ($P < 0.002$).

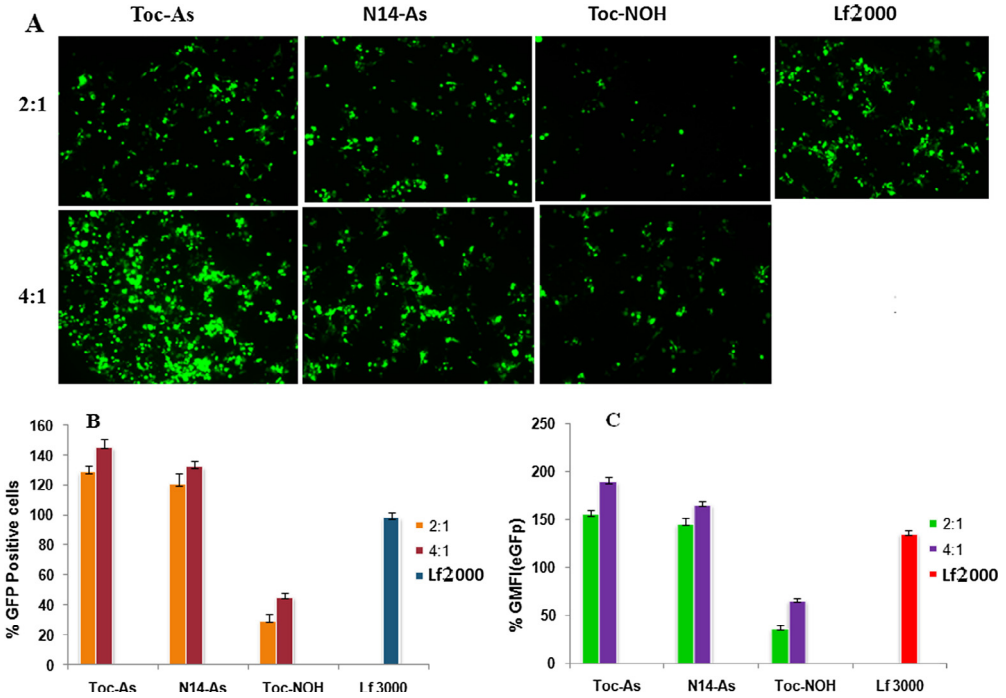


Fig. 7. Transient transfection using the reporter gene, eGFP in HepG2 cells: (A).

ization [49–51]. Hence, evaluation of gene transfer efficacies across a range of lipid:DNA charge ratios in multiple cultured cells in presence of increasing concentrations of added serum is needed for obtaining meaningful systemic potential of any *in vitro* efficient cationic transfection lipid. In order to know the effect of serum on the transfection efficiencies of the present lipids, transfection

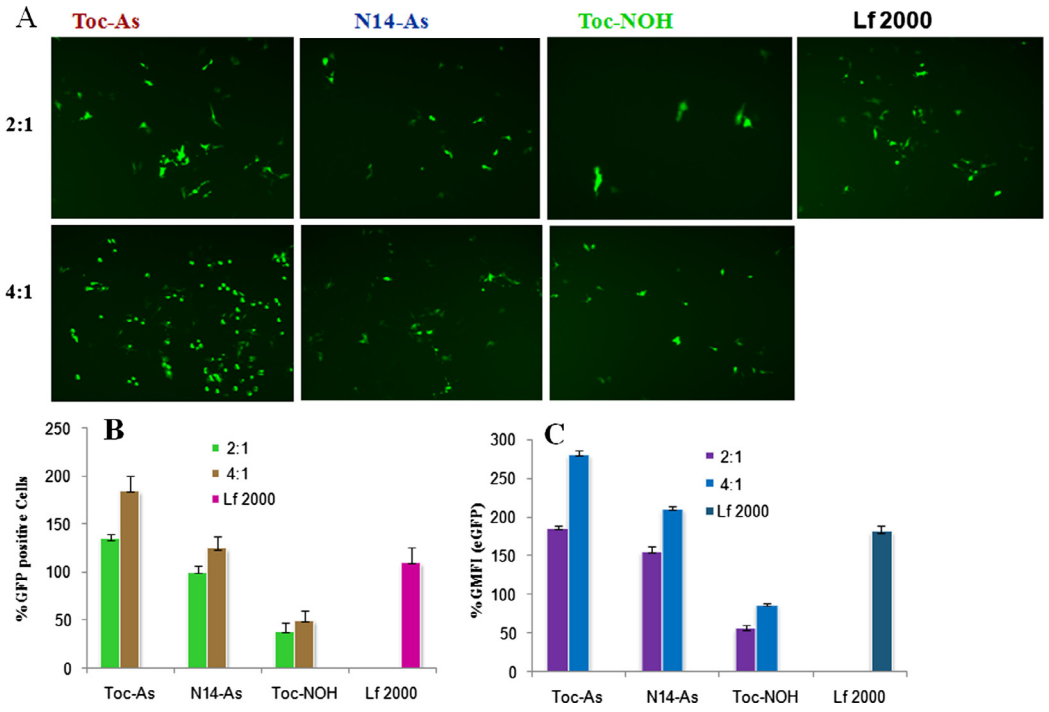


Fig. 8. Transient transfection using the reporter gene, eGFP in HEK-293T cells: (A) Representative fluorescence images of eGFP expression at the represents charge ratios. Liposomes of all cationic lipids formulated with colipid DOPE (1:1) lipid and were complexes with negatively charged GFP in the optimized charge ratios 2:1 and 4:1 were used. The liposome-DNA complexes were incubated for 4 h in presence of 10% serum. Images were acquired 48 h post transfection. (B) Representative graph depicts the transfection efficiencies in terms of percentage GFP positive cells. (C) The graph indicating geometric mean fluorescent intensity (GMFI) of transfected cells using eGFP as the reporter gene. The data shown is the mean and standard deviation of three different experiments.

