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Synthesis of the Anionic Hydroxypropyl- β -cyclodextrin:Poly(decamethylenephosphate) Polyrotaxane and Evaluation of its Cholesterol Efflux Potential in Niemann-Pick C1 Cells

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Abstract

Niemann–Pick type C disease (NPC) is a lysosomal storage disease that is characterized by a progressive accumulation of unesterified cholesterol in the lysosomes leading to organ damage from cell dysfunction. Hydroxypropyl- β -cyclodextrin (HP- β -CD) is an attractive drug candidate for treating NPC, as it diminishes cholesterol accumulation in NPC cells. Systemic HP- β -CD treatment, however, is limited by rapid renal clearance. We designed a new anionic HP- β -CD polyrotaxane to act as a slow release formulation based on a polyalkylene phosphate core to improve the pharmacokinetics. The polyalkylene phosphate comprises hydrophobic decamethylene spacers linked by biodegradable anionic phosphodiester bonds. HP- β -CD was threaded onto this polymer first and α -CD afterwards to prevent burst release of the threaded HP- β -CD. Our findings show that HP- β -CD was slowly released from the watersoluble polyrotaxane over a 30 days period. The polyrotaxane provided persistently diminished cholesterol levels in NPC1 cells by 20% relative to untreated cells. These results demonstrate the therapeutic potential of this novel HP- β -CD polyrotaxane for the mobilization of aberrantly stored cholesterol in NPC1 cells.

Graphical Abstract

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x SEC diagram of **1**, synthesis of **7**

Conflicts of interest

There are no conflicts to declare

1. Introduction

Cyclodextrins (CDs) are cyclic oligomers of glucose known to complex hydrophobic or amphiphilic guest molecules in aqueous environments due to hydrophobic interactions.¹ This binding ability has been widely exploited for the solubilization of active pharmaceutical ingredients (APIs) for improvement of their bio-availability and reduction of undesirable side effects.^{2,3} Hydroxypropyl- β -CD (HP- β -CD), a highly water-soluble derivate of β -CD, is already commercially used for several drug formulations, for example for itraconazole (“Sporanox”),⁴ hydrocortisone (“Dexocort”),⁵ and indomethacin (“Indocid”).⁶ Furthermore, HP- β -CD itself can exhibit pharmacological action due to solubilization and transport of cholesterol as well as influencing the lipid rafts of cells.^{7,8} HP- β -CD forms a water-soluble 2:1 complex with cholesterol as demonstrated by solubility measurements,^{9,10} thus offering therapeutic potential for the treatment of lysosomal metabolic disorders such as Niemann-Pick Type C (NPC).^{11–13} This rare genetic disease (incidence $\sim 1:120,000$) leads to an accumulation of cholesterol and sphingolipids in the lysosome because of mutations in either the NPC1 or NPC2 proteins. Aberrant accumulation of cholesterol as microcrystalline deposits leads to damage of liver, spleen and brain, causing progressive degeneration of the patient.¹⁴ Administration of HP- β -CD promotes cholesterol normalization within late endosomal/lysosomal compartments to enable its metabolism and excretion.¹⁵ Subcutaneous administration of 4000 mg/kg body weight of HP- β -CD 4 days a week produced a 72% reduction of total cholesterol content in the liver of *npc*^{-/-} mice after treatment for 77 d.¹⁶ Despite this success, the poor pharmacokinetics of HP- β -CD require frequent administrations that are cumbersome and potentially toxic (e.g., lung damage).^{17,18} These side effects can be mitigated by intracisternal administration; however there is still a risk of ototoxicity.^{15,19} Therefore, alternative methods for a safer administration of HP- β -CD are desirable. We report a new material for cyclodextrin delivery based on a water-soluble polyphosphate HP- β -CD polyrotaxane.

