Inclusion Polymers

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Functional Cyclodextrin Polyrotaxanes for Drug Delivery

Nobuhiko Yui, Ryo Katoono, and Atsushi Yamashita

Dedicated to Professor Naoya Ogata on the occasion of his 77th birthday (Kiju)

Abstract The mobility of cyclodextrins (CDs) threaded onto a linear polymeric chain and the dethreading of the CDs from the chain are the most fascinating features seen in polyrotaxanes. These structural characteristics are very promising for their possible applications in drug delivery. Enhanced multivalent interaction between ligand–receptor systems by using ligand–conjugated polyrotaxanes would be just one of the excellent properties related to the CD mobility. Gene delivery using cytocleavable polyrotaxanes is a more practical but highly crucial issue in drug delivery. Complexation of the polyrotaxanes with DNA and its intracellular DNA release ingeniously utilizes both CD mobility and polyrotaxane dissociation to achieve effective gene delivery. Such a supramolecular approach using CD-containing polyrotaxanes is expected to exploit a new paradigm of biomaterials.

Keywords CD mobility, Gene delivery, Multivalent interaction, Saccharide/protein interaction, Supramolecular dissociation

Contents

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1 Introduction

One of the structural features seen in polyrotaxanes is the absence of any covalent binding between cyclic compounds and a linear polymeric chain capped with bulky end-groups at both terminals [1]. It looks like a necklace: the cyclic compounds are mechanically locked by the linear polymeric chain. The cyclic compounds in a polyrotaxane can slide and/or rotate along the axial polymeric chain if the polyrotaxane is soluble in a certain solvent. Furthermore, such mechanical locking between the cyclic compounds and the linear polymeric chain will be opened once one of the terminal bulky end-groups is cleaved by any external conditions. These characteristics are only observable in and specific to polyrotaxanes and are never seen in conventional polymeric architectures (Fig. 1).

The first report on preparing a polymeric inclusion complex (polypseudorotaxane) using cyclodextrin (CD) was published by Ogata and his coworkers in 1976 [2]. They prepared several inclusion complexes consisting of an aromatic or aliphatic diamine with β-CD and then applied the complex to polycondensation reactions with an acid chloride such as isophthaloyl and terephthaloyl dichloride to prepare CD-included polyamides. In 1990, Harada and Kamachi reported a very straightforward method to prepare an inclusion complex (pseudopolyrotaxane) between α-CD and a linear polymeric chain such as poly(ethylene glycol) (PEG) of various molecular weights in aqueous conditions [3]. They found that one can obtain pseudopolyrotaxanes as a precipitate within a short period of time if the saturated aqueous solution of α-CD is directly mixed with aqueous solutions of PEG at room temperature. This unexpected finding led to explore quite a new paradigm of the world of polyrotaxane as functional polymers. At almost the same period of time, Wenz and his coworker reported the first polyrotaxane synthesis from α-CD and a polyamine [4]. Harada and Kamachi have analyzed not only the formation of a variety of pseudopolyrotaxanes, but also the preparation of polyrotaxanes and their related architectures, and their significant efforts contributed much to the progress in understanding polyrotaxanes using CDs [5].

From the viewpoint of biomaterials which are to be utilized in contact with a living body, the combination of α-CD and PEG as building-blocks allowed us to envision the novel design of biologically inert and/or biodegradable polymers. CDs have been approved by the FDA as food additives and as drug formulations, and PEG is a worldwide-used water-soluble polymer for conjugating biologically active agents such as drugs and proteins. It is thus easily established that biodegradable polyrotaxanes consisting of α-CD molecules and a PEG chain capped with bulky end-groups via biodegradable linkages have much potential in drug delivery.

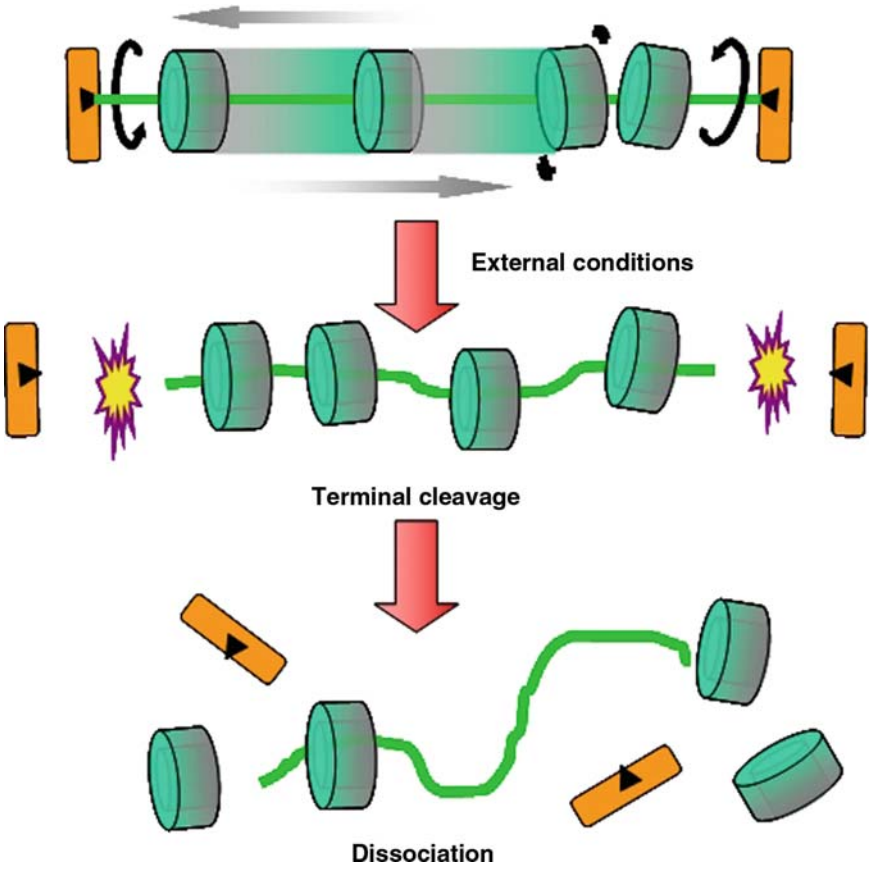

Fig. 1 Images of the mobility of mechanically interlocked cyclic compounds and stimuli responsive dissociation of the polyrotaxanes

Supramolecular dissociation of the polyrotaxanes into constituent molecules such as α-CD and PEG was quite a new image as a mode of biodegradation in a living body. This insight was the first step to initiate our studies on polyrotaxanes as biomaterials 15 years ago [6].

In general, the most important strategy when initiating the design of materials using polyrotaxanes lies in the effectiveness of such a supramolecular structure regarding their functionality. If the functionality of the polyrotaxanes were already achieved in conventional materials, the use of polyrotaxanes would of course not make any sense. Quite a new functionality which has never been achieved in previous studies should be thoroughly considered as it is strongly required for initiating and proposing a new concept. From this point of view, it should be noted that the characteristics of polyrotaxanes depicted in Fig. 1 are so fascinating that they create a paradigm shift in materials science [7]. Of course, one should thoroughly consider the following three issues when initiating the design of materials: (1) the strategy

of designing the functionality of biomaterials, (2) the tactics of preparing new architectures which fit the design concept, and (3) the logistics of cost performance and feasibility. When taking these issues into account, polyrotaxanes will constitute one of the greatest challenges to achieving far-reaching applications in the future. From these perspectives, the fact that polyrotaxanes have a lot of supramolecular characteristics including the mobility of cyclic compounds threaded onto a linear polymeric chain and the perfect dissociation at specific sites in a living body seems to constitute a sophisticated paradigm.

