Anticoagulant supramolecular-structured polymers: Synthesis and anticoagulant activity of taurine-conjugated carboxyethylester-polyrotaxanes

Yoon Ki Joung\textsuperscript{a}, Yoichi Sengoku\textsuperscript{a}, Tooru Ooya\textsuperscript{a}, Ki Dong Park\textsuperscript{b}, Nobuhiko Yui\textsuperscript{a,*,}\textsuperscript{a}

\textsuperscript{a}School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Tatsunokuchi, Ishikawa 923-1292, Japan
\textsuperscript{b}Department of Molecular Science and Technology, Ajou University, 5 Wonchon, Youngtong, Suwon 442-749, South Korea

Received 16 October 2004; revised 14 March 2005; accepted 14 March 2005
Available online 14 July 2005

Abstract

Polyrotaxanes with both sulfonyl and carboxyl groups were synthesized and characterized for mimicking the anticoagulant activity of heparin. A polyrotaxane consisting of \(\alpha\)-cyclodextrins (\(\alpha\)-CDs) and poly(ethylene glycol) (PEG) was synthesized, and carboxyethylester (CEE) groups and taurine were successively conjugated with the polyrotaxane to obtain taurine-conjugated carboxyethylester-polyrotaxanes (TAU-CEE-PRxs). The number of \(\alpha\)-CDs and the anionic groups could be varied by synthetic conditions. The structural factors of TAU-CEE-PRxs affecting anticoagulant activity were suggested as following: (i) relatively lower threading percentage of \(\alpha\)-CDs, (ii) the ratio of anionic groups similar to heparin, and (iii) lower molecular weight of PEG. The TAU-CEE-PRx that sufficiently meet the mentioned requirements showed enhanced antithrombin III (AT III) activity, indicating that the TAU-CEE-PRx interacts with AT III and/or thrombin. From these results, it is suggested that the sliding and rotation of free \(\alpha\)-CDs with anionic groups are related with enhancing anticoagulant activity.

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Keywords: Polyrotaxane; Anticoagulant activity; Heparin; Antithrombin III; Cyclodextrin; PEG; Supramolecular structure; Anionic polymer; APTT; Taurine

1. Introduction

After discovering heparin in 1916, many studies for molecular modeling of heparin’s structure have been investigated [1]. By the 1970s, chemical structure of heparin was identified as a highly sulfated polysaccharide that contains L-iduronic acid and glucosamine [2]. Understanding the chemical structure and its conformation is clinically important to clarify factors for anticoagulant activity. It is known that heparin binds to antithrombin III (AT III) with changing its conformation, resulting in the inhibition of blood coagulation through complexation with thrombin [3–6]. In order to clarify the mechanism, many studies have been focused on the modification of functional groups and removal of partial sequence [7–9]. Structural requirements for the heparin activity are suggested as the density and precise placement of negative charge, chain flexibility, topological conformation, and average molecular weight [10,11]. Mimicking heparin using synthetic polymers has been extensively studied by introducing sulfonyl and/or carboxyl groups to some polymeric backbones [12,13]. These synthetic polymers are focused on new anticoagulants instead of heparin in the standpoint of bio-safety. However, the design of the heparin mimicking polymers is not enough for showing high anticoagulant activity. This may be due to poor molecular design and synthesis of sulfonated polymers to mimic anticoagulant mechanism of heparin. In the anticoagulant mechanism, strong binding between AT III and the polymers should be important. It is reported that helical structure [1], and the density and position of sulfate and carboxyl groups [14] are the most considerable factors for the heparin activity.

