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NaCl improves siRNA delivery mediated by nanoparticles of hydroxyethylated cationic cholesterol with amido-linker

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ABSTRACT

We investigated the effect of amido- (NP-OH) and carbamate linkers (NP-HAPC) in nanoparticles composed of hydroxyethylated cationic cholesterol on siRNA transfection. The presence of NaCl in forming a NP-OH nanoplex increased the suppressive effect of gene expression by increasing the size of the nanoplex and changing the cellular uptake mechanism from membrane fusion and clathrin-mediated endocytosis to clathrin- and caveolae-mediated endocytosis.

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Non-viral vectors, such as cationic lipid-based[1,2] and polymer particles[2] have been focused on for their ease of synthesis, low immune response and safety. Many different cationic lipids have been synthesized for non-viral vectors. The general structure of a cationic lipid has three parts: (1) a hydrophobic lipid anchor group, which helps to form the micellar structure and can interact with cell membranes; (2) a linker group, such as an ester, amido or carbamate; and (3) a positively charged headgroup, mainly composed of cationic amine. Concerning the anchor group, cationic cholesterol derivatives could be justified by their high transfection activity and low toxicity. The linker group of a cationic cholesterol derivatives controls the conformational flexibility, degree of stability, biodegradability, and gene transfection efficacy.[3,4] Concerning the head group, cationic cholesterol derivative, such as cholesteryl-3β-carboxyamidoethylene-*N*-hydroxyethylamine (OH-Chol) having a hydroxyethyl group at the amine headgroup and an amido-linker (Fig. 1), achieved high transfection ability for plasmid DNA and synthetic small interfering RNA (siRNA) deliveries.[5–7] siRNAs, which are small double-stranded RNA, suppresses the expression of a target gene by triggering specific degradation of the complementary mRNA sequence. We have reported that cationic nanoparticles composed of OH-Chol (NP-OH) could deliver siRNA with high transfection efficiency at a charge ratio (±) of 3/1 in vitro when the nanoparticle/siRNA complex (nanoplex) was formed in 50 mM NaCl solution.[7] Cationic cholesterol derivatives with an ether linker have been demonstrated to exhibit superior

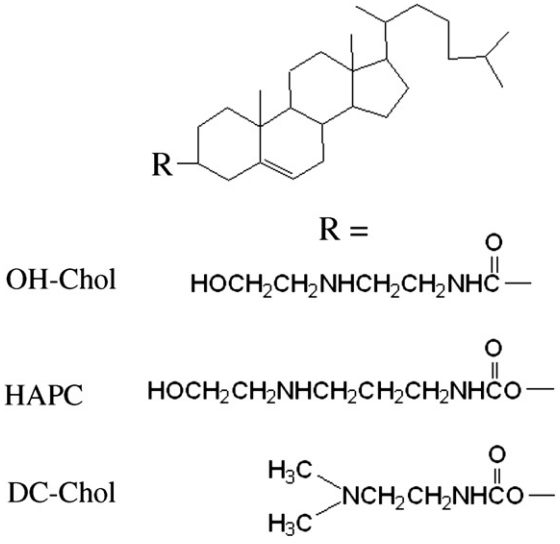

Figure 1. The structure of cationic derivatives of cholesterol.

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in vitro gene transfection efficacies to their ester counterparts;[8,9] therefore, in this study, to investigate the effect of a linker in cholesterol derivatives on transfection, we synthesized *N*-hydroxyethyl aminopropane carbamoyl cholesterol iodide (HAPC) with a carbamate linker (Fig. 1) and compared the physicochemical properties and siRNA transfection with NP-OH in forming a nanoplex in the presence or absence of NaCl.