This chapter deals with CD-based functional polyrotaxanes for drug delivery, our main achievements over the last decade. First, we present an overview of the perspectives of polyrotaxane preparations for biomaterials applications. Then we highlight recent topics of our studies on drug delivery using CD-based polyrotaxanes. In particular, we describe a concept for enhancing multivalent interaction of a ligand-mobile polyrotaxane with receptor proteins. This issue is significantly related to receptor-mediated drug delivery and to modulation of cellular and tissue metabolism. The efficiency of such ligand–conjugated polyrotaxanes is also demonstrated in the design of inhibitors which are recognized by intestinal transporters in mammalian tissues but neither absorbed into the tissue nor exhibiting any toxicity. Finally, in order to expand this concept to more practical applications, we also introduce our studies on cytocleavable polyrotaxanes for gene delivery as an ultimate modern therapy. The dynamic motion of polyrotaxanes would contribute significantly to forming a polyplex with DNA to be delivered into target cells, and dissociating the polyrotaxane structure in intracellular environments is an effective way to release DNA for transfection at the nucleus.

2 Preparation and Properties of Polyrotaxanes for Biomedical Use

A large number of methods for the preparation of polyrotaxanes using CDs have been made available, and reported elsewhere [8,9]. In particular, one of the common strategies to prepare polyrotaxanes has been the capping of both terminals of pseudopolyrotaxanes with bulky groups, since Harada and Kamachi reported a unique preparation method of pseudopolyrotaxanes and polyrotaxanes [3, 5]. In order to design polyrotaxanes for medical and pharmaceutical applications, special attention should be paid to their biocompatibility as well as to their nontoxicity. For instance, assuming that polyrotaxanes will be used as a drug carrier, they should be water-soluble as well as functional for drug conjugation. If their molecular weight is higher than 10,000, in order to be excreted from urine, their digestion or degradation in a living body may be considered. Of course, biological inertness during their usage is also one of the necessary conditions to perform their functionality in contact with a living body. In this section, we describe a common preparation of polyrotaxanes for use in a living body by capping pseudopolyrotaxanes with amino acid derivatives as bulky end-groups via a condensation reaction, and a chemical modification of CD

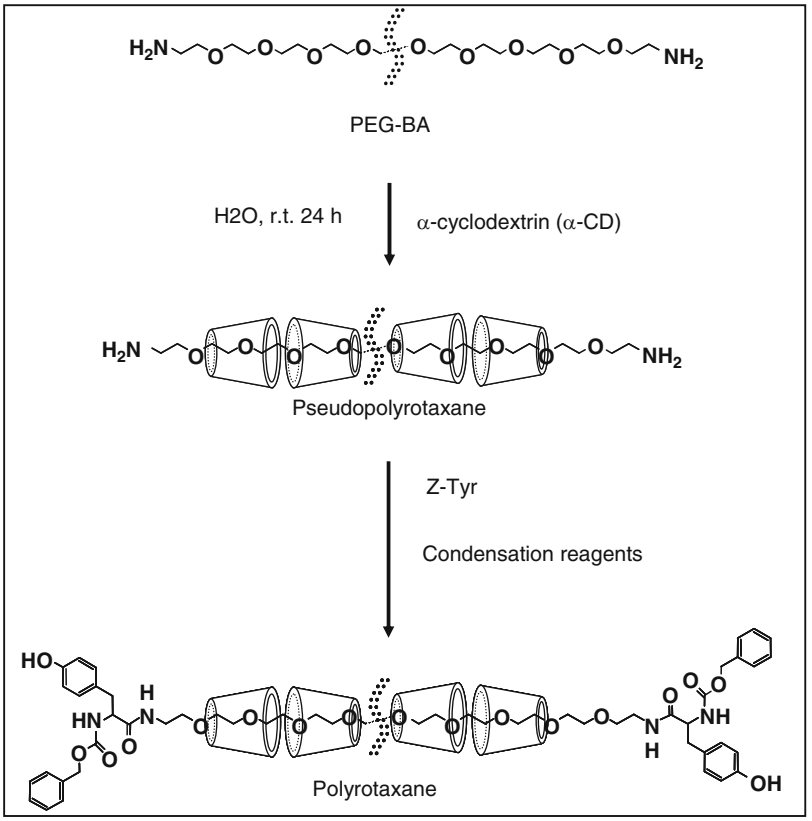

Fig. 2 Synthetic scheme of polyrotaxanes, in which α-CDs are threaded onto a PEG chain capped with Z-L-Tyr

molecules in polyrotaxanes for improving water-solubility and/or for functionalizing by conjugation of biologically-active agents.

Basically, the preparation of polyrotaxanes for biological applications consists of the following two steps: the preparation of a pseudopolyrotaxane by mixing α-CD and PEG-bis(amine) (PEG-BA) in water, and a subsequent capping reaction with amino acid derivatives such as *N*-benzyloxycarbonyl-L-tyrosine (Z-Tyr) (Fig. 2). In the first step, of course, pseudopolyrotaxanes consisting of α-CD and PEG–BA are commonly prepared according to the method reported by Harada and his coworkers as mentioned above. In the second step, Z-Tyr is allowed to react with the terminal amino groups of PEG–BA in pseudopolyrotaxanes by using a variety of suitable condensing agents in DMF or MeOH. When biologically labile linkages such as disulfide bond are introduced into the polyrotaxane, an SS-introduced PEG–BA is employed in place of PEG–BA mentioned in the above protocol.

Purification of the polyrotaxanes is one of the important aspects in preparing biomaterials. In the course of the polyrotaxane preparations mentioned above,

contamination from unthreaded CD and other chemicals is highly problematic and is to be completely removed. Usually, purification of polyrotaxanes by reprecipitation and dialysis in DMSO and water is very promising in removing these undesirable contaminants. Also, GPC measurements in DMSO or in suitable aqueous solutions are helpful to verify the purification of polyrotaxanes. Finally, chemical composition such as the number of threading CD molecules in the obtained polyrotaxane is calculated from the ratio of peak integrations for both C(1) protons in α-CD around 4.8 ppm and methylene protons in PEG around 3.5 ppm in the NMR spectrum measured in D_2O/NaOD, as shown later (Fig. 14).

Water solubility of polyrotaxanes is a critical issue when considering their medical and pharmaceutical applications, such as drug delivery. In general, polyrotaxanes prepared following the above-mentioned methods are poorly soluble in water, illustrated by the fact that the insolubility of pseudopolyrotaxanes in water enabled their isolation with ease. In order to endow intact polyrotaxanes with water solubility, a variation of the chemical modifications which have been previously reported for CD chemistry [10] is applicable. In particular, charged functional groups such as amino and carboxyl groups can be introduced at the hydroxyl groups of CDs through suitable spacers, since such chemical modifications are useful not only for improving water solubility but also for further functionalizing polyrotaxanes with biologically-active moieties in the following step. For the introduction of carboxyl groups, CD-containing polyrotaxanes are allowed to react with dicarboxylic acid anhydrides such as succinic anhydride in pyridine. A variety of biologically active agents can be introduced at these groups in the polyrotaxanes via condensation reactions. The dialysis against water, GPC and NMR measurements in water are conventional steps to ascertain purification and chemical compositions. These protocols are depicted in Fig. 3.

3 Enhancing Multivalent Ligand–Receptor Interactions Using Polyrotaxanes

Control of the binding of biologically active agents or ligands to receptor sites of proteins on the plasma membranes of cells is a crucial factor for modulating receptor-mediated cellular metabolism as well as endocytosis for drug delivery. One of the important aspects in this event is how effectively and specifically the binding on membrane proteins using very low quantities of the agents or ligands can be achieved. In this perspective, a "multivalent interaction" using a functional polymer has been proposed and extensively studied over the last decades [11]. The term of multivalency is defined as a way to bind simultaneously multiple copies of ligands with receptor sites of proteins. This approach is believed to be promising for enhancing the binding constant of ligand–receptor interaction, and is expected to exploit significant improvements of such applications as targeting drugs, drug-mediated drug delivery and tissue regenerations (Fig. 4).

