Published in final edited form as:
Biomacromolecules. 2013 December 9; 14(12): 4189–4197. doi:10.1021/bm400922a.

Synthesis of 2-Hydroxypropyl-β-cyclodextrin/Pluronic-Based Polyrotaxanes via Heterogeneous Reaction as Potential Niemann-Pick Type C Therapeutics

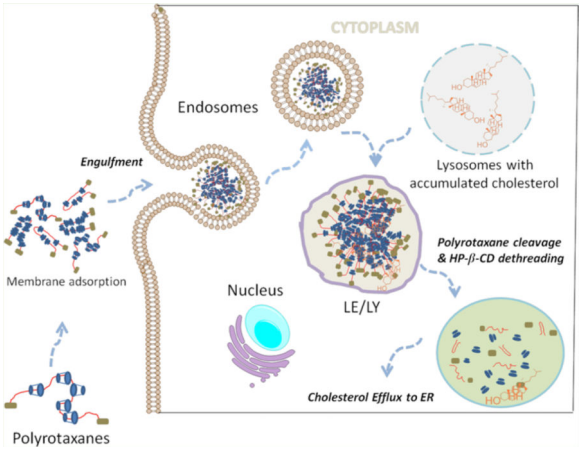
Yawo A. Mondjinou[‡,†], **Leslie A. McCauliff**[‖,†], **Aditya Kulkarni**[‡], **Lake Paul**[§], **Seok-Hee Hyun**[‡], **Zhaorui Zhang**[‡], **Zhen Wu**[‡], **Mary Wirth**[‡], **Judith Storch**[‖], and **David H. Thompson**[‡,*]

[‡]Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, Indiana 47907, United States

[§]Discovery Park - Bindley Biosciences Center, Purdue University, 1203 West State Street, West Lafayette, Indiana 47907, United States

[‖]Department of Nutritional Sciences, Rutgers University, 96 Lipman Drive, New Brunswick, New Jersey 08901-8525, United States

Abstract



Five polyrotaxanes were synthesized by threading 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) onto a variety of α,ω-ditriethylenediamino-*N*-carbamoyl-poly-(ethylene oxide)-*block*-poly(propylene oxide)-*block*-poly-(ethylene oxide) (Pluronic) triblock copolymers using a two-pot

[*]**Corresponding Author**. davethom@purdue.edu.
[†]These authors contributed equally to this work (Y.A.M. and L.A.M.).

ASSOCIATED CONTENT

S Supporting Information

^{1}H NMR and 2D NOESY spectra of all polyrotaxanes; UPLC chromatograms showing low free cyclodextrin levels in the polyrotaxanes; MALDI-MS; UV–visible spectra showing the shift of the TNBS end-cap absorbance, confirming the effectiveness of the end-capping reaction; AFM images with curvature profiles and size histograms; stability of the polyrotaxanes in buffer as depicted by HPLC analysis; and the quantitative filipin staining study of *npc*2$^{-/-}$ fibroblast cells after treatment with polyrotaxanes are reported. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

strategy under heterogeneous, nonaqueous conditions. The threaded HP-β-CD units were retained on the pseudopolyrotaxane precursors by end-capping the branched diamine termini with sodium 2,4,6-trinitrobenzene sulfonate. Inclusion of the Pluronic copolymers within the HP-β-CD cavities was more favorable in nonpolar solvents, such as diethyl ether and *n*-hexane, both of which gave better coverage ratios than polar solvents. ^{1}H NMR and MALDI-TOF were used to estimate the average molecular weights of the purified polyrotaxane products. A globular morphology of aggregated polyrotaxanes was observed by tapping-mode AFM imaging of dried samples. Treatment of Niemann-Pick C (NPC) type 2-deficient fibroblasts with the polyrotaxane derivatives produced substantial reductions in sterol accumulation, as seen by diminished filipin staining in these cells, suggesting that Pluronic-based polyrotaxanes may be promising vehicles for delivery of HP-β-CD to cells with abnormal cholesterol accumulation.

INTRODUCTION

Niemann-Pick type C (NPC) is a lysosomal storage disorder characterized by the accumulation of unesterified cholesterol and other lipids in the late endosome/lysosome (LE/LY) compartment. Aberrant accumulation of cholesterol in NPC cells has been shown to originate from mutation of the genes encoding either of two LE/LY proteins, membrane-bound NPC1 or soluble NPC2, both of which are required for cholesterol efflux from the lysosome.[1,2] Unfortunately, the treatment options are limited for this typically fatal disease.[3,4] Several studies have shown that β-cyclodextrin (β-CD) and its derivatives, including 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), are able to mobilize the accumulated cholesterol from the LE/LY compartment in NPC mouse models, resulting in extended lifespan.[5–9] Liu and co-workers reported that the subcutaneous injection of HP-β-CD (4.0 g/kg of body weight) into *npc*1$^{-/-}$ mice produced improvements in their survival, hepatopathology, and neuropathology.[6] Administration of HP-β-CD to either *npc*1$^{-/-}$ or *npc*2$^{-/-}$ patient fibroblasts has also been shown to stimulate efflux of the accumulated intracellular cholesterol, resulting in a reversal of the disease phenotype at the cellular level.[10,11] Although these results are promising, it is still unclear how HP-β-CD causes the release of cholesterol from cells in human NPC disease. Furthermore, in animals and humans, high dosages or continuous administration of HP-β-CDs are required since their persistence in the bloodstream is brief (>90% is cleared within 24 h) due to their appreciable water solubility and relatively low molecular weight (≈ 1460 g·mol^{-1}). Thus, the design of long circulating, biocompatible macromolecules capable of delivering multiple copies of HP-β-CD to the LE/LY of NPC cells could improve the cholesterol clearance properties of cyclodextrins for NPC therapy.

Polyrotaxanes are a new class of supramolecular materials that have been evaluated in cell culture systems for their gene delivery[12–14] and drug release[15,16] characteristics. Since the first syntheses of α-CD/poly(ethylene oxide) (PEO) polyrotaxanes,[17–20] many efforts have focused on developing new generations of these macromolecular assemblies using different stoppers and cyclodextrin monomers.[21–38] The common characteristic of a polyrotaxane architecture is the threading of macrocyclic host molecules onto a polymer chain of compatible dimensions via host–guest hydrophobic interactions, followed by capping the

ends of the polymeric chain with bulky molecules to form a molecular necklace type structure.[17]

Cyclodextrins are natural macrocyclic oligosaccharides produced by the enzymatic cyclization of 6, 7, or 8 (+)-glucopyranoside units linked by α-1,4-connections to generate α-, β-, or γ-CD, respectively. These glucopyranoside macrocycles possess a toroidal topology with a hydrophobic internal cavity. β-CD and its derivatives have garnered the most attention due to their widespread use in the pharmaceutical and food industry,[39] where they are used as solubilizing agents, permeability enhancers, and active ingredient stabilizers. 2-Hydroxypropyl-β-cyclodextrin is an attractive precursor for polyrotaxane synthesis; however, since HP-β-CD is substantially more water-soluble at room temperature (0.65 g/mL in water) than β-CD (0.018 g/mL) and several drug formulations using HP-β-CD as an inactive pharmaceutical ingredient have been FDA-approved. This high solubility in aqueous solution makes it a good candidate for designing well-tolerated HP-β-CD-based polyrotaxanes that could enhance the pharmacokinetics and biodistribution of HP-β-CD as an NPC therapeutic. Recently, we have shown that substantial reduction of cholesterol in *npc*$2^{-/-}$ fibroblasts occurs upon their treatment with β-cyclodextrin (β-CD) based polyrotaxanes.[40] Herein, we report the development of polymeric HP-β-CD-based complexes endowed with enzymatically cleavable linkages to control the rate of cyclodextrin release in cell culture as an initial step toward evaluation of these compounds in animal models of NPC disease.

