RESEARCH ARTICLE

[View Article Online](#)
[View Journal](#) | [View Issue](#) Cite this: *Med. Chem. Commun.*,
2017, 8, 989

Novel 1,2,3-triazolium-based dicationic amphiphiles synthesized using click-chemistry approach for efficient plasmid delivery†‡

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Herein, we report the synthesis, characterization and evaluation of the transfection efficiencies of a series of dicationic amphiphiles designed to construct quaternary ammonium ion-based cationic lipids varying in chain length of the hydrophobic back bone connected individually through head group to a 1,2,3-triazolium cation consisting of 2-hydroxy ethyl chain as substitution. Accordingly, three dicationic amphiphiles were synthesized by “click chemistry” approach and formulated to bilayered vesicles using DOPE as a co-lipid. The transfection efficacies of these novel lipid formulations were measured and correlated with the results obtained from various physicochemical techniques. Importantly, the observed gradient in the activity profile, where the transfection potential increased with decreasing chain length of the lipid hydrophobic back bone, highlights the synergistic interplay of the lipid alkyl chain length in coordination with charge delocalization in modulating the transfection potency of these 1,2,3-triazolium-based lipids.

Received 16th December 2016,
Accepted 18th February 2017

DOI: 10.1039/c6md00699j

rsc.li/medchemcomm

1. Introduction

The cutting edge technology for the delivery of a therapeutic gene and its expression has long been dominated by cationic lipid-based non-viral vectors.^{1,2} The ease in construction and characterization of such cationic lipids has been added to several intensive investigations for optimizing their performance.^{3,4} To date, cationic lipids are the non viral vector of choice for clinical trials worldwide.⁵ Nevertheless, numerous cytofectins, reported so far, still need modification to address the barriers in regard to direct *in vivo* transfection. In short, the preferred structure promoting stable transfection still remains unresolved.⁶ This has inspired the introduction of novel structural motifs in the lipid backbone with a view to surpass the current transfection standards. The majority of cationic lipids addressed in various studies,^{7,8} suggested that the common structural back bone of lipid consists of the following basic building blocks; head group in polar form exposed to an aqueous environment, hydrophobic back bone lying in the non-polar milieu of liposome and both are connected together either directly with a covalent bond or by a functional unit called a linker.

Considerable efforts have been made to increase the transfection efficiencies of non-viral cationic lipids through the systematic modification of individual domains, mainly the head group and hydrophobic region.^{9,10} In several recently-successful outcomes in this field, the head group has attracted considerable attention due to its crucial roles in self aggregation to develop unilamellar vesicles and charge-charge electrostatic interactions with DNA during complexation.^{11,12} In fact, the self aggregation and DNA complexation are the two characteristics that strongly influence the head group of a lipid by providing positive charge potentials, and the close proximity with DNA in the lipoplex assembly helps determine the efficiency of transfection.^{13,14} In addition, unshared positive charge-based mono cationic lipids and poly cationic lipids have been used thoroughly in different investigations to generate better reagents because the active involvement of the head group in endosomal buffering leads to endosomolysis.^{15–17} Apart from the several key steps in the lipofection pathway, endosomolysis plays significant role in successive nucleic acid delivery. Most recent reports have been developed in favor of endosomolysis by adding pH sensitive groups, such as 1*H*-imidazole to cationic head group for improved transfection results.^{18–20}

Ever since Solodin *et al.*²¹ reported the use of delocalisable heterocyclic cations in the head group of a cationic lipid (DOTIM) in liposomal mediated gene delivery in 1995, the interest of research has been shifted to the use of heterocyclic cations instead of unshared positive charges in the head group because their soft cationic nature provides smart

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† The authors declare no competing interests.

‡ Electronic supplementary information (ESI) available. See DOI: 10.1039/c6md00699j

wrapping of DNA and low toxicity.^{22,23} Consequently, the use of heterocyclic functionalities, such as pyridinium^{24,25} and imidazolium,^{26,27} as head groups provided better transfection efficiencies with low toxicity due to their delocalizable cationic charge and inspired to find better heterocyclic constructs as head groups for better potentials. In addition, the insertion of hydroxy functional units in the head group region increased the transfection efficiencies remarkably due to increasing interactions between the head group of the lipid and DNA with additional hydrogen bonding.^{28,29} This was further witnessed in our previous structure activity study that also proved that increasing the number of hydroxyl groups in the head group increased DNA delivery in proportion.³⁰ This has reinstigated the current introduction of additional heterocyclic cation having a hydroxyl substituted chain beside the head group of quaternary ammonium ion based series of cationic lipids.

Herein, we present the screening of the transfection potentials of novel dicationic amphiphiles, in which the head group of quaternary ammonium cation-based cationic lipids were conjugated to the 1,2,3-triazolium heterocyclic cation. Herein, we present the screening of transfection potentials of novel dicationic amphiphiles, in which head group of quaternary ammonium cation was conjugated to 1,2,3-triazolium heterocyclic cation. These novel dicationic lipids having 1,4 disubstituted 1,2,3-triazolium cation moiety are synthesized by following the strategy called Huisgen 1,3 dipolar cycloaddition. This is an important reaction of a well known strategy called "Click chemistry"^{31,32} that was followed to couple acetylene and azide functions to produce 1,2,3-triazole intermediate in high yield using an established protocol of copper(I) catalyzed click chemistry.³³ This five membered 1,2,3 triazole heterocyclic aromatic ring has already been used as a core ring in numerous biologically potent libraries and also as a linker in cationic amphiphilic vector design^{34,35} because of its unique properties, such as stability towards acidic and basic environments as well as not being viable to oxidative or reductive hydrolysis.³⁶ These features are the major reasons for the utilization of 1,2,3-triazole heterocyclic ring to develop an additional cation, and there are no reports using 1,2,3-triazolium as a head group for cationic lipid mediated gene delivery like pyridinium and imidazolium.