OH-Chol and HAPC were synthesized as previously described.[10,11] 3([*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol, Sigma Chemical Co., St. Louis, MO, USA) was used as a commercially available cationic cholesterol derivative with a carbamate linker (Fig. 1). All formulations of nanoparticles consisted of 1 mg/ml cationic cholesterol and 0.13 mg/ml Tween 80 (NOF Co. Ltd., Tokyo, Japan). For example, NP-OH nanoparticles were prepared with lipids in 10 ml of water by the modified ethanol injection method.[10,11] Nanoparticles consisting of DC-Chol and HAPC were prepared by the same procedure, and were named NP-DC and NP-HAPC, respectively. The nanoparticle/siRNA complex (nanoplex), at a charge ratio (±) of 3/1, was formed by the addition of each nanoparticle to 100 pmol siRNA in water or 50 μl of 50 mM NaCl solution with gentle shaking and leaving at room temperature for 15 min.[7] The average size of each nanoparticle was approximately 100–130 nm (Table 1).[12] We compared the physicochemical properties of nanoplexes formed in water and 50 mM NaCl solution. The size of NP-OH, NP-HAPC, and NP-DC nanoplexes formed in water slightly increased to about 200–400 nm, and those formed in 50 mM NaCl solution significantly increased to about 800–1000 nm (Table 1). The cationic charge on the surface of the cationic nanoparticles may be neutralized by the presence of NaCl, resulting in instability of the nanoplex and facilitating the size increase.[13] The ζ-potential of each nanoplex was approximately from +22 to +40 mV.

We measured the transfection efficiencies of NP-OH into human prostate tumor PC-3 cells stably expressing the luciferase gene (PC-3-Luc) by assaying luciferase activity 48 h after siRNA transfection in the presence of serum.[14] The siRNA targeting nucleotides of Luc and random siRNA as a negative control were synthesized by Hokkaido System Science (Hokkaido, Japan).[15] At 48 h after siRNA transfection, luciferase activity was measured as counts per second (cps)/μg protein using the luciferase assay system (Pica gene, Tokyo Ink Mfg. Co. Ltd., Tokyo, Japan) and BCA reagent (Pierce, Rockford, IL, USA) as previously reported.[6] As shown in Figure 2A, concerning the nanoplex formed in water, NP–HAPC was more effective in the down-regulation of luciferase activity than NP-OH and NP-DC. The presence of NaCl in forming a nanoplex increased the suppression of luciferase activity in NP-OH; however, it did not affect RNAi activity in NP-HAPC, indicating that the structure of the linker in cationic cholesterol derivatives influenced the transfection activity. A commercially available transfection reagent, Lipofectamine 2000, exhibited strong gene suppression by siRNA. We examined the cytotoxicities 48 h after transfection of the NP-OH, NP-HAPC, and NP-DC nanoplexes and Lipofectamine 2000 lipoplex in PC-3 cells with a cell proliferation assay kit (Dojindo, Kumamoto, Japan). The Lipofectamine 2000 lipoplexes exhibited significant toxicity (about 40% cell viability) (Fig. 2B). In contrast, NP-OH, NP-DC, and NP-HAPC nanoplexes did not actually

Table 1
Size and ζ-potential of nanoplexes formed in water or 50 mM NaCl solution at a charge ratio (±) of 3/1[a]

Nanoparticles	Cationic lipid	Size of nanoparticle (nm)	ζ-potential of nanoparticle (mV)	Size of nanoplexes formed (nm)		ζ-potential of nanoplexes[b] (mV)
				In water	In 50 mM NaCl	
NP-OH	OH-Chol	118.0 ± 3.1	40.7 ± 1.0	224.4 ± 1.0	1107.9 ± 72.7	22.1 ± 0.6
NP-HAPC	HAPC	120.0 ± 2.9	45.1 ± 0.5	227.5 ± 8.0	1093.0 ± 51.3	39.3 ± 0.6
NP-DC	DC-Chol	117.1 ± 11.8	45.3 ± 1.8	373.0 ± 9.9	836.7 ± 13.0	39.9 ± 1.9

[a] Particle size distributions and ζ-potentials were measured 10–15 min after forming nanoplex. Values represent means ± SD (n = 3).
[b] Nanoplex formed in water.