Pluronic surfactants, a family of poly(ethylene oxide)-*block*-poly(propylene oxide)-*block*-poly(ethylene oxide) (PEO-PPO-PEO) triblock copolymers, enjoy a wide range of applications due to their favorable biocompatibility and low toxicity.[41–43] A growing body of evidence shows that β-CD forms inclusion complexes with Pluronic surfactants via complexation with the central PPO blocks,[44–49] however, there are no reports to our knowledge describing HP-β-CD/Pluronic polyrotaxanes. This is likely due to the low threading efficiency of 2-hydroxylpropyl-β-cyclodextrin onto the Pluronic core if the conventional aqueous threading conditions are used. Recently, inclusions of lipophilic compounds with cyclodextrin in nonpolar solvents have been reported.[50,51] Takata et al. have reported an interesting heterogeneous synthesis of polyrotaxanes in a high-yield onepot reaction using permethylated α-CD and core polymers such as amine-terminated poly(tetrahydrofuran) and amine-terminated poly(ethylene oxide) in hydrocarbon solvents.

In this paper, we report the synthesis of HP-β-CD-based Pluronic polyrotaxanes under heterogeneous conditions using hexane as the solvent. Five different polyrotaxanes were prepared with various Pluronic triblock copolymer cores. 2,4,6-Trinitrobenzene (TNB) end-capping was chosen because the reaction is facile and generates a polyrotaxane product that is easily monitored by absorption spectroscopy. These complexes were designed to dethread in NPC cells by enzymatic cleavage of the TNB end-caps. This, in turn, would result in the release of the cyclodextrin monomers within the LE/LY compartment where they can facilitate cholesterol efflux. NMR, MALDI-TOF/ MS, and UV–visible spectroscopy methods were used to characterize the resulting materials and determine molecular weights of the polyrotaxanes. Tapping-mode atomic force microscopy (AFM) imaging of these materials was also performed. Filipin staining analysis of these cyclodextrin-carriers in

*npc*2$^{-/-}$ fibroblasts revealed that they promote the removal of aberrantly accumulated cholesterol from these cells.

EXPERIMENTAL SECTION

Materials

Pluronic triblock copolymers F127 (EO 200, PO 65), F68 (EO 153, PO 29), L35 (EO 22, PO 16), L64 (EO 26, PO 30), and L81 (EO 6, PO 43) were purchased from Sigma-Aldrich and dried by azeotropic distillation from benzene under vacuum before use. 2-Hydroxypropyl-β-cyclodextrin, carbonyldiimidazole (CDI), triethylamine (TEA), and tris(2-aminoethyl)amine (TAEA) were also purchased from Sigma-Aldrich and were used directly. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) solution, 10% *w/v* in water, was obtained from Research Organics and used as received. All solvents were distilled from an appropriate desiccant prior to use. Cellulose dialysis membranes were obtained from Spectrum Laboratories and immersed in deionized water for at least 30 min prior to use. Ultrapure water (resistivity ≈ 18.0 MΩ/cm^{-1}) was generated from a NANOpure Ultrapure water system. Human fibroblasts from an NPC2 patient (GM18455) were from Coriell Institute of Medical Research (Camden, NJ). Filipin was purchased from Sigma.

Synthesis of Bis-2,4,6-trinitrobenzene-End-Capped HP-β-CD/Pluronic Polyrotaxanes

Preparation of α,ω-Bis-tris(2-aminoethyl)amine Pluronic Triblock Copolymer (TAEA-Pluronic)—The typical synthetic procedure of the TAEA-Pluronic derivatives is described as follows. Dried Pluronic copolymer (0.400 mmol) was dissolved in 30 mL of dry CH_2Cl_2. Triethylamine (1.5 equiv) was slowly added over 30 min on an ice bath. The mixture was allowed to slowly warm up to 20 °C before addition of excess CDI (20.0 mmol). This mixture was then stirred under nitrogen for 24 h at 20 °C and concentrated with a rotary evaporator. The product was precipitated in 500 mL ether and filtered in the cases of the solid Pluronic surfactants F127 and F68. The crude product was washed with ether, filtered, and vacuum-dried to afford 70–98% of a white powder of α,ω-biscarbonylimidazole Pluronic triblock copolymer. In the case of liquid Pluronic surfactants (L35, L64, L81), the products were washed by centrifugation (8000 rpm, 5 min, 20 °C). The crude CDI-activated Pluronic intermediates (3.53g; 0.276 mmol) were dissolved in 30 mL dry CH_2Cl_2 before addition of tris(2-aminoethyl)amine (13.8 mmol). The mixture was then stirred under dry N_2 at 20 °C for 24 h. The product was precipitated in 300 mL diethyl ether and washed three times with diethyl ether by either centrifugation (liquid Pluronic materials) or filtration (solid Pluronic materials). The final product was dried under a 50 μm Hg vacuum for 72 h to yield either white powders or yellow liquids of α,ω-bis-tris(2-aminoethyl)amine Pluronic intermediates (Pluronic-TAEA). ^{1}H NMR (D_2O): δ 1.00 ppm (m, CH_3 of PPO), 2.60–2.80 ppm (m, 16H, CH_2 of TAEA), 3.54– 3.65 ppm (m, CH_2 of PEO, and PPO, CH of PPO).

Preparation of TNB-End-Capped Polyrotaxanes: General Protocol—Dried Pluronic-TAEAs (0.04 mmol) and 2-hydroxypropyl-β-cyclodextrin (i.e., ratio of CD/PPO unit = 1:2) were dissolved (or suspended) in 15 mL of hexane and the mixture vortexed for 3 min before vigorously stirring for 2 h. Then, bath sonication for 30 min at 30 °C, followed

by 5 min probe sonication (Model W-350, 50 W, 1/2″ probe) was performed to improve the threading efficiency of the Pluronic copolymers. Then, the mixture was stirred for 48 h at 20 °C and shaken on a rocking plate for an additional 24 h before removal of hexane and addition of water to make a slurry suspension to which 2,4,6-trinitrobenzenesulfonic acid solution (10% w/v in water, 0.24 mmol) and $NaHCO_3$ (0.24 mmol) were added. The mixture was stirred at 20 °C for 24 h and then mixed an additional 24 h on a rocking plate to allow the products to aggregate and precipitate. The unreacted reagents and unthreaded cyclodextrins were removed by twice dissolving the crude product in 10 mL of methanol and precipitating the product by addition of 500 mL diethyl ether. The product was purified by dialysis using 3500–14000 MWCO regenerated cellulose membranes in deionized water for 8 d and dried by lyophilization to generate yellow-orange powders of polyrotaxanes. ^{1}H NMR (DMSO-d_6): δ 8.7 ppm (s, 8H, Ph *m*-H), 5.0 ppm (b, C_1-H of CD), 4.0 ppm (t, 4H, phenyl C-NH), 3.5–3.8 ppm (m, $C_{3,5,6}$-H of CD), 2.6–2.8 ppm (m, 16H, CH_2 of TAEA), 1.0 ppm (d, CH_3 of PPO).

Nuclear Magnetic Resonance, NMR

^{1}H NMR and two-dimensional NOESY spectra were collected on either a Bruker Advance ARX400 or a DRX 500–1 NMR spectrometer equipped with Unishell-QNP and Topspin-TBI probes, respectively. Spectra were recorded at 25 °C using DMSO-d_6 as solvent unless otherwise indicated, using approximately 15 mg of each polyrotaxane dissolved in 1 mL of solvent.

Ultraviolet–Visible Spectroscopy

Absorption spectra, recorded using a HP8453 UV–vis spectrophotometer equipped with tungsten and deuterium lamps, were measured to confirm the effectiveness of the TNBS end-capping reaction with the pseudopolyrotaxane precursors. The samples were dissolved in water (1 mg/mL) and spectra were recorded at 20 °C.

Matrix-Assisted Laser Desorption Ionization Time-Of-Flight, MALDI-TOF

MALDI-MS spectra were acquired over a mass range of 1500–35000 Da in positive-ion reflector mode on an Applied Biosystems/MDS Sciex 4800 MALDI-TOF/TOF Analyzer with 4000 Series Explorer v3.5 software using a laser power of 6000 and 6500 laser shots in linear mode. The matrix consisted of a freshly prepared ionic liquid matrix (ILM) made using a previously described protocol with some modifications.[52] Briefly, 2′,4′,6′-trihydroxyacetophenone monohydrate (THAP) and 1,1,3,3-tetramethylguanidine (TMG) were mixed at a molar ratio of 1:2 in methanol. The solution was then sonicated for 15 min at 40 °C. After removal of methanol by centrifugal evaporation in a SpeedVac for 3 h at 20 °C, ILMs were left under a 50 μm Hg vacuum overnight. Final ILM solutions were then prepared at a concentration of 90 mg/mL in DMF for use as a matrix. The polyrotaxane samples were prepared at 3 mg/mL in DMF and then mixed in a 1:80 polyrotaxane/ILM ratio for MALDI-MS analysis. Then, 0.6 μL of the polyrotaxane/ILM mixture was deposited onto a mirror-polished stainless steel MALDI target and allowed to dry at 20 °C under atmospheric pressure overnight before analysis.