2. Results & discussions

2.1. Chemistry

In this report, a series of dicationic amphiphiles (L1, L2 and L3) having varying aliphatic alkyl chain lengths (C14, C16 and C18 respectively) were synthesized, where the head group of the quaternary ammonium cation was connected covalently to a heterocyclic cation (1,2,3-triazolium) having 2-hydroxy ethyl chain as substitution. Initially, acetylene derivatives of corresponding tertiary amines (1a–c) were obtained in high yields (approximately 90%) by the alkylation of propargyl amine using the respective alkyl bromides. The corresponding acetylene derivatives of the tertiary amine was coupled with 2-azidoethanol to yield the click adduct *via*

copper(I)-catalyzed 1,3 dipolar cyclo addition. Finally, the obtained click adduct (2a–c) was allowed to quaternize using excess methyl iodide in a sealed tube under reflux for approximately 7 days and resulted in ~50% 1,2,3-triazolium salts. The weak base character of 1,2,3-triazole may be quaternized, which leads to the consumption of more time to obtain the second cation on triazole ring, whereas poor yields of iodide salts may be expected due to the elimination of a water molecule (dehydration) from 2-hydroxy ethyl 1,2,3-triazole in the presence of residual copper (Cu) under reflux temperature. The obtained dicationic iodide salts were subjected to chloride ion exchange to replace the iodide counter ion with a chloride ion to afford titled lipids. All the novel lipids and intermediates were purified and characterized using spectral (¹H-NMR, ESI-MS) and elemental analytical data (Fig. 1).

Small unilamellar vesicles were prepared by mixing the dicationic amphiphiles with one of the well known neutral co-lipid (DOPE) for enhanced transfection results in an equimolar ratio (1:1) by following the lipid hydration technique, as described elsewhere.³⁷ These aqueous dispersions were stable and clear for up to three months by storing at 4 °C. Following the formulation, liposomal vesicles were characterized in terms of the size, surface potential and DNA binding.

2.2. Physicochemical characterization of the particles (lipid:DOPE) and complexes (lipid:DOPE:DNA)

The mean size of the dispersed vesicles in the aqueous medium was determined by dynamic light scattering. The technique that involves photon correlation spectroscopy produced the value of hydrodynamic diameter of the suspended particles, and the relative values of L1, L2 and L3 were obtained as 150 nm, 113 nm and 54.7 nm, respectively. This clearly represents a decreasing size from L1–L3 (Fig. 2A). To be specific, the vesicular diameter decreases in proportion with the length of the hydrophobic long chain present in lipid backbone. Liang C. H. *et al.* reported this trend of shorter alkyl chain comprising cationic lipids producing a larger hydrodynamic sphere owing to the higher surface potential and *vice*

Fig. 1 Molecular structures of the lipids used in this study.

Fig. 2 A) Hydrodynamic diameter (nm) of the lipid vesicles (light gray) and lipid:DNA complexes (dark gray) at 1:1 N/P charge ratio using 5 μ g pCMV- β gal DNA were measured by dynamic light scattering. B) Zeta-potentials of lipid formulations (1:0, L1-light gray, L2-gray and L3-black) and lipoplexes prepared from lipid:DNA at various N/P charge ratios (0.3:1–9:1) using a constant amount of pCMV- β gal DNA (10 μ g) in Milli-Q. C) Representative transmission electron microscopy images of negatively stained lipid:DOPE vesicles and liposome:DNA complexes at 1:1 N/P ratio.

versa.³⁸ Importantly, the size of the lipoplexes generated from these lipids could also be correlated. The size of the lipoplexes at 1:1 N/P charge ratio (TE optimized ratio) has increased an average ratio of 2/3 diameter, with respect to the corresponding liposome and indicated that an optimal condensation with the DNA molecule might facilitate maximum transfection efficiency.

To further confirm the size and reveal the supra molecular structure of these vesicles and complexes, transmission electron microscopy was performed using negative staining. Fig. 2C shows that the liposomes were observed as compacted vesicles with a distinct boundary. The corresponding complexes formed at the optimized transfection ratio also revealed a similar vesicular nature with an irregular margin.

Significantly, the zeta potential is one of the parameters used to determine the stability of the formulated complexes with DNA, and is mostly in proportion with the transfection

potential. Fig. 2B represents the zeta potential of liposomes (1:0) and lipoplexes at different charge ratios (0.3:1, 1:1, 3:1 and 9:1). The negative zeta potential observed at a 0.3:1 ratio transformed to a positive value at the next higher 1:1 ratio, most probably following complete charge neutralization of the DNA.

2.3. Gel electrophoresis

The extent of DNA binding is a significant parameter to elucidate the transfection potential of these dicationic lipid formulations. To investigate the DNA binding capacity, conventional gel electrophoresis was performed as a function of the lipid:DNA charge ratio (N/P), as depicted in Fig. 3. Fig. 3 confirms that the mobility of DNA was optimally retarded with all the lipid complexes, particularly at 1:1 and 3:1 N/P charge ratios. It is also understood that all three lipids could

Fig. 3 DNA binding patterns of dicationic liposome formulations (L1, L2 and L3) on 1% agarose gel electrophoresis. L1, L2 and L3 represents liposome formulations prepared from lipid:DOPE at 1:1 molar ratio. Lipoplexes were prepared in 20 μ L of 0.5 $\times$ PBS by mixing a constant amount of pCMV- β gal DNA (0.4 μ g per well) with varying lipid charge ratios (1:1, 3:1 & 9:1) mentioned on top of each panel. DNA alone, depicted as “C” on each panel served as the control.

retain the DNA in the wells at least 50–60% at 1:1 charge ratio and 80–90% of DNA at 3:1 charge ratio. Comparatively, at higher ratios, all three lipids were capable of completely inhibiting the electrophoretic mobility of the plasmid DNA, particularly at 9:1. The results are in corroboration with the pattern of the zeta potentials, as described in the previous section. Thus, the lipoplexes of lipids L1, L2 and L3 having optimal lipid–DNA interactions at 1:1 and 3:1 charge ratios may effectively facilitate the release of DNA into the cytoplasm after delivery. This is evident in the following reporter gene expression data.

