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Threaded macromolecules as a versatile framework for biomaterials

Atsushi Tamura[1], and Nobuhiko Yui[1,*]

[1]Department of Organic Biomaterials, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda, Tokyo 101-0062, Japan.

*Corresponding author. Prof. Nobuhiko Yui (Phone: +81-3-5280-8020, Fax: +81-3-5280-8027, E-mail: yui.org@tmd.ac.jp)

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polyrotaxane, pseudopolyrotaxane, cyclodextrin, supermolecule, hydrogel, nanoparticle, drug delivery system, gene delivery

Abstract

Polyrotaxanes (PRXs) are a class of supramolecular threaded macromolecules, in which cyclic molecules are threaded onto the main- or side-chain of polymers. To date, various studies have been conducted on the synthesis of PRXs, and various combinations of cyclic molecules and polymers that can form a PRX have been discovered. Among these combination, PRXs composed of cyclodextrins (CDs) and a linear polymer have attracted much attention and have been investigated by many researchers. Because of the non-covalently associated characteristic of PRXs, these supermolecules exhibit unique properties, such as the dynamic motion of the threaded cyclic molecules along a polymer axle and complete dissociation of the supramolecular structure, that are never observed in other synthetic polymers. These inherent properties of PRXs are of interest in the design of novel biomaterials, such as hydrogels, scaffolds in tissue engineering, drug delivery carriers, and polymer-drug conjugates. Thus, various studies have been conducted to utilize PRXs as a framework for biomaterials. In this review, we describe the recent progress in biomaterial application of PRXs such as drug delivery and gene delivery.

1. Introduction

The biomedical application of polymeric materials is recognized as an emerging topic of interest.[1,2] The molecular architecture of polymers is one of the main factors affecting the properties of polymeric biomaterials. Various polymers including synthetic polymers, biopolymers, hyper-branched polymers, and dendritic polymers have been used in tissue engineering, regenerative medicine, and drug delivery systems. Polyrotaxanes (PRXs) are a class of supramolecular threaded macromolecules, in which cyclic molecules are threaded onto the main- or side-chain of polymers.[3-8] Among these supermolecules, PRXs composed of many cyclic molecules threaded onto a polymer axle have extensively been investigated (**Figure 1**). The synthesis of PRXs composed of

α-cyclodextrins (α-CDs) and poly(ethylene glycol) (PEG) was first reported by Harada and co-workers.[9-11] Upon mixing of the two components in aqueous solution, the α-CDs are threaded onto the PEG chain via intermolecular interaction. The resultant complexes are called inclusion complexes or pseudopolyrotaxanes. The pseudopolyrotaxanes are assembled onto each other via an intermolecular interaction and are finally precipitated from the solution. To inhibit the dethreading of the cyclic molecules, both terminals of the linear polymer are capped with bulky stopper molecules, and the resultant interlocked polymers are called PRXs. Cyclic molecules and a linear polymer can combine in a complementary fit, and that inclusion complex formation is strongly related to the cross-sectional area of the polymer chain and the cavity size of the cyclic molecules.[3-6] Various polymers that form a pseudopolyrotaxane with α-, β-, or γ-CDs have been discovered, including stereoregular polymers, cationic polymers, and anionic polymers.[3-23] Additionally, various cyclic molecules have been observed to thread onto a polymer chain, including CDs, crown ethers, calixarenes, pillararenes, and other cyclic compounds.[24-29]

Because of the unique necklace-like structure of PRXs, it is expected that PRXs could perform new functions in current biomaterials. For example, PRXs are non-covalently assembled between a linear polymer and many cyclic molecules; thus, it is envisaged that the threaded cyclic compounds are freely mobile along the polymer axle.[30-32] This mobile nature of PRXs allows more multivalent interactions with receptor proteins if biological ligands are incorporated into the cyclic molecules.[31] This type of dynamic motion of threading molecules along a polymer axle is an intrinsic property that is observed only in the PRX structure. Additionally, taking advantage of the non-covalent association of PRXs, biocleavable PRXs bearing cleavable linkages between the polymer terminals and bulky stoppers can be synthesized to cause stimulus-responsive dissociation of PRXs.[33-35] This type of PRXs is regarded as a new class of biodegradable polymers and could be applied for the controlled release of drugs. Note that PRX-based biodegradable polymers can be completely degraded in physiological conditions because the cleavage of a single cleavable linker elicits the

dissociation of the entire supramolecular structure; in contrast, conventional biodegradable polymers degrade incompletely via hydrolysis. These unique characteristics of PRXs that are derived from their supramolecular structures are of special interest for designing novel biomaterials. In this review, we describe the recent progress of PRXs and pseudopolyrotaxanes as supramolecular biomaterials, with a particular focus on the CD-based PRXs.

2. Polyrotaxane hydrogels

2.1. Chemically cross-linked polyrotaxane hydrogels

Biocompatible and biodegradable polymeric materials have been widely used as implantable materials to modulate the release of drugs and as scaffolds for cell implantation.[36] In particular, aliphatic polyesters, such as poly(L-lactide) and poly(lactide-*co*-glycolide), are utilized as biodegradable materials.[35] However, the hydrolytic degradation of polyesters under *in vivo* conditions is not ideal, because the crystalline oligomers and unhydrolyzed polymers remain at implantation sites for a long time and thus elicit a chronic inflammatory response. Chemically cross-linked hydrogels have attracted much interest as alternative implantable biomaterials.[38] These chemically cross-linked hydrogels can incorporate various therapeutic payloads, such as macromolecular drugs and proteins, within the gel network. Additionally, biocompatible hydrogels can be transplanted into the body and act as an implantable drug reservoir under *in vivo* conditions. To date, various methods for constructing chemically cross-linked hydrogels have been developed, such as the polymerization of monomers and appropriate cross-linkers and the chemical cross-linking of hydrophilic polymers.[38,39]

Our group has reported biodegradable supramolecular hydrogels using hydrolyzable PEG/α-CD-based PRX-bearing ester linkages as a framework for a gel network.[40] Because the hydrolysis of any of the ester groups at either of the two terminals of PEG triggers the complete dissociation of a PEG/α-CD complex, these PRX-based hydrogels are anticipated to perfectly and

rapidly disappear in physiological conditions at the designated time. PRX-based hydrogels are obtained by the covalent cross-linking of α-CD moieties on the PRXs (**Figure 2A**). Briefly, the α-CD moieties on the hydrolyzable PRXs are first activated with *N*,*N*'-carbonyldiimidazole, followed by the cross-linking reaction with bisamino-PEG in organic media to obtain PRX gels. Because the hydrolyzable PRX-based hydrogels are composed of PEG and α-CDs, both of which are biocompatible and approved by the US Food and Drug Administration (FDA), the constituents of the hydrogels are essentially biocompatible. Additionally, the degradation kinetics of PEG-PRX hydrogels can be regulated by varying the molecular weight of the bisamino-PEG as a cross-linker and the content of hydrolyzable PRXs in the hydrogels.[41,42] Therefore, the application of PRX-based hydrogels as biocompatible and biodegradable drug carriers or scaffolds for tissue engineering has been suggested.

Alternatively, Ito and co-workers have developed supramolecular slide-ring hydrogels based on PRXs, in which threaded α-CDs are directly conjugated to each other to provide figure-of-eight cross-linking points in the three-dimensional network.[43,44] Although the cross-linking points of the hydrogels are typically fixed within the gel network, the figure-of-eight cross-linking points in the slide-ring hydrogels can freely slide along the polymer axles. Because of the existence of movable cross-linking points, the slide-ring hydrogel exhibits unique physical properties, such as high water absorption, stretchability, and a low Young's modulus.[44] This unique nature of PRXs in the slide-ring hydrogel can be applied to other hydrogels. Briefly, acryloyl groups-modified PRXs can be used as a cross-linker of poly(*N*-isopropylacrylamide) (PIPAAm) hydrogels, in which PRX acts as a movable cross-linker.[45,46] PIPAAm hydrogels cross-linked with PRXs also have grater softness, flexibility, and mechanical stability than conventional PIPAAm hydrogels. Thus, PRX-based cross-linking is a versatile method for modulating the mechanical properties of various hydrogels.