Atomic Force Microscopy

The topology (diameter, height) of the polyrotaxane particles was determined in air at 22 °C by tappingmode atomic force microscopy using a Multimode AFM equipped with Nanoscope IIIa controller (Veeco Instruments, U.S.A.), an uncoated probe tip of 10 nm or less (NSC15/A1BS, MikroMasch, U.S.A.), and cantilevers having a spring constant of 40 N/m. In a typical measurement, 7.0 μL of the polyrotaxane sample (1.0×10^{-9} mg/mL in water) was deposited onto a mica surface that was cleaned by probe sonication and dried using a TechSpray duster containing 1,1,1,2-tetrafluoroethane gas.

High-Performance and Ultra-Performance Liquid Chromatography, HPLC/UPLC

An Agilent series 1200 HPLC coupled with an ESA Corona detector was employed for the dethreading studies of the polyrotaxanes. In this assay, the cyclodextrin peak in the chromatogram was integrated and the concentration of HP-β-CD, obtained from polyrotaxane cleavage, was determined by comparison with a standard curve for 1 mL aliquots of aqueous polyrotaxane solution that was treated with one of two different buffers (pH 7.4 or 5.5) at 37 °C. The aqueous solutions of polyrotaxanes (2.0 mg/mL) were filtered through a 0.2 μm cellulose membrane filter before injection. The calibration curve was constructed by analyzing different concentrations of HP-β-CD standard dissolved in water. The separation was performed at 50 °C on an Agilent reversed-phase Zorbax Eclipse XDB-phenyl column (2.1×150 mm^2, particle size 5 μm). The mobile phase composition was a mixture of water (A) and acetonitrile (B) in a gradient elution at a flow-rate of 0.25 mL/min. The water/acetonitrile mixture composition was as follows: 0–9 min, water (100%), 9–11 min, water/acetonitrile (40/60, v/v), 11–12 min, water/acetonitrile (29/ 71, v/v), and 12–25 min, water (100%). UPLC-MS analysis was performed as an independent measurement to determine the percentage of free cyclodextrin in the samples using a Thermo Accela UPLC system (Thermo Fisher Scientific, Waltham, MA, U.S.A.) coupled to a Thermo LTQ Velos mass spectrometer. A homemade hydrophilic interaction column (2.1×30 mm, 700 nm nonporous silica particles coated with polyacrylamide) was used as the stationary phase. The temperature of the column oven was maintained at 25 °C. Stock aqueous solutions of HP-β-CD were prepared at different concentrations in the range of 0.05–2 mg/mL in water as calibration standards.

Effect of Polyrotaxanes on Cholesterol Accumulation in NPC2-Deficient Cells

To assess the therapeutic potential of HP-β-CD/Pluronic polyrotaxanes in an appropriate tissue culture model, human NPC2-deficient fibroblast cells (*npc*$2^{-/-}$) were treated with the polyrotaxanes. Cells were seeded on eight-well tissue culture slides at a density of 6×10^3 cells, in Eagle's minimum essential medium with Earle's salts and nonessential amino acids containing 15% fetal bovine serum and penicillin/streptomycin (MEM/FBS/pen/strep) at 37 °C and 5% CO_2. Each compound was solubilized in DMSO and diluted in cell culture media to a concentration yielding the equivalent of 25 μM free HP-β-CD and a final DMSO concentration of 0.001 (*v/v*). From 36 to 48 h after plating, medium was removed from the cells and the media containing each polyrotaxane sample was added. Cells were fixed at 0.5, 1.0, 3.0, and 6.0 h post-treatment with 10% buffered formalin, then stained with 0.05 mg·mL^{-1} filipin. Slides were prepared with Prolong Gold antifade reagent (Molecular

Probes) to suppress photobleaching. The reduction of cholesterol accumulation was monitored qualitatively by imaging the filipin stain in the cells, and quantitatively by the determination of filipin stain area to total cell area, as previously described.[10] Results are expressed relative to control untreated cells and are represented as mean ± SE (n = 3). No changes in cell number or viability (>95%), assessed by trypan blue exclusion, were found for any of the compounds under any of the treatment conditions.

RESULTS AND DISCUSSION

Preparation and Characterization of HP-β-CD/Pluronic Polyrotaxanes

The synthesis of HP-β-CD/Pluronic polyrotaxanes was performed via the sequence shown in Scheme 1.

The preparation of tris(2-aminothyl)amine-modified Pluronic was achieved by slight modification of the method reported by Li and co-workers.[53] Even though several studies have reported inclusion complex formation between unmodified β-CD and Pluronic surfactants in excellent yields,[45,46] our first attempts to thread HP-β-CD onto the various Pluronic copolymer precursors in water were unsuccessful even though multiple reports described high yield threading reactions between saturated aqueous solutions of β-CD in water containing Pluronic copolymer.[17–20] We infer from these observations that the low monomer solubility of the Pluronic surfactants in water keeps them in their micellar form, thus, preventing the HP-β-CD molecules from being able to access the hydrophobic polymer segments to initiate the threading reaction. Based on the work of Takata and co-workers,[54] we explored the use of organic solvents for the threading reaction. Several solvents (3 mL) were used to dissolve 100 mg of Pluronic F127-TAEA and HP-β-CD in a 2:1 PPO/CD ratio. The turbid solutions were sequentially bath- and probe-sonicated, followed by stirring at 20 °C for 48 h. Low boiling solvents (e.g., dichloromethane, methanol, diethyl ether, ethyl acetate, and hexane) were removed under reduced pressure to yield white pseudopolyrotaxane intermediates. Subsequent addition of an excess of TNBS as an aqueous slurry suspension in the presence of $NaHCO_3$, followed by stirring of the orange viscous solutions at 20 °C for 24 h, produced end-capped polyrotaxanes that were purified by solvent washing and dialysis. For rotaxanation reactions in higher boiling solvents such as water, DMSO, and DMF, the TNBS end-capping reagent was added directly, followed by a washing and dialysis purification procedure. A summary of the impact of solvent type on the reaction yield of polyrotaxane and corresponding percent coverage of the Pluronic PPO block is shown in Table 1.

^{1}H NMR spectroscopy analysis was used to determine the number of cyclodextrins threaded onto the Pluronic axle by comparing the integral intensities of the HP-β-CD C_1–H (5.05 ppm) and PPO CH_3 (1.0 ppm) signals. The coverage ratio was calculated based on the assumption that two PPO units are capable of inclusion per CD unit. Our data show that nonpolar solvents such as hexane and diethyl ether promote higher threading efficiencies than water, D_2O, methanol, DMSO, or DMF, which show little or no sign of HP-β-CD in the product NMR spectra. We infer from these findings that poor solvents for the cyclodextrins promote aggregation of HP-β-CD through hydrogen bond interactions between their wide and narrow faces, thereby forming hydrophobic tunnels that enable more facile threading by

the Pluronic chains. Additionally, we propose that the nonpolar solvents reduce self-association of the Pluronic copolymers by solvating their lipophilic PPO blocks. The polyrotaxane structure, obtained by threading HP-β-CD onto Pluronic F127 in hexane solution, was confirmed by ^{1}H NMR, as shown in Figure 1B. The proton peak at ~1.0 ppm is assigned to the PPO methyl groups on the copolymer, whereas the proton signals in the 3–3.5 ppm region are attributed to the methylene units (CH_2) of the PEO and some of the HP-β-CD protons. The broad signal displayed in the 4.5– 5.0 ppm region is assigned to the HP-β-CD C_1–H proton as well as the OH-8 proton of the 2-hydroxypropyl cyclodextrin modification. The aromatic TNB proton signals can be observed further downfield in the region of 8–9 ppm. The average number of HP-β-CD units threaded onto the PPO block was estimated from the relative intensities of the ^{1}H NMR signals attributed to the C_1–H, OH-8 HP-β-CD, and PPO methyl signals.