2.4. Transfection biology

To evaluate the transfection efficiencies of DOPE-mixed liposomal formulations of 1,2,3-triazolium-based dicationic lipids, a β -galactosidase reporter gene expression analysis was followed. Different cell lines (HEK-293, HepG2, CHO and B16F10) were utilized for the activity comparison of lipoplexes across various N/P charge ratios (0.3:1 to 9:1). Fig. 4 depicts the transfection efficiencies of the three different dicationic lipid formulations (L1, L2 and L3), and it demonstrates that the maximum transfection activity has

Fig. 4 Transient transfection *in vitro*: graph depicts the transfection efficiencies of dicationic lipid formulations (L1, L2 & L3) in four different cell lines A) HEK-293; B) CHO; C) B16F10; D) HepG2. The lipoplexes were prepared at various charge ratios (N/P) of lipids 0.3:1 (black); 1:1 (light gray); 3:1 (white); 9:1 (gray) using a constant amount of pCMV- β gal DNA (0.3 μ g per well). The complexes were incubated for 4 h in the presence of serumless DMEM, and the assay was performed 48 h post transfection. Representative data presents the normalized miller units of β -galactosidase/mg protein and were obtained from an average triplicate of three individual experiments (Effectene: Efctn).

materialized at 1 : 1 and 3 : 1 charge ratios, irrespective of the cell lines studied. The maximum efficiencies of these lipid formulations at specified charge ratios are clearly supported by the facts estimated in previous sections, such as DNA binding, zeta potentials of lipid:DNA complexes and hydrodynamic sizes of the DNA-derived complexes.

To investigate the effect of the designed construct (1,2,3-triazolium), which confers an additional delocalized cationic charge to the unshared positive charge present on the simple quaternary ammonium head group region, an interesting study was endeavored. For this purpose, an independent lipid that resembles the synthesized dicationic lipid in its structure except for the additional 1,2,3-triazolium group in the head group region is required. The commercially available cationic lipid, DDAB, can fit in as the required standard when compared along the rest of the synthesized lipids in the β -gal transfection activity profile.^{39,40} In addition, the transfection reagent Effectene was used as the positive control (Efctn). Herein, the charge ratio 1 : 1 has been considered due to its maximum activity at the minimal charge ratio for the reporter gene and the average of triplicate transfection results in the four different cell lines HEK-293, HepG2, CHO and B16F10, as depicted in Fig. 5. It becomes clear from the representative graph that lipid L1 is most effective in terms of the transfection potential in all the cell lines studied at 10% serum conditions. Lipid L1 with the chain length C14 is on an average, two times more active than both the control lipid DDAB and commercial transfection reagent, Effectene. In contrast, lipid, L2, having a chain length of C16, has relatively equal activity with the control and Effectene, and lipid L3 synthesized from the C18 chain back bone was found to be less active. Herein, the specificity of the cell lines also plays a role in deciding the transfection potential of the syn-

thesized lipids. In fact, Fig. 5 also illustrates the cell line specific activity and shows that the activity of lipid L1 is a maximum in the HEK-293 cell lines followed in order of activity by CHO, B16F10 and HepG2 cell lines.

In the present investigation, as the length of the alkyl chain was varied from C14 to C16 to C18 of the dicationic lipid formulations, the transfection activities decreased with increasing chain length of the anchoring group of the lipid. In fact, several reports established a clear connection between the length of the hydrophobic chain and the transfection efficiency and described that a shorter alkyl chain in the hydrophobic backbone increases the fluidity of the bilayer and helps in a higher intermembrane transfer rate, resulting in a potential disruption of the endosome and consequent release of DNA.³⁸ In addition, the interesting outcome of this study describes the least activity of lipid L3 among the lipids studied including control lipid having the same backbone length. In spite of the associated features, such as a delocalized cation along with hydroxyl functionality in the head group of L3, inferior activity of L3 is accounted in terms of plasmid delivery. This particular observation is supported by an earlier investigation⁴¹ on the basis of surface hydration of lipid membranes. Surface hydration is one of the parameters to determine the efficiency of liposome–DNA complexation that has further led to a better understanding of the transfection patterns. The complexation of lipid–DNA molecules is mainly due to the electrostatic interactions between the head group of cationic lipid and phosphate backbone of plasmid DNA. According to Bajaj *et al.* (2008), as the surface hydration of a cationic lipid increases, the transfection efficiency will decrease. Probably, the combination of dicationic and hydroxyl functionality in the head group of lipid L3 might be responsible for its higher surface hydration compared to the mono cationic DDAB lipid. This could be a reason behind the lower transfection activity of lipid L3 in spite of having the same back bone as that in the control lipid DDAB.

In addition, the results obtained from the β -gal reporter gene expression are further supported using another plasmid DNA in terms of fluorescent protein expression. Subsequently, the complexes were prepared using pEGFP-N₃ plasmid (0.8 μ g) at an optimized N/P charge ratio (1 : 1). The cells of both HEK-293 and CHO were incubated with the formulated lipoplexes in 10% FBS + DMEM for 4 h. The green fluorescent expression was visually observed under the fluorescent microscope; the images were captured 48 h post transfection for the qualitative analysis of gene expression. The representative fluorescent microscopy images in Fig. 6 clearly show that the fluorescent gene expression obtained from the HEK293 cells was relatively higher than that obtained from the CHO cell lines. The GFP expression obtained using the lipids L1, L2 and L3 compared to the control lipid DDAB and commercial cytofectin Efctn followed a rank order as follows: L1 > L2 $\approx$ DDAB > Efctn > L3. These results are consistent with the quantitative transfection data obtained from β -galactosidase gene expression.