We have focused on polyrotaxanes as supramolecular-structured and rod-like polymers. Polyrotaxane is defined as a molecular assembly in which many cyclic molecules are threaded onto a linear macromolecule capped with bulky-end groups. Representative polyrotaxane is based on a combination of \(\alpha\)-cyclodextrins (\(\alpha\)-CDs) and poly(ethylene glycol) (PEG) capped with some bulky-end groups, reported by Harada et al. [15]. Threading cyclic compounds in
the polyrotaxanes can slide or rotate along the axis, and the structure in solution is proposed as a rod-like one [16,17]. Moreover, hydroxyl groups of α-CDs in the polyrotaxanes are subjected to chemical modifications. Some conjugates of polyrotaxanes have been investigated by our group, and these results strongly suggested that the mobility of ligands in the conjugates enhances multivalent interaction with various proteins [18–21]. In our system, α-CDs are threaded onto a PEG chain capped with bulky-end groups via biodegradable linkages. On the basis of antithrombin-binding sequence in heparin and its helical conformation, we have proposed that new anticoagulants are possible to be designed by polyrotaxanes. In our previous study, sulfonated polyrotaxanes were synthesized and evaluated as anticoagulants. The sulfonated polyrotaxanes, in which α-CDs were threaded onto a tri-block copolymer (Pluronic®), followed by sulfonation of α-CDs, showed considerable anticoagulant activity as compared with the mother polyrotaxanes and pluronic® [22,23]. However, the effect of structural factors such as the number of α-CDs, the number of sulfonyl and/or carboxyl groups, and the molecular weight of PEG on the anticoagulant activity are unknown. These factors are likely to correspond to key requirements for anticoagulant activity such as the density and ratio of anionic groups, the conformational position of anionic groups, and the molecular weight of heparin.

In this study, polyrotaxanes with sulfonyl and carboxyl groups, taurine-conjugated carboxyethylester (CEE)-polyrotaxanes, were synthesized and their anticoagulant activity was evaluated. The ratio of anionic groups could be remove impurities including free α-CDs. The resulting precipitate was dried in vacuo at room temperature to obtain α-CD threading. The α-CD threading. The α-CDs in the polyrotaxane (Mn of PEG: 4000) was calculated to be 38, which was determined by comparing the integration of the peak at 4.8 ppm (C(1)H of α-CD) with that at 3.5 ppm (CH2 of PEG) from the 1H NMR spectrum. In the same manner, the number of α-CDs in the polyrotaxane (Mn of PEG: 35,000) was calculated to be 187.

2.2. Preparation of Z-L-Tyr-terminated polyrotaxanes

Polypseudorotaxanes (inclusion complex of α-CDs and diamino-PEG, the number of α-CDs: 39 and 260 when the Mn of PEG was 4000 and 35,000, respectively) were prepared according to the previously reported method [15]. Then, Z-L-Tyr-terminated polyrotaxanes were synthesized. Z-L-Tyr (12.2×10⁻² mol), BOP (12.2×10⁻² mol), HOBt (12.2×10⁻² mol), and DIEA (12.2×10⁻² mol) were dissolved in DMF (10 ml). The solution was added to a suspension of the polypseudorotaxane (6.1×10⁻⁴ mol) in DMF (20 ml), and the reaction mixture was stirred at room temperature for 24 h. In this process, DMSO was added to reaction mixture for controlling α-CD threading. The mixture was poured into excess acetone to precipitate crude products and to remove BOP, HOBt, DIEA, and unreacted diamino-PEG. The precipitate was collected by centrifugation and washed with ethanol and pure water to remove impurities including free α-CDs. The resulting precipitate was dried in vacuo at room temperature to obtain Z-L-Tyr-terminated polyrotaxane as a white powder. The average number of α-CDs in the polyrotaxane (Mn of PEG: 4000) was calculated to be 38, which was determined by comparing the integration of the peak at 4.8 ppm (C(1)H of α-CD) with that at 3.5 ppm (CH₂ of PEG) from the 1H NMR spectrum. In the same manner, the number of α-CDs in the polyrotaxane (Mn of PEG: 35,000) was calculated to be 187.