To further confirm the rotaxanation reaction between HP-β-CD and the F127 Pluronic axle, two-dimensional-NOESY ^{1}H NMR spectra were collected. As shown in Figure 2, the inner $C_{3,5}$-H protons of HP-β-CD display a spatial correlation with the PPO methyl groups. This result is consistent with previous reports for β-CD-polymer complexes,[53,55–57] suggesting that HP-β-CD molecules were threaded onto the F127 Pluronic chains and proximal to the PPO methyl substituents. Furthermore, to determine whether the end-capping reaction was effective, UV–visible spectroscopy was performed on aqueous solutions (0.5 mg/mL) of HP-β-CD/F127 Pluronic polyrotaxane and free TNBS (Figure SI5). The absorption maxima of the polyrotaxane complex (ca. 345 nm, 422 nm) differs from that of the unreacted TNBS precursor. This finding is in agreement with the work of Fleury et al.,[58] suggesting that the corresponding HP-β-CD/F127 Pluronic polyrotaxane product was fully end-capped.

Encouraged by these results, we implemented the same reaction conditions to prepare polyrotaxanes based on the other four Pluronic copolymers (F68, L35, L64, and L81) with differing PEO and PPO block lengths. In these cases, 0.04 mmol, dissolved in 15 mL of hexane was used for all the other Pluronic threading reactions.

Table 2 summarizes the effect of PPO block size on the percent coverage relative to the maximum theoretical coverage possible for the PPO block. These data show that the threading efficiency is inversely proportional to the hydrophilic–lipophilic balance (HLB) of the Pluronic axle, with high coverages observed for L81 and L64. These findings are consistent with our hypothesis that nonpolar solvents favor the rotaxanation reaction by promoting interactions between the hydrophobic Pluronic PPO block and the hydrophobic cavity of the self-associated HP-β-CD monomers. F127 is an exception to this trend, likely due to the large PEO blocks that flank the PPO core, thereby suppressing the rotaxanation reaction due to weaker hydrophobic interactions between the cyclodextrin cavity and the large PEO blocks that must be traversed before the HP-β-CD units reach the hydrophobic PPO block that stabilizes the HP-β-CD inclusion.

MALDI-TOF MS Analysis of HP-β-CD/Pluronic Polyrotaxanes

MALDI-TOF mass spectrometry was used to determine the distribution of molar masses of the polyrotaxane products formed by the sequence shown in Scheme 1. NMR spectroscopy and SEC chromatography are the most common methods employed for polyrotaxane

characterization, however, Jarroux et al. have recently reported the analysis of polydimethyl siloxane:cyclodextrin polyrotaxane compositions and molecular weights using MALDI-TOF.[59] A 1:80 polyrotaxane/ILM matrix composition, initially evaluated for HP-β-CD/F127 Pluronic polyrotaxane with a THAP/TMG mixture (1:2 ratio in methanol), was found to produce the best signal-to-noise ratios. Figure SI3 shows the spectra of all five polyrotaxanes. In each spectrum, a range of peaks corresponding to different degrees of HP-β-CD threading (n_{CD}) was observed. The peak intensity was found to decrease with increasing polyrotaxane *m/z* values, until the signal was no longer discernible from the baseline. For all polyrotaxanes, the observed *m/z* values corresponded to the sum of TNB-end-capped Pluronic chains +1460n_{CD}. Interestingly, the spectra reveal a stepwise increment of mass differing by 1460 Da, corresponding to the molar mass of the HP-β-CD monomer. Furthermore, the most intense ion peak families were in agreement with the values calculated by NMR as summarized in Table 2; however, the spectral profiles show that the polyrotaxane products are polydisperse compounds (Figure SI3).

AFM Imaging of Polyrotaxanes

Using tapping-mode AFM, we were able to observe the microstructures of aggregated polyrotaxanes. Figure 3 reveals that all the polyrotaxanes appear as large globular aggregates of different sizes, with average diameters ranging between 47 to 80 nm and heights varying between 0.5 and 2 nm. These data show that the polyrotaxane molecules cluster into spherical assemblies when dried from an aqueous solution onto the mica substrate, presumably due to lateral hydrogen bond interactions between the rotaxanated HP-β-CDs. The combination of low threading efficiencies and flexible, unthreaded PEO ends promotes the aggregation of the hydrophobic PPO/HP-β-CD domains into spherical particles that are surrounded by a PEO corona as reported by Zhang et al.[55,60] The spherical appearance of these nanoparticles, if persistent in blood, suggests that they may possess attractive long-circulation properties in vivo by avoiding their rapid clearance from blood via renal filtration.

npc2$^{-/-}$ Fibroblast Response to Polyrotaxane Exposure

The noncovalent association of HP-β-CD with Pluronic-based polyrotaxanes confers these polymers with the ability to readily dethread the cyclodextrin units from the polymer axles upon removal of the end-capping group. Because the main goal of this work is to design polyrotaxanes that will release HP-β-CD upon activation within the LE/LY compartment of NPC cells so as to promote free HP-β-CD-mediated cholesterol efflux from the cells, we initially investigated the end-cap cleavage reaction and dethreading kinetics of HP-β-CD/Pluronic polyrotaxane complexes that were exposed to buffers of different pH as a mimic of their response to neutral (pH 7.4) and acidic endosome compartments (pH 5.5). HPLC analysis of F127 based-polyrotaxane (07HP.F127, 2 mg/mL), exposed at 37 °C to either pH 7.4 PBS buffer or pH 5.5 citrate buffer, revealed that the HP-β-CD/F127 Pluronic polyrotaxane is stable for up to two days toward both mildly acidic and neutral pH conditions (Figure SI6). Although this result was encouraging in terms of the stability of the polyrotaxane particles under physiological conditions prior to endocytosis, it suggested that low pH-mediated end-cap cleavage from the polyrotaxane carrier within acidic late endosomes/lysosomes would be slow in those cellular compartments. Nonetheless, a

substantial and rapid decrease in filipin staining was observed after polyrotaxane treatment of *npc*2$^{-/-}$ fibroblasts that contained large pools of aberrantly stored cholesterol, providing a qualitative indication of cholesterol reduction within these cells (Figure 4). Time-dependent evaluation of filipin staining in these cells provided further evidence of reduced cholesterol accumulation for all the polyrotaxane compounds, to levels that were similar to the extent of cholesterol reduction that is produced by 25 μM free HP-β-CD (i.e., 60 to 90% of untreated controls).

These findings suggest that the polyrotaxanes were internalized and dethreaded within the *npc*2$^{-/-}$ cells, thereby releasing free HP-β-CD that could then mobilize aberrantly stored cholesterol. Given the stability of the compounds at pH 5.5, we believe that the TNB group is nevertheless cleaved from the polyrotaxane by either an enzymatic or reduction reaction occurring within the LE/LY compartment. There is a significant body of data indicating that nitrobenzene substrates can be reduced by nitroreductase enzymes[61–64] that are present in numerous human tissues.[65] Based on these findings and the sequential reduction mechanism of aromatic nitro compounds proposed by Biaglow et al.,[66] we infer that the carbamyl-linked trinitrobenzene end-caps of the polyrotaxanes may be reduced to sterically smaller amine substituents that enable the cyclodextrins to slip off the polymer axles. Enzymatic end-cap cleavage is another potential mode of HP-β-CD release;[67] however, enzyme-mediated prodrug activation has not been reported for NPC cells to our knowledge. Therefore, an alternative explanation is that the carbamate linkage attaching the end-cap to the polyrotaxane scaffold may serve as a substrate for tyrosinase hydrolysis in NPC cells, thus triggering end-cap removal and subsequent dethreading of the polyrotaxane. Work is in progress to delineate the operative mechanism of polyrotaxane dethreading and cholesterol efflux in NPC cells.

