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Acid-labile polyrotaxane exerting endolysosomal pH-sensitive supramolecular dissociation for therapeutic applications

Kei Nishida, Atsushi Tamura, and Nobuhiko Yui*

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 (Tel: +81-3-5280-8020, Fax: +81-3-5280-8027, E-mail: yui.org@tmd.ac.jp)

Keywords

pH-sensitivity, ketal linkage, polyrotaxane, cyclodextrin, Niemann-Pick type C disease

Abstract

pH-sensitive polymeric materials are rational design for therapeutics, because they can change their intrinsic properties selectively in response to pH changes in targeted tissues and organelles, such as tumor tissue, skin, inflammation site, and intracellular endosomes and lysosomes. Herein, we described a novel acid-labile Pluronic/β-cyclodextrin (β-CD)-based polyrotaxane (PRX) showing the dissociation of supramolecular interlocked structure in response to a pH change with a narrow acidic range for biomedical applications. Acid cleavable ketal linkages were introduced into both terminals of the axle polymer of the PRX (ket-PRX) to acquire acid sensitivity. The ket-PRX was sufficiently stable and maintain its supramolecular structure at physiologic condition (pH 7.4), whereas dissociation of the ket-PRX was observed at weakly acidic pH condition (pH 5.0) due to the cleavage of the ketal linkages. Concomitant with this supramolecular dissociation it was found that the ket-PRX released β-CDs to form an inclusion complex with guest molecules specifically at weakly acidic pH conditions. As one of the possible intracellular therapeutic applications, it was confirmed that the ket-PRX showed superior reduction of lysosomal cholesterols in Niemann-Pick type C disease in comparison to β-CD derivatives, presumably due to the local release of β-CDs from the ket-PRX in response to weakly acidic pH in endosomes and/or lysosomes. Altogether with these results, it is concluded that the ket-PRX exerting pH-sensitive dissociation is an attractive candidate as potential therapeutic biomaterials.

Introduction

Stimuli-sensitive polymeric biomaterials exerting a change in chemical structure, polarity, solubility, and assembly-disassembly state in response to environmental changes such as temperature, redox potential, pH, and the existence of specific molecules have received considerable attention, because they can convert their intrinsic functions and properties specifically in targeted site of action.[1-3] In this regard, various stimuli-sensitive therapeutic materials such as biodegradable hydrogels, nanoparticles, polyelectrolyte complexes containing biomolecules, and biocleavable drug-polymer conjugates have been developed for facilitating their therapeutic efficacy in response to specific environments.[4-7] We have proposed new modality of biocleavable polymeric materials based on polyrotaxanes (PRXs) consisting of inclusion complexes of cyclodextrins (CDs) and a linear polymer capped with bulky stopper molecules via cleavable linkages (*i.e.* ester, hydrazone, disulfide, and oligopeptide).[8-11] It should be noted that the biocleavable PRXs can more efficiently dissociate in response to physiological stimuli in comparison to conventional biodegradable materials, because the cleavage of a single site of the terminal cleavable linkages can induce the completion of supramolecular dissociation. Taking the advantages of this unique biocleavable character of PRXs into account, we have developed various supramolecularly biocleavable materials such as hydrogels, the intercellular delivery carriers for biomolecules, drug-PRX conjugates, and therapeutics for intractable disease.[12-15]

Among various stimuli-sensitive materials, pH-sensitive materials that can selectively be cleaved

in response to a pH change within a narrow acidic range will be considered useful and versatile, because a reduction in the pH was reported in various tissues and organelles such as tumor tissue, skin, inflammation site, and intracellular endosomes and lysosomes.[16-19] For example, the environmental pH in endosomes and lysosomes is approximately decreased to 4.5 - 6.5 due to the influx of the protons by vacuolar-type ATPase.[20] In this regard, we have designed pH-sensitive PRXs bearing ester or hydrazone linkages for the intracellular delivery of therapeutic biomolecules. However, one issue has been raised that the previously designed pH-sensitive PRXs require non-physiological pH change such as strong alkaline pH and extremely low pH (pH 2), and sometimes long time for the complete dissociation of the PRX structure.[10,21,22] It is still controversial for the development of acid-labile PRXs, most likely due to the difficulties in the synthesis of acid-labile PRXs, such as dissociation during the reaction and purification process.

Note that ketal linkages are known to be cleaved under weakly acidic condition, whereas they are stable under neutral and alkaline conditions.[23,24] Herein, we described a novel acid-labile Pluronic/β-cyclodextrin (β-CD)-based PRX bearing ketal linkages (ket-PRX) for expecting complete dissociation and β-CD release in weakly acidic environments such as endosomes and lysosomes (**Fig. 1**). In this study, the synthesis of acid-labile Pluronic/β-CD-based PRX bearing ketal linkages was established and its pH-sensitive dissociation behavior was investigated. Additionally, for one of the potential therapeutic applications of ket-PRX in lysosomes, the cholesterol reduction ability of the ket-PRX in fibroblasts derived from patients of Niemann-Pick type C disease was demonstrated.

Materials and methods.

Materials.

β-Cyclodextrin (β-CD) was obtained from Nihon Shokuhin Kako (Tokyo, Japan). Pluronic P123 (M_n: 6,350, PEG segment $M_{n,PEG}$: 1,100×2, PPG segment $M_{n,PPG}$: 4,150), 1,1'-carbonyldiimidazole (CDI), 2,2-bis(aminoehtoxy)propane, *N*-(triphenylmethyl)glycine (Trt-Gly-OH), 2-(2-hydroxyethoxy)ethylamine (HEEA), 6-(*p*-toluidino)-2-naphthalenesulfonic acid sodium salt (TNS), and 2-hydroxypropyl-β-CD (HP-β-CD, average molecular weight of 1,460) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) was obtained from Wako Pure Chemical Industries (Osaka, Japan). *N*-hydroxysuccinimide (NHS) was obtained from Acros Organics (Geel, Belgium). Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were obtained from Dojindo Laboratories (Kumamoto, Japan). Other solvents were obtained from Kanto Chemicals (Tokyo, Japan).

Characterization of polyrotaxanes

Size exclusion chromatography (SEC) was carried out on a HLC-8120system (Tosoh, Tokyo, Japan)

equipped with a combination of TSKgel α-4000 and α-2500 columns (Tosoh), eluted with *N,N*-dimethylformamide (DMF) containing 10 mM LiBr at a flow rate of 0.5 mL/min at 60 ºC. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance Ⅲ 400 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany).

