Cycloamylose-based Biomaterial: Nanogel of Cholesterol-bearing Cationic Cycloamylose for siRNA Delivery

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A new siRNA delivery system made of a functional cycloamylose nanogel is reported. Cholesterol-bearing cationic cycloamylose formed nanogels by self-assembly in water. The nanogels formed 20–40 nm nanoparticles that bound to 21 bp siRNA. The complexes exerted an RNAi effect on SCC7-GL3 cells that stably express the luciferase gene.

Cycloamylose (CA), which consists of α-1,4-glucose units, was produced by an enzymatic reaction of linear amylose. CA forms an inclusion complex with hydrophobic molecules inside a helical cavity of polymer chains. In comparison with amylose, CA has higher water solubility.[1] To our knowledge, the application of CAs to biomaterial such as drug carriers has not been reported. In this paper, we report the synthesis and characterization of a cationic CA nanogel, and its application to siRNA nanocarriers.

The intracellular delivery of nucleic acids has received considerable attention in gene therapy. In addition, RNA interference (RNAi) has emerged as a new therapeutic pathway for delivering functional RNA, such as short interfering RNA (siRNA) to target cells. To date, various siRNA carriers, especially polymer-based carriers, have been developed.[2] However, safe and efficient delivery carriers are required to achieve the desired RNAi effect.

Recently, nanogels have attracted growing interest for use in drug delivery systems.[3] We have developed physically cross-linked nanogels consisting of hydrophobically modified polysaccharides. For example, cholesterol-bearing pullulan (CHP) forms a stable nanogel (20–30 nm) in water by self-assembly.[4] Nanogels trap proteins inside the polymer network and show molecular chaperone-like activity.[4b] The cationic CHP nanogel acted as a nanocarrier for effective intracellular protein delivery and also showed nucleic acid chaperone-like activity.[5] We have applied nanogel engineering principles to biomedical applications, using various polysaccharides such as pullulan, mannan, and cluster dextrin.[6] In this study, we focus on CA as a new polysaccharide-based biomaterial.

Cationic spermine groups were attached to CA ($M_n = 1.9 \times 10^4$ g mol^{-1}, $M_w/M_n = 1.08$, DP ≈ 100, gifted from Ezaki Glico Co., Ltd.) by conventional 1,1′-carbonyldiimidazole (CDI) activation. Spermine derivatives showed superior activity for the transfection of siRNA.[7] The spermine-bearing CA (catCA) thus obtained was reacted with cholesteryl *N*-(6-isocyanatohexyl)/carbamate. The degrees of substitution of cationic groups and cholesterol in the CA derivative (CH-catCA) were 25 and 3.1, respectively, in 100 glucose units of CA (Figure 1a).

The hydrodynamic diameters of catCA and CH-catCA in Tris-HCl buffer (10 mM, pH 7.4) were 8.0 and 27.7 nm, respectively, and the corresponding ζ-potentials were +9.9 and +24.6 mV. The TEM image obtained by freeze-fracture showed that CH-catCA formed spherical nanoparticles (Figure 1b). CH-catCA formed monodispersive cationic nanogels in water in a manner similar to that of other cholesterol-bearing polysaccharides as reported previously.[5]

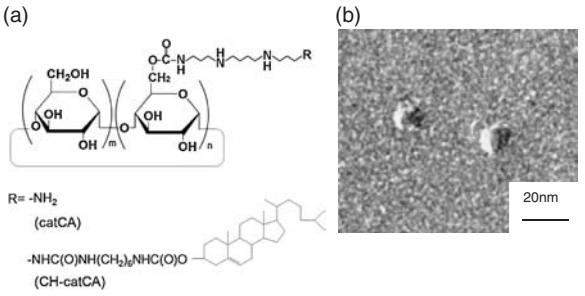

Figure 1. (a) Chemical structure of catCA and CH-catCA, (b) TEM image of CH-catCA.

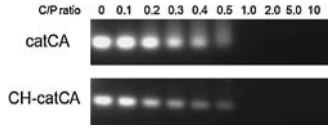

Figure 2. Agarose gel electrophoresis of catCA and CH-catCA/siRNA complex. CatCA or CH-catCA were mixed with 21 bp siRNA at various C/P ratios. Each sample was applied to 2% agarose gel. Gel electrophoresis was performed in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 100 V for 30 min.

