Effects of polyrotaxane structure on polyion complexation with DNA

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Abstract

Aminoethylcarbamoyl-polyrotaxanes (AEC-\textalpha/E35-TYR-Zs) were synthesized as a novel cationic polymer for DNA complexation and characterized in terms of physicochemical properties of polyion complex. Here, AEC groups were introduced to hydroxyl groups of \textalpha-cyclodextrins (\textalpha-CDs) that are threaded onto a poly(ethylene glycol) (PEG) chain capped with bulky end-groups (polyrotaxane). We examined the ability of DNA complexation of the AEC-\textalpha/E35-TYR-Zs compared with polyethylenimine (PEI). Agarose gel shift assay and ethydium bromide (EB) displacement analysis showed that the number of threading \textalpha-CDs was one of the dominant factors to enhance the ability of DNA complexation. In addition, increasing the number of AEC groups in the AEC-\textalpha/E35-TYR-Zs led to tighter complexation.

Keywords: Cyclodextrin; Polyrotaxane; Supramolecular structure; DNA complexation; Gene delivery

1. Introduction

Various synthetic polymers with cationic groups have been focused on DNA complexation that is one of the approaches to gene delivery. Although many efforts have been made to develop gene vectors using cationic polymers for gene delivery, their transfection activity is not enough for sufficient level [1]. These vectors are complexed with DNA and shown to exhibit ability for transfection [2–7]. Researchers have paid much attention to physicochemical properties, such as shape, surface charge, size and aggregation properties, that have been considered as important factors of gene expression [8].

In order to enhance the gene expressions, supramolecular structures of DNA/polycation complexes have been designed by assessing both the physicochemical properties and biocactivities. Polymeric architecture of the polycations is very important in terms of physicochemical nature of the DNA/polycation complexes and the following interaction with cell surface. Linear or branched polyethyleneimine (PEI) and hyperbranched polyamidoamine (PAMAM) have been extensively studied as gene carriers [9,10]. Szoka et al. reported that branched PEI forms stable DNA complexes without any aggregation, which leads to high gene expression [11]. It has been suggested that hydrophilic nature of DNA/polycation complexes is a significant factor for enhancing gene expression. Maruyama et al. have systematically studied comb-type copolymers, dextran-grafted poly(lysine). This copolymer significantly stabilizes DNA double strand, and the obtained DNA complex is soluble in physiological environments due to the hydrophilic dextran graft chain [12]. Yamaoka et al. have suggested that poly(lysine-co-serine) exhibits high gene expression, because the hydroxyl groups of serine residues improved transfection of DNA complex maintaining soluble states [13]. Thus, designing supramolecular structures of DNA/polycation complexes must be one of the promising approaches to investigating the architecture of polycation backbone.

Another important factor of gene expression is how to escape from endosomal entrapping after internalization into cells. It is currently considered that endocytosis is a major pathway for DNA entry mediated by DNA/polycation
complexes. However, a large fraction of the complexes taken up in endosome is delivered to lysosome and consequently degraded. Thus, many efforts have been made to establish strategies for the efficient escape of DNA from endosome into cytosol to improve their transfection efficiency. Linear PEI [3,14,15] and fractured polyamidoamine dendrimers [16,17] have been shown to achieve efficient transfection for various animal and human cells, because the amino groups of these polymers become protonated under weakly acidic conditions in endosome and suppress lowering of pH in endosome/lysosome by adsorbing proton, resulting in prohibiting degradation of DNA. In addition, the endosome buffering effects are thought to induce osmotic swelling of endosomal interior, resulting in the rupture of the endosome and subsequent release of DNA into cytoplasm [18]. However, despite of the excellent properties, PEI has not been applied to a clinical use because of the possibility of cytotoxicities, presumably due to the lack of biodegradabilities. Therefore, polycations having good DNA complexation ability, properties of endosomal escape and biodegradability should be designed for ideal gene vectors.