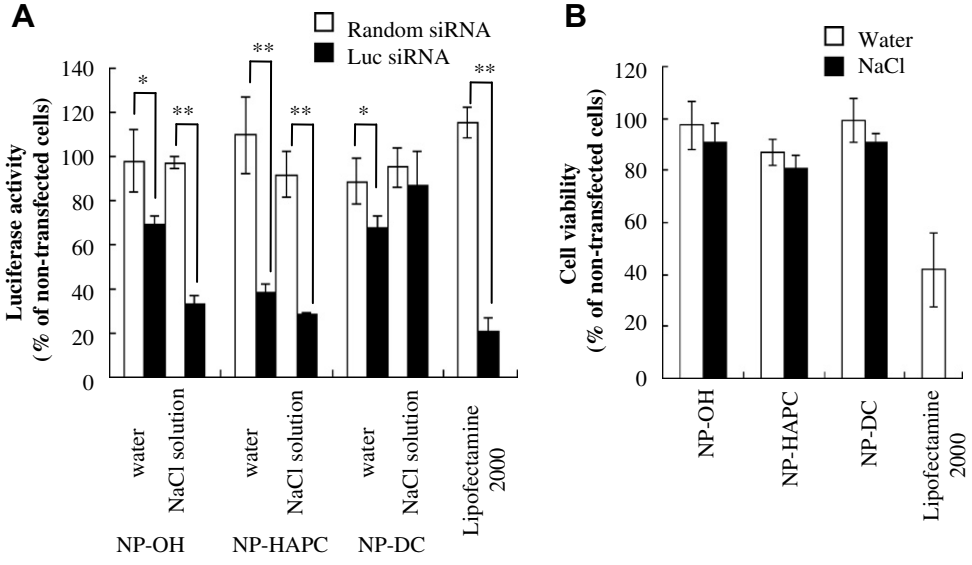

Figure 2. Effect of the difference between nanoparticles on transfection in PC-3-Luc cells 48 h after transfection (A). Nanoplexes of Luc or random siRNA dose at a charge ratio (±) of 3/1 were prepared in water or 50 mM NaCl. The value are expressed as means ± SD (n = 3). Cytotoxicity of nanoplexes was estimated by measuring cellular viability by WST-8 solution (B). Nanoplex of Luc siRNA was prepared at a charge ratio (±) of 3/1 in water or 50 mM NaCl. Assays were performed 48 h after PC-3-Luc cells were exposed to the nanoplex. Values are expressed as means ± SD (n = 4).

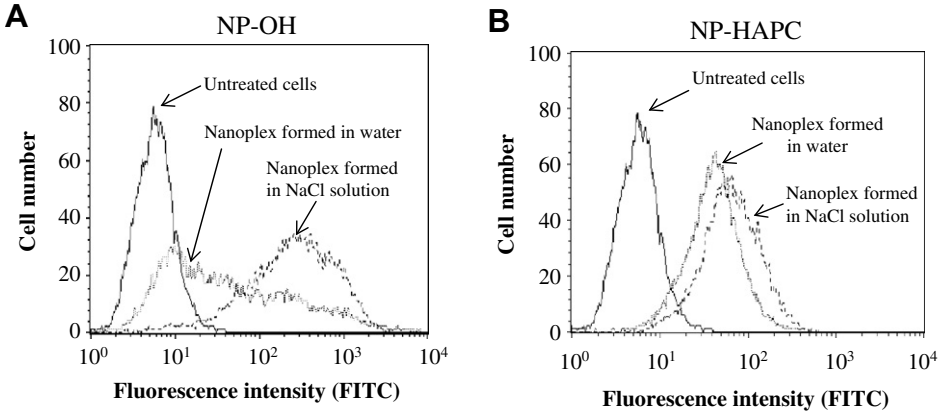

Figure 3. Association of NP-OH and NP-HAPC nanoplexes with PC-3 cells 24 h after transfection. NP-OH (A) or NP-HAPC (B) was mixed with 100 pmol of FAM-siRNA in water or NaCl solution for 10 min, and then diluted to 100 nM with medium.

exhibit cytotoxicity (about 80–100% cell viability). These findings indicated that NP-OH and NP-HAPC nanoplexes could efficiently suppress gene expression by siRNA without cytotoxicity.