Fig. 5 Relative transfection results from the HepG2, HEK-293, CHO and B16F10 cell lines at a 1 : 1 N/P charge ratio of three lipid formulations (L1, L2 & L3) compared to DDAB (control lipid) and Efctn. The cells were incubated with the complexes in 10% FBS + DMEM for 4 h and the assay was terminated after 48 h of complex addition. Efctn (Effectene) and DDAB:DOPE (1 : 1) formulations were served as controls.

Fig. 6 Representative fluorescence microscopy images obtained from pEGFP-N₃ transfection using co-liposomal formulations of L1, L2 and L3 in HEK-293 and CHO. The complexes were prepared from dicationic lipid formulations at a 1:1 N/P charge ratio using pEGFP-N₃ plasmid (0.8 μ g) in serum minus DMEM. For visual comparison, images were captured in Fioid fluorescent imaging station at 48 h post transfection. DDAB and Efctn served as the positive controls for relative activity comparison.

2.5. Serum compatibility *via* transfection

In spite of the numerous setbacks in cationic lipid mediated gene delivery, serum is one of the well-known modulating factors to disrupt the transfection efficiencies of novel cationic amphiphiles.^{42,43} However, a transfection reagent derived from any cationic lipid possessing superior transfection activity has to be serum-compatible to mandate/execute its application *in vivo*. To know the impact of serum on the stability of lipoplexes derived from dicationic lipid formulations, the assay was conducted in those two cell lines with the highest activity rank order (HEK-293, CHO). The complexes were prepared at an optimized N/P charge ratio (1:1) to formulate the final transfection complex using increasing concentrations of added serum (10–50%, v/v). Interestingly, as shown in Fig. 7, the β -galactosidase gene expression pattern of all the three lipids in both cell lines studied remains unaffected in the presence of 10% added serum. However, upon increasing the serum concentrations to 30% and 50%, respectively, a corresponding decrease in activity to 20% and 40% was observed for all the three lipids.

2.6. Cell viability

The significance of efficient synthetic gene carriers is related directly to the cell compatibility and survival measures. Thus,

the cationic lipid toxicity possess a major limiting factor for the clinical application of liposomal gene delivery.⁴⁴ The cell viabilities of the lipoplexes derived from novel dicationic lipids were assessed using an MTT cell based assay⁴⁵ following the same conditions as those followed in actual transfection studies. Fig. 8 displays the percentage of cell viability of the lipoplexes derived at varying lipid to DNA charge ratios, *i.e.* 0.3:1, 1:1, 3:1 and 9:1. The toxicity profile of the lipoplexes in all the cell lines used for transfection were compared with the control and standard formulations, DDAB & Efctn. The obtained data showed that the cell survivability is linked directly to the combined effects of both the lipid concentration and the dialkyl chain length of lipid backbone. Specifically, cell viability can be increased by decreasing the chain length and lipid concentration. To find the liposomal sensitivity towards cell cytotoxicity, MTT assay was repeated using the plain liposomal suspension (without DNA) at two different concentrations (25 & 50 μ m) in two cell lines (HEK-293 and CHO). The resulting % cell viabilities after 24 h of incubation showed (Fig. S19†) ~85% and ~45% cell viabilities at 25 μ m and 50 μ m concentrations of liposomes (L1, L2 & L3), respectively. Finally, the formulations of these 1,2,3-triazolium based lipids L1 and L2 were developed as potential transfection reagents having lower toxicity than the available commercial transfection reagent, Effectene (Efctn).

Fig. 7 Representative histograms depicting the *transient* transfection results conducted in HEK-293 (A) and CHO (B) in the presence of increasing concentrations of serum. The complexes were prepared at optimized N/P charge ratio (1:1) in serum-free DMEM, final transfection complex was formulated using (–FBS (black), +10% FBS (light gray), +30% FBS (white) and +50% FBS (gray)) increasing concentrations of serum containing DMEM. The results were measured in terms of the β -galactosidase units per mg protein 48 h of post transfection.

Fig. 8 % cell viability by an MTT assay resulting from A) HEK-293, B) CHO, C) B16F10 and D) HepG2. The cells were treated with the complexes prepared in 10% FBS + DMEM with various charge ratios (N/P; 0.3:1/light gray, 1:1/white, 3:1/black and 9:1/gray) of dicationic lipid formulations L1, L2 and L3 along with the control lipid (DDAB:DOPE/vertical stripes) and commercial transfection reagent (Effectene/checks) at a constant amount of pCMV- β gal DNA (0.3 μ g per well), as mentioned in the methods. The complexes were incubated for 4 h and the cells were assayed after 24 h of complex addition.

2.7. Conclusions

In summary, a series of aliphatic back bone-anchored dicationic lipids varying in chain length were synthesized by connecting the head group of quaternary ammonium to a heterocyclic cation (1,2,3-triazolium) having a 2-hydroxy ethyl chain, formulated to lipid vesicles using DOPE as a co-lipid (L1, L2 & L3) and their transfection potentials in various cell lines were evaluated using reporter gene activity with two different plasmids, such as pCMV- β gal and pEGFP-N₃. The study also focused on the effects of additional heterocyclic construct on transfection by comparing the activity with a control formulation DDAB:DOPE including in the transfection. Comparative biological studies indicated that enhancement of transfection due to additional heterocyclic cation is in good agreement with all the experimental results. The results also demonstrate that the activities of these dicationic lipids are critically dependent on the chain length of the hydrophobic back bone. The lipoplexes produced from these dicationic lipids were stable in serum, least toxic and mediated good transfection. Hence, the present study endorses

the triazole based dicationic head group with the C14 alkyl chain length as a better transfecting reagent for modulating the activity and toxicity profile.

