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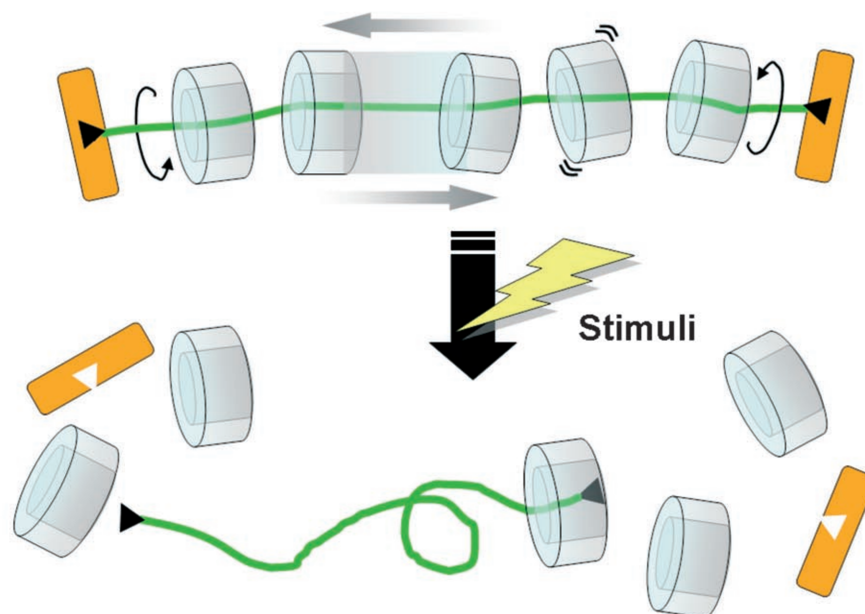
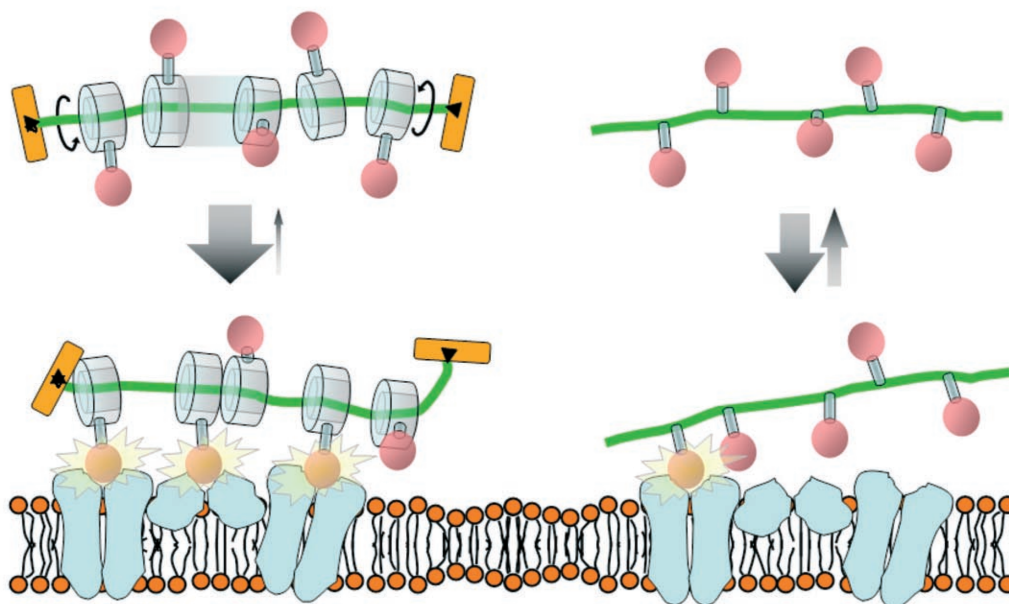


Polyrotaxanes Can Induce Multivalent Interactions with Biomacromolecules



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Molecular Mobility of Interlocked Structures Exploiting New Functions of Advanced Biomaterials

Nobuhiko Yui* and Tooru Ooya[a]

Abstract: Cyclic compounds can rotate and/or slide along a polymeric chain in a polyrotaxane structure, and the mobility of the ligands linked by the cyclic compounds is closely related to enhancing the multivalent interaction with binding sites on the receptor proteins. This concept is being exploited in more practical applications in the biomedical and pharmaceutical fields, such as a non-viral gene carriers.

