

SUPPLEMENTARY INFORMATION

Cationic cholesterol-modified gelatin as an *in vitro* siRNA delivery vehicle

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Materials and methods

Materials

6-carboxy fluorescein (FAM)-labeled siRNA directed against the firefly luciferase (sense 5' FAM-GUGCGCUGCUGGUGCCAACuu, antisense 5'-GUUGGCACCAGCAGCGCACuu) was purchased from Eurofins Operon (Huntsville, AL). 4', 6-Diamidino-2-phenylindole (DAPI) vectashield mounting medium was obtained from Vector Laboratories (Burlingame, CA). Sodium acetate, glycogen, Dulbecco's modified Eagle's medium (DMEM) low-glucose with L-glutamine and 4% paraformaldehyde in phosphate-buffered saline (PBS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lipofectamine® 2000 was obtained from Invitrogen (Carlsbad, CA). Passive lysis buffer (PLB) and luciferase assay system was purchased from Promega (Madison, WI). Bicinchoninic acid (BCA) protein assay kit was obtained from

Pierce, Thermoscientific (Rockford, USA). Cell counting kit-8 solution (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan).

Synthesis of cationic cholesterol-modified gelatin (cCMG)

cCMG was synthesized from recombinant human gelatin and amino-modified cholesterol (Ch-A) via the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) coupling method as described in detail previously.¹ Briefly, cholesterol was reacted with 1,6-hexyl diisocyanate to form the cholesterol isocyanate (Ch-I). The Ch-I so obtained was reacted with ethylene diamine to yield amino-modified cholesterol (Ch-A). The Ch-A was allowed to conjugate with the carboxyl groups of the Asp and Glu residues present in rhG by the EDC coupling method. This reaction was carried out overnight. The product was then dialyzed against distilled water and freeze-dried.

Synthesis of siRNA

RNA oligonucleotides were synthesized as described elsewhere² using the 2'-O-TOM protected – cyanoethyl phosphoramidites. siRNA utilized for the study targeted the GL3 luciferase and consisted of 21-nt with a 2-nt overhang at the 3'-end of each strand (luc siRNA: sense 5'-GUGCGCUGCUGGUGCCAACuu, antisense 5'-GUUGGCACCAGCAGCGCACuu).

Stability of the polymeric micelle, cCMG, at biological condition

1 mg of the polymeric micelle, cCMG, was dispersed in 1 mL phosphate buffer saline (PBS). This solution was incubated for 24 h at 37 °C so as to evaluate its stability. Stability was characterized in terms of size and zeta (ζ)-potential using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). Measurements were carried out at 25 °C. The size and ζ -potential are reported as the mean \pm standard deviation (SD) of 14 subruns.

Calculation of N/P ratio and formation of cCMG-siRNA polyplexes

The number of available amino groups in the polymeric micelle cCMG, was assessed by a 2, 4, 6- Trinitrobenzenesulfonic acid (TNBS) (Tokyo Chemical Industries Co. Ltd., Tokyo, Japan) colorimetric assay as explained elsewhere.³ Briefly, cCMG was dissolved in 0.1M sodium bicarbonate buffer (pH – 8.5) and were diluted to various concentrations. The solution containing 1mL of sample and 0.5mL of 0.01% TNBS diluted freshly in sodium bicarbonate was mixed and incubated at 37 °C for 2 h after which 0.5 mL of 10% sodium dodecyl sulfate and 0.25 mL of 1N HCl were added to the sample and the absorbance was measured at 335 nm. The number of free amino groups was determined by the molar absorption coefficient. The N/P ratios of the polyplexes were thus calculated. For the cCMG/ siRNA weight/ weight ratios (w/w in nM) of 0.5, 1, 5, 10, 15, 30, 40, 60 and 90, the N/P ratios were found to be 0.04, 0.2, 0.9, 1.3, 2.7, 3.8, 5, 8.7 and 11.3, respectively.