2.2. Physical gelation via the self-assembly of polyrotaxanes

Another method for preparing hydrogels is the physical gelation caused by non-covalent self-assembly, such as the assembly caused by hydrophobic interactions, hydrogen bonding, and electrostatic interactions.[39] During studies on the inclusion complexation between CD and linear polymers, Harada and co-workers first reported the unique sol-gel transition behavior of inclusion complexes.[47] In the formation of an inclusion complex between PEG and α-CD in aqueous media, the complexes exhibited physical gelation. This gelation is thought to occur with the intramolecular hydrogen bonding between α-CDs on the pseudopolyrotaxanes (**Figure 2B**). The time of gelation is dependent on the concentration of both PEG and α-CD in aqueous media and the molecular weight of PEG. In particular, when the concentration of PEG and α-CD is sufficiently high, the mixture exhibits rapid gelation. Because of the lack of a covalent cross-linking point and the rapid gelation of the pseudopolyrotaxane hydrogels, these molecules could be applied as injectable hydrogels.

These PEG/CD hydrogels allow physical entrapment of bioactive molecules, such as proteins.[48] For example, Arima and co-workers have demonstrated the preparation of insulin-loaded PEG/γ-CD hydrogels, in which the three-dimensional structure of insulin is maintained.[49] Li and co-workers have studied the release kinetics of fluorescein isothiocyanate-labeled dextran (FITC-dextran) from PEG/α-CD hydrogels.[50] The release rate decreases as the molecular-weight of PEG increases. This decrease is presumably due to the chain entanglement effect of the high molecular weight PEG. The hydrogels prepared from PEG with a molecular weight of 35,000 or 100,000 exhibit sustained release profiles, and almost all of the FITC-dextran is released from these hydrogels within 120 h. Although the release kinetics are regulated by varying the molecular weight of PEG, they appear to be relatively fast. Further molecular design of the supramolecular hydrogels is required to achieve long-term drug release under *in vivo* conditions.

Based on the self-assembly of α-CD-threaded polymers, various supramolecular hydrogel systems have been developed.[48] To achieve long-term drug release from supramolecular hydrogels, Li and co-workers have utilized amphiphilic triblock copolymers,

PEG-*b*-poly[(*R*)-3-hydroxybutyrate]-*b*-PEG (PEG-*b*-PHB-*b*-PEG), as an axle polymer to induce gelation with α-CDs.[51] The cooperation effect of hydrogen bonding between α-CD-threaded PEG segments (PRX segments) and the hydrophobic interaction between PHB segments resulted in the formation of supramolecular hydrogels with a stable macromolecular network. The *in vitro* release kinetics studies of FITC-Dex from the hydrogel revealed that the PEG-*b*-PHB-*b*-PEG/α-CD hydrogels achieved long-term sustained controlled release of macromolecular drugs over 26 days. Application of the hydrogel as a promising injectable drug delivery system has been suggested. Similarly, the use of other amphiphilic diblock or triblock copolymers, such as PEG-*b*-poly(ε-caprolactone), PEG-*b*-poly(propylene glycol) (PPG)-*b*-PEG, and PCL-*b*-PEG-*b*-PCL, as axle polymers for preparing supramolecular hydrogels with CDs has also been demonstrated.[52,53] Zhang and co-workers have encapsulated lysozymes in supramolecular hydrogels composed of PEG-*b*-PCL diblock copolymers and α-CDs.[54] The enzymatic activity of the lysozymes encapsulated in the supramolecular PEG-*b*-PCL/α-CD gel network was maintained for 14 days, whereas the activity of native lysozymes significantly decreased during 7 days of incubation. Accordingly, PRX-based supramolecular hydrogels are thought to be an excellent candidate for protein delivery.

To utilize such an inclusion complex as reversible crosslinks in stimulus-responsive hydrogel systems, our group has developed supramolecular hydrogels using PEG-grafted dextran (PEG-*g*-Dex) as an axle polymer for α-CDs.[55] α-CDs are threaded onto the PEG-tethered chain of PEG-*g*-Dex to induce gelation. The gelation time for PEG-g-Dex/α-CD is typically short compared with that of the PEG/α-CD system. In addition, PEG-*g*-Dex/α-CD hydrogels exhibit a unique temperature-dependent gel-sol transition based on supramolecular assembly and disassembly. Furthermore, our group has developed stimulus-responsive supramolecular pseudopolyrotaxane hydrogels, whose rheological properties can be modulated in response to pH changes.[56] Herein, poly(ethylenimine) (PEI)-*b*-PEG-*b*-PEI triblock copolymers form an inclusion complex with α- or γ-CDs under alkaline pH condition,[57] and the α-CD mobility along the PEI-*b*-PEG-*b*-PEI chain

capped with bulky end groups can be controlled by varying the pH.[58] Thus, PEG-*b*-PEI diblock copolymer-grafted dextran (PEG-PEI-Dex) has been designed to demonstrate the gelation with α- or γ-CDs. The addition of γ-CD to a PEG-PEI-Dex solution at pH 10 induced a significant increase in the viscoelastic properties. Furthermore, the viscoelastic properties of PEG-PEI-Dex/γ-CD changed with the pH. The PEG-PEI-Dex/γ-CD contains the full double-stranded inclusion complex along both the PEG and PEI segments of the grafted chains at pH 10. In contrast, the networks exhibit a loose nature at pH 4, presumably due to the formation of a partial double-stranded inclusion only on the PEG segments of the grafted chains. Therefore, the macroscopic rheological changes in PEG-PEI-Dex/γ-CD hydrogels result from molecular mechanical actuations of the double-stranded inclusion system in supramolecular networks. This system has a latent possibility for constructing contractile muscle systems.

2.3. Physical gelation between cyclodextrins and PEGylated proteins

PEG-modified proteins are currently regarded as essential proteins for pharmaceuticals.[59] PEG modification, often called PEGylation, of a protein provides high solubility in aqueous media, stability in physiological conditions, and a prolonged half-life in the bloodstream. To further expand the utility of the PEGylated protein pharmaceuticals, Uekama and co-workers have developed supramolecular hydrogels between PEGylated insulin and CDs.[60-62] These researchrs have demonstrated supramolecular gelation between PEGylated insulin and various CDs; α- and γ-CDs formed hydrogels with PEGylated insulin, whereas no gelation was observed with β-CD. The PEGylated insulin molecules were released from the hydrogels through the dissociation of the PEG/CD inclusion complexes. The release rate could be modulated by the concentration of hydrogels in the medium and the number of PEG chains on the insulin.[61,62] Additionally, these researchers subcutaneously administered the PEGylated insulin/CD hydrogels to rats.[60-62] When insulin or PEGylated insulin was subcutaneously administered, the time required to reach the

maximum level of insulin in the plasma was determined to be 0.25 or 0.42 h, and the plasma insulin level decreased to the basal level within 2 or 4 h, respectively. In contrast, the PEGylated insulin/CD hydrogels significantly maintained the plasma insulin level, and the insulin level decreased to the basal level after more than 6 h. Additionally, the PEGylated insulin/CD hydrogels exhibited a significantly prolonged reduction in the plasma glucose level comparison with that for insulin and PEGylated insulin. These results indicate that using PRX-based supramolecular hydrogels is a promising strategy to modulate the body disposition and to enhance the therapeutic efficacy of PEGylated proteins.

This supramolecular injectable hydrogel technique can be applied to other PEGylated proteins and to other PEGylated materials. PEGylated lysozyme and PEGylated and α-CD-conjugated poly(amidoamine) dendrimer (generation of dendrimer: G2) have also been observed to form supramolecular hydrogels upon addition of the α- or γ-CDs.[63,64] In these hydrogels, the supramolecular hydrogel formulations achieve sustained release of the payloads through the dissociation of pseudopolyrotaxanes. Sedlák and co-workers have reported PEG/α-CD hydrogels in which the terminal of the PEG chains is capped with low-molecular-weight drugs (prednisolone) via acid-labile hydrazone linkages.[65] The prednisolone is released from the hydrogels by the cleavage of the hydrazone linkages under the acidic conditions. Accordingly, the supramolecular hydrogel system based on the self-assembly of pseudopolyrotaxanes is a versatile method for delivering various PEGylated materials and drugs.