experiment was performed by complexing with eGFP plasmid at transfection efficient charge ratio 4:1 in HEK-293T and CHO cell lines with increasing added serum concentrations from 10 to 50%. The *in vitro* transfection studied with increasing concentrations of added serum results show that all the three lipids **Toc-As**, **N14-As** and **Toc-NOH** lipids were unaffected even in the presence of high concentration of serum up to50% of added serum (Fig. 9). The results demonstrate that **Toc-As** lipid showed slight increase in the transfection with increase in the concentration of added serum. **Toc-As** and **N14-As** exhibited higher transfection potentials even at higher concentration of added serum compared to **Toc-NOH,** which is found to be the least. It clearly indicates that lipoplexes having zeta potential values <10 mV, which greatly benefit the serum stability of lipoplexes and decrease the cytotoxicity, exhibited serum compatibility. The maximum serum compatibility of lipids **Toc-As** and **N14-As** expected to be due to enhanced surface charge shielding of the liposome-DNA complexes induced by hydroxyl functionalities of ascorbic acid in the head group region.

2.10. Cellular uptake

The transfection efficiencies of the non-viral gene delivery systems seem to be linked to the internalization (endocytosis) of DNA associated complexes. In order to verify the cellular uptake efficiency of lipoplexes of the present lipids, membrane green fluoresce-labeled CHO cell lines were incubated with the lipo-

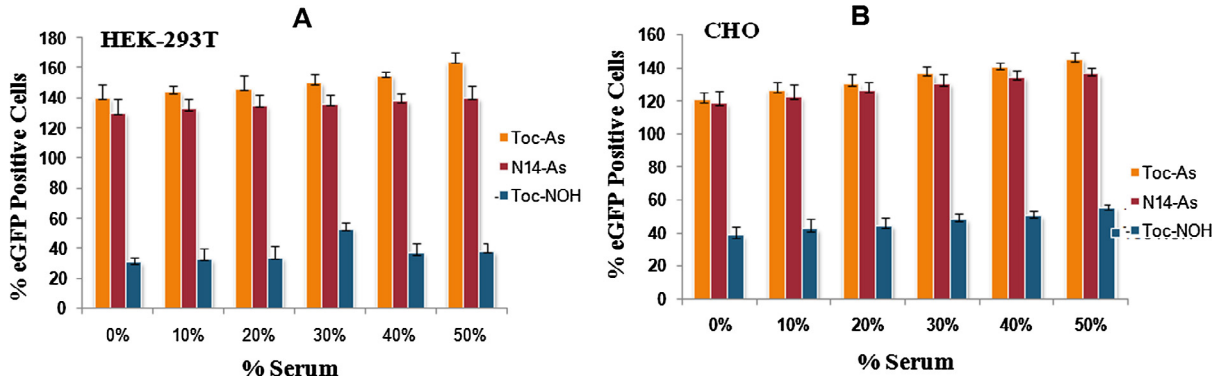


Fig. 9. Transfection efficacies of the cationic lipoplexes Toc-As, N14-As and Toc-NOH in the presence of increasing concentrations of added serum. *In vitro* transfection efficiencies of liposome-eGFP complexes prepared using GFP reporter gene at a lipid/DNA charge ratio of 4:1 were evaluated in the presence of enhancing concentrations of added serum in (A) HEK-293T and (B) CHO types of cells. The error bar indicates that the standard error. The difference in the data obtained is statistically significant in all charge ratios ($P < 0.003$).

plexes comprising of Rhodamine-PE labelled liposomes of lipids (**Toc-As**, **N14-As** and **Toc-NOH**) and *p*CMV-SPORT-β-gal plasmid at 4:1 charge ratio for 4 h. The internalization of rhodamine labelled lipoplexes was observed under epifluorescence microscope. The intracellular red fluorescence intensity of the cells was quantified using microplate fluorescent reader. Fig. 10 shows the red and green fluorescent images of CHO cells after incubation for 4 h. The results of the cellular uptake experiment show that the% of rhodamine positive cells follows the order: **Toc-As** > **N14-As** > **Toc-NOH**. The lipoplexes particle size may play a vital role in facilitating the cellular uptake. Hence, it is obvious that **Toc-As** lipoplexes having the least particle size at 4:1 is one of the reasons for its high potency for uptake by cell lines. The other reason for the higher uptake efficiency may be the multiple hydroxyl group functionality in its head group region, which may involve in the favourable hydrogen bonding interactions with the biological membrane components. Thus, the higher uptake efficiency of the lipid **Toc-As** may also an attribute to its superior *in vitro* transfection efficiency compared to **N14-As** and **Toc-NOH.**

2.11. Cytotoxicity assay

The cytotoxicity is a major limiting factor for the success of gene therapy in clinical applications. Especially, in liposomal gene delivery, the cytotoxicity of cationic liposomes is a result of apoptosis [22,18,21,21,23,27] and that cationic liposome-induced apoptosis exhibited due to the generation of reactive oxygen species (ROS) [23,24].Particularly neurons are vulnerable to increases in ROS levels, because these cells have a reduced capacity to detoxify ROS [24,23].Towards, this end to observe the cytotoxicities of the lipoplexes of the designed antioxidant lipid **Toc-AS** and control lipids, **N14-As** and **Toc-NOH,** MTT assay was performed in four different cell lines i.e. HepG2, HEK-293, Neuro-2a, and CHO across the charge ratios 1:1 to 8:1. The cell viability results are summarised in Fig. 11.The results indicate that the lipoplexes of **Toc-As**, **N14-As** and **Toc-NOH** lipids found to be non-toxic up to the charge ratios of 4:1–1:1 in HepG2, HEK-293T and CHO cell lines and slightly toxic (80%) at 8:1 charge ratio. Whereas in Neuro-2a cell lines the lipid **Toc-NOH** showed less viability i.e.83% at 4:1 charge ratio, the transfection efficient charge ratio, when compared to lipids (**Toc-As** and **N14-As**) containing ascorbic acid moiety in the head group region. It is clear that the neuro cell line which are vulnerable to ROS related apoptosis could survive in the presence lipids (**Toc-As** and **N14-As**) containing ascorbic acid moiety in the head group region than in the presence of lipid (**Toc-NOH**) not having ascorbic acid in the head group region. Hence, the delivery systems consisting of our designed antioxidant lipid i.e. **TOC-AS** having high radical scavenging ability along with maximum transfection efficiency and least cytotoxicity, may be helpful in treating ROS (reactive oxygen species) related diseases such as brain stroke/ischemia and malignancy.

