

Glutathione as the End Capper for Cyclodextrin/PEG Polyrotaxanes*

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Abstract A facile one-pot synthesis of α -cyclodextrin-based polyrotaxane (PR) in aqueous solution is reported, where the peptide glutathione was used as the end-capping agent and the thiol-ene Michael addition was used as the end-capping reaction. Both polyrotaxanes with low threading ratio and high threading ratio were successfully obtained. In contrast to the conventionally used multiple-step synthesis methods and hydrophobic end cappers, this one-pot aqueous synthesis as well as the biocompatibility of the end-capping agent could result in a much more biocompatible PR to be used as biomaterials.

Keywords: Polyrotaxane; Cyclodextrin; Polyethylene glycol; Glutathione; Biocompatibility.

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INTRODUCTION

Since it was first reported in 1990s, polyrotaxane (PR) consisting of α -cyclodextrin (α -CD) and polyethylene glycol (PEG) has attracted extensive interests due to its relatively convenient preparation and fascinating supramolecular structure^[1–5]. With many hydroxyl groups from the CDs for further chemical modification, PRs are also capable of multiple ligands incorporation, thus PRs represent an interesting non-covalent route to overcome one of the biggest limitations of PEG, *i.e.*, lack of functional groups. Moreover, being constrained on the axial PEG chains, the CDs may still be able to rotate and slide, which also renders incorporated ligands on PRs great flexibility and mobility^[6, 7]. With these characteristics, CD-based PRs have great potential to be used as novel biomaterials^[8–12].

Besides CD and PEG, a complete PR needs end capping groups at the PEG chain ends to prevent CD de-threading. Most of the synthesis methods reported in literature utilized bulky and hydrophobic moieties^[13, 14], such as di-nitrobenzene^[15], adamantane^[16] and cholesterol^[17]. Though both CD and PEG are biocompatible, the end capping groups may cause problems in future biomedical applications. These large and hydrophobic groups at the PEG chain ends make the PRs to be surfactant-like with undesirable interactions with cells. For example, di-nitrobenzene moieties were found to cause immunogenicity problem^[18], while adamantane and cholesterol at PEG chain ends interfere with the cell membranes and even cause cell death^[19–21]. In addition, most of end capping reactions can only be conducted in organic solvents, and hence the preparation uses a two-step strategy, *i.e.*, obtaining pseudo-polyrotaxane (PPR) in the aqueous solutions followed by capping the PPR in organic solvents^[13–15]. A few studies reported the one-pot preparation in aqueous solutions to make the PR synthesis more convenient^[22–24], but they still lack more biocompatible end-capping agents.

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Therefore, a method that caps the PPR in the aqueous solution under mild condition with cell-friendly end-capping reaction and moieties is highly desirable. Herein, we propose glutathione as an easily-available, biocompatible capping molecule, and thiol-ene Michael addition as an efficient reaction for end-capping of PPR. Meanwhile, using glutathione as end-capping agent, which is an endogenous peptide^[25], could possibly avoid the bio-compatibility issue and enable enzyme-responsive de-threading. Here we demonstrated that the tri-peptide structure of glutathione is large enough for restricting CDs on the PEG chain. The coupling reaction is efficient and PRs with both low and high α -CD threaded ratio were obtained by simply changing the time of addition of glutathione.

EXPERIMENTAL SECTION

Materials

PEG (MW 4000, PEG4K) and sodium hydride (NaH) were purchased from Alfa Aesar. Ethyl 2-(bromomethyl) acrylate (EBrMA) was synthesized according to the literature^[26]. All other reagents were used as received without further purification.

Analytical Techniques

NMR spectra were obtained from a 300 MHz NMR spectrometer (JOEL). MALDI-TOF MS was conducted using a BIFLEX III TOF-MS (Bruker Daltonics) and DHB was used as matrix. Gel permeation chromatography was performed on a Prominence LC system (Shimadzu), deionized water with addition of 0.05% NaN₃ was used as eluent and flow rate was kept as 0.5 mL/min. Powder X-ray diffraction (XRD) was conducted on an R-Axis Spider apparatus (Rigaku, Cu).

