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Solid phase synthesis of novel asymmetric hydrophilic head cholesterol-based cationic lipids with potential DNA delivery

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ABSTRACT

Twenty-four asymmetric divalent head group cholesterol-based cationic lipids were designed and synthesized by parallel solid phase chemistry. These asymmetric head groups composed of amino functionality together with trimethylamino, di(2-hydroxyethyl)amino or guanidinyl groups. Spacers between cationic heads and linker were both equal and unequal in length. These lipids were subjected to evaluation for DNA binding affinities by gel retardation assay and were screened for their transfection efficiency on HEK293 cells. Cationic lipids with equal chain length exhibited high transfection efficiency when polar part contained asymmetric polar heads. In contrast, lipids with unequal chain length exhibited high transfection efficiency when polar part contained symmetric heads. According to the optimal formulation, seven lipids exhibited higher transfection efficiency than the commercially available transfection agents, Effectene™, DOTAP and DC-Chol, to deliver DNA into PC3 human prostate adenocarcinoma cells. 3β-[*N*-(*N*′-Guanidinyl)-2′-aminoethyl)-*N*-(2-aminoethyl)carbamoyl] cholesterol (**5**) bearing amino and guanidinyl polar heads exhibited highest transfection efficiency with minimal toxicity. The morphology of active liposomes was observed by transmission electron microscopy (TEM) and size of liposomes were around 200–700 nm.

1. Introduction

Gene therapy is an approach to treat genetic disorders, AIDS and other acquired genetic defects. This method is the replacement of a defective gene with a normal one. Various techniques involving viral and non-viral vectors have been developed to insert genetic material into recipient cells.[1] Much effort has been devoted to the development of non-viral delivery due to the disadvantages of viruses used for gene delivery which include generation of immune responses.[2] Consequently, non-viral vector was chosen for DNA delivery. Non-viral vectors can be classified into two major categories: physical methods (e.g., microinjection,[3] hydrodynamic,[4] particle bombardment,[5] electroporation,[6] ultrasound[7] and encapsulated microsphere[8]) and chemical methods (e.g., DEAE–dextran,[9] calcium phosphate,[10] cationic lipid,[11] cationic polymer[12] and cationic dendrimer[13]). Of all the non-viral chemical vectors, cationic lipid is the chemical transfection agent for delivery of nucleic acids using liposomes which hold great promise as a safe and non-immunogenic approach to gene delivery.[14]

Cationic lipids form liposomes when formulated in water under optimal conditions.[15] The surface of these liposomes is positively charged and is attracted electrostatically to the phosphate backbone of DNA, as well as to the negatively charged surface of the cell membrane. Lipoplexes, which are also known as liposome–DNA complex, play a central role in current approaches to gene delivery, serving as potent transfection vectors.[16] The mechanism of DNA intake is not exactly known but is believed to be related to endocytosis.[17] To achieve DNA delivery, lipoplex complexes need to bind the cell surface, across the membrane, release DNA into the cytoplasm, and finally transport the DNA into the nucleus.[18] Most cationic liposomes have a common neutral phospholipid in addition to the cationic lipid component. The phospholipid is needed for stabilizing most type of cationic lipids in a lipid bilayer and may provide the cell penetration function of cationic liposomes.[19] In this report the neutral phospholipid, dioleoylphosphatidyl ethanolamine (DOPE), was used as a helper lipid.

Since the key invention of cationic liposome in DNA delivery,[11] several cationic lipids having various cationic headgroups, linkers and hydrophobic tails were reported. Cholesterol-based cationic lipids[20] are among the most promising agents that are potentially and safely delivery gene into cells. Cholesterol was often used as a lipid anchor because of its lipid bilayer stabilizing activity[21] and minimal

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toxicity to the treated cells.[20] Cholesterol-based cationic lipids with variation of headgroups, mono-, di-, and polyvalent, have been synthesized and tested for their transfection efficiency.[20,22–24] Cholesterylspermidine,[22] BGTC[23] and Lipid 67[24] are samples of cholesterol-based cationic lipids bearing symmetric terminal polar groups (Fig. 1). To our knowledge, cholesterol-based lipid with unsymmetric terminal cationic heads has not been reported. We report here the synthesis of asymmetric hydrophilic head cholesterol-based cationic lipids and transfection efficiency evaluation. To study structure–transfection activity relationship, cationic lipids with symmetric polar head were synthesized and compared the transfection activity of those asymmetric lipids (Fig. 2).

2. Results and discussion

2.1. Synthesis

The synthesis of symmetric and asymmetric terminal hydrophilic head lipids (**1**–**2** and **3**–**8**, respectively) having symmetry

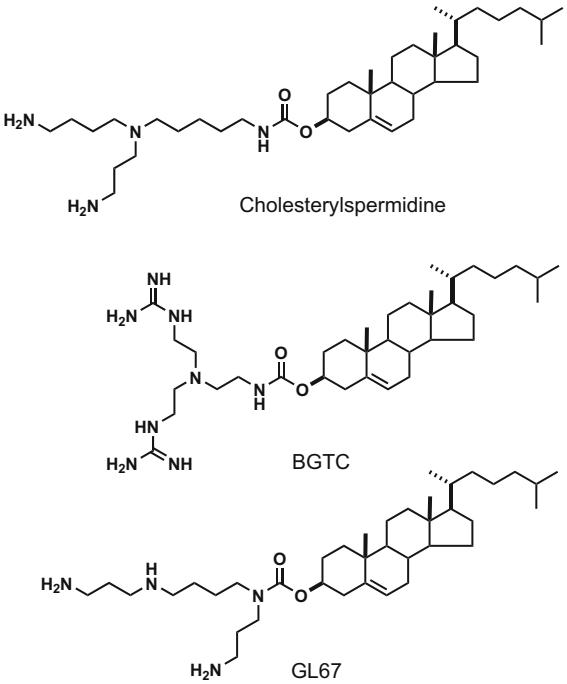
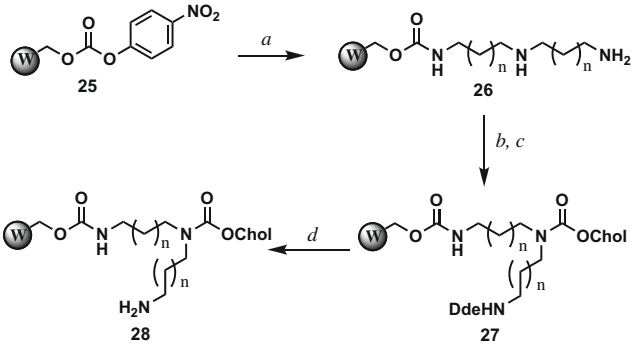


Figure 1. Structures of symmetrical edge-head cholesterol-based cationic lipids.



Scheme 1. Reagents and conditions: (a) diethylenetriamine or bis(3-aminopropyl)amine (excess), CH_2Cl_2, 6 h; (b) Dde-OH (excess), CH_2Cl_2, DMF, 12 h; (c) cholesteryl chloroformate (4 equiv), pyridine, CH_2Cl_2, 12 h; (d) 5% N_2H_4/DMF, 2×30 min.

spacer length (library 1, Fig. 2) was carried out as shown in Schemes 1 and 2. The key template **28** used for the synthesis of cationic lipids library 1 was synthesized as outlined in Scheme 1. The active carbonate resin **25**[25] was reacted with polyamine, diethylenetriamine or nor-spermidine, to afford the resin **26**. The primary amine was selectively protected with Dde-OH[26] and the secondary amine was capped with cholesteryl chloroformate to generate the resin **27**. The key intermediate **28** was obtained after treatment the resin **27** with 5% N_2H_4 in DMF. Treatment of the resin **28** with 50% TFA in CH_2Cl_2 gave the lipids **1** and **2**. The free primary amine on the resin **28** allowed different designs for the cationic head. Numbers of cationic heads, trimethyl quaternary amine,[11,20a] guanidinium[23,27] and amine having hydroxyethyl group[28,29] have been synthesized and evaluated for their transfection efficiency. Some of them exhibited remarkably high transfection efficiencies. Reaction of the resin **28** with methyl iodide in the presence of DIEA base for 18 h generated the resin **29**, which was treated with 50% TFA in CH_2Cl_2 to obtain the desired lipids **3** and **4**. The synthesis of the lipids **5** and **6** was accomplished by treatment the resin **28** with *N*′,*N*′-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea[27] for 18 h followed by cleavage with 50% TFA in CH_2Cl_2. The lipids **7** and **8** were also synthesized by reaction of the resin **28** with 2-bromoethanol for 18 h followed by cleavage with TFA.

To investigate the effect of the chain length between cationic head and linker on the transfection efficiency, cationic lipids library 2 was synthesized (Fig. 2) as shown in Schemes 3 and 4. The resin **32**, which was prepared by reacting the active carbonate **25** with appropriate diamine, was coupled with bromoacetic acid

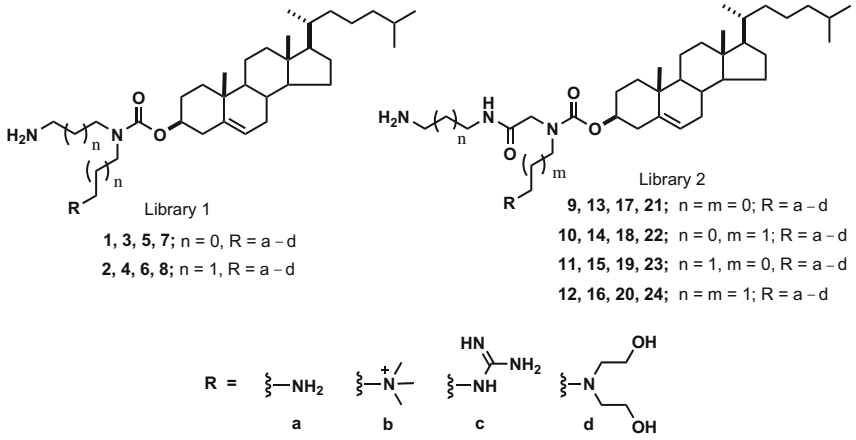
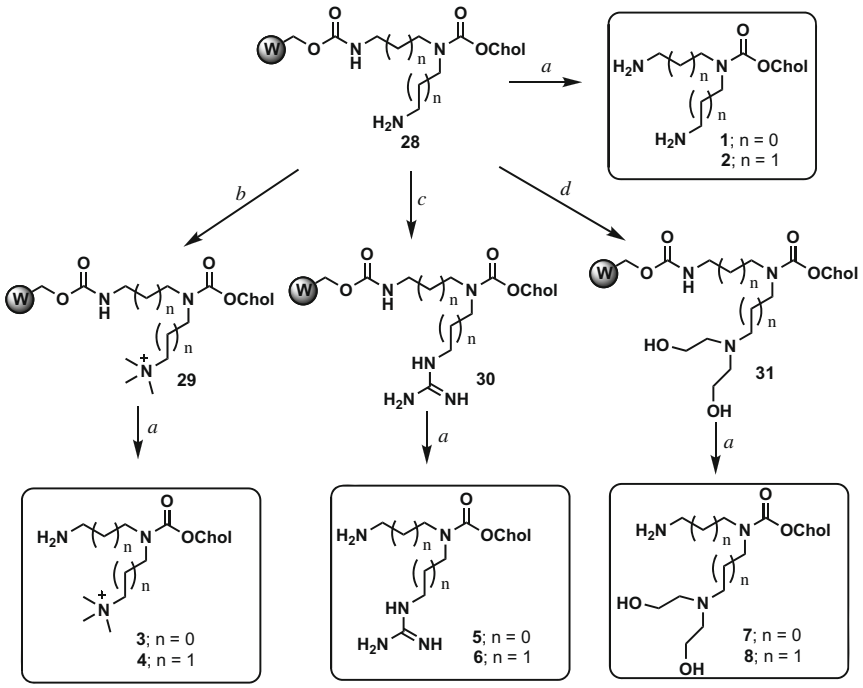
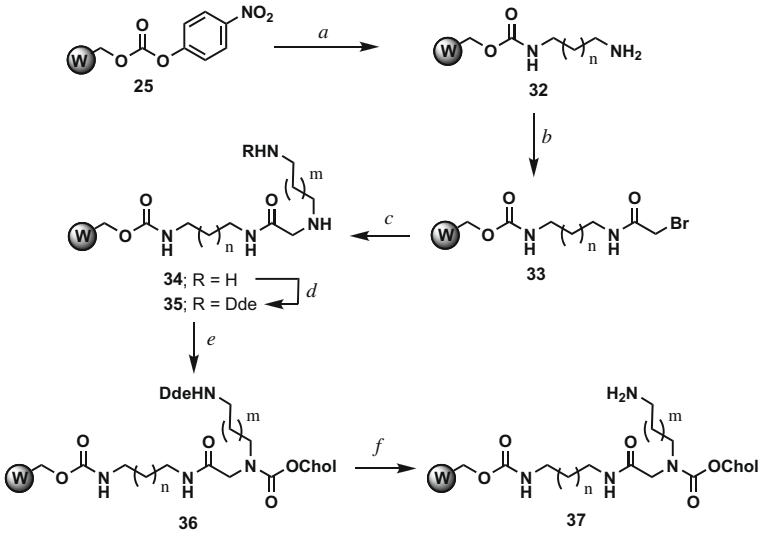


Figure 2. Structures of new asymmetrical polar head cholesterol-based cationic lipids.