For the formation of complexes, aqueous solutions of cCMG were diluted to different concentrations for the desired N/P ratio using Opti-MEM reduced serum medium (Invitrogen, US) and mixed with the siRNA solution. The solution was incubated for 20 minutes at room temperature (RT) to allow the formation of cCMG-siRNA complexes and thereafter used for characterization and gene knockdown studies.

Gel shift assay

To qualitatively determine if there was any free siRNA remaining after the formation of a complex between the polymer and siRNA, a gel retardation analysis was carried out on a 20% non-denaturing polyacrylamide gel in tris-borate EDTA (TBE) buffer. siRNA Ladder Marker (Takara Bio, Japan) was used as the size marker for the dsRNA. The gel was electrophoresed at

10 W for 3h, stained with SYBR Green I (Lonza Rockland, US) according to the manufacturer's protocol and visualized by scanning on a BioRad Molecular Imager (BioRad, CA).

Measurement of size and ζ -potential of the polyplexes

We analyzed the polyplexes formed by using various stoichiometric ratios of the polymeric micelles and siRNA by dynamic light scattering (DLS) for size as well as for the changes in ζ -potential using the Zetasizer Nano ZS. The size distribution results are shown as z-average size. All measurements were reported as the mean \pm standard deviation (SD) of 14 subruns and carried out at 25 °C.

Cell culture

The human cervical cancer cells or HeLa cells that stably expressed luciferase (HeLa-luc) were utilized for the study which was kindly provided by Dr. Hiroto Hatakeyama. The cells were cultured in DMEM medium that was supplemented with 10% fetal bovine serum (FBS), 1X penicillin and streptomycin and 0.4 mg/mL G-418 (Roche, Germany) at 37 °C in a humidified atmosphere containing 5% CO₂.

cCMG-mediated siRNA delivery

To validate the potential of cCMG to deliver siRNA to cells, FAM-labeled siRNA that targeted the firefly luciferase gene, was utilized. HeLa cells were seeded at a concentration of 1.4×10^4 cells/well in a glass bottom 96-well plate and cultured in complete DMEM medium for 24 h at 37 °C under 5% humidified CO₂. For siRNA delivery, polyplexes were formed by mixing the aqueous solutions of cCMG and siRNA for 20 min. The concentration of cCMG was kept constant at 600 nM while the siRNA concentration was varied from 50 nM, 20 nM and 3 nM.

Commercially available Lipofectamine® 2000 was used as a standard control and transfection was performed according to the manufacturer's protocol. The complexes were added to the cells to give a final volume of 100 µL and incubated for 24 h in complete medium without antibiotics. After 24 h, the cells were washed with PBS, fixed with paraformaldehyde for 1 h. The nuclei were stained with DAPI and the cells were observed using a fluorescence microscope (Carl Zeiss, Göttingen, Germany).

cCMG-mediated gene silencing

HeLa-luc cells were seeded on a 96-well plate at a concentration of 1×10^4 cells/well and grown for 24 h. After 24 h, the cells were treated with the polyplexes, formed at the N/P ratio of 3.8 (as described above) with a siRNA concentration of 20 nM. Lipofectamine® 2000 was used as a standard control and transfected according to the manufacturer's protocol. After 72 h of incubation in complete medium without antibiotics, the cells were rinsed with PBS and lysed by PLB for 30 min and subjected to luciferase assay system. The firefly luminescence was measured using a Mithras LB940 plate reader (Berthold technologies, Germany) after the addition of luciferin substrate. The relative light units (RLU) were normalized to the total protein concentration using the BCA protein assay kit and the results are shown as RLU/mg of protein. The data are expressed as mean \pm SD of three independent experiments.

