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Carbamate-linked cationic lipids for gene delivery

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Abstract—Series of cationic lipids **1a**–**p**, with variable length of hydrocarbon chains, alternative quaternary ammonium heads, carbamate linkages between hydrocarbon chains and quaternary ammonium heads, as well as different anion combined with them, were synthesized for liposome-mediated gene delivery. Two plasmid DNAs, pGL3-control and pGFP-N2, were transferred by cationic liposomes formed from the above cationic lipids into five mammalian cell lines, and the transfection efficiency of some of the cationic liposomes was superior or parallel to that of two commercial transfection agents, Lipofectamine2000™ and Sofast™.

1. Introduction

In 1987, Felgner and co-workers first reported the utilization of unnatural diether-linked cationic lipid (DOTMA) as a synthetic carrier to deliver gene into cells.[1] Since then, a number of published reports have described strategies for synthesis of versatile cationic lipids for gene delivery.[2] These studies covered diverse kinds of cationic lipids such as glycerol backbone based, cholesterol based, cationic peptide based, poly(ethylenimine) (PEI) based, and poly(L-lysine) (PLL) based structures.[3–19] Cationic lipids, with prominent non-immunogenic character and low cellular toxicity in delivering gene, have engendered considerable interest among the gene therapy community.[20,21] It is generally believed that electrostatic interaction brings cationic lipids and polyanion DNA together to form liposome/DNA complexes (lipoplexes). These lipoplexes, once exposed to cells, were then endocytosed by the cells and the inserted gene expressed.

Most of the spacers in the above-mentioned synthesized lipids were ether, ester or amide bond. Contrary to the above strategies, in our previous work we developed eight carbamate-linked cationic lipids, **1a**–**h**, with iodine anion combined with them.[3,4] In this paper, another eight carbamate-linked cationic lipids, **1i**–**p**, with chlorine anion combined were also synthesized. The chemical structures of the carbamate-linked cationic lipids **1a**–**p** ((2,3-bisalkylcarbamoyloxy-propyl)-trialkyl-ammonium halide) and their precursors **2a**–**d** (1-dimethylamino-2,3-bisalkylcarbamoyloxy-propane) were as shown below, and the biological performance of these cationic lipids was studied. Cationic liposomes were prepared with the above cationic lipids, and two plasmid DNAs, pGL3-control and pGFP-N2, were transferred into five mammalian cell lines by the formed cationic liposomes.

2. Results and discussion

2.1. Properties of cationic liposomes

TEM photographs of the cationic liposomes prepared with cationic lipids **1a**–**p** were similar to those formed with the diether-linked cationic lipids as reported previously,[22] which showed that cationic lipid **1a**–**p** could form cationic liposomes. Taking cationic liposome **1d** prepared with cationic lipid **1d** as an example, the TEM photograph of it is shown in Figure 1. It could be seen from the TEM photograph that the formed cat-

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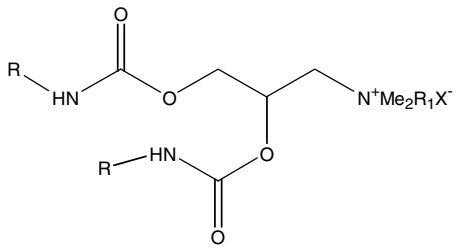
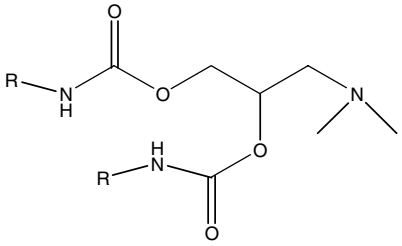


1a: R_1=Me, R=$C_{12}H_{25}$, X^-=I^-
1b: R_1=Me, R=$C_{14}H_{29}$, X^-=I^-
1c: R_1=Me, R=$C_{16}H_{33}$, X^-=I^-
1d: R_1=Me, R=$C_{18}H_{37}$, X^-=I^-
1e: R_1=Et, R=$C_{12}H_{25}$, X^-=I^-
1f: R_1=Et, R=$C_{14}H_{29}$, X^-=I^-
1g: R_1=Et, R=$C_{16}H_{33}$, X^-=I^-
1h: R_1=Et, R=$C_{18}H_{37}$, X^-=I^-

1i: R_1=Me, R=$C_{12}H_{25}$, X^-=Cl^-
1j: R_1=Me, R=$C_{14}H_{29}$, X^-=Cl^-
1k: R_1=Me, R=$C_{16}H_{33}$, X^-=Cl^-
1l: R_1=Me, R=$C_{18}H_{37}$, X^-=Cl^-
1m: R_1=Et, R=$C_{12}H_{25}$, X^-=Cl^-
1n: R_1=Et, R=$C_{14}H_{29}$, X^-=Cl^-
1o: R_1=Et, R=$C_{16}H_{33}$, X^-=Cl^-
1p: R_1=Et, R=$C_{18}H_{37}$, X^-=Cl^-



2a R=$C_{12}H_{25}$
2b R=$C_{14}H_{29}$
2c R=$C_{16}H_{33}$
2d R=$C_{18}H_{37}$

Chemical structures of carbamate-linked cationic lipids **1a-p** and their precursors **2a-d**

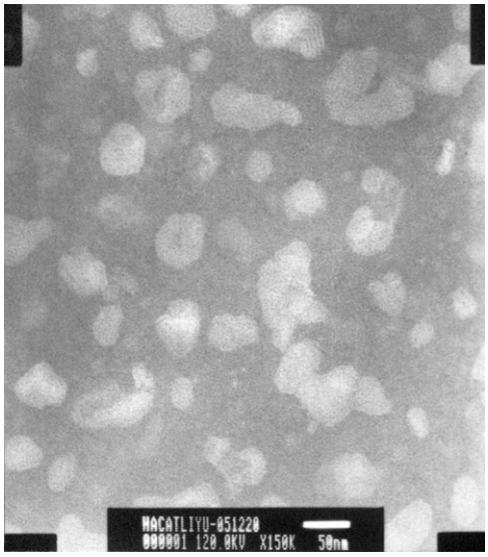


Figure 1. TEM photography of cationic liposome **1d** prepared with cationic lipid **1d**.

ionic liposomes were spherical structures, and some could further be observed as texture structures, which should be attributed to the lipid bilayers.

Mean diameters and zeta-potentials of the cationic liposomes prepared with cationic lipids **1a**–**p** were as shown in Table 1.

It was commonly believed that small distribution in diameter of cationic liposomes would benefit transfection.[23] The mean diameters of cationic liposomes prepared here were mostly between 100 and 200 nm, so it could be propitious for good transfection efficiency. The zeta-potentials of the prepared cationic liposomes were almost around 50 mV, which would benefit lipoplexes forming with the anionic DNA molecules.

2.2. DNA condensation

Because of the negative phosphate radicals in DNA molecules, the nucleic acid would migrate to the positive pole in electrophoresis. If the DNA molecules were exposed to cationic liposomes, owing to the cationic heads of the synthesized cationic lipids, the cationic lipids would combine the phosphate radicals in the DNA molecules to form lipoplexes. Thus the negative radicals in DNA molecules were neutralized by the cationic liposomes, whose migration in electrophoresis would be faded away. As for DNA condensation by the prepared cationic liposomes, taking cationic liposome **1c** as an example, the agarose gel electrophoresis of the lipoplexes is shown in Figure 2, which show the DNA molecules were gradually neutralized as the cationic liposome/DNA (positive/negative charge) ratio increased gradually.