Keywords: cyclodextrins · gene carriers · molecular recognition · polymers · polyrotaxanes

Introduction

The most characteristic feature in polyrotaxanes, in which many cyclic compounds are threaded onto linear polymeric chains capped with bulky end-groups, is the noncovalent bonds between the cyclic compounds and the polymeric chain.[1–5] These cyclic compounds may rotate and/or slide along the polymeric chain, and can be dethreaded from the chain, destroying the supramolecular structure, if the terminal capping groups are cleaved by any stimulus (Figure 1). In general, the mobility of cyclic compounds in polyrotaxanes is thermodynamically governed by the intermolecular forces between the cyclic compounds and the polymeric chain as well as the spatial allowance due to the movement along the polymeric chain.[6] In contrast, one of the crucial characteristics of conventional polymers is decreased chain mobility due to the high molecular weight of its repeating units. It is known that the majority of polymer properties are always accompanied by a low diffusivity and/or low mobility in the solution state and long relaxation time in the solid state. From this point of view, it is expected that the mobility of cyclic compounds in polyrotaxanes can be significantly maintained as long as the low-molecular-weight compounds are in the solution state.

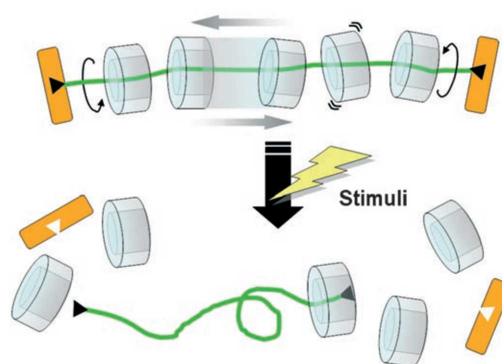

Figure 1. Characteristic image of polyrotaxanes. The cyclic compounds in the polyrotaxanes can rotate and/or slide along the polymeric chain in solution. The supramolecular structure can be dissociated by the terminal cleavage of capping groups by means of external stimuli

We have paid much attention to the characteristics, such as the "mobile" nature of cyclic compounds in polyrotaxanes in recent studies on the design of novel supramolecular biomaterials, which has never been achieved by using conventional polymer architectures.[7–14] Nature utilizes several supramolecular approaches to construct "mobile" molecular structures with hierarchical architectures in order to perform sophisticated biological functions. For instance, the fluid mosaic model consisting of proteins in lipid bilayers represents the mobility of cellular surfaces, and this model has been recognized in processes, such as the control and modulation of signal transduction across cell membranes by means of specific ligand–receptor recognition. We have postulated that our designed polyrotaxanes can be used to derive the thermodynamic benefits for modulating the interaction with biological systems; the enthalpic gain due to their polymeric architectures and the entropic gain due to

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their excellent mobility are similar to those of low-molecular-weight compounds.[8]

The most striking contribution of the "mobile" nature seen in polyrotaxanes to the design of biomaterials functions would be to enhance the multivalent interaction with biological receptors; "mobile" ligands conjugated to the cyclic compounds in polyrotaxanes can effectively bind receptor proteins in a multivalent manner (Figure 2a).[8–11,15,16] Stoddart and his co-workers have suggested "multivalency and cooperativity in supramolecular chemistry" using self-assembled polypseudorotaxanes. They have synthesized lactose-appended cyclodextrin derivatives, and then the derivatives were threaded onto hydrophobic polymers in aqueous solution to form dynamic multivalent lactosides for binding to lectins.[15] In addition, the polypseudorotaxane was stable in aqueous conditions in a solubilized state. In general, polypseudorotaxanes derived from cyclodextrins without any chemical modifications are precipitated because of crystallization occurring through strong hydrogen-bonding interactions.[4] After chemical modification, the hydrogen bonds should be eliminated, resulting in dissociation of polypseudorotaxanes. Stoddart et al. have developed a self-assembled strategy to produce multivalent interactions with garectin-1.[16] A self-assembled pseudopolyrotaxane consisting of lactoside-substituted cyclodextrin "beads" threaded onto a linear polyviologen "string" was investigated for its ability to inhibit galectin-1 mediated T-cell agglutination. The term multivalency is defined as a way to simultaneously bind multiple copies of ligands with receptor sites of proteins. This approach is very promising for the enhancement of the binding constant of the ligand–receptor interaction, and can exploit a variation of the possible applications, such as targeting drugs, receptor-mediated drug delivery systems, and tissue regeneration. A variety of multifunctional polymer architectures has been extensively studied as multivalent ligand–polymers. However, one of the major barriers in the course of multivalent interaction by polymers is the spatial mismatching between ligand-immobilized polymers and receptor sites. The increased number of ligands in these multifunctional polymers eventually causes an excessive increased density of the ligands,[17,18] and this situation is thermodynamically unfavorable for the multivalent interactions (Figure 2b).