In general, polymers are attractive as potential carriers for the controlled delivery of APIs. Polymeric delivery vehicles have been employed to improve both the solubility and bioavailability of drugs to improve their efficacy and/or diminish their toxicity.^{20,21} These vehicles can improve the retention of the drug in the human body due to reduced renal clearance.^{22–24} Targeting is also possible through incorporation of ligands that promote active transport into specific cells via receptor-mediated endocytosis.²⁵

Covalent linkage of APIs to degradable polymers produces polymeric pro-drugs that are new molecular entities, thus requiring extensive toxicity and efficacy testing.²⁶ More commonly, a strategy of physically mixing small molecules and polymeric carriers, such as PLGA or

modified starch, is employed to produce vehicles with improved delivery characteristics,^{27,28} although burst release of the API due to weak intermolecular interactions is often observed for these formulations.²⁷ Polyrotaxanes, molecules where many macrocyclic rings are strung onto a polymer chain, are interesting alternative carrier entities since they are kinetically less stable than polymeric prodrugs, but by far more stable than physical mixtures due to the topological bond between the threaded rings and the polymeric backbone.^{1,29} The dissociation rate of a polyrotaxane can be controlled by the type of stopper groups attached along the polymer or at the chain ends to allow slow release of the threaded rings.^{1,30}

Polyrotaxanes have been synthesized from various ring shaped molecules, such as crown ethers, cucurbiturils and cyclodextrins.³¹ Among these macrocyclic precursors, cyclodextrins have been the most widely explored since they are produced on an industrial scale, display very low toxicity under physiological conditions, and have been widely used as excipients in FDA-approved small molecule drug formulations.^{4,32} Amphiphilic polymers are suited best for the formation of CD polyrotaxanes since CDs spontaneously thread onto hydrophobic chain segments in water driven by hydrophobic interactions.^{1,33} So far, neutral and cationic polymers like polyethylene glycol,³⁴ polypropylene glycol,^{35,36} poly(oligomethylenimine)s³⁷ and derivatives of them, as well as polyamides³⁸ and polyesters³⁹ have been complexed by CDs to form polyrotaxanes. Among them, the neutral CD polyrotaxanes show very low water solubilities and require further derivatization.¹ Rapid normalization of cholesterol levels in NPC cells⁴⁰ and the mouse model of NPC disease⁴¹ has been reported for polyrotaxanes composed from HP- β -CD (or 2-(2-hydroxyethoxy)ethyl carbamate-modified β -CD) and PEG-PPG-PEG block polymers.^{40–43} Although the PEG backbone provides modest improvements in polyrotaxane solubility, it could potentially contribute to immune responses, liver accumulation, and poor bio-degradability.^{44,45} Cationic CD polyrotaxanes are known to be highly water-soluble; however, these materials are often accompanied by significant toxicity.^{46–48} These potential shortcomings in the development of an effective CD polyrotaxane-based NPC therapy led us to explore the synthesis and performance testing of anionic polyrotaxanes derived from poly(decamethylenephosphate), a nontoxic water-soluble polymer that is degradable by hydrolysis in aqueous media. Polymeric phosphoric acid diesters are synthetic analogs of polynucleotides that are known to be degradable by nucleases to generate non-toxic byproducts.^{49–52} They can be readily prepared by oxidation of polymeric phosphorous acid diesters. The latter are typically prepared by transesterification of diphenyl phosphite with α,ω -diols.⁵⁴ Polymeric phosphodiester have already been used as prodrugs, hydrogels and drug carriers.^{51,55,56} A pseudopolyrotaxane based on biodegradable polyphosphoester ionomers obtained by ring opening polymerization has been described by Tamura et al.⁵³ Polyrotaxane formation was only observed with α and γ -CD and the resulting polyrotaxanes formed precipitates in aqueous solution. No inclusion complexes were obtained with β -CD.

We herein describe for the first time the homogenous assembly of a water-soluble polyrotaxane from HP- β -CD and an anionic polymeric phosphodiester. The resulting HP- β -CD polyrotaxane displays cholesterol normalization activity in NPC cells.

2. Experimental

2.1. General

Hydroxypropyl- β -CD (HP- β -CD) with d.s. = 0.9 per glucose unit and α -CD were obtained from Wacker Chemie (Munich, Germany) and dried at 70 °C for 18 h in vacuum before use. 1,10-Decanediol, diphenylphosphite, fluorescein-isothiocyanate, toluidinylnaphthalene-2-sulfonate, Filipin III, and Triton X-100 were purchased from Sigma Aldrich (Steinheim, Germany). *N*-Chlorosuccinimide (NCS), imidazole, LiCl and solvents were purchased from commercial suppliers. Dichloromethane (DCM) was dried over P₂O₅ and distilled before use. The polyrotaxane products were purified via ultrafiltration in a 400 mL ultrafiltration cell from Millipore (Darmstadt, Germany); the 5 kDa regenerate cellulose membrane was obtained from Microdyn-NADIR (Wiesbaden, Germany).