3. Nanoassemblies of polyrotaxanes

3.1. Self-assembled nanoparticle formation of polyrotaxanes.

Nanoparticle formulations are regarded as a promising modality in the current drug delivery system (DDS).[66,67] In particular, self-assembled nanoparticles, such as polymeric micelles and liposomes, have attracted much attention as drug carriers.[68,69] Polymeric micelles are typically constructed from

the self-assembly of amphiphilic diblock copolymers composed of a hydrophilic segment and a hydrophobic segment. The hydrophobic segments in the diblock copolymers self-assemble in aqueous solution to form a hydrophobic core that is segregated from the hydrophilic segments that form the surrounding polymer layer. Hydrophobic drugs can be encapsulated in the core of polymeric micelles. The hydrophilic outermost layers of the polymeric micelles contribute to a reduction in the non-specific uptake by the reticuloendothelial system (RES), such as Kupffer cells of the liver and the macrophages of the spleen, resulting in the prolongation of the blood circulation time compared with that of free drugs. It should be noted that nanometer-scale long-circulating macromolecules and particles preferably accumulate in tumor tissue because of the vascular hyperpermeability of the tumor tissue and the impairment of lymphatic drainage.[70-72] This effect is called an enhanced permeability and retention (EPR) effect and is regarded as a general concept for the passive targeting of drugs to tumor tissues. To date, various polymeric micelles and other nanoparticles have been developed to achieve successful cancer therapy, and various molecular interactions have been utilized to construct nanoparticles and encapsulate various drugs, such as hydrophobic interactions, coordination, electrostatic interactions, hydrogen bonding, and host-guest interactions.[73-78]

The intermolecular interaction between PRXs has also been used to form self-assembled nanoparticles. A promising method of constructing polymeric micelles through the self-assembly of PRXs involves PRX-containing ABA-type triblock copolymers that comprise a central PRX segment and hydrophilic polymer chains, and various PRX-containing triblock copolymers have been developed (**Figure 3A**).[79-83] For example, Feng and coworkers have synthesized PRX-based triblock copolymers bearing hydrophilic polymers, such as poly(*N*-hydroxypropylmethacrylamide) (PHPMA), poly(ethylene glycol) methyl ether methacrylate (PEGMA), and temperature-responsive PIPAAm.[79-82] The central PRX segments self-assemble in aqueous solution to form a segregated core of nanoparticles, and the hydrophilic polymer segments form the outermost layer to provide

dispersion stability to the nanoparticles. The supramolecular triblock copolymers composed of Pluronic F127/β-CD PRX as a central block and hydrophilic PPEGMA chains (PPEGMA-*b*-PRX-*b*-PPEGMA) form core-shell-type spherical polymeric micelles in aqueous solution.[81] The hydrodynamic diameter of the PPEGMA-*b*-PRX-*b*-PPEGMA polymeric micelles ranges from 58 to 188 nm and depends on the number of threading β-CDs in the PRX segments. Additionally, the PPEGMA-*b*-PRX-*b*-PPEGMA micelles can incorporate amphotericin B in the core of the micelles to reduce the strong hemolytic activity of amphotericin B. Caruso and co-workers developed biodegradable supramolecular polymeric micelles.[83] The authors synthesized PEG/α-CD-based PRX with alkyne-containing bulky end caps via reducible disulfide linkages. Then, azido-terminated PEGs were modified at the terminal end of PRXs via Huisgen cycloaddition (PEG-*b*-SS-PRX-SS-*b*-PEG) to form polymeric micelles in aqueous solution. These polymeric micelles degrade upon the addition of reductive molecules, such as glutathione (GSH) and D,L-dithiothreitol (DTT), through the reductive cleavage of disulfide linkages in PEG-*b*-SS-PRX-SS-*b*-PEG.

In contrast to these PRX-based polymeric micelles self-assembled from the intermolecular interaction of PRX segments, our group has developed PRX-based triblock copolymers bearing hydrophobic polymer chains to form supramolecular polymeric micelles through the hydrophobic interaction of the polymers (**Figure 3B**).[84,85] These polymeric micelles display an outermost layer of movable PRX, which is expected to contribute to enhancing the multivalent interaction with receptor proteins if any biological ligands are incorporated into the CDs.[31,34] The supramolecular flower-like micelles have the appealing property of being able to incorporate hydrophobic drugs within the core of the micelles as well as being able to utilize the freely mobile PRX segments as a ligand installation moiety. In this regard, triblock copolymers that comprise PEG/α-CD PRX as a central block and hydrophobic poly(benzyl methacrylate) chains (PBzMA-*b*-PRX-*b*-PBzMA) have been designed to form supramolecular polymeric micelles with an outermost layer of PRX loops.[84] ^{1}H

NMR and transmission electron microscopic (TEM) studies revealed that PBzMA-*b*-PRX-*b*-PBzMA formed stable polymeric micelles with outermost PRX layers that were 15.3 nm in diameter. The hydrophobic core of the micelles allows the incorporation of hydrophobic anticancer drugs and can load 8.8 wt% of paclitaxel. The paclitaxel-loaded PBzMA-*b*-PRX-*b*-PBzMA polymeric micelles exhibited an anticancer effect against HeLa cells. Additionally, PLA-*b*-PRX-*b*-PLA has been observed to form supramolecular flower-like micelles.[85] The outermost PRX layer of these polymeric flower-like micelles has the potential to enhance target recognition via the installation of ligand molecules and could be applicable for the active targeting of the drugs.

Among the various PRX-based nano-assemblies, the morphological transition of the nanoparticles is observed. For example, the triblock copolymers composed of PEG/α-CD PRX as a central block and hydrophilic poly[(*N*,*N*-dimethylaminoethyl)methacrylate] chains (PDMAEMA-*b*-PRX-*b*-PDMAEMA) also form spherical micelles in aqueous solution with a diameter of 100 to 150 nm.[86] These polymeric micelles undergo a morphological change to form a bundle-like aggregate when the pH decreases to 2. A similar morphological change of supramolecular assemblies is also observed in pseudopolyrotaxanes between α-CDs and PEG-*b*-poly(acrylic acid) (PEG-*b*-PAA).[87] The α-CDs thread onto PEG segments of PEG-*b*-PAA in the first stage to form rod-like PRX aggregates, and then evolve into spherical assemblies after 7 days.

3.2. Polyrotaxane-based hollow nanoparticles

Hollow nanoparticles have attracted much attention as a new class of colloidal nanoparticles that has potential for application in drug delivery systems.[88] One method for constructing hollow nanoparticles such as polymersomes, is the self-assembly of well-defined diblock copolymers or surfactants through hydrophobic interactions and hydrogen bonding. The self-assembly structure of amphiphilic diblock copolymers can vary from spherical micelles to cylindrical micelle to

polymersomes, and these superstructures are theoretically governed by a packing parameter of amphiphilic diblock copolymers.[89,90] Typically, amphiphilic diblock copolymers with short hydrophilic segments (a packing parameter between 1/2 to 1) form polymersomes.

The host-guest inclusion complexation of cyclic molecules has also been employed to construct self-assembled hollow particles.[91] Jiang and co-workers reported on the preparation of supramolecular hollow particles formed from the host-guest inclusion complexation of β-CD-bearing polymers and adamantine-bearing polymers, in which β-CD and adamantine in the side-chain of each polymer form multivalent host-guest complexes.[92] Huang and co-workers successfully prepared various supramolecular particles, including hollow particles, through the self-assembly of diblock copolymers composed of a supramolecular polymer and a traditional hydrophilic polymer.[78] In this study, the supramolecular polymer segments were composed of a host-guest interaction of crown ethers and guest molecules to induce the self-assembly of diblock copolymers in aqueous solution. Wang and co-workers developed PRX-based hollow nanoparticles composed of Pluronic F127 (PEG-*b*-PPG-*b*-PEG) and α-CD via the hydrothermal treatment of the inclusion complex.[93] TEM observations clearly demonstrate the formation of hollow nanoparticles. The diameters and wall thicknesses of the hollow nanoparticles can be varied via the ratio of α-CD to F127.

Another method for constructing hollow nanoparticles is the template method. In this method, the polymers are deposited around template nanoparticles, followed by removal of the template core to obtain hollow nanoparticles. Li and co-workers reported on the preparation of PRX-based hollow nanoparticles using gold nanoparticles as a template.[94] These researchers layered α-CDs on the PEG-modified gold nanoparticles through the formation of an inclusion complex, and then the threaded α-CDs were cross-linked. Finally, the template gold nanoparticles were removed by cyan etching to obtain PRX-based hollow nanoparticles. Similarly, Caruso and co-workers prepared PRX-based degradable hollow nanoparticles on template silica nanoparticles.[95] PEG/α-CD PRX terminated with alkyne stoppers via disulfide linkages were grafted to azide-functionalized silica

particles (2.76 μm in diameter) using azide-alkyne click chemistry. Then, the PRXs modified on the surface of the silica particles were cross-linked with a degradable linker (cystamine). Finally, the silica particles were dissolved by hydrofluoric acid (HF) treatment to obtain a robust cross-linked hollow particle. The anticancer drug doxorubicin (DOX) was conjugated to the threaded α-CDs in PRX-based hollow particles. The conjugated DOX was released by the addition of GSH, which degraded the hollow particles via the cleavage of disulfide linkages in the PRXs and cross-linkers. The researchers also developed a PRX-based hollow particle using the layer-by-layer assembly of PRXs.[96] The PRX hollow particles were prepared by the sequential deposition of positively-charged and negatively-charged PRXs onto silica particles.[96,97] Then, the template silica particles were dissolved by HF treatment to obtain polyelectrolyte hollow particles. These PRX hollow particles are of interest for a broad range of applications.