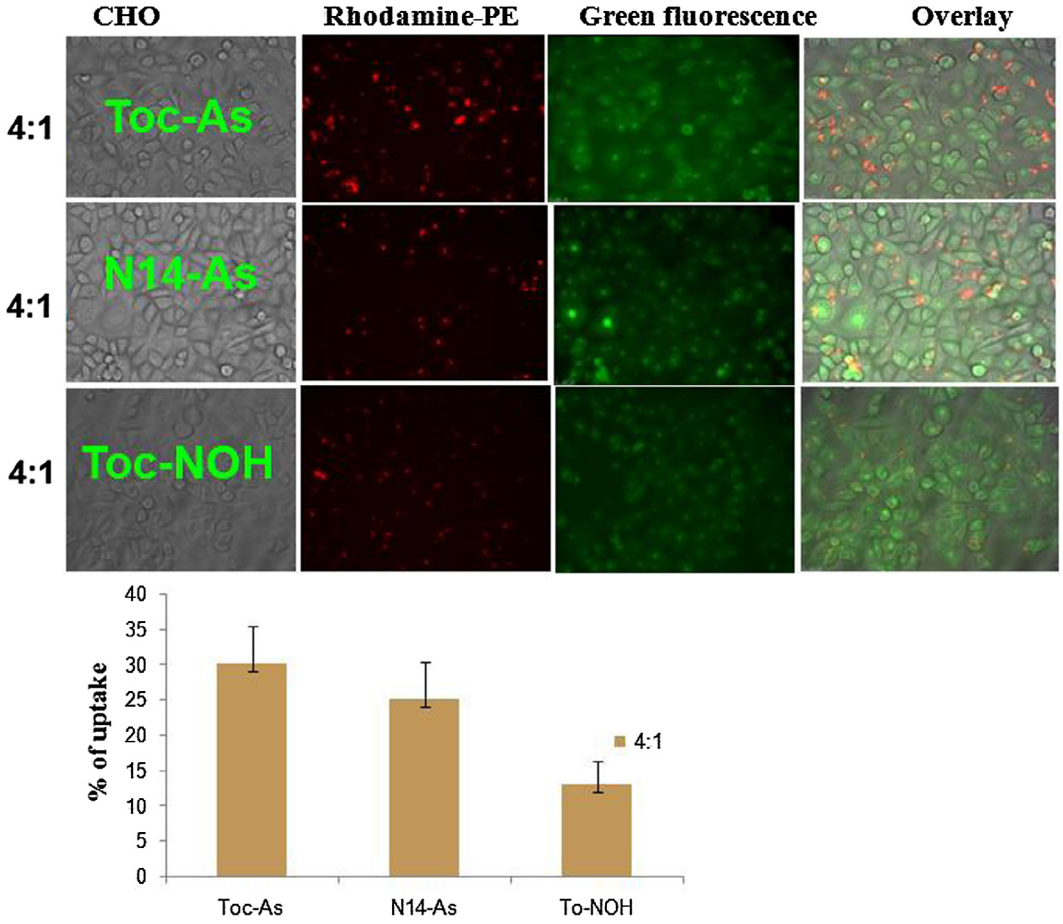


Fig. 10. Cellular Uptake of rhodamine labelled lipoplexes. Fluorescence images (A) and% uptake (B) of CHO cells incubated for 4 h with the complexes of rhodamine labeled liposomes of Toc-As, N14-As, and Toc-NOH and plasmid DNA. The percentage uptake was calculated using the formula % uptake = 100× (fluorescence intensity of the fluorescence lipoplex treated cell lysate-background)/(fluorescence intensity of lipoplex added to the cells-background). The details of the experiments are as described in the text. Data are showed as mean ± SD (n = 3). * represent $sp < 0.03$.