Synthesis of α -PEG-MA

The α -PEG-MA was synthesized as reported previously^[27]. Briefly, PEG4K (4.0 g, 1.0 mmol) was dissolved in 200 mL dry THF followed by adding NaH (0.3 g, 12.5 mmol). After that, EBrMA (0.7 mL, 4.0 mmol) was added dropwise. Avoiding light by wrapping the flask with aluminum foil, the reaction was carried out overnight at room temperature. Then, acetic acid was added dropwise to neutralize the reaction. After filtering off insolubles and evaporating most of the solvent, the condensed solution was poured into diethyl ether to precipitate the product. The solid was washed with diethyl ether three times, and then vacuum dried. ¹H-NMR (300 MHz, D₂O, δ): 1.19 (t, 6 H, CH₃—), 3.58 (s, 200 H, PEG backbone), 4.13–4.21 (t, s, 8 H, —CH₂—C(O)—O—, —O—CH₂—C(=CH₂)—), 5.84 (s, 1 H, —CH=CH₂), 6.28 (s, 1 H, —CH=CH₂).

¹H-NMR Monitoring of the α -PEG-MA-glutathione Reactions

The reaction of α -PEG-MA with glutathione was monitored with ¹H-NMR of mixed samples in D₂O. α -PEG-MA (5.0 mg, 0.0012 mmol) was dissolved in 0.50 mL D₂O and pH was adjusted to 8.65 with NaOD (30 wt%). Glutathione (3.8 mg, 0.012 mmol) was dissolved in 0.50 mL D₂O and pH was adjusted to 9.09 with NaOD (30 wt%). Reaction was conducted by mixing the two solutions in standard NMR tube. Measurements (16 scans, room temperature) were taken every 3.5 min.

End-capping Efficiency Study

Glutathione (0.5 g, 1.6 mmol) was dissolved in 2 mL deionized water, and 0.5 mL NaOH solution (31.7 %) was added to adjust the pH to 9.0. α -PEG-MA (0.2 g, 0.05 mmol) was dissolved in 6 mL deionized water while α -CD (0.5 g, 0.5 mmol) was dissolved in 4 mL deionized water. After mixing α -PEG-MA and α -CD solutions, the mixture was immediately divided into 4 portions, 2.5 mL each. 0.6 mL glutathione solution was then added to one of the mixtures at three different time points (15 min, 1 h and 2 h). As a control, the last one was added with 0.6 mL deionized water 2 h after mixing.

Synthesis of Glutathione-capped PR

To achieve a low α -CD threading ratio, α -PEG-MA (101.8 mg, 0.025 mmol) dissolved in 3 mL deionized water and α -CD (250.0 mg, 0.25 mmol) dissolved in 2 mL deionized water were mixed for 30 min. And 2.8 mL glutathione aqueous solution (253.1 mg, 0.82 mmol, pH = 9.5) was then added. The mixture was stirred for

another 1 h and transferred into to a dialysis tube (MWC 3,500 Da). After dialyzed against deionized water for 3 days, during which water was changed 3 times a day, the product was obtained by freeze-drying. As control, PEG-S-G which was α -PEG-MA reacting with glutathione without α -CD was also prepared according to the same molar ratio.

To obtain the high α -CD threading ratio, α -PEG-MA (50.0 mg, 0.013 mmol) dissolved in 3 mL deionized water and α -CD (1.00 g, 1.0 mmol) dissolved in 5 mL deionized water were mixed and stirred for 5 h. And 3.0 mL glutathione aqueous solution (366.0 mg, 1.2 mmol, pH = 9.5) was then added. The mixture was stirred overnight and then washed with deionized water until no free α -CD in supernatant could be detected on GPC. The solids was then collected and freeze dried.

