Surface Properties of Lipoplexes Modified with Mannosylerythritol Lipid-A and Tween 80 and Their Cellular Association

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The surface properties of cationic liposomes and lipoplexes largely determine the cellular association and gene transfection efficiency. In this study, we measured the surface properties, such as zeta potentials, surface pH and hydration levels of MHAPC- and OH-Chol-lipoplexes and their cellular association, without and with the modification of biosurfactant mannosylerythritol lipid-A (MEL-A) or Tween 80 (MHAPC=*N*,*N*-methyl hydroxyethyl aminopropane carbamoyl cholesterol; OH-Chol=cholesteryl-3β-carboxyamindoethylene-*N*-hydroxyethylamine). Compared to OH-Chol-lipoplexes, the higher cellular association of MHAPC-lipoplexes correlated with the significantly higher zeta potentials, lower surface pH levels and "drier" surface, as evaluated by the generalized polarization of laurdan. Both MEL-A and Tween 80 modification of MHAPC-lipoplexes did not significantly change zeta potentials and surface pH levels, while MEL-A modification of OH-Chol-lipoplexes seriously decreased them. MEL-A hydrated the liposomal surface of MHAPC-lipoplexes but dehydrated that of OH-Chol-lipoplexes, while Tween 80 hydrated those of MHAPC- and OH-Chol-lipoplexes. In all, cationic liposomes composed of lipids with secondary and tertiary amine exhibited different surface properties and cellular associations of lipoplexes, and modification with surfactants further enlarged their difference. The strong hydration ability of Tween 80 may relate to the low cellular association of lipoplexes, while the dehydration of MEL-A-modified OH-Chol-lipoplexes seemed to compensate the negative zeta potential for the cellular association of lipoplexes.

Key words cationic liposome; lipoplex; surfactant; surface pH; hydration

For effective gene transfection into mammalian cells, numerous cationic lipids have been synthesized and formulated into cationic liposomes.[1—3] In general, the diverse cationic lipids contain a hydrophobic region, a linker and a cationic headgroup, which are substantially quite variable in different cationic lipids. However, in the cationic headgroup, the basic element is amine, which is responsible for DNA complexation as a protonized form. All four amine types, from primary to quaternary amine, have been reported in diverse cationic lipids and exhibited substantially different cellular association and gene transfection capabilities.[4,5]

Understanding the key step in gene transfection, *i.e.* cellular association of lipoplexes, which is largely determined by the physiochemical properties of cationic liposomes and their lipoplexes, will provide valuable information for designing more effective cationic lipids and optimizing the liposomal formulation. Particle size and zeta potentials of lipoplexes are often reported when discussing their cellular association[6—8]; however, another physiochemical property, the surface hydration of lipoplexes, is always neglected. Although the hydration levels of lipoplexes containing different liposomal formulations have been well established,[9—11] their effect on the cellular association of lipoplexes is still lacking.

We reported previously that cationic liposomes, composed of dioleoylphosphatidylethanolamine (DOPE) and *N*,*N*-methyl hydroxyethyl aminopropane carbamoyl cholesterol (MHAPC) or cholesteryl-3β-carboxyamindoethylene-*N*-hydroxyethylamine (OH-Chol), demonstrated distinctive differences in their cellular association and gene transfection efficiencies *in vitro*.[12] The major differences between MHAPC and OH-Chol are the amine types and linkers shown in Fig. 1, with MHAPC having a tertiary amine linked to the cholesteryl skeleton by a carbamate ester, and OH-Chol having a secondary amine with an amido linker. To understand the different cellular association of lipoplexes composed of different cationic lipids, the effect of amine types and linkers on the surface properties, *e.g.* zeta potentials and surface hydration levels, was examined. Furthermore, mannosylerythritol lipid-A (MEL-A) is a newly developed biosurfactant[13] and modification of OH-Chol-liposomes with MEL-A markedly increased the gene transfection efficiency of plasmid DNA.[14,15] Recent findings found that MEL-A induced membrane fusion between the target cells and cationic liposomes[16,17]; however, the physiochemical mechanism of the fusion ability of MEL-A has not been fully understood. As a