NMR measurements were performed using a Bruker AVANCE Ultrashield 400 (400.2 MHz) instrument (Billerica, Massachusetts, USA).

SEC measurements were performed on a PSS Suprema Lux 30 column equipped with a RI Waters 2410 detector. The flow rate was 1 mL/min and calibrations were performed using pullulan standards. The eluent was phosphate buffer, pH 10 with 5 vol% acetonitrile (ACN).

Fluorescence measurements were performed on a Tecan Infinite 2000 microplate reader (Crailsheim, Germany). The data were processed with Tecan i-control software version 3.4.2.0.

2.2. Synthesis

2.2.1. Poly(decamethylenephosphite) 2—1,10-Decanediol (6.227 g, 26.5 mmol, 1.0 eq) was added under a N₂ atmosphere to diphenylphosphite (4.619 g, 26.5 mmol, 1.0 eq) in a vacuum distillation apparatus to remove the phenol byproduct by distillation. The mixture was initially heated over a 1 h period to 100 °C at 30 mbar and stirred 1 h at 100 °C before reducing the pressure to 0.06 mbar and stirring for an additional 1 h. Then, the reaction was heated to 150 °C over a 1 h period and stirred for an additional 1 h before raising the temperature over 1 h to 200 °C and stirring for another 1 h. The reaction was then cooled to 20 °C, giving 5.77 g (26.2 mmol, 99 %) of **2** as a white waxy solid. ¹H-NMR: δ /ppm (CDCl₃, 400 MHz) = 6.78 (d, 1H, PH, ¹J_{HP} = 346 Hz), 4.04 (sh, 4H, H-1), 1.70 – 1.54 (sh, 4H, H-2), 1.29 (sh, 12H, H-3/4/5). ¹³C-NMR: δ /ppm (CDCl₃, 100 MHz) = 65.8 (C-1), 30.4 (C-2), 29.4 (C-3), 29.1 (C-4), 25.5 (C-5). ³¹P-NMR: δ /ppm (CDCl₃, 100 MHz) = 6.06 ppm.

2.2.2. Poly(decamethylenephosphate) 1—To a solution of **2** (5.144 g, 23.38 mmol, 1.0 eq.) in 200 mL DCM was added *N*-chlorosuccinimide (3.43 g, 25.72 mmol, 1.1 eq). The mixture was stirred for 5 d at 20 °C to produce the polymeric phosphoryl chloride **3**. Then, a solution of imidazole (3.18 g, 46.08 mmol, 2.0 eq) in 70 mL DCM was added dropwise over 2 h at 20 °C and the solution stirred overnight at 20 °C to generate the polymeric phosphoryl imidazolid **4**. The reaction mixture was filtered and 500 mL of a 0.05 M Na₂CO₃ solution was added to the filtrate before evaporation of the DCM. The aqueous phase was stirred overnight and then filtered. The clear filtrate was purified by ultrafiltration via 5 kDa polyethersulfone membrane against 2 L 0.1 M phosphate buffer containing 0.1 M LiCl and

then against 2 L H₂O. After freeze drying, 2.13 g (8.85 mmol, 38 %) of white fluffy solid **1** was recovered. ¹H-NMR: δ /ppm (D₂O) = 3.82 (bs, 4H, H-1), 1.62 (bs, 4H, H-2), 1.32 (sh, 12H, H-3/4/5). ¹³C-NMR: δ /ppm (D₂O) = 66.0 (C-1), 30.3 (C-2), 29.9 (C-3), 29.3 (C-4), 25.7 (C-5). ³¹P-NMR: δ /ppm (D₂O) = 0.60 ppm.