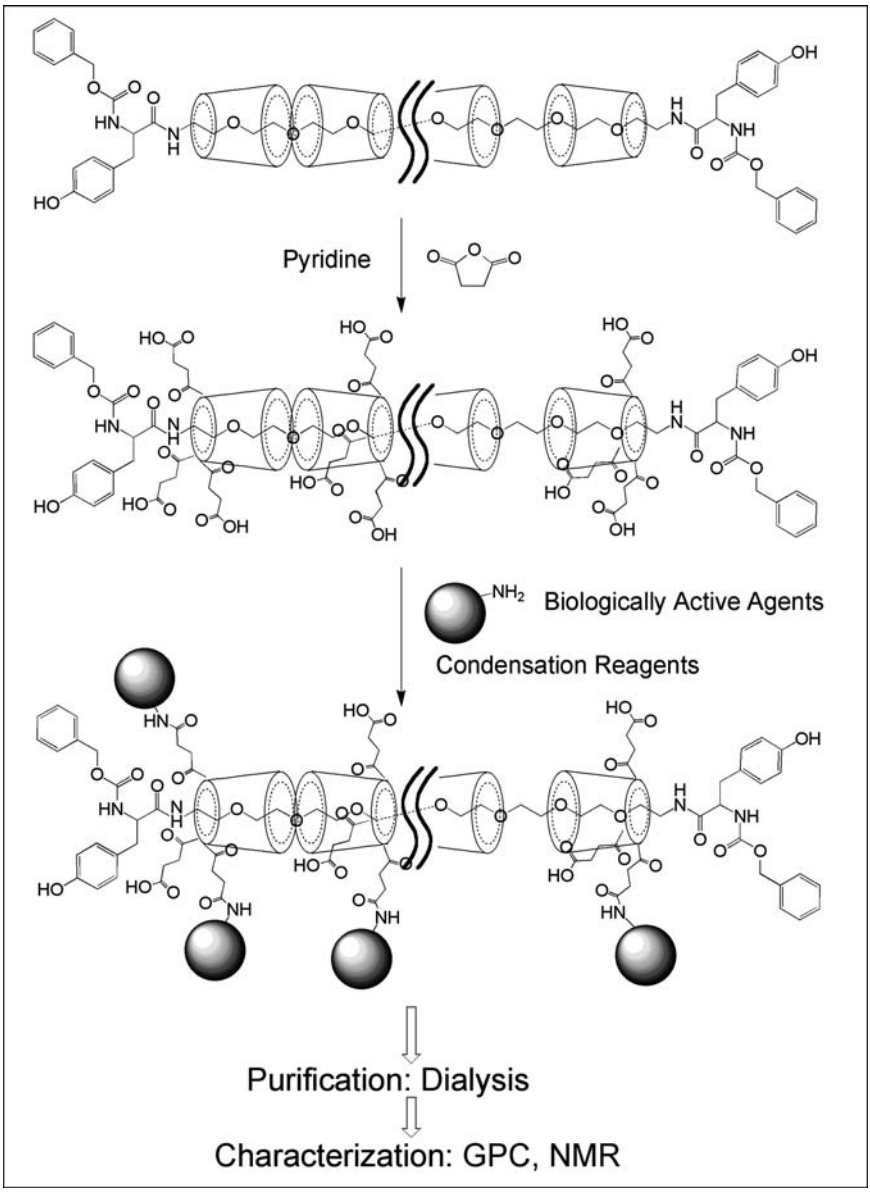

Fig. 3 Synthetic scheme of synthesis of functional polyrotaxanes, in which carboxyl groups were introduced into hydroxyl groups in CDs

A variety of functional polymers has been designed and demonstrated to be a tool of multivalent ligand-immobilized polymers. However, the binding constant using such polymers was not as enhanced as expected. Such unsatisfying results using a functional polymer were mainly attributed to a spatial mismatch between the

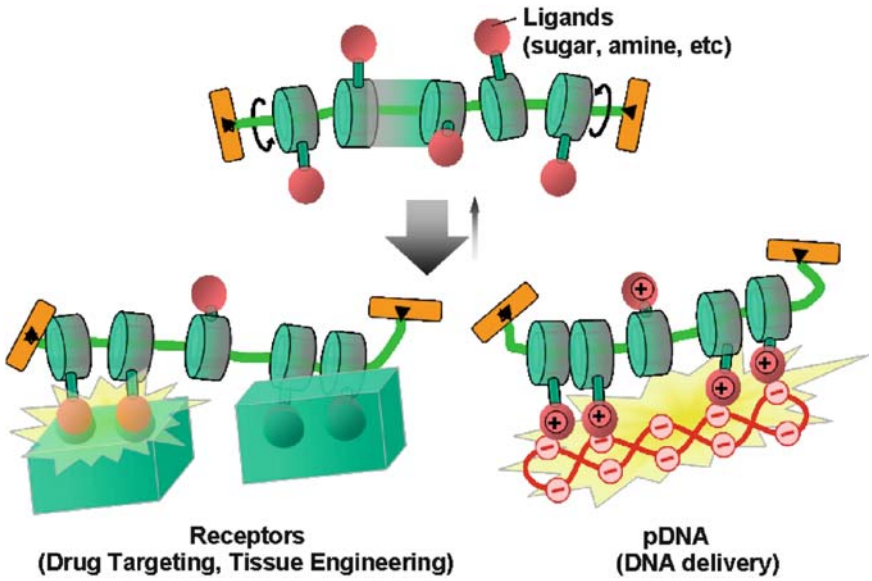

Fig. 4 Mobile cyclic compounds enhance molecular recognition. Cyclic compounds can rotate and/or slide along a polymeric chain in the structure of polyrotaxanes, and the mobility of ligands linked by the cyclic compounds play a key role in enhancing multivalent interaction with biomacromolecules. This concept can be used in sugar recognition and plasmid DNA polyplex formation [7]

ligand–polymers and receptor sites of proteins. Increasing the number of ligands in the polymer eventually causes an excessively large density of ligands, and this excess density is thermodynamically unfavorable to multivalent interactions between the ligands and receptors [12, 13].

From these perspectives, we tried to enhance the multivalent interactions using polyrotaxanes. As mentioned above, the most striking features observed in polyrotaxanes is the freely mobile nature of cyclic compounds threaded onto a linear polymeric chain capped with bulky end-groups. Thus, we believe that polyrotaxanes are advantageous in deriving thermodynamic benefits for enhancing multivalent interaction with biological systems. Freely mobile ligands conjugated to the cyclic compounds in polyrotaxanes would effectively bind to receptor proteins in a multivalent manner, which is based on the enthalpic gain due to enhanced opportunity of the binding for increasing internal energy of the bond molecules via their excellent mobility close to low molecular-weight compounds (Fig. **5 a,**ba). Although the multivalent event is entropically unfavorable, the enthalpic gain would overcompensate it in comparison with conventional ligand–polymer conjugates (Fig. **5 a,**bb).

In order to prove our hypothesis, we examined the interaction of ligand–immobilized polyrotaxanes with receptor proteins [14–17]. For instance, we studied the multivalent interaction of saccharide–conjugated polyrotaxanes with a lectin, a model receptor protein. A series of the maltose–conjugated polyrotaxanes with

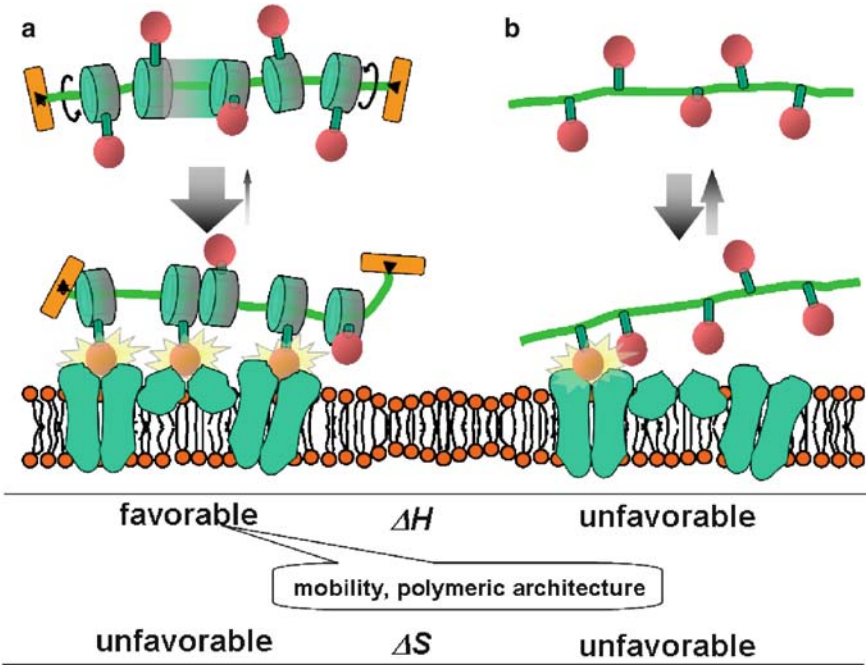

Fig. 5 a,b The effects of the mobile motion of the cyclic compounds in polyrotaxanes on binding receptor proteins in a multivalent manner: Image of binding/dissociating equilibrium **a** between a ligand–polyrotaxane conjugate and receptor sites, and **b** between a ligand–immobilized-polymer and receptors [7]