The size of both NP-OH and NP-HAPC nanoplexes formed in water or NaCl was similar, but their transfection activities were different. First, to examine the effect of the nanoplex formed in 50 mM NaCl on transfection efficiency, we measured the amount of nanoplexes associated with the cells by flow cytometric analysis (FACSCalibur, Becton–Dickinson, San Jose, CA, USA).[6] 5′-Carboxyfluorescein (FAM)-labeled random siRNA was used to determine the amount of cellular association. In nanoplexes formed in the presence of 50 mM NaCl, the association with the NP-OH nanoplex of FAM-labeled siRNA 24 h after transfection significantly increased (Fig. 3A) but that with NP-HAPC nanoplex slightly increased (Fig. 3B). Furthermore, to examine the difference of kinetics for siRNA uptake between the nanoplex forming in water

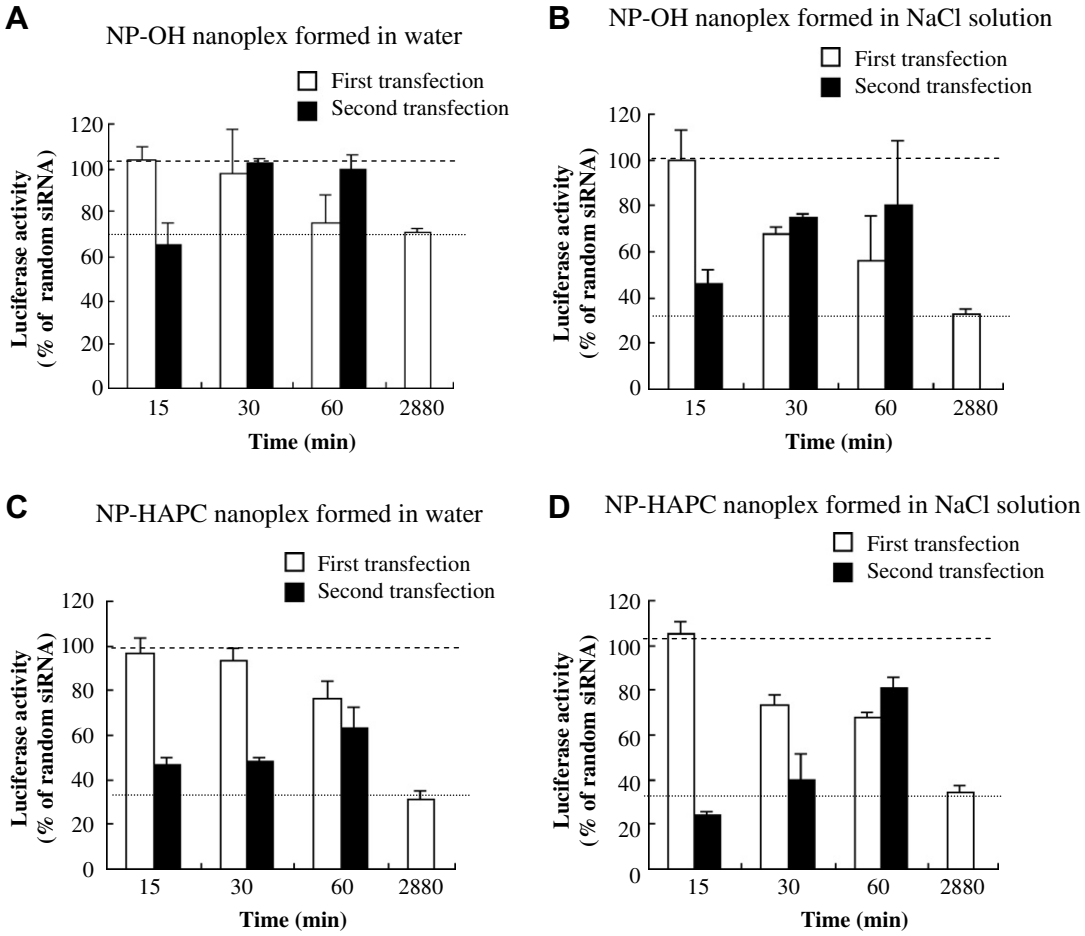

Figure 4. Kinetics of nanoplex-mediated gene transfer. Cells were incubated with NP-OH nanoplex formed in water (A), in NaCl (B), NP-HAPC nanoplex formed in water (C) or in NaCl (D) for different periods of time (first transfection), then washed, and further incubated in fresh medium. The removed nanoplexes were incubated with other fresh cells (second transfection). Incubation was finished 48 h after the first transfection. Open bars represent primary nanoplexes and filled bars secondary ones. Values are expressed as means ± SE ($n = 3$).

and that with NaCl solution, we investigated the time needed for internalization of the nanoplex. The NP-OH or NP-HAPC nanoplex was incubated with the cells for 15, 30, 60 min (first transfection) at 100 nM siRNA, and then the supernatant was used to transfect into other fresh cells (second transfection) for a total of 48 h. The NP-OH nanoplex formed in water exhibited a suppression of luciferase activity by the first transfection for 60 min, comparable to transfection for 48 h, and furthermore, no suppression by the second transfection (Fig. 4A), suggested that the nanoplex was completely internalized in the cell within 60 min. On the other hand, the NP-OH nanoplex