2.3. Synthesis of CEE-polyrotaxanes

Carboxyethylpolyeoxotaxanes (CEE-polyrotaxanes) were prepared according to our established method [20]. The Z-L-Tyr-terminated polyrotaxanes (The number of α-CDs: 38, 3.2×10⁻⁵ mol) and succinic anhydride (5.1×10⁻³ mol) (as the stoichiometric ratio of hydroxyl groups in the Z-L-Tyr-terminated polyrotaxanes) were dissolved in dried pyridine (20 ml) and stirred at room temperature for 24 h. The reaction mixture was poured into excess diethyl ether and washed with diethyl ether three times. The precipitate was collected by centrifugation and dried in vacuo to give the CEE-polyrotaxanes. The average numbers of carboxyl groups and α-CDs in the polyrotaxanes (Mn of PEG: 4000) were determined by calculating the integration ratio of the methylene peak of CEE (2.3 ppm) and C(1)H of α-CD (4.9 ppm) on 1H NMR spectrum in Fig. 1(a). In the same manner, the number of...
carboxyl groups and \( \alpha \)-CDs in the polyrotaxanes (\( M_n \) of PEG: 35,000) was calculated.

### 2.4. Synthesis of taurine-conjugated CEE-polyrotaxanes

The CEE-polyrotaxane (1.9 \( \times \) 10\(^{-5} \) mol) was dissolved in distilled water (20 ml), and then taurine (8.0 \( \times \) 10\(^{-3} \) mol) was added to the prepared solution. Aqueous WSC solution (3.9 \( \times \) 10\(^{-2} \) mol) was added to the solution in drops, and then the mixed solution was stirred at room temperature for 24 h. The result of the amounts of CEE and taurine groups remained carboxyl groups, and average molecular weight were calculated to be 76, 24, and 34,800 by comparing integrations of the methylene peak of taurine (2.9–3.1 ppm) and methylene peak of CEE (2.3 ppm) from \(^1\)H NMR spectra, respectively, in Fig. 1(b).

### 2.5. Anticoagulant activity of taurine-conjugated CEE-polyrotaxanes

Activated partial thromboplastin time (APTT) test was performed using Automated Blood Coagulation Analyzer (CA-50, Sysmex Corp., Kobe, Japan). Coagtrol IX–IIX and TAU-CEE-PRxs were thoroughly dissolved in pure water as to be 0.05 wt% solution, and then reaction tubes and reagents are set in the instrument that is previously warmed up to 37°C. Before the APTT test, a standard curve was checked to make sure that they are correctly set. Fifty microliters of all reagents are orderly inserted into the incubated tube according to procedure and finally an aqueous solution of TAU-CEE-PRxs (20 ml) is added.

### Table 1

| Code   | Composition\(^a\) | Threading of \( \alpha \)-CD | Conjugation of anionic groups\(^b\) | Anticoagulant activity (IU/ mg)±SD |
|--------|-------------------|-------------------------------|-----------------------------------|-----------------------------------|
| SP4-1  | 35TAU-19CEE-9\(\alpha\)/E4 | 9 10 | 2.1 3.9 1.8 | 1.74±0.049 |
| SP4-2  | 76TAU-24CEE-9\(\alpha\)/E4 | 9 10 | 2.7 8.4 3.2 | 2.16±0.016 |
| SP4-3  | 117TAU-13CEE-9\(\alpha\)/E4 | 9 10 | 1.4 13.0 9.0 | 0.281±0.028 |
| SP4-4  | 75TAU-56CEE-15\(\alpha\)/E4 | 15 33 | 3.7 5.0 1.3 | 0.365±0.030 |
| SP4-5  | 278TAU-15CEE-22\(\alpha\)/E4 | 22 49 | 0.7 12.6 18.6 | 0.883±0.015 |
| SP4-6  | 92TAU-30CEE-38\(\alpha\)/E4 | 38 84 | 0.8 2.4 3.1 | 0.456±0.009 |
| SP35-1 | 273TAU-30CEE-39\(\alpha\)/E35 | 39 10 | 0.8 7.0 9.1 | 0.027±0.005 |
| SP35-2 | 220TAU-155CEE-40\(\alpha\)/E35 | 40 10 | 3.9 5.5 1.4 | 0.694±0.021 |
| SP35-3 | 162TAU-27CEE-40\(\alpha\)/E35 | 40 10 | 0.7 4.1 6.0 | 0.610±0.017 |
| SP35-4 | 423TAU-201CEE-75\(\alpha\)/E35 | 75 19 | 2.7 5.6 2.1 | 0.767±0.024 |
| SP35-5 | 416TAU-76CEE-78\(\alpha\)/E35 | 78 20 | 1.0 5.3 5.5 | 0.013±0.002 |
| SP35-6 | 334TAU-336CEE-79\(\alpha\)/E35 | 79 20 | 4.3 4.2 1.0 | 0.074±0.019 |