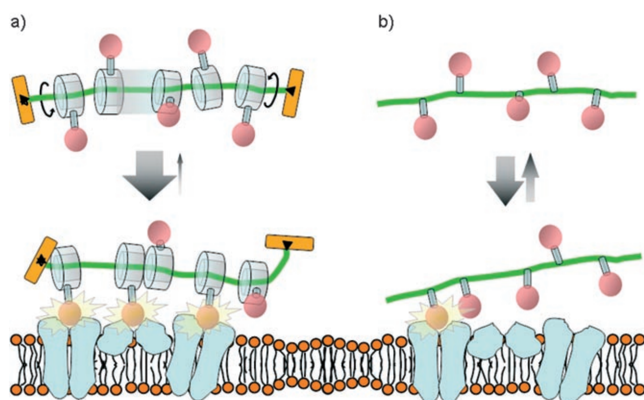

Figure 2. The effect of "mobile" motion of the cyclic compounds in polyrotaxanes on binding receptor proteins in a multivalent manner: Image of binging/dissociating equilibrium a) between a ligand–polyrotaxane conjugate and receptor sites and b) between a ligand–immobilized-polymer and receptor sites.

In contrast, the mobile nature of ligands conjugated to the cyclic compounds in polyrotaxanes is believed to significantly contribute to eliminating spatial mismatching upon the interaction between the ligands and the receptors in a multivalent manner. This article highlights our recent progress in such polyrotaxanes aimed at novel biomaterial functions that have never been achieved by covalently linked polymers with low molecular mobilities.

Theoretical Perspectives

Multivalent interactions of specific ligands with binding sites of receptors have been distinguished from nonspecific interactions by their larger equilibrium binding constant (K), although monovalent sugar–sugar and sugar–protein interactions are usually quite small in the range of 10^3–$10^4\,\mathrm{M}^{-1}$. Whitesides et al.[19] have proposed the following equations [Eq. (1)–(4)] for the calculation of the free energy and binding constants in multivalent interactions, in which N is the number of interacting units in a multivalent interaction, $\Delta G_{N,\mathrm{av}}$ is the average free energy of a single interaction in a multivalent interaction, ΔG_N is the free energy of binding in a multivalent interaction, K_N is the binding constant of a multivalent interaction, $K_{N,\mathrm{av}}$ is the average binding constant of a single interaction in a multivalent interaction, and ΔH_N and $T\Delta S_N$ are the enthalpy and entropy of a multivalent interaction, respectively.

$$\Delta G_{N,\mathrm{av}} = \Delta G_N / N \quad (1)$$

$$\Delta G_N = -RT \ln K_N \quad (2)$$

$$K_N = (K_{N,\mathrm{av}})^N \quad (3)$$

$$\Delta G_N = \Delta H_N - T\Delta S_N \quad (4)$$

In many cases, the free energy of binding for a multivalent interaction may not be proportional to the number of monoligand units. If the backbone of polymers conjugated with multiple copies of ligands is conformationally rigid, small spatial mismatches are likely to lead to a lower enthalpic energy. Multivalent interactions can also be considered in terms of their entropic energy. To prevent the entropic energy loss in the course of multivalent interactions, a key point is how to eliminate the spatial mismatching in ligand–receptor binding through the molecular design of multivalent ligand–polymer conjugates.

The free energy change in multivalent interactions is a function of K_N [Eq. (2)], which can be considered from the viewpoint of kinetics as being defined by Equation (5) in

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which k_a and k_d are the apparent binding rate constant and dissociation rate constant, respectively.

$$K_N = k_a / k_d \quad (5)$$

As can be seen from Equation (5), multivalent interactions favor a decrease in k_d, resulting in an increase in the K_N. However, in order to increase k_a, enhancing the simultaneous binding of multiple copies of ligands with receptors requires the elimination of the spatial mismatching between the ligands and receptors through the molecular design of multivalent ligand–polymer conjugates.