3.3. Polyelectrolyte complex formation of polyrotaxanes with nucleic acids

In molecular biology and cell biology research, plasmid DNA (pDNA) and small interfering RNA (siRNA) are currently used in essential methods for investigating specific gene functions. These technologies are also regarded as a novel therapeutic approach through the expression of deficient genes or the down-regulation of pathogenic genes.[98-100] In this regard, numerous pDNA and siRNA carriers based on polymers, liposomes, and other nanoparticles have been developed. Most of these carriers are based on polyelectrolyte complexes, which are self-assembled complexes formed from negatively charged nucleic acids and positively charged polymers, liposomes, or polypeptides through electrostatic interaction. Among the various cationic polymers, PEI, poly(L-lysine) (PLL), and PAMAM dendrimers have been employed as the cationic component for the delivery of pDNA and siRNA.[101-103]

There are various biological barriers involved in the intracellular delivery of pDNA and siRNA, such as cellular internalization, escape from the endosome to reach the cytosol, nuclear localization,

and release from complexes.[104,105] To overcome these obstacles, various cationic polymers that facilitates cellular internalization and endosomal escape have been extensively studied. Our group has focused on improving the release process from polyelectrolyte complexes using biocleavable PRXs. In this regard, *N*,*N*-dimethylamino (DMAE) group-modified PEG/α-CD PRXs bearing disulfide linkages (DMAE-SS-PRX) have been developed to form an polyelectrolyte complex with pDNA (**Figure 4**).[106-108] Although disulfide linkages are sufficiently stable in the extracellular environment, the selective cleavage of disulfide linkages occurs due to the thiol-disulfide exchange reaction with intracellular reductive molecules such as glutathione tripeptide (GSH).[109,110] Because the intracellular concentration of GSH (0.5 to 10 mM) is known to be 3 to 4 orders of magnitude higher than that in the extracellular environment and because most of the intracellular GSH is stored in the cytoplasm,[111] it is anticipated that the reduction-induced dissociation of PRX may be achieved under intracellular conditions. Although various reduction-sensitive polycations bearing disulfide linkages at the main or side polymer chains have been developed,[109,110] the cleavage of multiple disulfide linkage sites is required for the complete degradation and dissociation of polyelectrolyte complexes. In contrast, biocleavable DMAE-SS-PRXs have an appealing property among the disulfide-bearing polycations because their supramolecular structure can be efficiently dissociated by cleaving only a single site in the terminal disulfide linkages.

To validate the contribution of the supramolecular dissociation of DMAE-SS-PRXs to gene expression, *in vitro* luciferase gene expression using DAME-SS-PRX/pDNA complexes has been compared with that using linear PEI/pDNA and non-cleavable DMAE-PRX/pDNA.[106] The gene expression efficiency of the DMAE-SS-PRX/pDNA complex was observed to be independent of the N/P ratio (mixing ratio of amino groups in DMAE-SS-PRX to phosphate groups in pDNA), although that of the PEI/pDNA increased with the N/P ratio. These results suggest that the gene expression of the DMAE-SS-PRX is independent of the amount of free polycations. It is evident that the intracellular cleavage of disulfide linkages in DMAE-SS-PRXs plays a pivotal role in enhancing

gene expression because the gene expression efficiency of the DMAE-SS-PRX complex was much higher than that of the non-cleavable DMAE-PRX. Additionally, the DMAE-SS-PRX/pDNA exhibited no cytotoxicity regardless of the N/P ratio, whereas the PEI/pDNA exhibited definite cytotoxicity upon increasing the N/P ratio. This result indicates that the supramolecular dissociation of DMAE-SS-PRX can contribute to eliminating the cytotoxicity usually observed with high-molecular-weight polycations.

Taking advantage of the intracellular dissociation of DMAE-SS-PRX/pDNA complexes, lipid-enveloped nanoparticles containing DMAE-SS-PRX/pDNA complexes have been developed in our group in collaboration with Harashima and his co-workers.[112-114] The surface modification of lipid-enveloped nanoparticles with a cell-penetrating peptide, the octaarginine peptide, results in higher transfection activity than that of reported viral vectors.[115,116] The lipid-enveloped nanoparticles containing DMAE-SS-PRX/pDNA exhibit higher gene expression than those containing conventional polycation/pDNA, suggesting that the intracellular release efficiency plays a key role in transgene expression.[117] DMAE-SS-PRXs are appropriate model polycations for investigating the relationship between the pDNA release in the nucleus and the transfection activity because the cationic density in a DMAE-SS-PRX-molecule can be easily controlled, thus permitting the efficiency of DNA release to be adjusted. The lipid-enveloped nanoparticles encapsulating the complex of pDNA and DMAE-SS-PRXs with various numbers of threading α-CDs and DMAE groups were prepared, and the relationship between the pDNA release efficiency and transgene expression efficiency was investigated. The results indicated that the gene expression efficiency increased with efficient pDNA release from the complexes. A high efficiency of pDNA release resulted in a positive effect on transcription. However, the gene expression efficiency decreased, when the value for the efficiency of pDNA release was higher than a certain value. It has been suggested that an excess of polycations might inhibit post-transcriptional processes, such as nuclear mRNA export, translation, and related processes. These results provide important insight into the

development of polycation-based pDNA carriers. In addition to the biocleavable DMAE-SS-PRX, various PRXs or pseudopolyrotaxanes, such as Pluronic/β-CD, PEG/α-CD, PPG/β-CD, poly[(ethylene glycol)-*ran*-(propylene glycol)]/α-CD, ionene/α-CD, and linear PEI/γ-CD, have been studied for pDNA delivery.[118-125]

These cationic PRX-based carrier systems have been utilized in the delivery of siRNA. For example, PEG/α-CD and ionene/α-CD PRXs have been reported to demonstrate the potential ability of cationic PRXs in siRNA delivery.[126-129] Our group has performed a comprehensive study to verify the supramolecular structure–function relationship of biocleavable DMAE-SS-PRXs in siRNA delivery.[130] The binding ability of DMAE-SS-PRXs to siRNA and the stability of DMAE-SS-PRX/siRNA complexes against polyanion exchange are observed to improve when increasing the number of threading CDs in DMAE-SS-PRX. When the number of DMAE groups on DMAE-SS-PRXs increased, the PRXs form tightly associated complexes with siRNA. The intracellular uptake analysis of DMAE-SS-PRX/siRNA complexes by flow cytometry has revealed that the cellular uptake level of siRNA increases remarkably with both the number of threading CDs and DMAE groups on PRX. Note that the DMAE-SS-PRX/siRNA complexes with high numbers of threading CDs exhibited excellent gene silencing activities (down-regulation of the luciferase gene). These results suggest that the rigidity of the PRX structure, especially when increased by high numbers of threading CDs, is responsible for the stable complex formation with siRNA. Additionally, the gene silencing activity of DMAE-SS-PRX/siRNA complexes increases with the number of DMAE groups in PRXs, presumably because of the high cellular uptake of siRNA. For a better understanding of the effect of the number of threading CDs in DMAE-SS-PRXs, PEG5k (M_n = 4,550)/α-CD and PEG10k (M_n = 9,810)/α-CD PRXs with a comparable number of threading α-CDs were synthesized. The PEG5k/α-CD-based DMAE-SS-PRX/siRNA exhibited a higher siRNA intracellular uptake level and gene silencing activity than the PEG10k/α-CD-based DMAE-PRX/siRNA with comparable numbers of threading α-CDs and DMAE groups (**Figure 5A**).

This result strongly suggests that both the high percentage of threading α-CDs and the number of DMAE groups in DMAE-SS-PRX are predominant factors in the efficient intracellular delivery of siRNA. It should be noted that the biocleavable DMAE-SS-PRX/siRNA complexes exhibit superior gene silencing activity to that of non-cleavable DMAE-PRX with the same PEG chain length, number of threading CDs, and number of DMAE groups (**Figure 5B**). This result strongly suggests that the intracellular dissociation of DMAE-SS-PRX/siRNA complexes and the subsequent release of siRNA facilitates the gene silencing activity.