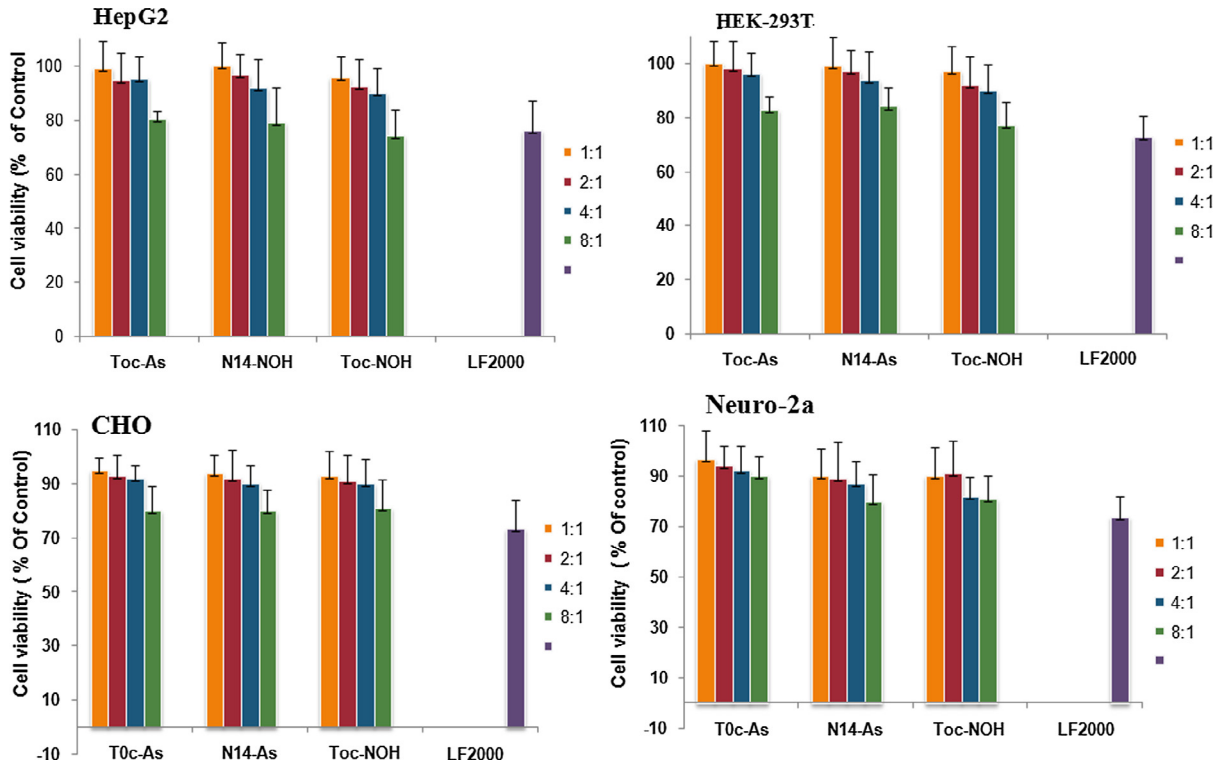


Fig. 11. Representative percent of cell viabilities up on treatment of HepG2 (A), HEK-293T (B), CHO (C), and Neuro-2a (D) cells with lipids Toc-As, N14-As and Toc-NOH using MTT assay. The absorbance obtained with reduced formazan with cell in the absent of cationic lipids was taken to be 100. The toxicity assays were evaluated as depicted in the text. The data obtained is the average values of three independent experiments (n = 3).

3. Conclusions

In summary, the synthesized α-tocopherol-ascorbic acid hybrid cationic amphiphile can efficiently deliver genes into multiple culture cells. Among the three cationic lipids studied (**Toc-As**, **N14-As** and **Toc-NOH**), lipid **Toc-As** with ascorbic acid head group showed superior transfection activity. This α-tocopherol-ascorbic acid hybrid lipid has significantly affected the physico-chemical and biological properties of cationic lipids. Hence, ascorbic acid head group might be attractive in designing new lipids for gene delivery and it might be useful for drug delivery. Based on the results it is suggested that **Toc-As** lipid will be a promising non-viral gene delivery vector with superior transfection efficiency, strong radical scavenger activity and least cytotoxicity for treating ROS related diseases in brain.

4. Experimental section

4.1. General procedure and chemicals reagents

Mass spectral data were acquired by using a commercial LCQ ion trap mass spectro-meter (Thermo Finnigan, SanJose, CA, U.S.) equipped with an ESI source.^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian FT400 MHz NMR spectrometer. α-Tocopherol was purchased from Sigma Co. Super negatively charged eGFP plasmid, and rhodamine-PE were ample gifts from IICT (Indian Institute of Chemical Technology, Hyderabad, India). Lipofectamine-2000 was purchased from Invitrogen Life Technologies, polyethylene glycol 8000, and o-nitrophenyl-β-d-galactopyranoside was purchased from Sigma (St. Louis, MO, U.S.). NP-40, antibiotics, and agarose were purchased from Hi-media, India. 1,2-dioleoyl-*sn*-*glycero*-3-phosphoethanolamine (DOPE) was purchased from Fluka (Switzerland). Unless otherwise stated, various organic solvents including α-tocopherol chloroform, acetone, ethanol, methylene chloride, phosphomolybdic acid spray reagent, ethyl bromo acetate, p-toluene sulfonylchloride, and *N*,*N*-dimethylformamide (DMF) were purchased from Sigma-Aldrich Co. and were used without further purification. The progressive of the reaction was monitored by thin-layer chromatography using 0.25 mm silica gel plates. Column chromatography technique was executed with silica gel (Acme Synthetic Chemicals, India; finer than 200 and 60-120mesh). Elemental analyses were performed by High Resolution Mass Spectrometry (HRMS) using QExactive equipment (Thermo Scientific) and purity of lipids were characterized by HPLC (Shimadzu LC Solution) and showed more than 95% purity. HepG2, CHO, Neuro-2a and HEK-293T cells were procured from the National Centre for Cell Sciences (NCCS), Pune, India. Cell were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS in humidified atmosphere containing 5% CO2/95% air.

4.2. Synthesis of O-acetic acid-α-tocopherol (1A, Scheme 1)

A solution of α-tocopherol (0.5 g, 1.16 mmol) in *N*,*N*-dimethylformamide (20 mL) was treated with ethyl bromo acetate (3.4 g, 8.3 mmol) and an excess of powdered KOH (1.2 g, 30 mmol). The resulting yellow residue was stirred vigorously for 24 h at room temperature. The reaction was acidified with 6 N HCl and extracted with ethyl acetate (3 × 30 mL). The combined ethyl acetate layers were washed with H_2O (3 × 30 mL) and brine (1 × 30 mL), and then dried with Na_2SO_4. The ethyl acetate solution was concentrated to yellow oil and purified by silica gel chromatography eluting with 20% (v/v) EtOAc and 1% acetic acid in hexanes. This yielded **1A** as yellow color (0.50 g, 88%).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm 0.8–0.90 (m, 12H), 1.00–1.50 (m, 25H), 1.77–1.88 (m, 2H), 2.08 (s, 3H), 2.13 (s, 3H), 2.17 (s, 3H), 2.55–2.50 (t, 2H), 4.3 (s, 2H), 7.98 (broad, 1H). **ESI-MS**: Calculated 488; found [M+18] 506.