3. Materials and methods

All the chemicals, reagents and solvents used for the synthesis were of the highest purity and procured from Alfa Aesar, Spectrochem and used without further purification. The mass spectral data were acquired using a commercial LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, U.S.) equipped with an ESI source. The ¹H NMR spectra were recorded on a Bruker (2001), SAINT (version 6.28a) & SMART (version 5.625) Bruker AXS Inc., Madison, Wisconsin, USA. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Calbiochem (Merck Millipore, Massachusetts, USA). Endotoxin-free plasmids pCMV- β gal and pEGFP-N₃ were extracted from competent *E. coli* bacteria; the extraction was carried out using Nucleo Bond Xtra Midi Plus EF (MACKAREY-NAGEL, Duren, Germany), following a previously described protocol. The zwitterionic lipid,

1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), and the control lipid, didodecyltrimethylammonium bromide (DDAB), were purchased from Avanti Polar Lipids. Fetal bovine serum (FBS) was procured from Thermo Fischer Scientific (Gibco®).

3.1. Synthesis

General procedure for the synthesis of acetylene derivatives (1a–c). In a 50 mL round bottom flask, 1 g (18.16 mmol) propargyl amine, 12.54 g (90.78 mmol) anhydrous K_2CO_3 and corresponding alkyl bromide (38.136 mmol) were placed in 15 mL of acetonitrile. The resulting mixture was allowed to reflux at 80 °C for about 2 days to achieve the maximum disappearance of starting materials on TLC. The contents were cooled and exposed to rota evaporation to remove the solvent. The resulting crude was dissolved in an excess of chloroform (100 mL), which was then washed three times with water (3 × 30 mL) and one time with brine (50 mL). The separated organic layer was removed under reduced pressure, followed by drying upon anhydrous sodium sulphate to remove the remaining water droplets. The resulting crude was subjected to 60–120 mesh silica gel column chromatography purification using 1% EtOAc: pet ether as the eluent (Scheme 1).

N-Tetradecyl-N-(prop-2-yn-1-yl)tetradecan-1-amine (1a). Colorless oil, yield (5.6 g, 69%). 1H NMR (400 MHz, $CDCl_3$) δ 3.40 (s, 2H, $-N-CH_2-C\equiv$), 2.44 (t, J = 8 Hz, 4H, $-N-(CH_2)_2-$), 2.15 (s, 1H, $H-C\equiv C-$), 1.43 (s, 4H, $-N-(CH_2)_2-(CH_2)_2-$), 1.26 (s, 44H, $-(CH_2)_{22}-(CH_3)_2$), 0.88 (t, J = 8 Hz, 6H, $-(CH_2)_{22}-(CH_3)_2$). ESI-MS: 447 $[M + H]^+$.

N-Hexadecyl-N-(prop-2-yn-1-yl)hexadecan-1-amine (1b). Colorless solid, yield (6.41 g, 70%). 1H NMR (400 MHz, $CDCl_3$) δ 3.41 (s, 2H, $-N-CH_2-C\equiv$), 2.44 (t, J = 8 Hz, 4H, $-N-(CH_2)_2-$), 2.15 (s, 1H, $H-C\equiv C-$), 1.44 (t, J = 8 Hz, 4H, $-N-(CH_2)_2-(CH_2)_2-$), 1.28 (s, 52H, $-(CH_2)_{26}-(CH_3)_2$), 0.88 (t, J = 8.0 Hz, 6H, $-(CH_2)_{26}-(CH_3)_2$). ESI-MS: 505 $[M + H]^+$.

N-Octadecyl-N-(prop-2-yn-1-yl)octadecan-1-amine (1c). Colorless solid, yield (7.48 g, 73.5%). 1H NMR (400 MHz, $CDCl_3$) δ 3.40 (s, 2H, $-N-CH_2-C\equiv$), 2.44 (t, J = 8 Hz, 4H, $-N-(CH_2)_2-$),

2.15 (s, 1H, $H-C\equiv C-$), 1.44 (s, 4H, $-N-(CH_2)_2-(CH_2)_2-$), 1.24 (s, 60H, $-(CH_2)_{30}-(CH_3)_2$), 0.88 (t, J = 8.0 Hz, 6H, $-(CH_2)_{30}-(CH_3)_2$). ESI-MS: 560 $[M + H]^+$.

General procedure for the click reaction (2a–c). To a stirred solution of an acetylene-derivative of 1 mmol (1a–c) and 2-azidoethanol (1 mmol) in *tert*-BuOH:H₂O (1:1; 20 mL) were added $CuSO_4 \cdot 5H_2O$ (0.1 mmol) and sodium ascorbate (0.2 mmol). The resulting mixture was stirred at room temperature until complete conversion of the starting material, which was then diluted with an excess of water (100 mL). The product was extracted in ethyl acetate (3 × 50 mL) and the solvent was removed under reduced pressure, followed by drying upon anhydrous $NaSO_4$. The compound showing the R_f in the range 0.4–0.5 in 9:1 $CHCl_3$:MeOH was purified using 60–120 mesh silica gel column chromatography using 2–3% MeOH: $CHCl_3$ as eluent.