formed in NaCl solution and NP-HAPC nanoplex formed in water and NaCl solution exhibited less suppression by the first transfection for 60 min, and suppression by the second transfection, suggesting that the nanoplexes were not sufficiently internalized into the cells by 60 min (Fig. 4B–D). From the results, we found that the NP-OH nanoplex formed in water was more rapidly taken up into the cells than the other nanoplexes. NP-OH and NP-HAPC might be internalized into the cells by a different uptake mechanism.

Next, we studied the effect of a cationic nanoplex on membrane fusion using anionic liposomes as a model of the plasma membrane by fluorescence of calcein leakage from anionic liposomes. Anionic liposomes, composed of dioleoylphosphatidylcholine (DOPC, NOF Co., Ltd), dioleoylphosphatidylethanolamine (DOPE, NOF Co., Ltd) and dioleoylphosphatidylglycerol (DOPG, NOF Co., Ltd), at a molar ratio of 1:2:1 were prepared by the thin film method and calcein was encapsulated into the liposome as previously reported.[16,17] The ζ-potential of all cationic nanoplexes was positive (Table 1), and that of anionic liposome was negative (−68.5 mV). When mixed with the anionic liposome, the NP-OH nanoplex formed in water rapidly leaked calcein from anionic liposome by 41% at 5.5 min (Fig. 5A).[18] In contrast, the nanoplex that formed in NaCl solution leaked calcein slowly and slightly (7.6% at 5.5 min) which was 4-fold lesser than that formed in water. NP-HAPC nanoplexes formed in the presence of NaCl slightly and similarly leaked calcein compared with that formed in water (19.9% and 18.4% at 5.5 min in the NP-HAPC nanoplex formed in water and NaCl solution, respectively) (Fig. 5B). The linker group between hydrophilic and hydrophobic lipid moiety influenced membrane fusibility.[4] In our study, OH-Chol with an amido-linker had the ability to fuse rapidly with anionic liposomes compared to HAPC with a carbamate linker when the nanoplexes were formed in water. This characteristic of the OH-Chol nanoplex was changed by formation of the nanoplex in NaCl solution.