In the last decade, a variety of multivalent ligand–polymer conjugates have been extensively studied, for example, neoglycopolymers,[17,18,20] glycodendrimers,[21,22] glycosylated poly(phenyl isocyanide),[23] glycosylated cyclodextrins,[24,25] glycosylated poly(glutamic acid),[26] and polystyrene derivatives.[27,28] These approaches have been successful in the enhancement of multivalent interaction to a certain extent, presumably due to the contribution of the increased number of ligands in decreasing the k_d. However, enhancement is not always realised, because further increase in the number of ligands in these conjugates usually causes an excess density of ligands, leading to the spatial mismatching between the ligands and receptors. Generally, if the geometry of receptors dose not fit the spacing between ligands, the multivalent ligands are distorted to fit the distance of binding sites in the receptor. This situation often causes the spatial mismatching. Thus, from the above-mentioned point of view, the ultimate goal for the design of multivalent ligand–polymer conjugates is how to eliminate the spatial mismatching between multiple copies of ligands conjugated to polymer backbone and binding sites of receptors.

Throughout the above-mentioned perspectives, cyclic compounds in polyrotaxanes are expected to exhibit much higher mobility than covalently immobilized molecules. We have assumed that specific ligands immobilized into cyclic compounds in polyrotaxanes are likely to behave close to the free ligands to eliminate the spatial mismatching in the bindings with receptors, resulting in extraordinary enhanced binding constant of the interaction.

Proof of Concept

In the last few decades, we have paid special attention to biomaterial design by using polyrotaxanes consisting of cyclodextrins (CDs) and a linear polymer capped with bulky end-groups linked through a biodegradable spacer. Biodegradable polyrotaxanes consisting of α-CDs and poly(ethylene glycol) (PEG) capped with phenylalanine by means of a peptide linkage were synthesized as our first trial biomaterials aiming at novel drug carriers.[29–32] The thermoresponsive mobile nature of β-CDs along a triblock-copolymer chain of PEG-poly(propylene glycol)-PEG was also observed in our studies on polyrotaxanes.[33,34] In the course of our studies, we have demonstrated that polyrotaxanes have excellent properties to enhance multivalent interactions in biological events. For instance, polyrotaxanes consisting of maltose-immobilized α-CDs and a PEG chain capped with tyrosine (Tyr) residue have been prepared and were used to examine the interaction with a sugar-recognizable protein, such as Concanavalin A (Con A).[8,9]

As a first step, we examined the inhibitory effect of the maltose–polyrotaxane conjugates on Con A induced hemagglutination. A series of maltose–polyrotaxane conjugates with different number of threading α-CDs (50, 85, and 120) was prepared with PEG with an average molecular-weight of 20000. Approximately 220 of the α-CDs can be theoretically threaded onto a PEG chain, assuming two repeating units of ethylene glycol are included into one α-CD cavity. The number of α-CDs units, that is, 50, 85, and 120, represents 23, 39, and 55%, respectively, of the coverage of PEG chain in these polyrotaxanes. In each polyrotaxane, the number of maltose groups introduced to α-CDs was varied from about 40 to 240.

These maltose–polyrotaxane conjugates exhibited much stronger inhibitory effects than maltose itself, in some cases up to 3000 times stronger than maltose.[8] If the potency is calculated per polyrotaxane unit, the inhibitory effect reaches over 700000 times higher than one maltose molecule. These data strongly emphasize the remarkable effect of polyrotaxane structure on multivalent interactions.

By increasing the number of maltose groups in the polyrotaxanes, the inhibitory effect was enhanced as expected by the cooperative effect multivalent interactions. However, the cooperative inhibition increased with the number of maltose groups in the polyrotaxanes up to a certain number of α-CDs, that is, up to 85 CDs, and a further increase in the number of CDs caused a decrease in the potency with increasing maltose groups per polyrotaxane (Figure 3). This result indicates that there is no straightforward relationship between the number of maltose groups and the inhibitory effect.

In addition, for the same number of maltose residues, stronger inhibition was observed for the polyrotaxane with an appropriate number of threading α-CDs. This is because the flexibility of polyrotaxanes is strongly related to the number of α-CDs, and one may expect that polyrotaxane mobility plays a dominant role in the inhibitory effect. We also found that maltose–poly(acrylic acid) conjugate has a lower inhibitory effect than the polyrotaxane conjugates (Figure 3).