\(^{a}\) xTAU-yCEE-z\(\alpha\)/En: taurine-conjugated carboxyethylster-polyrotaxane, x: number of sulfonyl group, y: number of carboxyl group, z: number of \( \alpha \)-CD, \( n \times 10^3 \): \( M_n \) of PEG.

\(^{b}\) The number and ratio of anionic groups per \( \alpha \)-CD were calculated by the integration of each peak in \(^1\)H NMR spectra.

\(^{c}\) The number of \( \alpha \)-CDs threaded onto polyrotaxane was calculated by the integration of each peak in \(^1\)H NMR spectra.

\(^{d}\) The threading percentage of \( \alpha \)-CDs (\%) = (real threading number/theoretical threading number) \times 100.
The result of the APTT test was obtained as time, in second. Standard curve of coagulation time was made using heparin as a reference. The anticoagulant activity of TAU-CEE-PRxs was calculated as heparin-related unit (IU/mg) by using the standard curve.

2.6. Antithrombin III activity test

AT III activity of a TAU-CEE-PRx was quantitatively determined by a chromogenic assay using the AT III Auto B kit. This method is based on the determination of free thrombin that does not participate in complex formation with AT III using a chromogenic substrate for thrombin. A thrombin reagent was dissolved with a buffer solution (Tris, 100 mmol/l; NaCl, 8.7 g/l; pH 8.2) and incubated for 30 min at room temperature. Substrate reagent (tosyl-gly-pro-arg-5-amino-2-nitrobenzoic acid isopropylamide, 4 mmol/l) was dissolved with distilled water before use. SP4-2 (in Table 1) or heparin was dissolved with distilled water, and these solutions were used as sample solution. One-microliter of thrombin reagent and 2 μl of standard plasma (Coagtrol IX–IX) were added to vial. The solution was mixed and incubated for 3 min at 37 °C.Twenty-two microliters of the substrate reagent was added to the solution and immediately incubated for 2 min at 37 °C. After that, the absorbance at 405 nm (A405) of the resulting solution was determined within 30 s to measure the initial A405. After 60 s, A405 was determined again to calculate the difference of A405 for 1 min in the samples (ΔA405/min). In the similar manner, the difference of A405 for 1 min in a blank (ΔA405/minb) and in a standard plasma (ΔA405/minsp) was determined by using saline solution and in a standard plasma instead of the sample solution, respectively. Finally, AT III activity (% of normal activity) was calculated by the following equation:

\[
AT III \text{ activity} \% = \left( \frac{\Delta A_{405/\text{min}} - \Delta A_{405/\text{minb}}}{\Delta A_{405/\text{minsp}}} \right) \times F_L 
\]

\[
F_L = \frac{AT III \text{ activity in standard plasma (92%)}}{\Delta A_{405/\text{min}} - \Delta A_{405/\text{minsp}}}
\]

According to the data of the used standard plasma, the AT III activity in the standard plasma was 92%.

3. Results and discussion

3.1. Synthesis of taurine-conjugated CEE-polyrotaxanes

TAU-CEE-PRxs were synthesized by conjugating taurine with CEE-polyrotaxanes as shown in Scheme 1. By using different batch of the Z-L-Tyr-terminated polyrotaxanes, the number of α-CDs was also varied. In case of PEG 4000, threading number of α-CDs was varied from 9 to 38, and the theoretical number is 45, assuming α-CD includes two repeating units of ethylene glycol. From this result, the threading percentage of α-CDs was calculated as 10–84%. In the same manner, the threading number and percentage of α-CDs in PEG 35,000 (the theoretical number: 398) was varied from 39 to 79 and was calculated to be 10–20%, respectively. The number of CEE per α-CD was found ca. 6–12 in both cases.