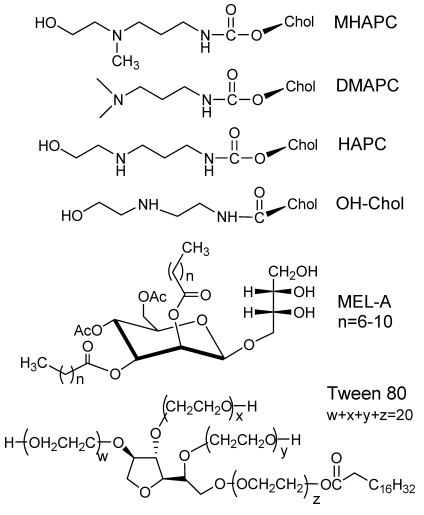

Fig. 1. Chemical Structures of Cationic Lipids and Surfactants

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control surfactant, Tween 80 was selected due to its popularity and wide application in drug delivery. Studying the surface properties of cationic liposomes and lipoplexes, without and with MEL-A or Tween 80, might help to clarify how they affect the cellular association of lipoplexes and gene transfection of MHAPC- and OH-Chol-lipoplexes.

To our knowledge, no comprehensive study has been performed to characterize the surface properties of surfactant modified liposomes and lipoplexes composed of different cationic lipids. In the present study, we measured the zeta potentials, surface pH and hydration levels of MEL-A or Tween 80-modified cationic liposomes and lipoplexes, and also discussed these findings relating to their cellular association.

Experimental

Materials MHAPC, DMAPC, HAPC and OH-Chol were synthesized as reported previously (DMAPC=*N*,*N*,*N*-dimethyl aminopropane carbamoyl cholesterol; HAPC=*N*-hydroxyethyl aminopropane carbamoyl cholesterol, Fig. 1).[5,18] DOPE and Tween 80 were obtained from NOF Co., Ltd. (Tokyo, Japan), and MEL-A was purified as reported previously.[13] 4-Heptadecyl-7-hydroxycoumarin (HC) and 6-dodecanoyl-2-demethylaminonaphthalene (laurdan) were purchased from Lambda (Graz, Austria). *N*-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (rhodamine-DHPE) was obtained from Invitrogen (CA, U.S.A.). The plasmid DNA pCytomegalovirus (pCMV)-luc encoding the luciferase gene under the control of the CMV promoter was constructed as described previously.[19] A protein-free preparation of the plasmid was purified following alkaline lysis using Maxiprep columns (Qiagen, Hilden, Germany).

Preparation of Liposomes and Lipoplexes MHAPC, DMAPC, HAPC and OH-Chol were formulated into liposomes with DOPE at a molar ratio of 1/1 by modified ethanol injection[18] with a concentration of 4.5 mM cationic lipid for each type of liposome, which were named MHAPC-, DMAPC-, HAPC- and OH-Chol-liposomes, respectively. For MEL-A and Tween 80-modified liposomes, 50% or 100% (molar % to DOPE, cationic lipid/DOPE/surfactant=1 : 1 : 0.5 or 1 : 1 : 1) of these surfactants was included in the lipids prior to preparation,[19] and expressed as MEL-A (0.5), MEL-A (1) and Tween 80 (0.5), respectively. To measure liposomal surface pH, 0.5% (molar % to total lipids) of HC was incorporated into lipids during preparation. To measure the hydration level of the liposomal surface, 0.2% (molar % to total lipids) of laurdan was incorporated into lipids. Liposomes were labeled with 0.2% (molar % to total lipids) of rhodamine-DHPE to measure cellular association of lipoplexes. Lipoplexes at a charge ratio (+/−, amine in cationic lipids/DNA phosphate ratio) of 3/1 were prepared by adding each liposome to an aliquot of DNA, with standing at room temperature for 5 min.