These results suggest that an important structural aspect of polyrotaxanes, that is, the mobility of α-CD molecules along the PEG chain, including sliding or rotational motion, plays a crucial role on binding with Con A. Indeed, NMR measurements on spin–lattice relaxation time (T_1) and spin–spin relaxation time (T_2) of α-CD and maltosyl C1 protons and PEG methylene protons in maltose–polyrotaxane conjugates revealed that the mobility of α-CDs in the polyrotaxane governs the molecular motion of maltosyl groups in the α-CDs in polyrotaxane.[9]

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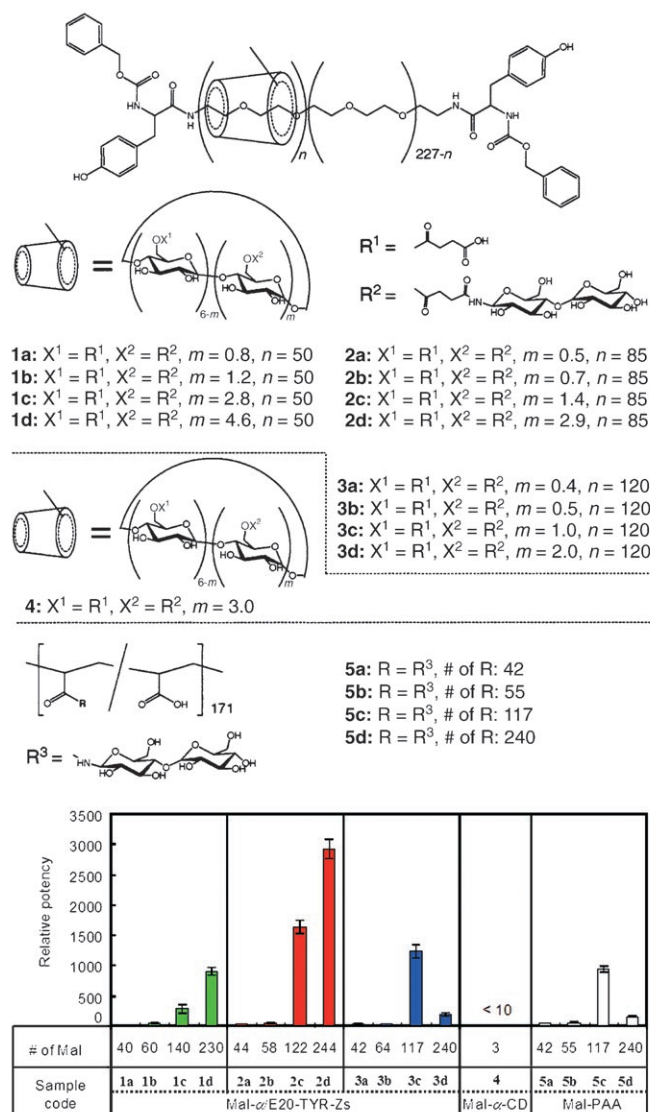

Figure 3. Chemical structure of maltose-polyrotaxane conjugates consisting of α-CDs, PEG, benzyloxycarbonyl-tyrosine and maltose (Mal-α/E20-TYR-Zs, **1**–**3**), maltose-α-CD conjugates (**4**), and maltose-poly(acrylic acid) conjugate (**5**). The graph shows that relative potency of Con A induced hemagglutination inhibition based on minimum inhibitory concentration (MIC) of maltose unit ($n=3$, mean ± S.E.M) (from reference [8], Copyright 2003 American Chemical Society).

It is worth mentioning that the inhibitory effect of maltose–polyrotaxane conjugates on Con A induced hemagglutination was found to be closely related to the T_2 values of maltosyl groups. This finding strongly supports our suggestion that the high mobility of maltose ligands on α-CDs along the PEG chain contributes much to the enhancement of multivalent binding with Con A.