Condensation of plasmid DNA by one of the commercial transfection agents, Sofast™, was also implemented and the agarose gel electrophoresis of Sofast™/DNA complex was similar to that of cationic liposome **1c**/DNA complex.

Either for the prepared cationic liposomes or for the commercial transfection agent, the negative charges in

Table 1. Mean diameters and zeta-potentials of cationic liposomes **1a**–**p**

	1a	**1b**	**1c**	**1d**	**1e**	**1f**	**1g**	**1h**	**1i**	**1j**	**1k**	**1l**	**1m**	**1n**	**1o**	**1p**
Diameter (nm)	166.1	280.3	276.2	90.0	158.0	102.7	133.1	283.7	143.3	174.4	169.0	209.3	144.1	122.3	146.5	160.1
PDI	0.55	1.00	0.89	0.34	0.59	0.47	0.32	0.87	0.35	0.47	0.59	0.74	0.37	0.51	0.60	0.57
ξ-potential (mV)	42.1	39.7	48.7	51.3	48.9	46.9	39.5	48.5	49.3	58.5	47.2	52.7	48.5	42.5	42.7	55.6

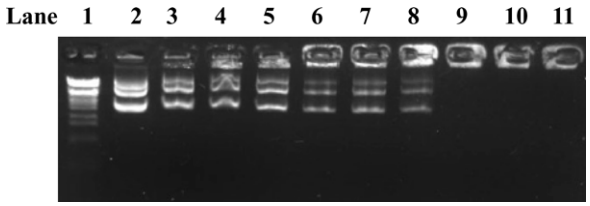


Figure 2. Complex formation of cationic liposome **1c**/pGFP-N2 at various positive/negative charge ratios by agarose gel electrophoresis using 1.0% agarose in Tris–acetate running buffer. (Lane 1) Marker (*λ-Eco*T14 I from Takara), (lane 2) naked pDNA (0.2 μg), and (lanes 3–11) pDNA (0.2 μg) with progressively increasing proportions of cationic liposome **1c** (on weight of cationic lipid **1c**: (lane 3) 2.57 μg, (lane 4) 5.14 μg, (lane 5) 7.71 μg, (lane 6) 10.28 μg, (lane 7) 12.85 μg, (lane 8) 15.42 μg, (lane 9) 17.99 μg, (lane 10) 20.56 μg, (lane 11) 23.13 μg).

the DNA molecules were gradually neutralized along with the gradual increase of the transfection agent/DNA ratio, which retarded the migration of DNA increasingly. It showed that the combination of transfection agents and plasmid DNA turned firmer and firmer on the gradual increase of the positive/negative charge ratio.

The commercial transfection agent Sofast™ was in nature a kind of polycations, which could form complexes with DNA by a small quantity. Complexes of DNA with another commercial transfection agent, Lipofectamine2000™, according to the optimal positive/negative charge ratio of Lipofectamine2000™ to DNA narrated by the Invitrogen instruction, were also prepared, but they could not give the expected results in agarose gel electrophoresis. That is, no retardation effect of DNA in the electrophoresis could be observed. The positive charge density of the prepared cationic liposomes and Lipofectamine2000™ was not so strong as that of the polycations, Sofast™. Also as a cationic liposome, the optimal positive/negative charge ratio of Lipofectamine2000™ had better references for the prepared cationic liposomes. And of course, the optimal positive/negative charge ratio of the prepared cationic liposomes to plasmid DNA should be determined by cell transfection experiments as shown in Section 2.3.1. It could not be deemed that firmer the combination of the transfection agent and the plasmid DNA was, the higher the transfection efficiency would be.

2.3. In vitro transfection

2.3.1. Optimal transfection condition (positive/negative charge ratio) selection. For mammalian cell lines transfection, optimal positive/negative charge ratio of the prepared cationic liposomes to plasmid DNA was determined. Liposome **1b**/pGL3-control complex transfected in COS-7 was chosen to seek the optimal transfection conditions. Four concentrations, 1.25 nmol/50 μL (**1b**1), 2.5 nmol/50 μL (**1b**2), 3.75 nmol/50 μL (**1b**3), and 5 nmol/50 μL (**1b**4), for cationic lipids/DOPE (1:1 in mol) of cationic liposome **1b** in serum-free DMEM were prepared. Plasmid DNA pGL3-control was diluted with serum-free DMEM to a final concentration of 0.5 μg DNA/50 μL DMEM. 50 μL of the above cationic liposome solutions was then added to the 50 μL of the pGL3-control solutions to form cationic liposome/plasmid DNA complexes (lipoplexes), respectively. Solutions of Lipofectamine2000™ and Sofast™ in 50 μL serum-free DMEM were used to form complexes with 0.5 μg pGL3-control/50 μL DMEM solution, according to the optimal positive/negative charge ratios in the suppliers' guides, respectively. Another 0.5 μg pGL3-control in 50 μL DMEM solution was diluted with 50 μL serum-free DMEM as a control. All tests were performed in triplicate.

Transfection results in COS-7 were as shown in Figure 3. It showed, after transfection, in COS-7 cell line that, no transfection occurred for the naked DNA, which meant naked DNA was digested after being endocytosed by the cells. All plasmid DNAs in carriers were expressed, among which the transfection efficiency of Lipofectamine2000™ was better than that of Sofast™, and cationic liposome **1b** in the four concentrations gave better transfection efficiency than the two commercial transfection agents. An U shape curve took shape from **1b**1 to **1b**4's concentrations, i.e., **1b**1 and **1b**4 gave better efficiency than **1b**2 and **1b**3. Transfection efficiency of **1b**1 and **1b**4 showed hardly any difference. As in the same transfection condition, another kind of cationic liposomes, the diether-linked cationic liposomes, also gave a better transfection efficiency for the **1b**4's concentration (data not shown, paper in preparation),[22] the concentration of **1b**4, 5 nmol of the cationic lipids/DOPE (1:1 in mol) in 50 μL serum-free DMEM, was selected as an optimal concentration in the following transfection experiments.

2.3.2. Transfection efficiency of cationic liposomes 1a–p. Cationic liposomes **1a**–**p**, Lipofectamine2000™, and Sofast™ were used to transfer pGL3-control into COS-7

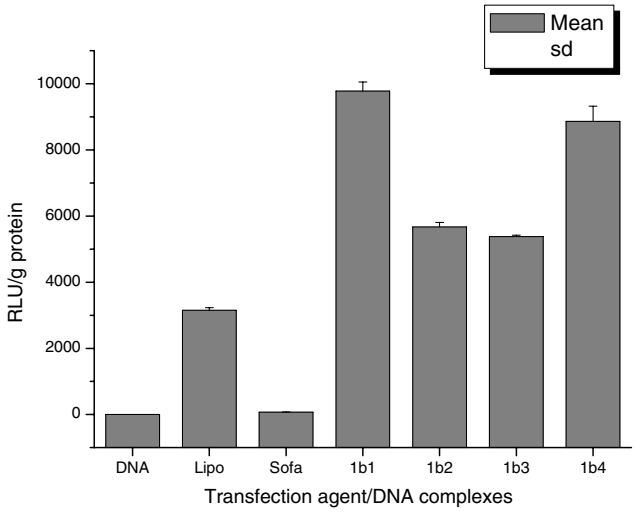


Figure 3. Transfection efficiency of transfection agent/pGL3-control complexes after transfection in COS-7 cell line. (From left to right: naked DNA, Lipofectamine2000™/DNA, Sofast™/DNA, cationic liposome **1b**1/DNA, **1b**2/DNA, **1b**3/DNA, and **1b**4/DNA complexes. Lipo means Lipofectamine2000™, and Sofa represents Sofast™.)

and Hep-2 cell lines according to the optimal positive/negative charge ratio of cationic liposome to plasmid DNA determined in Section 2.3.1 and the suppliers' guides, respectively. Naked plasmid DNAs were used as control. Transfection results in COS-7 and in Hep-2 were as shown in Figures 4 and 5, respectively.