Scheme 2. Reagents and conditions: (a) 50% TFA/CH_2Cl_2, 2 h; (b) MeI (10 equiv), DIEA, DMF, 18 h; (c) *N,N′*-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea (4 equiv), DIEA, DMF, 18 h; (d) 2-bromoethanol (8 equiv), DIEA, 18 h.

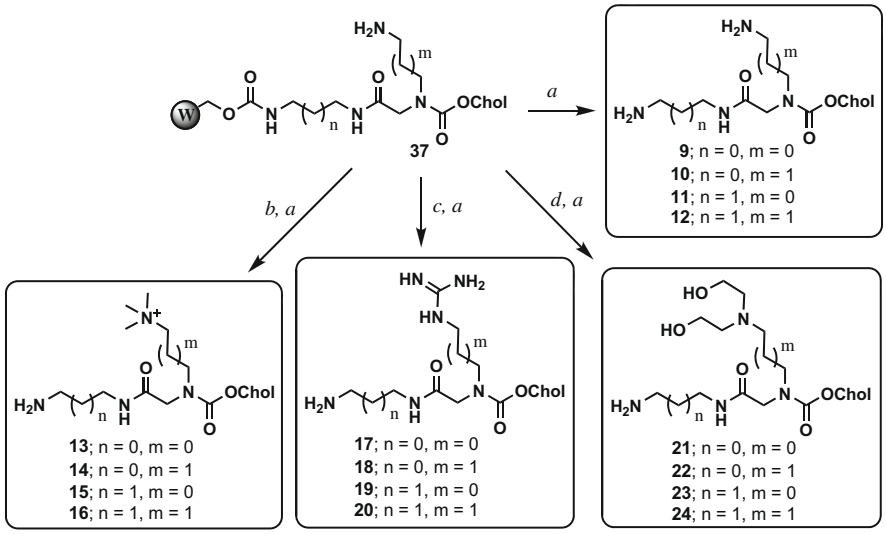


Scheme 3. Reagents and conditions: (a) 1,2-diaminoethane or 1,3-diaminopropane (excess), CH_2Cl_2, 6 h; (b) bromoacetic acid (4 equiv), DIC (4 equiv), DMF, 12 h; (c) 1,2-diaminoethane or 1,3-diaminopropane (excess), DMF, 12 h; (d) Dde-OH (excess), CH_2Cl_2, DMF, 12 h; (e) cholesteryl chloroformate (4 equiv), pyridine (20 equiv), CH_2Cl_2, 12 h; (f) 5% N_2H_4/DMF, 2 × 30 min.

to obtain the resin **33**, which was then reacted with different diamine to generate the resin **34**. The primary amine of the resin **34** was selectively protected with Dde-OH allowing the remaining amine to couple to the cholesterol tail. Thus, the resin **35** was reacted with cholesteryl chloroformate to provide the resin **36**. The Dde group was removed with 5% N_2H_4 in DMF to give the scaffold **37**. Cationic lipids **9**–**12** were obtained after TFA treatment (Scheme 4). The lipids **13**–**24** which contain various polar heads were prepared in the same manner as described for library 1. All synthesized lipids **1**–**24** were obtained in 32–70% yields (based on original loading of Merrifield resin and as TFA salts). Structure elucidation of these lipids was achieved by spectroscopic means (^1H and ^{13}C NMR, IR and mass spectra).

2.2. DNA binding affinity

The relative DNA binding affinities of cationic lipids were evaluated to determine whether transfection activities correlated with DNA binding. The DNA binding affinities were determined by gel retardation assay. Cationic lipids were mixed with plasmid DNA, known as lipoplex, at weight ratios of 1:20 (DNA/sample, w/w) and the lipoplexes were loaded on agarose gel (Fig. 3). The result indicated that most of lipids from library 1 interacted sufficiently with DNA to retard migration through the gel matrix except the lipid **5**. Lipids with equal chain length (library 1) were able to bind to DNA more sufficient than the lipids with unequal chain length (library 2) at the DNA/lipid weight ratio of 1:20. For library 2, the



Scheme 4. Reagents and conditions: (a) 50% TFA/CH_2Cl_2, 2 h; (b) MeI (10 equiv), DIEA, DMF, 18 h; (c) *N,N′*-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea (4 equiv), DIEA, DMF, 18 h; (d) 2-bromoethanol (8 equiv), DIEA, 18 h.

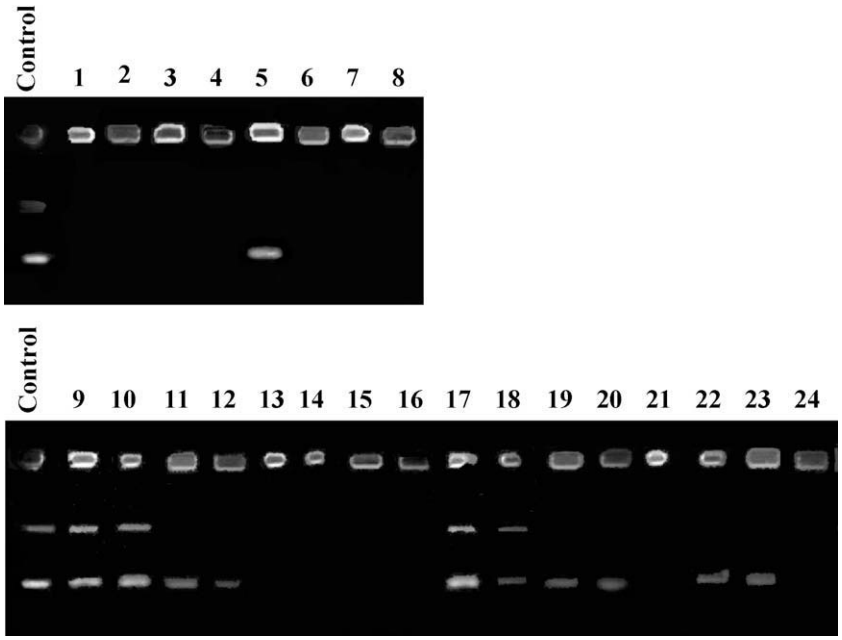


Figure 3. Gel retardation assay of DNA/cationic lipids complexes at a weight ratio of 1:20. Lanes marked 'Control' contained DNA alone and was used as a control. The presence of a lower band indicated that DNA has migrated and has not been bound by the transfection compound.

lipids **13–16** bearing trimethyl quaternary amine bound sufficiently with DNA whereas the lipids with the amino (**9–12**) and guanidinyl (**17–20**) head groups did not fully bind to DNA. It should be noted that the lipids **3–4** and **13–16** which possess permanent charge bound strongly to DNA to retard migration.

2.3. Transfection activity

2.3.1. Transfection screening

All the synthesized cationic lipids were tested for DNA delivery to HEK293 (Human embryonic kidney cell lines) using β-galactosidase as a reporter gene. Figure 4 shows the transfection screening results using plasmid DNA (0.1 μg/well) at DNA/liposome ratio 1:20 (w/w). The liposome formation in the preliminary transfection activity screening was with and without DOPE. In the case of liposome formation with DOPE, cationic lipid/DOPE ratio was 2:1 (w/w). The results indicated that eight lipids, **2**, **5–8**, **10–11** and **19**, exhibited relative transfection efficiency over 100% as compared to Effectene™ transfection (100%). Most the lipids in library 1 bearing unsymmetrical polar head and symmetrical chain length exhibited high transfection efficiency. In contrast, cationic lipid library 2 (**10** and **11**) with symmetric polar head and unsymmetric chain length showed higher transfection activity than Effectene™. From the screening result, eight lipids which exhibited higher transfection efficiency than the positive control were subjected to further optimization. To find out the optimized transfection efficiency of these lipids, the lipids/DOPE ratios, DNA/lipids ratios, and DNA per well were studied.

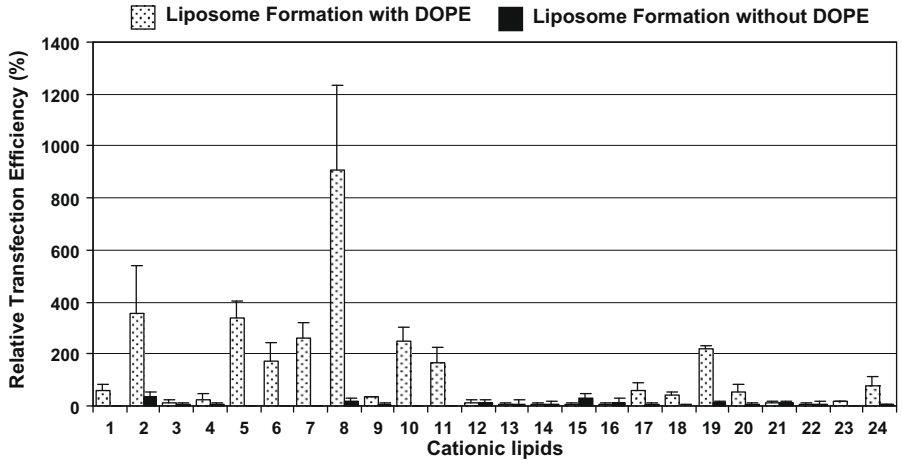


Figure 4. Transfection screening activity of synthesized cationic lipids employing pCH110-encoding β-galactosidase (0.1 μg/well). The liposome formation was both with DOPE and without DOPE. The lipoplexes was used at DNA/lipids (w/w) ratios of 1:20. The transfection efficiencies of the lipids were compared to that of commercially available reagent, Effectene™, which calculated as 100% transfection efficiency (data not shown).

2.3.2. Optimization of cationic lipid/DOPE ratios

Helper lipid, DOPE, has been known to increase the transfection efficiency of cationic liposome to transfer and release DNA into the cytoplasm.[30] In order to find out the most effective formulations, transfections with equal DNA/lipid weight ratio (1:20) and varying the weight ratio of lipids (**2**, **5–8**, **10–11** and **19**)/DOPE were performed (Fig. 5). The optimized lipid/DOPE ratio was found to vary for each lipid. Among the compound tested, at a lipid/DOPE ratio of 1:2, the lipid **8** was found to be the most effective compound to deliver DNA into the HEK293 cells (900%). The transfection efficiency of the lipid **8** decreased dramatically when the weight ratio of lipid/DOPE decreased. The lipids **10** and **19** showed the maximum efficiency at a lipid/DOPE ratio of 2:1 whereas the lipids **5** and **11** exhibited the highest efficiency at a lipid/DOPE ratio of 1:1. The optimal formulation of each lipid was used for the next experiment.

2.3.3. Optimization of DNA/amount of cationic lipids ratios

By using the respective optimized lipid/DOPE ratio for each lipid, all the selected lipids were tested at the equal amount of DNA (0.1 μg/well) and varying the amount of lipid. Three DNA/cationic lipid ratios, 1:10, 1:20 and 1:40 (w/w), were prepared and evaluated for transfection efficiency and the result is shown in Figure 6. The transfection efficiency of the lipids **2**, **7**, **8**, **11** and **19** was found to decrease at lower DNA/lipid ratio. In contrast, the lipids **5** and **6** exhibited highest transfection efficiency when lower DNA/lipids ratio was used. The optimal DNA/cationic lipid ratio for each lipoplex formation was used for further optimization.

2.3.4. Optimization of the amount of DNA

To see whether variation in the amount of DNA affected the transfection efficiency of these lipids, the experiments were performed using the optimal lipid/DOPE (Fig. 5) and DNA/lipid (Fig. 6) ratios. The amounts of DNA used in the experiment were 0.1, 0.2 and 0.4 μg/well. The lipids **2**, **6**, **8** and **11** exhibited higher transfection efficiency when the amount of DNA increased (Fig. 7). The results have indicated that these lipids are more efficient at high DNA concentration. At the highest amount of DNA (0.4 μg/well), the lipid **8** exhibited the highest transfection activity.

2.3.5. The effect of serum

One of the major draw backs of cationic lipids for their in vivo use is the inhibition of the transfection efficiency of cationic liposomes in the presence of serum. Most of cationic lipids which

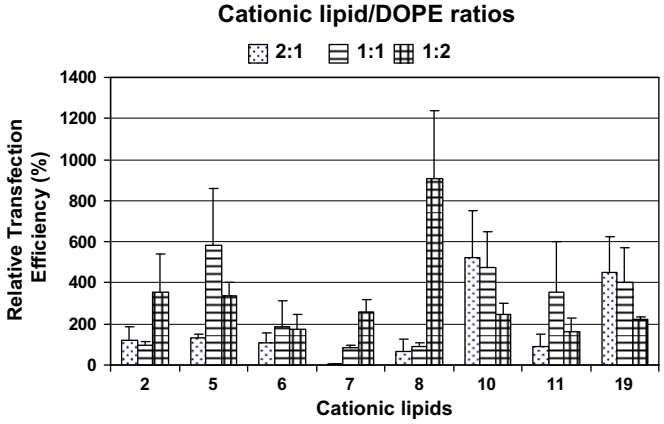


Figure 5. In vitro transfection efficiency of the selected lipids in HEK293 cells across the cationic lipid/DOPE weight ratio of 2:1, 1:1 and 1:2. Transfection efficiency of the lipids was compared to that of the commercial reagent, Effectene™, which calculated as 100% transfection efficiency (data not shown).