Aqueous solutions of catCA or CH-catCA were mixed with siRNA (siGL3, 21 bp) at various C/P ratios (number of cationic groups in CA/number of phosphate groups in siRNA) and maintained for 30 min at room temperature. The results of gel electrophoresis experiments revealed that siRNA complexed completely with both catCA and CH-catCA at C/P = 1 (Figure 2).

The sizes and ζ-potentials of the complex at various C/P ratios were measured by DLS. Both complexes showed a negative ζ-potential at a size of ca. 150 nm below a C/P ratio of 0.5. Above a C/P ratio of 1.0, positively charged 15–40 nm nanoparticles were formed (Table 1).

The stability of the complex in the presence of a polyanion was examined. Each complex (C/P = 10) was incubated for 15 min at 37 °C in the presence of a heparin solution (0.5–1.0 mg mL^{-1}), after which electrophoresis was carried out. In the case of the catCA–siRNA complex, almost all the siRNA was released in the presence of 1.0 mg mL^{-1} heparin. However, in the case of the CH-catCA–siRNA complex, the nanogels released only half of the siRNA, even at a heparin concentration of 1.0 mg mL^{-1}. This result suggested that CH-catCA nanogels may form a stable complex with siRNA through binding inside the nanogel matrix.

The cellular uptake of FITC-labeled siRNA was investigated by confocal laser scanning fluorescence microscopy (CLSFM).

Table 1. Particle size and ζ-potential of catCA/siRNA and CH-catCA/siRNA complexes at various C/P ratios

C/P ratio	Mean diameter/nm (PdI)		ζ-potential/mV	
	catCA	CH-catCA	catCA	CH-catCA
0.1	n.d.	157 ± 2.0 (0.06)	-20.3 ± 0.1	-27.3 ± 0.5
0.5	1233 ± 64 (0.26)	794 ± 15 (0.32)	-21.7 ± 0.3	-30.4 ± 0.2
1.0	170 ± 0.6 (0.11)	274 ± 17 (0.48)	$+23.5 \pm 0.6$	$+25.7 \pm 0.5$
2.0	20.4 ± 0.7 (0.46)	37.8 ± 1.7 (0.27)	$+18.4 \pm 0.8$	$+16.2 \pm 0.3$
5.0	16.8 ± 0.2 (0.37)	27.3 ± 0.6 (0.25)	$+9.4 \pm 0.9$	$+15.9 \pm 0.7$
10	14.3 ± 0.2 (0.31)	27.5 ± 0.1 (0.27)	$+19.7 \pm 1.0$	$+15.8 \pm 0.2$

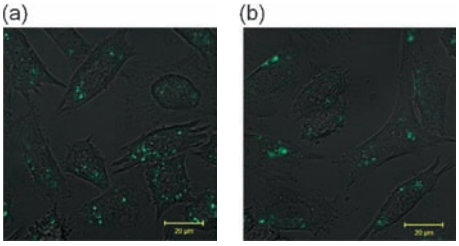

Figure 3. CLSFM images of transfected SCC7-GL3 cells. (a) catCA/FITC-siGL3 complex and (b) CH-catCA/FITC-siGL3 complex. SCC7-GL3 cells were cultured in DMEM-containing complex (C/P = 10) for 4 h. The concentration of FITC-siGL3 was 50 nM. The scale bar represents 20 μm.

CatCA or CH-catCA/FITC–siRNA complexes were incubated with squamous carcinoma cells (SCC7-GL3) for 4 h in DMEM (10% FBS, 1% antibiotic). As shown in Figure 3, both carrier systems were internalized inside the cell probably by endocytosis. Flow cytometric measurements indicated that the cellular uptake efficiencies of the two complexes (C/P = 10, 50 nM FITC-siGL3) were comparable (Figure 4).

To investigate the RNAi effects of the catCA/siRNA complex and CH-catCA/siRNA complex, luciferase activity was determined in SCC7-GL3 cells stably expressing the pGL3 luciferase gene. In this experiment, we used two types of siRNA, siGL3, and siGL2 (i.e., siGL2 with three mismatches compared with siGL3). After transfection of the complexes (C/P = 10, 50 nM siRNA) for 24 h, the luciferase expression level of the catCA or CH-catCA/siGL3 complex decreased, but that of the catCA or CH-catCA/siGL2 complex did not (Figure 5). These results indicate that the catCA or CH-catCA system induced sequence-specific silencing in the cells and showed no cytotoxicity under the transfection condition. Their silencing effects were approximately 28 and 47%, respectively. Higher activity in CH-catCA nanogel system was probably due to higher stability of the complex with siRNA (protection from enzymes).