RESULTS AND DISCUSSION

The strategy of synthesizing PR by using glutathione as end-capping reagent is shown in Fig. 1. The coupling reaction used to end-cap α -CD on axel PEG chain, Michael addition of glutathione to α -PEG-MA, was examined first. To serve as a capping reaction, both conversion ratio and reaction rate need to be efficient. The reaction was studied by $^1\text{H-NMR}$ and the peaks corresponding to the protons of the reactive vinyl groups of α -PEG-MA ($\delta = 6.28$ and 5.84) were monitored. The peak of CH_3 of the ethyl end groups of α -PEG-MA ($\delta = 1.19$) was used as an internal standard. After mixing the two components, the peaks of the vinyl groups were decreasing overtime (Fig. 2a). The ratio of unreacted vinyl groups normalized to their initial state ($t = 0$) was plotted as a function of time (Fig. 2b), which demonstrated that the reaction is efficient and the chain ends were completely capped in about 30 min.

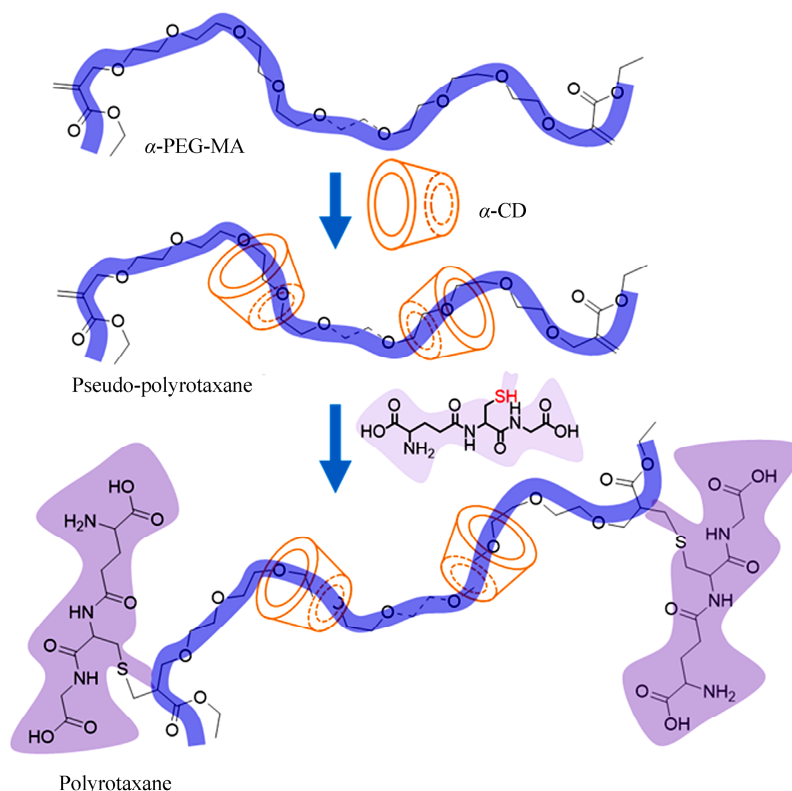


Fig. 1 Scheme of one-pot synthesis of glutathione-capped PR

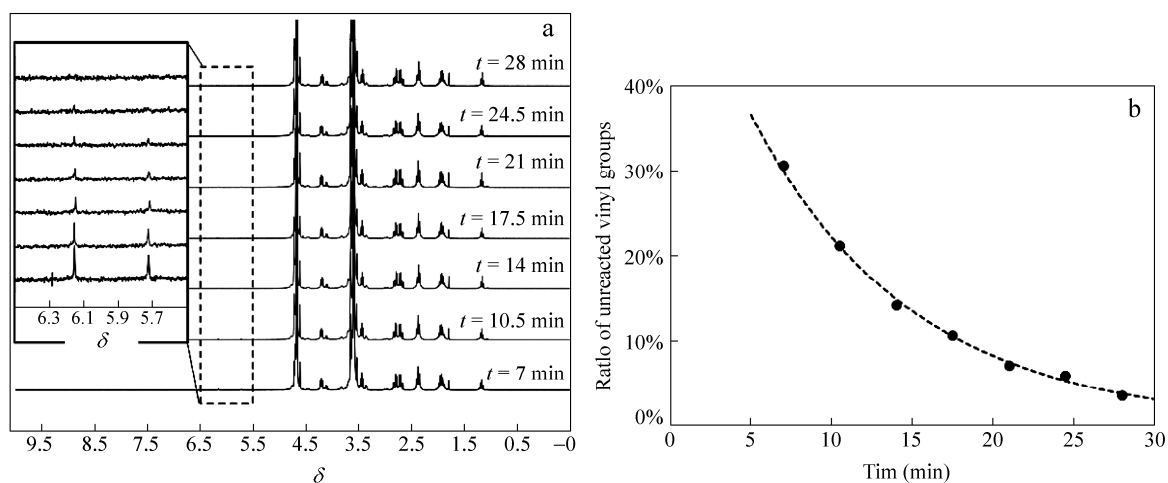


Fig. 2 (a) ¹H-NMR spectra of α -PEG-MA with glutathione in D₂O at different time after mixing; (b) Ratio of unreacted vinyl groups over time calculated from ¹H-NMR

Besides the coupling reaction, the major requirement for an end-capping reagent of PR is being large enough to prevent cyclodextrins de-threading from the PEG chain. The minimum distance from the thiol group to the end of glutathione molecule was estimated as 0.62 nm in ChemBioDraw (CambridgeSoft®, Version 12.0) (Fig. S1), while the internal cavity diameter of α -CD is 0.47 nm^[28]. Therefore, glutathione should have a sufficient size for blocking α -CD in PEG chain. Further confirmation of the end-capping efficiency was performed by capping α -PEG-MA with glutathione followed by testing the threading behavior of α -CD. As described in experimental section, α -CD was mixed with the reaction mixture of α -PEG-MA and glutathione at different