Synthesis of polyrotaxane bearing ketal linkages (ket-PRX)

Pluronic P123 (10.0 g, 1.57 mmol) and CDI (9.9 g, 61.1 mmol) were dissolved in of tetrahydrofuran (THF) (135 mL), and the solution was stirred for 24 h at room temperature. After the reaction, the polymer was purified by dialysis against THF for 3 days (Spectra/Pro 6, molecular weight cut-off of 3,500) and evaporated to obtain α,ω-biscarbonylimidazolyl Pluronic P123 (P123-CI) (9.26 g, 89.9%). Then, P123-CI (1.0 g, 152.2 μmol) and 2,2-bis(aminoethoxy)propane (0.49 mL, 3.04 mmol) were dissolved in of THF (9.25 mL), and the solution was stirred 24 h at room temperature. After the reaction, the polymer was purified by dialysis against methanol (Spectra/Pro 6, molecular weight cut-off of 3,500) for 3 days and evaporated to obtain 2,2-bis(aminoethoxy)propane-conjugated Pluronic P123 (P123-ket-NH_2) (0.67 g, 67.2% yield).

A saturated solution of β-CD was prepared by dissolving β-CD (20 g, 17.6 mmol) in 100 mM carbonate buffer (pH 9.0, 1 L). Then, P123-ket-NH_2 (0.5 g, 76.3 μmol) dissolved in small aliquot of methanol was added to the β-CD solution, and mixture was stirred for 24 h at room temperature. After the reaction, the precipitate was collected by centrifugation, and freeze-dried to obtain a

pseudopolyrotaxane. To form activated ester of Trt-Gly-OH (Trt-Gly-NHS), Trt-Gly-OH (485.0 mg, 1.53 mmol), NHS (175.6 mg, 1.53 mmol), and EDC (322.3 mg, 1.68 mmol) were dissolved in dehydrated DMF (5.34 mL), and the solution was stirred for 3 h at room temperature. To this solution, 200 mM carbonate buffer (pH 9.0, 7.12 mL) and the pseudopolyrotaxane dissolved in methanol (14.2 mL) were successively added. The reaction mixture was stirred for 24 h at room temperature. Then, the precipitate was collected by centrifugation and successively washed with methanol/DMF, followed by ammonia solution (pH 10.0)/DMF. These washing processes were repeated three times to remove free β-CD and unreacted reagents. Finally, the recovered precipitate was freeze-dried to obtain a PRX bearing ketal linkages in the axle polymer (1.06 g, 34.1% yield based on P123). The number of β-CDs threaded onto Pluronic P123 was determined from the ^{1}H NMR spectra in D_2O by comparing peak area between 4.84 ppm (H_1 proton of β-CD) and 1.06 ppm (-CH_2-CH(C***H*****$_3$**)-O- of Pluronic P123). The number-averaged molecular weight (M_n) of the ket-PRX was calculated from the chemical composition determined from the ^{1}H NMR spectra in D_2O.

Modification of (2-hydroxyethoxy)ethyl groups onto ket-PRX (HEE-ket-PRX)

The ket-PRX (250 mg, 10.5 μmol) and CDI (246.3 mg, 1.52 mmol) were dissolved in dehydrated DMF (20 mL), and stirred for 24 h at room temperature. Then, HEEA (1.05 mL, 10.5 mmol) was added to the reaction mixture and stirred for 24 h at room temperature. After the reaction, the PRX was purified by dialysis against methanol (Spectra/Pro 6, molecular weight cut-off of 25,000) for 3

days. The recovered PRX was evaporated and dissolved in ammonia solution (pH 10.0). Finally, this aqueous solution was freeze-dried to obtain (2-hydroxyethoxy)ethyl group-modified ket-PRX (HEE-ket-PRX) as a white powder (238.0 mg, 63.3% yield). The number of HEE groups modified on the ket-PRX was determined from the ^{1}H NMR spectra in D_2O by comparing peak area between 3.36 ppm (-C***H*****$_2$**-CH_2-O-CH_2-CH_2-OH of HEE group) and 1.06 ppm (-CH_2-CH(C***H*****$_3$**)-O- of Pluronic P123). The number-averaged molecular weight of the HEE-ket-PRX was calculated from the chemical composition determined from the ^{1}H NMR spectra in D_2O.

Hydrolysis of HEE-ket-PRX

The HEE-ket-PRX (2 mg) was dissolved in 10 mM HEPES buffer solutions at pH 5.0, 6.5, and 7.4 (1 mL), and these solutions were incubated at 37 ºC. After incubation for prescribed time period, small aliquots of the solutions were collected and ^{1}H NMR spectra was recorded in D_2O. The hydrolysis of the HEE-ket-PRX was calculated from the ^{1}H NMR spectra by comparing the peak area of the ketal linkages (1.45 ppm) and PPG main chain (1.0 to 1.3 ppm).

Formation of inclusion complexes between TNS and dethreaded β-CDs from HEE-ket-PRXs

The HEE-ket-PRX (100 μM of β-CD) was dissolved in acetate or HEPES buffer at pH 4.5 to pH 9.0 (pH 4.5: acetate buffer/pH 5.0 to 9.0: HEPES buffer) (1 mL) and incubated for 1 h at 37 ºC. Then, TNS (20 μM) was added to each solution and the solutions were incubated for 10 min at 37 ºC. The

fluorescence spectra were recorded on an FP-8000 spectrofuluorometer (Jasco, Tokyo, Japan) at the excitation wavelength of 325 nm.

Cell culture

Human skin fibroblasts derived from a Niemann-Pick type C disease patient (NPC1) (GM03123; P237S and I1061T mutations in NPC1) and normal human dermal fibroblasts (GM05659) were obtained from the Coriell Institute for Medical Research (Camden, NJ, USA). These cells were grown in Dulbecco's modified eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco), 100 units/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco) in a humidified 5% CO_2 atmosphere at 37 ºC.