The zeta potential of liposomes and lipoplexes was measured by the dynamic light scanning method (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan), both in Milli Q water (Elix® equipment, Millipore, MA, U.S.A.) and 1/10 phosphate-buffered saline (pH 7.4, 1/10 PBS).

Circular Dichroism (CD) Measurement Lipoplexes were prepared by adding 100 μl liposomes (4.5 mM cationic lipid) to 50 μl DNA (1 mg/ml), and diluted to 1 ml with PBS (pH 7.4) 5 min after the addition of DNA; the final concentration of DNA was 50 μg/ml for each sample. CD spectra were measured as the average of two scans from 220 to 320 nm at a scan rate of 50 nm/min at 25 °C on a spectropolarimeter (J-800, Jasco Co., Tokyo, Japan).

Fluorescence Measurement Twenty microliters of HC-labeled liposomes were diluted to 2 ml with PBS to a concentration of 0.045 mM of cationic lipid. Lipoplexes were prepared by adding 20 μl liposomes to 100 μl DNA (100 μg/ml), and diluted to 2 ml with PBS 5 min after adding DNA. HC fluorescence was measured by scanning the excitation wavelength between 300 and 400 nm at an emission wavelength of 450 nm (bandwidth 5 nm) at 25 °C on a fluorescence spectrometer (RF-5300PC, Shimadzu Co., Kyoto, Japan). The dissociation degree of HC, *i.e.* liposomal surface pH, can be monitored by the ratio of excitation fluorescence intensities at 380 and 330 nm (380/330).[20] A higher value of 380/330 indicates a higher liposomal surface pH.

Twenty microliters of laurdan-labeled liposomes were diluted to 2 ml with PBS to a concentration of 0.045 mM of cationic lipid. Lipoplexes were prepared by adding 20 μl liposomes to 100 μl DNA (100 μg/ml), and diluted to 2 ml with PBS 5 min after adding DNA. Laurdan fluorescence was measured by scanning the emission wavelength between 400 and 500 nm at an excitation wavelength of 340 nm (bandwidth 5 nm) at 25 °C on a Shimadzu RF-5300PC. The hydration level of the liposomal surface was monitored by the generalized polarization (GP) value, which was calculated as follows:

$$GP(Ex340)=(I_{440}-I_{490})/(I_{440}+I_{490})$$

wherein I_{440} and I_{490} are the emission intensities at wavelengths of 440 nm and 490 nm at an excitation wavelength of 340 nm.[9,21] A higher GP (Ex340) value represents a lower hydration level (dehydration) on the liposomal surface. Both the intensity ratio of 380/330 and GP (Ex340) were calculated from the absolute values of fluorescence intensity of one measurement, which was run in the same day with strictly controlled conditions. The repeated experiments showed different values, but similar trend.

Flow Cytometry Human lung adenocarcinoma A549 cells were maintained in RPMI-1640 medium supplemented with 10% FBS and kanamycin at 37 °C in a 5% CO_2 humidified incubator. The cells were prepared by plating in a 35-mm culture dish 24 h prior to each experiment. Each rhodamine-labeled liposome was mixed with 2 μg pCMV-luc at a charge ratio (+/−) of 3/1, and then diluted in 1 ml PBS (pH 7.4). After incubation with lipoplexes for 2 h, all cells were collected with FACS buffer (PBS containing 0.1% BSA and 1 mM EDTA). After centrifugation at 1500 rpm for 3 min, the supernatant was discarded and the cell pellets were resuspended with FACS buffer for 2 rounds. The suspended cells were directly introduced into a FACSCalibur flow cytometer (Becton Dickinson, CA, U.S.A.). Data for 10000 fluorescent events were obtained by recording forward scatter (FSC), side scatter (SSC) and red fluorescence. Mean intensity values of rhodamine inside cells were calculated to compare the association amount of lipoplexes.

