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A SYMMETRICAL AND BIODEGRADABLE CATIONIC LIPID. SYNTHESIS AND APPLICATION FOR EFFICIENT GENE TRANSFECTION

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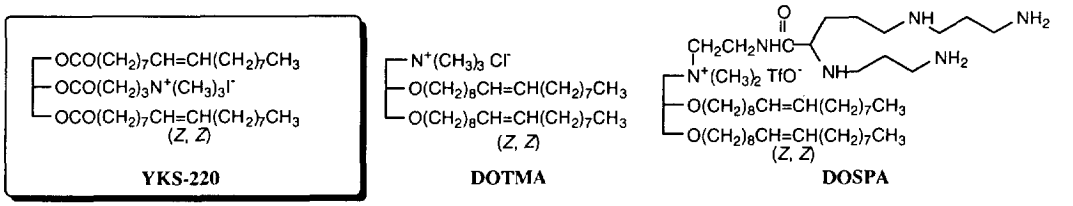
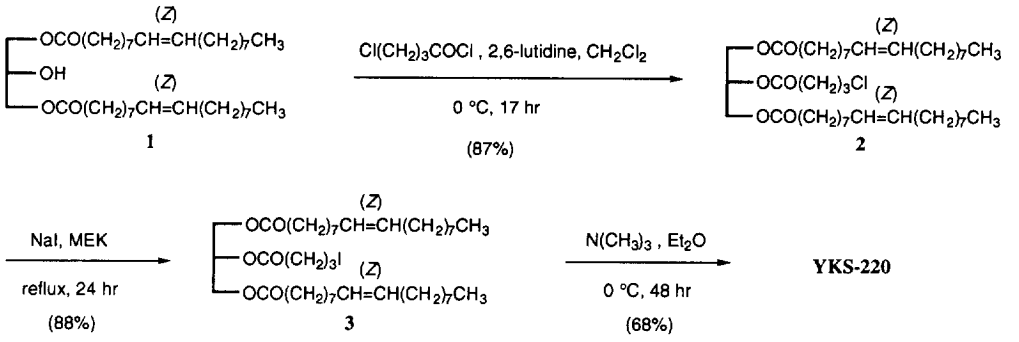
Abstract: A novel cationic lipid with a symmetrical and biodegradable structure was synthesized, and the transfection efficiency and the cytotoxicity of the cationic liposomes composed of the cationic lipid and dioleoylphosphatidylethanolamine (DOPE) were studied. © 1997 Elsevier Science Ltd.

Introduction of genes into mammalian cells is essential for therapeutic and molecular biological tools. Many techniques of transfection have been developed to date. The methods utilizing viral vectors are now well established and widely used.[1] For the present purpose, a possibility still remains that infection by a virus particle disorders the normal cell function and/or produces unexpected deleterious viruses. In spite of lower efficiency, transfection with artificial liposomes would not entail such risks. This method, if the transfection efficiency is high enough and does not have notable cytotoxicity, would serve as a potential vector for the safest transfection into the living system. Since cationic lipid DOTMA (LIPOFECTIN®) was first synthesized and employed for a gene transfection vector by Felgner and his colleagues,[2] some other cationic lipids, such as DOSPA (LIPOFECTAMINE™), have been synthesized and they show improvements in transfection efficiency and cytotoxicity.[3-9] Most of the cationic lipids previously reported have the quaternary ammonium and/or polyamine head group which connects with the hydrophobic tail group by ether linkage. As is generally known, ether linkage is stable *in vivo* and may cause cytotoxicity.[10] Here, we have designed a novel cationic lipid *N*-[3-[2-[1,3-bis(oleoyloxy)]propoxycarbonyl]propyl]-*N,N,N*-trimethylammonium iodide (YKS-220), which features a glycerol backbone connecting two ester-linked hydrophobic tails at the 1 and 3 positions and a cationic head group at the 2 position. The biodegradable ester linkages instead of an ether linkage are introduced into YKS-220 for the fast hydrolysis after transfection in order to prevent its cytotoxicity. Moreover, our lipid has a symmetrical structure. Therefore, preparation of our compound is relatively easy compared to asymmetrical lipid molecules. In this report, we describe the preparation of YKS-220 and bioavailability of the cationic liposome composed of YKS-220 and DOPE.