time points. According to the literature, the threading of α -CD onto PEG chain can be visually demonstrated since the complex will precipitate from the solution^[1, 2]. Consistently, without glutathione capping, the mixture of α -CD and α -PEG-MA did become turbid and white precipitation was observed (Fig. S2. E). In contrast, if glutathione was added before precipitation (Fig. S2. B, C, D), the solution remained clear, demonstrating the efficient end-capping of glutathione. In addition, the sample with shortest mixing time (15 min) also indicates that the end-capping reaction is fast and efficient.

Due to the convenience of Michael addition, which can be carried out in mild condition in aqueous solution, which is preferable for inclusion forming between PEG and α -CD, the one-pot synthesis of PR could be performed readily. In addition, because of the fast and efficient reaction rate, it is possible to end-cap the PPR at different stage during α -CD threading to adjust the threading number of α -CD. To demonstrate that this can be done, the coupling reaction was performed at two time points in the following studies.

To obtain a low threading ratio, the glutathione was added to cap the PPR *in situ* 30 min after the mixing of α -CD and α -PEG-MA, when the solution was still clear. After dialyzed against water to remove the free α -CDs and unreacted residuals, the product was collected and the average number of threaded α -CDs per PEG chain (N_{CD}) was characterized by using ¹H-NMR. The peak at $\delta = 4.98$ in ¹H-NMR spectrum (**l** in Fig. 3a) corresponds to the proton of H-1 of α -CD, while that at $\delta = 1.19$ corresponds to the CH₃ residual of the methyl end groups of α -PEG-MA (**g** in Fig. 3a). With dividing the peak area of **l** by that of **g** mentioned above, N_{CD} was calculated to be about 1.2. To further verify the structure, ¹³C-NMR was also obtained (Fig. S3). All the peaks were assigned in the spectrum and confirmed the structure of the obtained PR product.

