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ARTICLE TYPE

New Dimeric Lipids Derived from α-Tocopherol as Efficient Gene Transfection Agents. Mechanistic Insights into Lipoplex Internalization and Therapeutic Induction of Apoptotic Activity

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In this report, we present cationic dimeric (gemini) lipids for significant plasmid DNA (*p*DNA) delivery to different cell lines without any marked toxicity in the presence of serum. Six gemini lipids based on α-tocopherol were synthesized which differ in their spacer chain lengths. Each of these gemini lipids mixed with a helper lipid 1, 2-dioleoyl phosphatidyl ethanolamine (DOPE), was capable of forming stable aqueous suspensions. These co-liposomal systems were examined for their potential to transfect pEGFP-C3 plasmid DNA in to nine cell lines of different origins. The transfection efficacies noticed in terms of EGFP expression levels using flow cytometry were well corroborated using independent fluorescence microscopy studies. Significant EGFP expression levels were reported using the gemini co-liposomes which counted significantly better than one well known commercial formulation lipofectamine 2000 (L2K). Transfection efficacies were also analyzed in terms of the degree of intracellular delivery of labeled plasmid DNA (*p*DNA) using confocal microscopy which revealed an efficient internalization in the presence of serum. The cell viability assays performed using optimized formulations demonstrated no significant toxicity towards any of the cell lines used in the study. We also had a look at the lipoplex internalization pathway to profile the uptake characteristics. A caveolae/lipid raft route was attributed to their excellent gene transfection capabilities. The study was further advanced by using a therapeutic p53-EGFP-C3 plasmid and the apoptotic activity was observed using FACS and growth inhibition assay.

Introduction

Gene therapy has opened up new frontiers to deal with several challenging tasks pertaining to the current medicinal practices.[1,2] Unlike viral vectors, the non-viral carriers for the accomplishment of gene therapy have increasingly been updated, coming up with relevant solutions to key issues in therapy.[3-5] Firm establishment of a successful platform still needs further honing to achieve desirable outcome.[6,7] Since the introduction of liposomal vectors as non-viral carriers for gene delivery, considerable attention has been paid towards the optimization of vectors to achieve therapeutic goals.[8,9] Cationic lipids, being a safe alternative are advantageous due to the ease of their synthesis, storage, excellent capability to bind nucleic acids and regulation of transfection levels while avoiding any adverse immunogenic reactions.[10-12] A cationic lipid is typically made up of the polar head group(s), hydrophobic backbone and a linker that connects the two segments of opposing polarity.[13] Numerous efforts have been made for the modification of molecular structural constitution of cationic lipids to attain the desirable activity.[14,15] The variations and modifications in hydrophobic backbone, head group and linker have been reported to be fruitful endeavors for the improved efficacies.[16-25] Some of the efficient cationic lipid formulations developed over the years include 3β-[*N*,(*N*,*N*-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) with diether lipids, *N*-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*-trimethyl ammonium chloride (DOTMA), or N′,N′-dioctadecyl-N-4,8-diaza-10-aminodecanoylglycine amide (DODAG) *etc.* in combination with the helper lipid DOPE are considered to be among the successful outcomes.[26-28] These types of cationic lipids have enabled exercise of structure-activity relation (SAR) studies towards dealing with different segments of cationic lipids for the improvisation leading to desirable goals.[16,29] Recent developments in this area also contain a prominent class of cationic lipids, *i.e. gemini* lipids, reported to be efficient cytofectins from various laboratories including us.[30-46] The typical lineament of these gemini lipids is generally represented having two polar headgroups linked by a flexible or rigid spacer and two hydrophobic segments. Towards developing synthetic cationic lipid formulations for elevating efficacy levels with no encircling limitations, we report here a new class of gemini lipids while exploiting a biologically active α-tocopherol based hydrophobic backbone. Tocopherol has earlier been employed while being chemically conjugated to ribonucleic acids and polymers for effective RNAi action.[47,48] Tocopherol, in particular as a lipid backbone, in the form of monomeric cationic lipids with unmodified ammonium head groups has not been able to induce gene transfection at the desirable efficacy levels.[49]