2.2.3. Polyrotaxane 5— α -CD (11.6 mg, 16 mmol, 1.3 eq) and **1** (4.1 mg, 12 mmol, 1.0 eq) were each dissolved in 1 mL D₂O. Aliquots (350 μ L) of both solutions were mixed and incubated at 60 °C for 22 d. ¹H-NMR: δ /ppm (D₂O) = 5.06 (s, 0.25 H, α -CD threaded), 5.05 (d, 0.75 H, free α -CD) 3.99 – 3.57 (m, 6 H, H-1 + α -CD), 1.81 – 1.23 (sh, 16H, H-3/4/5).

2.2.4. Polyrotaxane 6—2-HP- β -CD (ds: 0.9, average MW 1500 g/mol; 29.75 g, 198 mmol, 6.0 eq) and **1** (802 mg, 3.30 mmol, 1.0 eq) were added stepwise to 100 mL of a 0.05 M NaCl solution to produce a clear solution. The stirred solution was heated to 40 °C for 5.5 h. Then, a solution of α -CD (3.20 g, 3.3 mmol, 1 eq) in 30 mL H₂O was added and the mixture was stirred at 70 °C for 16 h. To separate unthreaded CD from the polyrotaxane, the solution was purified via ultrafiltration using a 5 kDa polyethersulfone membrane against a 0.05 M LiCl solution and H₂O in sequence before freeze drying. A white, fluffy solid (1.278 g) was recovered. ¹H-NMR: δ /ppm (D₂O) = 5.22 (s, 0.6 H, HP-CD), 5.08 (s, 0.7 H, HP-CD), 5.04 (s, 0.16 H, α -CD), 4.12 – 3.41 (m, 14.3 H, H-1 + α -CD + HP-CD), 1.64 (bs, 4H, H-2), 1.35 (sh, 12H, H-3/4/5), 1.16 (s, 2.8H, HP-CD).

2.2.5. FITC tagged Polyrotaxane 6a—Polymer **1** (27.0 mg, 0.11 mmol, 1.0 eq) and fluorescent CD **7** (44.0 mg, $2.8 \cdot 10^{-5}$ mol, 0.25 eq.) (synthesis in Supporting Information) were dissolved in 2 mL of a 0.05 M LiCl solution and the solution was heated to 40 °C. After 1 h, HP- β -CD (500 mg, 0.33 mmol, 3.0 eq.) was added and the solution was kept at 40 °C. After 6 h, a solution of α -CD (150 mg, 0.15 mmol, 1.3 eq in 1 mL 0.05 M LiCl) was added and the mixture was heated to 70 °C for 16 h. To separate unthreaded CD from the polyrotaxane, the solution was purified via ultrafiltration using a 5 kDa polyethersulfone membrane against a 0.05 M LiCl solution and H₂O in sequence before freeze drying. A yellow, fluffy solid (62 mg) was recovered. ¹H-NMR: δ /ppm (D₂O) = 8.05–6.20 (bs, 0.67 H, Fluo-CD), 5.14 (s, 0.76 H, HP-CD), 5.08–4.97 (sh, 1.33 H, HP-CD + Fluo-CD), 4.96 (s, 0.26 H, α -CD), 4.06 – 3.32 (m, 22 H, H-1 + α -CD + HP-CD + Fluo-CD), 1.78–1.13 (sh, 12H, H-2/3/4/5), 1.09 (s, 4 H, HP-CD).

2.3. TNS Assay

The assay was performed in 50 mM PBS (8 g/L NaCl, pH 7.35). Polyrotaxane **6** (29.0 mg) was dissolved in 11.2 mL PBS and incubated at 37 °C. In defined intervals over a 50 d period, 1 mL samples of the solution were removed and frozen. To purify the free HP- β -CD from the polyrotaxane, 500 μ L of the samples were purified by ultrafiltration using Vivaspin 2, MWCO 3000 (Sartorius, Göttingen, Germany) devices. Free HP- β -CD was collected in the filtrate. The filtrates were freeze dried and redissolved in 200 μ L water. These solutions (10 μ L) were mixed with 15 μ L water (to reach again a salt concentration of 50 mM), 225 μ L PBS, and 50 μ L of a 0.72 mM TNS solution in PBS. After 30 min incubation at 20 °C, the fluorescence intensities of the samples were measured at an excitation wavelength of 366 nm and a detection wavelength of 448 nm at 27 °C.