As could be seen from Figure 4, most lipoplexes of cationic liposome **1a**–**p**/pGL3-control implemented transfection in COS-7 cell line, what' more, some lipoplexes showed better transfection efficiency than Lipofectamine2000™/pGL3-control and Sofast™/pGL3-control complexes. Among the two commercial transfection agents, the transfection efficiency of Lipofectamine2000™/pGL3-control complex was better than

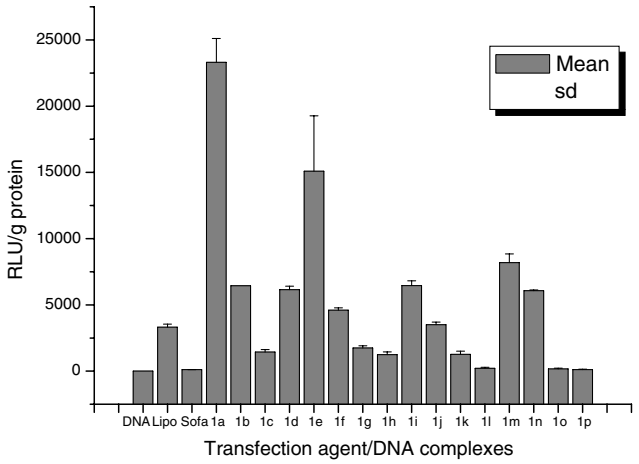


Figure 4. Transfection efficiency of transfection agent/pGL3-control complexes in COS-7 cell line. (From left to right: naked DNA, Lipofectamine2000™/DNA, Sofast™/DNA, and cationic liposome **1a**–**p**/DNA complexes. Lipo means Lipofectamine2000™, and Sofa represents Sofast™.)

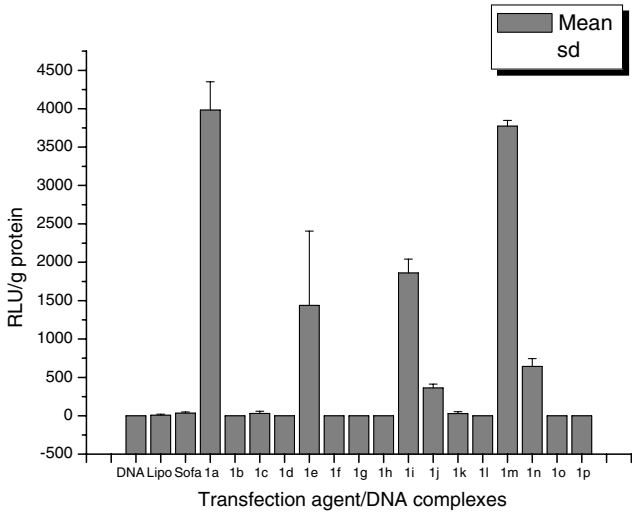


Figure 5. Transfection efficiency of transfection agent/pGL3-control complexes in Hep-2 cell line. (From left to right: naked DNA, Lipofectamine2000™/DNA, Sofast™/DNA, and cationic liposome **1a**–**p**/DNA complexes. Lipo means Lipofectamine2000™, and Sofa represents Sofast™.)

that of Sofast™/pGL3-control complex. In Hep-2 cell line, as could be seen from Figure 5, most lipoplexes of cationic liposome **1a**–**p**/pGL3-control showed no transfection, but there were several cationic liposomes that had transfection activity, such as cationic liposome **1a**, **1e**, **1i**, and **1m**. It should be noted that the two commercial transfection agents, Lipofectamine2000™ and Sofast™, showed hardly any transfection activities in Hep-2 cell line either, which indicated that discrepancy between different cell lines was rather large, and that transfection efficiency of certain transfection agent depended on different cell lines.

It should also be noted that, cationic lipids with carbamate-linkage were better in transfection efficiency than cationic lipids with diether-linkage (data not shown, paper in preparation).[22] For the above 16 carbamate-linked cationic liposomes, nine of 16 showed better transfection efficiency than Lipofectamine2000™, while only five of 16 for the 16 diether-linked cationic liposomes were better in transfection efficiency than Lipofectamine2000™ in COS-7. And in Hep-2 cell line, the number was six of 16 and three of 16, respectively, which showed that the carbamate-linked cationic lipids had better transfection efficiency than that of the diether-linked cationic lipids.

Cationic lipids **1a**–**p** could be divided into four groups, **1a**–**d**, **1e**–**h**, **1i**–**l**, and **1m**–**p**, taking hydrocarbon chain length as a criterion. In each group, the length of the double hydrocarbon chains of the four cationic lipids ranged from 12- to 18-carbon. In COS-7 cell line, it could be seen obviously from Figure 4 that the transfection efficiency of each group almost decreased gradually, namely cationic lipids with hydrocarbon chain length of 12-carbon had the best transfection efficiency, while that with the hydrocarbon chain length of 18-carbon had the worst. The transfection efficiency of cationic lipids with hydrocarbon chain length of 14- and 16-carbon was between that of 12- and 18-carbon, and cationic lipids with 14-carbon length hydrocarbon chains were better in transfection efficiency than their 16-carbon counterparts. The trend was almost the same in Hep-2 cell line as shown in Figure 5. Although transfection efficiency in Hep-2 was not as good as that in COS-7, it could also be seen that cationic lipids with the hydrocarbon chain length of 12-carbon had the best transfection efficiency, and the longer the hydrocarbon chain was, the worse the transfection efficiency exhibited.

As was reported previously, the length of hydrophobic hydrocarbon chains could influence the stability and fluidity of the formed cationic liposomes. Generally speaking, the transfection efficiency decreased as the length and saturation degree of the hydrocarbon chains increased. Short hydrocarbon chains redounded to the penetration of cationic liposomes into cell membrane, thus was propitious to the fusion between cationic lipids and the cell membrane, and finally helped cell transfection.[24,25] The results in our experiments agreed with the above conclusion. As about the sizes and zeta-potentials of the prepared cationic liposomes, as shown in Table 1, the sizes and zeta-potentials of cationic

liposomes **1a**–**p** ranged in a definite range, and hardly any regularity could be drawn, which suggested that the length of hydrocarbon chain was an independent variable among factors influencing the transfection efficiency of the cationic lipids, whose influence on transfection efficiency was independent of the sizes and zeta-potentials of the formed cationic liposomes.