Synthetic Chemistry. The synthesis of YKS-220 is shown in Scheme 1. The diglyceride **1**[11] was treated with 4-chlorobutanoyl chloride in the presence of 2,6-lutidine to give the symmetrical triglyceride **2**, which was converted to the corresponding iodo compound **3** on treatment with sodium iodide in methylethylketone. Formation of ammonium salt, YKS-220,[12] was accomplished by reaction of **3** with an excess amount of trimethylamine.



Scheme 1

Preparation of cationic liposome. The cationic liposomes were prepared by mixing chloroform solutions of YKS-220 and DOPE in a vial (total lipids 1 μmol, chloroform 0.5 ml) and removing the chloroform by rotary evaporation to produce the dried lipid films. The vial was placed under vacuum for 4 hr to remove the remaining solvent and then deionized water (0.5 ml) was added. The vial was sealed and vortexed for 10 min at room temperature to afford multilamellar vesicles (MLV), which were sonicated for 5 min under a nitrogen atmosphere by using a probe-type sonicator to give small unilamellar vesicles (SUV). The SUV liposomes were filtered through an 0.2 μm filter to provide sterilization. The size of the SUV liposomes was determined with a submicron laser light scattering instrument to be 128 ± 65 nm (YKS-220:DOPE=1:4). No change in the size of the liposomes was observed at 4 °C over 6 months.

Formation of the liposome-DNA complex and transfection into a CHO cell line. Plasmid PGV-C[13] encoding the luciferase gene under the SV-40 large T antigen promoter/enhancer and liposome, both of which had been diluted with OPTI-MEM® I reduced-serum medium, were mixed and the whole was allowed to stand for 15 min. CHO cells were inoculated at a density of 1 x 10^5 cells per 35-mm plate, and incubated at 37 °C for 18 hr in 2 ml of Ham's F-12 medium supplemented with 10% fetal bovine serum and 2% antibiotics under 5% CO_2 atmosphere. After washing the cell culture plates with PBS, 2 ml of the liposome-DNA complex solution containing 20 nmol total lipids and 0.5 μg plasmid DNA was added. Transfection efficiency was measured by luciferase assays[13] after incubation for 24 hr at 37 °C.

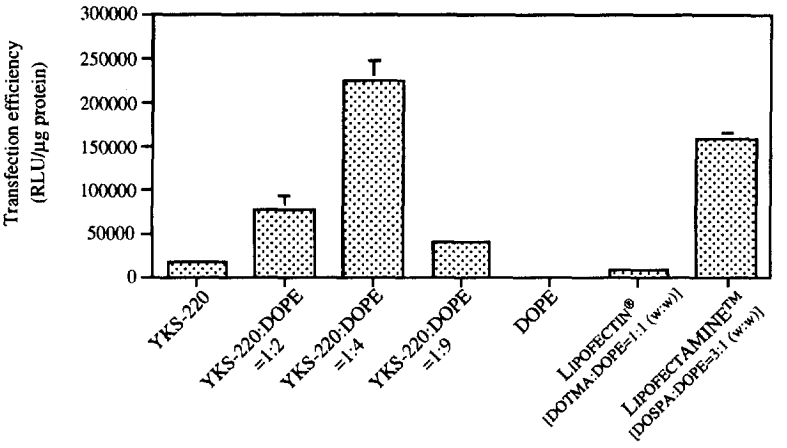


Fig. 1. Transfection efficiency of our cationic liposomes, LIPOFECTIN® and LIPOFECTAMINE™ to CHO cell line. The plasmid PGV-C (0.5 μg) complexed with our cationic liposomes [20 nmol total lipids (4 nmol YKS-220 for YKS-220:DOPE=1:4)], LIPOFECTIN® [8 nmol (4 nmol DOTMA)] and LIPOFECTAMINE™ [10 nmol (7 nmol DOSPA)] were used for transfection. Each value is the mean ± SD of triplicate transfection.