2.4. Filipin Staining of NPC1 cells

NPC1-deficient cells (Coriell Institute) were seeded in 96 well tissue culture dishes at a density of 1600 cells/well in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were incubated for 24 h at 37 °C in 5% CO₂ before treatment with 6 or free HP- β -CD solution in triplicate. A 100 μ L solution containing 0.24 mg of 6 to give a HP- β -CD equivalent concentration of 800 μ M in the cell culture media was used per well as the highest concentration. HP- β -CD equivalent concentrations of 400, 200, 100, 25, 12.5 μ M and untreated control were used to evaluate the dose-response relationship of 6 in NPC1-deficient cells. α -CD (0.4 μ g) and 3.4 μ g polydecamethylene phosphate 1 per well (amount of 1 present in 25 μ M HP- β -CD equivalent of 6) were additional controls. After 24 h, the cells were washed with PBS and fixed using 3.7% paraformaldehyde solution. Total unesterified cholesterol content within the cells was determined by staining with 50 μ g/mL Filipin III in PBS for 1.5 h. Next, the cells were washed and the nuclei were stained with a solution of 16 μ M Nuclear Red DCS1 (AAT Bioquest) in PBS. Cells were imaged using the 5 $\times$ objective on an Opera Phenix High-Content Imaging System (Perkin Elmer). Harmony Software (v4.1, Perkin Elmer) was used to quantify the data collected from one field per well while excluding border cells. Nuclei were identified in the Cy5.5 channel and unesterified cholesterol content was evaluated using the mean filipin intensity above the background in the DAPI channel. Mean filipin intensity in treated cells were compared to that of untreated cells and expressed as % Filipin stain $\pm$ SEM. One-way ANOVA analysis was performed in GraphPad Prism (v7.03, GraphPad Software). MTS assays were performed as per manufacture's protocol to evaluate the apparent toxicities of 6 at the concentrations used.

2.5. Cellular Tracking of α -CD End-capped Polyrotaxanes

NPC1-deficient cells were treated with 25 μ M CD equivalent FITC-tagged polyrotaxane 6a in Opti-MEM media and incubated for either 4 h or 24 h at 37°C, 5% CO₂ and 95% relative humidity. After 24 h treatment, the polyrotaxane solution was removed and the cells washed with 1X PBS to remove non-internalized polyrotaxane 6a and incubated for another 24 h under the same conditions. For imaging, the nuclei were stained with Hoechst 33342 and the acidic endosomal and lysosomal compartments were stained with LysoTracker Red DND 99. Confocal images were acquired using a Nikon A1R multiphoton confocal microscope with a 60X oil objective equipped with 405 nm, 488 nm and 561 nm lasers for Hoechst 33342, FITC and LysoTracker Red DND 99, respectively.

2.6. FACS Analysis

NPC1-deficient cells treated with 25 μ M CD equivalent FITC tagged PR 6a in Opti-MEM media and incubated for 4 h or 24 h at 37 °C, 5% CO₂ and 95% relative humidity. After 24 h treatment, the PR solution was removed by aspiration and cells were washed with 1X PBS to remove non-internalized PR 6a and incubated for another 24 h under the same conditions. FACS analysis for cellular association was performed using a BD LSR Fortessa flow cytometer equipped with a 488 nm laser to quantify mean fluorescence of 5000 NPC1 cells (n=3, one-way ANOVA, ****p<0.0001).

3. Results & Discussion

3.1. Polymer Synthesis

Poly(decamethylene phosphate) **1** was chosen as the guest polymer, because it is less toxic ($LC_{50} = 900 \mu\text{g/mL}$ for HEPG2 cells) than the previously used cationic polymers.⁵⁷ It was obtained through oxidation of poly(decamethylene phosphite) **2**. This was performed in three steps via the polymeric phosphoryl chloride **3** and polymeric phosphoryl imidazolidine **4** intermediates as shown in Scheme 1. This three step procedure was chosen to avoid the formation of pyrophosphates that are known to occur during the more direct one step oxidation with aqueous iodine.⁵⁸ Li^+ counterions appeared to be superior to Na^+ because of their positive influence on the aqueous solubility of **1**. Ultrafiltration processing of the material gave poly(decamethylenephosphate) **1** of an average molar mass of M_w 18 kDa polymer, as determined by SEC, indicating an average degree of polymerization of 76 (SEC diagram in Supporting Information). The solubility of the polymer in water is 110 mg/mL.