Results and Discussion

Zeta Potentials of Liposomes and Lipoplexes As efficient gene transfection vectors, cationic liposomes and lipoplexes realize their applications through electrostatic interaction with the plasma membrane.[1] With the notion that a positively charged particle facilitates interaction with the cell membrane, and *vice versa*, the zeta potentials of liposomes and lipoplexes were measured to elucidate their cellular association.

As shown in Fig. 2, all the liposomes formulations of MHAPC and OH-Chol exhibited a high positive zeta potential ranging from 50 to 75 mV. MHAPC-lipoplexes exhibited much higher zeta potentials than OH-Chol-lipoplexes both in Milli Q and PBS. Interestingly, MEL-A did not change the zeta potential of MHAPC-lipoplexes, while it markedly decreased that of OH-Chol-lipoplexes to a negative value of −14.6 and −21.7 mV in Milli Q and PBS, respectively. Tween 80 modification slightly decreased the positive zeta potential of MHAPC-lipoplexes, while it decreased the zeta potential of OH-Chol-lipoplexes to a negative value, but to a lesser extent than MEL-A.

The zeta potentials of lipoplexes can be affected by many factors, such as lipid compositions, the size of lipoplexes, the measurement conditions, *etc.* The non-modified MHAPC- and OH-Chol-lipoplexes were about 300 and 400 nm, respectively, but those with MEL-A and Tween 80 modification became smaller and similar (200—250 nm) (data not shown). Since the lipoplex sizes were similar and the measurement conditions were the same, the differences in zeta potential between MHAPC- and OH-Chol-lipoplexes may be ascribed to their structural differences (Fig. 1). MHAPC has a tertiary amine in the headgroup, while OH-Chol possesses a secondary amine. The differences in zeta potential may arise from different protonation levels of the amines. Furthermore, MEL-A modification also generated totally different results on the zeta potentials of MHAPC- and OH-Chol-lipoplexes, suggesting that MEL-A might interact with OH-Chol and affect its protonation level in lipoplexes.

CD Spectra Since MEL-A and Tween 80 affected the

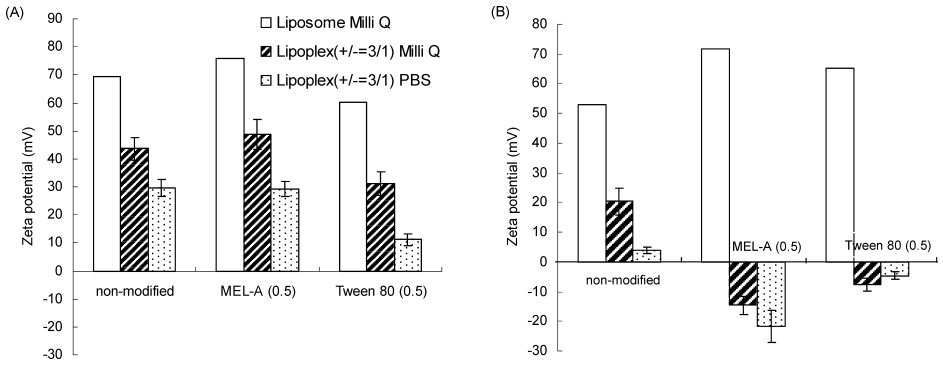

Fig. 2. Zeta Potentials of MHAPC- (A) and OH-Chol- (B) Liposomes and Lipoplexes in Milli Q and PBS

Each lipoplex result represents the mean±S.D. (n=3).

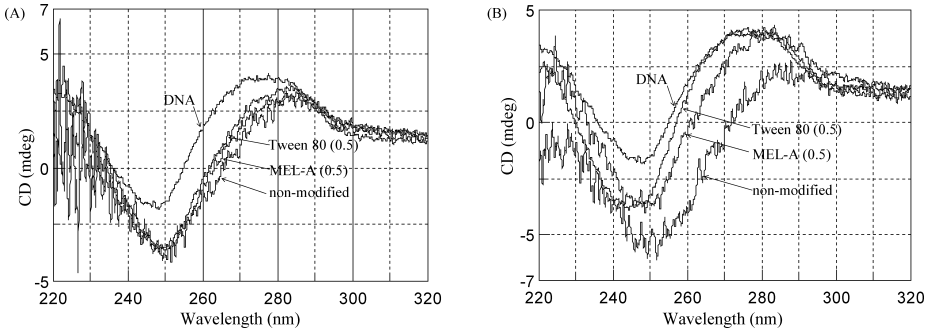

Fig. 3. CD Spectra of DNA in MHAPC- (A) and OH-Chol- (B) Lipoplexes in PBS at a Charge Ratio (+/−) of 3/1