Here we demonstrate the usefulness of cationic gemini lipids based on tocopherol backbone as highly potent gene carriers in combination with helper lipid, DOPE in the presence of serum without any obvious cytotoxicity. We synthesized six new gemini lipids differing in the spacer chain length and used each of them to deliver *p*DNA (pEGFP-C3 plasmid DNA) in nine different cell lines of diverse origins in the presence of serum (FBS). The EGFP expression profile revealed high transfection capability of the dimeric co-liposome derived lipoplexes significantly better than a commercial formulation, lipofectamine 2000 (L2K) in all the cell lines. Significant intracellular entry of fluorescein labeled *p*DNA was also evidenced using these gemini co-liposomes. The optimized co-liposomal formulations were well characterized using a number of physical methods e.g. dynamic light scattering (DLS), zeta potential and atomic force microscopy (AFM) measurements. Gene transfection capability of these cationic tocopheryl gemini co-liposomes was further bolstered using an efficient p53 expression plasmid fused with GFP gene (p53-EGFP-C3) recently reported from our laboratory[35] and the apoptotic activities were investigated.[35,50] The apoptosis induced by such transfection due to the expression of p53 in cell lines, H1299 ($p53^{-/-}$) and HEK 293T ($p53^{+/+}$) of varied p53 status was examined using annexin-V-PI dual staining assay and the effect on cellular proliferation was also assessed. Overall, in the present report we come up with gemini lipids bearing α-tocopherol as hydrophobic backbone as excellent cytofectins which could have significant potential to heave the liposomal gene delivery platform to higher levels.

Results and discussion

Synthesis

The dimeric lipids with different lengths of spacers were prepared as described.[51] Briefly, an appropriate α,ω-dibromoalkane (1 eq.) was reacted with *O*-aminoethyl [*N*, *N*-dimethyl]-α-tocopherol in a solvent mixture of EtOAc-MeOH (1:1) upon heating in a closed pressure tube for about two weeks until the TLC revealed the complete disappearance of the dibromide. The resultant crude solid was then repeatedly precipitated using a mixture of $CHCl_3$ and EtOAc to obtain pure dimeric lipids (Scheme 1, ESI†), acronymed as 'TnT' where 'T' stands for the tocopherol and 'n' stands for the length of the polymethylene spacer (Fig. 1). The spectroscopic and analytical data of the each dimeric lipid were consistent with its given structure, cf. Supporting Information (ESI†).

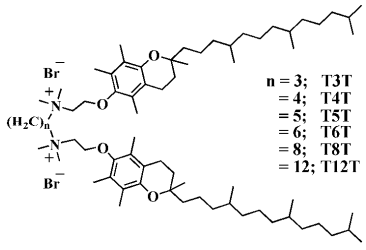

Fig. 1 Molecular structure of tocopheryl gemini lipids.

Gel Electrophoresis

We characterized the *p*DNA binding ability of transfection optimized co-liposomal formulations (DOPE/gemini lipid, 4:1) of different gemini lipids by running the lipoplexes on 1% agarose gel electrophoretically at five different N/P ratios (0.25:1-1.25:1, Lipid/DNA). Gemini co-liposomes could efficiently bind the *p*DNA molecules at an N/P ratio of 0.5:1 but complete retardation was observed at an N/P ratio of 0.75:1 except for T5T co-liposomes which showed complete retardation at N/P ratio of 0.5:1 itself (Fig. 2). This result revealed that co-liposomes derived from each of the gemini lipids could easily condense *p*DNA molecules leading to lipoplex formation.

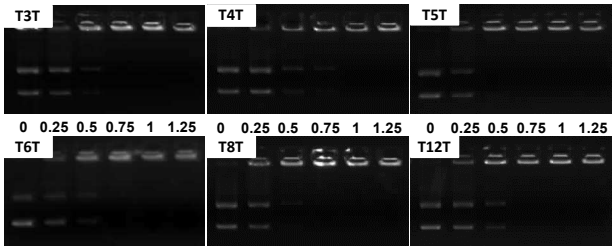

Fig. 2 Gel electrophoretic patterns depicting *p*DNA binding ability of different gemini co-liposomes. Lipoplexes were prepared at five N/P ratios (Lipid/*p*DNA) using 200 ng of *p*DNA and run on 1% agarose gel for about 30 min. Numbers between the gel panels show N/P ratios used.

Particle Size and Zeta Potential Measurements

The observations made under DLS experiments revealed that the transfection optimized co-liposomes in all the gemini lipid formulations were in the ~120-150 nm range. Complexation with *p*DNA led to an increase in size where the lipoplexes generated following optimized transfection N/P ratios ranged from ~250 to 300 nm in size (Fig. 3B). On the other hand, the zeta potential values well corroborated with the observations of the gel retardation assay. Positive zeta potentials were noticed at N/P ratio of 0.75:1 for all the gemini formulations. Lipoplexes of optimal transfection conditions possessed zeta potential values of ~30 mV (Fig. 3A).