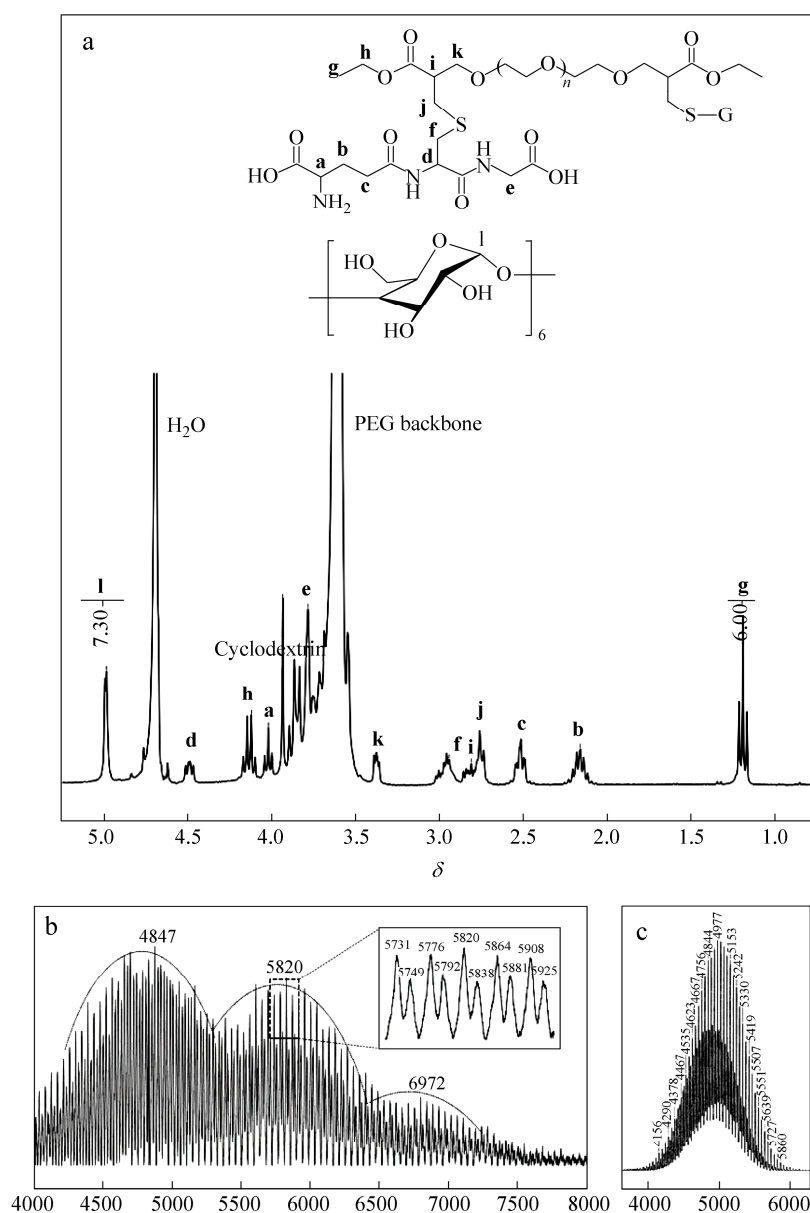


Fig. 3 PR with low α -CD threading ratio characterized by (a) ^1H -NMR (D_2O , 300 M), (b) MALDI-TOF (DHB as matrix) and (c) MALDI-TOF of PEG-S-G as control

MALDI-TOF MS was performed (Fig. 3b) to directly confirm that the obtained PR product was inclusion of α -CDs with PEG instead of the mixture of free α -CDs and PEG. Compared to PEG-S-G (Fig. 3c), it was clear that there were two extra clusters of peaks besides the main PEG-S-G clusters differed by about 972/973 Da, which corresponds to the molecular weight of one α -CD. Within each cluster, peak intervals are 44 Da, corresponding to the repeat unit of PEG. Considering the molecular weight of PEG used here was 4000 Da, these three clusters can be assigned to PEG with no α -CD, PR containing 1 and 2 α -CD(s) on PEG chains, respectively. Specifically, the peak of 5820 Da (from the enlarged detail in Fig. 3b) corresponds to PR composed of 1 α -CD and glutathione-S-PEG-S-glutathione with 89 PEG repeat units (theoretically calculated value: $[\text{C}_{248}\text{H}_{472}\text{N}_6\text{O}_{137}\text{S}_2]\text{Na}^+$, 5817 Da). This result of low threading ratio was also consistent with the ^1H -NMR result.

On the other hand, the PR with high α -CD threading ratio can be synthesized by mixing the α -CD and α -PEG-MA for longer time (5 h) to let threading proceeded adequately. Because now the end capping reaction needs to be done with pseudo-polyrotaxane as precipitate, longer reaction time was used (overnight) to maximize the end capping yield. To optimize the purification procedure, instead of dialysis, the obtained solid was washed thoroughly with water until no free α -CD can be obviously detected in supernatant by GPC. As a result, the obtained product should be the glutathione capped PR without free α -CD, and then the N_{CD} was calculated from $^1\text{H-NMR}$ (Fig. 4a) as around 35. According to the literature, the α -CD cavity accommodates two ethylene glycol units of PEG^[29] and so the full coverage on PEG4K would be 45 α -CDs per chain. This means around a 78% coverage was obtained here. Further characterization of this high coverage PR was conducted by powder XRD. The characteristic diffraction peak at around $2\theta = 20^\circ$ (Fig. 4b) demonstrated the extended column structure of α -CDs due to the inclusion formation, which was in consistent with the literature^[1].