To investigate the internalization mechanism of the NP-OH nanoplex formed in water and NaCl solution, we examined the effect of cellular uptake by endocytosis inhibitors such as sucrose and filipin. Hypertonic treatment with sucrose typically blocks clathrin-mediated internalization and filipin treatment blocks caveolae-mediated internalization. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled nanoparticles was prepared by incorporating 0.05 mol % of DiI (Lambda Probes & Diagnostics, Graz, Austria) into the total lipid. The uptake amount of DiI-labeled NP-OH nanoplex formed in water decreased to about 90% and 70% in the presence of filipin and sucrose, respectively, and that formed in NaCl was significantly decreased to about 50–60% in

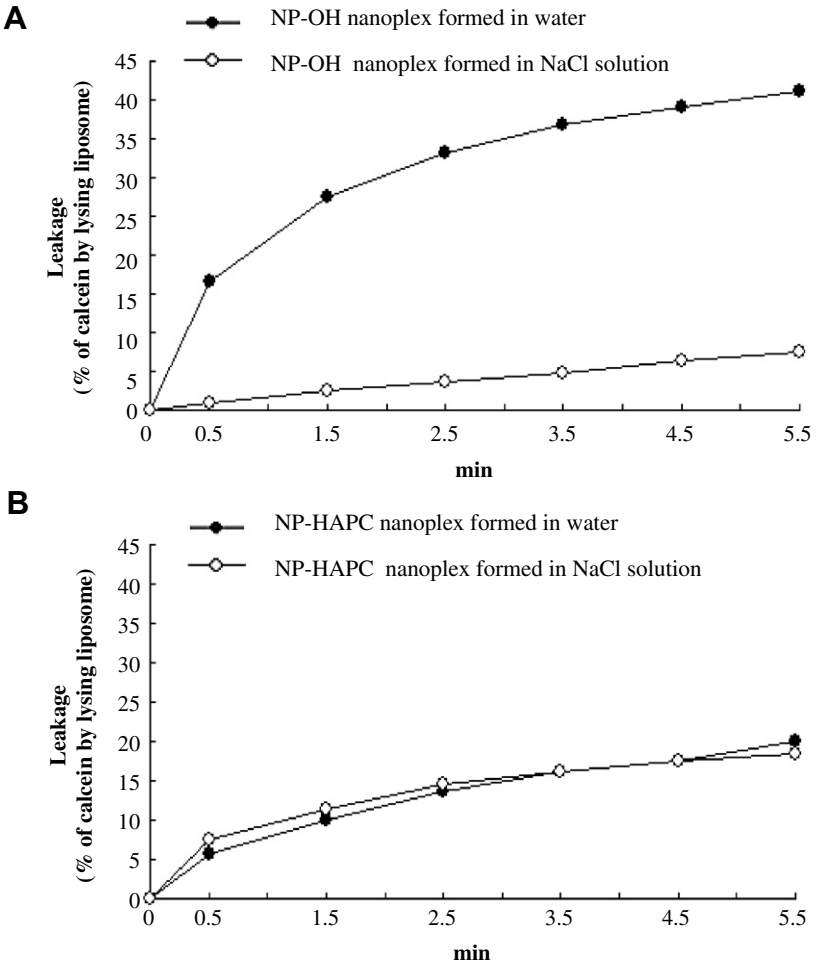

Figure 5. NP-OH or NP-HAPC nanoplex-induced calcein leakage from negatively charged DOPC/DOPE/DOPG liposomes in the temporal pattern.