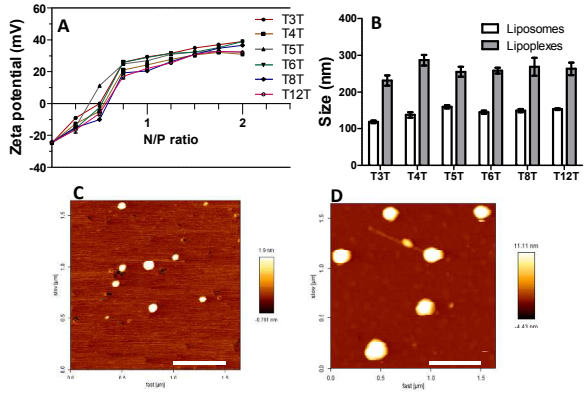

Fig. 3 Zeta potentials (A) of complexes of different gemini co-liposomal formulations with *p*DNA at different N/P ratios and particle sizes (B) of the transfection efficient lipoplexes in comparison with respective liposomes. Representative AFM images of co-liposomes (C) and lipoplexes (D) derived from T3T gemini co-liposomes. Scale bar = 0.5μm.

Atomic Force Microscopy (AFM) Analysis

We also investigated the size and morphology of the transfection optimized co-liposomes derived from the gemini lipids and their complexes with *p*DNA at optimized ratios using AFM.[52] The AFM revealed that the co-liposomes derived from any of the gemini lipid were small vesicles of ~120-160 nm in size. However, the complexes with *p*DNA were relatively larger vesicles of 230-290 nm in size (Fig. 3C and 3D). These observations affirmed the size measurements made under DLS studies. Therefore, the co-liposomes could efficiently condense the *p*DNA and gave rise to the lipoplexes of relatively smaller sized nicely packed vesicles.

Gene Transfection Studies

The evaluation of gene transfection capability of these tocopherol based dimeric cationic co-liposomes was performed through an analysis of EGFP expression produced by means of pEGFP-C3 *p*DNA transfection. The data obtained from flow cytometry was used to generate EGFP expression profile in terms of the geometric means of fluorescence intensity (MFI) of enhanced green fluorescence protein (EGFP) expression in transfected cells.[31] All the transfection experiments were conducted in 10% serum conditions as serum is known to often disrupt the gene transfection capabilities of lipoplexes derived from cationic liposomes.[53] Transfection experiments were first conducted in Caco-2 cells at a fixed N/P (cationic lipid/DNA) ratio of 0.5 to optimize the best helper lipid concentration which turned out to be 4:1 (molar ratio, DOPE/gemini lipid) almost consistently for all the gemini lipids (Fig. S1, ESI†).

Using the optimized DOPE based formulations, transfection experiments were followed at four different N/P ratios (0.25:1, 0.5:1, 0.75:1 and 1:1) to get an insight of maximum gene expression levels. All the gemini co-liposomes (T3T, T4T, T5T, T6T, T8T and T12T) could transfect Caco-2 cells efficiently (Fig. S2, ESI†). For gemini co-liposomal formulations T3T, T5T and T8T, the EGFP expression levels were even better than the commercially available transfection reagent Lipofectamine 2000 (L2K) (**P* <0.05) while T4T and T6T remained comparable to it. It was only T12T which appeared to exhibit relatively lower expression levels than that of L2K in Caco-2 cells. We moved ahead and also looked at the gene transfection capability of optimized formulations in eight more cell lines namely HeLa (cervical carcinoma), U251 (glioma), A549 (lung adenocarcinoma), HepG2 (hepatocellular carcinoma), COS-7 (monkey kidney fibroblast like cells), CHO (chinese hamster ovary), HEK 293T (human embryonic kidney, transformed), BT-474 (human breast carcinoma). The gene expression results obtained in these cell lines almost corroborated the pattern of gene transfection capabilities of the gemini co-liposomes as observed in Caco-2 cells (Fig. 4).

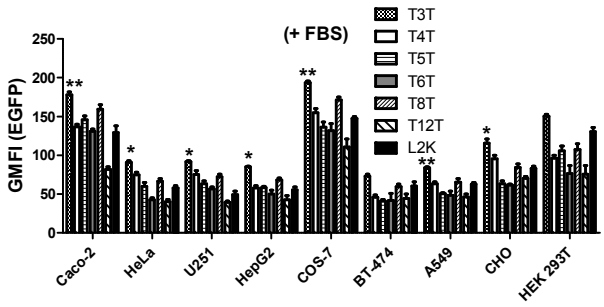

Fig. 4 Maximum EGFP expression obtained from transfections performed by different gemini co-liposomal formulations at optimized N/P ratios in the presence of serum in numerous cell lines. The *p*DNA (pEGFP-C3, 0.8 µg) was used in transfections and expression levels were analyzed 48 h post transfection. EGFP expression levels of most efficient co-liposomes (T3T) were analyzed for statistical significance in comparison with L2K (**P* <0.05 and ***P* <0.01, Two-tailed Student's t-test).