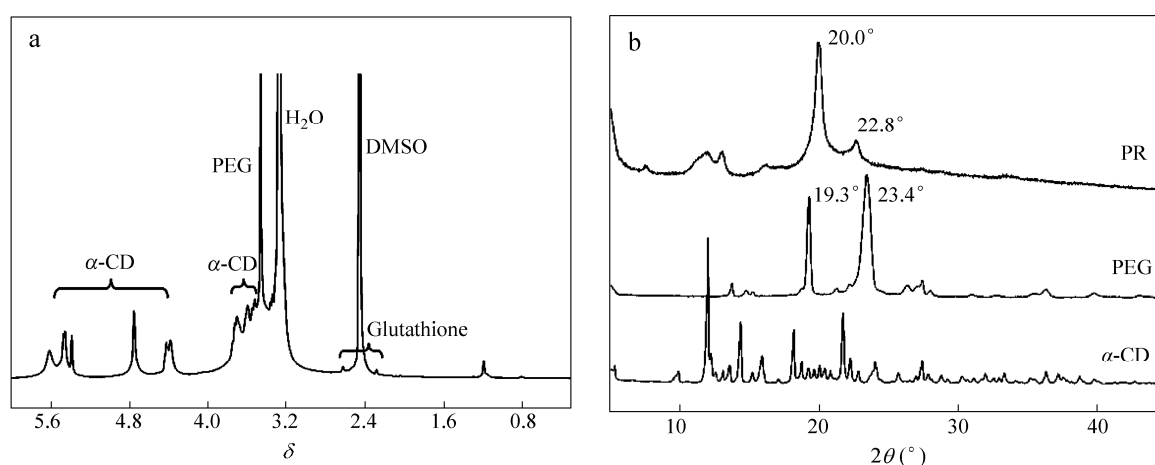


Fig. 4 PR with high α -CD threading ratio characterized by (a) $^1\text{H-NMR}$ (DMSO-d_6 , 300 M) and (b) powder XRD

The PRs synthesized with two different threading ratios could potentially be used for two kinds of applications. PR with lower threading ratios (1–2 CDs/chain) can benefit more from the mobility due to the loose distribution of the entrapped α -CDs along the axial polymer chains. On the other hand, the one with higher threading ratios (35 CDs/chain) may benefit from the high functionality due to the abundant hydroxyls introduced by α -CDs. However, unfortunately, the PR with intermediate threading ratio was not obtained. It may be due to the formation mechanism of inclusion between α -CDs with PEG. A possible explanation is that at lower threading ratio stage, the PPR is in the equilibrium condition with free α -CDs and PEG chains, where the whole solution is homogenous and clear. This resulted in that the *in situ* end-capping just obtained PR with low threading ratios. When more α -CDs thread onto the chain, the PEG chains will then be fully threaded at once without a “one-by-one” stepwise procedure^[30, 31], and hence only a nearly fully covered PR can be obtained after end-capping the precipitated pseudo-polyrotaxane.

CONCLUSIONS

In summary, a biocompatible endogenous peptide glutathione was utilized to end-cap the α -CDs based PR *in situ* in aqueous solutions. This thiol-ene Michael addition is shown to be fast and efficient, and the size of glutathione was sufficient to block the α -CDs on PEG axles. By adding glutathione after different time of mixing α -CDs with PEG, both PRs with low threading ratio ($N_{CD} \sim 1.2$) and high threading ratio ($N_{CD} \sim 35$) were successfully obtained. Compared with the conventional multiple-step synthesis methods, this one-pot aqueous synthesis as well as the biocompatibility of the end-capping agent could result in a more biocompatible PR to be used as biomaterials.

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