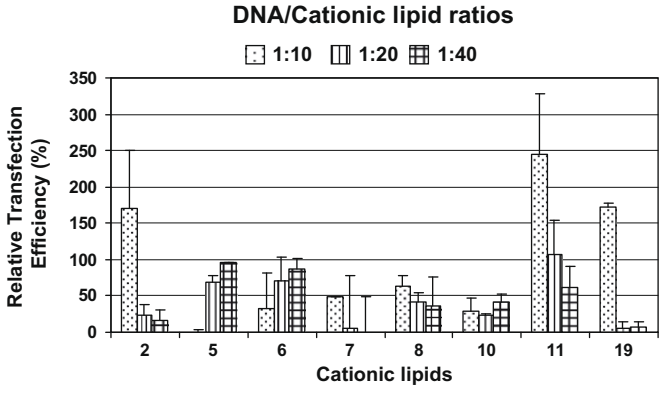


Figure 6. In vitro transfection efficiency of the selected lipids in HEK293 cells across the DNA/cationic lipid weight ratios of 1:10, 1:20 and 1:40 (w/w). The optimal liposome formation of each lipid from Figure 5 was used as described in the text. Transfection efficiency of the selected lipids was compared to that of the commercial reagent, Effectene™, which calculated as 100% transfection efficiency (data not shown).

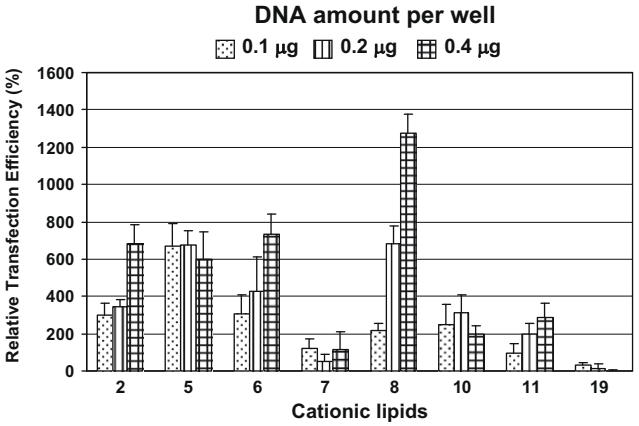


Figure 7. Effect of DNA amount for gene delivery. The optimal liposome formation (Fig. 5) and DNA/lipids complex (Fig. 6) of each lipid were used to mix with various amount of DNA from 0.1 to 0.4 μg. Transfection efficiency of the lipids was compared to that of the commercial reagent, Effectene™, which calculated as 100% transfection efficiency.

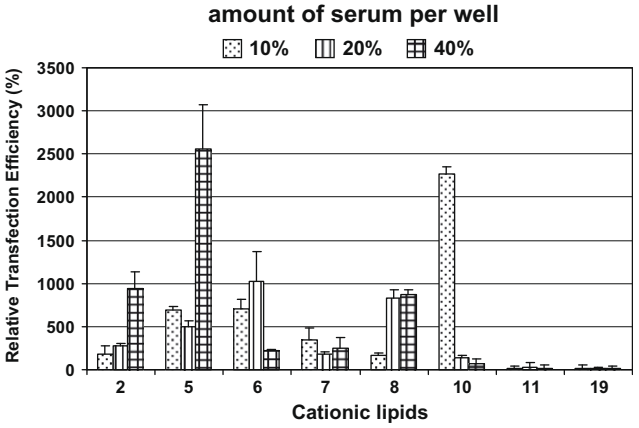


Figure 8. Effect of serum for transfection efficiency. The optimal liposome formation (Fig. 5), DNA/cationic lipid (Fig. 6) and amount of DNA per well (Fig. 7) of each lipid were used to transfer gene to HEK293 cell at various amount of serum. Transfection efficiency of the lipids was compared to that of the commercial reagent, Effectene™, which calculated as 100% transfection efficiency.

exhibited high transfection activity in the absence of serum lost their efficiency when transfected in the presence of serum.[20b,c] The selected lipids with the optimal condition of each lipid from Figures 5–7 were tested for the effect of serum on gene transfection efficiency. These experiments were carried out in the presence of 10%, 20% and 40% serum. The results are shown in Figure 8. The lipids **2** and **5** exhibited highest transfection efficiency (1000% and 2600%, respectively) when the experiment was performed under 40% serum. Transfection efficiency of the lipid **10** reached its maximum when 10% serum was present. Transfection efficiency of this lipid was significantly decreased when the cells were tested in the presence of 20% and 40% serum. At 20% and 40% serum, the lipid **8** showed similar activity to transfer DNA into cells. The lipids **11** and **19** could not deliver DNA into cells when the experiment was carried out under serum containing condition.

2.3.6. Transfection efficiency toward different cell lines

It is well-known that transfection agents have ability to specifically deliver DNA into different cell types. To evaluate the transfection efficiency of these lipids toward the different mammalian cell lines, COLO 205, D-17, HeLa and PC3 cells, the experiments were performed using optimum conditions of each lipid (Figs. 5–7). These four cells were chosen as representing cancers of importance to human health. Three commercially available transfection agents, Effectene™, DOTAP and DC-Chol, were tested under identical conditions for comparison. The experiment was carried out under serum-free condition. The transfection results are shown in Figure 9. All of lipids, except **6**, exhibited higher transfection efficiency to deliver DNA into PC3 cells than Effectene™, DOTAP and DC-Chol. Lipid **5** exhibited highest activity having relative transfection efficiency of 3200%. All the compounds tested could not reach their transfection efficiency of 500% against COLO 205, D-17 and HeLa cells. The results indicated that transfection efficiency of these newly synthesized lipids was cell dependent.

2.3.7. Transfection toxicity

Cytotoxicity of synthesized cationic lipids is very important for gene delivery. To assess the relationship between cytotoxicity and transfection efficiency, the toxicity of synthesized lipids on HEK293, COLO 205, D-17, HeLa and PC3 cell lines using optimal condition (Figs. 5–7) were determined by measuring changes in cell metabolic activity (MTT assay). The results were shown as % cell viability as compared to the control cells in the presence of

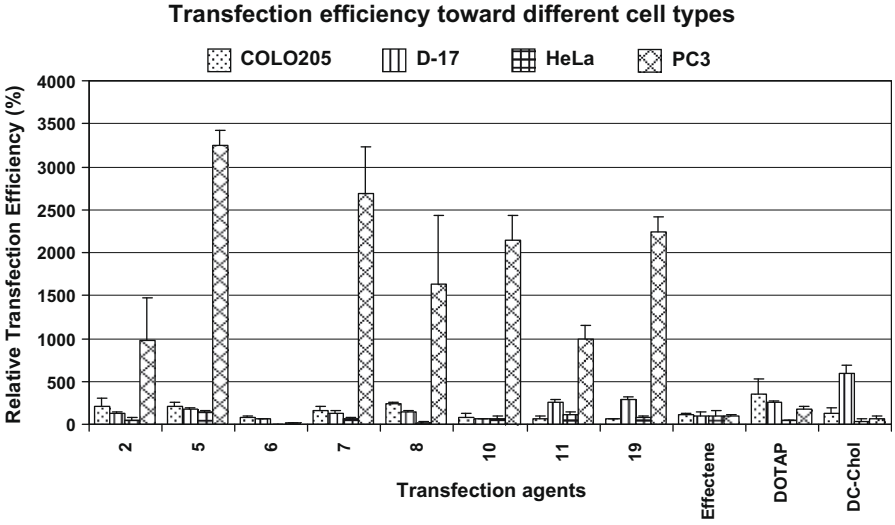


Figure 9. Transfection efficiency of selected lipids toward COLO 205, D-17, HeLa and PC3 cell using optimum conditions from Figures 5–7. Transfection efficiency of the lipids was compared to that of the commercial reagent, Effectene™, DOTAP and DC-Chol. Transfection efficiency of Effectene™ for each cell line was calculated as 100%.

DNA (Fig. 10). Most of the lipids including the commercially available agents showed no cytotoxicity to all cell types. The lipid **7** was toxic to the cells tested and cell viability was lower than 50%. This result was contrast to the previous experiment which the lipid **7** exhibited relative transfection efficiency of 2700% against PC3 cells.

2.4. Transmission electron microscopy (TEM)

The morphology of the prepared liposome from the synthesized cationic lipids, which were used to study transfection optimization, was observed under transmission electron microscopy (TEM) after negative staining. The liposome of each lipid was prepared at its optimal lipid/DOPE ratio. The morphology of liposomes was shown in Figure 11. The diameter of the aggregates spanned from 200 to 700 nm. Most of the aggregates showed vesicle-like organizations. Morphology of liposomes/DNA complexes (lipoplexes) was also visualized under transmission electron microscopy as shown in Figure 12. Most of the lipoplexes were found to be large spherical aggregates which the sizes are within the range 300–1000 nm. In this study, sizes of lipoplexes prepared from these lipids are unlikely to play important role in modulating for transfection efficiency.

In summary, cholesterol-based cationic lipids have been of interest to many researchers for its use in gene delivery applications. Cholesterol-based cationic lipids can be easily synthesized with different polar head to enhance transfection efficiency. Toward this end, we have synthesized asymmetric divalent polar head groups cholesterol-based cationic lipids bearing amino group together with either trimethylamino, di(2-hydroxyethyl)amino or guanidinyl functionality. Based on the results reported here, new cationic lipids required neutral lipid, DOPE, to enhance transfection efficiency. It was found that cationic lipids library 1, which possessed equal spacer length, exhibited high transfection efficiency when bearing the asymmetric polar heads. However, when the unequal spacer length was introduced into cationic lipid library 2 the

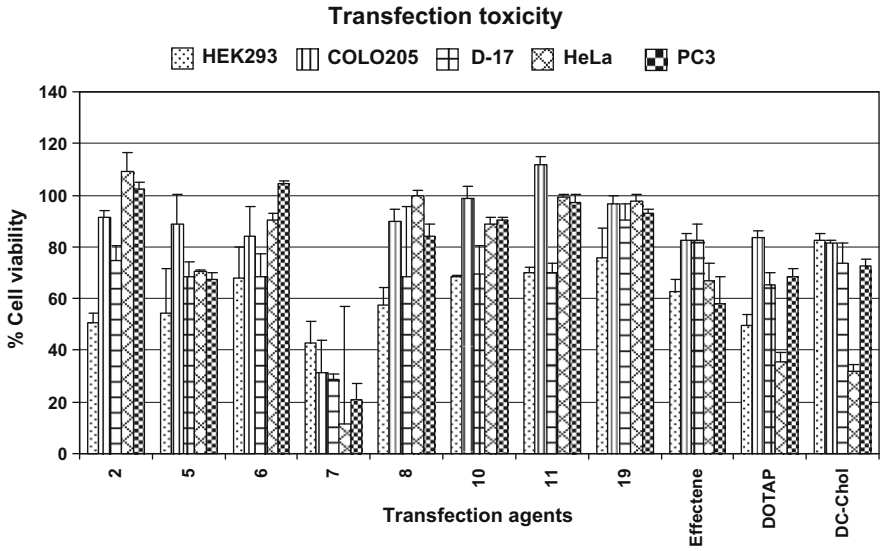


Figure 10. Effect of transfection synthesized lipids on cell metabolic activity. Liposomes of cationic lipid with helper lipid, DOPE, were formed and added to DNA to form lipoplexes. These complexes were added to HEK293, COLO 205, D-17, HeLa and PC3 cell. Cell metabolic activity was determined by a MTT assay.

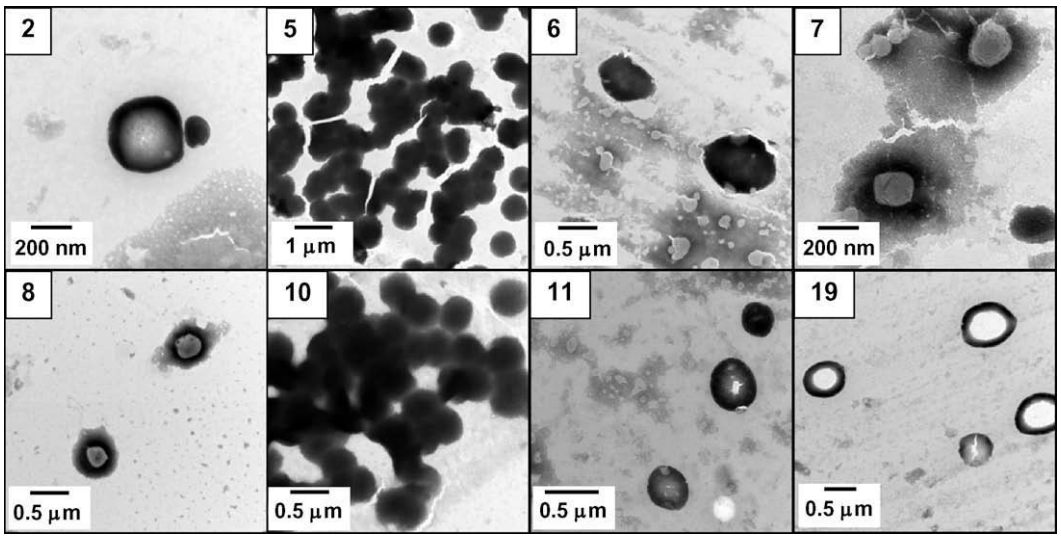


Figure 11. Transmission electron microscopic images of cationic liposomes **2**, **5**–**8**, **10**, **11** and **19**.

