



# Synthesis, Characterization, and Potential Application of Cyclodextrin-Based Polyrotaxanes for Reinforced Atelocollagen Threads

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**Abstract:** Preparing strong and flexible atelocollagen-based materials for biomedical applications is still a challenging task. To address this challenge, this study describes the synthesis and characterization of water-soluble polyrotaxanes (PRs) with different coverage ratios and molecular weights of axle polymers, and their potential applications for PR-reinforced atelocollagen threads (PRATs). A novel method was established for the syntheses of PRs with relatively low coverage ratio at the sub-gram scale, in which the aldehyde groups were employed as crosslinking sites for preparing the PRATs via reductive amination. The aldehyde groups were successfully quantified by <sup>1</sup>H nuclear magnetic resonance spectroscopy using 1,1-dimethylhydrazine as an aldehyde marker. Fourier-transform infrared and thermogravimetric analysis measurements supported the characterization of the PRs. Interestingly, tensile testing demonstrated that coverage ratio affected the mechanical properties of the PRATs more strongly than molecular weight. The insights obtained in this study would facilitate the development of soft materials based on atelocollagens and PRs.

Keywords: atelocollagen thread; crosslinking; cyclodextrins; polyrotaxane; slide-ring features



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# 1. Introduction

Polyrotaxanes (PRs) are essential components of composite materials [1–6]. Since the discovery of the first PR in 1992, various types of macrocyclic compounds and polymers have been evaluated in the formation of supramolecular architectures [1–6]. Currently, PR consisting of cyclodextrin (CD) and/or an amphiphilic polymer (e.g., polyethylene glycol (Peg)) is regarded as one of the promising slide-ring materials for various functionalities [7–11]. Multiple attempts were made to apply PRs as therapeutic and diagnostic tools in the field of biomedical chemistry [12–17]. For example, PRs are utilized for crosslinking with biomacromolecules, delivery of bioactive molecules, and surface modification of cell-adhesive materials [18–23], which indicates that selecting proper design and synthetic methods is important for implementing their desired functionalities as biomaterials. However, some of the previously used synthetic approaches for PR crosslinking remain controversial [24–30].

Collagen is a natural protein and major component of mammalian organs and tissues [31,32]. Owing to the abundance and biological benefits of collagens, collagen-based biomaterials have been widely employed in various fields [33–36]. Pepsin digestion produces an atelocollagen lacking N- and C-terminal telopeptides, which reduces the potential immunogenicity of collagen [37,38]. Atelocollagens are promising materials utilized together with collagens in advanced therapeutics [39–41]. We have previously discussed the benefits of applying atelocollagens in oligonucleotide therapeutics, drug development, and regenerative medicine [23,42–44]. However, the preparation of flexible and strong atelocollagens remains a challenging task despite their potential applicability in human treatment procedures (e.g., ligament and tendon repair). Herein, we report the synthesis, characterization, and potential applications of CDthreaded PRs for reinforced atelocollagen threads. Two different types of PRs were synthesized and crosslinked to investigate the effects of their molecular weight and coverage ratio on the mechanical properties of the PR-reinforced atelocollagen threads (PRATs) (Figure 1).



Figure 1. Schematic illustrations of PRs for PRATs.

## 2. Materials and Methods

# 2.1. Chemicals

Hydroxyl group-terminated polyethylene glycols (Peg<sub>10k</sub>-OH and Peg<sub>20k</sub>-OH), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP), and βCD were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). iodobenzene diacetate (PhI(OAc)<sub>2</sub>), *N*,*N*-dimethylformamide αCD, (DMF), dichloromethane, diethyl ether, dry acetonitrile (MeCN), and 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) were procured from Kanto Chemical Co., Inc (Tokyo, Japan). DMF was distilled under reduced pressure and stored with a molecular sieve prior to use. CD monoaldehydes (i.e.,  $\alpha$ CD-CHO and  $\beta$ CD-CHO) were synthesized according to the previous method [23]. N,N-diisopropylethylamine (DIPEA), hexamethylphosphoric triamide (HMPA), triphenylmethylamine  $(Trt-NH_2),$ [bis(trifluoroacetoxy)iodo]benzene (PhI(OAcTf)<sub>2</sub>), 1,1-dimethylhydrazine (DMHZ), and sodium cyanoborohydride (NaBH<sub>3</sub>CN) were obtained from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Amino groupterminated Pegs (Peg<sub>10k</sub>–NH<sub>2</sub> and Peg<sub>20k</sub>–NH<sub>2</sub>) were synthesized from Peg<sub>10k</sub>–OH and Peg<sub>20k</sub>-OH, respectively, via a typical Gabriel amine synthesis procedure described elsewhere [8]. PRs with a coverage ratio of 20 mol% were synthesized using Peg and  $\alpha$ CD  $(Peg_{10k}PR\alpha CD1 and Peg_{20k}PR\alpha CD1)$ .  $\alpha CD$ -threaded *pseudo*-PR (PegPR\alpha CD3) and adamantane end-capped PR (PegPR $\alpha$ CD2) were prepared according to a previously developed method [45]. To produce PRs with lower coverage ratios, triblock copolymer Pluronic and