Dimeric co-liposomes derived from T3T and T8T turned out to be the best cytofectins, better than L2K while others were comparable to that of L2K. Representative flow cytometry histograms that depict the difference in EGFP expression levels between the transfections of most efficient formulation, T3T and L2K are also shown in two different cell lines (Caco-2 and COS-7 cells) in Fig. S3, ESI†. The visual analysis of the EGFP expression was also made under fluorescence microscopy using optimized transfection conditions in all the cell lines studied. EGFP expressions corroborated well with the observations made in flow cytometry (Fig. 5). The gemini co-liposomes presented here are capable enough to transfect cells belonging to different origins in the presence of serum. Thus, these cationic geminis could be exploited as promising gene carriers.

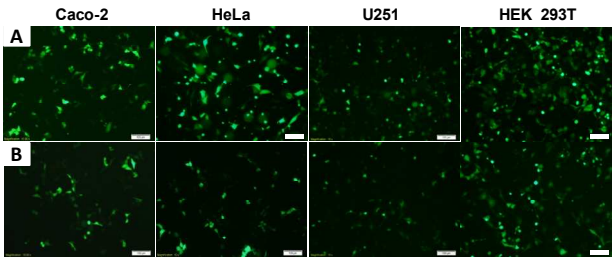

Fig. 5 Representative fluorescence microscopic images depicting EGFP expression (48 h post transfection) due to pEGFP-C3 (0.8 µg) transfections performed by T3T gemini co-liposomes (panel A, left to right) and L2K as a positive control (panel B, left to right) in the presence of serum in different cell lines. Sacle bar = 100 µm.

We also looked into the differences in relative transfection activity of different dimeric lipids by means of circular dichroism (CD) measurements where the lipid-DNA interaction could be probed. Interestingly, the formulations T3T and T8T perturbed the plasmid DNA structure (presumably due to the compaction of *p*DNA by the respective dimeric lipid) more than other counterpart dimeric formulations (Fig. S4, ESI†). This was consistent with reported studies with other gemini surfactants.[44] Such altered mode of compaction of pDNA by T3T and T8T may be responsible for their relatively higher transfection activity, though it needs to be further corroborated by other physical

methods to provide a comprehensive model of the role of spacer of the present class of dimeric lipids in gene transfection activity.

Cellular uptake of labeled *p*DNA

To get an insight in to the gene transfection levels at the platform of *p*DNA intracellular delivery, transfection experiments were conducted using a fluorescein labeled *p*DNA. After 6 h of T3T co-liposome based lipoplex (N/P, 0.75) incubation with cells, a visual analysis was made using confocal microscopy and the quantification of *p*DNA transfection was performed with the help of FACS analysis. The *p*DNA internalization based on confocal microscopy analysis appeared to be more pronounced for the cells transfected with optimized formulation (T3T) than L2K mediated cellular transfections (Fig. 6).

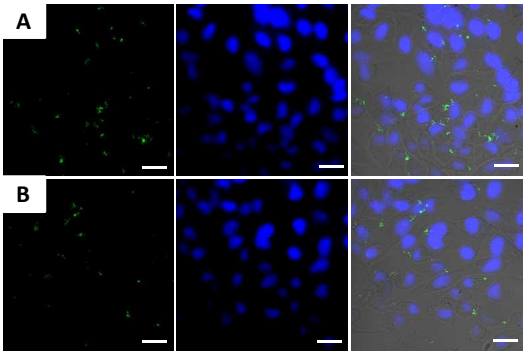

Fig. 6 Representative confocal microscopy images depicting labeled *p*DNA (1 μg) internalization in HeLa cells after 6 h of lipoplex (panel A, T3T lipoplex and panel B, L2K lipoplex) incubation in the presence of serum. Panel A and B represent (left to right) *p*DNA fluorescence (green), DAPI nuclear stain (blue) and merge of previous two images along with brightfield. Scale bar = 20 μm.

This observation was further substantiated when the transfected HeLa and Caco-2 cells were analyzed using flow cytometry where significantly higher number of the *p*DNA fluorescence positive cells were detected with the T3T co-liposome mediated transfections than L2K. Representative flow cytometry histograms and quantification for labeled *p*DNA transfection by T3T co-liposomes in comparison with L2K are shown in Fig. 7A and 7B respectively. Thus, the efficient internalization of *p*DNA mediated by the gemini co-liposomes could be marked for their excellent gene transfection capabilities.