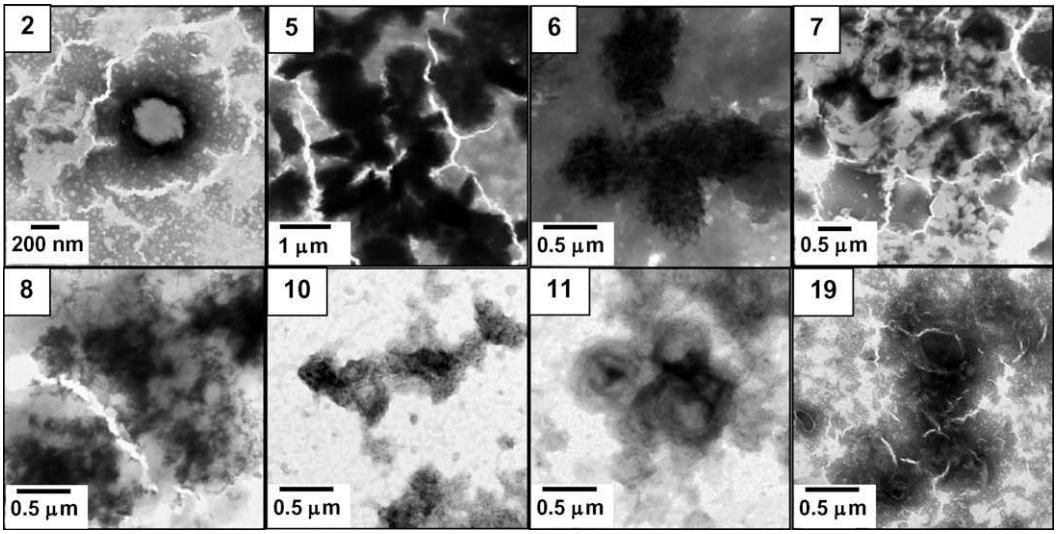


Figure 12. Transmission electron microscopic images of lipoplexes prepared from optimized liposomes (**2**, **5**–**8**, **10**, **11** and **19**)/DNA complexes.

transfection efficiency decreased. The optimal formulation (DOPE/lipid ratio, DNA/lipid ratio, amount of lipid) of each lipid for highest transfection efficiency was dependent on each compound. Seven cationic lipids exhibited greater transfection efficiency than commercially available transfection agents, Effectene™, DOTAP and DC-Chol, against PC3 (human prostate adenocarcinoma) cells. We have found that cationic lipid containing amino and guanidinyl polar headgroup with equal chain length (lipid **5**) is the most efficient one. The guanidinyl group might contribute to high transfection efficiency. It has been known that guanidinyl group processes several interesting features especially the high pK_a nature of guanidinyl group.[31] This makes it remain protonated at a broad range of pH and transfection efficiency should be insensitive to variations of pH during in vitro transfection. The optimal conditions for lipid **5** to exhibit highest transfection efficiency against PC3 cells included lipid/DOPE at weight ratio of 1:1, DNA/liposome ratio of 1:40 and the amount of DNA 0.1 μg/well. Most importantly, this lipid was compatible for high serum condition make it promising non-viral transfection vector for further in vivo study.

3. Experimental

3.1. General

NMR spectra were recorded on a Bruker AVANCE 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. All coupling constants (*J* values) were measured in hertz. ES mass spectra were recorded with a Finnigan LCQ mass spectrometer. Infra-red spectra were recorded on a Perkin–Elmer Spectrum GX60237. The size and morphology of the cationic liposomes were recorded on JEM-2100, JEOL electron microscope. Starting materials and reagents were purchased from commercial suppliers and used without further purification.

3.2. Solid phase synthesis of transfection agent

3.2.1. Synthesis of lipids library 1

To the active carbonate resin **25**[25] (1 equiv, 1.1 mmol/g) was added an excess of diethylenetriamine or bis(3-aminopropyl)amine in CH_2Cl_2 (10 mL). The suspension was shaken for 6 h. The resin was filtered and washed successively with CH_2Cl_2, DMF, MeOH, DMF and CH_2Cl_2 (3 × 10 mL each). The resulting resin **26** was dried under vacuum for 2 h and gave a positive ninhydrin test.[32] The solution of Dde-OH (excess) in CH_2Cl_2/DMF was added to the resin **26**. The reaction was shaken for 12 h. The resulting resin was filtered and washed successively with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3 × 10 mL each) to afford the negative ninhydrin test resin. After that, the resin was dried under vacuum for 2 h and reacted with cholesteryl chloroformate (4 equiv) in CH_2Cl_2 using pyridine (2 mL) as a base for 12 h. The resulting resin was filtered and washed successively with CH_2Cl_2, DMF, MeOH, DMF and CH_2Cl_2 (3 × 10 mL each) to give the desired resin **27**. The Dde-protecting group was removed with 5% N_2H_4 in DMF for 2 × 30 min to give free amino resin **28**. The resin **28** gave a positive ninhydrin test. The resin was washed successively with CH_2Cl_2 and MeOH (3 × 10 mL each) before cleavage at the last step. The dried resin **28** was treated with 50% TFA/CH_2Cl_2 for 2 h. The resin was filtered and the solution was collected. The solvents were removed under a stream of nitrogen and evaporated under reduced pressure to give the desired product **1** and **2**.

3.2.1.1. 3β-[*N*,*N*-(2,2′-Diaminoethyl)carbamoyl]cholesterol (1). Yield: (resin: 1.1 mmol/g, 214.5 mg) 45.8 mg, 38%; IR (CH_2Cl_2): ν_{max} 3436, 2918, 2849, 1773, 1682, 1541, 1507, 1463, 1381, 1202, 1021 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$ + 9 drops of CD_3OD): δ 0.53 (s, 3H, CH_3-18), 0.765 and 0.769 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27, overlapping signal), 0.80 (d, J = 6.4 Hz, 3H, CH_3-21), 0.91 (s, 3H, CH_3-19), 0.91–2.26 (m, 30H, protons in cholesteryl skeleton), 3.05 (m, 4H, ($H_2NCH_2CH_2$)$_2$N), 3.38 (m, 4H, ($H_2NCH_2CH_2$)$_2$N), 3.48 (br s, 1H, H-3-Chol), 5.26 (br s, 1H, H-6-Chol), 8.00–8.4 (br s, 6H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 9 drops of CD_3OD, 100 MHz): 11.6, 18.5, 18.9, 20.8, 22.3, 22.5, 23.7, 24.1, 27.6, 27.8, 28.0, 31.6, 31.7, 35.6, 35.8, 36.0, 36.3, 38.0, 39.3, 39.5, 42.1, 49.8, 56.0, 56.5, 76.7, 122.7, 139.4 (carbons in cholesteryl skeleton), 38.5 (($H_2NCH_2CH_2$)$_2$N), 46.7 (($H_2NCH_2CH_2$)$_2$N), 156.9 (C=O carbamoyl); MS (ES$^+$): *m*/*z*: 516 ([*M*+H]$^+$, 100%).

3.2.1.2. 3β-[*N*,*N*-(3,3′-Diaminopropyl)carbamoyl]cholesterol (2). Yield: (resin: 1.1 mmol/g, 123.9 mg) 52.4 mg, 70%; IR (CH_2Cl_2): ν_{max} 3355, 2918, 2845, 2280, 1675, 1600, 1509, 1468, 1429, 1202 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$ + 5 drops of CD_3OD): δ 0.62 (s, 3H, CH_3-18), 0.811 and 0.815 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27, overlapping signal), 0.86 (d, J = 6.2 Hz, 3H, CH_3-21), 0.90 (s, 3H, CH_3-19), 0.90–2.28 (m, 32H, protons in cholesteryl skeleton and ($H_2NCH_2CH_2CH_2$)$_2$N), 2.88 (br s, 4H, (H_2NCH_2 CH_2CH_2)$_2$N), 3.26 (br s, 4H, ($H_2NCH_2CH_2CH_2$)$_2$N), 4.39 (br s, 1H, H-3-Chol), 5.30 (br s,

1H, H-6-Chol), 7.94 (br s, 6H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 5 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.1, 20.9, 22.4, 22.7, 23.9, 24.2, 27.9, 28.1, 31.7, 35.8, 36.1, 36.4, 36.8, 38.2, 39.4, 39.6, 42.2, 49.9, 56.2, 56.6, 75.8, 122.6, 139.4 (carbons in cholesteryl skeleton), 25.7 and 26.4 (($H_2NCH_2CH_2CH_2)_2N$), 27.2 and 38.4 (($H_2NCH_2CH_2$-$CH_2)_2N$), 44.2 and 44.6 (($H_2NCH_2CH_2CH_2)_2N$), 156.5 (C=O carbamoyl); MS (ES^+): *m/z* 544.7 ($[M+H]^+$, 100%).

3.2.2. Synthesis of lipids 3 and 4

To the free amino resin **28** (1 equiv, 148.3 and 139.3 mg, 1.1 mmol/g) was added methyl iodide (10 equiv, 83 and 78 μL) and DIEA (10 equiv, 279 and 261 μL) in DMF (2 mL). The suspension was shaken for 18 h. The resin was washed successively with CH_2Cl_2, DMF, MeOH, DMF and CH_2Cl_2 (3 × 2 mL each) to afford resin **29**. The resin **29** was cleaved in the same manner of the synthesis of lipids **1** and **2** to give lipids **3** and **4**.

3.2.2.1. 3β-[*N*-((*N′*,*N′*,*N′*-Trimethyl)-2′-aminoethyl)-*N*-(2-aminoethyl)carbamoyl]cholesterol (3). Yield: (resin: 1.1 mmol/g, 148.3 mg) 48.0 mg, 53%; IR (CH_2Cl_2): ν_{max} 3436, 2932, 2840, 1681, 1469, 1426, 1202, 1132 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 6 drops of CD_3OD): δ 0.60 (s, 3H, CH_3-18), 0.788 and 0.792 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27, overlapping signal), 0.84 (d, J = 6.4 Hz, 3H, CH_3-21), 0.94 (s, 3H, CH_3-19), 0.96–2.30 (m, 30H; protons in cholesteryl skeleton), 3.06 (s, 9H, (CH_3)$_3N^+$), 3.10 (br s, 2H, $H_2NCH_2CH_2N$), 3.48–3.77 (m, 6H, $CH_2CH_2NCH_2$-$CH_2N(CH_3)_3$), 4.40 (br s, 1H, H-3-Chol), 5.30 (br s, 1H, H-6-Chol), 8.00–8.30 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 6 drops of CD_3OD, 100 MHz): 11.6, 18.5, 19.0, 20.9, 22.3, 22.6, 23.7, 24.1, 27.8, 28.0, 31.8, 35.6, 36.0, 36.4, 36.7, 39.3, 39.5, 42.1, 49.9, 56.0, 56.5, 76.6, 122.8, 139.1 (carbons in cholesteryl skeleton), 38.1 and 38.4 ($CH_2CH_2NCH_2CH_2$), 45.0 and 46.0 ($CH_2CH_2NCH_2CH_2$), 53.6 and 53.7 ($N(CH_3)_3$), 155.7 (C=O carbamoyl); MS (ES^+): *m/z* 559.4 ($[M+H]^+$, 100%).

3.2.2.2. 3β-[*N*-((*N′*,*N′*,*N′*-Trimethyl)-3′-aminopropyl)-*N*-(3-aminopropyl)carbamoyl]cholesterol (4). Yield: (resin: 1.1 mmol/g, 139.3 mg) 57.4 mg, 64%; IR (CH_2Cl_2): ν_{max} 3436, 2931, 1677, 1468, 1423, 1379, 1202, 1130 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 6 drops of CD_3OD): δ 0.61 (s, 3H, CH_3-18), 0.797 and 0.801 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27, overlapping signal), 0.85 (d, J = 6.3 Hz, 3H, CH_3-21), 0.94 (s, 3H, CH_3-19), 0.94–2.28 (m, 32H, protons in cholesteryl skeleton, $CH_2CH_2CH_2NCH_2CH_2CH_2$), 2.89 (br s, 4H, $H_2NCH_2CH_2CH_2NCH_2CH_2CH_2N(CH_3)_3$), 3.02–3.03 (br s, 9H, $N(CH_3)_3^+$), 3.27 (br s, 4H, CH_2NCH_2), 4.39 (br s, 1H, H-3-Chol), 5.29 (br s, 1H, H-6-Chol), 7.98 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 6 drops of CD_3OD, 100 MHz): 11.7, 18.5, 19.0, 20.9, 22.4, 22.6, 23.7, 24.1, 27.8, 28.1, 31.7, 35.7, 36.1, 36.4, 36.8, 38.3, 39.4, 39.6, 42.2, 49.9, 56.1, 56.6, 75.8, 122.6, 139.4 (carbons in cholesteryl skeleton), 25.7 and 26.4 ($CH_2CH_2NCH_2CH_2$), 37.0 ($H_2NCH_2CH_2CH_2N$), 38.4 ($CH_2CH_2CH_2N(CH_3)_3^+$), 44.3 ($NCH_2CH_2CH_2N(CH_3)_3$), 44.5 (H_2NCH_2 CH_2CH_2N), 53.1 ($N(CH_3)_3$), 156.1 (C=O carbamoyl); MS (ES^+): *m/z* 587.1 ($[M+H]^+$, 100%).