2-(4-((Ditetradecylamino)methyl)-1H-1,2,3-triazol-1-yl)-ethanol (2a). Yellowish gummy solid, yield (0.485 g, 91%) 1H NMR (400 MHz, $CDCl_3$) δ 7.66 (s, 1H, $-N-HC\equiv C-(CH_2)-$), 4.48 (t, J = 4 Hz, 2H, $HO-CH_2-CH_2-$), 4.07 (t, J = 4 Hz, 2H, $HO-CH_2-CH_2-$), 3.83 (s, 2H, $=C-CH_2-N-$), 2.50 (t, J = 8 Hz, 4H, $-N-(CH_2)_2-$), 1.51 (s, 4H, $-N-(CH_2)_2-(CH_2)_2-$), 1.25 (s, 44H, $-N-(CH_2)_{22}-$), 0.86 (t, J = 12 Hz, 6H, $-(CH_2)_{22}-(CH_3)_2$). ESI-MS: 535 $[M + H]^+$.

2-(4-((Dihexadecylamino)methyl)-1H-1,2,3-triazol-1-yl)-ethanol (2b). Yellowish gummy solid, yield (0.548 g, 93%) 1H NMR (400 MHz, $CDCl_3$) δ 7.61 (s, 1H, $-N-HC\equiv C-(CH_2)-$), 4.50 (t, J = 4 Hz, 2H, $HO-CH_2-CH_2-$), 4.09 (t, J = 4 Hz, 2H, $HO-CH_2-CH_2-$), 3.81 (s, 2H, $=C-CH_2-N-$), 2.47 (t, J = 8 Hz, 4H, $-N-(CH_2)_2-$), 1.51 (s, 4H, $-N-(CH_2)_2-(CH_2)_2-$), 1.27 (s, 52H, $-(CH_2)_{26}-(CH_3)_2$), 0.90 (t, J = 8 Hz, 6H, $-(CH_2)_{26}-(CH_3)_2$). ESI-MS: 591 $[M + H]^+$.

2-(4-((Diocetadecylamino)methyl)-1H-1,2,3-triazol-1-yl)-ethanol (2c). Yellowish gummy solid, yield (0.591 g, 91.5%) 1H NMR (400 MHz, $CDCl_3$) δ 7.65 (s, 1H, $-N-HC\equiv C-(CH_2)-$), 4.48 (t, J = 4 Hz, 2H, $HO-CH_2-CH_2-$), 4.06 (t, J = 4 Hz, 2H, $HO-CH_2-CH_2-$), 3.82 (s, 2H, $=C-CH_2-N-$), 2.50 (t, J = 4 Hz, 4H, $-N-(CH_2)_2-$), 1.51 (s, 4H, $-N-(CH_2)_2-(CH_2)_2-$), 1.25 (s, 60H, $-(CH_2)_{30}-(CH_3)_2$), 0.88 (t, J = 4 Hz, 6H, $-(CH_2)_{30}-(CH_3)_2$). ESI-MS: 647 $[M + H]^+$.

Synthesis of dicationic lipid (L1–3). To an obtained click intermediate 2a–c (1 mmol) in 5 mL of acetonitrile was added 10 mmol of excess methyl iodide in a screw cap sealed tube. The resulting mixture was refluxed for about 7 days at 80 °C until the complete conversion of the starting material monitored by TLC. Consequently, the residue was subjected to column chromatography purification to yield a pure quaternized iodide salt using 3–4% MeOH: $CHCl_3$ as eluent. The pure dicationic iodide lipid was further subjected to repeated anion exchange procedure to afford pure titled lipids (L1–3).

4-((Ditetradecyl(methyl)ammonio)methyl)-1-(2-hydroxyethyl)-3-methyl-1H-1,2,3-triazol-3-ium, (lipid L1). Brown gummy solid, yield (0.264 g, 47%) 1H NMR (400 MHz, $CDCl_3$) δ 8.81 (s, 1H, $-N-CH\equiv C-(CH_2)-$), 5.16 (s, 2H, $-CH\equiv C-CH_2-N^+$), 4.59 (t, J = 8 Hz, 2H, $HO-CH_2-CH_2-$), 3.81 (t, J = 8 Hz, 2H, $HO-CH_2-CH_2-$), 3.46–3.37 (m, 7H, $-N^+-(CH_3)(CH_2)_2-$,

Scheme 1 Synthetic route and molecular structures of dicationic lipids. Reagents and conditions: a) alkyl bromide, K_2CO_3 , acetonitrile, 80 °C, 48 h; b) 2-azidoethanol, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, *tert*-BuOH:H₂O, RT, 16 h; c) MeI, acetonitrile, sealed tube, 80 °C, 72 h, amberlite anion exchange resin.

$-N^+(\text{CH}_3)=\text{N}-N-$, 3.20 (s, 3H, $-N^+(\text{CH}_3)(\text{CH}_2)_{2-}$), 1.82–1.74 (m, 4H, $-N^+(\text{CH}_3)(\text{CH}_2)_{2-}(\text{CH}_2)_{2-}$), 1.36–1.26 (m, 44H, $-(\text{CH}_2)_{2-}(\text{CH}_2)_{22-}(\text{CH}_3)_2$), 0.88 (t, $J = 8$ Hz, 6H, $-(\text{CH}_2)_{22-}(\text{CH}_3)_2$). ESI-MS: m/z : 564 $[\text{M}^+]$ for $[\text{C}_{35}\text{H}_{72}\text{N}_4\text{O}^{2+}]$. Elemental analysis: calculated: % C: 74.41; % H: 12.85; % N: 9.92; % O: 2.83. Observed: % C: 74.01; % H: 12.5; % N: 9.98; % O: 2.63.