Recently, Uekama et al. investigated α-CD-dendrimer conjugates as a non-viral gene vector [19]. The α-CD-dendrimer conjugates have membrane-disruptive ability, which suggests a possibility of endosome membrane disruption. However, high concentration of the α-CD-dendrimer conjugates is necessary for the membrane disruption, so that cytotoxicity of the conjugates should be concerned because of hemolytic activity of α-CDs. We have studied biodegradable polyrotaxanes, in which many α-cyclodextrins (α-CDs) are threaded onto a poly(ethylene glycol) (PEG) chain capped with bulky end-groups via biodegradable linkages [20]. The terminal hydrolysis with or without catalysts leads to the dissociation of the supramolecular structure, resulting in the release of α-CDs [21,22]. Eliminating hydrogen bonds between hydroxyl groups of α-CDs by some chemical modification enables entropy-driven α-CD release at the terminal hydrolysis. The introduction of hydrophilic groups such as hydroxypropyl [23], hydroxyethylcarbamoyl [24], and carboxyethyl groups [25] is good methods to obtain the driving force. The released α-CD derivatives expose their hydrophobic cavities that can incorporate phospholipids from cell membranes [26]. Thus, we hypothesized that biodegradable polyrotaxanes are good candidates of new gene vectors in the following strategy. Many hydroxyl groups of α-CDs in polyrotaxanes can be chemically modified into amino-groups that should ionically complex with phosphate anions of DNA. One can design the terminal biodegradable linkages as to be hydrolyzed in a weakly acidic environment of endosome. Since hydrophobic cavity of α-CD is occupied by PEG chain in the polyrotaxane, possibility of hemolysis should be negligible in this system. DNA/amino-group-introduced polyrotaxane complexes are expected to interact with cell surfaces and then internalize in a similar way of general DNA/polycation complexes. Once the complexes are taken up in endosome, the terminal labile linkages will be hydrolyzed, resulting in the release of amino-group-introduced α-CDs. It is imagined that the hydrolysis-triggered release of the amino-group-introduced α-CDs changes the way of interaction with DNA. We have clarified that ligand-introduced polyrotaxanes enhance multivalent interaction with complement binding proteins due to both many ligands [27] and high mobility of ligand-introduced α-CDs in the polyrotaxane [28]. In this system, ligand-introduced α-CD itself does not show the enhanced binding because of monovalent interaction with the binding
proteins. Since the amino groups of the polyrotaxanes multivalently interacts with phosphate groups of DNA, the terminal hydrolysis of the polyrotaxanes and the following release of the amino group-introduced $\alpha$-CDs can convert the multivalent interaction into monovalent interaction, resulting in the release of free DNA. In addition, the released amino group-introduced $\alpha$-CDs are expected to disrupt endosomal membrane due to incorporating phospholipids of the endosomal membrane (Fig. 1). Thus, the design of biodegradable polyrotaxanes as a gene vector has a great potential of DNA complexation, interaction with cell surfaces, internalization, endosomal escape and release of DNA to cytoplasm by dissociation of the polyrotaxane structure via the terminal hydrolysis.

In this study, amino group-introduced polyrotaxanes (aminoethylcarbamoyl polyrotaxanes) were synthesized and evaluated their physical complexes with plasmid DNA. The numbers of $\alpha$-CDs and the aminoethylcarbamoyl groups were varied, and those effects on the physicochemical properties of the complexes were investigated.

2. Methods

2.1. Materials

$\alpha$-CD was purchased from Bio-Research Corporation of Yokohama (Yokohama, Japan). Poly (ethylene glycol) (PEG: $M_n = 35,000$) was purchased from Fluka (Kyoto, Japan). $p$-Toluenesulfonyl chloride, potassium phthalimide and hydrazine hydrate were obtained from nakanai tesque Inc. (Kyoto, Japan). Methylene chloride, pyridine and dimethylformamide (DMF) were purchased from Wako Pure Chemical Co. Ltd, and nakanai Tesque Inc., respectively, and distilled by used methods. Z-L-Tyrosine dihydrate ($\alpha$-L-TYR) was purchased from Kokusan Chemical Co. Ltd (Tokyo, Japan). $D_2$O (Kanto chem. Co.) and NaOD (Aldrich chem. Co.) were used as solvents for NMR measurements. $\alpha$, $\omega$-Diamino-PEG (PEG-BA) was prepared according to a method similar to that described by Harada et al. [29].