4.3. Synthesis of O-ethyl alcohol -α-tocopherol (1B, Scheme 1)

To a solution of lithium aluminium hydride (0.08 g, 1.1 mmol) in 10 mL of dry THF, a solution of **1A** (2.0 g, 1 mmol) in 5 mL dry THF was added slowly at 0 °C with the help of addition funnel about 10 min. The reaction mixture was stirred for 6 h at room temperature and then the excess $LiAlH_4$ was quenched with ethyl acetate. The ethyl acetate layer was washed with water (3 × 50 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography to give *O*-ethyl alcohol-α-tocopherol **1B** (1.89 g), 96% as a clear oil. Rf = 0.2 (20% EtOAc/hexane, 2:8).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm 0.8–0.90 (m, 12H), 1.00–1.5 (m, 25H), 1.77–1.88 (m, 2H), 2.08 (s, 3H), 2.13 (s, 3H), 2.17 (s, 3H), 2.52–2.59 (t, 2H), 3.66 (t, 2H), 3.85 (t, 2H).). **ESI-MS**: Calculated 474; found [M^+] 474.

4.4. Synthesis of O-ethyl-O-sulfonyl benzyl -α-tocopherol (1C, Scheme 1)

To a solution of **1B** (4.0 g, 9.0 mmol) in 10 mL of dry DCM were added tosyl chloride (2.12 g, 18 mmol), pyridine (1.46 g, 18 mmol), and a catalytic amount of DMAP. The reaction mixture was stirred at room temperature for about 12 h. The solvent was evaporated in vacuum to dryness. The residue was dissolved in 25 mL of ethyl acetate and washed twice with 2 × 30 mL of copper sulphate solution to remove any excess pyridine. The organic layer was dried on anhydrous sodium sulphate, the solvent was evaporated, and the residue was purified by column chromatography by eluting with 2–4% (v/v) ethyl acetate in *n*-hexane to obtain 4.0 g (yield 85.10%, Rf = 0.4, 10% ethyl acetate in hexane).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm 0.8–0.90 (m, 12H), 1.00–1.5 (m, 25H), 1.77–1.88 (m, 2H), 2.12 (s, 3H, -$SO_2C_6H_5C\underline{H}_3$), 2.13 (s, 3H), 2.18 (s, 3H), 2.22 (s, 3H), 2.59 (s, 3H), 3.82 (t, 2H), 3.92 (t, 2H), 7.3 (d, 2H), 7.8 (d, 2H).

ESI-MS: calculated 628; found [M + NH_4^+] = 646

4.5. Synthesis O-Amino ethyl-[N,O-(hydroxyethyl), N methyl]-α-tocopherol (1D & 1E, Scheme 1)

A mixture of *N*-methyl ethanolamine (0.2 g, 8.18 mmol) and **1C** (1.9 g) taken in 10 mL of toluene in around bottomed flask and is refluxed for 24 h. The reaction mixture was poured into ethyl acetate (100 mL), washed with water (2 × 100 mL), dried over anhydrous sodium sulphate, and filtered. Ethyl acetate is removed from the filtrate on a rotary evaporator. The column chromatographic purification of the resulting residue using 60–120 mesh size silica gel and eluting with 1–2% methanol (v/v) in chloroform afforded 2.4 g (63.15% yield Rf = 0.4, MeOH/$CHCl_3$; 1:19 v/v) of the tertiary amine **1D**. After conformation of **1D** compound and it was dissolved in 7 mL of 1 N Hydrochloric acid in methanol. The reaction mixture was stirred for 12 h and the solvent was evaporated under rota evaporator, then afford pure lipid **Toc-NOH** (**1E**, Scheme 1) appears as light yellow color.

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm 0.8–0.90 (m, 12H), 1.00–1.5 (m, 25H), 1.77–1.88 (m, 2H), 1.95 (s, 3H, –N$C\underline{H}_3$), 2.15 (s, 3H), 2.17 (s, 3H), 2.20 (s, 3H), 2.42 (s, 3H), 3.84 (t, 4H), 4.33 (t, 4H), **ESI-MS**: Calculated 531; found [M^+] 531.

4.6. Amino ethyl-[N,O-(hydroxyethyl), N methyl] benzene sulfonate-α-tocopherol (3A, Scheme 3)

To a solution of *O*-Amino ethyl-[N, *O*-(hydroxyethyl), N methyl]-α-tocopherol (4.0 g, 9.0 mmol) in 10 mL of dry DCM were added p-toluene sulfonyl chloride (2.12 g, 18 mmol), pyridine (1.46 g, 18 mmol), and a catalytic amount of DMAP. The reaction mixture was stirred at room temperature 12 h. The total solvent was evaporated in vacuum to dryness. The residue was dissolved in 25 mL of ethyl acetate and washed twice with 2 × 40 mL of copper sulphate solution to remove any excess pyridine. The organic layer was dried on anhydrous sodium sulphate, the solvent was evaporated, and the sample was purified by column chromatography, eluting with 2–5% (v/v) ethyl acetate in *n*-hexane to obtained 4.0 g (yield 85.10%, Rf = 0.5, Ethyl acetate in hexane; 1:9, v/v) of *O*-tosylation ethyl-α-tocopherol.

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm 0.8–0.90 (m, 12H), 1.00–1.5 (m, 25H), 1.77–1.88 (m, 2H), 2.01 (s, 3H, –N$C\underline{H}_3$), 2.10 (s, 3H), 2.11 (s, 3H), 2.14 (s, 3H), 2. 15 (s, 3H, -$SO_2C_6H_5C\underline{H}_3$), 2.49–2.55 (t, 2H), 3.84 (t, 4H), 4.33 (t, 4H), 7.33 (d, 2H), 7.83 (d, 2H);

ESI-MS: Calculated 686; found [M^+] 686.

