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Cerasome as an Infusible, Cell-Friendly, and Serum-Compatible Transfection Agent in a Viral Size

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As the function of nucleic acids continues to expand, there is growing need for efficient delivery of functional nucleic acids. Since the discovery of lipofection,[1] cationic lipids have been widely used as transfection agents in gene delivery.[2] They form liposomal bilayers in water. Polyanionic DNAs are readily bound to the resulting cationic liposomes to give complexes (lipoplexes) which are taken in the cells via endocytosis to ultimately result in expression of the encoded gene. We are concerned about lipoplex formation. The bilayer-keeping forces are not very strong, and liposomes are not so rigid or robust. They are potentially fusible with cell membranes and hence toxic. They also easily undergo DNA-mediated/induced cross-linking/fusion to give larger particles (≫100 nm) that have lower endocytosis susceptibility[3] and poorer vascular mobility. This problem has been challenged, occasionally in the context of artificial viruses, with different strategies of mostly multicomponent surface coverage[4] such as stabilized plasmid−lipid particles,[4a] saccharide-manipulated particles,[4b] dimerizable cationic detergents,[4c,g] liposome−μ-DNA systems,[4d,4e] glycoviruses,[4f] and PEG-stabilized plasmid nanoparticles.[4h] The Nara group of the present authors recently reported what they called cerasome (ceramic-coated liposome).[5] We have focused on the consequence of surface rigidification of the cerasome as a gene carrier. We report here that the cerasome retains the integrity of such in the complexation with plasmid DNA, and the resulting DNA complex of infusible or monomeric cerasome in a viral size (∼70 nm) exhibits a remarkable transfection performance (high activity, minimized toxicity, and serum-compatibility).

Reference lipid **2**[6] is a derivative of L-alanine, whose carboxyl and amino groups are converted to amides with dihexadecylamine and ω-trimethylammoniohexanoic acid, respectively. It forms bilayer liposomes (vesicles) in water after sonication.[6] Lipid **1** is a derivative of **2**, having a (triethoxysilyl)propyl group on the ammonium nitrogen (see Supporting Information (SI) for preparation). As detailed before,[5] it also self-assembles with concomitant hydrolysis (Si−OEt + H_2O → Si−OH + EtOH) followed by condensation (2Si−OH → Si−O−Si + H_2O) to give partially ceramic- or silica-coated (see SI for mass analysis) liposomes (cerasomes), which are otherwise similar to the **2**-derived liposomes in the DLS size (∼60 nm, Figure S1) and the freeze-fracture TEM appearance (60−70 nm, Figure 1a (**1**) and 1b (**2**)).

Complexation of cerasome lipid **1** with plasmid DNA was monitored by agarose gel electrophoresis. The plasmid used was pGL3 (5256 base pairs) encoding the firefly protein luciferase. The DNA duplex (pGL3) was rendered immobile at N/P ≥ 1.0 (Figure 2a) as a result of complexation[7] (N and P stand for the ammonium group of **1** ([N] = [**1**]) and a phosphate moiety of DNA,

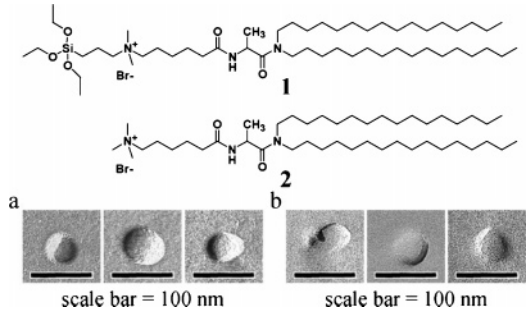

Figure 1. Structures of lipids **1** and **2** and freeze-fracture TEM images of the liposomes formed with lipid **1** (a) or **2** (b) in water at [**1**] = [**2**] = 12.5 μM. Approximately 10 independent images were taken for each lipid; three of them, including the smallest one and the largest one, are shown.