CONCLUSION

We have demonstrated that HP-β-CD/Pluronic polyrotaxanes can be synthesized under nonaqueous heterogeneous conditions, with nonpolar solvents such as hexane and diethyl ether favoring the formation of polyrotaxane products with high coverage ratios of HP-β-CD. The structures of the polyrotaxane products were confirmed by NMR and UV–visible spectroscopy analysis, with tapping-mode AFM serving to determine the topology of the polymeric particles. It was also shown that the compounds were stable for hours under mildly acidic and neutral pH conditions. Results obtained from the treatment of *npc*2$^{-/-}$ fibroblasts with various HP-β-CD/Pluronic polyrotaxane species showed a substantial reduction of the accumulated cholesterol that was equivalent to the unmodified HP-β-CD monomer. The chemical basis of the dethreading mechanism remains unclear at this time. Taken together, our results suggest that HP-β-CD/Pluronic polyrotaxanes are promising vehicles for delivery of HP-β-CD as a potential NPC therapeutic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by the National Niemann-Pick Disease Foundation BreakThrough Fund (D.H.T.), the Ara Parseghian Medical Research Foundation (J.S.), and an American Heart Association predoctoral fellowship (L.A.M.).

REFERENCES

1. Patterson, MC.; Vanier, MT.; Suzuki, K.; Morris, JA.; Carstea, E.; Neufeld, EB.; Blanchette-Mackie, JE.; Pentchev, PG. The metabolic and molecular bases of inherited disease. 8th ed.. Scriver, C.; Beaudet, A.; Sly, W.; Vale, D., editors. New York, New York, U.S.A.: McGraw-Hill Professional Publishing; 2001. p. 3611
2. Kwon HJ, Abi-Mosleh L, Wang ML, Deisenhofer J, Goldstein JL, Brown MS, Infante RE. Cell. 2009; 137:1213. [PubMed: 19563754]
3. Wraith, JE.; Imrie, J. Understanding Niemann-Pick disease type C and its potential treatment. U.K.: Blackwell Publishing; 2007.
4. Patterson MC, Platt F. Biochim. Biophys. Acta. 2004:1685–1677.
5. Camargo F, Erickson RP, Garver WS, Hossain GS, Carbone PN, Heidenreich RA, Blanchard J. Life Sci. 2001; 70:131. [PubMed: 11787939]
6. Liu B, Li H, Repa JJ, Turley SD, Dietschy JM. J. Lipid Res. 2008; 49:663. [PubMed: 18077828]
7. Liu B, Turley SD, Burns DK, Miller AM, Repa JJ, Dietschy JM. Proc. Natl. Acad. Sci. U.S.A. 2009; 106:2377. [PubMed: 19171898]
8. Lope-Piedrafita S, Totenhagen JW, Hicks CM, Erickson RP, Trouard TP. J. Neurosci. Res. 2008; 86:2802. [PubMed: 18512758]
9. Davidson CD, Ali NF, Micsenyi MC, Stephney G, Renault S, Dobrenis K, Ory DS, Vanier MT, Walkley SU. PLoS One. 2009; 4:e6951. [PubMed: 19750228]
10. McCauliff LA, Xu Z, Storch J. Biochemistry. 2011; 50:7341. [PubMed: 21740003]
11. Anton IR, Zhang G, Warren JD, Maxfield FR, Breslow JL. Proc. Natl. Acad. Sci. U.S.A. 2010; 107:5477. [PubMed: 20212119]
12. Li J, Loh XJ. Adv. Drug Delivery Rev. 2008; 60:1000.
13. Kulkarni A, DeFrees K, Schuldt RA, Hyun S-H, Wright KJ, Yerneni CK, VerHeul R, Thompson DH. Mol. Pharmacol. 2013; 10:1299.
14. Kulkarni A, DeFrees K, Schuldt RA, Vlahu A, VerHeul R, Hyun S-H, Deng W, Thompson DH. Integr. Biol. 2013; 5:115.
15. Yui, N.; Katoono, R.; Yamashita, A. Inclusion Polymers. Wenz, G., editor. Vol. 222. Berlin: Springer-Verlag; 2009. p. 55
16. Zhang X, Ke F, Han J, Ye L, Liang D, Zhang A-y, Feng Z-g. Soft Matter. 2009; 5:4797.
17. Harada A, Kamachi M. Macromolecules. 1990; 23:2821.
18. Harada A, Li J, Kamachi M. Nature. 1992; 356:325.
19. Harada A, Li J, Kamachi M. Macromolecules. 1993; 26:5698.
20. Wenz G, Keller B. Angew. Chem. Int. Ed. Engl. 1992; 31:197.
21. Araki J, Ito K. Soft Matter. 2007; 3:1456.
22. Huang FH, Gibson HW. Prog. Polym. Sci. 2005; 30:982.
23. Loethen S, Kim JM, Thompson DH. Polym. Rev. 2007; 47:383.
24. Okada M, Takashima Y, Harada A. Macromolecules. 2004; 37:7075.
25. Riess G. Prog. Polym. Sci. 2003; 28:1107.
26. Rodriguez-Hernandez J, Checot F, Gnanou Y, Lecommandoux S. Prog. Polym. Sci. 2005; 30:691.
27. Mayumi K, Osaka N, Endo H, Yokoyama H, Sakai Y, Shibayama M, Ito K. Macromolecules. 2008; 41:6480.
28. Ren LX, Ke FY, Chen YM, Liang DH, Huang J. Macromolecules. 2008; 41:5295.
29. Farcas A, Ghosh I, Jarroux N, Harabagiu V, Guégan P, Nau WM. Chem. Phys. Lett. 2008; 465:96.
30. Iguchi H, Uchida S, Koyama Y, Takata T. ACS Macro Lett. 2013; 2:527.

31. Akae Y, Arai T, Koyama Y, Okamura H, Johmoto K, Uekusa H, Kuwata S, Takata T. Chem. Lett. 2012; 41:806.
32. Jang K, Miura K, Koyama Y, Takata T. Org. Lett. 2012; 14:3088. [PubMed: 22676854]
33. Wang J, Gao P, Ye L, Zhang A-y, Feng Z-g. J. Phys. Chem. B. 2010; 114:5342. [PubMed: 20373770]
34. Chelli S, Majdoub M, Aeiyach S, Jouini M. J. Polym. Sci. Part A: Polym. Chem. 2009; 47:4391.
35. Yang C, Ni XP, Li J. Eur. Polym. J. 2009; 45:1570.
36. Huang J, Ren L, Chen Y. Polym. Int. 2008; 57:714.
37. Kalashnikov PA, Sokolov VI, Topchieva IN. Russ. Chem. Bull. 2005:54.
38. Beirne J, Truchan H, Rao L. Anal. Bioanal. Chem. 2011; 399:717. [PubMed: 20838778]
39. Szente L, Szejtli J. Trends Food Sci. Technol. 2004; 15:137.
40. Collins CJ, McCauliff LA, Hyun S-H, Zhang Z, Paul LN, Kulkarni A, Wirth MJ, Storch J, Zick K, Thompson DH. Biochemistry. 2013; 52:3242. [PubMed: 23560535]
41. Marin A, Sun H, Husseini GA, Pitt WG, Christensen DA, Rapoport NY. J. Controlled Release. 2002; 84:39.
42. Cohn D, Lando G, Sosnik A, Garty S, Levi A. Biomaterials. 2006; 27:1718. [PubMed: 16310849]
43. Ahmed F, Alexandridis P, Neelamegham S. Langmuir. 2001; 17:537.
44. Fujita H, Ooya T, Kurisawa M, Mori H, Terano M, Yui N. Macromol. Rapid Commun. 1996; 17:509.
45. Fujita H, Ooya T, Yui N. Macromol. Chem. Phys. 1999; 200:706.
46. Fujita H, Ooya T, Yui N. Macromolecules. 1999; 32:2534.
47. Gaitano GG, Brown W, Tardajos G. J. Phys. Chem. B. 1997; 101:710.
48. Joseph J, Dreiss CA, Cosgrove T, Pedersen JS. Langmuir. 2006; 23:460. [PubMed: 17209594]
49. Loethen S, Ooya T, Choi HS, Yui N, Thompson DH. Biomacromolecules. 2006; 7:2501. [PubMed: 16961310]
50. Kida T, Iwamoto T, Fujino Y, Tohnai N, Miyata M, Akashi M. Org. Lett. 2011; 13:4570. [PubMed: 21819092]
51. Uccello-Barretta G, Sicoli G, Balzano F, Schurig V, Salvadori P. Tetrahedron: Asymmetry. 2006; 17:2504.
52. Michels JJ, O'Connell MJ, Taylor PN, Wilson JS, Cacialli F, Anderson HL. Chem. —Eur. J. 2003; 9:6167. [PubMed: 14679528]
53. Li J, Yang C, Li H, Wang X, Goh SH, Ding JL, Wang DY, Leong KW. Adv. Mater. 2006; 18:2969.
54. Nakazono K, Takashima T, Arai T, Koyama Y, Takata T. Macromolecules. 2009; 43:691.
55. Qin J, Meng X, Li B, Ha W, Yu X, Zhang S. J. Coll. Interface Sci. 2010; 350:447.
56. Ikeda T, Ooya T, Yui N. Macromol. Rapid Commun. 2000; 21:1257.
57. Luo H, Liu Y, Yu Z, Zhang S, Li B. Biomacromolecules. 2008; 9:2573. [PubMed: 18798668]
58. Fleury G, Brochon C, Schlatter G, Bonnet G, Lapp A, Hadziioannou G. Soft Matter. 2005; 1:378.
59. Przybylski, Cd; Blin, Fo; Jarroux, N. Macromolecules. 2011; 44:1821.
60. Zhang X, Zhu X, Ke F, Ye L, Chen E-q, Zhang A-y, Feng Z-g. Polymer. 2009; 50:4343.
61. Bray HG, Thorpe WV, Wood PB. Biochem. J. 1949; 44:39.
62. Bueding E, Jolliffe N. J. Pharm. Exp. Ther. 1946; 88:300.
63. Channon HJ, Mills GT, Williams RT. Biochem. J. 1944; 38:70. [PubMed: 16747752]
64. Parker VH. Biochem. J. 1952; 51:363. [PubMed: 12977737]
65. Green MN, Josimovich JB, Tsou K-C, Seligman AM. Cancer. 1956; 9:176. [PubMed: 13284714]
66. Biaglow JE, Varnes ME, Roizentowle L, Clark EP, Epp ER, Astor MB, Hall EJ. Biochem. Pharmacol. 1986; 35:77. [PubMed: 2934068]
67. Mammen M, Choi S-K, Whitesides GM. Angew. Chem. Int. Ed. Engl. 1998; 37:2754.