4.7. Synthesis of O-Amino ethyl-[N,O-(hydroxyethyl), N methyl] 5, 6 isopropylidine ascorbic acid -α-tocopherol (3B)

To a solution of 5,6 isopropylidine ascorbic acid (4.0 g, 1 mmol) in 10 mL of dry DCM were added triethylamine (1.46 g, 1.1 mmol), and a catalytic amount of DMAP. The reaction mixture was stirred at room temperature for 15 min. The solution *of O*-Amino ethyl-[N, *O*-(hydroxyethyl), N methyl] benzene sulfonate-α-tocopherol (2.12 g, 18 mmol), in dry DCM were added drop wise about 20 min. The reaction mixture is dissolved in 25 mL of ethyl acetate and washed twice with 2 × 30 mL of 1 N HCl solution to remove any excess triethylamine. The organic layer was dried on anhydrous magnesium sulphate, the solvent was evaporated, and the sample was purified by column chromatography, eluting with 5–8% (v/v) ethyl acetate in *n*-hexane to obtain 4.0 g of *O*-Amino ethyl-[*N,O*-(hydroxyethyl), *N* methyl] 5, 6 isopropylidine ascorbic acid -α-tocopherol. (Yield 85.10%, Rf = 0.3, Ethyl acetate in hexane, 1:9 v/v).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm 0.8–0.90 (m, 12H), 1.00–1.5 (m, 25H), 1.77–1.88 (m, 14H), 2.08 (s, 3H, –N$C\underline{H}_3$), 2.14 (s, 3H), 2.17 (s, 3H), 2.18 (s, 3H), 2.56–2.82 (t, 2H), 3.84 (t, 4H), 4.33 (t, 7H), 4.99 (m, 1H); **ESI-MS**; Calculated 730; found [M^+] 730.

4.8. Synthesis of O-Amino ethyl-[N,O-(hydroxyethyl), N methyl] ascorbic acid -α-tocopherol (3C, Scheme 3) (Toc-As lipid)

To a solution of *O*-Amino ethyl-[*N,O*-(hydroxyethyl), *N* methyl] 5, 6 isopropylidine ascorbic acid -α-tocopherol was dissolved in 10 mL of 1 N hydrochloric acid in methanol. The reaction mixture was stirred at room temperature for overnight. The solution was evaporated vacuum to dryness. The residue was dissolved in hexane, then add diehtylether drop wise and kept it for overnight at 0–4 °C to get the white solid. No further purification techniques were performed as the compound decomposes on silica gel. To obtain yield 65.5%, Rf = 0.3 ($CHCl_3$/MeOH; 9:1v/v).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm: 0.8–0.90 (m, 12H), 1.00–1.5 (m, 25H), 1.77–1.88 (m, 2H), 2.08 (s, 3H), 2.14 (s, 3H), 2.17 (s, 3H), 2.18 (s, 3H), 3.6 (t, 2H), 3.66 (t, 2H), 3.79 (2H,t), 3.95 (t, 2H), 4.42–4.48 (m, 2H), 4.38–4.41 (m, 1H),5.13(m, 1H).

^{13}C NMR (400 MHz, $CDCl_3$) δ 165.80, 147.99, 147.59, 127.71, 125.74, 124.57, 123.01, 117.65, 74.87, 73.64, 70.35, 65.13, 63.35, 62.47, 40.06, 39.38, 37.58, 37.47, 37.40, 37.29, 32.80, 32.70, 31.18, 29.72, 28.00, 24.82, 24.45, 23.89, 22.74, 22.65, 21.05, 20.65, 19.76, 19.70, 12.74, 11.88, 11.81. ESI-Mass: Calculated 690, found 690+M^+, **ESI-HRMS**: Calculated; 690 found: 690 [M^+].

4.9. Synthesis of 5,6 isopropylidine ascorbic acid (2A, Scheme 2)

A mixture of l-Ascorbic acid compound 1 g (2 mmol) and acetone (15 mL) was stirred for 15 min. 2,2-Dimethoxy propane (1.25 mL) and catalytic amount of tin chloride was added to the reaction mixture and it was refluxed for 6 h. Cooled the reaction mixture to 5–10 °C and stirred for 45 min at room temperature. The precipitated was filtered, washed with acetone and dried to get the compound **2A**. Yield: 82%; M.P: 206 °C. TLC: 100 DCM: 20 EA: 10 ethanol: 1 Acetic acid.

4.10. Synthesis of N, N-di tetradecyl ethanolamine (2B, Scheme 2)

A mixture of ethanolamine (6.6 mmol) and tetradecyl bromide (0.066 mmol) in 1:1 ratio in 20 mL methanol–acetonitrile solvent mixture (30:70) was refluxed for 48 h on a water bath. The solvent was evaporated on a water bath followed by cooling up to 5 °C to obtain a milky white mass. It was washed with ether several times to remove any trace of the tetradecyl bromide and recrystallized from methanol/ethyl acetate mixed solvent to obtain a white solid product. (Yield; 90%).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 0.863–0.88 (s, 6H), 1.31 (m, 40H), 2.2 (m, 2H), 2.62 (m, 4H), 2.88(t, 2H), 2.91 (t, 2H) **ESI-MS**: Calculated: 453 found [453 + M^+].

4.11. Synthesis of N, N-di tetradecyl 2-chloro ethyl ammonium (2C, Scheme 2)

To a stirred solution of *N, N*-di tetradecyl ethanolamine 0.1 g (1 mmol) in chloroform 100 mL a solution of thionyl chloride in chloroform 10 mL was added drop wise at −5 °C. After removing an ice bath the reaction mixture was allowed to reach the room temperature and refluxed for 150 min. After cooling methanol 20 mL was added drop wise and solution was stirred for 20 min. Then the solvents were evaporated in vacuum and the product was treated with 20 mL of methanol and evaporated to dryness again. The crude product was recrystallized from ethyl acetate (Yield: 92%).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 0.863–0.88 (s, 6H), 1.31 (m, 40H), 2.2 (m, 2H), 2.62 (m, 4H), 2.88(t, 2H), 2.91 (t, 2H) **ESI-MS**: Calculated; 458 found: 460 + [M + 2].