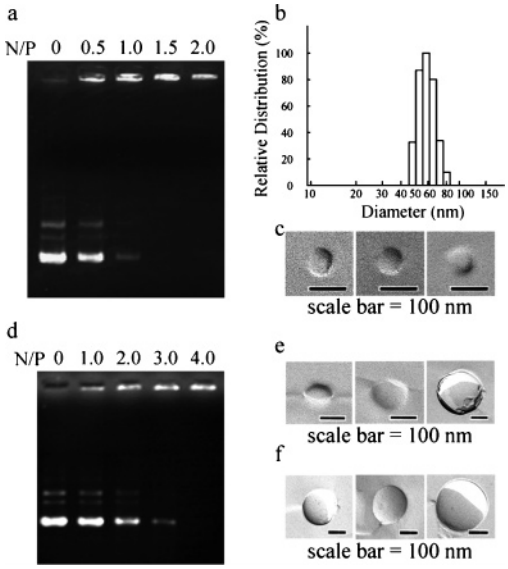

Figure 2. Electrophoretic gel shifts for pGL3 (160 ng) in the absence and presence of increasing amounts of lipid **1** (a) or **2** (d) using 0.7% agarose gel in 40 mM Tris-acetate buffer, size distribution profile for the **1**−pGL3 complex at 2 μM P and N/P = 2.0 in reference to number of particles as evaluated by Nicomp analysis of the DLS data (b), and freeze-fracture TEM images of an aqueous solution of **1**−pGL3 (c) and **2**−pGL3 (e) complexes at 6.25 μM P and N/P = 2.0 and **2**−pGL3 complex (f) at 62.5 μM P and N/P = 8.0. Approximately 10 independent images were taken for each sample; three of them, including the smallest one and the largest one, are shown.

respectively). DLS (2.0 μM P) and freeze-fracture TEM (6.25 μM P) at N/P = 2 (Figure 2b and 2c, respectively) revealed that the particles present had a rather uniform size of ∼70 nm, which was essentially the same as that for the DNA-free cerasome itself (60−70 nm, Figure 1a).[7] Non-ceramic reference lipid **2** turned out to be

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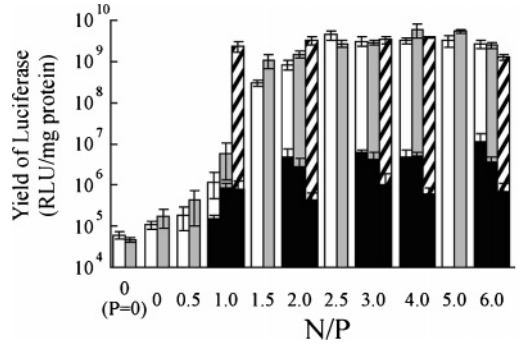

Figure 3. Normalized yields of luciferase in the serum-free transfection of HeLa (white bars) and HepG2 (gray bars) cells using pGL3 (200 ng) and variable amounts of lipid **1** or **2** (black bars) as a gene carrier. The data for transfection of HepG2 in the presence of serum (10% FCS) are shown by slashed bars for **1** and black bars therein for **2**.

a somewhat weaker complexer and required N/P $\geq$ 3.0 for its electrophoretic immobilization (Figure 2d). The TEM image at 6.25 μM P and N/P = 2 revealed the presence of particles of various sizes (100−300 nm) (Figure 2e), and the particle sizes became more uniform in the range of 200−300 nm at 62.5 μM P and N/P = 8 (Figure 2f). These results indicate that the normal liposomes formed by non-ceramic lipid **2** undergo fusion upon interaction with pGL3, while the surface-rigidified or -sewed cerasome derived from ceramic lipid **1** binds to the plasmid more strongly without fusion to give DNA complexes of *monomeric* cerasome of the original size (see SI).[7]