Taurine was conjugated with the CEE-polyrotaxanes using WSC as a condensation reagent. Substitution of the CEE group to the sulfonyle group was controlled by the feed content of WSC. The conjugation of taurine was determined from characteristic peaks in 1H NMR spectrum as shown in Fig. 1(b). The peaks of taurine on the 1H NMR spectrum at 3.1 ppm (adjacent CH2 of amine group) and 3.3 ppm (adjacent CH2 of sulfonic acid) were shifted to 3.4 and 2.9 ppm after the conjugation with CEE group, respectively. This result indicates that taurine was conjugated to the CEE group of α-CDs in the CEE-polyrotaxanes. The result of the synthesis was summarized in Table 1. The feed ratio of WSC and the CEE-polyrotaxanes was varied to control the number of activated carboxyl groups, and the feed of taurine was fixed. As a consequence, the number of taurine was randomly controlled due to different chemical environments such as the number of the CEE groups and α-CDs in the CEE-polyrotaxanes. Carboxyl groups in the CEE-polyrotaxanes were substituted to the sulfonyle groups by the conjugation of taurine and the ratio of these anionic groups (SO3−/COO− in Table 1) was varied.

3.2. Anticoagulant activity of taurine-conjugated CEE-polyrotaxanes

The coagulation time obtained from APTT was converted to the unit related with anticoagulant activity of heparin (International Unit (IU)/mg). This process makes it possible to compare the anticoagulant activity of TAU-CEE-PRxs with that of heparin. Heparin activity was accurately calibrated against coagulation time (data not shown).

The anticoagulant activity of TAU-CEE-PRxs is summarized in Table 1. Anticoagulant activity of heparin is mainly derived from the interval and density of the anionic groups, and the molecular weight. It may be possible to control these factors in our supramolecular system in terms of the number of α-CDs, the conjugation degree of anionic groups in α-CDs, and the molecular weight of PEG. Therefore, the evaluation of TAU-CEE-PRxs was focused on the correlation between these factors and the anticoagulant activity.

3.3. The effect of the threading percentage of α-CDs on anticoagulant activity

Anticoagulant activity of TAU-CEE-PRxs with various numbers of α-CDs was analyzed. Fig. 2 shows the anticoagulant activity of TAU-CEE-PRxs as the threading percentage of α-CDs. Higher values of anticoagulant activity were seen in lower threading percentage, and relatively lower
ones were found as increasing the threading percentage of α-CDs. Our previous studies have clarified that the ligand mobility of α-CDs in polyrotaxanes in relation to the threading percentage of α-CD contributes to enhancing multivalent interaction with proteins [18–21]. A decrease in the threading percentage of α-CDs gives broader spaces between adjacent α-CDs, and these are provided as space for α-CD sliding along the PEG chain. It is considered that the enough space for sliding of α-CDs results in the enhancement of mobility of α-CDs, leading to the positional change of anionic groups in α-CDs as to easily bind with AT III. However, since molecular weight of PEG in TAU-CEE-PRxs is one of the important factors, it is estimated that samples with PEG 35,000 do not correspond to above-mentioned results.

3.4. The effect of the ratio of anionic groups on anticoagulant activity

The number and ratio of anionic groups in heparin is thought to be an important factor for anticoagulant activity.
Fig. 2 shows the importance of the ratio of anionic groups in TAU-CEE-PRxs for anticoagulant activity. A variation of anticoagulant activity was seen in the polyrotaxanes with relatively low threading percentage of \( \alpha \)-CDs. This indicates that another factor related with anionic groups in TAU-CEE-PRxs quite influences the anticoagulant activity. These polyrotaxanes had the ratio of anionic groups of 1.8, 3.2, and 9.0 per \( \alpha \)-CDs, respectively, and showed significantly different anticoagulant activity (Table 1). This implies that the ratio of anionic groups is an important factor as much as the number of \( \alpha \)-CDs. Fig. 3 shows how the ratio of anionic groups in TAU-CEE-PRxs affects the anticoagulant activity. In both cases of TAU-CEE-PRxs with PEG 4000 and 35,000, the ratio required for high anticoagulant activity was found to be around 3 and 2, respectively. Although this result is under influence of the threading percentage of \( \alpha \)-CDs, it is coincident with the description mentioned in Fig. 2. It was reported that the ratio of sulfate and carboxyl groups in heparin molecule is important for high anticoagulant activity [14] and especially, the ratio of anionic groups in heparin is calculated ca. 3 (sulfate/carboxyl) [25]. These results may suggest that the similar ratio of anionic groups to that of heparin is an alternatively crucial requirement for the anticoagulant activity.