4.12. Synthesis of N, N-di tetradecyl 2-(5, 6 isopropylidine ascorbic acid) ethyl ammonium (2D, Scheme 2)

5,6-isopropylidene ascorbic acid 0.05 mg (0.1 mmol) was dissolved in 15 mL of dry DMSO followed by addition of dry K_2CO_3 (0.06 mmol) and *N, N*-di tetradecyl 2-chloro ethyl ammonium (0.1 mmol) at the room temperature. The reaction mixture was stirred for 1 h and refluxed to 90 °C for 16 h. After cool to the room temperature the reaction mixture was diluted with 100 mL of DCM. The dilution solution was washed with 50 mL of 10% aqueous sodium chloride solution three times and then dried over anhydrous sodium chloride, followed by filtration. The filtrate was concentrated under reduced pressure and purified on column chromatography and followed by recrystallized from ether and hexane. Rf: 0.2 (MeOH/DCM: 1:19 v/v).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 0.863–0.88 (s, 6H), 1.31 (m, 40H), 2.2 (m, 2H), 2.62 (m, 4H), 2.88(t, 2H), 2.91 (t, 2H), 3.40 (d, 1H), 3.50 (m, 2H), 4.18 (m, 1H) **ESI-HRMS**: Calculated; 652 found: 652 + [M + 1].

4.13. Synthesis of N, N-di tetradecyl 2-(ascorbic acid) ethyl ammonium (2E, Scheme 2)

1 g of the *N,N*-di tetradecyl-2-(5,6-isopropylidine ascorbic acid) ethyl ammonium was dissolved in 10 mL of methanol, to which 3 mL of 2 N hydrochloric acid solution was added. The mixture was refluxed for 2 h and concentrated under reduced pressure to give an adhesive liquid product. Ethanol was added to the product, which was then concentrated to give crude crystalline *N,N*-di tetradecyl 2-(ascorbic acid) ethyl ammonium, it was recrystallized in ethyl acetate/ethanol (8:2) to give of white crystalline solid (yield: 75.3%).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 0.863–0.88 (s, 6H), 1.31 (m, 40H), 2.18 (m, 2H), 2.42 (m, 4H), 2.88 (t, 2H), 2.91 (t, 2H), 3.40 (d, 1H), 3.50 (m, 2H), 4.18 (m, 1H). **ESI-HRMS**: Calculated; 611 found: 611 [M + 1].

4.14. Antioxidant assay

The inherent antioxidant activity of the **Toc-As**, **N14-As** and **Toc-NOH** lipids were carried out by means of the scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals. The ability of **Toc-As**, **N14-As** and **Toc-NOH** to scavenge radicals was evaluated by incubating different concentrations of the **Toc-As**, **N14-As** and **Toc-NOH** with DPPH (100 μM) radicals in aqueous ethanol for different periods of time and the amount of unscavenged radicals was calculated by measuring the intensity at 490 nm using a microplate reader (Infinite 200 PRO, TECAN). Ascorbic acid (AA, vitamin C) was used as a positive control and untreated DPPH was used as a negative control to measure the antioxidant efficiencies of the **Toc-As**, **N14-As** and **Toc-NOH**. The antioxidant efficiencies of the **Toc-As**, **N14-As** and **Toc-NOH** are expressed in terms of the percentage of free-radical scavenging activity as a function of concentration and incubation time according to Eq. (1)

$$\%Scavenger\ activity = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100 \quad (1)$$

4.15. Preparation of liposomes and plasmid DNA

The cationic lipids and the co-lipid DOPE (1 mmol each) were dissolved in chloroform in a glass vial, and a thin film of lipids was made on the wall of the glass tubes while evaporating the total solvent with a thin flow of moisture free nitrogen gas and dried under high vacuum for 2 h, then 1 mL of sterile deionized water was added to the vacuum-dried lipid film and the mixture was allowed to swell overnight. The vial was then vortexed for 4 min at room temperature to get a transparent or translucent solution and sonicated in a bath sonicator and probe sonicator respectively to produce small unilamellar vesicles (SUVs) from multilamellar vesicles (MLVs). The resulting clear aqueous liposomes were used to form liposome-DNA complexes. eGFP plasmid was amplified in DH5α-strain of *Escherichia coli*.

4.16. Lipoplex preparation and DNA binding assay

Positively charged liposomes were complexed with negatively charged DNA to form lipid-DNA complexes. 1:1–8:1 lipid charge ratios were used. 1% agarose gel (pre-stained with ethidium bromide) was used to elucidate the DNA binding capability of liposomes with Lipid: DNA charge ratios from 1:1 to 8:1.plasmid DNA (0.3 μg) was complexed with the varying amount of cationic liposomes in that order (from 0.9 μL to 7.2 μL) in a total volume of 30 μL in HEPES buffer (pH 7.4) and incubated at room temperature for 20–25 min. 4 μL of 6x loading buffer (0.25% Bromophenol blue in 40% (w/v) sucrose with sterile H_2O) was added to it and from the resulting solution 30 μL was loaded on each well. The samples were electrophoresed at 80 V for 45 min and the agarose

gel images for DNA bands were visualized using a Bio-Rad Gel Doc XR + imaging system (Bio-Rad, Hercules, CA, USA).

4.17. Heparin displacement assay

Heparin was used to study the anionic dislocation of DNA within the lipoplexes. The lipid: pDNA complexes were prepared as described in the above section (DNA binding assay) and incubated for 20 min. Following the incubation, 0.1 μg of sodium salt of heparin was added and incubated for another 30 min. The samples were electrophoresed in an agarose gel (1.5%) for heparin displacement analysis and DNA bands were visualized as mentioned in the above section.

4.18. Zeta potential (ξ) and size measurements

The sizes and the surface charges (zeta potentials) of liposomes and lipoplexes with varying charge ratios (8:1–1:1) were measured by photon correlation spectroscopy and electrophoretic mobility on a Zetasizer 3000HSA (Malvern, U.K.). The sizes were measured in DMEM media with a sample refractive index of 1.59 and a viscosity of 0.89. The system was calibrated by using the 2005 nm polystyrene polymer (Duke Scientific Corps., Palo Alto, CA, U.S.). The diameters of liposomes and lipoplexes were calculated by using the automatic method. The zeta potential was also measured using the following parameters: viscosity, 0.89 c P; dielectric constant, 79; temperature, 25 °C; F (Ka), 1.50 (Smoluchowski); maximum voltage of the current, V. The system was calibrated by using DTS0050 standard from Malvern. Measurements were done 10 times with the zero-field correction. All the liposomes and lipoplexes of the size measurements were done 10 times in triplicate with the zero field correction and values represented as the average of triplicate measurements. The potentials were measured 10 times and represented as their average values as calculated by using the Smoluchowski approximation.

