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RNAi gene silencing using cerasome as a viral-size siRNA-carrier free from fusion and cross-linking

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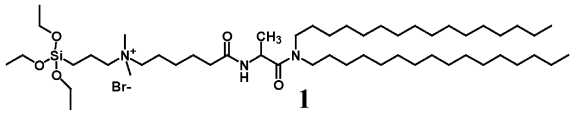
Abstract—Surface-rigidified cerasomes (ceramic-coated liposomes) are neither fused nor cross-linked when bound to siRNA (short duplex RNA) but not to plasmid DNA (long duplex DNA) which induces cross-linking. Non-ceramic reference liposomes are easily fused by the siRNA. The cerasome can thus be used as a viral-size siRNA-carrier in a wide range of concentration for RNAi silencing of exogenous and endogenous genes.

Since the discovery of lipofection,[1] cationic lipids have been widely used as transfection agents in gene delivery in vitro.[2] They form cationic liposomes, to which anionic DNAs (plasmids) are bound electrostatically. This is, however, an over-simplified formulation. Liposomes (vesicles) easily undergo fusion (aggregation) as induced not only by hydrophobic species[3] but also by simple ions (divalent metal cations[4] and organic dianions[5] for anionic and cationic liposomes, respectively). There is no doubt that DNAs (and RNAs as well) are potential fusion-inducers to lead to transfection-irrelevant (low efficiency and high toxicity) big particles.[6] Thus, we are often forced to use the plasmid and lipid in low 'protocol' concentrations. A real problem of artificial (non-viral) gene delivery, as far as its low efficiency is concerned, seems to lie in this high-concentration intolerance.

We recently introduced the cerasome strategy.[7,8] Trialkoxysilylated quaternary ammonium lipid **1** (Fig. 1) forms single-walled liposomes with concomitant sol-gel processes on the surface (Si-OEt + H_2O → Si-OH + EtOH followed by 2Si-OH → Si-O-Si + H_2O) to give surface-rigidified cerasomes (ceramic- or silica-coated liposomes),[9] which can be used as an infusible transfection agent of high performance at ~10 μM lipid concentration. Unfortunately, as shown below, however, the cerasomes are still not high-concentration tolerant, because they are still cross-linkable by the plasmid as a huge template. What if we use much shorter oligonucleotides? This is how we started the present work on siRNA delivery. We report here that the cerasome is neither fused nor cross-linked by the siRNA and can thus



5' CUUACGCUGAGUACUUCGATT 3'
3' TTGAAUGCGACUCAUGAAGCU 5'

$siRNA_{firefly}$

5' GUGGGAGCGCGUGAUGAACTT 3'
3' TTCACCCUCGCGCACUACUUG 5'

$siRNA_{DsRed2}$

Figure 1. Lipids **1** and **2** and silencers $siRNA_{firefly}$ and $siRNA_{DsRed2}$.

Keywords: Liposome; RNA; Cerasome; siRNA delivery; RNA interference.
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be used as a viral size siRNA-carrier in a wide concentration range.[10]

Complexation of cerasome lipid **1**[11] and siRNA was monitored by gel electrophoresis. The siRNA used was a 21-mer duplex RNA ($siRNA_{firefly}$, Fig. 1)[11] homologous in sequence with the gene of firefly protein luciferase encoded in plasmid pGL3. The RNA duplex was rendered immobile at N/P $\geqslant$ 1.5 (Fig. 2a) as a result of complexation with the lipid (N and P stand for the ammonium group of **1** ([N] = [**1**]) and a phosphate moiety of RNA, respectively). Freeze-fracture TEM (transmission electron microscopy) revealed that the particles present at 100 μM N and 33 μM P (N/P = 3.0) had a rather uniform and compact size of ~70 nm (Fig. 3a), which was essentially the same as that of the RNA-free cerasome itself (60–70 nm, Fig. 3b). When plasmid pGL3 was used in place of $siRNA_{firefly}$ in a similar concentration range of 100 μM N and 50 μM P (N/P = 2.0), a quite different morphology was observed (Fig. 3c), a number of cerasome particles being bound to, and hence cross-linked by, the plasmid in an extended form to give a mean DLS (dynamic light scattering) size of the complex of 205 nm.[12] Non-silylated reference lipid **2**[11] turned out to be a somehow weaker complexer and required N/P $\geqslant$ 3.0 for immobilizing $siRNA_{firefly}$ (Fig. 2b). The TEM images at 100 μM N and 33 μM P (N/P = 3.0) revealed the presence of much bigger particles with various sizes (150–500 nm, Fig. 3d), in marked contrast to the liposomes (60–70 nm, Fig. 3e) formed by lipid **2** in the absence of $siRNA_{firefly}$.