The T_2 value for the methylene protons of the PEG chain tends to become larger with decreasing the number of α-CDs in polyrotaxanes, suggesting that the PEG chain is more flexible in the regions in which their ethylene glycol units is exposed to the aqueous medium. In addition, from the fluorescence measurements, the K_N values for maltose–polyrotaxane conjugates with 22, 38, and 53 % threading α-CDs were 5.7×10^4, 1.1×10^6, and $5.3\times10^5\,\text{M}^{-1}$, respectively. The largest K_N value of the maltose–polyrotaxane conjugate with 38 % threading α-CDs correlates well with the T_1 and T_2 values of maltosyl groups and α-CDs, suggesting that the mobility of maltose groups conjugated with α-CDs affects its affinity for Con A.[9]

These findings suggest that the mechanically locked structure of polyrotaxane with a controlled number of threading α-CDs can have favorable thermodynamic effects on multivalent interactions. Finally, we have established one concept that the combination of multiple copies of ligands and their supramolecular mobility along the mechanically locked polyrotaxane structure should contribute to the novel design of polymeric architectures aiming at enhanced multivalent interactions.

Recently, we have studied the kinetic analysis on interaction of maltose–polyrotaxane conjugates with Con A immobilized surfaces by surface plasmon resonance spectroscopy.[35] The magnitude of k_d in polyrotaxanes is usually much lower than in maltose itself, indicating an easily observable event in the case of multivalent ligand–polymer conjugates. Special interest should be paid to the fact that the greatest magnitude of k_a was observed for the polyrotaxane exhibiting the highest potency in Con A induced hemagglutination experiments. This result indicates that the greatly improved K_N value observed at the polyrotaxane is derived from increased value of k_a, and strongly supports our hypothesis that the "mobile" nature of ligands in conjunction with the polyrotaxane structure can contribute much to eliminating the spatial mismatching between ligands and binding site of receptors.

Current and Future Works

To expand our concept of "mobile" ligands seen in polyrotaxanes to more practical applications, we have focused on biocleavable polyrotaxanes as a non-viral gene carrier.[7] Gene delivery using polycations is one of the greatest challenges for inventing non-viral gene carrier systems instead of toxic virus-based vector systems.[36–39] Polycations have been believed to make a polyion complex (polyplex) with anionic DNA to deliver the DNA to target cells by means of endocytosis, eventually leading to the nucleus. However, several difficulties have arisen in this strategy: how, when, and where are the DNA polyplexes decondensed to release and deliver the DNA?[36] Of course, another serious problem is the removal of the polyplex from endosomal/lysosomal digestion, releasing the DNA into the cytoplasm, and finally reaching the nucleus through the nucleus membrane. In addition the cytotoxicity of polycations has been investigated. For instance, high-molecular-weight polycations, such as polyethyleneimine (PEI) have been studied as a non-viral gene vector that effectively condenses plasmid DNA (pDNA) to give a stable polyplex, whereas low-molecular-weight polycations are more favored in terms of deconden-

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sation of the polyplex as well as high transfection and low cytotoxicity.[40] To solve such controversy, introducing biodegradable moieties into polycations to decondense the polyplex has been recently proposed.[41–43] For example, introducing many disulfide linkages into the main chain of polycations has been reported as a key for controlling intracellular gene delivery, because the pDNA polyplex is decondensed in cytoplasm through the cleavage of disulfide linkages.[41] However, gene-transfer efficiency in relation to endosomal escape decreases with increasing the number of disulfide linkages due to over stabilization of the polyplex, resulting in insufficient cleavage.[44]

From these perspectives, we designed a biocleavable polyrotaxane that has a necklace-like structure between many cationic α-CDs and a disulfide-introduced PEG chain. Here, dimethylaminoethyl-modified α-CDs (DMAE-α-CDs) are threaded onto a PEG ($M_n = 4000$) chain capped with benzyloxycarbonyl-Tyr through disulfide (S–S) linkages that exist only at both terminals of the PEG chain (DMAE-SS-PRX).[7] The mobility of cationic α-CDs along the PEG chain in the polyrotaxanes is favorable for efficient polyplex formation with anionic DNA, as expected from our previous findings on the multivalent interaction mentioned above. Also, it is expected that the polyrotaxane will show sufficient cleavage of S–S linkages under reducing conditions, because the introduction of only two S–S linkages avoids the over stabilization of the polyplex. The S–S cleavage will trigger the pDNA decondensation (pDNA release) through the dissociation of the noncovalent linkages between α-CDs and PEG, looking like a broken necklace (Figure 4).