3.5. The effect of molecular weight on anticoagulant activity

As shown in Fig. 3, TAU-CEE-PRxs with PEG 4000 generally present higher anticoagulant activity than those with PEG 35,000. This indicates that shorter chain length of TAU-CEE-PRxs is more effective for higher anticoagulant activity. From this result, it is presumed that TAU-CEE-PRxs with PEG 35,000 are too long to interact with AT III for specific binding. Moreover, it is known that low-molecular-weight (LMW) heparin fractions have better biological properties, and penta-saccharide sequence is required for specific binding with AT III [1]. As these reasons, it may be suggested that TAU-CEE-PRxs with lower molecular weight PEG are more effective than those with higher molecular weight PEG for high anticoagulant activity, though the series of different molecular weight is not enough. For adequate result, further experiment should be performed using many kinds of molecular weight of PEG.

3.6. Interaction of TAU-CEE-PRx with antithrombin III

Table 2 shows the effect of heparin and SP4-2 on AT III activity. As a control, AT III activity in plasma was measured (95.2%) and this value was similar with that of standard plasma (92%). Compared with the control, SP4-2 showed remarkable enhancement in the AT III activity. This result indicates that SP4-2 interacts with AT III to form a complex, which can lead to AT III–thrombin complex. Therefore, it is suggested that high anticoagulant activity of SP4-2 is due to complex formation with AT III, which enhances binding with thrombin. Presumably, it is inferred that similar ratio of anionic groups in SP4-2 to heparin leads

| Sample | \( SO_3^- \) content (mmol/g) | \( COO^- \) content (mmol/g) | \( SO_3^- / COO^- \) | AT III activity (%) |
|--------|-----------------------------|-----------------------------|---------------------|---------------------|
| Control | –                          | –                          | –                   | 95.2\( ^b \)         |
| Heparin | 4.6                        | 1.5                        | 3.0                 | 102.8               |
| SP4-2  | 2.2                        | 0.7                        | 3.2                 | 101.3\( ^b \)       |

\( ^a \) Control: pure buffer solution; heparin and SP4-2: 0.1\( \times 10^{-3} \) wt% solution.

\( ^b \) Significantly different AT III activity at \( P \leq 0.0001 \), where calculated using a \( t \)-test.
to AT III binding site, and the residual anionic groups act as thrombin-binding site. In addition, sliding motion of α-CDs may make the bound thrombin slide along the TAU-CEE-PRx backbone until it hooks itself on the exposed loop of the activated AT III [8].

4. Conclusions

Various TAU-CEE-PRxs were synthesized and their anticoagulant activity was evaluated in terms of the threading percentage of α-CDs, the ratio of anionic groups, and molecular weight of PEG. Based on all results, following conclusions are presented. Factors controllable for TAU-CEE-PRxs with high anticoagulant activity are: (i) the threading percentage of α-CDs, (ii) the ratio or number of anionic groups, and (iii) the molecular weight of PEG. It can be presumably assumed that lower threading percentage of α-CDs could maintain average space between adjacent α-CD molecules, resulting in the high anticoagulant activity. Presumably, it is assumed that synergistic effect of mobile anionic groups by sliding motion of α-CDs in TAU-CEE-PRxs contributes to specific binding with AT III. Further experiments about AT III binding are now in progress and will be reported in our forthcoming paper.

Acknowledgements

This study was supported by Grants-in-Aid for JAPS-KOSEF Joint Research Projects from Japan Society for the Promotion of Science (JAPS) and Korea Science and Engineering Foundation (KOSEF), and JAIST International research grant.

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