zeta potentials of MHAPC- and OH-Chol-lipoplexes differently, we suppose that the interaction between DNA and cationic liposomes would be substantially different. In Fig. 3A, all MHAPC formulations showed enhanced negative ellipticity, a flattening of the positive bands and an overall red shift of the bands, that is, the typical features of the C form of DNA.[10,22] This suggested that the MHAPC-liposomes had strong complexation with DNA and surfactants did not alter the interaction. As shown in Fig. 3B, non-modified OH-Chol-lipoplexes also exhibited a complete C form of DNA, while modification by MEL-A and Tween 80 only generated modest negative ellipticity. These data suggested that MEL-A- and Tween 80-modified OH-Chol-liposomes were not effective in DNA complexation, correlating to the negative zeta potentials of the lipoplexes as shown in Fig. 2B.

Liposomal Surface pH as Monitored by Dissociation Degree of HC 4-Heptadecyl-7-hydroxycoumarin (HC) is a weak acid fluorophore. At a pH $>pK_a$, the maximum excitation is shifted to the wavelength of about 380 nm. Fluorescence intensity at the excitation wavelength of 330 nm is the pH-independent isosbestic point, which reflects the actual level of HC in liposomes; therefore, the dissociation degree of HC in liposomes can be monitored by the ratio of fluorescence intensities when excited at 380 and 330 nm (380/330). A high value of 380/330 indicates a more dissociated form of HC, *i.e.* a higher liposomal surface pH.[20] The different cationic lipids in liposome formulations adopted different protonation levels due to high pH values on the liposomal surface.[20]

As shown in Fig. 4, the liposomes and lipoplexes showed the same trend of change of liposomal surface pH after surfactant modifications. All lipoplexes showed decreased pH compared to liposomes; in particular, OH-Chol formulations showed a remarkable decrease of pH. This suggested that DNA complexation may further increase proton concentration on the liposomal surface and the protonation of cationic lipids. MHAPC-lipoplexes demonstrated much lower pH values than OH-Chol-lipoplexes (values of 380/330 are <1 and >1 for MHAPC- and OH-Chol-lipoplexes, respectively), indicating rich protons on the surface of MHAPC-lipoplexes while deficient on OH-Chol-lipoplexes. The different proton levels on the liposomal surface would substantially affect the protonation levels of cationic lipids. In the proton-rich environment, MHAPC with the tertiary amine should have a higher protonation level than OH-Chol, corresponding to the higher zeta potentials of MHAPC-lipoplexes (Fig. 2A). The relatively low proton environment of OH-Chol-lipoplexes made the secondary amine in OH-Chol a major free amine form with lower zeta potential (Fig. 2B).

MEL-A modification of MHAPC-lipoplexes did not greatly influence their surface pH (Fig. 4A), while it evidently decreased that of OH-Chol-lipoplexes with an increased amount of MEL-A (Fig. 4B). This suggested that MEL-A did not change the protonation of the tertiary amine of MHAPC; but significantly affected the protonation of the secondary amine in OH-Chol. These results corresponded well with the striking negative zeta potential of MEL-A modified OH-Chol-lipoplexes (Fig. 2B). The slight effect of Tween 80 on the surface pH of both MHAPC- and OH-Chol-lipoplexes reflected the structural differences between MEL-A and Tween 80. As shown in Fig. 1, MEL-A bears hydroxyl groups in the hydrophilic part and Tween 80 has polyoxyethylene groups. We suppose that the hydroxyl groups in MEL-A may have hydrogen bond interactions with the secondary