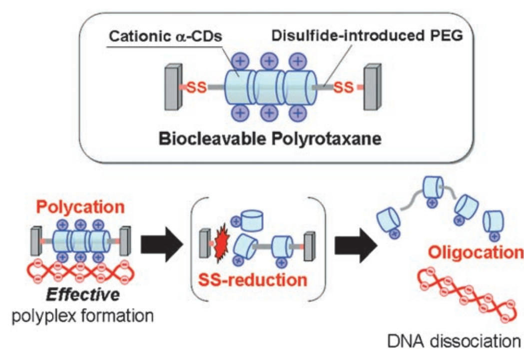

Figure 4. Schematic structure and pDNA complexation/dissociation image of biocleavable polyrotaxane (DMAE-SS-PRX) as a gene carrier.

In our designed polyrotaxanes, the number of threading α-CDs and DMAE groups per polyrotaxane was estimated to be approximately 23–30 and 40, respectively, from the ^{1}H NMR spectra. The pDNA complexation of the DMAE-SS-PRX was compared with a linear polyethyleneimine with an M_n of 22000 (LPEI22k) in terms of gel electrophoresis and ζ potentials. The ζ potential of the DMAE-SS-PRX polyplexes was +4.8 mV at an N/P ratio of 0.5, and the DMAE-SS-PRX formed a tightly packed pDNA polyplex (the diameter: ca. 178–189 nm from a dynamic light scattering measurement). In contrast, LPEI22k did not form any tightly packed polyplex at this N/P ratio. The ζ potential of the DMAE-SS-PRX polyplexes became positive when the N/P ratio was 0.25–0.5, although the LPEI22k polyplex showed still negative value. These data suggest that the DMAE-SS-PRX polyplex can condense pDNA more efficiently than LPEI22k, even at a low charge ratio. The pK_a value of the DMAE-SS-PRX (7.5) and LPEI22k (8.0) indicates that only the half of DMAE groups were protonated and participated in the pDNA complexation in the buffer (pH 7.4). Therefore, it is likely that the driving force for condensing the pDNA with the DMAE-SS-PRX should involve not only electrostatic interaction, but several other factors. Presumably, the structural characteristics of polyrotaxanes, such as a rodlike structure and the association of the terminal benzyloxycarbonyl groups, may have an effect on this event. It is assumed that the mobile motion of α-CDs in the necklace-like structure of the DMAE-SS-PRX polyplex prevents spatial mismatching in multivalent interactions between the cationic groups in the polyrotaxane and the anionic groups in pDNA, resulting in the effective condensation of pDNA with much smaller amount of the polyrotaxane.

In vitro pDNA decondensation experiments in the presence of 10 mM dithiothreitol (DTT) as a reducing agent confirmed that pDNA was perfectly released from the DMAE-SS-PRX polyplex in the presence of the counter polyanion. In contrast, the polyplex of DMAE-introduced polyrotaxane (DMAE-PRX), which has no S–S linkages, was stable under the same conditions. Since the DMAE-α-CD release was confirmed in the same reducing conditions, it is considered that the S–S cleavage under reducing conditions led to the destabilization of the polyplex by means of PEG dethreading from DMAE-α-CD cavities, and interexchange with polyanions caused the decondensation of pDNA.

The positively charged surface of polyplex and supramolecular dissociation of the DMAE-SS-PRX is expected to improve the intracellular trafficking and transfection efficiency. The intracellular trafficking with NIH3T3 cells was evaluated by quantitative three-dimensional analysis by using a confocal laser scanning microscope (CLSM) technique. It is surprising that the DMAE-SS-PRX polyplex (N/P ratio = 5) was completely removed from endosome and/or lysosome 90 min after the transfection (Figure 5a). Presumably, the much more positively charged surface of the DMAE-SS-PRX polyplex than the LPEI22k polyplex and the good buffering capacity are advantageous for a proton-sponge effect. It was noted that approximately 30% of the pDNA cluster was found in nucleus (Figure 5b), being clearly confirmed by the CLSM image (Figure 5c). In the case of the LPEI22k polyplex, the pDNA cluster seemed to be located above the nucleus (Figure 5d), and approximately 30% of the pDNA was still located in endosome and/or lysosome even after the same incubation time (Figure 5a). Therefore, it is considered that the high localization of pDNA cluster in the nucleus is due to rapid endosomal removal of the DMAE-SS-PRX polyplex. Furthermore, the DMAE-SS-PRX polyplex affected the transfection efficien-