When the terminal cleavable-linkers in PRXs are transformed into acid-degradable ester linkages (DMAE-COO-PRX), the gene silencing activity increases further.[131] In response to the acidic pH in endosomes, the ester linkages of the DMAE-COO-PRXs are hydrolyzed, and an abundance of DMAE-modified CDs is thought to be released from the PRXs. In this situation, the released α-CDs might undergo endosomal membrane destabilization through the removal of phospholipids from the membrane, resulting in the endosomal escape of siRNA and enhanced gene silencing. Indeed, confocal laser scanning microscopic (CLSM) observations have revealed that the colocalization ratio of siRNAs to endosomes decreased for DMAE-COO-PRX compared with that for DMAE-SS-PRX. Thus, the cellular uptake and intracellular trafficking of the PRX/siRNA complexes can be modulated by varying the supramolecular structure and cleavable linkers in PRXs.

3.4. Polyelectrolyte complex formation of polyrotaxanes with proteins

Our group has also applied biocleavable DMAE-SS-PRX to protein delivery.[132] The intracellular delivery of proteins is an essential methodology to extend the therapeutic application of protein therapeutics. Because the biocleavable DMAE-SS-PRXs induce significantly higher gene expression of pDNA or gene silencing efficiency of siRNA than the non-cleavable DMAE-PRX through the degradation of the DMAE-SS-PRXs and the efficient intracellular release of their cargos, it is strongly anticipated that DMAE-SS-PRXs have great potential as vehicles to achieve the efficient

intracellular release of proteins (**Figure 6A**). To verify this hypothesis, anionic β-galactosidase (β-gal) (464.5 kDa, pI = 4.6) was selected as a model enzyme to form a polyelectrolyte complex with the DMAE-SS-PRXs. The polyelectrolyte complex formation between anionic enzymes and DMAE-SS-PRX is clearly confirmed by native agarose gel electrophoresis. However, when β-gal forms a polyelectrolyte complex with DMAE-SS-PRXs, the enzymatic activity of β-gal decreases to approximately 20%, most likely because of the masking of the active site of the enzymes. Upon the addition of reductive molecules (GSH), the PRXs dissociate into their constituent molecules and release the enzymes, resulting in complete recovery of the enzymatic activity.

To investigate the activity of the delivered enzymes in living cells, membrane-permeable TokyoGreen-β-Gal (TG-β-gal) was used as a substrate to monitor the enzymatic activity via the fluorescence intensity.[134] CLSM images of the HeLa cells after incubation with TG-β-gal for 30 min revealed that in contrast to non-cleavable DMAE-PRX/β-gal, the cleavable DMAE-SS-PRX/β-gal exhibits a remarkably bright green fluorescence derived from the enzymatic conversion of TG-β-gal (**Figure 6B**). This result suggests that the enzymatic activity of the delivered β-gal under intracellular environments for cleavable DMAE-SS-PRX is higher than that of non-cleavable DMAE-PRX. To quantify the fluorescence intensity and to compare the kinetics of the intracellular enzymatic reaction, the time-course of the fluorescence intensity change was monitored after the addition of TG-β-gal. In the intracellular environment, the PRX-based enzyme complexes exhibited a higher intracellular fluorescence intensity compared with that of the non-cleavable DMAE-PRX/β-gal complexes (**Figure 6C**). This result suggests that the intracellular dissociation of DMAE-SS-PRXs and the subsequent release of β-gal contribute to enhancing the activity of the delivered enzymes. Accordingly, the biocleavable DMAE-SS-PRXs provide an attractive technique for delivering various therapeutic bioactive molecules into living cells and enhancing their biological activity through supramolecular dissociation in the intracellular environment.

4. Polyrotaxanes as a macromolecular drug

4.1. Polyrotaxane-drug conjugates

Polymer-drug conjugates are a class of promising drug delivery candidates for modulating the drug distribution in the body and the therapeutic efficacy of drugs. In particular, drugs are covalently conjugated to the side chain or terminal of hydrophilic polymers, such as poly(2-hydroxypropyl methacrylamide) (PHPMA), poly(styrene-co-malic acid), poly(glutamic acid), and PEG, via cleavable linkers.[134] Biocleavable PRXs have potential as macromolecular drugs that use supramolecular dissociation to release drug-conjugated CDs. In this regard, PRX-drug conjugates comprising theophylline, an asthma drug, and PEG/α-CD PRXs bearing enzymatically-degradable stoppers have been synthesized.[135] Upon the addition of an enzyme (papain), theophylline-immobilized α-CDs were completely released from PRX via hydrolysis of the terminal stoppers. It is suggested that the PRX-drug conjugates that can release drugs via the dissociation of the supramolecular structure could be applied in the delivery of various drugs.

Another type of supramolecular drug conjugates is PRX-drug conjugates coupled with hydrolysable ester linkages. Based on our achievement in biodegradable PRX studies, Yang and coworkers reported on PEG/α-CD PRX-camptothecin and PEG/α-CD PRX-doxorubicin conjugates, in which the anticancer drugs are covalently coupled to α-CD moieties on PRX via ester linkages.[136,137] The conjugated drugs were observed to be released from PRX under physiological conditions. Additionally, the researchers demonstrated that both PRX-camptothecin and PRX-doxorubicin conjugates exhibit cytotoxic effects against cancer cells *in vitro*. Jiang and co-workers developed PPG/β-CD PRX-paclitaxel conjugates and investigated their drug distribution in mice and their *in vivo* antitumor activity.[138] The half-life of PRX-paclitaxel conjugates in mice after intravenous administration was determined to be 5.8 h, which is significantly longer than that of free paclitaxel (18.8 min). Therefore, PRX-drug conjugates are beneficial for prolonging the circulation time and drug distribution. Additionally, the intravenous administration of PRX-paclitaxel

conjugates to tumor-bearing mice resulted in delayed tumor growth compared with that of free paclitaxel. This result is presumably due to the preferential accumulation of PRX-paclitaxel conjugates in tumor tissues through the EPR effect.

4.2. Biocleavable polyrotaxanes as therapeutics for lysosomal storage disorders

As mentioned above, our group has paid special attention to the supramolecular dissociation of PRX in the design of biomaterials. Accompanied by the dissociation of PRX, the abundant threading CDs are released from PRXs. Next, we have attempted to utilize the stimulus-responsive release property of CDs for the treatment of lysosomal diseases, which are a family of intracellular metabolic diseases caused by the defective function of lysosomal proteins.[139] Among various lysosomal diseases, Niemann-Pick type C (NPC) disease is an autosomal recessive lysosomal trafficking disorder, in which the cholesterols are abnormally accumulated in lysosomes.[140,141] Recently, hydroxypropyl-β-cyclodextrin (HP-β-CD) has received much attention as a potential therapeutics for NPC disease.[142,143] It has been revealed that the administration of HP-β-CD reduces the cholesterol content in various organs, leading to a remarkable life-span prolongation of mice with a model of NPC disease. However, despite the significant therapeutic effect of HP-β-CD, a high dose is required for NPC disease therapy due to its non-specific inclusion of plasma components and its short blood-circulation time. Additionally, CDs are known to have a toxic effect at high concentration.[144] To address these drawbacks, we focused on biocleavable Pluronic/β-CD PRXs bearing terminal disulfide linkages (**Figure 7A**).[145] Biocleavable PRXs have tremendous advantages that allow them to overcome all of the problems of β-CDs in NPC disease therapy, such as masking the toxic effect of β-CD derivatives, retardation of renal clearance, preferential accumulation in endosomes/lysosomes through endocytosis, and site-specific exposure of the hydrophobic cavity of β-CDs via cleavage of the terminal linker of PRXs in intracellular environments to achieve the therapeutic effect.

The CLSM observation of the FITC-labeled biocleavable PRXs revealed these supermolecules exhibit negligible interaction with the plasma membrane and mainly accumulate in the late endosomes, whereas HP-β-CDs preferentially accumulate at the periphery of cells (**Figure 7B**). Additionally, HP-β-CDs removed the cholesterol from the plasma membrane, leading to a disruption of the integrity of the plasma membrane. In contrast, biocleavable PRX causes negligible removal of cholesterols from the plasma membrane because the hydrophobic cavity of β-CDs is occupied by a polymer chain. Accordingly, HP-β-CDs exhibit higher hemolytic activity and cytotoxicity than those of biocleavable PRXs. To validate the cholesterol removal ability of PRXs, the amount of intracellular cholesterol in NPC patient-derived fibroblasts was investigated. Notably, the lysosomal release of threaded β-CDs from biocleavable PRXs by the intracellular cleavage of terminal disulfide linkages achieved approximately 100-fold higher cholesterol removal ability from NPC disease-derived cells than that of β-CD derivatives (**Figure 7C**). In contrast, non-cleavable PRXs caused a negligible reduction in the cholesterol amount in NPC-disease-derived cells. Consequently, the biocleavable PRXs are considered a noninvasive and effective therapeutic for NPC disease.