As can be seen in Fig. 1, the transfection efficiency of our cationic liposome (YKS-220:DOPE=1:4) was higher than that of LIPOFECTAMINE™ and *ca.* 10-fold higher than that of LIPOFECTIN®. To the best of our knowledge, it is one of the most effective cationic liposomes for the gene transfer into cells. Figure 1 also shows that the transfection efficiency of our cationic liposome is influenced by the composition ratio of YKS-220 and DOPE. The neutral lipid DOPE could destabilize the endosome membrane and enhance the transfection efficiency because of its ability to form a hexagonal structure,[14] while high content of DOPE would reduce the stability of liposome.[15] In our experiments, we found that the cationic liposome composed of YKS-220:DOPE in a molar ratio of 1:4 was the most effective for gene transfection and sufficiently stable, but YKS-220 or DOPE alone showed little transfection efficiency.

Cytotoxicity Assay. As shown in Fig. 2, the cytotoxicity of cationic liposome (YKS-220:DOPE=1:4), LIPOFECTIN® and LIPOFECTAMINE™ (without plasmid DNA) was evaluated on CHO cells by using WST-1 assay.[13] Generally, the cationic lipids with a quaternary ammonium structure was known to be cytotoxic,[16] and in our experiments LIPOFECTIN® showed notable cytotoxicity at 40 μM and LIPOFECTAMINE™ at 80 μM. On the contrary, our cationic liposome was devoid of cytotoxicity up to 160 μM total lipids, as we expected.

In conclusion, a novel cationic lipid YKS-220 with a symmetrical and biodegradable structure has been designed and synthesized. The cationic liposome composed of YKS-220 and DOPE was demonstrated to show high transfection efficiency and have no cytotoxicity up to 160 μM. These results indicate that the cationic lipid YKS-220 is one of the most promising candidates as a gene transfection vector *in vivo*.

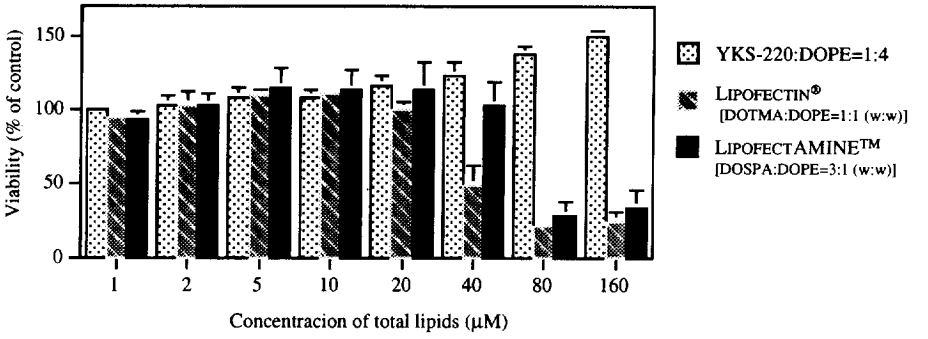


Fig. 2. Cytotoxicity of our cationic liposomes, LIPOFECTIN® and LIPOFECTAMINE™ to CHO cell line. 5 x 10^3 CHO cells were treated with indicated reagent for 24 hours. Each value is the mean ± SD of triplicate experiments.

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12. mp 44-46 °C ($CHCl_3$). ^1H NMR ($CDCl_3$) δ: 0.88 (6H, t, J = 7 Hz), 1.17-1.40 (40H, m), 1.61 (4H, m), 2.02 (8H, m), 2.12 (2H, m), 2.33 (4H, t, J = 8 Hz), 2.54 (2H, t, J = 7 Hz), 3.48 (9H, s), 3.75 (2H, m), 4.14 (2H, dd, J = 5, 12 Hz), 4.38 (2H, dd, J = 4, 12 Hz), 5.16 (1H, m), 5.34 (4H, m). *Anal.* Calcd for $C_{46}H_{86}INO_6$: C, 63.06; H, 9.89; N, 1.60. Found: C, 62.92; H, 9.60; N, 1.51.
13. The luciferase assays and cytotoxicity assays were performed by using commercially available kits (PicaGene™, Toyo Ink, Tokyo, Japan; Cell Counting Kit, Wako Pure Chemical Industries, Ltd., Osaka, Japan).
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