Filipin staining

NPC1 or normal fibroblast fibroblasts were plated on a 35-mm glass-based dish (Iwaki Glass, Tokyo, Japan) at a density of 2×10^4 cells/dish and incubated overnight. Then, the HEE-ket-PRX (1 mM of β-CD) and HP-β-CD (1 mM) solutions were applied to each dish. After incubation for 24 h at 37 ºC, the cells were washed twice with phosphate buffer saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were stained with filipin (PolyScience, Warrington, PA, USA) (50 μg/mL) for 45 min at room temperature. The confocal laser scanning microscopic (CLSM) images were auquired on a FluoView FV10i confocal laser scanning microscope (Olympus, Tokyo,

Japan) equipped with a 60× water-immersion objective lens (numerical aperture of 1.2) and a diode laser.

Analysis of total cholesterol content

NPC1 or normal fibroblasts were plated on a 12-well plate (Nunc, Roskilde, Denmark) at a density of 1.0×10^5 cells/well and incubated overnight. Then, HEE-ket-PRX and HP-β-CD solutions prepared at various concentrations were applied to each well. After incubation for 24 h at 37 ºC, the cells were washed twice with PBS. Then, the cells were harvested with trypsin-EDTA, washed three times with PBS, and lysed with cell lysis buffer (100 mM phosphate buffer, 1 M NaCl, 50 mM cholic acid, and 1% Triton X-100). The cellular total cholesterol was determined by Amplex Red Cholesterol Assay Kit (Molecular Probes). Briefly, the sample solution (50 μL) was combined with the assay solution (50 μL) containing cholesterol esterase (2 U/mL), cholesterol oxidase (2 U/mL), horseradish peroxidase (2 U/mL), and Amplex Red reagent (fluorescent substrate) (300 μM) and incubated for 30 min at 37 ºC. The fluorescence intensities were measured on an ARVO MX multilabel counter (Perkin Elmer, Wellesley, MA, USA) equipped with a filter set for excitation and emission at 560 ± 10 nm and 590 ± 10 nm, respectively. Protein content in the lysate was also determined with a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Cellular cholesterol content was normalized to protein content and expressed as nmol/mg of protein.

Results and discussion

Synthesis of acid-labile PRX bearing ketal linkages

For achieving the dissociation of PRXs in response to weakly acidic environments, a Pluronic/β-CD-based PRXs bearing ketal linkages was designed (**Fig. 1**). In this regard, 2,2-bis(aminoethoxy)propane-conjugated Pluronic P123 (P123-ket-NH_2) was utilized as an axle polymer for the acid-labile PRX. In the ^{1}H NMR spectrum of P123-ket-NH_2, singlet peak at 1.36 ppm assignable to the methyl protons of ketal linkages was clearly observed (**Fig. 2A**). Also, it was found that the ketal linkages were almost quantitatively introduced into both terminals of Pluronic P123 without degradation. In the preparation of pseudopolyrotaxanes, β-CD and P123-ket-NH_2 were allowed to react in mild alkaline solution to avoid the hydrolysis of ketal linkages, because the ketal linkages were stable at the alkaline conditions. The capping reaction of the pseudopolyrotaxanes was performed with Trt-Gly-OH in the presence of DMT-MM as previously described.[15] However, the expected PRXs were not obtained at this reaction condition, presumably due to the hydrolysis of ketal linkages by acidic Trt-Gly-OH (data not shown).

To avoid the degradation of ketal linkages during the capping reaction, a carboxyl group of Trt-Gly-OH was converted to active ester by conventional NHS and EDC, and the Trt-Gly-NHS was used as a capping reagent for the synthesis of PRX. From the SEC charts, the peak of ket-PRXs was unimodal and the peak top was shifted toward high molecular weight region in comparisons to

P123-ket-NH_2 and β-CD, suggesting that the ket-PRX was obtained without the contamination of free β-CDs (**Fig. 3**). Also, the ^{1}H NMR spectrum of the ket-PRX showed a peak at 1.36 ppm assignable to the methyl protons of ketal linkages accompanied with the peaks corresponding to the Pluronic P123 (1.23 ppm), β-CD (5.10 ppm), and Trt end groups (7.35, 7.41, 7.49 ppm) (**Fig. 2B**). The number of threaded β-CDs in the ket-PRX and the M_n were calculated to be 14.4 and 23,700 from the ^{1}H NMR spectrum, respectively. The theoretical maximum number of threaded β-CDs onto PPG segment of Pluronic P123 is 30.1, assuming one β-CD molecule forms an inclusion complex with two repeating units of propylelne glycol. Altogether with these results, the ket-PRX was successfully synthesized without the degradation of ketal linkages.

Because the Pluronic/β-CD PRX shows negligible solubility in aqueous media, hydroxyl groups of the threaded β-CDs in the PRX (HEE-ket-PRX) were modified with (2-hydroxyethoxy)ethyl (HEE) groups to impart water solubility. From the SEC charts, the peak of HEE-ket-PRX was slightly shifted to high molecular weight region in comparison to unmodified ket-PRX, and negligible degradation of the PRX was observed after the reaction (**Fig. 3**). In the ^{1}H NMR spectrum of the HEE-ket-PRX, the peaks assignable to ketal linkages and modified HEE groups were observed (**Fig. 2C**). From the ^{1}H NMR pectrum of HEE-ket-PRX, the number of HEE groups modified on the ket-PRX was 90.7, which corresponds to 6.3 molecules of HEE groups were modified on a β-CD molecule. Also, the M_n of the HEE-ket-PRX was calculated to be 35,700. The obtained HEE-ket-PRX showed excellent solubility in water and dissolved at least 20 mg/mL of

concentration.

To roughly confirm the dissociation character of the HEE-ket-PRX in acidic condition, the SEC was measured after the addition of small aliquots of HCl (**Fig. 3**). As a result, the peak of the HEE-ket-PRX was disappeared, while the peaks of Pluronic P123, HEE group-modified β-CD (HEE-β-CD), and bulky capping groups as the constituent molecules of the HEE-ket-PRX were observed.

Hydrolysis of HEE-ket-PRX

To evaluate the pH-dependency of the dissociation of the HEE-ket-PRX, the hydrolysis kinetics of the HEE-ket-PRX at pH 5.0, 6.5, and 7.4 was investigated by ^{1}H NMR. At pH 5.0, the peak area of ketal linkages gradually decreased with time, whereas the peak of ketal linkages remained at pH 7.4 (**Fig. 4A**). Interestingly, at the pH of 5.0, the peaks of PPG segment of the axle polymer in the HEE-ket-PRX were shifted 1.23 ppm to high field (1.13 ppm) synchronizing with the decline of peak of ketal linkages. In our previous reports, the location of β-CD on PEG-*b*-PPG-*b*-PEG is changed by temperature accompanied with the peak shift of methyl protons of the PPG segment.[25] According to this fact, it is considered that the peak shift of the PPG segment in acidic conditions reflects the dethreading of β-CDs from the PRX.