5. Conclusions

In this review, we described the recent progress in using threaded macromolecules, including PRXs and pseudopolyrotaxanes, as supramolecular biomaterials, such as hydrogels, nanoparticles, gene delivery carriers, and supramolecular drug conjugates. The unique supramolecular framework of PRX provides new functionality to current biomaterials, such as the free mobility of threaded cyclic molecules and the self-assembly of PRXs via intermolecular interactions. In particular, the stimulus-responsive dissociation of biocleavable PRXs is a fascinating characteristic for the design of a new class of biodegradable materials that can be utilized for the controlled release of drugs. Additionally, the integration of multiple components in PRXs, such as the modification of hydrophilic or hydrophobic polymers, chemical functionalization of threading cyclic molecules, and

multiple assemblies with bioactive molecules, increases the potential for using PRXs in various biomedical applications. To date, many proof-of-concept studies of supramolecular biomaterials have been performed, especially under *in vitro* conditions. The practical application of supramolecular biomaterials faces many challenges, such as biocompatibility and performance under *in vivo* conditions. Although sustained research to address these challenges is required, we believe that supramolecular biomaterials possess a bright future.

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Figure captions

Figure 1. Schematic illustration of polyrotaxanes (PRX) showing the sliding motion of cyclic molecules along a polymer axle and stimuli-induced supramolecular dissociation via the cleavage of bulky stopper molecules.

Figure 2. (A) Chemical cross-linking of CD moieties in PRXs to form gel. (B) Physical gelation of PRXs via the self-assembly of threading CDs.

Figure 3. (A) Polymeric micelle formation of triblock copolymers of PRX and hydrophilic polymers. (B) Flower micelle formation of triblock copolymers of PRX and hydrophobic polymers. Reproduced with permission from ref. 84. © 2014 Royal Society of Chemistry.

Figure 4. (A) Chemical structure of *N*,*N*-dimethylaminoethyl (DMAE) group-modified PRX bearing terminal disulfide linkages (DMAE-SS-PRX). (B) Schematic illustration of an electrostatic interaction between DMAE-SS-PRX and pDNA, and the supramolecular dissociation via the cleavage of the terminal disulfide linkages in DMAE-SS-PRXs in an intracellular environment.

Figure 5. (A) Gene silencing efficiency of firefly luciferase expressed in HeLa cells incubated with PEG10k/α-CD DMAE-SS-PRX/siRNA (N/P 10) and PEG5k/α-CD DMAE-SS-PRX/siRNA (N/P 10) for 48 h. (B) Gene silencing efficiency of firefly luciferase expressed in HeLa cells incubated with cleavable DMAE-SS-PRX/siRNA (N/P 10) and non-cleavable DMAE-PRX/siRNA (N/P 10) for 48 h. The concentration of siRNA in the medium was adjusted to 100 nM. The data are expressed as means ± S.D. (n = 3) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$). Reproduced with permission from ref. 130. © 2014 Elsevier.

Figure 6. (A) Schematic illustration of the polyelectrolyte complex formation between the DMAE-SS-PRX and the anionic β-galactosidase (β-gal) and the intracellular dissociation of the complexes by the cleavage of the terminal disulfide linkages. (B) CLSM images of HeLa cells after 24 h incubation with cleavable DMAE-SS-PRX/β-gal and non-cleavable DMAE-PRX/β-gal, followed by 30 min incubation after the addition of TG-β-gal (green) (scale bars: 50 μm). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). (C) Time-course of the fluorescence intensity change of TG-β-gal in HeLa cells after 24 h incubation with β-gal (closed

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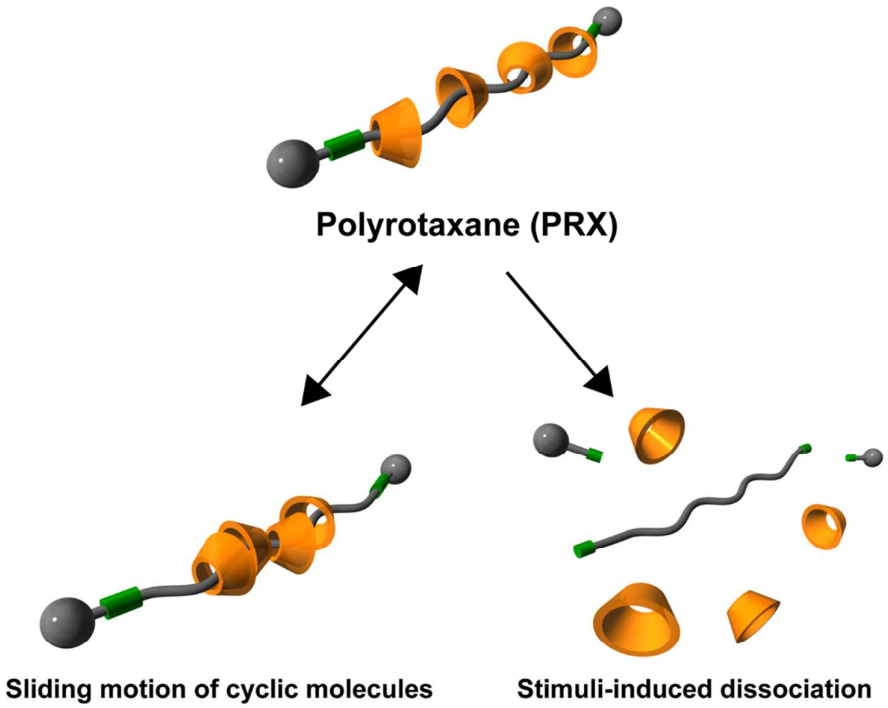
Figure 7. (A) Schematic illustration of the intracellular dissociation of the PRXs and the subsequent lysosomal release of threaded β-CDs to the improve cholesterol accumulation in Niemann-Pick type C (NPC) disease-derived cells. (B) Phase contrast and CLSM images of NPC1 cells incubated with FITC-HP-β-CD (green) and FITC-HE-SS-PRX (green) (bar: 20 μm). The nuclei were stained with DAPI (blue). The early and late endosomes were stained with anti-EEA1 and anti-CD63 antibodies, respectively (red). (C) The amount of intracellular total cholesterol in the NPC1 cells incubated with HP-β-CD (open squares), non-cleavable HE-PRX (closed triangles), and cleavable HE-SS-PRX (closed diamonds) at various β-CD concentrations for 24 h. The dashed lines represent the amount of intracellular total cholesterol in non-treated normal and NPC1 cells. Reproduced with permission from ref. 145. © 2014 Nature Publishing Group.



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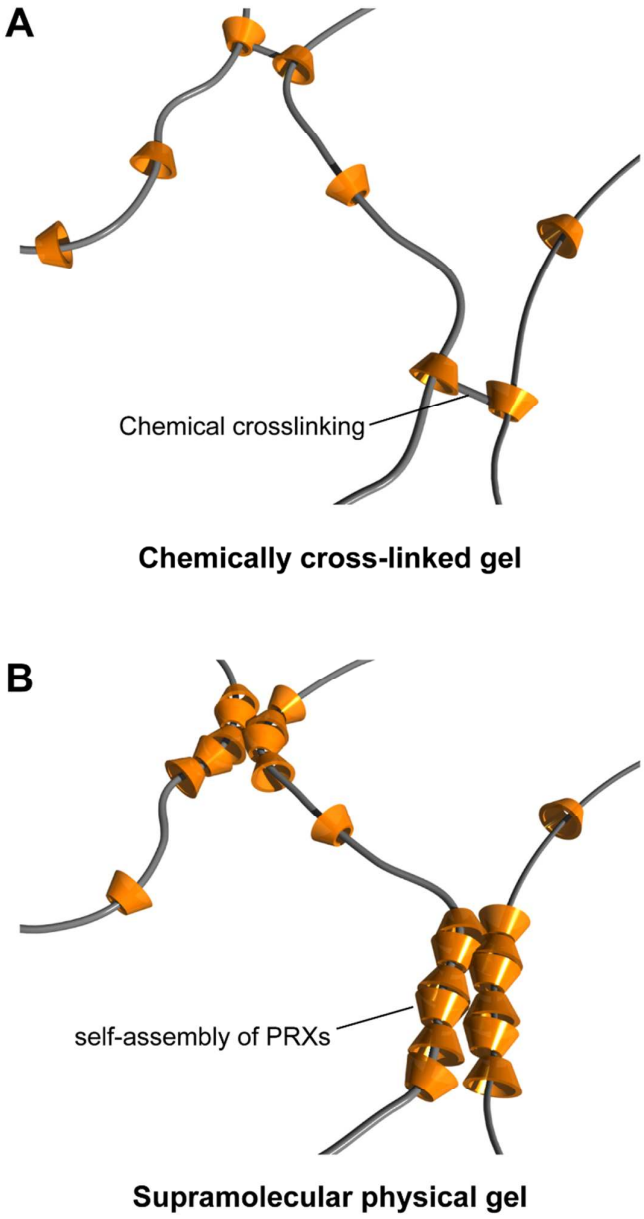