3.2. Polyrotaxane Formation and Stability

The spontaneous formation of polyrotaxane **5** was observable by $^1\text{H-NMR}$ spectroscopy during incubation of an aqueous solution of 8 mM **1** with 6 mM $\alpha\text{-CD}$ at 60°C (Figure 1a). New signals at 5.06 ppm were indicative of threaded $\alpha\text{-CD}$ as observed for other ionic polymers.⁵⁹ From the integrals at 5.060 and 5.035 ppm, the occupancy y of the host $\alpha\text{-CD}$ by the polymer backbone was determined and plotted vs. time (Fig. 2). The very low complexation rate observed was attributed to the pronounced steric hindrance between the bulky solvated phosphate groups and the rigid $\alpha\text{-CD}$ ring. The kinetics followed a stretched exponential time law (Equation 1) as is typical for one-dimensional diffusion.^{60,61} The occupancy y approaches a limiting value of $y_\infty = 48.5\%$ for infinite complexation time t and a time constant τ of 2.9 d.

The equilibrium constant K was calculated from y_∞ according to equation 2, with the total molar concentration of cyclodextrin $[\text{CD}]_0$, the molar ratio of total concentrations of guest repeats vs. host x , and the occupancy of cyclodextrin y defined as the molar ratio of occupied cyclodextrin vs. total cyclodextrin. The result, $K = 200 \text{ M}^{-1}$, was in reasonable agreement with the binding constants of other guest polymers with decamethylene binding sites.⁵⁹

$$y = y_\infty \left(1 - e^{-\sqrt{t/\tau}}\right) \quad (1)$$

$$K = \frac{y_\infty}{[\text{CD}]_0(x - y_\infty)(1 - y_\infty)} \quad (2)$$

Although steric hindrance between the bulky phosphate groups and the $\alpha\text{-CD}$ cavity impedes the rotaxanation process (e.g., polyrotaxane formation takes more than a week), the resulting polyrotaxane is kinetically stable and survives extended dialysis. Conversely, HP-

β -CD does not form stable complexes with the polymer since steric hindrance of the phosphate groups is negligible due to the larger internal diameter of β -CD cavity (0.65 nm) compared to the α -CD cavity (0.44 nm).⁶² Nevertheless, HP- β -CD polyrotaxanes are obtainable after subsequent incorporation of α -CD as a supramolecular stopper on the chain ends. Assembly of polyrotaxanes through subsequent threading of large and small rings was already shown to be practicable as reported previously.^{57,63} The HP- β -CD polyrotaxane **6** could be isolated in nearly quantitative yield after sequential threading of HP- β -CD and α -CD onto polyphosphate **1** and removal of excess α -CD and HP- β -CD by ultrafiltration. The solubility of **6** in water is 160 mg/mL. The composition of **6**, determined by the ¹H-NMR (Figure 1b), indicated HP- β -CD and α -CD coverages of 18% and 3%, respectively. Consequently, 18 % of repeating units of the polymer (14 in total) are complexed by HP- β -CD and the polyphosphate chain **1** is stoppered by around 2 to 3 α -CD rings situated at the chain ends. The proposed polyrotaxane structure was confirmed by the DOSY spectrum, shown in Fig. 3, where the signals of all constituents appear in one line at the same low diffusion coefficient $D = 1.55 \cdot 10^{-11} \text{ m}^2 \text{ s}^{-1}$.

Since polyrotaxane **6** was designed for slow release of HP- β -CD into aqueous media, its dissociation was investigated in a biological buffer. For this purpose, solutions of the polyrotaxane **6** were incubated in PBS at 37 °C for 50 d and the liberation of free HP- β -CD was followed by TNS assay. Toluidinylnaphthalene-2-sulfonate (TNS) is very useful for the detection of β -CD derivatives because it forms strongly fluorescing complexes whereas fluorescence of the free dye is negligible.^{64,65} Polyrotaxane **6** was removed by ultrafiltration from the analyte prior to this determination to avoid any interference of the fluorescence probe with the polymer. As shown in Fig. 4, HP- β -CD was slowly released from the polyrotaxane over a 30 d period. Only 50% of the threaded rings were liberated because of the limited dilution of the system; release *in vivo* would be more complete due to the larger volume of dilution.