different numbers of threading α-CD molecules (50, 85, and 120) was prepared from an inclusion complex consisting of α-CD and an α,ω-diamino-PEG with an average molecular weight of 20,000. Here, approximately 220 molecules of α-CD can be theoretically threaded onto this PEG chain, assuming that two repeating units of ethylene glycols are included into the cavity of an α-CD molecule. At first, polyrotaxanes with different number of threading α-CDs were prepared by capping both amino groups in the inclusion complex with Z-protected L-tyrosine in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophospate (BOP reagent), 1-hydroxybenzotriazole (HOBt), and *N*,*N*-diisopropylethylamine (DIEA) in DMSO/DMF, and were then allowed to react with succinic anhydride, resulting in carboxypropanoyl-modified polyrotaxanes (C-PRxs). Then, β-maltosylamine was conjugated with the carboxyl groups of C-PRxs in the presence of BOP reagent, HOBt, and DIEA. In these polyrotaxanes, the number of maltose groups conjugated to α-CD molecules was varied to some extent.[1]

[1] The number of maltose groups was not determined exactly due to the lack of attention to some issues about the purification and assignment.

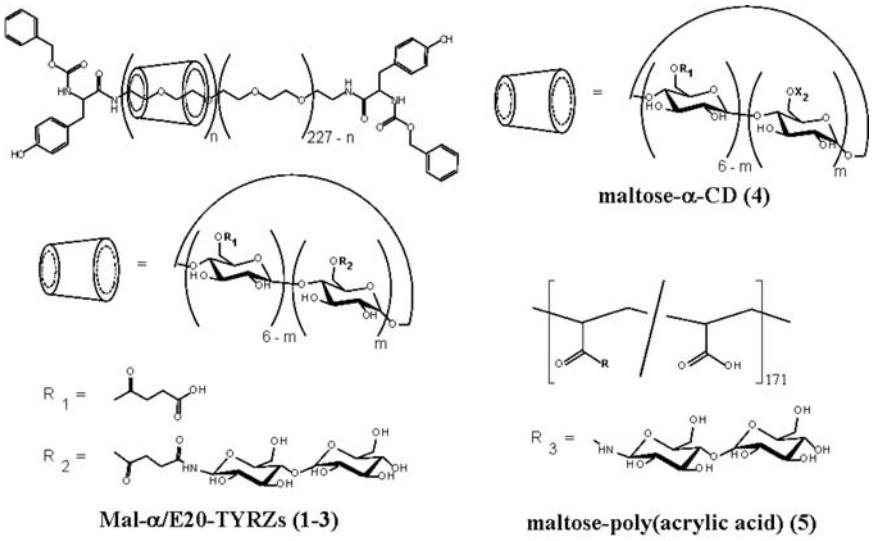

Fig. 6 Chemical structure of maltose–polyrotaxane conjugates consisting of α-CDs, PEG, benzyloxycarbonyl–tyrosine and maltose (Mal-α/E20-TYRZs, **1–3**), maltose-α-CD (**4**), and maltose-poly(acrylic acid) (**5**) conjugates [14]

The interaction of maltose–conjugated polyrotaxanes with their receptor proteins was evaluated by estimating the inhibitory effect of the polyrotaxanes on Concanavalin A-induced hemagglutination of red blood cells (Fig. 6). The minimum inhibitory concentration (MIC) of the maltose unit was determined as a measure of the relative potency vs the lectin. The maltose–conjugated polyrotaxanes were found to exhibit a much stronger inhibitory effect than maltose itself, up to 3,000 times larger. We also found that maltose-conjugated poly(acrylic acid)s have a much smaller inhibitory effect than the polyrotaxane conjugates. These results highlight the remarkable effect of polyrotaxanes on ligand–receptor multivalent interactions.

These results suggest the importance of structural aspects of polyrotaxanes, such as the mobility of α-CD molecules along the PEG chain including sliding or rotational motion, play a crucial role in binding of Con A. Indeed, we examined NMR measurements of the spin-lattice relaxation time (T_1) and the spin-spin relaxation time (T_2) for C(1) protons of α-CD, maltosyl C(1) and PEG methylene protons in maltose-polyrotaxane conjugates, which reveal that the mobility of α-CD in the polyrotaxane governs the molecular motion of maltosyl groups in α-CD molecules in polyrotaxane.

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 (Fig. 7). The T_2 value of maltosyl C(1) proton in the polyrotaxane exhibiting the greatest potency was almost the same as that in maltose–conjugated α-CD. This finding strongly supports our suggestion that the high mobility of maltose ligands on α-CD threaded onto the PEG chain contributes significantly to enhancing the multivalent binding with Con A. The T_2 of methylene

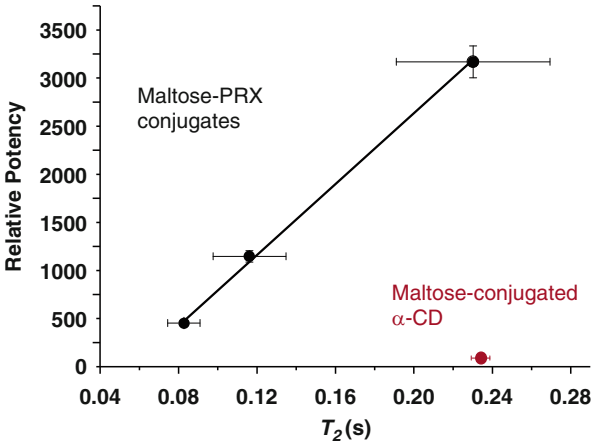

Fig. 7 Relationship between the T_2 value of maltosyl C(1) protons in the polyrotaxane and the relative potency of Con-A-induced hemagglutination inhibition based on the MIC of the maltose unit

protons in the PEG chain tends to become longer with a decrease of the number of α-CD molecules in polyrotaxanes, suggesting that the PEG chain is more flexible in the regions where their ethylene glycol units are exposed to the aqueous medium.

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

Alternatively, Stoddart and his coworkers prepared water-soluble pseudopolyrotaxanes consisting of lactose-appended α-/β-CDs threaded onto hydrophobic polymers such as poly(tetrahydro)pyrane and poly(propylene)glycol, respectively [18], and subsequently examined the binding of these self-assembled pseudopolyrotaxanes with lectins [19]. In their studies, they demonstrated great enhancement on multivalent interaction between lactose and lectin by using the lactose–conjugated pseudopolyrotaxanes, and suggested a flexible and dynamic ligand, including highly mobile ligands as a result of the CD rotating about the polymer chain, creating a new dimension for the study of protein–carbohydrate interactions.

4 Inhibitory Effect of Ligand–Conjugated Polyrotaxanes on Intestinal Transports

One of the possible applications directly related to multivalent ligand–receptor interactions will be the design of polymeric inhibitors which specifically bind receptors existing on cellular membranes to inhibit the uptake of biological substrates via the receptors. For example, there are many kinds of intestinal membrane transporters for

the specific uptake of digested food (proteins and carbohydrates) as well as drugs such as antibiotics. The restriction of these uptakes is strongly required for patients suffering from chronic renal diseases. In this sense, it is useful, for improving their quality of life, to achieve temporal inhibition of the uptake using an inhibitor. In particular, we note that such an inhibitor should be specifically recognized by a transporter without being absorbed to preclude kidney damage. From these points of view, it is obvious that ligand–conjugated polyrotaxane can be advantageous as a multivalent ligand–conjugated polymer.