Cytotoxicity analysis

The relevance of efficient synthetic gene carriers is directly liable to cell viability measures.[38,54] Thus the practical application of these delivery vehicles lies in being congenial to the biological system. We checked out the cell viabilities in response to cellular transfections mediated by efficient formulations in all the cell lines used for gene expression studies using MTT based assay.[31]

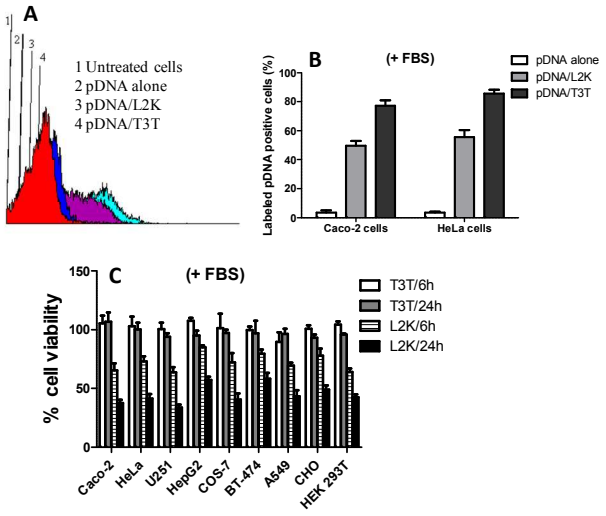

Fig. 7 Representative flow cytometry histograms (A) and quantification (B) for cellular uptake of labeled *p*DNA transfected by T3T gemini co-liposomal formulation while using L2K as a positive control. (C) Cell viability assessment (MTT assay) made 48 h post transfection initiation after two different lipoplex incubation periods (6h and 24h) following actual transfection conditions for the most efficient formulation (T3T) and L2K as a positive control.

Two different time points (6h and 24h) of lipoplex incubations with cells were selected and cell viabilities were measured at 48 h from the initiation of the transfection period. Interstingly, no loss in cell viabilities was noticed for the cell lines transfected with efficient gemini co-liposomes during both the time periods of incubation. On the other hand, L2K mediated transfections led to substantial losses in cell viabilities for both the incubation periods (Fig. 7C). Taken together the cell viability and gene expression profile, it may be concluded that these co-liposomes are indeed promising candidates for practical use.[54]

Cellular uptake pathway of lipoplexes

As the transfection efficiency of the non-viral gene delivery vectors seems to be linked to their internalization (endocytosis) pathway, we have attempted to get an insight in to the pathway that was involved in trafficking of lipoplexes derived from these cationic dimeric co-liposomes using flow cytometry. We studied the uptake of lipoplexes in three different cell lines used in transfection experiments while applying different endocytosis inhibitors, amiloride (macropinocytosis), chlorpromazine (clathrin pathway inhibitor), genistein (caveolae pathway inhibitor).[55-57] We also used methyl-beta-cyclodextrin (m-β-CD) to look at the sensitivity of lipoplex uptake towards the cholesterol content in cell membranes. In a typical experiment, first lipoplex (T3T) uptake was checked out in COS-7 cell line using fluorescein labeled *p*DNA in the presence of different inhibitors. Amiloride and chlorpromazine did not show any reduction in uptake of lipoplexes. Cells pretreated with genistein and m-β-CD showed a significant reduction (***P* <0.01 and ****P* <0.001) in lipoplex uptake while latter being more prominent (Fig. 8A). Subsequently, the next experiment was conducted to have a look at the EGFP expression levels in the presence of the above mentioned inhibitors. Interestingly, the gene expression

profile followed the same pattern where no EGFP reduction was observed in amiloride and chlorpromazine treated cells while pre-treatment with genistein and m-β-CD almost completely abolished (***P <0.001) the gene expression (Fig. 8B). Since the cholesterol depletion from the cell membranes using m-β-CD is known to perturb both clathrin and caveolae routed internalizations therefore the distinction was elicited using specific inhibitors of clathrin and caveolae routes. The caveolae route (genistein pretreatment) only mediated the transfer of the lipoplexes while clathrin and macropinocytosis being totally ineffective. We studied the lipoplex uptake and subsequent gene expression in two more cells line (HeLa and A549) where interestingly similar observations were noticed (Fig. 8).

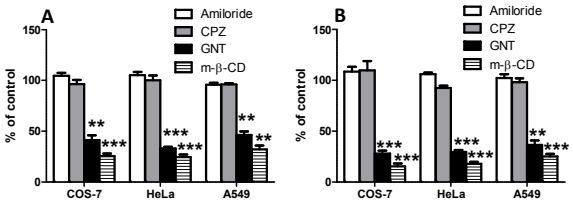

Fig. 8 Effect of different inhibitors on (A) cellular internalization (labeled *p*DNA) and (B) transfection (EGFP expression) of T3T co-liposomes derived lipoplexes in three different cell lines. The data set for inhibitor untreated cells was set as 100%. Inhibitor concentrations were as follows, chlorpromazine (CPZ, 10 μg/ml), genistein (GNT, 50 μg/ml), methyl-β-cyclodextrin (m-β-CD, 10 mg/ml) and amiloride (200 μM). The transfection efficacies were analyzed for statistical significance in comparison with untreated controls (***P <0.001 and **P <0.01, Two-tailed Student's t-test).