The time-course of hydrolysis of the HEE-ket-PRX was determined by the remained peak area of ketals at 1.45 ppm after incubation for prescribed time periods (**Fig. 4B**). Under the physiological pH

condition (pH 7.4) at 37 °C, 20% of ketal linkages were hydrolyzed after 3 h of incubation. On the other hand, under acidic pH condition (pH 5.0), the ketal linkages in the HEE-ket-PRX underwent drastic hydrolysis, and approximately 70% of ketal linkages were hydrolyzed within 15 min, and complete hydrolysis was observed after 3 h. Although the hydrolysis at the pH 6.5 was slower than pH 5.0, and approximately 60 to 70% of ketal linkages were hydrolyzed after 3 h. These results indicated that the ketal linkages in the HEE-ket-PRX were sufficiently stable and the supramolecular structure of the HEE-ket-PRX was maintained under physiological pH condition (pH 7.4), whereas they were readily cleaved to dissociate PRX structure at weakly acidic condition.

Inclusion complexation of dethreaded β-CD from the PRX with a fluorescent probe

Accompanied with the dissociation of the HEE-ket-PRX under acidic conditions, the dethreaded β-CDs have the ability to form an inclusion complex with guest molecules. To confirm the inclusion complexation of β-CDs dethreaded from the HEE-ket-PRX, the variation of fluorescence intensity of TNS was measured, because TNS forms inclusion complex with β-CD at 1:1 stoichiometry to show strong fluorescence when it incorporates into the hydrophobic cavity of the β-CD.[26] Although the free TNS showed negligible fluorescence, the inclusion complexation with highly water soluble β-CD derivative (HP-β-CD) resulted in the significantly increment of fluorescence intensity regardless of pH (pH 5.0 and 7.4) (**Fig. 5A**). In sharp contrast, the fluorescence intensity of TNS in the HEE-ket-PRX solution was increased only at pH 5.0, and the fluorescence spectra was nearly

identical to HP-β-CD/TNS complexes.

For further evaluating the fluorescence intensity change, pH-dependency of the fluorescence intensity at 440 nm was plotted for the HEE-ket-PRX and HP-β-CD (**Fig. 5B**). The fluorescence intensity of free TNS and HP-β-CD/TNS did not change with pH. In sharp contrast, the fluorescence intensity of TNS in the HEE-ket-PRX solution gradually increased with decreasing the pH below 7.0 and eventually reached constant value at pH 5.0. Because the hydrolysis of ketal linkages in the HEE-ket-PRX was promoted by decreasing pH (**Fig. 4B**), the large amount of β-CDs was released from the HEE-ket-PRX with decreasing pH below 7.0 resulting in the increment in fluorescence intensity of TNS through the inclusion complexation. Accordingly, it is clear that the HEE-ket-PRX have unique ability to allow pH-dependent formation of the inclusion complex between threaded β-CDs with guest molecules.

Lysosomal cholesterol reduction ability of HEE-ket-PRX in Niemann-Pick type C (NPC) disease

For applying the unique pH-dependent β-CD release and inclusion complexation characters of the HEE-ket-PRX, the therapeutic approach of the HEE-ket-PRX to Niemann-Pick type C (NPC) disease was demonstrated. In the cells of NPC disease, the unesterified cholesterols are chronically accumulated in the endosomes and lysosomes, due to the mutation of NPC1, a transmembrane cholesterol transporter.[27,28] Recently, it is reported that HP-β-CD has a potential in reducing the

intracellular lysosomal cholesterol content of NPC disease to improve the pathologies such as hepatosplenomegaly, neurodegeneration, and prolonging the life-span.[29,30] Meanwhile, we have previously reported that β-CD-threaded biocleavable PRXs show more excellent lysosomal cholesterol reduction than HP-β-CD through the intracellular local release of threaded β-CD.[15] However, the detailed site of releasing β-CDs from the PRXs is unknown in the previously reported systems (*i.e.*, PRXs bearing disulfide linkages). Because the HEE-ket-PRX can specifically dissociate under acidic conditions, the release of β-CD would occur in weakly acidic endosomes and lysosomes.

In this regard, the normal and NPC disease patient-derived fibroblasts (NPC1) were treated with the HEE-ket-PRX and HP-β-CD at the β-CD concentration of 1 mM for 24 h, and the cholesterols accumulated in the endosomes and lysosomes were visualized with filipin staining (**Fig. 6A**). The NPC1 fibroblasts showed strong fluorescence of filipin compared to normal fibroblasts. The treatment of NPC1 fibroblasts with HP-β-CD resulted in slight decrease in the fluorescence intensity. However, the fluorescence intensity of filipin in the HEE-ket-PRX-treated NPC1 cells was remarkably declined to almost normal level, suggesting that the lysosomal cholesterol accumulation was improved by the HEE-ket-PRX.

To quantitatively evaluate the cholesterol reduction ability of the HEE-ket-PRX, the quantification of total cholesterol (summation of esterified and unesterified cholesterols) in NPC1 fibroblasts was performed (**Fig. 6B**). The total cholesterol amount in NPC1 fibroblasts was 1.4-fold higher than

normal fibroblasts. After the treatment with HP-β-CD at various concentrations, the total cholesterol amount in NPC1 fibroblasts was declined in a concentration-dependent manner, which was consistent with the previous reports.[15] Note that the HEE-ket-PRX reduced total cholesterol at 10-fold lower concentration than HP-β-CD. The cholesterol content in NPC1 fibroblasts was decreased to nearly comparable level to normal fibroblasts by the treatment with the HEE-ket-PRX (1 mM), while the same concentration of HP-β-CD (1 mM) did not reduce cholesterols. This result strongly suggest that the lysosomal local release of β-CDs from the HEE-ket-PRX contributes to achieving the superior ability in reducing the lysosomal cholesterol level in NPC disease. Similar to the NPC1 fibroblasts, the treatment of normal fibroblasts with the HEE-ket-PRX and HP-β-CD resulted in the reduction of cholesterol content to some extent. Mondjinou and co-workers have reported the PRXs composed of HP-β-CD and Pluronic showed the removal of lysosomal cholesterol from NPC fibroblasts.[31] In this reports, the authors described that the ability of their PRXs to reduce lysosomal cholesterols was comparable to HP-β-CD. In our experimental result, the cholesterol reduction ability of our HEE-ket-PRX was significantly higher than HP-β-CD, indicating that the acid-labile character of the HEE-ket-PRX might contribute to enhancing the cholesterol reduction in NPC1 fibroblasts.