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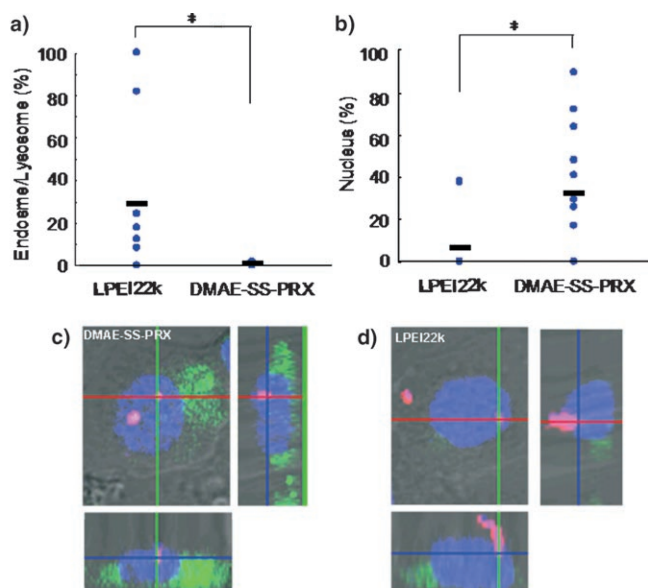

Figure 5. Effects of DMAE-SS-PRX/pDNA polyplexes on intracellular trafficking of pDNA. Comparison of a) the endosome/lysosome and b) the nucleus distributions of pDNA transfected with DMAE-SS-PRX and LPEI22k at N/P=5. Black bars represent the mean values ($n=10$). Asterisks indicate significant differences between two polymers, determined by the Mann–Whitney test ($P<0.05$). CLSM images for c) the DMAE-SS-PRX and d) the LPEI22k polyplexes (N/P=5) after 90 min transfections are shown below the quantified graph. Endosome/lysosome (green) was stained by Lysosensor and the blue fluorescence shows the Hoechst 33258-stained nuclei. The rhodamine-labeled pDNA shows a red fluorescence (from reference [7] Copyright 2006 American Chemical Society).

cy: the transfection efficiency of the DMAE-SS-PRX polyplex was independent of the N/P ratio, although that of the LPEI polyplex apparently was not. These results suggest that the transfection of the DMAE-SS-PRX polyplex is independent of the amount of free polycations. It is evident that the S–S cleavage plays a key role for the gene expression, since the transfection efficiency of the DMAE-SS-PRX polyplex was much higher than that of DMAE-PRX. The DMAE-PRX polyplex exhibited no cytotoxicity regardless of the N/P ratio, whereas the LPEI showed higher cytotoxicity with increasing N/P ratio. This result indicates that the supramolecular dissociation of the polyrotaxane into the constituent molecules with low molecular weights can contribute to eliminating the cytotoxicity usually observed with high-molecular-weight polycations.

It is not so easy to imagine that a stable polyplex with positively charged surface can be formed by mixing a very small amount of the biocleavable and cationic polyrotaxane with pDNA. This finding is likely to be due to the mobile motion of α-CDs in the necklacelike structure of the polyrotaxane. The pDNA decondensation of the polyplex occurs through the S–S cleavage in the polyrotaxane and the subsequent interexchange with polyanions. This is presumably due to reducing the potency of multivalent interaction between cationic polyrotaxane and anionic pDNA. Rapid endosomal escape and pDNA delivery to nucleus can be achieved by the polyrotaxane-based polyplex. Further studies on the design of polyrotaxanes exploit not only a far-reaching technology on non-viral gene delivery, but also a variety of practical applications for beneficial biomedical devices and tools.

Stimuli-responsive controlled mobility of cyclic compounds along a polymeric chain is distinguished as one of our future interests for supramolecular biomaterials. We have already demonstrated pH-responsive mobility control of α-CDs along PEI-PEG-PEI triblock-copolymer capped with bulky end-groups.[13] Controlling the mobility of ligands along the polymeric chain by a physiologically allowed pH change may lead to either modulating the K_N value in interaction with receptor proteins, or controlling the fluidity (clustering) of receptor proteins on plasma membranes. We believe that this can exploit the extracellular modulation of cytoplasmic metabolism at target cells and/or tissues.

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