2.2. Synthesis of aminoethylcarbamoyl-polyrotaxanes ($AEC-\alpha/E35-TYR-Zs$)

The synthetic route of $AEC-\alpha/E35-TYR-Zs$ was shown in Scheme 1. Polyrotaxanes, in which many $\alpha$-CDs are threaded onto a PEG-BA ($M_n = 35,000$) capped with $Z$-$\alpha$-TYR, was prepared according to our method [23]. The obtained polyrotaxanes with different number of $\alpha$-CD (the number of $\alpha$-CDs: 100 and 200, 300 determined by $^1H$-NMR measurements) and $\alpha$, $\omega$-carboxyldimidazole (CDI) were dissolved in DMSO and stirred at room temperature for 3 h under nitrogen atmosphere. The mixture was poured into excess ether to precipitate CDI-activated polyrotaxanes. Then, the CDI-activated polyrotaxanes dissolved in DMSO was slowly dropped into excess ethylenediamine under stirring at room temperature for 8 h. The reaction mixture was poured into excess ether and the obtained crude products were washed with ether and acetone two times, respectively. The washed products were collected by centrifuging and dried in vacuo to give the AEC-$\alpha/E35-TYR-Zs$. The degree of substitution of AEC groups in a polyrotaxane molecule was determined by $^1H$-NMR spectra. $^1H$-NMR ($D_2O + NaOD$, ppm): $\delta$ 7.04, 6.65 (aromatics of Z-$\alpha$-TYR), 5.74–4.80 (O(2)H and O(3)H of $\alpha$-CD), 4.88 (C(1)H of $\alpha$-CD), 4.53–4.36 (O(6)H of $\alpha$-CD), 4.19 (CH of L-TYR), 3.82–3.25 (C(3)H, C(5)H, C(6)H, C(4)H and C(2)H of $\alpha$-CD), 3.52 (CH$_2$CH$_2$O of PEG), 3.34–2.90 (CH$_2$ of AEC groups), 2.85–2.44 (CH$_2$ of AEC groups).

2.3. Plasmid DNA

The reporter gene plasmid, pEGFP-C1 encoding enhanced green fluorescence protein [30] was amplified in Escherichia coli, extracted by alkaline lysis, and purified either by column separation (Qiagen plasmid midi. and maxi. kits, Qiagen, Osaka, Japan). Plasmid integrity was confirmed by agarose gel electrophoresis using 1.0 wt% agarose gel. DNA concentrations were determined by absorbance at 260 nm [50 $\mu$g/(ml/absorbance unit)].

2.4. Formation of $AEC-\alpha/E35-TYR-Zs$ / plasmid DNA complexes and electrophoresis

$AEC-\alpha/E35-TYR-Zs$/plasmid DNA complexes were formed at defined ratio of the highly charged amino groups to phosphate groups of DNA (N/P ratio) by mixing in phosphate buffered saline (PBS) (pH 7.4). After incubating
for 10 min at room temperature, the samples were electrophoresed using 1.0 wt% agarose gel in 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA buffer (pH8.0) at 100 V for 15 min. Sybar-green stained bands were visualized using an UV illuminator (Bio-Rad Lab. Inc., Osaka, Japan) and photographed. As a reference, the same experiments were carried out using poly(lysine) instead of the AEC-α/E35-TYR-Zs.

2.5. Inhibition of ethydium bromide (EB)/DNA fluorescence by AEC-α/E35-TYR-Zs

The AEC-α/E35-TYR-Zs/DNA complexes were assessed by monitoring loss of ethydium bromide fluorescence [29]. Salmon sperm DNA dissolved in PBS were mixed with EB in a cuvette to be their final concentrations of 0.5 and 5.0 μg/ml, respectively. Fluorescence intensity was measured (λ<sub>ex</sub> 260 and λ<sub>em</sub> 600 nm) using a spectrofluorimeter (FP-6500, Jasco, Tokyo, Japan), and aliquots (1.0 ml) of AEC-α/E35-TYR-Zs (100 μM of AEC) were sequentially added to the cuvettes, mixed by inversion, and the residual fluorescence measured. Reading of the fluorescence were carried out in triplicate. As a reference, the same measurements were carried out using PEI instead of the AEC-α/E35-TYR-Zs. The N/P ratio of each cationic polymers decreasing EB fluorescence to 50% was designated as IF50.

2.6. ζ potential measurements

AEC-α/E35-TYR-Zs or PEI was mixed with plasmid DNA (2.5 μg/ml) in PBS. Concentrations of these cationic polymers were varied (1.9–75 μM of AEC groups) to be the N/P ratio from 0.25 to 10. The mixed solution was allowed to stand for 15 min at room temperature, and then 500 μl of PBS was added. ζ-Potential was measured by using an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka electronics, Osaka, Japan) at 25 °C. Measurements were performed five times with zero field correction. The potential was calculated by using the Smoluchowsky approximation.

3. Results and discussion

3.1. Synthesis and characterization of AEC-α/E35-TYR-Zs

The synthetic results were summarized in Table 1. From the 1H-NMR spectra of the obtained AEC-α/E35-TYR-Zs, all the peaks attributed to α-CDs, PEG, Z-L-TYR and AEC groups were observed. This result indicates that the polyrotaxane structure was maintained after introducing AEC groups. As shown in Table 1, the number of AEC groups was controllable by the CDI activation. Feed ratio of the hydroxyl groups of α-CDs in the polyrotaxane and ethylenediamine was not a dominant factor to control the number of AEC (data not shown). The number of the AEC groups per one polyrotaxane molecule was maximally ca. 890. Thus, a variation of AEC groups in the polyrotaxanes with different number of α-CDs was prepared as follows.