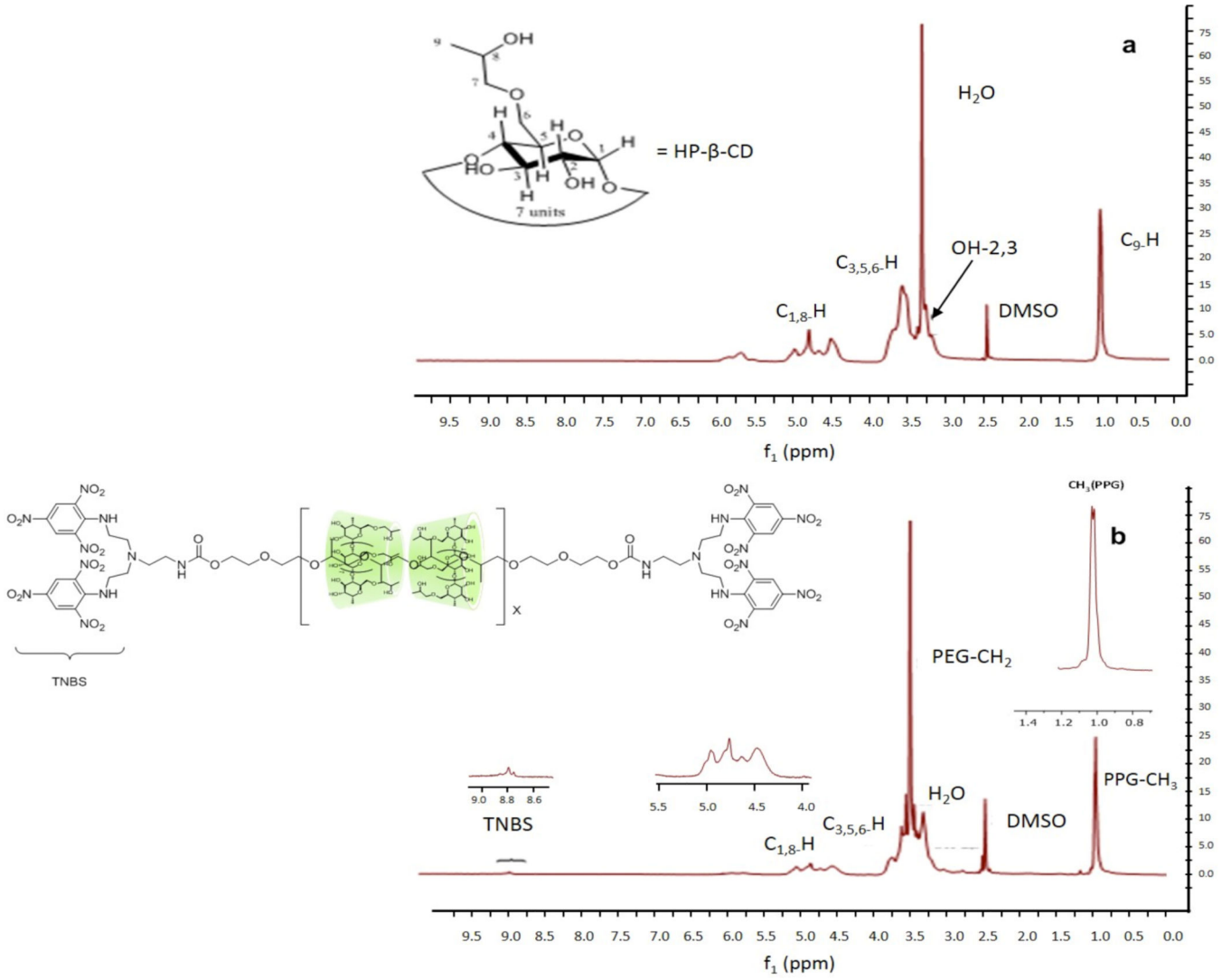


Figure 1.

^{1}H NMR spectra (400 MHz) in DMSO-d_6 at 25 °C. (a) HP-β-CD; (b) HP-β-CD/Pluronic F127 polyrotaxane (threaded in hexane).