βCD were employed as shown in Scheme 1 (Plu<sub>9k</sub>PRβCD1 and Plu<sub>15k</sub>PRβCD1). Hydroxyl group-terminated Pluronic reagents (Plu<sub>9k</sub>–OH and Plu<sub>15k</sub>–OH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Carboxyl group-terminated Pluronic compounds (Plu<sub>9k</sub>–COOH and Plu<sub>15k</sub>–COOH) were synthesized via bleach oxidation as described previously [46]. A Spectra/Por dialysis membrane (MWCO: 1 kDa) was used for purification in each step.



**Scheme 1.** Synthetic scheme for PluPRβCD1.

# 2.2. PegPRaCD1 General Synthesis Procedure

Synthesis of PegPR $\alpha$ CD1 from PegPR $\alpha$ CD2 is described in Scheme 2. PegPR $\alpha$ CD2 (0.1 g) and DIPEA (0.2 mL) were dissolved in HMPA (3 mL) and cooled to 4 °C. PhI(OAcTf)<sub>2</sub> (36 mg, 0.084 mmol) and a catalytic amount of TEMPO (3.9 mg, 0.025 mmol) were added to the obtained solution. The resulting mixture was stirred at 4 °C for 10 days and then added dropwise to diethyl ether. The obtained precipitate was repeatedly centrifuged in cold MeCN and dialyzed in deionized water. The dialyzed aqueous solution was freeze-dried to produce Peg<sub>10k</sub>PR $\alpha$ CD1 or Peg<sub>20k</sub>PR $\alpha$ CD1 with a nearly quantitative yield. IR (KBr)  $\tilde{v} = 3377, 2911, 1645, 1152, 1082, and 1032 \text{ cm}^{-1}$ . See Section 2.4 regarding the aldehyde marking with DMHZ for <sup>1</sup>H NMR measurement.



**Scheme 2.** Synthetic scheme for PegPRαCD1.

# 2.3. Synthesis of PluPRβCD1

2.3.1. PluPRβCD3 General Synthesis Procedure

Plu–COOH (1 g) and βCD (1 g, 0.88 mmol) were dissolved in deionized water (56 mL) and stirred at 25 °C for 7 days. A white precipitate was gradually formed during the reaction, which was thoroughly centrifuged (14,000× g, 20 min) and washed with cold water. Freeze drying the resulting precipitate produced PluPRβCD3 as a white powder. The further addition of βCD (0.36 g) to the supernatant and repetition of the above-mentioned procedure generated more PluPRβCD3. In total, approximately 0.7 g of Plu<sub>9k</sub>PRβCD3 and Plu<sub>15k</sub>PRβCD3 was obtained from one gram of Plu–COOH.  $\delta_{\rm H}$  (DMSO-d<sub>6</sub>): 1.01–1.05 (m, 3H, C<sub>*j*</sub>-H), 3.30–3.38 (m, C<sub>*b*,*d*</sub>-H and C<sub>*k*,*l*</sub>-H), 3.41–3.67 (m, 32H, C<sub>*c*,*e*,*f*</sub>-H and C<sub>*m*</sub>-H), 3.94 (br, 4H, C<sub>*n*</sub>-H), 4.37–4.40 (m, 7H, OH<sub>*g*</sub>), 4.83–4.84 (m, 7H, C<sub>*a*</sub>-H), 5.63–5.68 (m, 14H, OH<sub>*h*,*i*</sub>) (Figure S2).