First we prepared dipeptide–conjugated polyrotaxanes and studied their inhibitory effect on digested peptide uptake by intestinal human peptide transporter (hPEPT1) [20]. Here, Val–Lys as a dipeptide was conjugated to α-CD threaded onto a PEG 4,000 in polyrotaxanes, and we examined the inhibitory effect of the polyrotaxanes on the uptake of a model dipeptide via hPEPT1 using hPEPT1-expressing HeLa cells. The uptake of the model dipeptide was significantly inhibited by the polyrotaxanes, and the inhibition was much greater than dipeptide–conjugated reference samples such as dextran and α-CD. Also, we found that the effect of the polyrotaxanes was significantly enhanced by preincubation with hPEPT1-expressing cells (30 min before adding the model dipeptide), although the inhibitory effect of dipeptide–conjugated α-CD on the uptake was reduced by the preincubation. These results suggest that the supramolecular structure of the polyrotaxanes contributes to inhibiting the uptake via hPEPT1 in a multivalent manner. Furthermore, we confirmed that the inhibitory effect is dependent upon the molecular weight of the PEG chain in the polyrotaxanes when the average molecular weight of PEG was changed between 4,000 and 100,000. At the same concentration of dipeptide, the highest molecular weight of PEG (M_n: 100,000) showed the best inhibitory effect. After selecting the optimum molecular weight, dependence of hPEPT1 expression was also examined by changing the amount of the expressed hPEPT1 (10, 20, 60 μg per 15-cm dish). When the amount of expressed hPEPT1 on HeLa cell surface was the highest (60 μg per 15-cm dish), the dipeptide–conjugated polyrotaxane was found to exhibit the greatest inhibitory effect on the uptake of [^{3}H]Gly–Sar. This result suggests that high expression level of hPEPT1 was essential for inducing multivalent interaction [21].

In *vivo* performance of biomaterials after in vitro studies is one of the important steps for proving their applicability. When we applied the dipeptide–conjugated polyrotaxanes to an in *vivo* model using mice, the result was not as striking as expected. We considered this failure is due to insufficient blockage of the binding sites of the hPEPT1 located on the intestinal membrane: it is not so easy to occupy fully all the binding sites of the transporter with dipeptide molecules conjugated to the polyrotaxane. It is also well known that the activity of hPEPT1 is governed by proton concentration at the membrane. In order to maintain the binding with hPEPT1 via the multivalent interaction and then decrease the surrounding proton concentration on the intestinal membrane to complete the inhibition of the uptake, a polyrotaxane conjugated with dipeptide as well as sodium carboxylate was prepared as a revised sample. This polyrotaxane was found to decrease the pH of the intestinal tract significantly and to cause the inhibition of the dipeptide uptake in mice [21].

Presumably, the polyrotaxane can bind the intestinal membranes via multivalent interaction with hPEPT1 and effectively contribute to decreasing the local pH on the membrane, resulting in a significant inhibition of the uptake in mice. These results clearly indicate the efficacy of the ligand–conjugated polyrotaxanes as an inhibitor in intestinal uptake in *vivo*.

Furthermore, we prepared 2-(*N*,*N*-dimethylamino)ethylcarbamoyl (DMAEC)-polyrotaxanes and examined their inhibitory effect on the uptake of cations via carnitine/organic cation transporter, OCTN2, existing on the intestinal membrane and in tissues [22]. The DMAEC-polyrotaxanes were prepared by treating polyrotaxane with carbonyldiimidazole (CDI) in DMSO, followed by condensation with *N*,*N*-dimethylethylenediamine. The inhibitory effect was evaluated in terms of the uptake of L-carnitine in OCTN2-transfected HEK293/PDZK1 cells. The DMAEC-polyrotaxanes prepared from a PEG 20,000 were found to inhibit completely the uptake, although DMAEC-CD, the constituent molecule, and the polyrotaxanes with a shorter PEG 4,000 showed far less inhibition (Fig. 8). This result indicates that DMAEC groups along the polyrotaxanes are actually recognized simultaneously with multiple copies of OCTN2 on the cell surfaces, resulting in the complete inhibition of L-carnitine uptake.

Another interesting feature seen in the cationic polyrotaxanes in this study is their very low cytotoxicity. In general, cationic polymers such as polyarylamine (PAAm, Nitoo Boseki Co. Ltd., Japan; -$(CH_2CH(CH_2NH_2))_n$-) have been known to show high cytotoxicity when applied to native tissues and cells, due to nonspecific interaction with plasma proteins, sometimes leading to intracellular

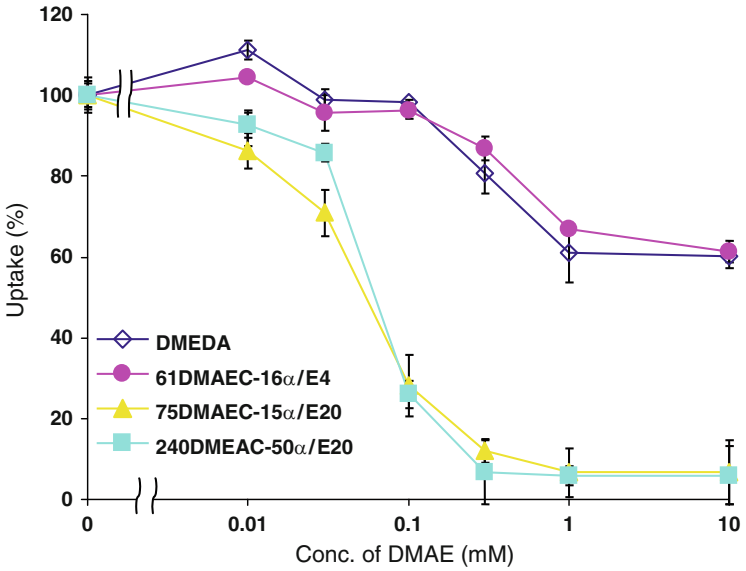

Fig. 8 Inhibitory effects of cationic polyrotaxanes (61DMAEC-16α/E4, 75DMAEC-15α/E20, 240DMAEC-50α/E20) on $OCTN_2$-mediated carnitine uptake in HEK293/$PDZK_1$ cells: HEK293/$PDZK_1$ cells were transiently transfected with YFP-$OCTN_2$, and the uptake of L-[^{3}H]carnitine was measured for 3 min in the presence of each compound [22]

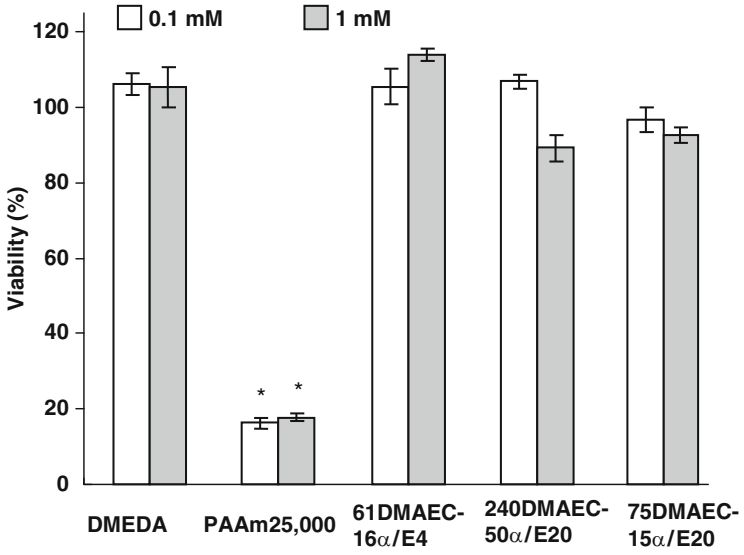

Fig. 9 Viability of HEK293/PDZK$_1$ cells in the presence of cationic polyrotaxanes (61DMAEC-16α/E4, 75DMAEC-15α/E20, 240DMAEC-50α/E20) and of cationic polymer (PAAm25,000) assessed by MTT assay. *Statistically significant difference from the control ($p < 0.05$)