Also could be seen from Figures 4 and 5 that, cationic lipids with the best transfection efficiency in both COS-7 and Hep-2 all had the iodine anion combined with it though the discrepancy between the highest transfection efficiency of cationic lipids with iodine (**1a**) and with chlorine (**1m**) anion was little in Hep-2 cell line. It could be calculated that, liposomes formed from cationic lipids with chlorine anion had larger average zeta-potential, i.e. 49.6 mV, than that of the iodine-counterion cationic lipids, i.e. 45.7 mV, which might make their bonding with the nucleic acid too firm to be detached, thus decreasing the corresponding tranfection efficiency.

According to the transfection results in COS-7 and Hep-2 cell lines as shown in Figures 4 and 5, three cationic liposomes, **1a**, **1e**, and **1m**, whose transfection efficiency was all higher than Lipofectamine2000™ and Sofast™, were selected to be further studied.

2.3.3. Transfection in other cell lines. To examine the biological activity of the cationic liposomes with relatively higher transfection efficiency, the three selected cationic liposomes plus Lipofectamine2000™ and Sofast™ were used to transfer pGL3-control into B16, MCF-7, and Hepa1-6 cell lines. Transfection results were as shown in Figure 6.

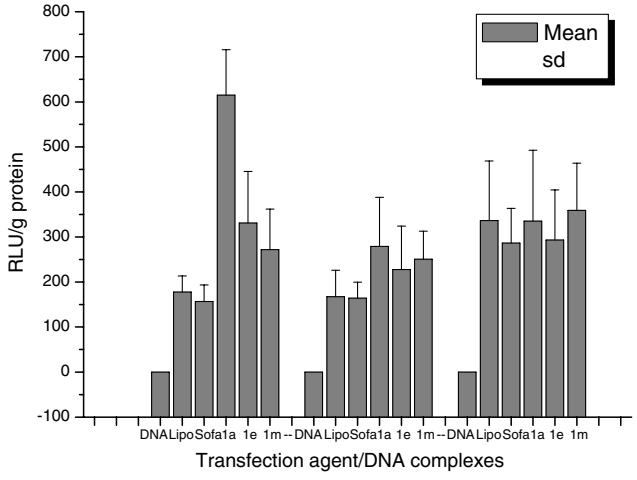


Figure 6. Transfection efficiency of transfection agent/pGL3-control complexes in B16 (the left group), MCF-7 (the middle group), and Hepa1-6 (the right group) cell lines. In each group, all the 6 columns from left to right represents naked DNA, Lipofectamine2000™/DNA, Sofast™/DNA, cationic liposome **1a**/DNA, **1e**/DNA, and **1m**/DNA complexes. Lipo means Lipofectamine2000™, and Sofa represents Sofast™.

As could be seen from Figure 6, transfection efficiency of the three selected cationic liposomes were higher than that of Lipofectamine2000™ and Sofast™ in B16 and MCF-7 cell line. In Hepa1-6, as show in Figure 6, transfection efficiencies of the selected cationic liposomes and the two commercial transfection agents were comparable.

Studying the RLU/g protein values of all the transfection agents among the above five cell lines, it could be seen that, transfection efficiency of the same cationic liposome, including the two commercial transfection agents, may range broadly in different cell lines. All the transfection agents gave the highest transfection efficiency in COS-7 among the five cell lines. This showed the structural discrepancy in different cell lines, and also suggested the different tissue specificity when gene therapy was applied. It should be taken into account the sensitivity of different target organs or tissues to transfection agents, or gene therapy for the inflammation tissues would be beyond discussion. Many research group and corporation searched for the universality of some transfection agents, yet the insensitivity of some tissues to some transfection agents was not bad. People may purposefully utilize the insensitivity of most tissues to certain transfection agents, which might lower the cytotoxicity of the transfection agents to the irrelative organs while treating the sensitive tissues. It would be a significative job to summarize the sensitive and insensitive tissues for transfection agents, and it would be even significative to add some target-ligand for the sensitive tissues in the cationic lipids when designing cationic lipid molecular.

2.3.4. Delivery of other plasmid DNA. To further verify the transfection activity of the selected cationic liposomes, the liposomes were applied to transfer another plasmid pGFP-N2 into mammalian cell lines. Also Lipofectamine2000™ and Sofast™ were used as contrast transfection agents. Plasmid DNA pGFP-N2 comprised a gene sequence encoding green fluorescence protein, and when it was successfully transfected in mammalian cells, the expressed fluorescence protein would give green fluorescence under excitation, and the intensity of the fluorescence could represent for the transfection degree.

No green fluorescence could be observed for naked pGFP-N2 and Sofast™/pGFP-N2 complex after transfection in COS-7 cell line, while Lipofectamine2000™/pGFP-N2, cationic liposome **1a**/pGFP-N2, **1e**/pGFP-N2, and **1m**/pGFP-N2 complexes all gave relatively strong green fluorescence after transfection, and the fluorescence intensity of cationic liposome **1a**/pGFP-N2 and **1m**/pGFP-N2 complexes was stronger than that of Lipofectamine2000™/pGFP-N2 complex. The green fluorescence in COS-7 cell line after transfection with cationic liposome **1m**/pGFP-N2 lipoplex was as shown in Figure 7.

Naked pGFP-N2 without any protection could not give green fluorescence after transfection, which indicated that naked DNA was digested by DNase after being

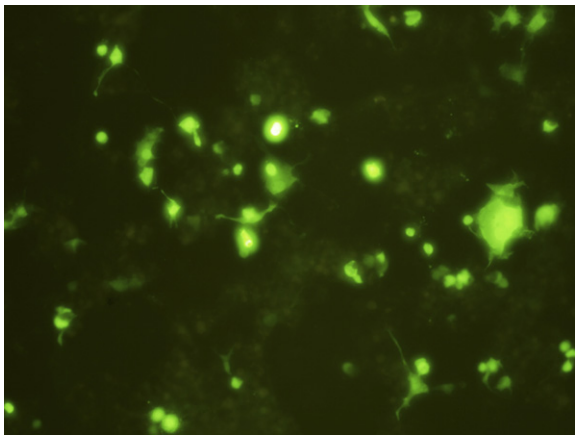


Figure 7. Green fluorescence emitted in COS-7 cell line after transfection with cationic liposome **1m**/pGFP-N2 complex (60×).

endocytosed into the cells, thus could not implement transfection. No green fluorescence was observed after transfection with one of the two commercial transfection agents, Sofast™, which showed no transfection for this agent. Taking into account of the cytotoxicity results in Section 2.4, it might be due to the high cytotoxicity of the polycations, Sofast™, which caused multitude perish of the treated cells, thus could not implement transfection. Strong green fluorescence could be observed after transfection for the other commercial transfection agent, Lipofectamine2000™, which meant it had relatively high transfection efficiency in COS-7. The selected cationic liposomes also gave strong green fluorescence after transfection, especially for cationic liposomes **1a** and **1m**, and it could be observed that both the area and the intensity of their fluorescence were larger than that of Lipofectamine2000™. This matched the transfection results acquired in pGL3-control delivery.