Stability of the polyplexes against FBS

In order to assess the stability of the siRNA in FBS, 10 pmol siRNA was mixed with cCMG at the N/P ratio of 3.8. The experimental protocol was performed as explained previously with slight modifications.⁴ To the resulting polyplex solution, four volumes of 10% FBS was added and incubated at 37 °C for the indicated time intervals. After incubation, the solution was diluted

with 100 μL water. 100 μL of Isogen (Nippon gene, Japan) was added, vortexed and incubated at RT for 15 min. 50 μL chloroform was added, mixed vigorously and incubated for another 2-3 min at RT and centrifuged at 12,000 g at 4 $^{\circ}\text{C}$ for 15 min. The aqueous layer so obtained was recovered. To this, 1 μL (20 μg) of glycogen, 0.1 volume of 3M sodium acetate (pH 5.5) and 2.5 volumes of 99.5% ethanol were added to precipitate the siRNA. The solution was mixed thoroughly and incubated at -20 $^{\circ}\text{C}$ for 1 h and centrifuged for 30 min at maximum speed. The supernatant was removed and the pellet was washed twice with 70% ethanol and redissolved in 20 μL of RNase free water. The RNA was run on 20% Native PAGE as described above. The percentage of RNA was analyzed by Image J.

Cell viability

The cytotoxicity of the polymeric micelles was determined using a water-soluble tetrazolium (WST) assay. HeLa-luc cells were seeded in a 96-well plate at a concentration of 1×10^4 cells/well and cultured in complete medium without antibiotics for 24 h at 37 $^{\circ}\text{C}$ under 5% humidified CO_2 . The culture medium was then replaced with fresh complete medium without antibiotics containing cCMG or Lipofectamine® 2000 alone or the polyplexes (N/P ratio 3.8) and lipoplexes of cCMG and Lipofectamine® 2000, respectively. The cells were incubated for 72 h after which they were washed with PBS and medium was added. Aliquots of 10 μL of CCK-8 solution were added to the medium and shaken for 30 s to mix the solution thoroughly and incubated at 37 $^{\circ}\text{C}$ for 25 minutes. The absorbance of each well was measured using a microplate reader (Model-680, Bio-Rad, USA) at 450 nm. The percentage of cell viability was calculated from the absorbance values.

SUPPLEMENTARY FIGURES

Before incubation at 37 °C		After incubation at 37 °C for 24 h	
Size (d.nm)	ζ-potential (mV)	Size (d.nm)	ζ-potential (mV)
417	+9.1	420	+9.0

Table S1:

Stability of the polymeric micelle, cCMG, at 37 °C for 24 h in PBS measured in terms of size (d.nm) and zeta (ζ)-potential (mV) by dynamic light scattering.

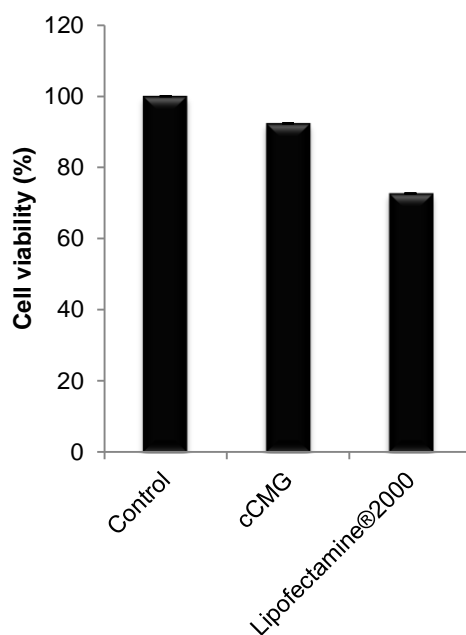


Fig. S1: Cytotoxicity of cCMG and Lipofectamine®2000 alone as estimated by WST assay after 72 h.

Supporting information references:

1. P.A. Kadengodlu and T. Hebishima, *et al.*, *Int. J. Nanomed.*, 2012, **7**, 5437.
2. N. Abe and H. Abe, *et al.*, *J. Am. Chem. Soc.*, 2007, **129**, 15108.
3. G.T Hermanson, *Bioconjugate Techniques*, ed. G.T. Hermanson, Elsevier, San Diego, 2nd edn., 2008, ch. 4, pp. 127-128.
4. M. Chen and S. Gao, *et al.*, *ACS Nano*, 2012, **6**, 4835; B. R. McNaughton and J. J. Cronican, *et al.*, *Proc. Natl. Acad. Sci.*, 2009, **106**, 6111.