damage, including mitochondrial energy transfer. Indeed, the PAAm inhibited the uptake markedly, but showed very high cytotoxicity in terms of the MTT assay. The MTT assay used here measures the reduction of a tetrazolium compound (MTT) to an insoluble formazan product by the mitochondria of living cells [23]. Thus, this result suggests that the inhibition observed for PAAm is not due to the specific binding with OCTN2 but to a nonspecific binding with plasma proteins, resulting in undesirable cytotoxicity through mitochondrial metabolism. However, our designed DMAEC-polyrotaxanes showed less cytotoxicity in term of the MTT assay, and the cell viability was almost the same as that of a control (Fig. 9). Presumably, the lower density of DMAEC groups along the polyrotaxane structure is considered to prevent intracellular uptake (endocytosis) of the polyrotaxane accompanied by electrostatic interactions with plasma proteins. The mobility as well as low density of DMAEC groups along the polyrotaxane structure appears to prevent cytotoxicity. These results strongly suggest that the cationic polyrotaxanes which we designed are promising candidates for an effective inhibition of cation transport via OCTN2 with less cytotoxicity.

5 Designing Cytocleavable Polyrotaxanes for Intracellular Gene Delivery

In order to expand our concept of "mobile" ligands seen in polyrotaxanes to more practical applications, we have focused on the design of cytocleavable polyrotaxanes as a nonviral gene carrier. Gene delivery using polycations is one of the greatest

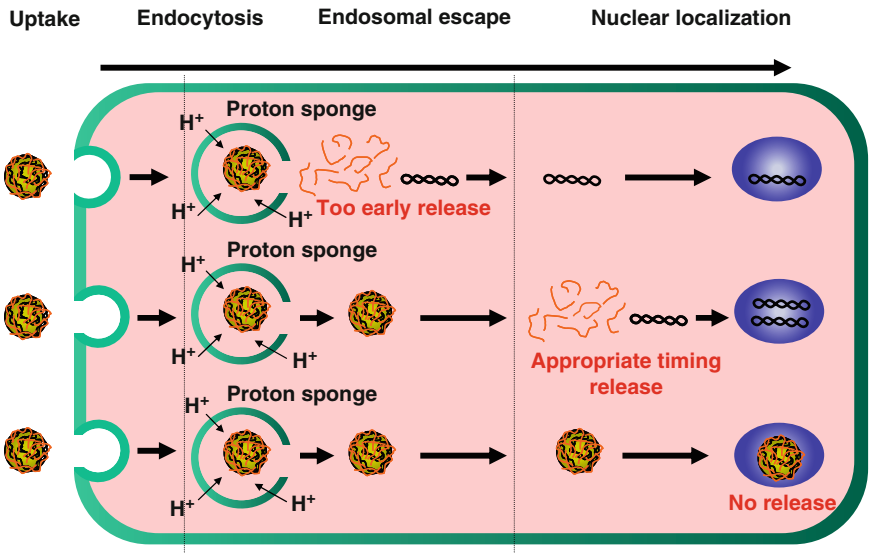

Fig. 10 Images of differences in intracellular trafficking and pDNA release timing

challenges for inventing nonviral gene carrier systems instead of toxic virus-based vector systems [24–26]. Polycations have been widely believed to form a polyion complex (polyplex) with anionic DNA to deliver it to target cells via endocytosis, eventually leading to the nucleus. However, several difficulties have arisen in this strategy: how, when, and where the DNA polyplexes must be dissociated to deliver and release the DNA (Fig. 10), in particular for the polyplex to escape from endosomal/lysosomal digestion to release the DNA into the cytoplasm, and finally to reach the nucleus through the nucleus membrane. The cytotoxicity of polycations has also been seriously pointed out during this research. For instance, high molecular-weight polycations such as polyethyleneimine (PEI) have been studied as nonviral gene vectors that effectively form a polyplex with plasmid DNA (pDNA), whereas low molecular-weight polycations are very advantageous in terms of pDNA release from the polyplex as well as of a low cytotoxicity [27].

In order to solve such a controversy, the introduction of biodegradable moieties into polycations to dissociate the polyplex has been recently proposed elsewhere [28–31]. For example, introducing many disulfide (SS) linkages into the main chain of polycations has been reported as a key for controlling intracellular gene delivery, because the pDNA polyplex is dissociated in the cytoplasm through the cleavage of disulfide linkages. However, excess amount of the disulfide linkers can induce the over stabilization of polyplex. Kissel and his coworkers reported that the transfection activity decreases with an increase in the SS crosslinking degree in a polyplex, due to decreasing the amount of released DNA [32].

From these perspectives, we designed a cytocleavable polyrotaxane that has a necklace-like structure between many α-CDs with attached cationic groups and

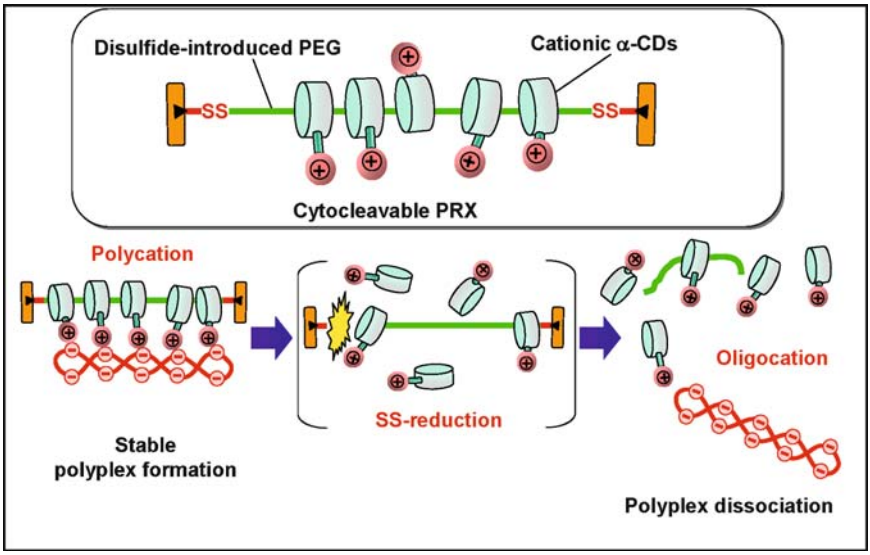

Fig. 11 Polyplex formation and pDNA release accompanied by the supramolecular dissociation of DMAEC-SS-polyrotaxanes

an SS-terminated PEG chain [33, 34]. Here, DMAEC-α-CDs are threaded onto a PEG chain ($M_n = 4{,}000$) capped with Z-Tyr via SS linkages that exist only at both terminals of the PEG chain (DMAEC-SS-PRX). Also, we expect that the polyrotaxane shows sufficient cleavage of SS linkages under reducible conditions. The SS cleavage will lead to triggering pDNA release via the dissociation of the noncovalent linkages between α-CDs and PEG, looking like a necklace broken into pieces (Fig. 11).

In our design of polyrotaxanes here, the respective numbers of threaded α-CDs and DMAEC groups per polyrotaxane were estimated to be around 23 and 40 for DMAEC-SS-PRx, 30 and 40 for DMAEC-PRx from their ^{1}H-NMR spectra. The complexation of DMAEC-(SS)-PRXs with pDNA was compared with that of a linear PEI with a M_n of 22,000 (LPEI22k) in terms of gel electrophoresis. A band for free pDNA in the electrophoresis was disappeared upon complexation with a smaller quantity of amino groups in the polyplex with the polyrotaxane than with the LPEI.