In Hep-2 cell line, it could be observed green fluorescence for cationic liposomes **1e** and **1m** after transfection, and no fluorescence could be observed for cationic liposome **1a** and the two commercial transfection agents. But the fluorescence intensity in Hep-2 was weaker than that in COS-7, which agreed with the results in pGL3-control delivery, showing the discrepancy of different cell lines in liposome-mediated transfection.

For the rest three cell lines, i.e., in B16, MCF-7, and Hepa1-6 cell lines, Lipofectamine2000™ and cationic liposome **1a** had activity in B16 cell line, Lipofectamine2000™ had activity in MCF-7 cell line, and only cationic liposome **1a** had transfection activity in Hepa1-6 cell line. No green fluorescence could be observed for another commercial transfection agent, Sofast™, in all the five cell lines. Like the results in pGL3-control delivery, transfection efficiency of both Lipofectamine2000™ and the selected cationic liposomes in other cell lines was all lower than that in COS-7, which confirmed that the transfection efficiency of transfection agents might vary much in different cell lines.

It could be seen from the transfection results of pGL3-control and pGFP-N2 delivery in the five mammalian cell lines that the transfection efficiency of the selected cationic liposomes was better than or parallel to that of the two commercial transfection agents. Also, according to the transfection results for the two plasmid DNAs, pGL3-control and pGFP-N2, it was found that COS-7, the African green monkey kidney cells, had the best transfection sensitivity among the five mammalian cell lines. As a primate animal, Africa green monkey had the most affinity appropinquity to mankind, so further research for the selected cationic liposomes should be proceeded with the kidney of the primate animals.

2.3.5. Morphology and zeta-potentials of cationic liposome/DNA complexes. Some research showed that the properties of the liposome/DNA complexes had much relation with the transfection efficiency of the cationic liposomes,[26] so some of the properties of the transfection agent/DNA complexes were studied. Zeta-potentials of the selected cationic liposome/pGFP-N2 complexes as well as those of Lipofectamine2000™/pGFP-N2 and Sofast™/pGFP-N2 complexes are shown in Table 2. Taking cationic liposome **1m**/pGFP-N2 complexes as an example, TEM photographs of them as well as those of Lipofectamine2000™/pGFP-N2 and Sofast™/pGFP-N2 complexes were also acquired, as shown in Figure 8.

It could be seen from Table 2 that zeta-potentials of the three selected liposomes/pGFP-N2 complexes as well as Lipofectamine2000™ (which was also in nature a cationic liposome)/pGFP-N2 complex were around 10 mV versus that of the Sofast™ (in nature a kind of polycations)/pGFP-N2 complex, which was 42.17 mV. As shown in Table 1, zeta-potentials of cationic liposomes **1a**–**p** before forming liposome/DNA complexes were around 50 mV. It could be seen that partial positive charge in cationic liposomes was neutralized after their combination with DNA, but the zeta-potential values of the formed cationic liposome/DNA complex still kept positive, thus were easy to combine with the negative cell surface. This was the precondition of successful transfection.

As for Sofast™, which was in nature a kind of polycations, it still had higher positive charge after forming Sofast™/pGFP-N2 complex with plasmid DNA. So its lower transfection efficiency might be due to the tightness of its combination with DNA, which made it harder for the combined plasmid DNA to be released from the complex. On the other hand, taking into account the cytotoxicity of the transfection agents as discussed in

Table 2. Zeta potentials of complexes formed from pGFP-N2 and some of the transfection agents (mV)

Lipofectamine2000™/pGFP-N2	Sofast™/pGFP-N2	**1a**/pGFP-N2	**1e**/pGFP-N2	**1m**/pGFP-N2
9.26	42.17	12.11	4.25	2.12

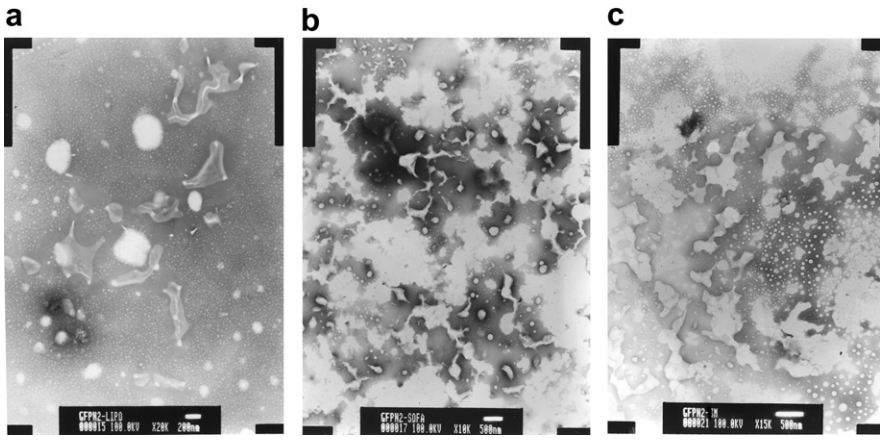


Figure 8. TEM photographs of some of the transfection agent/pGFP-N2 complexes. (a) Lipofectamine2000™/pGFP-N2 complex. (b) Sofast™/pGFP-N2 complex. (c) Cationic liposome **1m**/ pGFP-N2 complex.

Section 2.4, the cytotoxicity of Sofast™ was higher than its cationic liposome counterparts, and this might also be due to the higher positive charge of it.

As could be seen from Figure 8, it was relatively loose the combination of Lipofectamine2000™ with plasmid DNA, and there were still some dissociative DNA and cationic liposomes after complex forming, while the complex formed from Sofast™ and DNA tended to be spherical, which indicated that the polycations had more tendency to compress the plasmid DNA. The compactness degree of cationic liposome **1m**/DNA complexes was between that of Lipofectamine2000™/DNA and Sofast™/DNA complexes. Some research showed that, as for the positive cationic liposome/DNA complexes, there were some coexistence phenomena of dissociated cationic liposomes and the cationic liposome/DNA complexes, and the dissociated cationic liposomes had some effect on in vivo gene transfection.[27] This effect might be due to the increasing stay time of the lipoplexes, and protected DNA from DNase degradation. But if the lipoplexes were prepared at high positive/negative ratio, the dissociated cationic liposomes might be associated with cytotoxicity. Some research suggested that the cytotoxicity associated with cationic liposome/DNA complexes prepared at high positive/negative ratio might be essentially associated with the dissociated cationic liposomes.[28]

2.4. Cell viability/cytotoxicity of the selected cationic liposomes

Two criterions were mostly concerned for gene delivery carriers, which were transfection efficiency and their cytotoxicity. So the cytotoxicity of the selected cationic liposomes with relatively higher transfection efficiency was assayed, and the cytotoxicity results were as shown in Table 3.

As could be seen from Table 3, cytotoxicity of Lipofectamine2000™ and the selected cationic liposomes were lower, and the cell viability for the above transfection agents was all above 90%. While the cytotoxicity of another commercial transfection agent, Sofast™, was relative higher, for which the cell viability in COS-7 and Hep-2 was only more than 70%. Sofast™ was in nature a kind of polycations whose positive charge was much higher, thus caused higher cytotoxicity, while Lipofectamine2000™, cationic liposome **1a**, **1e**, and **1m** were all cationic liposomes whose positive charge was not so high, which gave relatively lower cytotoxicity. This could be proved in the agarose gel electrophoresis experiments of the transfection agents/DNA complexes as discussed in Section 2.2. That is, only small quantity of Sofast™ would compress DNA well, while for Lipofectamine2000™ and cationic liposomes **1a**–**p**, DNA was retarded only under the condition of relatively larger quantity of the cationic liposomes to compress it. Taking into account the transfection results of all the transfection agents, it was proved that cationic liposomes had better transfection performance than polycations.