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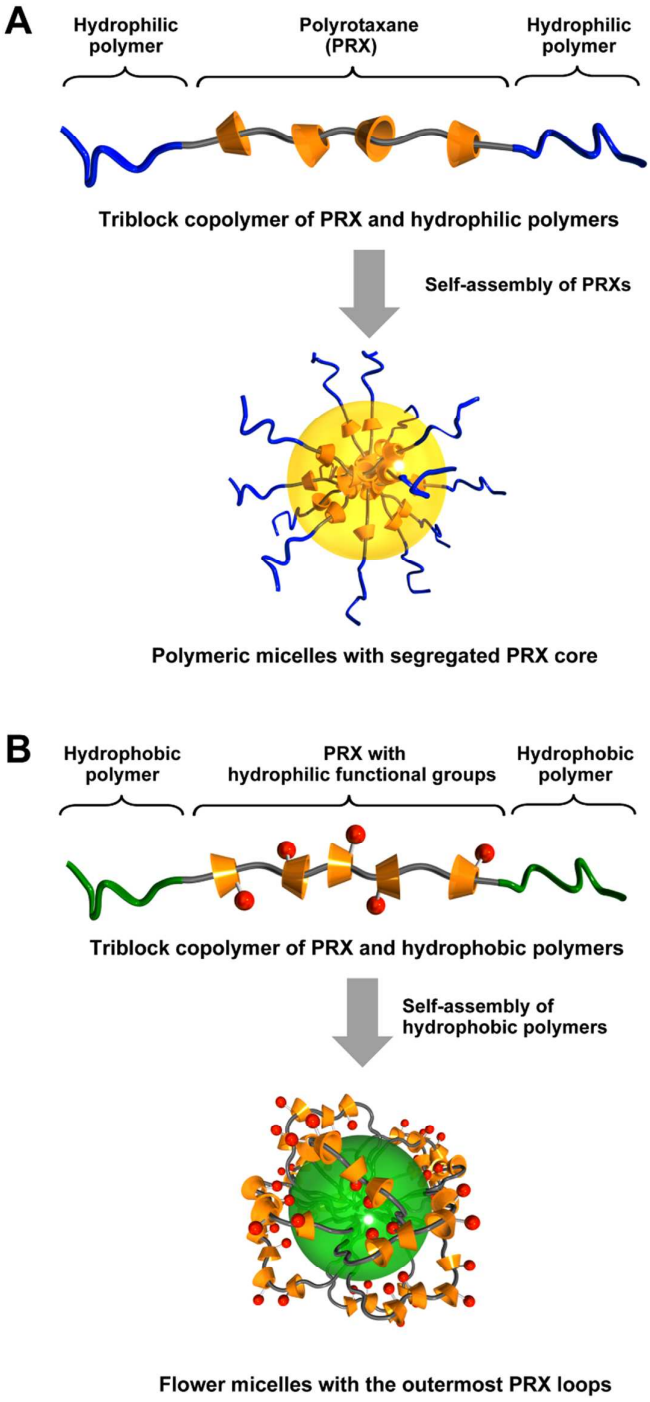

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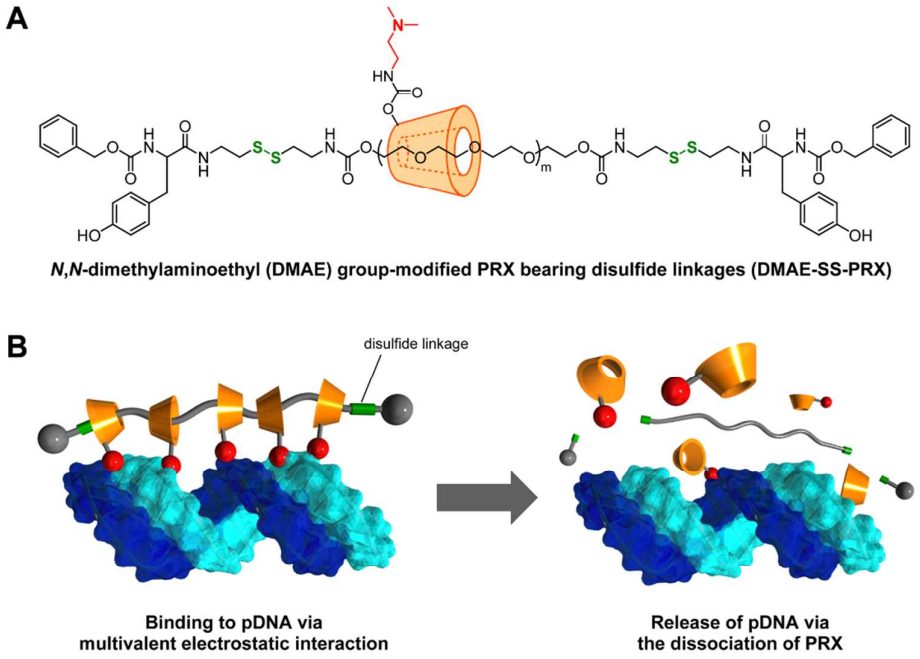

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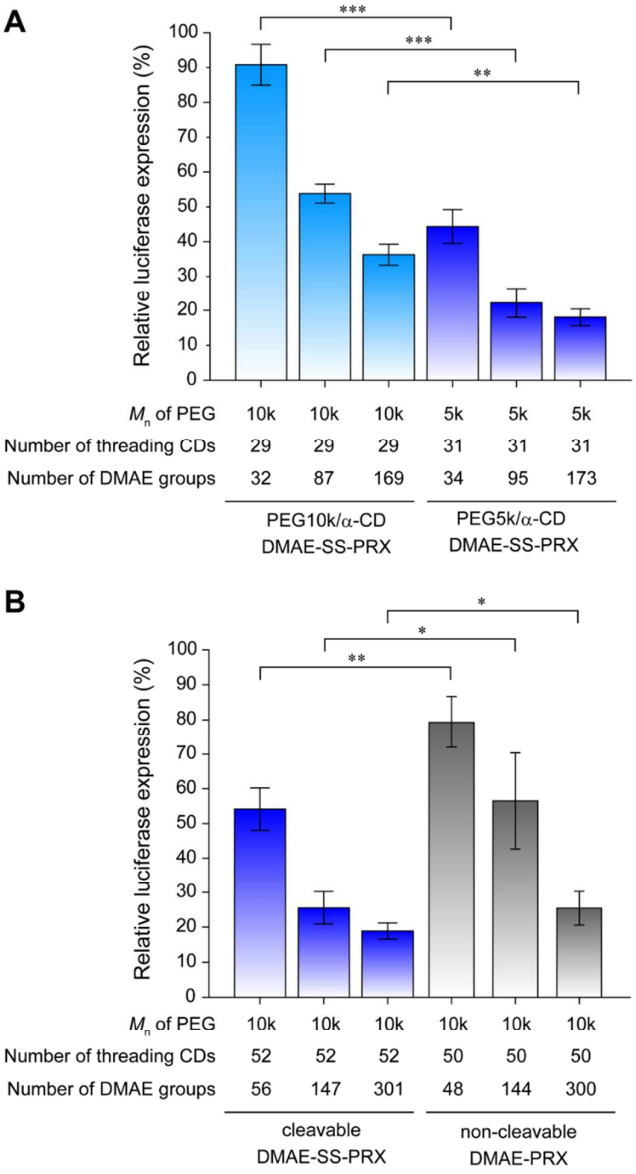