In conclusion, a cationic CA formed stable complexes with 21 bp siRNA. The complexes were effectively internalized by the cells. In comparison with catCA, CH-catCA nanogel exerted an effective RNAi effect in cells stably expressing the luciferase gene. These results demonstrate that self-assembled cationic CA nanogels act as novel siRNA delivery systems.

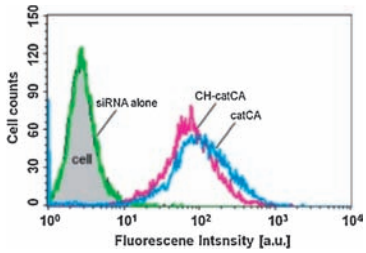

Figure 4. Histograms of the fluorescence intensity of SCC7-GL3 cells during uptake of FITC-labeled siRNA by catCA and CH-catCA. SCC7-GL3 cells were plated in a 12 well-culture dish at a density of 4×10^4 cells/well and cultured for 24 h before transfection. Complex solution (C/P = 10, siRNA 50 nM) were incubated with DMEM for 4 h. Subsequently, the cells were trypsinized and suspended in stain beffer. Flow cytometry was performed on FACScalibur.

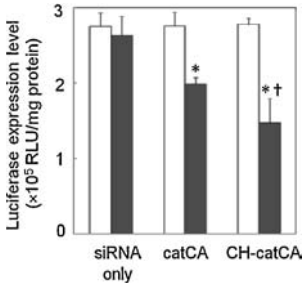

Figure 5. Gene silencing effects of siRNA (noncarrier), catCA/siRNA complex, and CH-catCA/siRNA complex on luciferase activity in cells stably expressing the pGL3 luciferase gene (SCC7-GL3). The cells were plated in a 12 well-culture dish at a density of 4×10^4 cells/well, and cultured for 24 h before transfection. The cells were incubated in a DMEM-containing complex solution (C/P = 10) for 4 h. Luciferase activities in cell lysates were assayed after transfection for 24 h. The final siRNA concentration was 50 nM. The experiment was carried out in quintuplicate; each value represents the mean ± SD. $^*p < 0.05$, compared with siGL2.

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References

1 a) W. Saenger, J. Jacob, K. Gessler, T. Steiner, D. Hoffmann, H. Sanbe, K. Koizumi, S. M. Smith, T. Takaha, *Chem. Rev.* **1998**, *98*, 1787. b) T. Takaha, M. Yanase, H. Takata, S. Okada, S. M. Smith, *J. Biol. Chem.* **1996**, *271*, 2902.
2 a) D. J. Gary, N. Puri, Y.-Y. Won, *J. Controlled Release* **2007**, *121*, 64. b) M. E. Davis, *Mol. Pharm.* **2009**, *6*, 659.
3 A. V. Kabanov, S. V. Vinogradov, *Angew. Chem., Int. Ed.* **2009**, *48*, 5418.
4 a) K. Akiyoshi, S. Deguchi, N. Moriguchi, S. Yamaguchi, J. Sunamoto, *Macromolecules* **1993**, *26*, 3062. b) Y. Nomura, M. Ikeda, N. Yamaguchi, Y. Aoyama, K. Akiyoshi, *FEBS Lett.* **2003**, *553*, 271.
5 a) H. Ayame, N. Morimoto, K. Akiyoshi, *Bioconjugate Chem.* **2008**, *19*, 882. b) N. Morimoto, J. Tamada, S. Sawada, N. Shimada, A. Kano, A. Maruyama, K. Akiyoshi, *Chem. Lett.* **2009**, *38*, 496.
6 a) E. Akiyama, N. Morimoto, P. Kujawa, Y. Ozawa, F. M. Winnik, K. Akiyoshi, *Biomacromolecules* **2007**, *8*, 2366. b) Y. Ozawa, S. Sawada, N. Morimoto, K. Akiyoshi, *Macromol. Biosci.* **2009**, *9*, 694.
7 X.-B. Xiong, H. Uludag, A. Lavasanifar, *Biomaterials* **2009**, *30*, 242.

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