3. Conclusion

The synthesized carbamate-linked cationic lipids could be prepared into cationic liposomes, which could be used as an efficient gene delivery carrier. Several cationic liposomes with relatively higher transfection efficiency were selected after in vitro transfection studies of the

Table 3. Relative cell viability of the selected cationic liposomes in different cell lines (%)

	Control	Lipofectamine2000™	Sofa™	**1a**	**1e**	**1m**
COS-7	100	97.0	76.0	96.2	94.9	93.8
Hep-2	100	97.5	70.5	93.8	92.4	93.0
B16	100	98.7	87.9	96.5	97.3	96.8
Hepa1-6	100	99.2	93.6	99.2	98.5	98.3
MCF-7	100	99.7	99.4	99.6	99.5	98.3

prepared cationic liposomes, whose biological performance was superior or parallel to that of the two commercial transfection agents, Lipofectamine2000™ and Sofast™. And the cytotoxicity of the selected cationic liposomes was acceptable.

4. Experimental

4.1. Materials and instruments

3-*sn*-Phosphatidylethanolamine, 1,2-dideoyl (DOPE) was purchased from Fluka Co. Ltd. (USA). Lipofectamine2000™ was from Invitrogen Co. Ltd. (USA); Sofast™ was from Xiamen Sunma Biotechnology Co. Ltd. (China). Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma Co. Ltd. (USA); bovine serum albumin (BSA) and fetal bovine serum (FBS) were purchased from Shanghai Weiqun Bio-tech Co. Ltd. (China). Cell Lysis buffer (25 mM Tris–phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, and 1% Triton® X-100) and luciferase assay system were purchased from Promega Co. Ltd. (USA). 3-(4,5-Dimethylthiazol-3-yl)-2,5-diphenyltetrazolum bromide (MTT) was purchased from Fluka Co. Ltd. (USA). Coomassie brilliant blue G250, phosphate buffered saline (PBS), NaCl, dimethylsulfoxide (DMSO) and other chemicals and reagents were of analytical grade, purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

A model HP1100 electrospray ionization mass spectrometer, a Micromass LC/Q-TOF high-resolution mass spectrometer, and a Varian INOVA 400-MHz nuclear magnetic resonance spectrometer were used to analyze the cationic lipids. A model JEM-2000EX transmission electron microscope (JEOL, Tokyo, Japan) was used for transmission electron microscopy (TEM) study. A KQ218 ultrasonic instrument (Kunshan Ultrasonic Instrument Co. Ltd., Kunshan, China) was used to prepare the cationic liposomes. A Brookhaven Zetaplus zeta potential analyzer and a Malvern Zetasizer 1000 system were used to measure the zeta-potentials and mean diameters of the cationic liposomes, respectively. A Luminometer (Perkin Elmer LS-55, US) was used to assay the RLU, and a 721 spectrophotometer (Shanghai Instruments Co. Ltd., China) was used to assay the contents of BSA. An Olympus IX71 fluorescence microscope (Japan) was used to record the green fluorescence. A Bio-RAD 550 microplate reader (Japan) was used to assay the cytotoxicity of the cationic liposomes.

4.2. Preparation of cationic lipids

Cationic lipids **1a**–**h** were synthesized as per previous report,[3,4] cationic lipids **1i**–**p** were synthesized similarly by quaternization of precursors **2a**–**d** with methyl chloride or ethyl chloride at 100 °C and then recrystallized from petroleum ether, respectively. Characterization data of **1a**–**h** were as reported in the previous report,[4] and characterization data of cationic lipids **1i**–**p** were as follows and the purity of them was greater than 98%.