We then examined the **1**-mediated transfection (see SI) of HeLa (uterine) and HepG2 (hepatic) cells using a fixed amount (200 ng, 0.6 nM or 6.25 μM P) of pGL3 and a variable amount of **1**.[8] Figure 3 shows the normalized yields of luciferase expressed in the HeLa (white bars) and HepG2 (gray bars) cells, which increase with increasing N/P ratio until saturation is reached at N/P $\cong$ 2. MTT assay (see SI) as a measure of cytotoxicity revealed that the cell viability (cell growth rate) remained $\geq$100% for both types of cells even at N/P = 6. These results indicate that neither the transfection-responsible cerasome−plasmid complex (~70 nm) nor the DNA-free cerasome (60−70 nm) is toxic under the present transfection conditions. Reference lipid **2**, on the other hand, turned out to be far less active and far more toxic. Under the above conditions (6.25 μM P, N/P $\geq$ 2) where lipid **1** gave an efficiency of 10^9−10^{10}, that of lipid **2** was only in the range of 10^6−10^7 (Figure 3, black bars) with cell viabilities at **2**/P = 3.0 of 63% for HeLa and 54% for HepG2. Remarkably, the cerasome−plasmid complexation is compatible with serum (Figures S2 (gel assay) and S3 (TEM)). The efficiency of **1**-mediated transfection of HepG2 cells is hardly affected (10^{9-10}) by the presence of 10% FCS (slashed bars in Figure 3). In marked contrast, that of **2**-mediated transfection is lower by one order of magnitude in the presence of the serum (black bars in the slashed ones, ~10^6) than that of the serum-free transfection; the cerasome factor becomes more pronounced (**1**/**2** = 10^{3-4}). Interestingly, the big difference in activity of **1**/**2** = 10^{2-3} (serum-free) fits on the size−activity correlation revealed for glycoviruses[4f] and can thus be explained primarily in terms of difference in the size of *monomeric* **1**−plasmid complex (~70 nm) and *fused* **2**−plasmid complex ($\geq$200 nm), although possible differences in aggregation behaviors should also be taken into account.

This work reveals the profound effects of an otherwise simple alkoxysilyl substituent in gene delivery. Lipid **1** forms liposomes which self-rigidify via in situ sol−gel processes on the surface to give cerasomes that retain their integrity in the complexation with plasmid DNA. The resulting serum-compatible DNA complex of infusible or monomeric cerasome in a viral size (~70 nm) exhibits a high transfection performance with minimized toxicity. Non-ceramic lipid **2** forms fewer self-confined and more mobile liposomes that undergo DNA-induced fusion into endocytosis-irrelevant and larger, more toxic particles; the transfection efficiencies differ by a factor of **1**/**2** = 10^{2-3} (−serum) or 10^{3-4} (+serum). The defects of lipid **2** are more or less common to any type of cationic lipids and probably polymers as well. The presented *mono-component* silicon strategy provides a simple and widely applicable new tool to overcome these general problems inherent in the current technology of artificial gene delivery. Size control is particularly crucial for in vivo applications. Particles of the viral size (mostly 30−100 nm) have not only good diffusion in vascular periphery but also good permeation into malignant tissues by the so-called EPR effect.[9]

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Supporting Information Available: Preparation of **1**, preparation of cerasome and mass analysis, DLS profiles for liposomes formed with lipid **1** and **2** (Figure S1), complexation of cerasome and plasmid in the presence of serum (Figures S2 and S3), stoichiometry of cerasome-plasmid complex, and transfection and luciferase/MTT assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Judging from the recent claim that it is the amino group that is responsible for the binding of DNA with aminoalkylated silica nanoparticles (He, X.-x.; Wang, K.; Tan, W.; Liu, B.; Lin, X.; He, C.; Li, D.; Hung, S.; Li, J. *J. Am. Chem. Soc.* **2003**, *125*, 7168−7169), the present cerasome−plasmid complexation must be primarily driven by electrostatic (N−P) forces, although contribution of the Si−O−Si and Si−OH moieties could not be ruled out. Calculation suggests that one cerasome binds ~3 molecules of plasmid pGL3 (see SI).

(8) For the transfection behavior (luciferase yield and toxicity) of lipofectamine 2000 under the present conditions, see: Matsui, K.; Horiuchi, S.; Sando, S.; Sera, T.; Aoyama, Y. *Bioconjugate Chem.* **2006**, *17*, 132−138.

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