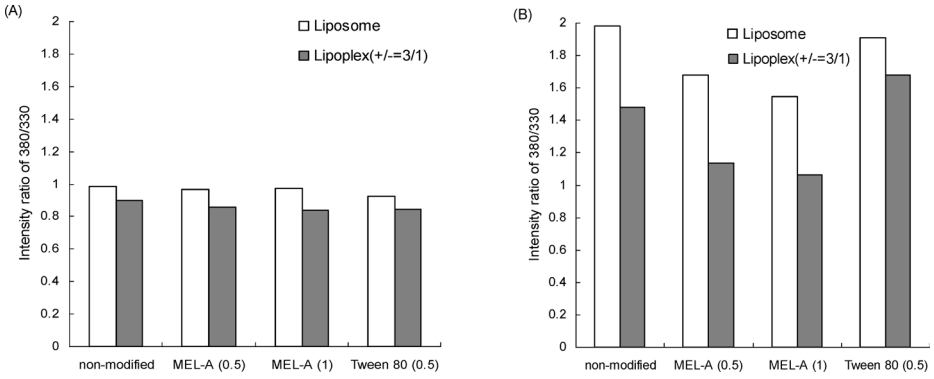

Fig. 4. The Change of Liposomal Surface pH (Dissociation Degree of HC) in MHAPC- (A) and OH-Chol- (B) Liposomes and Lipoplexes as Monitored by the Intensity Ratio of 380/330 nm in PBS

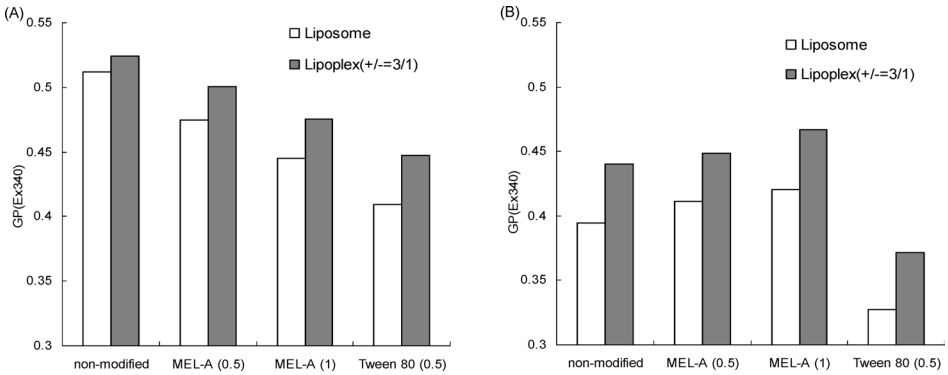

Fig. 5. The Change of Surface Hydration of MHAPC- (A) and OH-Chol- (B) Liposomes and Lipoplexes as Monitored by Laurdan Generalized Polarization (GP) in PBS

amine of OH-Chol, but the polyoxyethylene in Tween 80 does not, thereby affecting the liposomal surface pH of OH-Chol-lipoplexes. For MHAPC, due to the propane in the cationic part, the amine proton may form hydrogen-bond with the amine in the carbamate linker (inner molecular), thereby forming a stable hexacyclic structure. For OH-Chol, the proton of amine may be difficult to form an unstable pentacyclic when involved with hydrogen-bond. Therefore, the neighboring hydroxyl group in MEL-A may prefer to form hydrogen-bond with the amine of OH-Chol. The hydrogen bond interaction between MEL-A and OH-Chol may induce proton dissociation from protonized amine in OH-Chol, thereby decreasing the liposomal surface pH of MEL-A-modified OH-Chol formulations (Fig. 4B) and decreasing the protonation level of secondary amine of OH-Chol, which caused the negative zeta potentials of MEL-A-modified OH-Chol-lipoplexes (Fig. 2B).