3.3. Biological Evaluation of **6** in NPC1-deficient Cells

The therapeutic potential of polyrotaxane **6** was evaluated in primary NPC1 fibroblasts. In brief, NPC1-deficient cells (1600 cells/well in 96-well plates) were incubated with **6** for 24 h at 37 °C with different equivalent concentrations of free HP- β -CD in the cell media. The cells were then rinsed, fixed, stained with filipin, and the relative degree of cholesterol accumulation in the treated cells compared with untreated controls. Filipin staining correlates with the relative cholesterol accumulation in cells. A significant decrease in filipin staining (~10%) was observed in treated NPC1 cells relative to untreated cells upon incubation for 24 h with a single dose of polyrotaxane **6** at an equivalent HP- β -CD concentration of 25 μM (i.e., polyrotaxane **6** concentration = 25 μM HP- β -CD $\div$ 14 HP- β -CD/PR = 1.825 μM **6**). Additionally, treatment monomeric α -CD (at a concentration present in 1.825 μM **6**) did not result in any cholesterol efflux (Fig. 5 and Fig. S2). This is most likely because the hydrophobic cavity of α -CD is too small of accommodate cholesterol molecule as reported by Davidson *et al.*⁶⁶ Increasing the concentration of **6** results in enhanced cholesterol efflux with about 20% and 40% decrease in filipin staining at 200 μM and 800 μM HP- β -CD equivalent concentrations, respectively. The observed smaller effect of polyrotaxane **6** compared to free HP- β -CD was attributed to the slow release of the

threaded rings leading to a much lower bioavailability of the polyrotaxane-borne HP- β -CD. A cytotoxicity profile evaluation of **6** using MTS assay (Fig. 6), however, reveals a sharp increase in toxicity of **6** above 200 μ M HP- β -CD equivalent dose. This acute toxicity of the polyrotaxane might be caused by a surfactant effect of the amphiphilic polyphosphate backbone. Nevertheless, its degradation products (phosphoric acid and 1,10-decanediol) only show low toxicities.

We infer from these findings that **6** is dethreaded after internalization by the cells to release the HP- β -CD payload to effectively mobilize the aberrantly stored cholesterol deposits in NPC1-deficient cells as previously reported by Collins *et al.*⁶⁷ Although monomeric dose of HP- β -CD performs better in cholesterol efflux from the cells after a single 24 hours treatment in comparison of **6** (Fig. 7), the potential of **6** as a long circulating and slow HP- β -CD releasing platform makes them an attractive candidate for NPC therapeutics.

3.4 Cellular Tracking of α -CD End-capped Polyrotaxanes

A FITC tagged polyrotaxane **6a** was prepared for cellular tracking studies by co-threading a small amount of fluorescein-labeled β -CD **7** (shown in Fig. 8 synthesis described in Supporting Information) with HP- β -CD and subsequently stoppered by α -CD.

NPC1-deficient cells begin to internalize polyrotaxane **6a** within 4 h, however, the polyrotaxane appears only within non-acidic vesicles (i.e., compartments that are not stained by LysoTracker). These are most likely to be endosomes at an earlier stage of maturation.^{68,69} Extensive association of the polyrotaxane with the extracellular matrix of NPC1-deficient cells was also observed (Fig S3). Significant cellular uptake and localization within the acidic late endosomal/lysosomal compartment was observed after 24 h of treatment (Fig. 9C) Next, the non-internalized polyrotaxane was removed and cells were washed to prevent further uptake of **6a** before incubating for another 24 h to monitor the cellular fate of the already internalized polyrotaxane (Fig. 9D). The latter images show decreased fluorescein signal intensity indicating that the cyclodextrins escape the endosomal/lysosomal compartments within the cell leading to high dilution of the fluorophore.