Conclusions

A novel acid-labile HEE-ket-PRX bearing ketal linkages was successfully synthesized without the degradation and dissociation during the reaction and purification process. Hydrolysis study showed that ketal linkages of the HEE-ket-PRX were readily cleaved to dissociate PRX structure at weakly acidic pH condition which is comparable to the endosomal and lysosomal pH conditions. The fluorescent intensity of TNS was increased by decreasing pH, indicating that the dethreaded β-CDs formed inclusion complexes with TNS. The HEE-ket-PRX showed significant lysosomal cholesterol reduction in NPC disease model cells at 10-fold lower concentration than HP-β-CD, suggesting that the lysosomal release of β-CDs in response to the acidic lysosomal environments contribute to enhancing to therapeutic efficiency. Thus, the acid-labile HEE-ket-PRX exerting endolysosomal pH-sensitive dissociation would be applied for the various biomaterials that can facilitate the therapeutic efficacy in acidic tissues and organelles such as the delivery carriers for biomolecules and acid-labile hydrogels.

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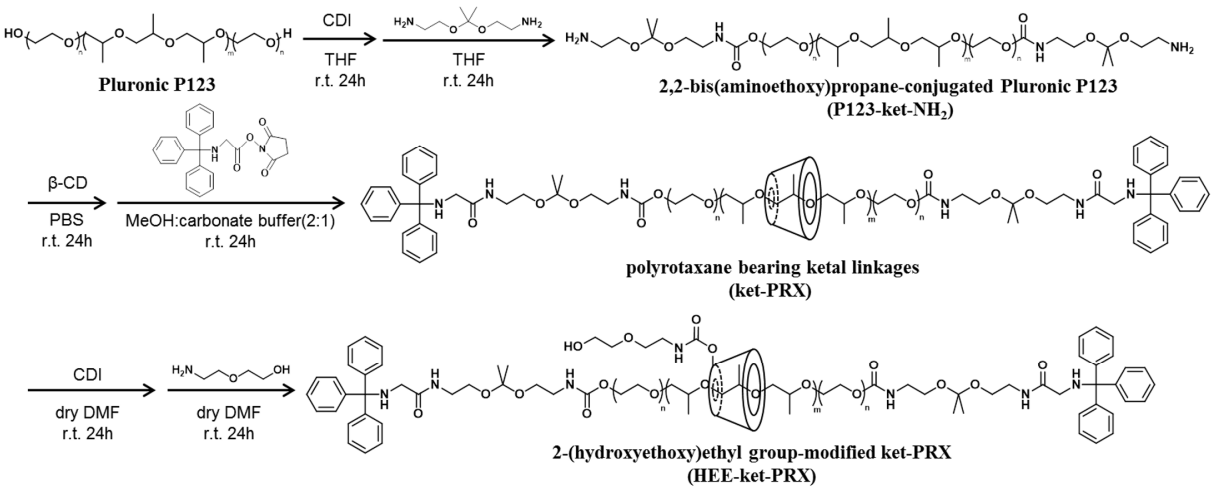

Scheme 1. Synthesis of an acid-labile PRXs bearing ketal linkages and the following (2-hydroxyethoxy)ethyl group modification.

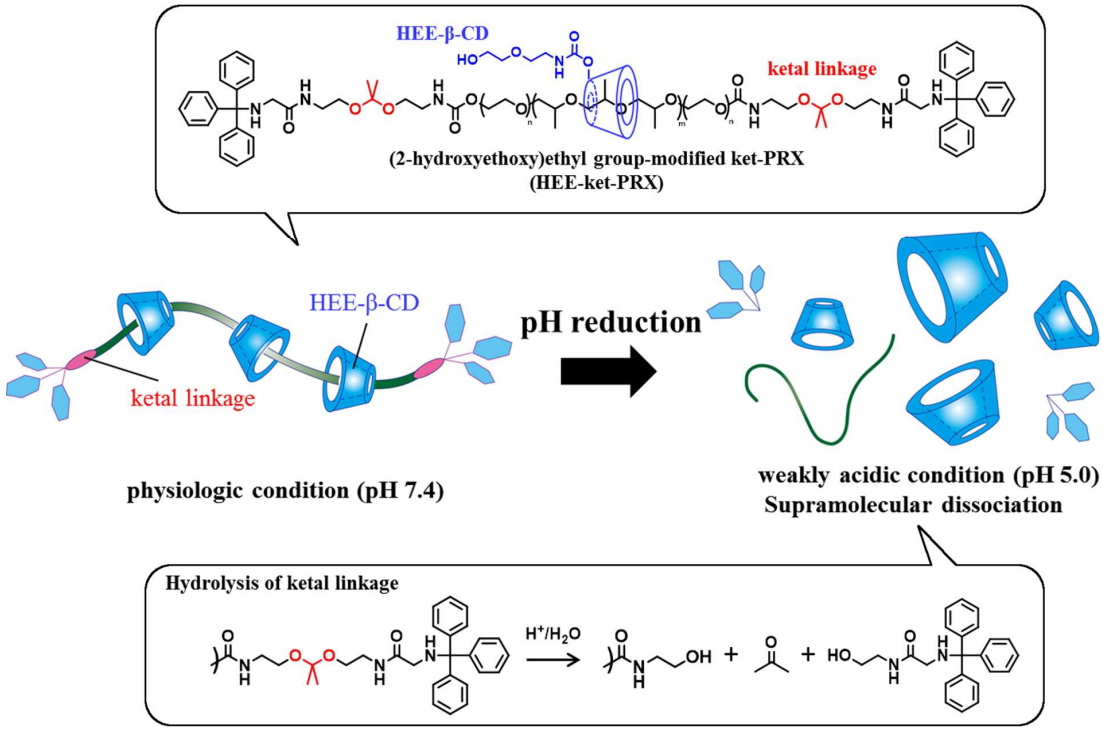

Fig. 1. Schematic diagram of acid-labile polyrotaxanes and pH-sensitive dissociation of the supramolecular structure.