The above results, obtained at a relatively high (100 μM) lipid (N) concentration, provide a couple of interesting comparisons. (1) The normal liposomes formed by non-ceramic lipid **2** undergo fusion upon interaction with $siRNA_{firefly}$, while the surface-rigidified or -sewed cerasomes derived from ceramic lipid **1** bind to the siRNA without fusion. This is supported also by fluorescence studies.[13] (2) The infusible cerasome particles are cross-linked when interacting with plasmid pGL3 but remain monomeric as such when binding to $siRNA_{firefly}$. The different behaviors of pGL3 and $siRNA_{firefly}$ may be understood in terms of their different chain-lengths. pGL3 contains 5256 base-pairs with an extended strand length of $l \cong 1800$ nm, which is long enough to assemble cerasome particles (d = 60–70 nm) on. The siRNA, on the otherhand, is composed of 19 base-pairs and two-base overhangs at both ends and is too short ($l \cong 7$ nm) to do so.

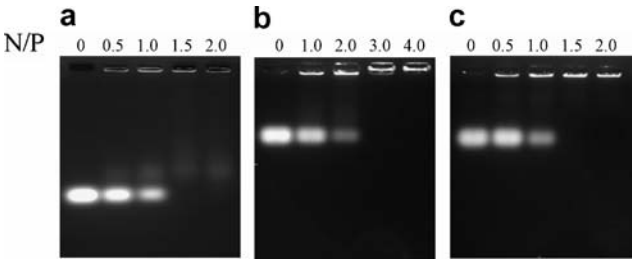

Figure 2. Electrophoretic gel shifts for $siRNA_{firefly}$ in the absence and presence of increasing amounts of lipid **1** (a, without FCS; c, with FCS (10%); FCS, fetal calf serum) or lipid **2** (b, without FCS) using 0.7% agarose gel in 40 mM Tris–acetate buffer.