Compound **1i**: Yield 95%; IR (KBr) ν_{max} 3338 (ν_{NH}), 1726 (ν_{CO}), 1711 (ν_{CO}), 1265–1241 (ν_{COC}, ν_{CN}); ^{1}H NMR (400 M, $CDCl_3$) δ 5.98 (s, 1H, OCH), 5.46 (s, 2H, 2× NH), 4.33–4.16 (OCH_2, NCH_2), 3.50 (s, 9H, $N(CH_3)_3$), 3.13 (d, J = 6.4, 4H, 2× $NHCH_2$), 1.51 (d, J = 6.4, 4H, 2× $NHCH_2CH_2$), 1.26 (s, 36H, 2× $(CH_2)_9$), 0.88 (t, J = 6.4, 6H, 2× CH_3), ^{13}C NMR (400 M, $CDCl_3$) δ 155.79 154.69 (C═O), 66.72 (OCH), [66.48, 63.23 (OCH_2, NCH_2)], 54.99 ($N(CH_3)_3$), 41.54 41.34 $NHCH_2$, 32.06–22.83 ($(CH_2)_{10}$), 14.27 (CH_3); MS *m*/*z*. Found $[M-Cl]^+$ 556.5, $C_{32}H_{66}ClN_3O_4$ calcd for $[M-Cl]^+$ 556.5053. *Compound* **1j**: Yield 97%; IR (KBr) ν_{max} 3344 (ν_{NH}), 1725 (ν_{CO}), 1711 (ν_{CO}), 1270–1257 (ν_{COC}, ν_{CN}); ^{1}H NMR (400 M, $CDCl_3$) δ [6.38, 6.31, 5.41 (OCH, 2× NH)], 4.18, 4.09 (OCH_2, NCH_2), 3.40 (s, 9H, $N(CH_3)_3$), 3.09 (d, J = 6.0, 4H, 2× $NHCH_2$), 1.49 (s, 4H, 2× $NHCH_2CH_2$), 1.25 (s, 44H, 2× $(CH_2)_{11}$), 0.88 (t, J = 6.4, 6H, 2× CH_3), ^{13}C NMR (400 M, $CDCl_3$) δ 156.18, 155.12 (2× C═O), 66.75 (OCH), [66.25, 63.64 (OCH_2, NCH_2)], 54.65 ($N(CH_3)_3$), [41.85, 41.36 (2× $NHCH_2$)], 32.08–22.84 ($(CH_2)_{12}$), 14.25 (CH_3); MS *m*/*z*. Found $[M-Cl]^+$ 612.7, $C_{36}H_{74}ClN_3O_4$ calcd for $[M-Cl]^+$ 612.5679. *Compound* **1k**: Yield 94%; IR (KBr) ν_{max} 3345 (ν_{NH}), 1712 (ν_{CO}), 1266–1245 (ν_{COC}, ν_{CN}); ^{1}H NMR (400 M, $CDCl_3$) δ [6.47, 6.31, 5.41 (OCH, 2× NH)], 4.18, 4.14 (OCH_2, NCH_2), 3.41 (s, 9H, $N(CH_3)_3$), 3.10 (s, 4H, 2× $NHCH_2$), 1.49 (s, 4H, 2× $NHCH_2CH_2$), 1.25 (s, 52H, 2× $(CH_2)_{13}$), 0.88 (s, 6H, 2× CH_3), ^{13}C NMR (400 M, $CDCl_3$) δ 156.24, 155.15 (2× C═O), 66.76 (OCH), [66.33, 63.67 (OCH_2, NCH_2)], 54.64 ($N(CH_3)_3$), 41.52 ($NHCH_2$), 32.10–22.86 ($(CH_2)_{14}$), 14.27 (CH_3); MS *m*/*z*. Found $[M-Cl]^+$ 668.9, $C_{40}H_{82}ClN_3O_4$ calcd for $[M-Cl]^+$ 668.6305. *Compound* **1l**: Yield 95%; IR (KBr) ν_{max} 3422 (ν_{NH}), 1711 (ν_{CO}), 1264–1258 (ν_{COC}, ν_{CN}); ^{1}H NMR (400 M, $CDCl_3$) δ [6.45, 6.16, 5.40 (OCH, 2× NH)], [4.24, 4.16 (OCH_2, NCH_2)], 3.41 (s, 9H, $N(CH_3)_3$), 3.10 (s, 4H, 2× $NHCH_2$), 1.48 (s, 4H, 2× $NHCH_2CH_2$), 1.24 (s, 60H, 2× $(CH_2)_{15}$), 0.87 (t, J = 6.4, 6H, 2× CH_3),^{13}C NMR (400 M, $CDCl_3$) δ 156.15, 155.08 (2× C═O), 66.77 (OCH), [66.38, 63.56 (OCH_2, NCH_2)], 54.67 ($N(CH_3)_3$), 41.52, 41.39 (2× $NHCH_2$), 32.10–22.86 ($(CH_2)_{16}$), 14.28 (CH_3); MS *m*/*z*. Found $[M-Cl]^+$ 724.5, $C_{44}H_{90}ClN_3O_4$ calcd for $[M-Cl]^+$ 724.6931. *Compound* **1m**: Yield 94%; IR (KBr) ν_{max} 3338 (ν_{NH}), 1614 (ν_{CO}), 1574 (ν_{CO}), 1265–1241 (ν_{COC}, ν_{CN}); ^{1}H NMR (400 M, $CDCl_3$) δ [6.03, 5.51, 5.46 (OCH, 2× NH)], 4.25, 4.19 (OCH_2, NCH_2), 3.72 (m, 2H, NCH_2CH_3), 3.41 (s, 6H, $N(CH_3)_2$), 3.14 (m, 4H, 2× $NHCH_2$), 1.53 (m, 4H, 2× $NHCH_2CH_2$), 1.48 (m, 3H, NCH_2CH_3), 1.26 (s, 44H, 2× $(CH_2)_{11}$), 0.88 (t, J = 6.8, 6H, 2× CH_3), ^{13}C NMR (400 M, $CDCl_3$) δ 155.78 154.66 (C═O), 66.41 (OCH), [64.07, 63.33, 61.34 (OCH_2, NCH_2, NCH_2CH_3)], 51.77 51.61 ($N(CH_3)_2$), 41.47, 41.28 ($NHCH_2$), 32.01–22.78 ($(CH_2)_{10}$), 14.23 (CH_3), 9.03 (NCH_2CH_3); MS *m*/*z*. Found $[M-Cl]^+$ 570.8, $C_{33}H_{68}ClN_3O_4$ calcd for $[M-Cl]^+$ 570.5210. *Compound* **1n**: Yield 93%; IR (KBr) ν_{max} 3320 (ν_{NH}), 1691 (ν_{CO}), 1275–1250 (ν_{COC}, ν_{CN}); ^{1}H NMR (400 M, $CDCl_3$) δ [6.48, 6.19, 5.42 (OCH, 2× NH)], 4.24, 4.16 (OCH_2, NCH_2), 3.65 (m, 2H, NCH_2CH_3), 3.33 (d, J = 4.8, 6H, $N(CH_3)_2$), 3.14 (d, J = 6.4, 4H, 2× $NHCH_2$), 1.48 (s, 3H, NCH_2CH_3), 1.42 (s, 4H, 2× $NHCH_2CH_2$), 1.26 (s,

44H, 2× $(CH_2)_{11}$), 0.88 (t, $J = 6.2$, 6H, 2× CH_3), ^{13}C NMR (400 M, $CDCl_3$) δ 156.37 (C═O), 66.43 (OCH), [64.98, 63.90 (OCH_2, NCH_2)], 59.58 (NCH_2CH_3), 51.41 ($N(CH_3)_2$), 41.29 ($NHCH_2$), 32.08–22.84 ($(CH_2)_{12}$), 14.27 (CH_3), 8.67 (NCH_2CH_3); MS *m/z*. Found $[M-Cl]^+$ 626.5, $C_{37}H_{76}ClN_3O_4$ calcd for $[M-Cl]^+$ 626.5836. *Compound* **1o** :Yield 92%; IR (KBr) ν_{max} 3332 (ν_{NH}), 1712 (ν_{CO}), 1266–1245 (ν_{COC}, ν_{CN}); ^1H NMR (400 M, $CDCl_3$) δ [6.48, 6.10, 5.41 (OCH, 2× NH)], 4.20, 4.16 (OCH_2, NCH_2), 3.66 (m, 2H, NCH_2CH_3), 3.34 (s, 6H, $N(CH_3)_2$), 3.13 (m, 4H, 2× $NHCH_2$), 1.50 (s, 4H, 2× $NHCH_2CH_2$), 1.43 (m, 3H, NCH_2CH_3), 1.25 (s, 52H, 2× $(CH_2)_{13}$), 0.88 (t, $J = 6.6$, 6H, 2× CH_3), ^{13}C NMR (400 M, $CDCl_3$) δ 156.14, 155.04 (C═O), 66.46 (OCH), [64.27, 63.57 (OCH_2, NCH_2)], 60.89 (NCH_2CH_3), 51.41 ($N(CH_3)_2$), 41.51, 41.38 ($NHCH_2$), 32.10–22.86 ($(CH_2)_{14}$), 14.29 (CH_3), 8.72 (NCH_2CH_3); MS *m/z*. Found $[M-Cl]^+$ 682.7, $C_{41}H_{84}ClN_3O_4$ calcd for $[M-Cl]^+$ 682.6462. *Compound* **1p**: Yield 94%; IR (KBr) ν_{max} 3417 (ν_{NH}), 1712 (ν_{CO}), 1260–1253 (ν_{COC}, ν_{CN}); ^1H NMR (400 M, $CDCl_3$) δ [6.46, 6.23, 5.41 (OCH, 2× NH)], 4.20, 4.15 (OCH_2, NCH_2), 3.64 (s, 2H, NCH_2CH_3), 3.32 (d, $J = 6.4$, 6H, $N(CH_3)_2$), 1.50 (s, 3H, NCH_2CH_3), 1.43 (s, 4H, 2× $NHCH_2CH_2$), 1.26 (s, 60H, 2× $(CH_2)_{15}$), 0.88 (t,$J = 6.4$, 6H, 2× CH_3), ^{13}C NMR (400 M, $CDCl_3$) δ 156.18, 155.12 (C═O), 66.43 (OCH), [64.50, 63.65 (OCH_2, NCH_2)], 60.85 (NCH_2CH_3), 51.33 ($N(CH_3)_2$), 41.49, 41.38 ($NHCH_2$), 32.10–22.86 ($(CH_2)_{16}$), 14.28 (CH_3), 8.72 (NCH_2CH_3); MS *m/z*. Found $[M-Cl]^+$ 738.7, $C_{45}H_{92}ClN_3O_4$ calcd for $[M-Cl]^+$ 738.7088.