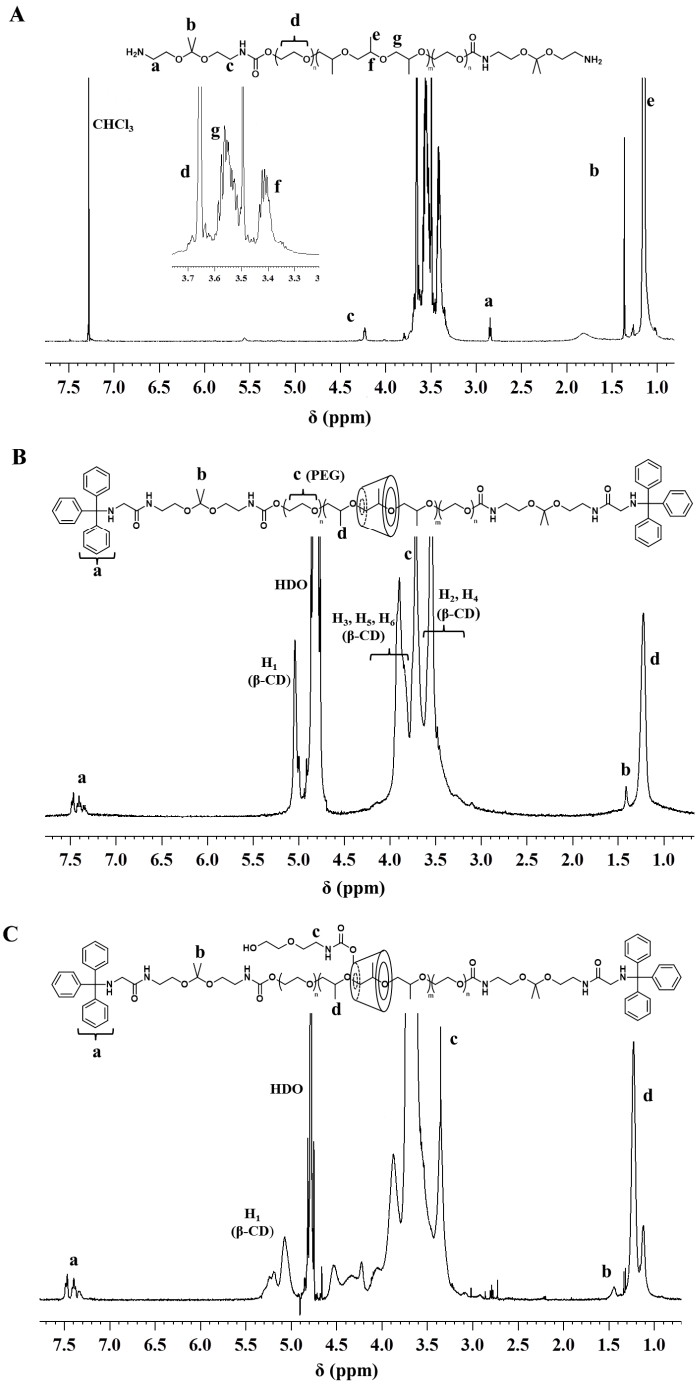

Fig. 2. ^{1}H NMR spectra of P123-ket-NH_2 in $CDCl_3$ (A), ket-PRX in D_2O (B), and the HEE-ket-PRX in D_2O (C).

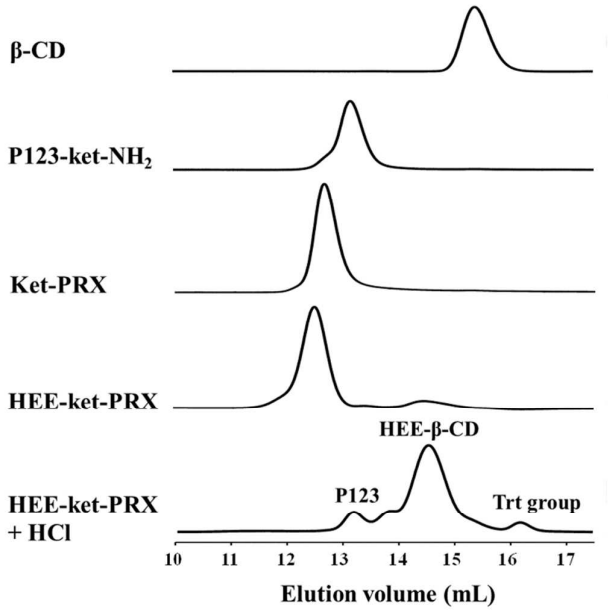

Fig. 3. SEC charts of β-CD, P123-ket-NH_2, ket-PRX, the HEE-ket-PRX, and the HEE-ket-PRX after the treatment with HCl.