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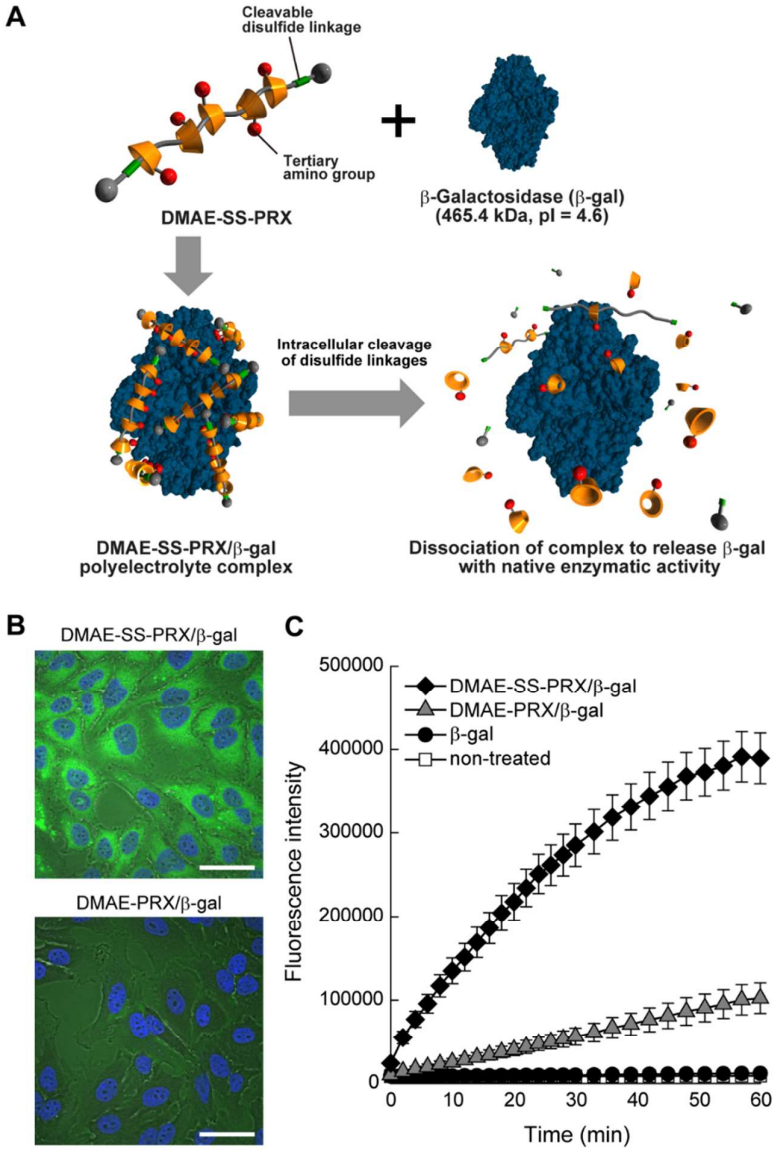

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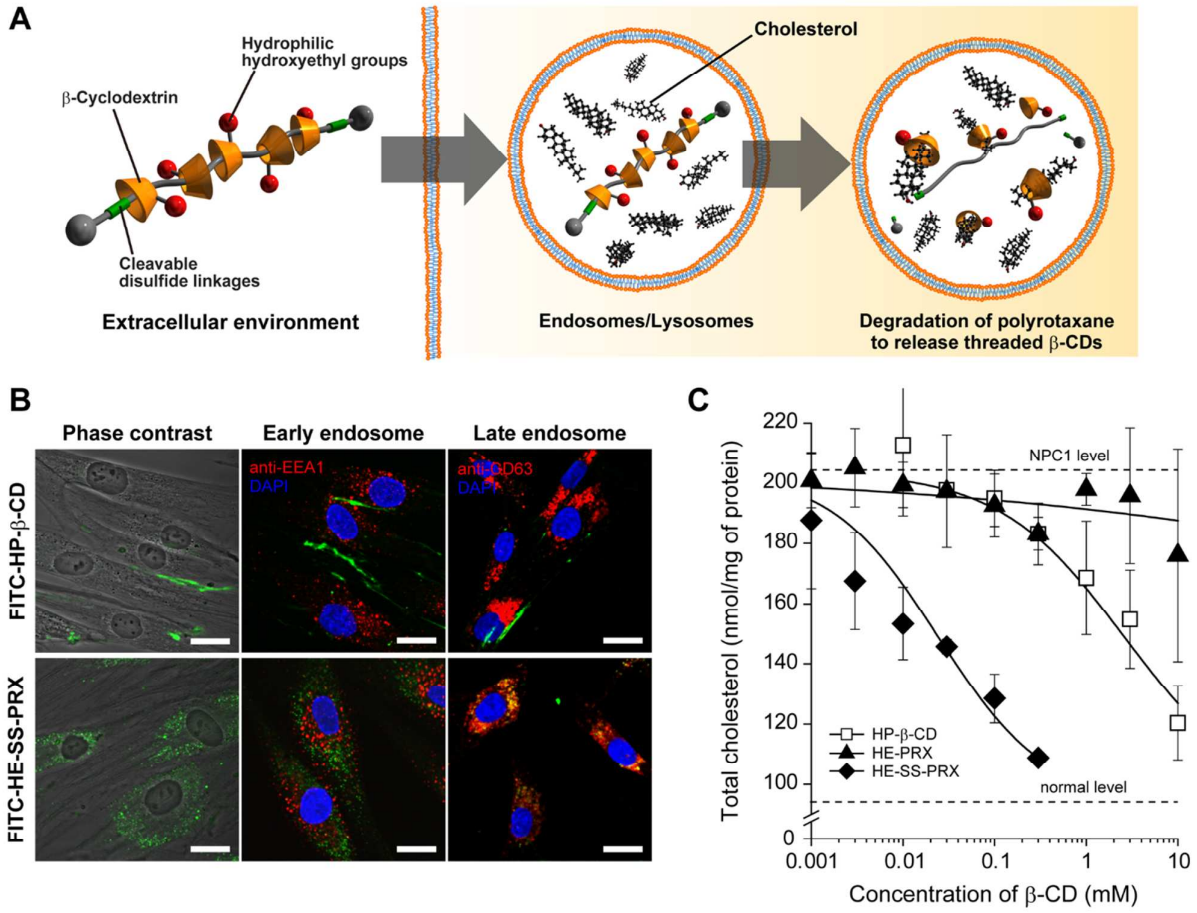

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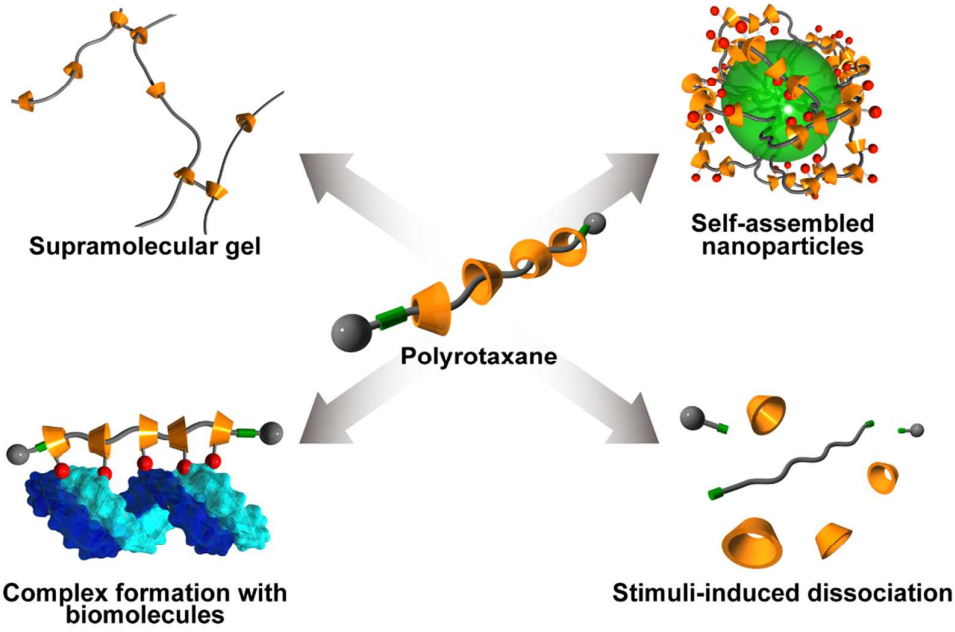
Graphical abstract



In this feature article, the recent progress in biomaterial application of threaded macromolecules including polyrotaxanes such as drug delivery and gene delivery are described.