3.2.3. Synthesis of lipids 5 and 6

The resin **28** (1 equiv, 148.4 and 145.3 mg, 1.1 mmol/g) was added a solution of *N,N′*-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea (4 equiv, 190 and 186 mg) and DIEA (4 equiv, 111 and 109 μL) in DMF (2 mL). The suspension was shaken for 18 h to give resin **30**. The resin was successively washed with CH_2Cl_2, DMF, MeOH, DMF and CH_2Cl_2 (3 × 2 mL each). The resin **30** was cleaved in the same manner for the lipids **1**–**2**.

3.2.3.1. 3β-[*N*-((*N′*-Guanidinyl)-2′-aminoethyl)-*N*-(2-aminoethyl)carbamoyl]cholesterol (5). Yield: (resin: 1.1 mmol/g, 148.4 mg) 42.1 mg, 46%; IR (CH_2Cl_2): ν_{max} 3355, 3182, 2934, 2278, 1779, 1674, 1509, 1468, 1432, 1376, 1203, 1138, 1018 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 3 drops of CD_3OD): δ 0.63 (s, 3H, CH_3-18), 0.821 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27), 0.87 (d, J = 5.7 Hz, 3H, CH_3-21), 0.95 (s, 3H, CH_3-19), 1.00–2.30 (m, 30H, protons in cholesteryl skeleton), 3.07 (br s, 2H, $H_2NCH_2CH_2N$), 3.29 and 3.36 (br s, 4H, $H_2NCH_2CH_2NCH_2CH_2$), 3.51 (br s, 2H, NCH_2CH_2NHC-$(NH)NH_2$)), 4.39 (br s, 1H, H-3-Chol), 5.30 (br s, 1H; H-6-Chol), 8.01 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 3 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.0, 20.9, 22.4, 22.7, 23.7, 24.2, 27.6, 27.9, 28.1, 31.7, 31.8, 35.7, 36.1, 36.4, 36.8, 38.0, 39.4, 39.6, 42.2, 49.9, 56.0, 56.5, 76.6, 122.7, 139.3 (carbons in cholesteryl skeleton), 38.5 ($CH_2CH_2NCH_2CH_2$), 47.4 (CH_2NCH_2), 156.9 (C=O carbamoyl and C=N guanidine); MS (ES^+): *m/z* 558.6 ($[M+H]^+$, 100%).

3.2.3.2. 3β-[*N*-((*N′*-Guanidinyl)-3′-aminopropyl)-*N*-(3-aminopropyl)carbamoyl]cholesterol (6). Yield: (resin: 1.1 mmol/g, 145.3 mg) 52.4 mg, 56%; IR (CH_2Cl_2): ν_{max} 3350, 2918, 1673, 1509, 1465, 1429, 1203, 1134 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 6 drops of CD_3OD): δ 0.62 (s, 3H, CH_3-18), 0.805 and 0.809 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27, overlapping signal), 0.86 (d, J = 6.3 Hz, 3H, CH_3-21), 0.95 (s, 3H, CH_3-19), 1.00–2.28 (m, 32H, protons in cholesteryl skeleton and $CH_2CH_2NCH_2CH_2$), 2.87 (br s, 2H, $H_2NCH_2CH_2CH_2N$), 3.08 (br s, 2H, $CH_2CH_2NHC(NH)NH_2$), 3.19 (br s, 2H, $H_2NCH_2CH_2CH_2N$), 3.25 (br s, 2H, NCH_2CH_2 CH_2N $HC(NH)NH_2$), 4.38 (br s, 1H, H-3-Chol), 5.30 (br s, 1H, H-6-Chol), 7.97 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 6 drops of CD_3OD, 100 MHz): 11.7, 18.5, 19.0, 20.9, 22.4, 22.6, 23.8, 24.1, 27.9, 28.1, 31.7, 35.7, 36.1, 36.4, 36.8, 38.2, 39.4, 39.6, 42.2, 49.9, 56.1, 56.5, 76.0, 122.6, 139.4 (carbons in cholesteryl skeleton), 25.7 and 26.4 ($CH_2CH_2NCH_2CH_2$), 37.3 ($H_2NCH_2CH_2CH_2N$), 38.5 (NCH_2CH_2 CH_2N $HC(NH)NH_2$), 44.1 ($NCH_2CH_2CH_2NHC(NH)NH_2$), 44.6 (H_2NCH_2 CH_2CH_2N), 157.2 (C=O carbamoyl and C=N guanidine); MS (ES^+): *m/z* 586.7 ($[M+H]^+$, 100%).

3.2.4. Synthesis of lipids 7 and 8

The resin **28** (1 equiv, 152.8 and 159.3 mg, 1.1 mmol/g) was added 2-bromoethanol (8 equiv, 95 and 99 μL) and DIEA (8 equiv, 230 and 240 μL) in DMF (2 mL) and the suspension was shaken for 18 h. The resulting resin **31** was successively washed with CH_2Cl_2, DMF, MeOH, DMF and CH_2Cl_2 (3 × 2 mL each). The resin **31** was cleaved in the same manner of the lipids **1** and **2** to give lipids **7** and **8**.

3.2.4.1. 3β-[*N*-(*N′*,*N′*-Di(2″-hydroxyethyl)-2′-aminoethyl)-*N*-(2-aminoethyl)carbamoyl]cholesterol (7). Yield: (resin: 1.1 mmol/g, 152.8 mg) 41.5 mg, 41%; IR (CH_2Cl_2): ν_{max} 3335, 2932, 2840, 2279, 1674, 1597, 1541, 1509, 1413, 1310, 1203, 1132 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 5 drops of CD_3OD): δ 0.62 (s, 3H, CH_3-18), 0.809 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27), 0.86 (d, J = 6.2 Hz, 3H, CH_3-21), 0.95 (s, 3H, CH_3-19), 0.90–2.28 (m, 30H, protons in cholesteryl skeleton), 3.12 (br s, 4H, CH_2 $CH_2NCH_2CH_2$), 3.36 (br s, 4H, CH_2NCH_2), 3.54 (br s, 4H, $N(CH_2$ $CH_2OH)_2$), 3.88 (br s, 4H, $N(CH_2CH_2OH)_2$), 4.37 (br s, 1H, H-3-Chol), 5.30 (br s, 1H, H-6-Chol), 8.12 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 5 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.0, 20.9, 22.4, 22.6, 23.8, 24.1, 27.7, 27.9, 28.1, 31.7, 31.8, 35.7, 36.1, 36.4, 36.8, 38.0, 39.4, 39.6, 42.2, 49.9, 56.60, 56.69, 76.6, 122.9, 139.2 (carbons in cholesteryl skeleton), 38.5 ($CH_2CH_2NCH_2CH_2$), 46.9 (CH_2CH_2 NCH_2 CH_2), 55.5, 55.8, 56.1 ($N(CH_2CH_2OH)_2$), 157.0 (C=O carbamoyl); MS (ES^+): *m/z* 604.4 ($[M+H]^+$, 100%).

3.2.4.2. 3β-[*N*-(*N′*,*N′*-Di(2″-hydroxyethyl)-3′-aminopropyl)-*N*-(3-aminopropyl)carbamoyl]cholesterol (8). Yield: (resin: 1.1 mmol/g, 159.3 mg) 64.1 mg, 58%; IR (CH_2Cl_2): ν_{max} 3383, 2932, 2840, 1676, 1468, 1432, 1202, 1132 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 7 drops of CD_3OD): δ 0.61 (s, 3H, CH_3-18), 0.80 (d, J = 6.5 Hz, 6H, CH_3-26

and CH_3-27), 0.85 (d, J = 6.3 Hz, 3H, CH_3-21), 0.95 (s, 3H, CH_3-19), 0.95–2.28 (m, 32H, protons in cholesteryl skeleton and CH_2CH_2N-CH_2CH_2), 2.88 (br s, 4H, H_2NCH_2 $CH_2CH_2NCH_2CH_2CH_2N$), 3.26 (br s, 8H, $CH_2NCH_2CH_2CH_2N$ ($CH_2CH_2OH)_2$), 3.84 (br s, 4H, $N(CH_2CH_2OH)_2$), 4.37 (br s, 1H, H-3-Chol), 5.30 (br s, 1H, H-6-Chol), 7.92 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 7 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.0, 20.9, 22.4, 22.6, 23.8, 24.2, 27.9, 28.1, 31.7, 35.7, 36.1, 36.4, 36.8, 38.2, 39.4, 39.6, 42.2, 50.0, 56.1, 56.6, 75.9, 122.6, 139.5 (carbons in cholesteryl skeleton), 25.7 ($H_2NCH_2CH_2CH_2N$), 26.4 ($CH_2CH_2CH_2$ $N(CH_2$ $CH_2OH)_2$), 37.1 ($H_2NCH_2CH_2CH_2N$), 38.3 ($CH_2CH_2CH_2$ $N(CH_2$ $CH_2OH)_2$), 44.3 ($CH_2CH_2CH_2N(CH_2CH_2OH)_2$), 44.5 ($H_2NCH_2CH_2$ CH_2N), 55.2, 55.3, 55.4 ($N(CH_2CH_2OH)_2$), 156.3 (C=O carbamoyl); MS (ES^+): m/z 632.8 ($[M+H]^+$, 100%).

3.2.5. Synthesis of lipids library 2

Active carbonate resin **25** (1 equiv) was added excess 1,2-diamonoethane or 1,3-diaminopropane in CH_2Cl_2. The suspension was shaken overnight. The resulting resin was washed with CH_2Cl_2, DMF and CH_2Cl_2 (three times each) to provide the corresponding resin **32**. Amino resin **32** (1 equiv, 1.1 mmol/g) was reacted with a solution of bromoacetic acid (4 equiv) and DIC in DMF (10 mL). The suspension was then shaken for 12 h. The resulting resin was filtered and washed successively with DMF, CH_2Cl_2, MeOH, CH_2Cl_2, DMF and CH_2Cl_2 (3 × 10 mL each) to give the desired resin **33**. The excess of 1,2-diaminoethane or 1,3-diaminopropane in DMF was reacted with the resin **33** for 12 h to give the desired resin **34** after successively washed with DMF, CH_2Cl_2, MeOH, DMF and CH_2Cl_2 (3 × 10 mL each). The resin **34** was dried under vacuum for 2 h. The resin **34** was reacted with Dde-OH (excess) in CH_2Cl_2 for 12 h. The resin was filtered and washed successively with CH_2Cl_2 and MeOH (3 × 10 mL each) and dried under reduced pressure to give the desired resin **35**. A solution of cholesteryl chloroformate (4 equiv) in CH_2Cl_2 was added to the resin **35** and pyridine (2 mL) added in the reaction mixture. The suspension was shaken overnight. The resulting resin was filtered and washed successively with MeOH and CH_2Cl_2 (3 × 10 mL each) to afford the desired resin **36**. The Dde protecting groups was removed by treating resin **36** with 5% N_2H_4 in DMF (2 × 30 min). The resin was washed successively with DMF, CH_2Cl_2, MeOH, DMF and CH_2Cl_2 (3 × 10 mL each) to give the resin **37**.

Resin **37** was treated with 50% TFA in CH_2Cl_2 for 2 h to give symmetric polar head lipids **9–12**. The asymmetric cationic head lipids **13–16**, **17–20** and **21–24** were prepared from resin **37** using the same manner as the synthesis of lipids **3–4**, **5–6** and **7–8**, respectively.

3.2.5.1. 3β-[*N*-(2′-Aminoethyl)-*N*-(*N*-glycine(*N*-(2-aminoethyl) amide))carbamoyl]cholesterol (9). Yield: (resin: 1.1 mmol/g, 177.0 mg) 46.3 mg, 42%; IR (CH_2Cl_2): ν_{max} 3346, 2918, 2279, 1675, 1597, 1509, 1413, 1310, 1203, 1124, 1015 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 5 drops of CD_3OD): δ 0.60 (s, 3H, CH_3-18), 0.794 and 0.797 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27, overlapping signal), 0.84 (d, J = 6.2 Hz, 3H, CH_3-21), 0.92 (s, 3H, CH_3-19), 0.92–2.27 (m, 28H, protons in cholesteryl skeleton), 3.04 (br s, 2H, $H_2NCH_2CH_2NHCO$), 3.11 (br s, 2H, $NHCOCH_2NCH_2CH_2NH_2$), 3.45 (br s, 2H, $H_2NCH_2CH_2NHCOCH_2N$), 3.54 (br s, 2H, $NHCOCH_2NCH_2CH_2NH_2$), 3.93 (br s, 2H, $NHCOCH_2N$), 4.37 (br s, 1H, H-3-Chol), 5.28 (br s, 1H, H-6-Chol), 8.05–8.40 (br s, 6H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 5 drops of CD_3OD, 100 MHz): 11.7, 18.5, 19.0, 20.9, 22.4, 22.6, 23.7, 24.1, 27.7, 27.8, 28.1, 31.7, 35.7, 36.0, 36.4, 36.7, 38.1, 39.4, 39.6, 42.2, 49.9, 56.0, 56.5, 76.9, 122.7, 139.2 (carbons in cholesteryl skeleton), 36.9 ($H_2NCH_2CH_2NHCO$), 38.1 ($NHCOCH_2NCH_2CH_2NH_2$), 39.1 ($H_2NCH_2CH_2NHCO$), 47.5 ($NHCOCH_2N$ $CH_2CH_2NH_2$), 51.0 ($NHCOCH_2N$), 156.0 (C=O carbamoyl), 173.0 (C=O amide); MS (ES^+): m/z 573.2 ($[M+H]^+$, 100%).