4.19. Cytotoxicity assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide) based reduction cytotoxicity assays of cationic lipids **Toc-As**, **N14-As** and **Toc-NOH** were carried out in CHO, Neuro-2a, HEK-293T, and HepG2 cells across the lipid: DNA charge ratios of 1:1–8:1in 96-wells plate. Briefly, 24 h after the adding of lipoplexes, MTT (0.5 mg/ml in DMEM) was added to cells and incubated for 4 h at 37 °C. Results were expressed as percent viability = [A540 (treated cells)-background/A540 (untreated cells)-background] × 100.

4.20. Transfection biology

Eukaryotic cells were seeded at a density of 10,000 (for CHO) and Neuro-2a, HEK-293T, and HepG2) respectively per well in a 96-well plate, 18–24 h before the transfection. Then 0.3 μg (0.91 nmol) of plasmid DNA was complexed with different concentrations of cationic lipids i.e. 1:1 to 8:1 in DMEM medium (total volume made up to 100 μL) for 30 min. Just prior to transfection, cells plated in the 96-well plate were washed twice with PBS (100 μL) followed by the addition of lipid-DNA complexes. After 4 h of incubation, 100 μL of DMEM with 20% FBS was added to the cells. The medium was changed to 10% complete medium after 24 h, and the reporter gene activity was estimated after 48 h. The cells were washed twice with PBS (100 μL each) and lysed in 50 μL lysis buffer [0.25 M Tris-HCl (pH 8.0) and 0.5% NP40]. The β-galactosidase activity per well was estimated by adding 50 μL of 2 substrate solution [1.33 mg/ml ONPG, 0.2 M sodium phosphate (pH 7.3), and 2 mM magnesium chloride] to the lysate. Absorbance of the product ortho-nitrophenol at 405 nm was converted to β-galactosidase units by using a calibration curve constructed using pure commercial β-galactosidase enzyme. Each transfection experiment was repeated 3 times on 3 different days. The transfection values were noted as an average of three replicate transfection plates, performed on three different days. The values of β-galactosidase units in replicate plates assayed on the same day varied by less than 20%.

4.21. Cellular eGFP expression study

For cellular α5GFP expression experiments in HEK-293T& HepG2 50,000 cells were cultured in each well of 24-wells plate 18–24 h before the transfection. Then 0.9 μg of eGFP plasmid DNA encoding green fluorescent protein was complexed with liposomes of lipids **Toc-As**, **N14-As** and **Toc-NOH** at charge ratio (lipid/DNA) 2:1 in DMEM medium (total volume made up to 100 μL) for 30 min. Just prior to transfection, cells plated in the 24-wells plate were washed with PBS (2 × 100 μL) followed by addition of lipoplexes. The media 400 μL was added after 4 h incubation of the cells. After 24 h, the complete medium was removed from each well, and the total cells were washed with PBS (2 × 200 μL). Finally 200 μL of PBS was added to each per well cells and visualized under the epifluoroscent microscope to observe the cells expressing the green fluorescent protein.

4.22. Circular dichroism

Circular dichroism spectra were recorded for naked plasmid and lipid/pDNA complexes in 5% dextrose solution. Since dextrose is a chiral molecule, CD spectra of dextrose alone was subtracted from that of pDNA in dextrose solution to obtain the profile for pDNA. Similarly, the spectra of complexes were recorded with background normalization to the profiles of corresponding liposomes alone in dextrose solution. Samples were scanned through a wavelength range of 200–500 nm at 25 °C using a 0.5 mL quartz cuvette, with a path length of 1 cm. Each sample was scanned four times with an integration time of 5 s, and the values were averaged. Scanning was done at 1 nm steps, and the slit width was 1 nm. Liposome-pDNA complexes were prepared at 4:1 ± charge ratio at 50 μg/mL pDNA concentration.

4.23. Transfection biology in presence of serum

Cells were cultured at a density of 15,000 cells (HEK-293T and CHO) per well in a 96-well plate, 18–24 h before the transfection. Then 0.3 μg (0.91 nmol) of eGFP was complexed with lipids (**Toc-As**, **N14-As** and **Toc-NOH**) in DMEM medium in the presence of increasing concentrations of added serum (10–30% v/v and total volume made up to 100 μL) for 30 min. The charge ratios of lipid/eGFP plasmid was maintained as 4:1, at which all the three lipids exhibited their highest transfection ability in two types of cells used for transfection viz. HEK-293T and CHO. The experimental procedure and determination of eGFP activity per well are similar to that reported for the *in vitro* transfection experiments.

4.24. Cellular uptake examine by epifluorescence microscopy

Cells were cultured at a density of 10000 cells/ well in a 96-well plate usually 16–24 h earlier to the treatment in 200 μL of growth medium such that the well became 30–50% confluent at the time of transfection. Double helix DNA (0.3 μg of pDNA diluted to 50 μL with serum-free DMEM media) was complexed with rhodamine-PE labeled cationic liposomes (diluted to 50 μL with DMEM) of lipids **Toc-As**, **N14-As** and **Toc-NOH** using4:1 lipid to DNA charge ratio. The cells were washed with PBS (1 × 200 μL), then treated with lipoplexes, and incubated at a humidified atmosphere con-

taining 5% CO2 at 37 °C. After 4 h of incubation, the cells were washed with PBS (3 × 200 μL) to remove the dye and fixed with 3.8% paraformaldehyde in PBS at room temperature for 10 min. The red fluorescent cells were detected under an epifluorescence microscope (Nikon, Japan).

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