1. α-CD 100 series [# of α-CD threading: 100, # of AEC: 450 and 890]
2. α-CD 200 series [# of α-CD threading: 200, # of AEC: 220, 300 and 420]
3. α-CD 300 series [# of α-CD threading: 300, # of AEC: 210, 240 and 300]

3.2. Influence of structural factors of AEC-α/E35-TYR-Zs on polyion complex

The plasmid DNA complexation with the polyrotaxanes was assessed by agarose gel electrophoresis. By changing the N/P ratio, the ability of DNA complexation with the AEC-polyrotaxanes could be quantitatively assessed in terms of the numbers of AEC and α-CDs. For all the cases, the amount of free plasmid DNA decreased with increasing the amount of the AEC-α/E35-TYR-Zs added, indicating that these polyrotaxanes have an ability to form a polyion complex.
complex with plasmid DNA. In the case of AEC-100α/E35-TYR-Z series, free DNA band was disappeared above the N/P ratio of 5.0. On the other hand, the critical N/P ratios, at the point disappeared free DNA band, of AEC-200α/E35-TYR-Z and AEC-300α/E35-TYR-Z series were 0.5 and 0.25, respectively. Changing the number of AEC in all the AEC-polyrotaxanes did not affect the critical N/P values. These results indicate that the number of AEC is likely to be a minor factor of DNA complexation. It should be noted that the critical N/P values seem to be dependent on the number of α-CDs in the AEC-polyrotaxanes. The critical N/P value decreased with increasing the α-CD threading, suggesting that increasing the number of α-CDs enhanced the DNA complexation ability. Furthermore, both AEC-200α/E35-TYR-Z and AEC-300α/E35-TYR-Z series showed greater complexation than PEI (Fig. 2). Since the number of secondary amino groups per one PEI molecule is 547, it is suggested that smaller number of the AEC groups contributes to DNA complexation. This fact suggests that the amino groups of the AEC-polyrotaxane participates in

![Fig. 2. Agarose gel electrophoretic analysis of the complexes in TBE buffer (pH7.5). The solutions containing the complexes were incubated for 15 min at room temperature after slight agitation. The electrophoresis was performed at 100 V for about 20 min. (A) PEI; (B) 450AEC-α100/E35-TRY-Z; (C) 890AEC-α100/E35-TRY-Z; (D) 220AEC-α200/E35-TYR-Z; (E) 300AEC-α200/E35-TYR-Z; (F) 420AEC-α200/E35-TYR-Z; (G) 210AEC-α300/E35-TYR-Z; (H) 240AEC-α300/E35-TYR-Z; (I) 300AEC-α300.](image)

![Fig. 3. Ethydium bromide displacement by the addition of PEI or AEC-α/E35-TYR-Zs to perform polymer /DNA complexes. Complexes were formed at various N/P ratios in the presence of ethydium bromide. Fluorescence was determined at λex 260 nm and λem 600 nm. (A) polyethylenimine; (B) AEC-α200/E35-TYR-Z; (C) AEC-α300/E35-TYR-Z. PEI (●), 220AEC-α200/E35-TYR-Z (●), 300AEC-α200/E35-TYR-Z (■), 420AEC-α200/E35-TYR-Z (▲), 210AEC-α300/E35-TYR-Z (○), 240AEC-α300/E35-TYR-Z (□).](image)
the interaction with much larger number of phosphate anions of DNA even before neutralization of the cations with the phosphate anions of DNA. Presumably, the supramolecular and red-like structure of the polyrotaxane [32] contributes to enhancing the DNA complexation.

In order to estimate the extent of complexation with DNA, EB displacement assay was carried out using 220AEC-200/E35-TYR-Z, 300AEC-200a/E35-TYR-Z, 420AEC-200/E35-TYR-Z, 210AEC-300a/E35-TYR-Z, 240AEC-300a/E35-TYR-Z and PEI (Fig. 3). It is known that decreased fluorescence of intercalated EB into DNA double strand indicates DNA condensation when polycations are complexed with DNA [28,30,31]. As shown in Fig. 3, the addition of cationic polymers to DNA/EB complex resulted in decreasing the fluorescence due to the displacement of EB by the cationic polymers. The degree of the displacement was depended on the types of cationic polymers, the numbers of AEC and α-CDs in the polyrotaxanes. Table 2 showed IF50 values calculated from the fluorescence inhibition curve in Fig. 3. Relative intensities of 220AEC-200/E35-TYR-Z and 300AEC-200a/E35-TYR-Z were not reached to half of the relative intensity and thus, there were no IF50 values at any N/P ratio (Fig. 3B). IF50 values decreased with increasing the number of AEC. It is reported that decreasing cation charge density along polymer backbones leads to diminishing the decreasing relative fluorescent values [31].