The Hydration Level of Liposomes and Lipoplexes as Monitored by Laurdan GP Value It is well-known that polyethyleneglycolylated liposomes and lipoplexes substantially prohibit their association with the cell membrane due to the PEG watery layer.[23—25] This information suggested that the liposomal hydration level is another important factor affecting its association with the cell membrane. Here we examined the effect of MEL-A and Tween 80 on hydration levels of liposomes and lipoplexes. Laurdan was firstly used to follow changes in the hydration level of liposomes by Parasassi *et al.*[21] In solvents with high polarity, laurdan shifts its emission spectrum to higher wavelengths due to dipolar relaxation processes.[21] When associated with liposomes, laurdan emission spectra depend strongly on the different hydration levels of lipid bilayers.

As shown in Fig. 5, liposomes and lipoplexes showed the same trend of change in hydration levels after surfactant modification. All lipoplexes demonstrated higher GP values, *i.e.* lower hydration levels, than the corresponding liposomes formulations. This suggested that the liposomal surface was further dehydrated after complexation with DNA.[9,26,27] By comparing the GP values of MHAPC- and OH-Chol-lipoplexes, MHAPC-lipoplexes showed relatively higher GP values than their counterparts, suggesting that the surfaces of MHAPC-lipoplexes were “drier” than those of OH-Chol-lipoplexes. The carbamate linker in MHAPC might contribute to the “dry” surface of MHAPC-lipoplexes. As shown in Fig. 6, cationic liposomes and their lipoplexes containing lipids with carbamate linker (DMAPC, MHAPC and HAPC) showed higher GP values than those of OH-Chol, which has an amido linker. For MHAPC-lipoplexes, both MEL-A and Tween 80 modification hydrated the liposomal surface by decreasing the GP values (Fig. 5A), with Tween 80 more effective than MEL-A. For OH-Chol-lipoplexes, in contrast, MEL-A dehydrated the liposomal surface, while Tween 80 maintained its effective wetting capability (Fig. 5B). The structural difference between MEL-A and Tween 80 may help to clarify the effective wetting ability of Tween 80. In one molecule of Tween 80, twenty oxyethylene residues were present in the hydrophilic region, while MEL-A possessed only three hydroxyl groups. The water molecules bound

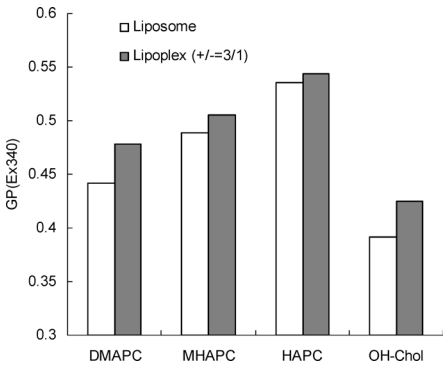

Fig. 6. The Surface Hydration of Cationic Liposomes Containing Cationic Lipids with Carbamate or Amido Linker as Monitored by Laurdan Generalized Polarization (GP) in PBS

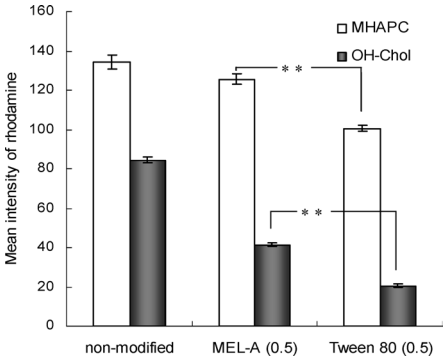

Fig. 7. Cellular Association of Rhodamine-Labeled Lipoplexes (+/−=3/1) in A549 Cells Incubated for 2 h in PBS

Each result represents the mean±S.D. (n=3). ** $p<0.01$ (Student's t test).

per Tween 80 would be much higher that those of MEL-A. The strong wetting ability of Tween 80 on MHAPC- and OH-Chol-lipoplexes might mask the positive charge of lipoplexes, thereby slightly decreasing the zeta potentials (Fig. 2).