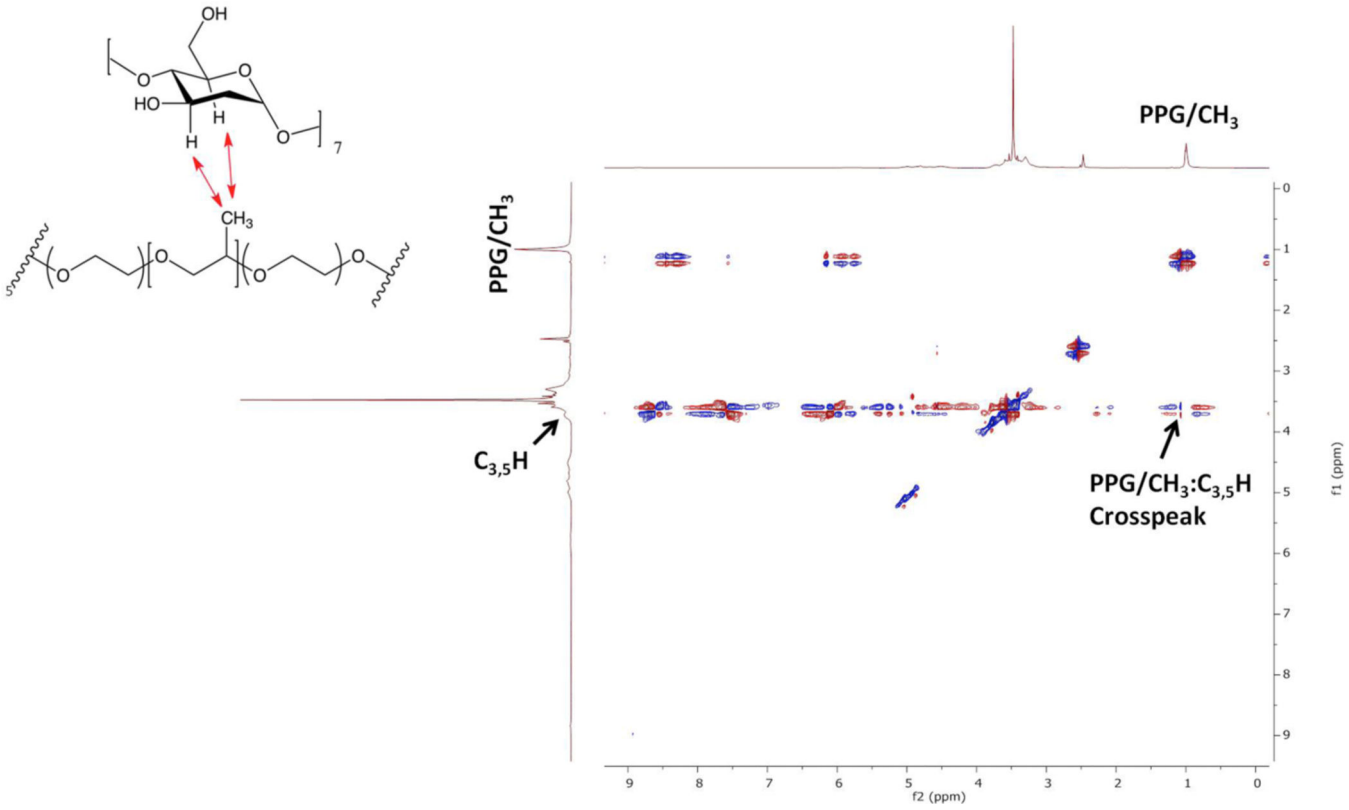


Figure 2.

Two-dimensional 400 MHz NOESY ^{1}H NMR spectrum of 07HP.F127 in DMSO-d_6 at 25 °C.

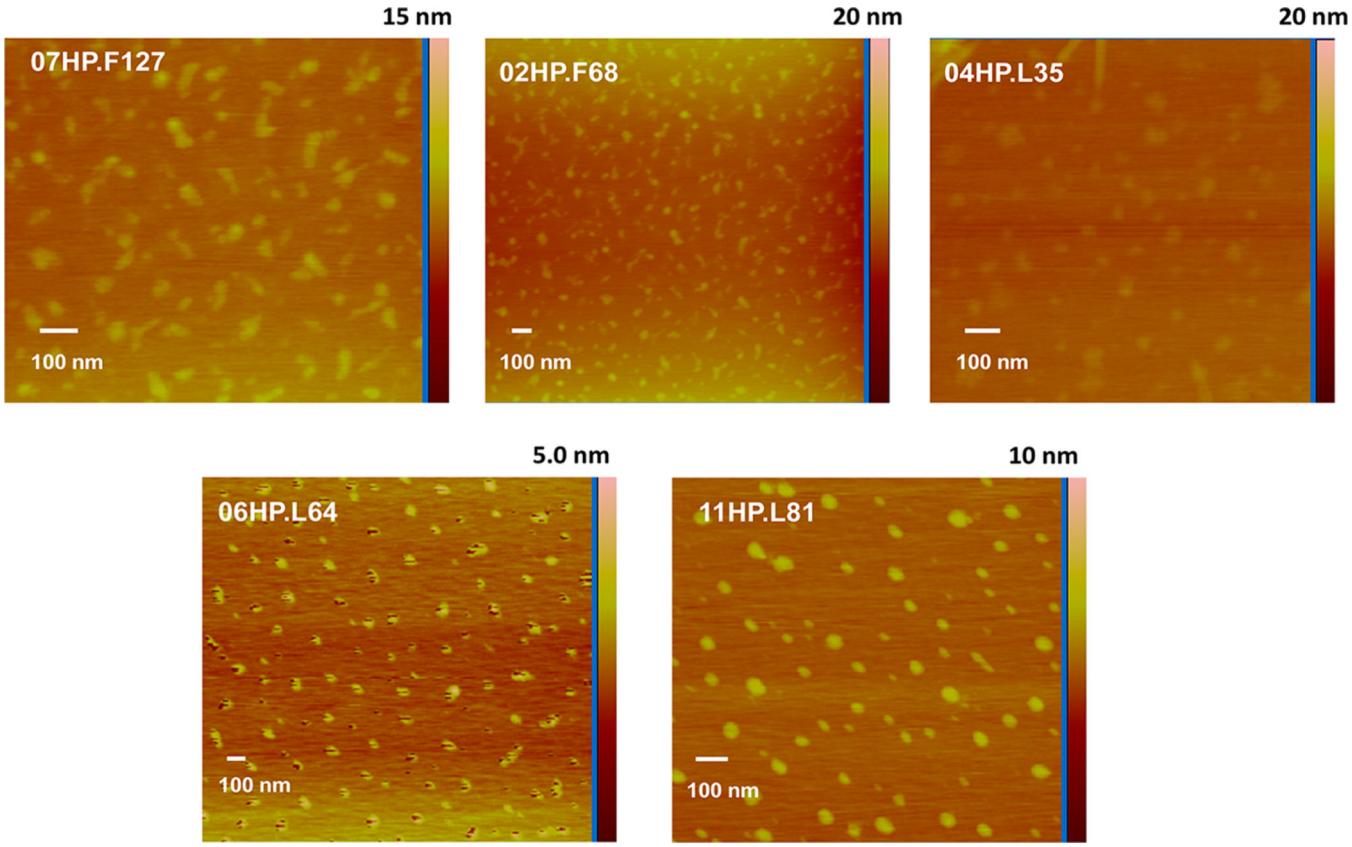


Figure 3.

Tapping multimode AFM images of polyrotaxanes on mica at 10^{-9} mg/mL and 20 °C after solvent (H_2O) removal.