In vitro pDNA dissociation experiments in the presence of 10 mM dithiothreitol (DTT) as a reducing agent confirmed that pDNA is perfectly released from the DMAEC-SS-PRX polyplex in the presence of a counter polyanion (Dextran sulfate, $M_n = 25{,}000$). However, the polyplex of DMAEC-PRX, which has no SS linkages, is stable in the same conditions. Since the DMAEC-α-CD release was confirmed in the same reducible condition by GPC, we consider that the SS cleavage under the reducible condition led to the dissociation of the polyplex via PEG dethreading from DMAEC-α-CD cavities, and an interexchange with polyanions caused the pDNA release.

The intracellular trafficking was measured through a quantitative three-dimensional analysis using a confocal laser scanning microscope (CLSM) technique. It is surprising that the DMAEC-SS-PRX polyplex (the N/P ratio was around 5) completely escaped from the endosome and/or the lysosome 90 min after the transfection. We note that ca. 30% of the pDNA cluster was found in the nucleus, as it was clearly shown by the CLSM image. In the case of the LPEI22k polyplex, the pDNA cluster was not located in the nucleus, and ca. 30% of the pDNA was still located in the endosome and/or the lysosome for the same incubation time. Therefore, the high localization of the pDNA cluster in the nucleus is due to rapid endosomal escape of the DMAEC-SS-PRX polyplex.

Furthermore, the DMAEC-SS-PRX polyplex affects the transfection of pDNA using NIH3T3 cells. The transfection of the DMAEC-SS-PRX polyplex is likely to be independent of the N/P ratio, whereas that of the LPEI polyplex is apparently dependent on this ratio. These results suggest that the transfection of the DMAEC-SS-PRX polyplex is independent of the amount of free polycations. It is obvious that the SS cleavage played a key role in the gene expression, since the transfection of the DMAEC-SS-PRX polyplex was much higher than that of the DMAEC-PRX (Fig. 12). Also, we found that the DMAEC-SS-PRX exhibited low cytotoxicity independently of the N/P ratio, whereas the LPEI showed a higher cytotoxicity with an increase in the N/P ratio (Fig. 13). This result indicates that the supramolecular dissociation of the polyrotaxane into the constituent molecules with low molecular weights can contribute to the elimination of the cytotoxicity that is usually observed for high molecular weight polycations.

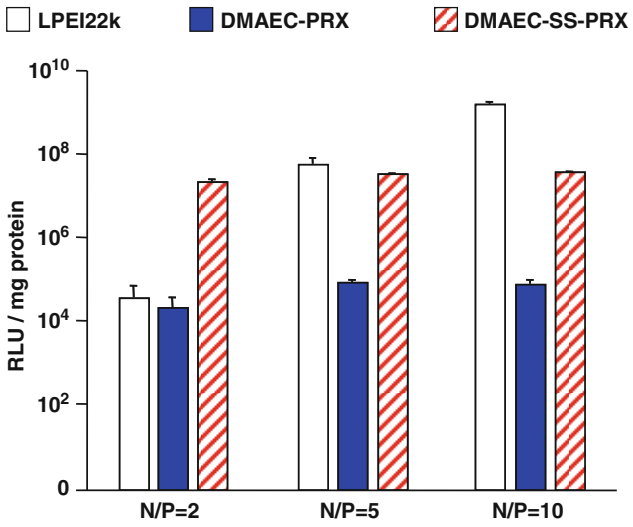

Fig. 12 Transfection activities of the DMAEC-SS-polyrotaxanes, DMAEC-polyrotaxanes, and the LPEI22k at different N/P ratios in NIH/3T3 cells. Luciferase activity in the NIH/3T3 cells was measured at 48 h after the addition of the polyplexes. Results were expressed as relative lights units (RLU) per mg of cell protein

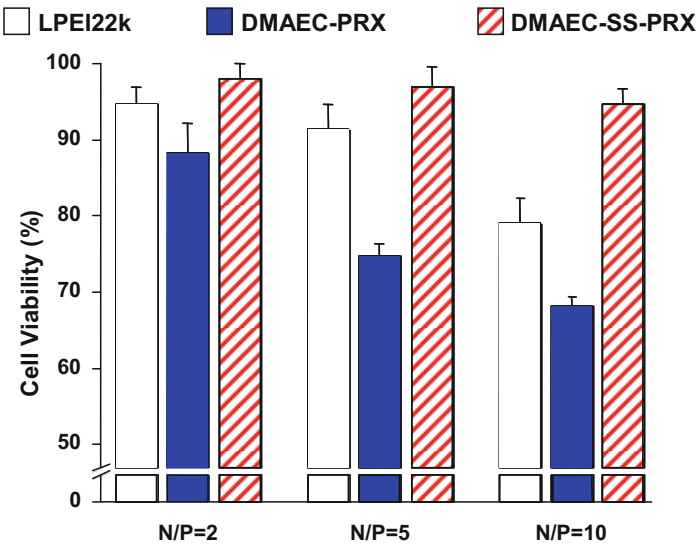

Fig. 13 Cells viability of the DMAEC-SS-polyrotaxanes, DMAEC-polyrotaxanes and LPEI22k at different N/P ratios measured via the MTT assay

The finding as observed above is likely due to the mobile motion of α-CDs in the necklace-like structure of the polyrotaxane. The pDNA dissociation of the polyplex occurred through the SS cleavage in the polyrotaxane and the subsequent interexchange with polyanions. This is presumably due to a reduction on the potency of the multivalent interaction between the cationic polyrotaxane and the anionic pDNA through the supramolecular dissociation. A rapid endosomal escape and pDNA delivery to the nucleus using such cytocleavable polyrotaxanes can be achieved through systematic analyses of polyrotaxane structures.

From this point of view, we further investigated pDNA transfection as well as polyion complexation with pDNA using a variety of DMAEC-SS-PRXs with different numbers of DMAEC groups (the number of α-CDs in the polyrotaxane was fixed to be 18) (Fig. 14) [35]. Positively charged polyplex with pDNA was observed for all the DMAEC-SS-PRXs when the N/P ratio was over 1, although the LPEI22k polyplex showed still negative value. This finding seems to be one of the unique characteristics of the polyrotaxanes related to their structures (Fig. 15). These data suggest that the DMAEC-SS-PRXs can form a polyplex with pDNA in small quantity of cationic groups. Therefore, it is likely that the driving force for complexation of the DMAEC-SS-PRX with the pDNA should be not only an electrostatic interaction but also any other factors.

The ethidium bromide assay revealed that the compaction of pDNA was significantly influenced by the number of DMAEC groups in DMAEC-SS-PRXs: a larger number of DMAEC groups in the DMAEC-SS-PRXs resulted in more tightly packed polyplex formation (Fig. 16). However, the highest transfection of pDNA was observed for a DMAEC-SS-PRX at appropriate number of DMAEC groups

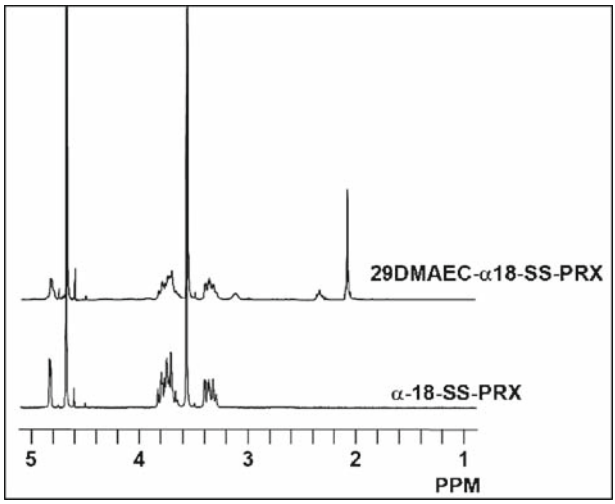

Fig. 14 NMR spectrums of α 18-SS-polyrotaxane and 29DMAEC-α 18-SS-polyrotaxane in $NaOD/D_2O$