Taking together all the observations, it may be concluded that the caveolae/lipid-raft pathway is involved in the gene expression mediated by these cationic dimeric co-liposomes.[55,57-59] It may be further emphasized here that this pathway which does not depend on the release of nucleic acids from the acidic endosomal compartments as in clathrin pathway could be attributed to the excellent gene transfection capabilities of these formulations.

Delivery of p53 gene

Studies were further advanced by carrying out experiments for investigating the anti-proliferative activity using a p53-EGFP-C3 fusion construct, recently reported from our group for inducing p53 mediated anti-tumor activity using a cationic cholesteryl gemini lipid.[35] Loss of normal p53 activity is quite a prevalent phenomenon in human cancer and this fact has reinforced the recent scientific efforts towards p53 gene delivery in cancer therapy. Accordingly, the p53-EGFP-C3 induced apoptosis and the anti-proliferative activity was checked in two cell lines of differentiated p53 status namely H1299 ($p53^{-/-}$) and HEK 293T ($p53^{+/+}$) after transfection using the best co-liposomes (T3T) and L2K as control. The apoptotic population in transfected cells was measured using annexin-V-Alexa Fluor-PI dual staining assay under flow cytometry. T3T co-liposome mediated transfections showed a significantly higher percentage of apoptotic cells (~80%) which was much higher than L2K (~40%). Seemingly, this could be attributed to the excellent gene transfection capability of the T3T co-liposomes (Fig. 9A-9C).

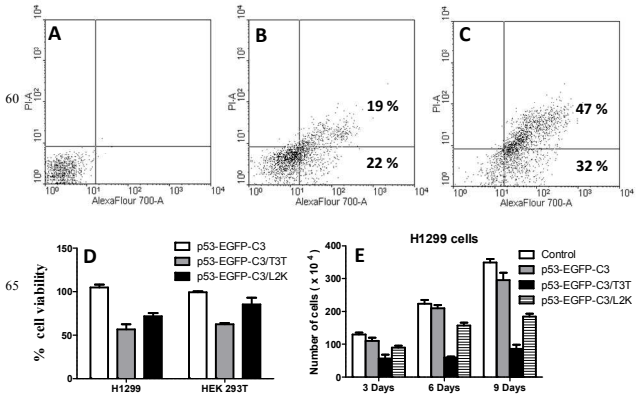

Fig. 9 Representative flow cytometry dot plots for apoptosis assay of untreated (A), L2K (B) and T3T (C) transfected H1299 cells. Cell viability assay (D) 48 h post transfection of HEK 293T ($p53^{+/+}$) and H1299 ($p53^{-/-}$) cells and (E) the effect of p53 expression on growth of H1299 cells. Transfections were performed using optimized conditions involving L2K as a positive control.

The cytotoxicity due to the p53 expression was measured in both cell lines using MTT assay and a growth assay was also performed to determine growth inhibition in H1299 cells.[35,50] The p53 transfections mediated by T3T co-liposomal formulation led to a substantial loss in cell viability in H1299 cells (~ 50%) and HEK 293T cells (~40%). On the other hand L2K transfected H1299 cells showed <30% cell viability loss while it was only ~10% in HEK 293T cells (Fig. 9D). The growth inhibition assay carried out in H1299 cells, also revealed similar effects where a significant inhibition in growth rate was seen in T3T co-liposome mediated transfections in comparison with untreated cells and p53-EGFP-C3 alone transfections. The growth inhibition rate again was more pronounced with T3T co-liposome transfected cells than that by L2K (Fig. 9E). These results suggest that the p53 expression induced apoptosis in cells and the anti-proliferative activity was a measure of the transfection status of cells which was well performed by the T3T co-liposomes.

Conclusions

Numerous efforts have been made so far towards the improvisation of cationic liposomal carriers. Yet the need to meet the challenges in gene therapy provokes us to come up with newer lipids that are efficient and have no encircling limitations. In the panorama, we report herein the elicitation of excellent gene transfer capabilities from the new cationic dimeric lipids based on tocopherol. All the six dimeric co-liposomal formulations are shown to be efficient enough to condense *p*DNA and deliver significantly into numerous cell lines belonging to different origins. The co-liposomes are of ~120-160 nm in size and optimized transfection efficient lipoplexes are in the 230-290 nm size range and with optimal positive zeta potential values. High EGFP expression levels are reported for transfections performed in the presence of serum which is significantly better than the commercial formulation lipofectamine 2000. The preliminary flow cytometry data were also well substantiated using fluorescence microscopy for visual EGFP expression analysis and fluorescein labeled *p*DNA cellular internalization by means of