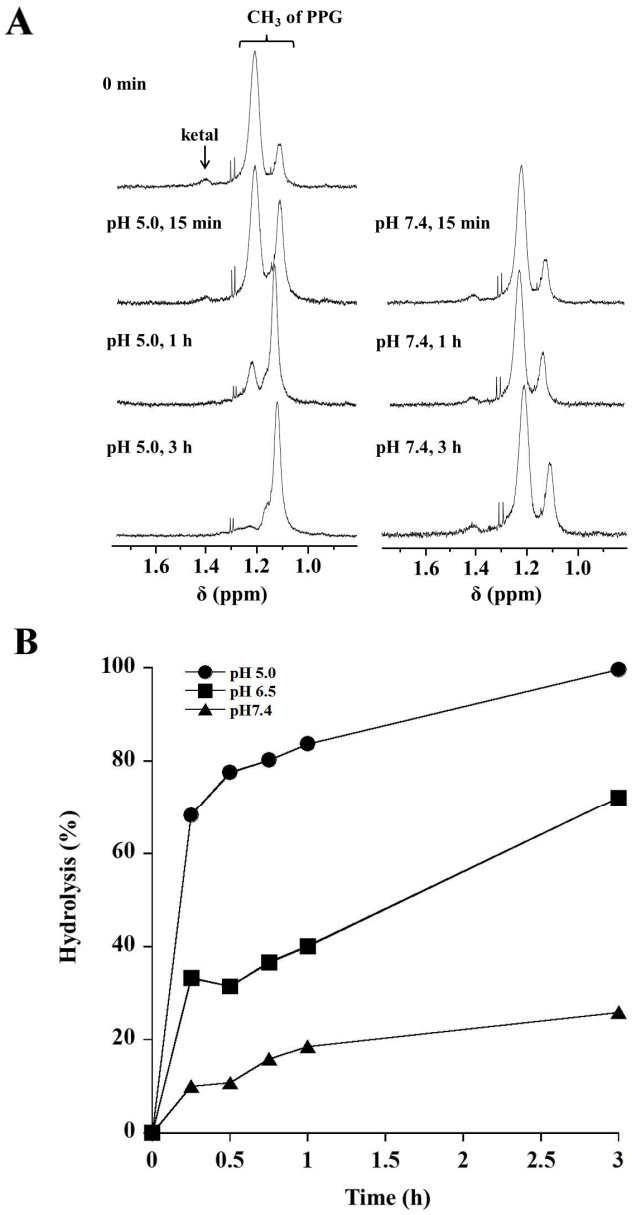

Fig. 4. (A) ^{1}H NMR spectra of ketal linkages and PPG segment of the axle polymer at pH 5.0 and 7.4 at various incubation time periods. (B) The time-course of hydrolysis of the HEE-ket-PRX at pH 5.0 (circles), 6.5 (triangles), and 7.4 (squares), as determined by ^{1}H NMR.

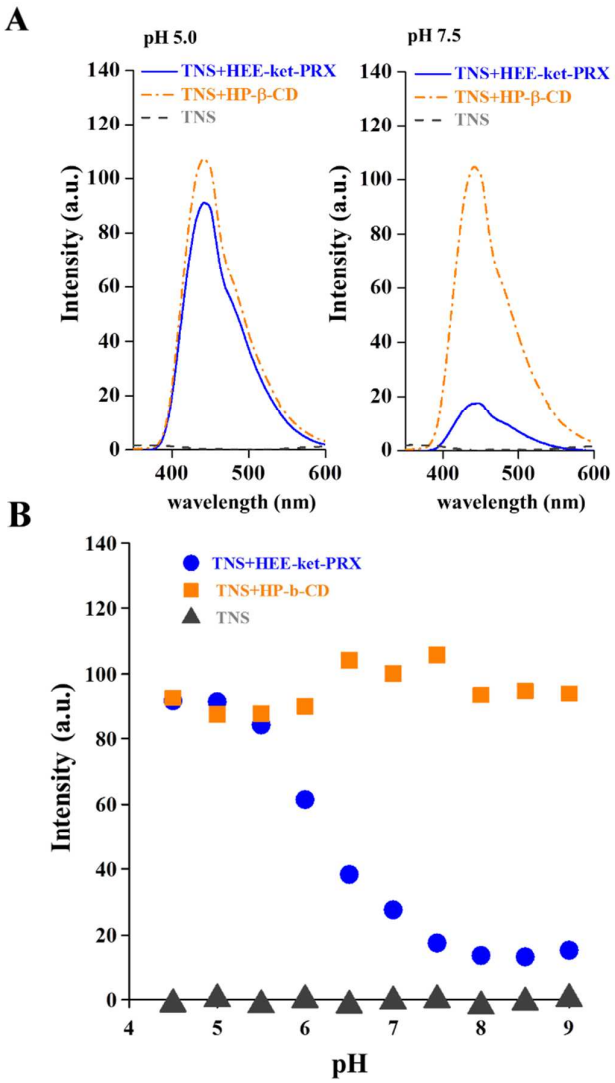

Fig. 5. (A) Emission spectra of TNS (20 μM) (dash), TNS + HP-β-CD (100 μM) (dash-dot), and TNS + HEE-ket-PRX (100 μM of β-CD) (solid) at pH 5.0 and pH 7.4. (B) pH-dependency of the fluorescence intensity at 440 nm for TNS (triangles), TNS + HP-β-CD (squares), and TNS + HEE-ket-PRX (circles).

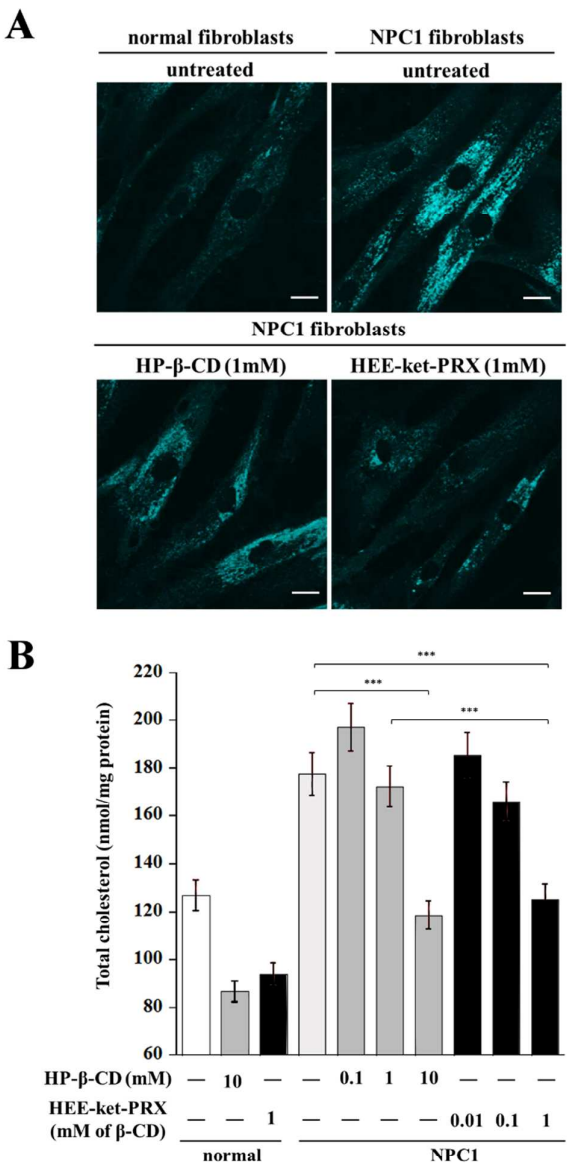

Fig. 6. (A) Filipin staining for cholesterol in the normal and NPC1 fibroblasts treated with HP-β-CD (1 mM) and the HEE-ket-PRX (1 mM of β-CD) for 24 h (scale bars: 20 μm). (B) The amount of total cholesterol in the normal and NPC1 fibroblasts treated with HP-β-CD and the HEE-ket-PRX at various concentration for 24 h. The values are expressed as the mean ± S.D. (n = 3) (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.005$.).