4-((Dihexadecyl(methyl)ammonio)methyl)-1-(2-hydroxyethyl)-3-methyl-1H-1,2,3-triazol-3-ium, (lipid L2). White gummy solid, yield (0.275 g, 44.35%) ^1H NMR (400 MHz, CDCl_3) δ 8.78 (s, 1H, $-N-\text{CH}=\text{C}-(\text{CH}_2)-$), 5.10 (s, 2H, $-\text{CH}=\text{C}-\text{CH}_2-\text{N}^+$), 4.59 (t, $J = 4$ Hz, 2H, $\text{HO}-\text{CH}_2-\text{CH}_2-$), 3.81 (t, $J = 8$ Hz, 2H, $\text{HO}-\text{CH}_2-\text{CH}_2-$), 3.46–3.37 (m, 7H, $-N^+(\text{CH}_3)(\text{CH}_2)_{2-}$, $-(\text{CH}_3)-\text{N}^+=\text{N}-\text{N}-$), 3.20 (s, 3H, $-N^+(\text{CH}_3)(\text{CH}_2)_{2-}$), 1.83–1.76 (m, 4H, $-N^+(\text{CH}_3)(\text{CH}_2)_{2-}(\text{CH}_2)_{2-}$), 1.39–1.26 (m, 52H, $-(\text{CH}_2)_{2-}(\text{CH}_2)_{26-}(\text{CH}_3)_2$), 0.88 (t, $J = 8$ Hz, 6H, $-(\text{CH}_2)_{26-}(\text{CH}_3)_2$). ESI-MS: m/z : 620 $[\text{M}^+]$ for $[\text{C}_{39}\text{H}_{80}\text{N}_4\text{O}^{2+}]$. Elemental analysis: calculated: % C: 75.42; % H: 12.98; % N: 9.02; % O: 2.58. Observed: % C: 75.41; % H: 12.85; % N: 9.2; % O: 2.38.

4-((Diocetadecyl(methyl)ammonio)methyl)-1-(2-hydroxyethyl)-3-methyl-1H-1,2,3-triazol-3-ium, (lipid L3). Creamy solid, yield (0.335 g, 49.5%) ^1H NMR (400 MHz, CDCl_3) δ 8.77 (s, 1H, $-N-\text{CH}=\text{C}-(\text{CH}_2)-$), 5.09 (s, 2H, $-\text{CH}=\text{C}-\text{CH}_2-\text{N}^+$), 4.59 (t, $J = 4$ Hz, 2H, $\text{HO}-\text{CH}_2-\text{CH}_2-$), 3.81 (t, $J = 4$ Hz, 2H, $\text{HO}-\text{CH}_2-\text{CH}_2-$), 3.46–3.42 (m, 7H, $-N^+(\text{CH}_3)(\text{CH}_2)_{2-}$, $-N^+(\text{CH}_3)=\text{N}-\text{N}-$), 3.37 (s, 3H, $-N^+(\text{CH}_3)(\text{CH}_2)_{2-}$), 1.86–1.79 (m, 4H, $-N^+(\text{CH}_3)(\text{CH}_2)_{2-}(\text{CH}_2)_{2-}$), 1.39–1.26 (bs, 60H, $-(\text{CH}_2)_{2-}(\text{CH}_2)_{30-}(\text{CH}_3)_2$), 0.88 (t, $J = 4$ Hz, 6H, $-(\text{CH}_2)_{30-}(\text{CH}_3)_2$). ESI-MS: m/z : 676 $[\text{M}^+]$ for $[\text{C}_{43}\text{H}_{88}\text{N}_4\text{O}^{2+}]$. Elemental analysis: calculated: % C: 76.27; % H: 13.10; % N: 8.27; % O: 2.36. Observed: % C: 76.41; % H: 13.15; % N: 8.2; % O: 2.38.

3.2. Preparation of liposomes

Cationic liposomes were prepared by mixing the desired quantities of cationic lipid and co-lipid (DOPE) from separately dissolved chloroform stocks. The solvent was evaporated uniformly using a thin flow of moisture-free nitrogen gas. The resulting thin films were rehydrated using appropriate volume of deionized water to swell the films overnight. These films were converted to multilamellar vesicles using a frequent vortex. Finally, optically clear aqueous suspensions were obtained by subjecting the MLVs to sonication in an ice bath using SONICS Vibra cell (25% Amplitude, pulse mode, 9 s on/off). The newly prepared clear aqueous suspensions were stored at 4 °C until use.

3.3. Size & zeta (ζ) potential measurements

Cationic liposomes derived from dicationic lipid and co-lipid (DOPE) and, complexes derived from liposome and DNA both were characterized with respect to their size and zeta potential using the instrument called SZ-100 NEXTGEN (HORIBA) equipped with a diode-pumped solid-state laser at $\lambda = 532$

nm calibrated at 25 °C. The particle size was obtained by an average of three different measurements in general mode. To obtain information regarding the stability and charge of the dispersed particles, the zeta potentials were measured. These measurements were also carried out in the same instrument using laser Doppler electrophoresis analysis provided by SZ-100 software. Herein, Milli-Q served as the blank control for instrument autocorrelation and sample dilutions.

3.4. Transmission electron microscopy (TEM)

The particle size distribution and morphological appearance of both SUVs and their derived complexes were examined under the transmission electron microscope. Briefly, 6 μL of a sample was spread onto a Cu grid coated with Formvar-film. After 60 s, the excess liquid was absorbed onto filter paper and allowed to stain using 5 μL of 2% uranyl acetate. This was followed by the removal of excess stain using fresh filter paper and dried at atmospheric pressure at room temperature and visualized under a JEOL JEM 2100 transmission electron microscope operated at 120 kV. The images were acquired on a Gatan camera using Digital Micrograph software.

3.5. Agarose-gel electrophoresis

To assess the DNA binding abilities of the optimized dicationic co-liposomal formulations (lipid/DOPE, 1:1), an agarose gel retardation assay was performed. In a typical experiment, 400 ng of pCMV- β gal was complexed with each dicationic formulation by maintaining three different N/P (lipid/plasmid) charge ratios, 1:1, 3:1 and 9:1, in 0.5 $\times$ PBS (20 μL). These complexes were loaded on to a freshly prepared 1% agarose gel after adding 6 $\times$ loading dye to the sample, followed by 25 min of complex incubation. Electrophoresis was done for about 90 min in 1 mM TAE running buffer in an electrophoresis chamber set to 70 V. The gel images were captured under a gel documentation system in transillumination mode, followed by EtBr staining for 30 min post electrophoresis.