3.2.5.2. 3β-[*N*-(3′-Aminopropyl)-*N*-(*N*-glycine(*N*-(2-aminoethyl)-amide))carbamoyl]cholesterol (10). Yield: (resin: 1.1 mmol/g, 208.0 mg) 69.0 mg, 51%; IR (CH_2Cl_2): ν_{max} 3428, 2918, 2834, 1674, 1462, 1376, 1202, 1130, 1018 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 4 drops of CD_3OD): δ 0.62 (s, 3H, CH_3-18), 0.816 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27), 0.87 (d, J = 6.2 Hz, 3H, CH_3-21), 0.96 (s, 3H; CH_3-19), 0.96–2.27 (m, 30H, protons in cholesteryl skeleton and $NHCOCH_2NCH_2CH_2CH_2$), 3.08 (br s, 4H, $CH_2CH_2NHCOCH_2NCH_2$-CH_2CH_2), 3.47 (br s, 4H, $CH_2NHCOCH_2$ NCH_2), 3.84 (br s, 2H, $NHCOCH_2N$), 4.39 (br s, 1H, H-3-Chol), 5.29 (br s, 1H, H-6-Chol), 8.01 (br s, 6H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 4 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.1, 20.9, 22.4, 22.7, 23.8, 24.2, 27.9, 28.0, 28.1, 31.7, 35.7, 36.1, 36.4, 36.8, 38.2, 39.4, 39.6, 42.2, 49.9, 56.1, 56.6, 76.6, 122.7, 139.3 (carbons in cholesteryl skeleton), 30.0 ($NHCOCH_2NCH_2CH_2$), 37.0 ($H_2NCH_2CH_2NHCO$), 38.2 ($NHCOCH_2N$-$CH_2CH_2CH_2$), 39.1 ($H_2NCH_2CH_2NHCO$), 45.8 ($NHCOCH_2NCH_2CH_2$), 52.2 ($NHCOCH_2N$), 156.0 (C=O carbamoyl), 173.0 (C=O amide); MS (ES^+): m/z 587.5 ($[M+H]^+$, 100%).

3.2.5.3. 3β-[*N*-(2′-Aminoethyl)-*N*-(*N*-glycine(*N*-(3-aminopropyl)-amide))carbamoyl]cholesterol (11). Yield: (resin: 1.1 mmol/g, 142.0 mg) 37.9 mg, 42%; IR (CH_2Cl_2): ν_{max} 3338, 2918, 2851, 1676, 1594, 1535, 1460, 1202, 1130 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 3 drops of CD_3OD): δ 0.63 (s, 3H, CH_3-18), 0.81 (d, J = 6.0 Hz, 6H, CH_3-26 and CH_3-27), 0.87 (d, J = 4.6 Hz, 3H, CH_3-21), 0.97 (s, 3H, CH_3-19), 0.97–2.90 (m, 30H, protons in cholesteryl skeleton and $H_2NCH_2CH_2CH_2NHCO$), 2.94 (br s, 2H, $H_2NCH_2CH_2CH_2NHCO$), 3.05 (br s, 2H, $NHCOCH_2NCH_2CH_2NH_2$), 3.29 (br s, 2H, $H_2NCH_2CH_2$-CH_2NHCO), 3.58 (br s, 2H, $NHCOCH_2NCH_2CH_2NH_2$), 3.93 (br s, 2H, $NHCOCH_2N$), 4.38 (br s, 1H, H-3-Chol), 5.30 (br s, 1H, H-6-Chol), 7.94 (br s, 6H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 3 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.1, 20.9, 22.4, 22.7, 23.8, 24.2, 27.9, 28.0, 28.1, 31.8, 35.7, 36.1, 36.4, 36.8, 39.4, 39.6, 42.2, 49.9, 56.1, 56.6, 76.6, 122.7, 139.4 (carbons in cholesteryl skeleton), 26.7 (H_2NCH_2-CH_2CH_2NHCO), 38.3 (H_2NCH_2 $CH_2CH_2NHCOCH_2NCH_2CH_2NH_2$), 52.4 ($NHCOCH_2N$), 156.5 (C=O carbamoyl), 172.5 (C=O amide); MS (ES^+): m/z 587.3 ($[M+H]^+$, 100%).

3.2.5.4. 3β-[*N*-(3′-Aminopropyl)-*N*-(*N*-glycine(*N*-(3-aminopropyl)-amide))carbamoyl]cholesterol (12). Yield: (resin: 1.1 mmol/g, 261.3 mg) 70.9 mg, 41%; IR (CH_2Cl_2): ν_{max} 3299, 3064, 2935, 1682, 1541, 1468, 1379, 1202, 1134 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 4 drops of CD_3OD): δ 0.62 (s, 3H, CH_3-18), 0.813 (d, J = 6.4 Hz, 6H, CH_3-26 and CH_3-27), 0.86 (d, J = 6.0 Hz, 3H, CH_3-21), 0.95 (s, 3H, CH_3-19), 0.95–2.30 (m, 32H, protons in cholesteryl skeleton and $CH_2CH_2CH_2NHCOCH_2NCH_2CH_2CH_2$), 2.91 (br s, 4H, $H_2NCH_2CH_2CH_2NHCOCH_2NCH_2CH_2CH_2NH_2$), 3.27 (br s, 4H, CH_2N-$HCOCH_2NCH_2CH_2$), 3.83 (br s, 2H, $NHCOCH_2N$), 4.39 (br s, 1H, H-3-Chol), 5.29 (br s, 1H, H-6-Chol), 7.96 (br s, 6H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 4 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.1, 20.9, 22.4, 22.7, 23.8, 24.2, 27.9, 28.1, 31.8, 35.7, 36.1, 36.4, 36.8, 38.3, 39.4, 39.6, 42.2, 49.9, 56.1, 56.6, 75.9, 122.6, 139.4 (carbons in cholesteryl skeleton), 26.9 ($H_2NCH_2CH_2CH_2NHCO$ and CH_2NCH_2-$CH_2CH_2NH_2$), 37.1 ($H_2NCH_2CH_2CH_2NHCO$), 38.4 ($H_2NCH_2CH_2CH_2$-NHCO), 47.5 ($CH_2NCH_2CH_2CH_2NH_2$), 50.5 ($NHCOCH_2N$), 156.5 (C=O carbamoyl), 171.6 (C=O amide); MS (ES^+): m/z 601.5 ($[M+H]^+$, 100%).

3.2.5.5. 3β-[*N*-((*N*′,*N*′,*N*′-Trimethyl)-2′-aminoethyl)-*N*-(*N*-glycine (*N*-(2-aminoethyl)amide))carbamoyl] cholesterol (13). Yield: (resin: 1.1 mmol/g, 192.0 mg) 76.1 mg, 59%; IR (CH_2Cl_2): ν_{max} 3436, 2934, 1678, 1541, 1468, 1378, 1202, 1131, 1023 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 10 drops of CD_3OD): δ 0.56 (s, 3H, CH_3-18), 0.749 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27), 0.80 (d, J = 6.2 Hz, 3H, CH_3-21), 0.88 (s, 3H, CH_3-19), 0.88–2.20 (m, 28H, protons in cholesteryl skeleton), 3.02–3.08 (m, 11H, $H_2NCH_2CH_2$

and $CH_2CH_2N(CH_3)_3$), 3.40 (br s, 2H, $CH_2N(CH_3)_3$), 3.50 (br s, 2H, $H_2NCH_2CH_2$), 3.64 (br s, 2H, $CH_2CH_2N(CH_3)_3$), 3.97 (br s, 2H, $NHCOCH_2$), 4.36 (br s, 1H, H-3-Chol), 5.21 (br s, 1H, H-6-Chol), 7.90–8.10 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 10 drops of CD_3OD, 100 MHz): 11.6, 18.4, 18.9, 20.3, 22.2, 22.5, 23.6, 24.0, 27.7, 27.8, 28.0, 31.6, 35.6, 35.9, 36.3, 36.4, 38.2, 39.3, 39.5, 42.1, 49.8, 55.9, 56.5, 76.5, 122.7, 139.1 (carbons in cholesteryl skeleton), 36.7 ($H_2NCH_2CH_2$), 38.0 ($CH_2N(CH_3)_3$), 39.1 ($H_2NCH_2CH_2$), 42.8 ($CH_2CH_2N(CH_3)_3$), 50.4 ($NHCOCH_2$), 53.4 and 53.5 ($CH_2CH_2N(CH_3)_3$), 156.1 (C=O carbamoyl), 170.6 (C=O amide); MS (ES^+): *m/z* 615.8 ($[M+H]^+$, 100%).

3.2.5.6. 3β-[*N*-((*N*′,*N*′,*N*′-Trimethyl)-3′-aminopropyl)-*N*-(*N*-glycine-(*N*-(2-aminoethyl)amide))carbamoyl] cholesterol (14). Yield: (resin: 1.1 mmol/g, 173.1 mg) 62.7 mg, 52%; IR (CH_2Cl_2): ν_{max} 3334, 3042, 2930, 2851, 2279, 1677, 1594, 1541, 1462, 1203, 1132 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$ + 6 drops of CD_3OD): δ 0.60 (s, 3H, CH_3-18), 0.795 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27), 0.84 (d, J = 5.3 Hz, 3H, CH_3-21), 0.93 (s, 3H, CH_3-19), 0.95–2.27 (m, 30H, protons in cholesteryl skeleton and $CH_2CH_2CH_2N(CH_3)_3$), 3.03 and 3.07 (s, 11H, $H_2NCH_2CH_2NHCO$ and $N(CH_3)_3$, partially overlapping signal), 3.17 (br s, 2H, $CH_2N(CH_3)_3$), 3.44 (br s, 4H, $H_2NCH_2CH_2NHCO$ and $NCH_2CH_2CH_2N(CH_3)_3$), 3.91 (br s, 2H, $NHCOCH_2N$), 4.38 (br s, 1H, H-3-Chol), 5.28 (br s, 1H, H-6-Chol), 8.02 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 6 drops of CD_3OD, 100 MHz): 11.7, 18.5, 19.0, 20.9, 22.4, 22.6, 23.7, 24.1, 27.8, 27.9, 28.1, 31.7, 35.6, 36.0, 36.4, 36.7, 38.2, 39.4, 39.6, 42.2, 49.9, 56.0, 56.5, 76.6, 122.7, 139.1 (carbons in cholesteryl skeleton), 30.0 ($CH_2CH_2N(CH_3)_3$), 37.0 ($H_2NCH_2CH_2NHCO$), 38.2 (CH_2 $N(CH_3)_3$), 39.2 ($H_2NCH_2CH_2NHCO$), 45.5 (NCH_2CH_2 $CH_2N(CH_3)_3$), 52.3 ($NHCOCH_2N$), 53.3 ($N(CH_3)_3$), 156.1 (C=O carbamoyl), 170.6 (C=O amide); MS (ES^+): *m/z* 630.8 ($[M+H]^+$, 100%).

3.2.5.7. 3β-[*N*-((*N*′,*N*′,*N*′-Trimethyl)-2′-aminoethyl)-*N*-(*N*-glycine (*N*-(3-aminopropyl)amide))carbamoyl] cholesterol (15). Yield: (resin: 1.1 mmol/g, 177.8 mg) 48.4 mg, 39%; IR (CH_2Cl_2): ν_{max} 3333, 2918, 2840, 1678, 1538, 1509, 1456, 1202, 1121 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$ + 3 drops of CD_3OD): δ 0.62 (s, 3H, CH_3-18), 0.809 (d, J = 5.8 Hz, 6H, CH_3-26 and CH_3-27), 0.86 (d, J = 4.8 Hz, 3H, CH_3-21), 0.95 (s, 3H, CH_3-19), 0.95–2.80 (m, 30H, protons in cholesteryl skeleton and $H_2NCH_2CH_2CH_2NHCO$), 2.83 (br s, 2H, $H_2NCH_2CH_2CH_2NHCO$), 2.93, 3.04 and 3.10 (br s, 9H, $N(CH_3)_3$), 3.27 (br s, 2H, $CH_2CH_2N(CH_3)_3$), 3.58 (br s, 2H, CH_2NHCO), 3.70 (br s, 2H, $CH_2CH_2N(CH_3)_3$), 4.01 (br s, 2H, $NHCOCH_2N$), 7.99 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 3 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.1, 20.9, 22.4, 22.7, 23.8, 24.2, 27.9, 28.0, 28.1, 31.7, 35.7, 36.1, 36.4, 36.8, 38.3, 39.4, 39.6, 42.2, 49.9, 56.1, 56.6, 76.5, 122.7, 139.3 (carbons in cholesteryl skeleton), 26.9 ($H_2NCH_2CH_2$-CH_2NHCO), 38.3 ($H_2NCH_2CH_2CH_2NHCO$ and $NCH_2CH_2N(CH_3)_3$), 52.4 ($NHCOCH_2N$), 53.3 and 53.5 ($N(CH_3)_3$), 156.1 (C=O carbamoyl), 170.6 (C=O amide); MS (ES^+): *m/z* 629.6 ($[M+H]^+$, 100%).