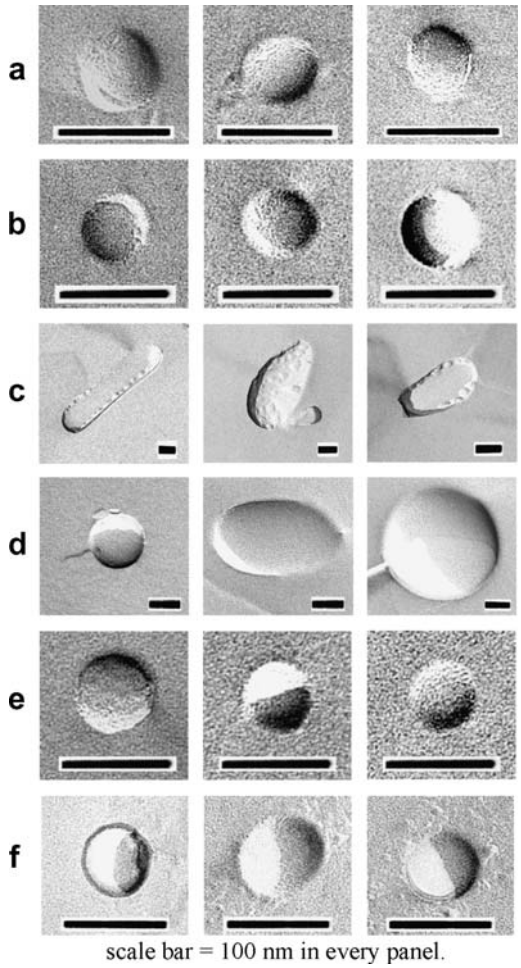

Figure 3. Freeze-fracture TEM images (scale bar = 100 nm) at 100 μM N of **1** $siRNA_{firefly}$ complex (a, N/P = 3.0), **1** (b), **1**-pGL3 complex (c, N/P = 2.0), **2**-$siRNA_{firefly}$ complex (d, N/P = 3.0), and **2** (e) in water and **1**-$siRNA_{firefly}$ complex (f, N/P = 3.0) in water containing 10% FCS (fetal calf serum). Approximately 10 independent images were taken for each sample; three of them, including the smallest one and the largest one, are shown.

RNA interference (RNAi) is a posttranscriptional gene silencing process.[14] The $siRNA_{firefly}$-mediated silencing of target firefly luciferase in pGL3 was investigated using *Renilla* luciferase contained in plasmid pRL-TK as a non-silenced control gene under dual-assay conditions.[10a] HeLa cells were cotransfected with a mixture of plasmids pGL3 (or pGL2 as a non-target control[15]) (180 ng per well) and pRL-TK (20 ng per well) and silencer $siRNA_{firefly}$ (0–126 ng) using lipid **1** as a common carrier at $N/P_t = 3.0$ ($P_t = P_{pGL3(2)} + P_{pRL\text{-}TK}$).[11] The normalized yields ($Y_{firefly}/Y_{Renilla}$) of pGL3-derived firefly luciferase (black bars), but not pGL2-derived ones[15] (slashed bars), sharply decrease in the presence of $siRNA_{firefly}$ (Fig. 4), showing that the effective (~90%) RNAi silencing of the present exogenous luciferase gene is achieved by a tiny amount (~10 ng, ~0.3 μM P) of the siRNA complexed with **1** as a carrier.[16]

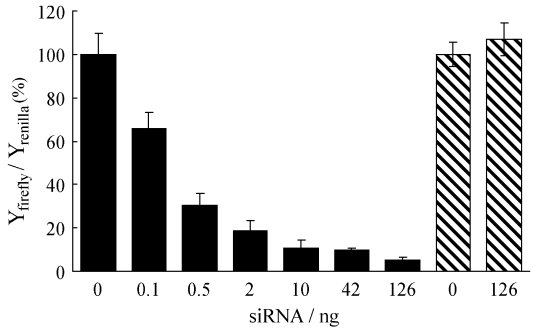

Figure 4. RNAi silencing of exogenous firefly luciferase gene in HeLa cells by the increasing amounts of $siRNA_{firefly}$ complexed with lipid **1** under cotransfection conditions. Chemiluminescence intensity ratios of target to control (pGL3 firefly to pRL-TK *Renilla*, black bars) or control to control (pGL2 firefly to pRL-TK *Renilla*, slashed bars) luciferase are normalized to a buffer control (siRNA = 0 ng). Data are averages of about five experiments and error bars represent standard deviations.

We then moved on to silencing of an endogenous gene using HeLa cells stably expressing fluorescent protein DsRed2.[17] They constantly fluoresce at 580 nm (excitation at 563 nm). Silencing of this endogenous DsRed2 gene in $HeLa_{DsRed2}$ was monitored by fluorescence intensity using the DsRed2-specific siRNA ($siRNA_{DsRed2}$, Fig. 1) ($\leqslant$500 ng or $\leqslant$15 μM P) as well as $siRNA_{firefly}$ as a non-silencing reference siRNA; both being complexed with lipid **1** at N/P = 2.0 ($\leqslant$30 μM N). The cell viability based on MTT assay was ~100% at least up to 252 ng (7.6 μM P and 15.2 μM N) of siRNA, where silencing was ~70%[18] with $siRNA_{DsRed2}$ and ~0% with $siRNA_{firefly}$. The silencing profile in the whole range is shown in Figure 5 (black bars). The smooth and [$siRNA_{DsRed2}$]-dependent decrease in the yields of DsRed2, coupled with the lack of activity of $siRNA_{firefly}$, confirms that the silencing is indeed due to specific RNAi by the $siRNA_{DsRed2}$ delivered from the bulk medium where, referring to Figure 3a (taken at 100 μM N), the