3.6. Amplification and purification of plasmid DNA

pCMV- β gal and pEGFP-N₃ plasmids were used in the *in vitro* gene transfection studies, which are β -galactosidase reported gene expression and green fluorescent gene expression, respectively. Both plasmids, which were prepared from laboratory stocks, were initially transformed to competent *E. coli* DH-5 α cells and amplified in LB broth media at 37 °C overnight. The endotoxin-free pure plasmids were isolated using the NucleoBond[®] Xtra Midi kit (MACKAREY-Nagel). The resultant plasmids were then dissolved in TE buffer solution and stored at –20 °C. The integrity, purity and concentrations of the plasmids were confirmed by agarose-gel electrophoresis and NanoDrop 2000 respectively.

3.7. Cells & cell culture

B16F10 (human melanoma carcinoma derived), CHO (Chinese hamster ovary), HEK-293 (Human embryonic kidney) and HepG2 (human hepatocarcinoma derived) cell lines were used from our laboratory maintenance cultures. The cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM, invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS), 10 mM NaHCO₃, 60 µg mL⁻¹ penicillin, 50 µg mL⁻¹ streptomycin and 30 µg mL⁻¹ kanamycin in a humidified atmosphere containing 5% CO₂.

3.8. Transfection biology

To investigate the transfection abilities of dicationic copolysomal formulations beta galactosidase assay was performed using pCMV-βgal plasmid in four different cell lines, from different origins. Briefly, all the cell lines were seeded onto separate 96-well plates at a density of 10 000 cells per well a day prior to transfection. On the day of transfection, lipoplexes were prepared using dicationic liposome and pCMV-βgal DNA (0.3 µg per well) at three different charge ratios ranging from 1:1 to 9:1 in 100 µL of serum-free media. These resulting complexes were incubated at room temperature for about 25 min, and the serum containing media from the cells was removed and washed with 1× PBS. After 25 min of incubation, the complexes were diluted to formulate the final transfection complex using serum-free media. This was followed by the addition of complexes to the wells in a triplicate manner, and the cells were allowed to incubate in the CO₂ incubator for about 4 h. The complex media were replaced by 10% serum media (0.3 mL per well) and the incubation was continued for about 48 h in a CO₂ incubator and assayed by following the protocol, as mentioned earlier.⁴⁶

Furthermore, to support the transfection patterns obtained from beta gal transfection, one more transfection was performed using the pEGFP-N₃ plasmid in HEK-293 and CHO cells. Briefly, the cells were seeded onto a 12-well plate at a density of 4×10^4 cells per well a day before the transfection. Prior to complete 24 h incubation of the cells, the lipoplexes were prepared with pEGFP-N₃ plasmid (0.8 µg per well) using the procedure as mentioned in prior sections. Subsequently, the lipoplexes were formulated to final transfection complex and the cells were incubated in CO₂ incubator for 4 h after the addition. The complex media were replaced with 10% serum-containing media and continued to incubate for 48 h. DMEM (without a complex), lipoplex formulated with Effectene (commercially available formulation) and DDAB:DOPE:DNA served as the controls. The transfection efficiencies were analyzed by capturing the images under a fluorescence microscope Flouid cell imaging station by a visual comparison.

3.9. Transfection in presence of serum

To determine the sensitivity of these synthesized lipid formulations towards the serum, we performed the transfection in the presence of increasing concentrations of serum using

pCMV-βgal. Briefly, the cells were seeded at a density of 10 000 cells per well in a 96-well plate 18–24 h before transfection. The complexes were then prepared using 0.3 µg per well of plasmid DNA mixed with the lipids (L1–3) at the maximum transfection optimized charge ratio (1:1) in DMEM in the presence of increasing concentrations of added serum (10–50% v/v). The final transfection complex was formulated using the appropriate volume of DMEM and allowed to incubate for about 25 min. The assay was then performed similarly, as mentioned in earlier sections.

3.10. Cytotoxicity studies

The dicationic lipid formulations were screened for their toxicities following the MTT-based assay, which involves the reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by viable cells to produce purple-colored insoluble formazan granules. The intensity of the color is related to the number of viable cells and is measured by colorimetric analysis. To measure the viabilities of the complexes derived from these lipids, the cells were seeded onto a 96-well plate 18 to 20 h prior to the complex addition. Furthermore, cells were incubated for 4 h in CO₂ incubator, and the complexes were replaced by complete DMEM to continue the incubation for a further 24 h. The MTT dye (0.5 mg mL⁻¹) was prepared freshly in serum-free DMEM and 100 µL was added to each well and allowed to incubate for 3 h prior to termination of the assay. Finally, the media were removed and 100 µL of MeOH:DMSO (1:1, v/v) was added to each well. This dissolves the purple colored dye, was then measured spectroscopically at 540 nm in a multiplate reader, Multiscan spectrum, and the untreated cells served as the controls. The results were expressed as percent viability = $A_{540}(\text{treated cells}) - \text{background} / A_{540}(\text{untreated cells}) - \text{background} \times 100$.

Acknowledgements

Financial support from the Department of Science and Technology, DST (to P. V. Srilakshmi), Government of India, New Delhi is gratefully acknowledged. MG thanks Council of Scientific and Industrial Research for the senior research fellowship. HR thanks DST for the senior research fellowship. We greatly acknowledge Dr. Vijaya Gopal, principal scientist in the Center for Cellular and Molecular Biology and her group for extending their lab facilities to us and also for their immense help in carrying out the experiments.

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