3.2.5.8. 3β-[*N*-((*N*′,*N*′,*N*′-Trimethyl)-3′-aminopropyl)-*N*-(*N*-glycine-(*N*-(3-aminopropyl)amide))carbamoyl] cholesterol (16). Yield: (resin: 1.1 mmol/g, 186.1 mg) 61.8 mg, 47%; IR (CH_2Cl_2): ν_{max} 3313, 3042, 2934, 2274, 1678, 1541, 1468, 1202, 1133 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$ + 5 drops of CD_3OD): δ 0.61 (s, 3H, CH_3-18), 0.80 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27), 0.85 (d, J = 6.1 Hz, 3H, CH_3-21), 0.94 (s, 3H, CH_3-19), 0.98–2.30 (m, 32H, protons in cholesteryl skeleton, $H_2NCH_2CH_2CH_2$ and $CH_2CH_2CH_2N(CH_3)_3$), 2.90 (br s, 4H, $H_2NCH_2CH_2CH_2$ and CH_2CH_2 $CH_2N(CH_3)_3$), 3.02, 3.07 and 3.10 (s, 9H, $N(CH_3)_3$, partially overlapping), 3.83 (br s, 4H, CH_2NHCO and $CH_2CH_2CH_2N(CH_3)_3$), 3.90 (br s, 2H, $NHCOCH_2N$), 4.39 (br s, 1H, H-3-Chol), 5.29 (br s, 1H, H-6-Chol), 7.96 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 5 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.1, 20.9, 22.4, 22.6, 23.7, 24.1, 27.9, 28.1, 31.7, 35.7, 36.1, 36.4, 36.8, 38.3, 39.4, 39.6, 42.2, 49.9, 56.1, 56.6, 75.9, 122.6, 139.4 (carbons in cholesteryl skeleton), 26.9 ($H_2NCH_2CH_2CH_2$ and $CH_2CH_2CH_2N(CH_3)_3$), 37.1 ($H_2NCH_2CH_2CH_2$), 38.3 ($CH_2N(CH_3)_3$), 38.4 ($H_2NCH_2CH_2$), 47.5 ($CH_2CH_2CH_2N(CH_3)_3$), 50.9 ($NHCOCH_2N$), 53.2 ($N(CH_3)_3$), 156.4 (C=O carbamoyl), 171.5 (C=O amide); MS (ES^+): *m/z* 644.6 ($[M+H]^+$, 100%).

3.2.5.9. 3β-[*N*-(*N*′-Guanidinyl-2′-aminoethyl)-*N*-(*N*-glycine(*N*-(2-aminoethyl)amide))carbamoyl]cholesterol (17). Yield: (resin: 1.1 mmol/g, 159.5 mg) 50.5 mg, 47%; IR (CH_2Cl_2): ν_{max} 3347, 2920, 2850, 2279, 1672, 1597, 1509, 1465, 1311, 1203, 1132 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$ + 5 drops of CD_3OD): δ 0.60 (s, 3H, CH_3-18), 0.796 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27), 0.84 (d, J = 5.8 Hz, 3H, CH_3-21), 0.92 (s, 3H, CH_3-19), 0.93–2.28 (m, 28H, protons in cholesteryl skeleton), 3.02 (br s, 2H, $H_2NCH_2CH_2$), 3.30, 3.35 and 3.43 (br s, 6H, $H_2NCH_2CH_2NHCOCH_2NHCH_2CH_2NH$-$C(NH)NH_2$), 3.93 (br s, 2H, $NHCOCH_2N$), 4.35 (br s, 1H, H-3-Chol), 5.27 (br s, 1H, H-6-Chol), 8.05 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 5 drops of CD_3OD, 100 MHz): 11.7, 18.5, 19.0, 20.9, 22.4, 22.6, 23.7, 24.1, 27.8, 28.1, 31.7, 35.7, 36.0, 36.4, 36.7, 38.1, 39.4, 39.6, 42.2, 49.9, 56.0, 56.5, 76.9, 123.6, 139.3 (carbons in cholesteryl skeleton), 36.9 ($H_2NCH_2CH_2$), 38.5 ($H_2NCH_2CH_2$), 47.5 ($CH_2CH_2NHC(NH)NH_2$), 51.0 (CH_2CH_2NHC $(NH)NH_2$), 156.5 (C=O carbamoyl and C=N guanidine, overlapping), 173.0 (C=O amide); MS (ES^+): *m/z* 615.4 ($[M+H]^+$, 100%).

3.2.5.10. 3β-[*N*-(*N*′-Guanidinyl-3′-aminopropyl)-*N*-(*N*-glycine(*N*-(2-aminoethyl)amide)) carbamoyl]cholesterol (18). Yield: (resin: 1.1 mmol/g, 173.4 mg) 51.4 mg, 43%; IR (CH_2Cl_2): ν_{max} 3327, 3070, 2918, 2834, 1674, 1574, 1467, 1432, 1202, 1134 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$ + 3 drops of CD_3OD): δ 0.63 (s, 3H, CH_3-18), 0.82 (d, J = 6.0 Hz, 6H, CH_3-26 and CH_3-27), 0.87 (d, J = 4.4 Hz, 3H, CH_3-21), 0.98 (s, 3H, CH_3-19), 0.98–2.29 (m, 30H, protons in cholesteryl skeleton and CH_2CH_2 $CH_2NHC(NH)NH_2$), 3.08 (br s, 2H, $H_2NCH_2CH_2$), 3.20 and 3.30 (br s, 4H, $H_2NCH_2CH_2$-$NHCO$ and $CH_2CH_2CH_2NHC(NH)NH_2$), 3.47 (br s, 2H, $CH_2CH_2CH_2N$-$HC(NH)NH_2$), 3.84 (br s, 2H, $NHCOCH_2N$), 4.40 (br s, 1H, H-3-Chol), 5.29 (br s, 1H, H-6-Chol), 8.04 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 3 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.2, 20.9, 22.4, 22.7, 23.8, 24.2, 27.9, 28.0, 28.1, 31.7, 35.7, 36.1, 36.4, 36.8, 38.2, 39.4, 39.6, 42.2, 49.9, 56.1, 56.6, 76.6, 122.7, 139.4 (carbons in cholesteryl skeleton), 30.0 (CH_2CH_2 $CH_2NHC(NH)NH_2$), 37.0 ($H_2NCH_2CH_2$-$NHCO$), 38.2 (CH_2CH_2 CH_2N $HC(NH)NH_2$), 39.1 ($H_2NCH_2CH_2NHCO$), 46.0 (CH_2CH_2 CH_2NHC $(NH)NH_2$)), 52.5 ($NHCOCH_2N$), 156.5 (C=O carbamoyl and C=N guanidine, overlapping), 173.0 (C=O amide); MS (ES^+): *m/z* 629.4 ($[M+H]^+$, 100%).

3.2.5.11. 3β-[*N*-(*N*′-Guanidinyl-2′-aminoethyl)-*N*-(*N*-glycine(*N*-(3-aminopropyl)amide)) carbamoyl]cholesterol (19). Yield: (resin: 1.1 mmol/g, 161.7 mg) 36.3 mg, 33%; IR (CH_2Cl_2): ν_{max} 3327, 2918, 1673, 1535, 1462, 1202, 1133 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$ + 4 drops of CD_3OD): δ 0.61 (s, 3H, CH_3-18), 0.805 (d, J = 6.0 Hz, 6H, CH_3-26 and CH_3-27), 0.85 (d, J = 5.0 Hz, 3H, CH_3-21), 0.95 (s, 3H, CH_3-19), 0.95–2.80 (m, 30H, protons in cholesteryl skeleton and $H_2NCH_2CH_2CH_2$), 2.92 (br s, 2H, $H_2NCH_2CH_2$), 3.26 and 3.33 (br s, 4H, $H_2NCH_2CH_2CH_2$ and $CH_2CH_2NHC(NH)NH_2$), 3.48 (br s, 2H, $CH_2CH_2NHC(NH)NH_2$), 3.92 (br s, 2H, $NHCOCH_2N$), 4.37 (br s, 1H, H-3-Chol), 5.28 (br s, 1H, H-6-Chol), 7.99 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 4 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.1, 20.9, 22.4, 22.7, 23.8, 24.1, 27.9, 28.0, 28.1, 31.7, 35.7, 36.1, 36.4, 36.8, 38.2, 39.4, 39.6, 42.2, 49.9, 56.1, 56.5, 76.2, 122.8, 139.2 (carbons in cholesteryl skeleton), 26.8 (H_2NCH_2-CH_2CH_2), 38.2 ($H_2NCH_2CH_2CH_2$ and $CH_2CH_2NHC(NH)NH_2$)), 52.5 ($NHCO$ CH_2N), 156.1 (C=O carbamoyl and C=N guanidine, overlapping), 170.5 (C=O amide); MS (ES^+): *m/z* 629.5 ($[M+H]^+$, 100%).

3.2.5.12. 3β-[*N*-(*N′*-Guanidinyl-3′-aminopropyl)-*N*-(*N*-glycine (*N*-(3-aminopropyl)amide))carbamoyl]cholesterol (20). Yield: (resin: 1.1 mmol/g, 170.3 mg) 62.1 mg, 52%; IR (CH_2Cl_2): ν_{max} 3320, 2933, 1678, 1543, 1468, 1202, 1134 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 5 drops of CD_3OD): δ 0.63 (s, 3H, CH_3-18), 0.82 (d, J = 6.4 Hz, 6H, CH_3-26 and CH_3-27), 0.86 (d, J = 5.9 Hz, 3H, CH_3-21), 0.95 (s, 3H, CH_3-19), 0.95–2.30 (m, 32H, protons in cholesteryl skeleton, $H_2NCH_2CH_2CH_2$ and CH_2CH_2 $CH_2NHC(NH)NH_2$), 2.92 (br s, 2H, $H_2NCH_2CH_2CH_2$), 3.22 (br s, 2H, $CH_2NHC(NH)NH_2$), 3.28 (br s, 4H, $H_2NCH_2CH_2CH_2$ and CH_2CH_2 $CH_2NHC(NH)NH_2$), 3.83 (br s, 2H, $NHCOCH_2N$), 4.39 (br s, 1H, H-3-Chol), 5.30 (br s, 1H, H-6-Chol), 7.91 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 5 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.1, 20.9, 22.4, 22.7, 23.8, 24.2, 27.9, 28.1, 31.8, 35.7, 36.1, 36.4, 36.8, 38.3, 39.4, 39.6, 42.2, 49.9, 56.1, 56.6, 75.9, 122.6, 139.4 (carbons in cholesteryl skeleton), 26.9 ($H_2NCH_2CH_2CH_2$ and $CH_2CH_2CH_2NHC(NH)NH_2$), 37.1 (H_2NCH_2 CH_2CH_2), 38.3 $CH_2CH_2CH_2NHC(NH)NH_2$), 38.4 ($H_2NCH_2CH_2CH_2$), 47.5 ($CH_2CH_2CH_2NHC(NH)NH_2$), 50.5 ($NHCO$ CH_2N), 156.5 (C=O carbamoyl and C=N guanidine, overlapping), 171.6 (C=O amide); MS (ES^+): m/z 643.3 ($[M+H]^+$, 100%).

3.2.5.13. 3β-[*N*-(*N′*,*N′*-Di(2″-hydroxyethyl)-2′-aminoethyl)-*N*-(*N*-glycine(*N*-(2-aminoethyl)amide))carbamoyl] cholesterol (21). Yield: (resin: 1.1 mmol/g, 185.7 mg) 71.8 mg, 53%; IR (CH_2Cl_2): ν_{max} 3334, 2933, 2279, 1678, 1541, 1509, 1432, 1203, 1133 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 5 drops of CD_3OD): δ 0.63 (s, 3H, CH_3-18), 0.818 and 0.821 (d, J = 6.5 Hz, 6H, CH_3-26 and CH_3-27, overlapping signal), 0.86 (d, J = 6.1 Hz, 3H, CH_3-21), 0.93 (s, 3H, CH_3-19), 0.93–2.32 (m, 28H, protons in cholesteryl skeleton), 3.10 (br s, 2H, $H_2NCH_2CH_2$), 3.37 (br s, 4H, $N(CH_2CH_2OH)_2$), 3.51 (br s, 4H, $H_2NCH_2CH_2$ and $CH_2N(CH_2CH_2OH)_2$), 3.70 (br s, 2H, $CH_2CH_2N(CH_2CH_2OH)_2$), 3.87 (br s, 4H, $N(CH_2CH_2OH)_2$), 3.99 (br s, 2H, $NHCOCH_2N$), 4.39 (br s, 1H, H-3-Chol), 5.31 (br s, 1H, H-6-Chol), 7.89 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 5 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.0, 20.9, 22.4, 22.7, 23.8, 24.2, 27.7, 27.9, 28.1, 31.7, 35.8, 36.1, 36.4, 36.8, 38.1, 39.4, 39.6, 42.2, 49.9, 56.1, 56.6, 76.6, 122.4, 139.2 (carbons in cholesteryl skeleton), 37.2 ($H_2NCH_2CH_2$), 38.1 ($CH_2N(CH_2CH_2OH)_2$), 39.2 (H_2NCH_2 CH_2NHCO), 47.5 ($CH_2CH_2N(CH_2CH_2OH)_2$), 50.5 ($NHCOCH_2N$), 55.3, 55.5 and 55.8 ($N(CH_2CH_2OH)_2$), 156.2 (C=O carbamoyl), 173.1 (C=O amide); MS (ES^+): m/z 661.5 ($[M+H]^+$, 100%).