4.3. Liposome preparation

Cationic liposomes were prepared with the synthesized cationic lipids **1a**–**p** in a final concentration of 3.36 mM as a mother liquid for lipoplexes agarose gel electrophoresis. A solution of cationic lipid (6.72 μmol) in chloroform/methanol (1 mL) was evaporated to dryness under a stream of nitrogen, and the residual solvent was removed under vacuum overnight. Liposomes were prepared by resuspending the lipid film in sterile pyrogen-free distilled water (2 mL) with sonication at 45 °C for 2 h in a closed vial.[3,4,22,29] For diameter and zeta-potential measurements, the above cationic liposome solutions were 5-fold diluted. For in vitro transfection experiments, cationic lipids **1a**–**p** were prepared together with DOPE (1:1 by mol) into cationic liposomes similarly in a final concentration of 1.68 mM as a mother liquid.

4.4. Transmission electron microscopy (TEM) studies

Electron micrographs of the prepared cationic liposomes were acquired using a JEM-2000EX transmission electron microscope at a voltage of 120 kV. The aqueous dispersion of the cationic liposomes was drop-cast onto a carbon-coated copper grid. A drop of 1% w/v phosphotungestic acid was added to the surface of the grid. After 5 min, excess fluid was removed and the grid surface was air-dried at room temperature before loading into the microscope.

4.5. Zeta-potential and size measurements

The zeta-potentials and mean diameters of the cationic liposomes were measured at 25 °C on a Brookhaven Zetaplus zeta potential analyzer and a Malvern Zetasizer 1000 dynamic light scattering system, respectively. For zeta-potential measurements, average values were calculated with the data from eight runs. For size measurements, average values were calculated with the data from ten runs.

4.6. Plasmid DNA

Plasmid DNA pGL3-control coding for the photinus pyralis luciferase gene was from Promega (USA). Plasmid pGFP-N2 coding for green fluorescence protein gene was from Takara (Dalian, China). Each was propagated in *Escherichia coli*, respectively (for pGL3-control in JM-109 and for pGFP-N2 in DH5α), isolated using PEG precipitation. The DNA concentration was determined by measuring UV absorbance at 260 nm, and the purity was confirmed by agarose gel electrophoresis and $OD_{260/280}$ measurement.[22]

4.7. Formation of lipoplexes

Complexes of cationic liposomes and plasmid DNA were formed by first diluting plasmid DNA and the appropriate amount of cationic liposomes separately with serum-free DMEM to equal volumes. The entire cationic liposome solution was then added to the plasmid DNA solution and vortexed immediately. This was vortexed on a low speed, to avoid DNA shearing, for 20 s and allowed to form lipoplexes for 20 min. The cationic liposomes' concentrations were calculated from the desired positive/negative charge ratio. About 330 g/mol was assumed to correspond to each repeating unit of DNA containing one phosphorus atom.[30–34]

4.8. Agarose gel electrophoresis

The condensation of plasmid DNA by the cationic liposomes was characterized by their ability to retard DNA migration through a 1% agarose gel electrophoresis. About 0.2 μg of plasmid DNA was complexed with cationic liposomes by a continuously increased amount separately. The formed lipoplexes were loaded into wells of an ethidium bromide (EB) containing 1% agarose gel and electrophoresed at 30 mA for 60 min in TAE buffer. Bands corresponding to each lipoplex were detected under UV light and photographed.[30–34]

4.9. Cell culture

COS-7 (African green monkey kidney cell), Hep-2 (Human larynx epidermoid carcinoma cell), MCF-7 (human breast cancer cell), B16 (Mouse melanoma cell), and Hepa1-6 (Murine Hepatocellular Carcinoma Cell) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) supplemented with 2% v/v penicillin/streptomycin and 2 mM glutamine. The cell lines were cultured at 37 °C under a 5% CO_2 atmosphere.[35,36]

4.10. In vitro transfection studies

Cells were seeded at designated density (in Section 2.3.1, the cell density was 5.2×10^4 cells/well, in other transfection experiments, the cell density was all 3.0×10^5 cells/well) in a 96-well plate and grown overnight in DMEM supplemented with 10% FBS, 2% v/v penicillin/streptomycin and, 2 mM glutamine at 37 °C under 5% CO_2. The media were removed and the plates were washed with PBS. Following this, 100 μL of lipoplexes in DMEM in the absence of serum was added slowly to each well in triplicate, the plates were incubated for 6 h, and transfection took place in the absence of serum. Lipoplexes were then removed, washed with PBS 2 times, and replaced with DMEM plus 10% FBS supplemented with 2% v/v penicillin/streptomycin and 2 mM glutamine. Plates were incubated for 48 h to allow protein expression. For pGL3-control, the media were then removed, washed with PBS 3 times, and 100 μL cell lysis buffer (Promega) was added to each well. Lysates were cleared by centrifugation for 5 min at 12000 rpm. 20 μL supernatants were assayed for the activity of the expressed luciferase.[30] Another 20 μL supernatants were assayed for protein content (Coomassie brilliant blue assay).[37] Transfection efficiency was measured using a luminometer (Perkin Elmer LS-55, US) according to the Promega's protocol.[38] Briefly, 20 μL of cell lysate was added to 100 μL of luciferase substrate and the light emitted measured over 10 s after a 5 s delay. Results were expressed as relative light units (RLU)/g protein and presented as means ± SD[30] For pGFP-N2, the plates were used for fluorescence microscope photography directly with an excitation wavelength of 460–490 nm after 48 h protein expression. Transfection condition of Lipofectamine2000™ and Sofast™ was according to the supplier's instructions correspondingly.

4.11. In vitro cell viability/cytotoxicity studies

To determine cell cytotoxicity/viability, the cells (COS-7, Hep-2, MCF-7, B16, and Hepa1-6) were plated at a density of 3.0×10^5 cells/well in 96-well plate at 37 °C under 5% CO_2 atmosphere. After 24 h of culture, the medium in the well was removed and the plate was washed with PBS and replaced with fresh medium containing 5 nmol cationic liposomes in 100 μL DMEM in the absence of FBS. The concentration of Lipofectamine2000™ and Sofast™ was as in the transfection condition. Cells cultured with DMEM free of cationic liposome were used as a control. After 24 h, 20 μL of MTT dye solution (5 mg/mL in phosphate buffer pH 7.4, MTT, Fluka) was added to each well. After 4 h of incubation at 37 °C and 5% CO_2 for cells exponentially growing and 15 min for cells steady-state confluent, the medium was removed and the generated formazan crystals were solubilized with 200 μL of DMSO, and the solution was vigorously mixed to dissolve the reacted dye. The absorbance of each well was read on a microplate reader (BioRAD model 550, Japan) at 595 nm. The spectrophotometer was calibrated to zero absorbance using culture medium without cells. The relative cell viability (%) related to control wells containing cell culture medium without transfection agents was calculated by $[A]_{test}/[A]_{control} \times 100$. Where $[A]_{test}$ was the absorbance of the test sample and $[A]_{control}$ was the absorbance of control sample.[39,40]

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