Of great interest are the different behaviors of MEL-A on the hydration levels of MHAPC- and OH-Chol-lipoplexes. As shown in Fig. 5, MEL-A hydrated MHAPC-lipoplexes while it dehydrated OH-Chol-lipoplexes with an increased MEL-A amount. MEL-A always showed its wetting ability on MHAPC-lipoplexes since there was no hydrogen bond interaction with MHAPC. However, the interaction of sugars with lipid membranes may reflect on the dehydrating phenomenon of MEL-A on OH-Chol-lipoplexes.[28,29] According to the water replacement hypothesis, sugars interact with lipid headgroups through hydrogen bonds and exclude water molecules from the lipid membrane, maintaining the "dry" membranes in a physical state similar to that seen in the presence of water.[30] In our case, the hydroxyl groups in MEL-A would form hydrogen bonds with the secondary amine of OH-Chol, thereby excluding water molecules from the liposomal surface and finally dehydrating OH-Chol-lipoplexes.

Cellular Association of Lipoplexes The cellular association of lipoplexes in A549 cells was studied to establish a relationship with zeta potentials and hydration levels of lipoplexes. As shown in Fig. 7, MHAPC-lipoplexes demonstrated much higher cellular association of lipoplexes than OH-Chol-lipoplexes. This phenomenon corresponded well with the higher zeta potentials (Fig. 2A) and "drier" surface (Fig. 5A) of MHAPC-lipoplexes as compared to OH-Chol-lipoplexes. Modification by MEL-A and Tween 80 could diminish the cellular association of both MHAPC- and OH-Chol-lipoplexes in PBS. We suppose that the relative hydrated surface of MEL-A-modified MHAPC-lipoplexes (Fig. 5A) may be responsible for the slightly decreased cellular association, since the zeta potentials of MEL-A-modified and -non-modified MHAPC-lipoplexes are similar (Fig. 2A). Modification of MHAPC-lipoplexes by Tween 80 resulted in a slightly wet surface (Fig. 5A) and lower positive charge (Fig. 2A), which may be reasons for the extremely low cellular association. In terms of the cellular association of OH-Chol formulations (Fig. 7), although MEL-A modification also decreased cellular association, it showed significant higher association than the Tween 80-modified formulation. The decreased cellular association of MEL-A modified OH-Chol-lipoplexes was mainly due to the strongly negative zeta potential (Fig. 2B) as compared to non-modified formulations, however MEL-A modification significantly increased gene transfection efficiency of OH-Chol-lipoplexes. It might be due to membrane fusion between plasma membrane and cationic liposomes induced by MEL-A.[12,14—17] It is hard to image how MEL-A-modified OH-Chol-lipoplexes exhibited higher cellular association than Tween 80-modified OH-Chol-lipoplexes, since the former had much lower zeta potential (Fig. 2B); however, the relatively dehydrated surface of MEL-A-modified OH-Chol-lipoplexes (Fig. 5B) seemed to compensate for the negative zeta potential of the interaction with the cell membrane and exhibited higher cellular association of lipoplexes than Tween 80-modified OH-Chol-lipoplexes.

Summary

The higher positive zeta potential and greater dehydrated surfaces may relate to the higher cellular association of MHAPC-lipoplexes than OH-Chol-lipoplexes. Modification by MEL-A and Tween 80 demonstrated that the tertiary amine in MHAPC was relatively "stable" for the zeta potential and surface pH, while the secondary amine in OH-Chol was greatly affected. Tween 80 had high wetting ability independent of cationic lipids, while MEL-A dehydrated the liposomal surface of OH-Chol-lipoplexes, possibly by hydrogen bond interaction with the secondary amine. The dehydration of MEL-A-modified OH-Chol-lipoplexes might compensate for the negative zeta potential for its cellular association of lipoplexes. We found that the hydrated surface of lipoplexes may be less effective than the dehydrated surface in the cellular association of lipoplexes.

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