Cationic Amphiphilic Macromolecules (CAMs)-Lipid Complexes for Intracellular siRNA Delivery

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ABSTRACT SUMMARY
Cationic amphiphilic macromolecules (CAMs) were non-covalently blended with lipids (DOPE and DOTAP) to serve as a siRNA delivery vehicle. By varying weight ratio of CAM to lipid, catonic complexes with different compositions were obtained and their properties were evaluated. Certain CAM-lipid (9N-lipid) with weight ratio of 1:10 was found to have significantly higher transfection efficiency than Lipofectamine. The intracellular trafficking was also applied to elucidate that siRNA escaped endosome at 24 hrs after transfection.

INTRODUCTION
Small interfering ribonucleic acids (siRNA) have been widely demonstrated as a promising gene-based therapeutic for many diseases since the discovery of RNA interference (RNAi) by Fire et al.1 Due to the anionic nature of siRNA and the presence of RNases in the blood stream, delivery of naked siRNA is limited by inadequate cellular uptake and poor stability in physiological condition, respectively. Therefore, efficient siRNA delivery vehicles are necessary.2 Although numerous non-viral delivery systems including cationic polymer-based3 and lipid-based4 systems, cell-penetrating peptides5, and chemically modified siRNAs6 have been developed to address these issues, they are still limited in clinical trials due to the lack of gene-silencing efficacy for in vivo delivery.

A mixed system containing cationic amphiphilic macromolecules (CAMs) and lipid was developed to obtain improved gene-silencing efficacy. Two species of CAMs, differing by the number of amine groups in their backbone (Scheme 1, 7N and 9N), were prepared as previously published.7 It has been previously demonstrated that the CAMs have moderate gene-silencing efficacy with low cytotoxicity in vitro.7 However, for practical applications, the gene silencing efficiency still needs to be improved. A mixed lipid system consisting of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, Scheme 1) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, Scheme 1) was introduced to create an CAM-lipid system as an siRNA delivery vehicle. DOPE was chosen for its ability to destabilize endosomal membrane and enhance siRNA release; DOTAP was utilized due to its cationic feature of protecting siRNA. The CAM-lipid complexes were prepared using a previously published method.8 Transfection efficiency and endosomal escape of CAM-lipid complexes were evaluated using an in vitro assay with a human primary glioblastoma cell line (U87) and anti-luciferase siRNA or Cy5-scrambled siRNA.

EXPERIMENTAL METHODS
CAM-lipid complexes were prepared based on different weight ratios of CAM to DOPE:DOTAP (weight-to-weight 1:1) lipids. The CAM and lipid were co-evaporated in a glass vial and solvent removed in vacuo. The remaining films were hydrated with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer overnight at room temperature. The materials were then extruded 21 times through a mini-extruder to give unilamellar structures.8 For siRNA delivery assay, U87 cells were plated at a density of 5000 cells/well in 96-well plates approximately 20 hours prior to transfection. Immediately prior to transfection, CAM-lipid/siRNA complexes were prepared in 20 μL of HEPES (N/P=50). Lipofectamine was used as a positive control. The CAM-lipid/siRNA complexes were brought to a total volume of 100 μL in OptiMEM medium to obtain a final siRNA concentration of 100 nM. The serum containing culture medium was aspirated from the cells, and each well treated with 100 μL of the CAM-lipid/siRNA complexes in OptiMEM medium. After a 4 hr incubation period, the transfection mixture was replaced with serum-containing growth medium and maintained under normal growth conditions until the cells were assayed for firefly luciferase expression after 48 hours.

For intracellular trafficking, U87 cells were seeded in 24-well plates at 70% confluency and allowed to adhere overnight. After 4 h or 24 h of incubation with Cy5-siRNA (scrambled) and 1:10 CAM-lipid, 10:1 CAM-lipid, or Lipofectamine control, U87 cells were washed twice with HEPES and stained with LysoTracker Red.
(Molecular Probes). After fixation in 4% paraformaldehyde for 15 min and counterstaining with 4',6-diamidino-2-phenylindole (DAPI), images were taken on a confocal microscope to view siRNA localization within the cells.

RESULTS AND DISCUSSION

To evaluate the transfection efficiency of CAM-lipid system, the delivery of anti-luciferase siRNA to U87 cells expressing luciferase was monitored. Anti-luciferase siRNA was used as the cargo of CAM-lipid complexes at various weight ratios in U87-LUC cells. It was found that increasing CAM content in the complex resulted in a decreased transfection efficacy, as shown in Figure 1. In contrast, increasing lipid content improved the transfection efficacy. Among those conditions, the 9N-lipid with a weight ratio of 1:10 showed significantly higher gene silencing than Lipofectamine.

Figure 1. Luciferase reporter gene down-regulation assay over 48 hrs performed in U87 luciferase cell line using complexes formulated from CAM-lipid complexes and anti-luciferase siRNA at N/P ratio of 50. Lipofectamine is used as a control. Asterisks represent that 9N-lipid with weight ratio of 1:10 has significantly higher efficiency than Lipofectamine (p < 0.05).

To further investigate the observed enhanced siRNA delivery, intracellular trafficking of CAM-lipid/siRNA system was conducted using 9N-lipid with Cy5-siRNA as the cargo. As shown in Figure 2, siRNA was co-localized with LysoTracker Red after 4 h of incubation, suggesting that the complexes have not been released from endosomes or early lysosomes. After 24 h, only minimal co-localization is occurring, suggesting that Cy5-siRNA has undergone endosomal escape. The same trends were observed when using Lipofectamine as the carrier. For the less effective carrier (9N-lipid with weight ratio of 10:1), siRNA appeared to aggregate on the cell surface after 4 h. After 24 h, some CAM-lipid complexes were internalized, however, much more complex remained on the cell surface as compared to the 1:10 formulation. These results suggest that transfection efficiency is lower at 10:1 weight ratio due to the insufficient cell uptake.

Figure 2. Confocal microscope images of Cy5-siRNA (blue) and endosomal (red) distribution in U87 cells when delivered by the indicated 9N-lipid complexes or Lipofectamine, 4 h and 24 h post-transfection.

CONCLUSION

CAM-lipid complexes were designed to deliver siRNA in vitro. In vitro transfection assays showed that 9N-lipid with weight ratios of 1:10 was more effective than Lipofectamine in transfecting cells. Intracellular trafficking study revealed that siRNA can escape from endosomes and be released from CAM-lipid complexes to perform gene down-regulation.

REFERENCES

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