confocal microscopy. All the co-liposomes derived from the present set of dimeric lipids and their complexes with *p*DNA molecules do not seem to impart any obvious cytotoxicity. The route (caveolae/lipid-raft) taken up by the lipoplexes for cellular entry could be suggested to be the reason for their excellent capability of gene transfection because this route, unlike the clathrin dependent pathway, does not involve the cellular endosomal compartments. The transfection efficient formulation could easily transfect p53 expression plasmid in to cells of varied p53 status and characteristic activities (apoptosis) were noticed due to p53 expression. Concisely, these cationic dimeric co-liposomes could be considered as safe alternatives for nucleic acid delivery *in vivo*. Currently, the evaluation of potential of these gemini lipid formulations at *in vivo* level and role in RNAi mediated gene therapy is underway in our laboratory.

Materials and methods

Cell culture

All the cell lines were cultured in cell culture medium DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum (FBS) and pen strep (100 units/ml penicillin and 100 µg/ml streptomycin). Cell culture was maintained in 37 ˚C incubators with a CO_2 level of 5% and relative humidity >95%. Cells in culture dishes (Whenever proper confluency levels were attained) were passaged at regular intervals by properly washing with DPBS (Dulbecco's Phosphate-Buffered Saline) and subsequent treatments using 0.5 % trypsin-EDTA to detach the cells followed by resuspension in DMEM with 10% FBS.

Preparation of liposomes

Each dimeric lipid was mixed with a helper lipid (DOPE) at desired mol ratios (1:1-1:5) in chloroform in autoclaved wheaton glass vials. Chloroform was evaporated under a steady stream of nitrogen gas which resulted in the formation of thin films. They were kept under vacuum for at least 6 h to remove the remaining traces of solvent. After a complete drying of each film, sterile milli-Q water was added to each vial while maintaining the stock lipid concentration as 0.3 mM and kept for hydration of thin films at 4 ˚C for overnight. Samples were then, vortexed intermittently while performing freeze-thaw cycles repeatedly. Finally, the samples were sonicated at 60 ˚C which yielded clear liposomal suspensions and the samples were stored in sterile environment for further use.

Plasmid

Transfection experiments were performed using pEGFP-C3 (Clontech, U.S.A.) plasmid DNA, which encodes for an enhanced green fluorescence protein (EGFP) under a CMV promoter, was amplified in *Escherichia coli* (DH5α) and purified using Qiagen Maxi Prep plasmid purification protocol (Qiagen, Germany). The purity of the plasmid was checked by electrophoresis on 1.0 % agarose gel. The concentration of DNA was estimated spectroscopically by measuring the optical absorption at 260 nm and confirmed by gel electrophoresis. Plasmid preparations showing a value of OD_{260}/OD_{280} >1.8 were used for the experiments.

Gel Electrophoresis

The *p*DNA complexation ability of different gemini lipid formulations was examined using gel retardation assay. In a typical gel experiment, lipoplexes were first prepared using 0.2 µg of *p*DNA with co-liposomal formulation (stock, 0.3 mM) at five different N/P ratios (0.25:1-1.25:1, lipid/DNA)in autoclaved MQ water maintaining a final volume of 20 µl. Lipoplexes were run on a 1% agarose gel electrophoretically (100 V) for a period of about 45 min. The *p*DNA complexation ability of each liposomal formulation was determined by assessing the *p*DNA complete retardation in wells loaded with successively increasing N/P ratios. Finally, gel images were captured using a gel documentation system.

Dynamic Light Scattering and Zeta Potential

A Malvern model Zetsizer Nano ZS (Malvern Instruments Inc., Westborough, MA) with a He-Ne ion laser of 633 nm was used to measure hydrodynamic particle size and zeta potential of the gemini co-liposomal formulations and their lipoplexes. For all experiments, dust-free deionized water with a sample refractive index of 1.59 and viscosity of 0.89 cP was used. Mean hydrodynamic diameters of each sample were recorded three times in every single experiment. Zeta potential measurements of suspensions (co-liposomes and lipoplexes) were performed at 25 ˚C with a fixed angle of 173˚. Smoluchowski approximation was used to calculate the zeta potential. A zeta dip cell was used for measuring the zeta potential values. For each sample, zeta potential values were measured five times. In the zeta titration runs, *p*DNA (0.05 mg/mL) was added to solution of HEPES buffer at pH = 7.4 (total volume 800 µl) and appropriate amount of gemini co-liposomal suspension at different N/P ratios was mixed and kept for incubation for 15 min. Finally, zeta potentials were recorded and the data were plotted against N/P ratios studied.