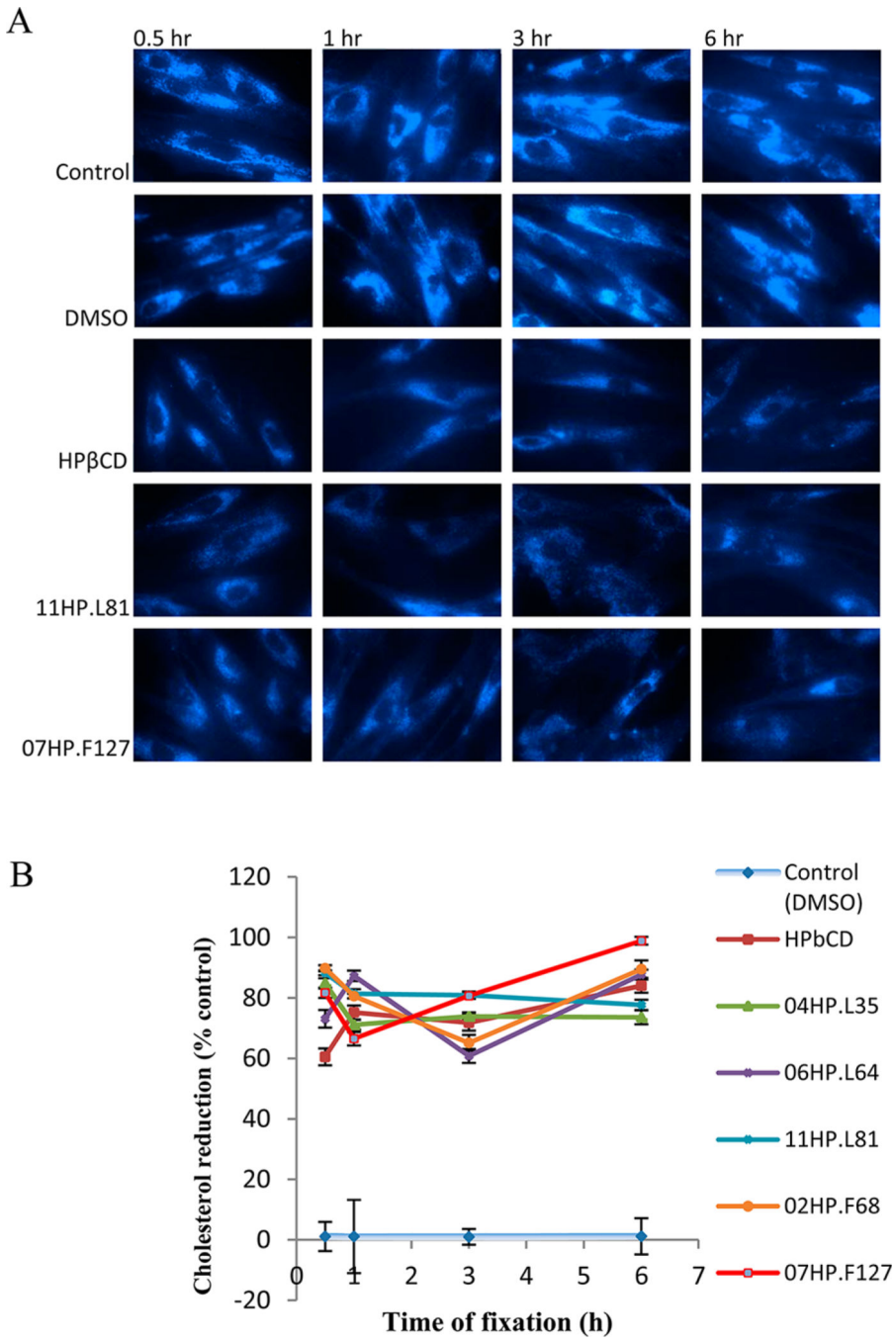
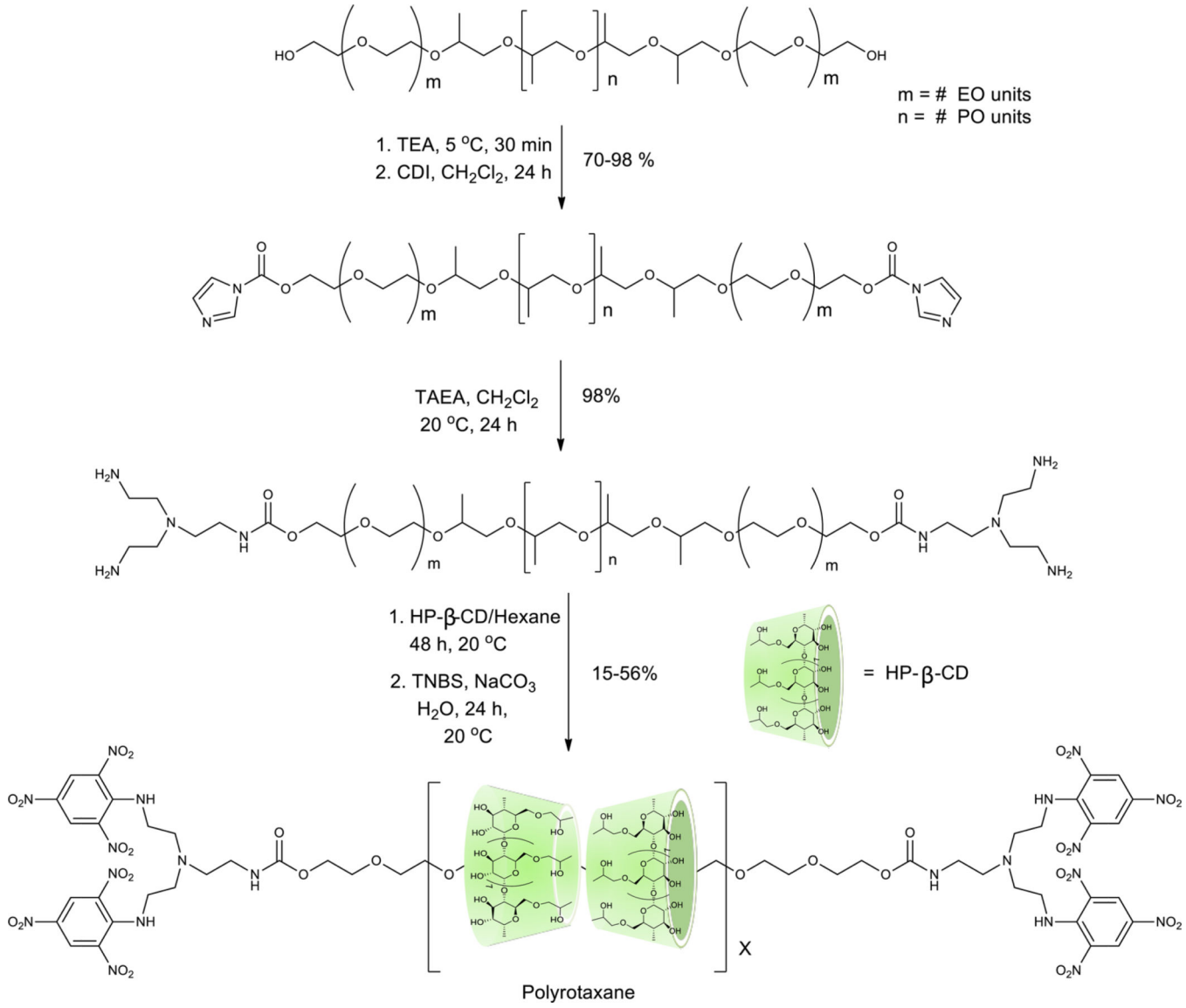


Figure 4.

Reduction of accumulated cholesterol in *npc*2$^{-/-}$ cells treated with polyrotaxanes. Polyrotaxane compounds were dissolved in DMSO and diluted in *npc*2$^{-/-}$ fibroblast culture media (MEM/FBS/pen/strep) to yield 25 μM of HP-β-CD. Cells were fixed after 0.5, 1, 3, and 6 h exposure to polyrotaxane or monomeric HP-β-CD before staining with filipin. (A) Representative images of cholesterol accumulation over time; (B) Quantitative estimation of accumulated cholesterol reduction within the cell bodies by determining the areas staining positive for filipin relative to untreated (control) cells, reported as a percentage.



Scheme 1.

Synthesis of HP-β-CD/Pluronic Polyrotaxane with 2,4,6-Trinitrobenzene (TNB) End-Caps

Table 1

Effect of Solvent on the Yield of HP-β-CD/Pluronic F127 Polyrotaxane[a]

solvent	ε[b]	mg	No. of CD[c]	coverage ratio[d] (%)
H_2O	79	2.2	0	0
D_2O	78	5.2	0	0
DMSO	46	14	0	0
DMF	37	39	0	0
methanol	33	1.4	0	0
dichloromethane	9.1	6.8	1	3
ethyl acetate	6.0	24	4	12
diethyl ether	4.2	17	9	28
hexane	1.9	86	11	34

[a] Pluronic F127 (M_n 12600, 100 mg), HP-β-CD (MW 1,460, 0.34 g, 1 CD/2 PO units), stirred in solvent (3 mL) for 48 h at 20 °C before addition of TNBS (0.046 mmol, 0.14 mL) and stirring at 20 °C for 24 h.

[b] ε: dielectric constant.

[c] Number of HP-β-CD units threaded.

[d] Determined by ^1H NMR integration, based on the ratio of the C_1–H protons of HP-β-CD and the methyl protons of PPO (assuming 1 CD/2 PPO units).

Table 2

Molecular Weight and Purity of Polyrotaxanes[a]

polyrotaxane	HLB[b]	CAC[b] (%)	EO/PO[b]	% free CD (UPLC)	n_{CD}	threading efficiency	MW (NMR) ($\times 10^3$)	MW (MALDI) ($\times 10^3$)	avg height (nm)	avg diameter (nm)
07HP.F127	22	0.004	400/65	3.3	7	22	24.0	25.0	2.20	80
02HP.F68	29	0.04	152/27	0.90	2	15	12.5	13.1	1.30	50
04HP.L35	19	1	43/16	2.3	4	44	8.93	8.1	0.81	61
06HP.L64	15	0.14	53/30	6.0	6	43	12.9	13.4	1.30	70
11HP.L81	2	0.0063	13/43	1.5	11	52	20.1	17.6	0.39	48

[a]The threading efficiency was calculated based on a presumed 1 HP-β-CD:2 PO unit ratio. n_{CD} refers to the number of HP-β-CD molecules threaded onto the Pluronic core as determined by ^{1}H NMR integration. The free CD values (*w/v*) were determined by UPLC chromatography using HP-β-CD as standard. The average size and height of the polyrotaxane products were determined from AFM images of the final products.

HLB: hydrophilic–lipophilic balance, CAC: critical aggregation concentration.

[b]Values adapted from Laibinis et al., *J. Coll. Interface. Sci.* **1991**, *142*, 74.