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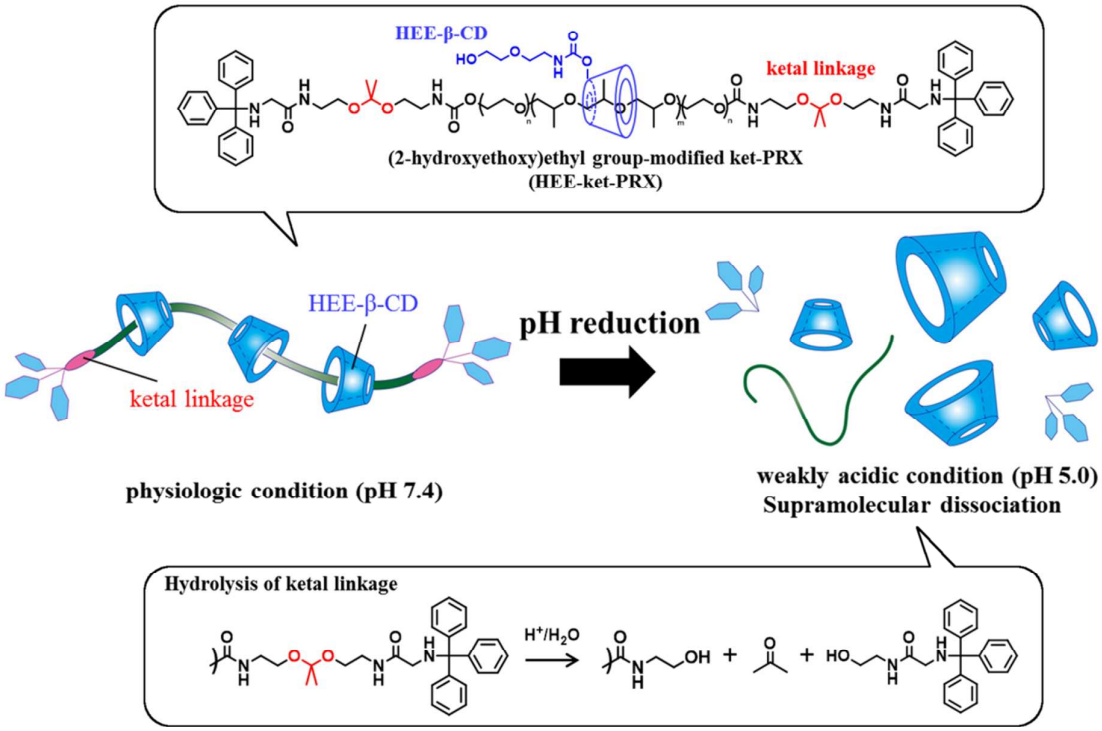

For achieving pH-sensitive complete dissociation and the subsequent release of threaded cyclic molecules from polyrotaxanes (PRXs) in weakly acidic environments such as endosomes and lysosomes, a novel acid-labile Pluronic/β-cyclodextrin (β-CD)-based PRX bearing ketal linkages was designed and its potential biomedical application was demonstrated.

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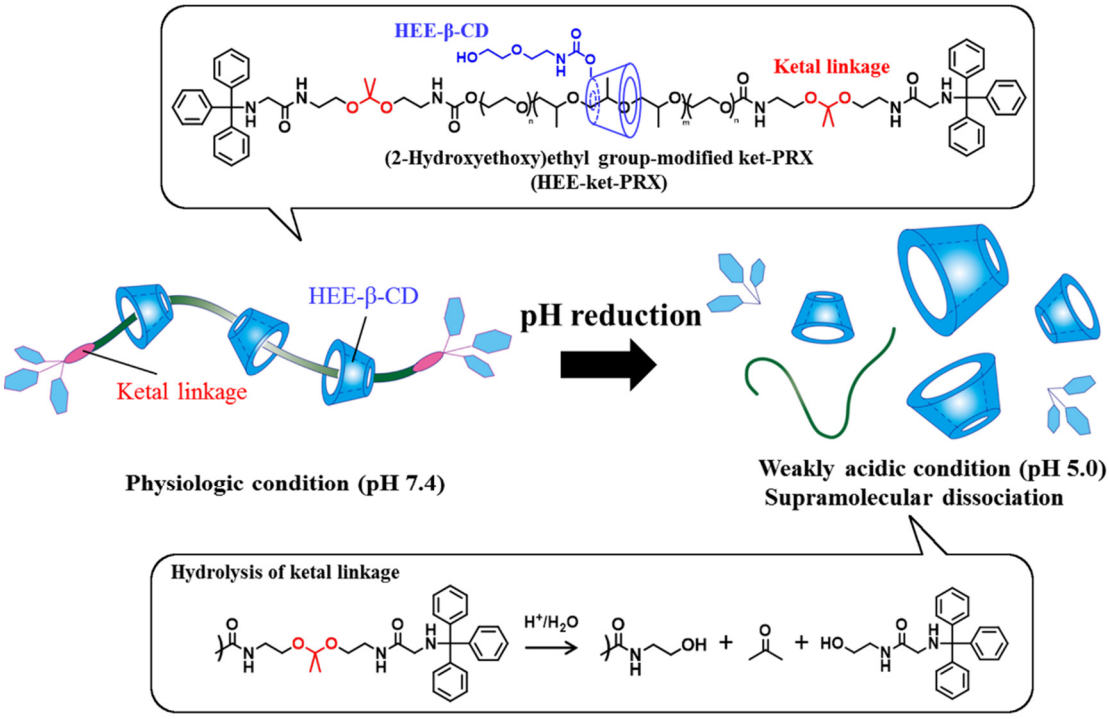

For achieving pH-sensitively complete dissociation and the subsequent release of threaded cyclic molecules from polyrotaxanes (PRXs) in weakly acidic environments such as endosomes and lysosomes, a novel acid-labile Pluronic/β-cyclodextrin (β-CD)-based PRX bearing ketal linkages was designed and its potential in biomedical application was demonstrated.