Such the polycations with low density of amino groups can complex with DNA, and the shape of the complex is extended in comparison with those with high density of amino groups. Taking this report into account, it is considered that large number of AEC groups in AEC-α/E35-TYR-Zs contributes to forming tighter complexes with plasmid DNA. Interestingly, any minimum fluorescent intensities of AEC-α/E35-TYR-Zs were larger than those of PEI (Fig. 3). This result suggests that complexation mechanism of AEC-α/E35-TYR-Zs is not likely to be neutralization-driven complexation. Since the slope of the intensity curve decreased with increasing the number of α-CDs below the N/P ratio of 1.0 (Fig. 3), it is considered that the rod-like structure of the polyrotaxane enhances complexation with DNA in the lower concentration. However, over the critical N/P ratio, relative intensity showed almost the same level (Fig. 3B and C). This result indicates that the obtained complex at the critical N/P ratio was stable despite of existing the other AEC-polyrotaxane molecules around the complex.

The surfaces of the complexed AEC-α/E35-TYR-Zs with DNA were assessed by measuring ζ-potentials (Fig. 4). ζ-Potentials of 220AEC-200a/E35-TYR-Z and 240AEC-300a/E35-TYR-Z reached to 0 mV at the N/P ratio of 1.4, at which free plasmid DNA disappeared in the buffer medium (Fig. 2). When the N/P ratio was over 0.6, the relative intensity of EB has reached to plateau region (Fig. 3). These results suggest that excess amount of AEC-α/E35-TYR-Zs does not participate in the plasmid DNA complex surrounds the surface of the complexes, resulting in neutralization. On the other hand, the ζ-potentials of 420AEC-200a/E35-TYR-Z gradually increased and reached to 0 mV at the N/P ratio of 8.0. It is unclear why only 420AEC-200a/E35-TYR-Z showed such the different ζ-potential property. PEI showed similar tendency with 420AEC-200a/E35-TYR-Z although the ζ-potential was reached to 0 mV at the N/P ratio of 4.2. Presumably, the high number of AEC in 420AEC-200a/E35-TYR-Z can participate into the complexation with plasmid DNA based on neutralization-driven mechanism as well as PEI.

### 4. Conclusion

Various types of AEC-α/E35-TYR-Zs were synthesized and evaluated in terms of structural factors of the AEC-α/E35-TYR-Zs on polyion complexation with plasmid DNA. The complexation at low N/P ratio was mainly dominated by the threading number of α-CDs. It was found that the introduction of AEC groups to the polyrotaxane

| Sample          | IF50 (N/P ratio) |
|-----------------|------------------|
| 220 AEC-200a/E35-TYR-Z | Not detected     |
| 300 AEC-200a/E35-TYR-Z | Not detected     |
| 420 AEC-200/E35-TYR-Z  | 1.3              |
| 210 AEC-300a/E35-TYR-Z | 2.5              |
| 240 AEC-300a/E35-TYR-Z | 0.9              |
| PEI              | 1.3              |

![Fig. 4. ζ-Potential of the complexes of plasmid DNA/PEI and plasmid DNA/AEC-α/E35-TYR-Zs. The electrophoretic mobility of the complexes of plasmid DNA/PEI and plasmid DNA/AEC-α/E35-TYR-Zs in PBS (pH 7.4) was determined by electrophoretic light scattering spectrophotometer at 25 °C. The PEI or AEC-α/E35-TYR-Zs was added to the plasmid DNA solution (2.5 mg/ml) at various charge ratios. PEI (○), 220AEC-α/200/E35-TYR-Z (●), 420AEC-α/200/E35-TYR-Z (▲), 240AEC-α/300/E35-TYR-Z (□).](image-url)
backbone enhanced the opportunity of complexation with phosphate groups of plasmid DNA in spite of lower number of cationic groups than that of PEI. It is suggested that increased number of AEC contributes to the formation of tighter complex. All the AEC-α/E35-TYR-Zs formed extended complex with plasmid DNA, which may be complexed at lower N/P ratio. Cellular uptake of these complexes and those transfections are now in progress and will be reported in our forthcoming paper.

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