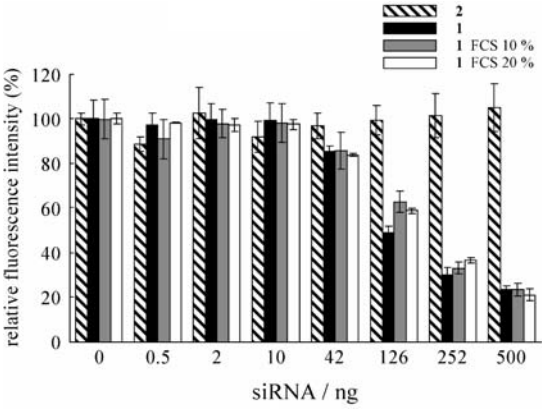

Figure 5. RNAi silencing of endogenous DsRed2 gene in $HeLa_{DsRed2}$ cells by the increasing amounts of $siRNA_{DsRed2}$ complexed with lipid **1** in the absence (black bars) or presence of 10% FCS (gray bars) or 20% FCS (white bars). Slashed bars are for control runs using lipid **2** in place of **1** in the absence of FCS. Fluorescence intensities for DsRed2 are normalized to a buffer control (siRNA = 0 ng). Data are averages of about five experiments and error bars represent standard deviations.

$siRNA_{DsRed2}$ ($\leqslant$30 μM N) must remain attached to the non-fused cerasome. The cell viability was >90% in the whole range. Control runs showed that non-ceramic reference lipid **2** was completely inactive (slashed bars) under the present conditions, where, referring to Figure 3d, the $siRNA_{DsRed2}$ must be contained in fused huge particles irrelevant for cellular uptake. Furthermore, the present system is serum-compatible. The **1**-$siRNA_{DsRed2}$ complex survives in the presence of FCS (fetal calf serum), as evidenced by electrophoresis (Fig. 2c) and TEM (Fig. 3f), and displays similar silencing performance (Fig. 5, gray bars (10% FCS) and white bars (20% FCS)) as it does in the absence of the serum (black bars).

Size control of particles is crucial for their efficient cellular uptake[19] and smooth vascular diffusion. This work reveals that the surface-rigidified, infusible cerasomes (d = 60–70 nm) are not cross-linked when interacting with short siRNAs ($l \cong 7$ nm), thus keeping the cerasome–siRNA complexes in a compact viral-size for their better RNAi performance.[20] The size stability and serum-compatibility of the complexes suggest a potential in vivo application of the cerasome-mediated siRNA delivery. siRNAs can be encoded in plasmid DNAs and delivered as such.[21] However, the size problem in lipofection-based gene (plasmid) delivery appears to be rather serious. The monomeric cerasome complexes of plasmid DNA (pGL3, $l \cong 1800$ nm) in a viral size ($d \cong 70$ nm) survive at ~10 μM N[7,8] but, as shown here, collapse into cross-linked multi-cerasome complexes at a 10-fold higher concentration (100 μM N). Plasmid-induced, concentration-dependent cross-linking of liposomes, even when rendered infusible, seems to be unavoidable as far as the huge plasmid is simply bound on the outer surface of the liposome and not encapsulated therein as in real viruses. Further work is now under way along the above line.

Acknowledgments

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Supplementary data

Supplementary data for the DLS size distribution profile for the cerasome–pGL3 complexes (Fig. S1), fluorescence study for fusion (Figs. S2 and S3), zeta potential study, and optical and fluorescence microscopic images of the $HeLa_{DsRed2}$ cells (Fig. S4) (3 pages). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.04.097.

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