3.2.5.14. 3β-[*N*-(*N′*,*N′*-Di(2″-hydroxyethyl)-3′-aminopropyl)-*N*-(*N*-glycine(*N*-(2-aminoethyl)amide))carbamoyl] cholesterol (22). Yield: (resin: 1.1 mmol/g, 199.3 mg) 61.6 mg, 42%; IR (CH_2Cl_2): ν_{max} 3315, 2934, 2840, 2274, 1677, 1541, 1467, 1429, 1202, 1134 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 5 drops of CD_3OD): δ 0.60 (s, 3H, CH_3-18), 0.792 (d, J = 5.6 Hz, 6H, CH_3-26 and CH_3-27), 0.85 (d, J = 5.3 Hz, 3H, CH_3-21), 0.94 (s, 3H, CH_3-19), 0.97–2.26 (m, 30H, protons in cholesteryl skeleton and CH_2CH_2 $CH_2N(CH_2CH_2OH)_2$), 3.05 (br s, 2H, $H_2NCH_2CH_2$), 3.27 (m, 10H, $CH_2NHCOCH_2NCH_2CH_2CH_2N(CH_2CH_2OH)_2$), 3.44 (br s, 4H, $N(CH_2CH_2OH)_2$), 3.84 (br s, 2H, $NHCOCH_2N$), 4.38 (br s, 1H, H-3-Chol), 5.28 (br s, 1H, H-6-Chol), 8.02 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 5 drops of CD_3OD, 100 MHz): 11.7, 18.5, 19.1, 20.9, 22.4, 22.6, 23.7, 24.1, 27.8, 28.0, 28.1, 31.7, 35.7, 36.1, 36.4, 36.8, 38.2, 39.4, 39.6, 42.2, 49.9, 56.1, 56.6, 76.1, 122.6, 139.4 (carbons in cholesteryl skeleton), 29.9 ($CH_2CH_2CH_2N(CH_2CH_2OH)_2$), 37.0 ($H_2NC$ H_2CH_2), 38.2 ($CH_2CH_2CH_2N(CH_2CH_2OH)_2$), 39.1 ($H_2NCH_2CH_2$), 46.0 ($CH_2CH_2CH_2N(CH_2CH_2OH)_2$), 52.5 ($NHCOCH_2N$), 55.4 and 55.6 ($N(CH_2CH_2OH)_2$), 156.0 (C=O carbamoyl), 171.5 (C=O amide); MS (ES^+): m/z 675.7 ($[M+H]^+$, 100%).

3.2.5.15. 3β-[*N*-(*N′*,*N′*-Di(2″-hydroxyethyl)-2′-aminoethyl)-*N*-(*N*-glycine(*N*-(3-aminopropyl)amide))carbamoyl] cholesterol (23). Yield: (resin: 1.1 mmol/g, 177.0 mg) 41.9 mg, 32%; IR (CH_2Cl_2): ν_{max} 3389, 3081, 2933, 1677, 1467, 1202, 1133 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 5 drops of CD_3OD): δ 0.62 (s, 3H, CH_3-18), 0.809 (d, J = 6.0 Hz, 6H, CH_3-26 and CH_3-27), 0.86 (d, J = 5.0 Hz, 3H, CH_3-21), 0.96 (s, 3H, CH_3-19), 0.96–2.28 (m, 30H, protons in cholesteryl skeleton and $H_2NCH_2CH_2CH_2$), 2.91 (br s, 2H, $H_2NCH_2CH_2CH_2$), 3.29 and 3.36 (br s, 4H, $N(CH_2CH_2OH)_2$), 3.50 (br s, 2H, $CH_2CH_2N(CH_2CH_2OH)_2$), 3.75 (br s, 2H, $H_2NCH_2CH_2CH_2$), 3.85 (br s, 2H, $CH_2CH_2N(CH_2CH_2OH)_2$), 3.92 (br s, 4H, $N(CH_2CH_2OH)_2$), 3.97 (br s, 2H, $NHCOCH_2N$), 4.39 (br s, 1H, H-3-Chol), 5.30 (br s, 1H, H-6-Chol), 7.89 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 5 drops of CD_3OD, 100 MHz): 11.7, 18.6, 19.1, 20.9, 22.4, 22.7, 23.8, 24.1, 27.9, 28.0, 28.1, 31.7, 35.7, 36.1, 36.4, 36.8, 38.2, 39.4, 39.6, 42.2, 49.9, 56.1, 56.5, 76.2, 122.8, 139.2 (carbons in cholesteryl skeleton), 26.8 ($H_2NCH_2CH_2CH_2$), 38.2 (H_2NCH_2 CH_2CH_2 and $CH_2CH_2N(CH_2CH_2OH)_2$), 52.5 ($NHCOCH_2N$), 55.1 and 55.3 ($N(CH_2CH_2OH)_2$), 156.1 (C=O carbamoyl), 170.5 (C=O amide); MS (ES^+): m/z 675.5 ($[M+H]^+$, 100%).

3.2.5.16. 3β-[*N*-(*N′*,*N′*-Di(2″-hydroxyethyl)-3′-aminopropyl)-*N*-(*N*-glycine(*N*-(3-aminopropyl)amide))carbamoyl]cholesterol (24). Yield: (resin: 1.1 mmol/g, 192.6 mg) 65.1 mg, 44%; IR (CH_2Cl_2): ν_{max} 3308, 3070, 2934, 1678, 1467, 1202, 1134 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$ + 4 drops of CD_3OD): δ 0.63 (s, 3H, CH_3-18), 0.820 and 0.824 (d, J = 6.0 Hz, 6H, CH_3-26 and CH_3-27, overlapping signal), 0.86 (d, J = 6.1 Hz, 3H, CH_3-21), 0.93 (s, 3H, CH_3-19), 0.93–2.30 (m, 32H, protons in cholesteryl skeleton, $H_2NCH_2CH_2CH_2$ and $NCH_2CH_2CH_2N(CH_2CH_2OH)_2$), 2.93 (br s, 2H, $H_2NCH_2CH_2CH_2$), 3.10 (br s, 4H, $N(CH_2CH_2OH)_2$), 3.29 (br s, 2H, $H_2NCH_2CH_2CH_2$), 3.57 (br s, 4H, $CH_2CH_2CH_2N(CH_2CH_2OH)_2$), 3.87 (br s, 4H, $N(CH_2CH_2OH)_2$), 3.99 (br s, 2H, $NHCOCH_2N$), 4.39 (br s, 1H, H-3-Chol), 5.31 (br s, 1H, H-6-Chol), 7.80 (br s, 3H, NH_3^+); ^{13}C NMR ($CDCl_3$ + 4 drops of CD_3OD, 100 MHz): 11.8, 18.6, 19.2, 21.0, 22.4, 22.7, 23.9, 24.2, 27.9, 28.1, 31.8, 35.8, 36.1, 36.5, 36.8, 38.3, 39.4, 39.7, 42.2, 49.9, 56.2, 56.6, 76.0, 122.6, 139.5 (carbons in cholesteryl skeleton), 26.9 ($H_2NCH_2CH_2CH_2$ and $NCH_2CH_2CH_2$ $N(CH_2CH_2OH)_2$), 37.3 ($H_2NCH_2CH_2CH_2$), 38.3 ($CH_2CH_2CH_2$ $N(CH_2CH_2OH)_2$), 38.4 (H_2NCH_2-CH_2CH_2), 50.5 ($NHCOCH_2N$), 55.4 ($N(CH_2CH_2OH)_2$), 156.5 (C=O carbamoyl), 171.5 (C=O amide); MS (ES^+): m/z 689.7 ($[M+H]^+$, 100%).

3.2.6. DNA binding affinities

The DNA binding ability of synthesized lipids was performed by gel retardation assay. The DNA/sample complexes 1/20 (w/w) were prepared by transferring 2.4 μL (10 μg/μL) of sample to an Eppendorf tube. Each sample was further diluted with 3 μL of PBS (phosphate-buffered saline) buffer. An aqueous solution of plasmid DNA (4 μL, 0.3 μg/μL) was added to each sample and the solutions were successively mixed by inverting several times. The DNA complexes were incubated at 25 °C for 30 min. Bromophenol blue-free gel-loading buffer (3 μL, 2 × 40% w/v sucrose in water) was added to the complexes. The solutions were mixed by inverting each tube and each sample (10 μL) was loaded onto a 1.0% agarose gel (0.5× TBE buffer). The gel was run at 135 V for 5 min and 50 V for 2 h at 400 mA. DNA bands were visualized by ethidium bromide staining.

3.2.7. Liposome preparation

Dioleoyl-L-α-phosphatidylethanolamine (DOPE) (Sigma) (1 μL, 50 μg/μL in CH_2Cl_2) and cationic lipid (2 μL, 50 μg/μL in abs. ethanol) were mixed (weight ratio 1:2). The organic solvents were evaporated under a stream of nitrogen and further dried under high vacuum (>2 h). The resulting thin film was hydrated with phosphate-buffer saline (PBS, pH 7.4, 100 μL) at room temperature for 1 h. The mixture was vortexed for one minute and sonicated (2 × 15 min) with 1 h rest between sonications using a bath-type sonicator. The liposomes were stored at 4 °C for 24 h prior to use. The liposome without DOPE was prepared as the same manner for DOPE-contained liposome excepted DOPE was not incubated.

3.2.8. Transfection procedure

Human embryonic kidney cells (HEK293), colorectal adenocarcinoma (COLO 205), canine osteosarcoma (D-17), human cervical adenocarcinoma (HeLa) and human prostate adenocarcinoma (PC3) were grown in DMEM medium supplemented with 10% fetal calf serum (FCS), penicillin (100 units/mL), streptomycin (100 mg/mL) and L-glutamine (4 mM) at 37 °C, 5% CO_2. For transfection, the cells were seeded up to 1×10^4 cells/well in a 96-well plate, to give 50–70% confluence to be used on the next day. The growth medium was removed and the cells were washed with PBS and replaced with 100 μL of fresh serum-free DMEM medium. DNA (pCH110-encoding β-galactosidase)/cationic liposome complexes (lipoplexes) were prepared as follows. An appropriate volume of each cationic liposome (1 μg/μL) was added to the plasmid DNA (0.4 μL, 0.5 μg/μL) and the complex was incubated at room temperature for 30 min before being diluted with phosphate-buffered saline to make a final DNA concentration of 0.1 mg/mL. The lipoplexes (10 μL) were then added to the cells and left to be incubated at 37 °C, 5% CO_2. The cells were then washed with PBS and fresh growth medium was added and further incubated for 48 h. For Effectene™ transfection, the method was carried out according to the manufacturer's instruction and the same ratios of plasmid DNA:Effectene™ were used. After transfection, the β-galactosidase activity per well was estimated by adding 100 μL of substrate solution (4 mg/mL of o-nitrophenyl-galactopyranoside (ONPG),[33] 0.2 M sodium phosphate (pH 7.3) and 2 mM magnesium chloride) to the lysate in a 96-well plate. Absorbance of the product *ortho*-nitrophenol at 405 nm was converted to% relative transfection by compared with positive control, Effectene™.

3.2.9. Transfection cytotoxicity

Cytotoxicities of the lipids **4**, **7**, **8**, **9**, **10**, **12**, **13** and **25** were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described previously.[34] The experiment was performed in 96-well plates. The amount of cationic liposome and commercially available transfection agents, Effectene™, DOTAP and DC-Chol, per well was used the same as that used in the transfection experiments. After 24 h. incubation, the medium was removed and replaced with a phenol red-free medium (90 μL). MTT (3 mg/mL) was added (10 μL/well) to the cells, followed by MTT solubilization solution (Sigma) (100 μL) to dissolve the resulting crystals, and the absorbance was measured at 520 nm on a microplate reader. The change in metabolic activity was calculated as A_{520} with compound/A_{520} without compound.

3.2.10. Electron microscopy

The liposomes or their lipoplexes solution (10–20 μL) was dropped on the formvar grid and left it stands at room temperature for 1 min. The water was removed by touching the edge of the droplet to the edge of a filter paper, leaving the thin aqueous film on the grid. 1% Potassium phosphotungstate (PTA) (10 μL) was applied to the grid and left it stands for 1 min. The extra solution was similarly removed by touching to the edge of a filter paper. The negative stain liposomes were allowed to dry at room temperature for 1 night by placing onto a filter paper in a covered Petri dish. The samples were observed under transmission electron microscope operated at 200 kV.

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