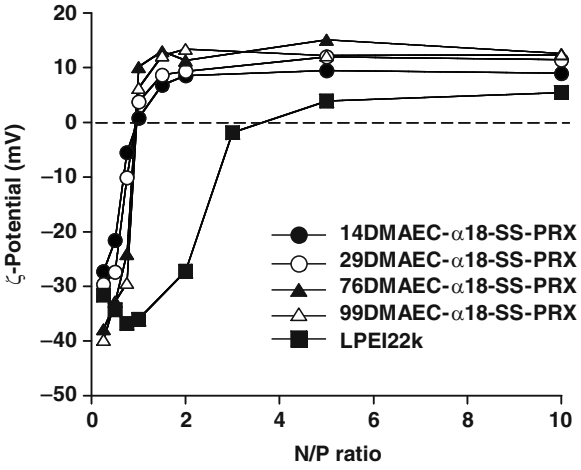

Fig. 15 ζ-Potential of the pDNA polyplexes with DMAEC-α 18-SS-polyrotaxanes for various N/P ratios

(Fig. 17). In particular, from the CLSM analysis it was observed that the DMAEC-SS-PRX with the lowest number of DMAEC groups exhibited a much faster pDNA release in cytoplasm. These results strongly suggest the importance of timing for DNA release under a reducible condition as well as of the stability of the pDNA polyplex before lysosomal and/or endosomal escape in the course of DNA delivery into the nucleus. Finally, we conclude that both polyplex stability and intracellular pDNA release for transfection were achieved by optimizing the number of DMAEC groups in the DMAEC-SS-PRXs [35].

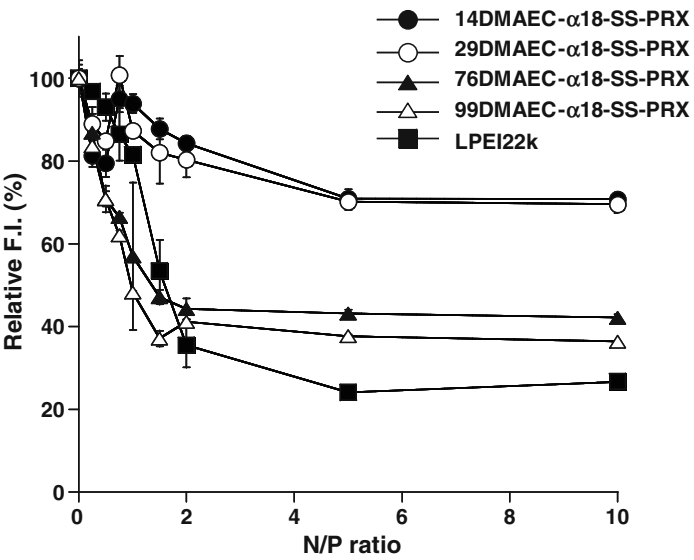

Fig. 16 Ethidium bromide displacement assay of the DMAEC-α 18-SS-polyrotaxanes polyplex and the LPEI22k polyplex. The recorded fluorescent intensities (F.I.) were expressed relative to the fluorescence intensity of the DNA–EtBr solution in the absence of polycation, after subtracting the fluorescence of EtBr in the absence of DNA under the same buffer conditions

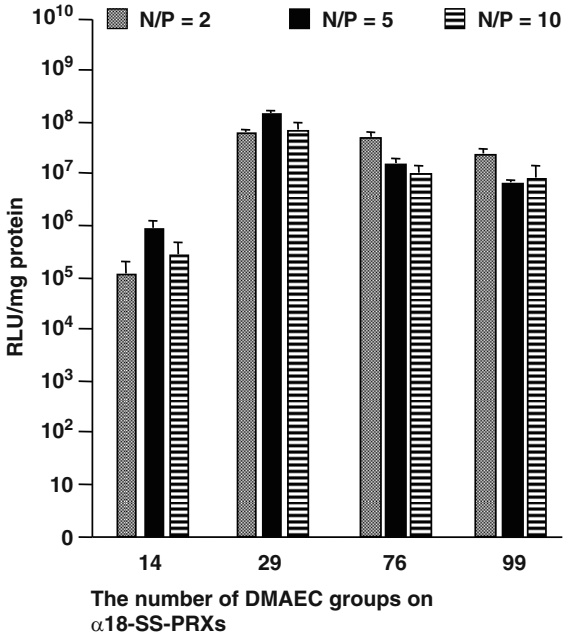

Fig. 17 Transfection activities of the DMAEC-α 18-SS-polyrotaxanes. Luciferase activity in the NIH/3T3 cells was measured 48 h after the addition of polyplexes. Results were expressed as relative light units (RLU) per mg of cell protein

6 Future Aspects

Further studies on the design of polyrotaxanes exploit not only a far-reaching technology of nonviral gene delivery, but also a variety of practical applications for beneficial biomedical devices and tools. Controlling the mobility of cyclic compounds threaded onto a linear polymeric chain and the interlocked structure seen in polyrotaxanes will convincingly lead to a paradigm shift in biomaterials design, and such a supramolecular approach becomes more important not only in biomaterials but also in any other functional materials.

Stimuli-responsive control of the mobility of cyclic compounds along a polymeric chain is distinctive as one of our future works on supramolecular biomaterials. We have already demonstrated the pH-responsive control of the α-CD mobility along a PEI–PEG–PEI triblock-copolymer capped with bulky end-groups [36–38]. The majority of α-CDs were found to be located at the center-block (PEG) in the copolymer in acidic conditions, although they were freely mobile along the copolymer in basic environment. Not only the location but also the mobility of α-CDs could be controlled by a pH change within a physiological range of pH 5–8. Such a control of the mobility of ligands along the polymeric chain can lead to either modulate the *K* value in the interaction with receptor proteins, or to control the fluidity (clustering) of receptor proteins on plasma membranes. This method can exploit the extracellular modulation of cytoplasmic metabolism at target cells and/or into tissues.

Another important aspect of the design of polyrotaxanes as biomaterials will be to create two- or three-dimensional architectures for cellular and tissue modulation. Regarding this issue, we are now preparing polyrotaxane surfaces which exhibit the mobility of CDs along a linear polymeric chain fixed both terminals to a solid substrate such as Si and/or Au. We strongly expect this unique surface to enhance specific binding with certain proteins and cells through a multivalent ligand–receptor interaction as well as to prevent nonspecific interaction with

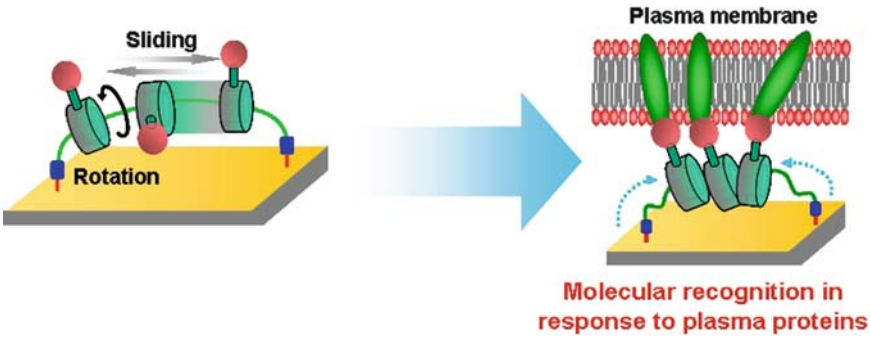

Fig. 18 Modulation of cellular metabolism and tissues via a multivalent binding of plasma membrane proteins with ligand conjugated polyrotaxanes immobilized on solid substrates at the terminal groups

biological molecules such as proteins, as illustrated in Fig. 18. In such a case, any reactive groups toward the Au surface, such as thiol, are introduced into the end-capping molecule of Z-Tyr, prior to the capping reaction of the pseudopolyrotaxanes.

7 Conclusions

Our recent studies on polyrotaxanes as biomaterials have been summarized in this chapter. The mobility of cyclic compounds along a linear polymeric chain is one of the fascinating characteristics seen in polyrotaxanes, and we have clarified that a modulating multivalent interaction with biological systems is strongly related to this unique feature. Another important issue regarding polyrotaxanes is the easiness in dissociating the supramolecular structures into pieces when cleaving one of the bulky end-groups at the terminals. This allows us to modulate the binding constant with biological systems, and is actually useful when designing cytocleavable polyrotaxanes as nonviral DNA vectors.

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