3.5 FACS Analysis of Cellular Association of α CD Endcapped Polyrotaxane with NPC1-deficient Cells

As suggested by our confocal data, NPC1-deficient cells show cellular association by 4 h. Mean fluorescence intensities peaked at 24 h after treatment. The cells were washed to remove non-internalized PR and prevent further uptake of PR before incubating for another 24 h to monitor the cellular fate of the already internalized PR. This is referred to as the 48 h timepoint. The 48 h time point images show decreased mean FITC intensity, indicating that all cells internalize the PR. The data also suggest that the PR are capable of escaping from the endosomal/lysosomal compartments of the cell or are degraded within them (Fig. 10).

4. Conclusion

A new HP- β -CD polyrotaxane containing a decamethylenephosphate core has been synthesized and its cholesterol normalization capacity evaluated in NPC1-deficient cells. The degree of polymerization in preparation of the decamethylenephosphate polymeric

precursor was determined to be 76. The degree of α -CD threading to produce **5** was estimated to be 48.5% after 2.9 d, whereas the HP- β -CD coverage was 18% and α -CD endcapping was approximately 3% in polyrotaxane **6**. Reduction in filipin staining, indicative of cholesterol depletion, was found to be approximately 20% at a polyrotaxane concentration of 0.3 g/L after 24 h incubation, similar in magnitude to the reductions observed by Mondjinou *et al.*⁷⁰ with mixed HP- β -CD:SBE- β -CD Pluronic polyrotaxanes that were used in 6 h exposures to NPC1 cells. Taken together, the findings suggest that polyrotaxane **6** may be a useful tool for the delivery of HP- β -CD as a therapeutic payload for normalizing cholesterol levels in Niemann-Pick Type C cells. The main advantages of our system is the simple release of HP- β -CD from the polymer by slippage⁷¹ and the expected slow renal clearance due to the high molecular weight of the polyrotaxane **6** of around 35 kDa. There is no need for any cleaving enzymes to release the drug in the cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1:
 ^1H NMR spectra of polyrotaxanes a) **5** and b) **6**.

Fig. 2:
Complexation kinetics of α -CD with guest polymer **1** at 60 °C. The data were fitted with equation (1), where $y_{\infty} = 48.5\%$ and $\tau = 2.9$ d.

Fig. 3:
DOSY NMR spectrum of polyrotaxane **6** in D_2O , signal at 4.8 ppm due to HOD.

Fig. 4:
Release of HP-β-CD from 2.59 mg polyrotaxane **6** in dissolved in 1 mL PBS over 50 d. The data were fitted with equation (1), wherein $y_{\infty} = 697 \mu\text{g}$ and $\tau = 10.5 \text{ d}$.

Fig. 5:
Filipin staining of primary fibroblasts after treatment with α -CD, poly-(decamethylenephosphate) **1**, HP- β -CD and **6** for 24 h (25 μ M equivalent HP- β -CD dose, Filipin was used at 50 μ g/mL).

Fig. 6:
Cytotoxicity of **6** evaluated via MTS assay after 24 h exposure to NPC1-deficient cells, concentration plotted in μM of HP- β -CD equivalents.

Fig. 7:
Dose-response curve in NPC1 cells after a single 24 h treatment with **6** and HP-β-CD.

Fig. 8:
Structures of FTIC-tagged polyrotaxane **6a** and the fluorescent CD-derivative **7**.

Fig. 9:

NPC1-deficient cells treated with 25μM CD-equivalent FITC tagged polyrotaxane **6a** in Opti-MEM media. (A) untreated cells, (B) 4 h **6a** treatment (c) 24 h **6a** treatment, and (D) C + 24 h post-treatment. i : all fluorescence channel; ii : DAPI channel; iii : fluorescein channel, and iv : Lysotracker channel. Localization within acidic endosomes/lysosome (red, stained with Lysotracker) peaked at 24 h after addition of **6a**, after which the cells were washed to remove **6a** and incubated for another 24 h in complete MEM media. Confocal images acquired 24 h post treatment show a decrease in the FITC signal indicating either release of cyclodextrins into the cytoplasm where it is diluted below detectable levels, or fluorescein degradation within them. The scale bar = 100 μm.

Fig. 10:

a) Histogram Plot for FITC intensity distribution among NPC1-deficient cell population. b) Cellular association of Polyrotaxane **6a** with NPC 1-deficient cells

Scheme 1:

Synthesis of the guest polymer poly(decamethylene phosphate) 1.