Atomic Force Microscopy (AFM)

To characterize the morphological features of co-liposomal formulations and their corresponding lipoplexes, AFM studies were performed. Aliquots of 30 µl of suspended aqueous co-liposomes and lipoplexes were drop-coated over freshly cleaved mica sheets and gently dried at room temperature for 24 h. The experiments were performed in tapping mode by JPK instruments at room temperature using NanoWizard JPK00901 software.

Transfection Biology (EGFP Expression)

Plasmid DNA (pEGFP-C3) was used to assess the gene transfection capability of gemini co-liposomal formulations. In a typical experiment, cells were seeded at the density of 40,000 cells/well in a 24 well cell culture plate. Cells were allowed to adhere and grow for 24 h. On the day of transfection, a constant amout of 0.8 µg of plasmid DNA was complexed with different gemini lipids at four different N/P (Lipid/*p*DNA) ratios (0.25:1, 0.5:1, 0.75:1 and 1:1) in cell culture media and allowed for half an hour of incubation. The old media were removed from the wells and the lipoplexes were added to cells while maintaining 10% serum conditions for transfection. Cells were incubated with lipoplexes for 6 h and the cultures were continued after addition of fresh cell culture medium for another 42 h for the expression of EGFP. The transfections were terminated by washing the cells properly with DPBS and trypsinization using 0.5 % trypsin-

EDTA to detach the cells. Cells were then collected in 5% FBS containing DPBS and EGFP expression was assessed using FACS Calibur flow cytometer (Becton-Dickinson). The data obtained from the flow cytometry was put under analysis using WinMDI 2.9 software and the transfection levels were expressed in terms of the intensity of EGFP fluorescence (Mean fluorescence intensity). To visually monitor the expression of EGFP levels, fluorescence microscopic observations were made (Olympus IX-81).

Cellular internalization of *p*DNA

The *p*DNA cellular entry was examined using a fluorescein labeled *p*DNA. The representative gemini co-liposomal formulation (T3T) mediated transfection experiments were performed as discussed in the transfection biology section. FACS (flow assisted cell sorting) and confocal microscopy were used to analyze the samples 6 h post transfection. In a typical experiment of confocal microscopy, cells were seeded on glass coverslips placed in 12 well cell culture plates. Next day, post transfection the wells were washed three times with DPBS (Dulbecco's Phosphate-Buffered Saline) properly and fixed with 4% paraformaldehyde. Cells were then incubated with DAPI (4', 6-diamidino-2-phenylindole) for nuclear counterstaining purpose for 5 min at a concentration of 300 nM. The cells were again washed properly for the removal of excess dye. The coverslips were removed from wells and placed over ProLong Gold antifade reagent (molecular probes) on a glass slide. Confocal laser scanning microscope (LSM meta, Zeiss) was used to analyze the samples. For quantitation of *p*DNA internalization, transfected cells were collected in 5% FBS containing DPBS and analyzed using flow cytometry.

Cell viability assay

Each of the cell lines was transfected with lipoplexes using the efficient co-liposomal formulations and L2K lipoplexes as well after 24 h of cells seeding (10,000 cells/well) in 96 well tissue culture plates. Lipoplexes were incubated with cells for two different time periods, *i.e.*, 6 h and 24 h. Cell viabilities were analyzed at 48 h from the transfection experiment initiation period. The cell viabilities were determined using conventional MTT based assay. The percentage data interpretation was made by taking viabilities of control cells (untreated cells) as 100%.

Treatment with inhibitors

For inhibition experiments, cells were incubated with chlorpromazine (10 μg/ml), genistein (50 μg/ml), methyl-β-cyclodextrin (m-β-CD, 10 mg/ml) and amiloride (200 μM) (all from Sigma) in normal cell culture medium for 30 min at 37 ˚C. Subsequently, the lipoplexes derived from the gemini co-liposomal formulation were incubated for 2 h. Finally, the cells were trypsinized and collected in 5% FBS containing DPBS followed by analysis using FACS.

Transfection of p53 gene and apoptotic activity

The lipoplexes were prepared using p53-EGFP-C3 *p*DNA and experiments were conducted as discussed in transfection biology section above. For the evaluation of cytotoxicity, ~10,000 cells/well were seeded in a 96 well plate while growth inhibition assays were conducted in 6 well plates with cells seeded at the density of 5 x 10^4 cells/well. The apoptosis in transfected cells was evaluated using annexin-V-Alexa Fluor and PI dual staining protocol (Invitrogen).

Notes and references

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† Electronic Supplementary Information (ESI) available: Preparative scheme, spectroscopic and analytical characterization details of the dimeric lipids; Optimization of DOPE content in liposomal formulations to obtain relatively higher gene expression levels in representative cell line; optimization of N/P ratios to achieve maximum gene expression levels in representative cell line; representative flow cytometry histograms for EGFP expressions in different cell lines; CD spectra. See DOI: 10.1039/b000000x/

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