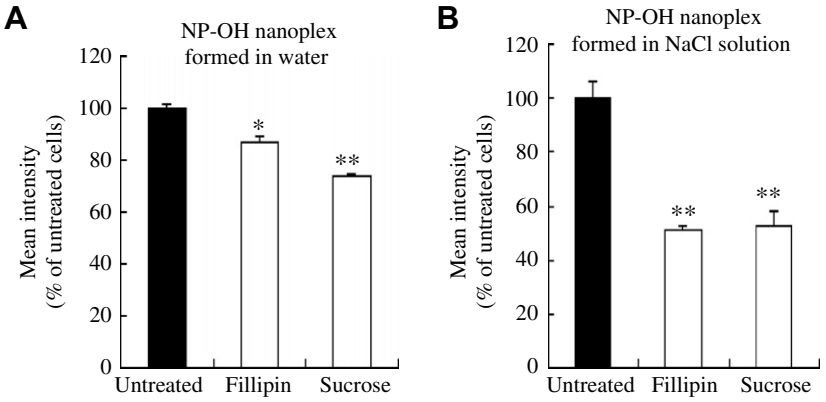

Figure 6. Effect of pharmacological reagents on the cellular uptake of NP-OH nanoplexes. Cells were pretreated with 50 μg/ml filipin or 100 mM sucrose at 37 °C for 30 min. The medium was replaced with culture medium containing DiI-labeled NP-OH nanoplex formed in water (A) or NaCl solution (B), and then incubated with the cells for 2 h at 37 °C. Each column represents the mean ± SD (n = 4).

the presence of filipin or sucrose (Fig. 6A and B). The results in Fig. 5 and 6 suggested that the NP-OH nanoplex formed in water was taken up via mainly clathrin-mediated endocytosis as well as fusion; in contrast, NP-OH nanoplexes formed in NaCl solution were taken up via both clathrin- and caveolae-mediated endocytosis.

The difference in the linker group between carbamate and amido in cationic cholesterol derivatives influenced membrane fusibility (Fig. 5A and B). NP-OH nanoplex formed in water had the ability to fuse with the cellular membrane, and produced nearly maximal levels of gene suppression after only a 60-min incubation period (Fig. 4A); however, it was not clear why the cationic cholesterol derivative with an amido-linker had membrane fusibility but that with carbamate linker did not. In the hydroxyethylated cationic cholesterol derivative with secondary amine, the amido-linker might induce more conformational flexibility than the carbamate linker, and therefore the positive charge of NP-OH could interact with the anionic membrane. On the other hand, for the NP-OH nanoplex formed in NaCl solution, the level of transfection was still very low at up to 1 h incubation compared with 24-h incubation. Such slow kinetics is characteristic of internalization via a mechanism involving caveolae, comfirming our observations on the effect of inhibitors (Fig. 6B). Since the composition of NP-OH nanoplexes formed in water and NaCl solution was identical, the only parameter determining their gene transfer might be their size. Microspheres with a diameter of <200 nm were internalized via clathrin-mediated endocytosis, while 500-nm particles entered cells via caveolae.[19] The NP-OH nanoplex forming in water was 220 nm and was rapidly taken up by membrane fusion and clathrin-mediated endocytosis. In contrast, the NP-OH nanoplex formed in NaCl solution was 1000 nm, and the large nanoplex lost the ability to fuse with the cellular membrane. As a result, the nanoplex was taken up by both caveolae- and clathrin-mediated pathways and induced efficient suppression by siRNA transfection. This finding corresponded to large aggregates (>500 nm), even though internalized very slowly, having more efficient gene transfection activity than small aggregates,[19,20] which were taken up very rapidly.[21] However, it was not clear why siRNA transfection via membrane fusion could not suppress gene suppression. Acidification of the NP-OH nanoplex in clathrin- or caveolae-mediated endosome might be needed for efficient dissociation of siRNA from NP-OH in the cells.

In this study, hydroxyethylated cationic cholesterol derivative OH-Chol with an amido-linker for synthetic siRNA transfection vector was affected by a nanoplex-forming condition, but HAPC with a carbamate linker was not. The presence of NaCl solution in forming the nanoplex increased the size, leading to caveolae-mediated endocytosis, which increased siRNA transfection efficiency. The results provided important information to develop efficient cationic lipid-based vectors for synthetic siRNA.

Acknowledgments

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