#### 2.3.2. PluPRβCD2 General Synthesis Procedure

BOP (0.34 g, 0.76 mmol), Trt–NH<sub>2</sub> (0.2 g, 0.76 mmol), and DIPEA (0.14 mL, 0.82 mmol) were dissolved in dry MeCN (3 mL). Powdered PluPRCD3 (0.5 g) was added to the resulting solution under vigorous stirring. The obtained reaction mixture was stirred at 25 °C for 2 days. Subsequently, it was repeatedly centrifuged ( $1500 \times g$ , 20 min) in cold MeCN until the supernatant became colorless. The precipitate was thoroughly washed with dichloromethane and filtered. The dialysis of the solid residue followed by freeze drying produced PluPRβCD2 as a white powder. The yields of Plu<sub>9k</sub>PRβCD2 and Plu<sub>15k</sub>PRβCD2 were 0.24 g (48%) and 0.20 g (40%), respectively.  $\delta_{\rm H}$  (DMSO-d<sub>6</sub>): 1.03–1.05 (br, 3H, C<sub>*j*</sub>-H), 3.28–3.38 (br, C<sub>*b*,*d*</sub>-H and C<sub>*k*,*l*</sub>-H), 3.42–3.66 (br, 32H, C<sub>*c*,*e*,*f*</sub>-H, C<sub>*m*,*n*</sub>-H), 4.45–4.47 (m, 7H, OH<sub>*g*</sub>), 4.82–4.83 (m, 7H, C<sub>*a*</sub>-H), 5.68–5.75 (m, 14H, OH<sub>*h*,*i*</sub>), 7.16–7.32 (m, trityl group) (Figure S3). IR (KBr)  $\tilde{v}$  = 3362, 2905, 1649, 1367, 1157, 1082, and 1030 cm<sup>-1</sup>.

#### 2.3.3. PluPRβCD1 General Synthesis Procedure

PluPRβCD2 (0.12 g) was dissolved in HMPA (3 mL) and cooled to 4 °C. PhI(OAc)<sub>2</sub> (22 mg, 0.065 mmol) and a catalytic amount of TEMPO (3.3 mg, 0.0195 mmol) were added to the prepared solution, and the resulting mixture was stirred at 4 °C for 10 days. The reaction mixture was added dropwise to diethyl ether, and the obtained precipitate was centrifuged ( $1500 \times g$ , 20 min). Subsequently, the precipitate was washed and centrifuged using cold MeCN and dialyzed in deionized water. Freeze drying the inner dialysis solution produced Plu<sub>9k</sub>PRβCD1 or Plu<sub>15k</sub>PRβCD1 with a nearly quantitative yield. IR (KBr)  $\tilde{v} = 3389$ , 2934, 1649, 1456, 1155, 1082, and 1030 cm<sup>-1</sup>. See the section below regarding the aldehyde marking with DMHZ for <sup>1</sup>H NMR measurement.

#### 2.4. Quantification of Aldehyde Groups Using DMHZ

PegPRαCD1 or PluPRβCD1 (10 mg) was dissolved in DMHZ-buffered water (0.2 M, pH = 8.2) and stirred at 37 °C for 48 h. NaBH<sub>3</sub>CN was added to the obtained solution at a final concentration of 0.2 M. The resulting mixture was stirred at 37 °C for another 48 h and subsequently dialyzed in deionized water for 24 h. Freeze drying the inner dialysis solution and thoroughly washing with ethanol produced the DMHZ-labeled PegPRαCD1 or PluPRβCD1 with a nearly quantitative yield. For PegPRαCD1-DMHZ  $\delta_{\rm H}$  (DMSO-d<sub>6</sub>): 2.54 (s, 3H, C<sub>k</sub>-H), 3.24 (br, N<sub>j</sub>-H, C<sub>b,b',d,d'</sub>-H), 3.40–3.52 (m, 4H, C<sub>l,m</sub>-H), 3.59–3.81 (br, 28H, C<sub>n</sub>-H, C<sub>c,e,f</sub>-H and C<sub>c',e',f'</sub>-H), 4.31 (br, 6H, OH<sub>g</sub>), 4.80–4.81 (br, 6H, C<sub>a,a'</sub>-H), 5.51 (br, 6H, OH<sub>h,h',i,i'</sub>) (Figure 2a).

For PluPRβCD1-DMHZ  $\delta_{\rm H}$  (DMSO-d<sub>6</sub>): 1.04–1.05 (br, 3H, C<sub>l</sub>-H), 2.54 (s, 3H, C<sub>k</sub>-H), 3.25 (br, N<sub>j</sub>-H, C<sub>b,b',c,c'</sub>-H and C<sub>m,n</sub>-H), 3.40–3.52 (m, 4H, C<sub>o</sub>-H), 3.64–3.82 (br, 32H, C<sub>p</sub>-H, C<sub>c,e,f</sub>-H and C<sub>c',e',f'</sub>-H), 4.34 (br, 7H, OH<sub>g</sub>), 4.83 (br, 7H, C<sub>a,a'</sub>-H), 5.59 (br, 14H, OH<sub>h,h',i,i'</sub>) (Figure 2b).



**Figure 2.** <sup>1</sup>H NMR spectra of (**a**) Peg<sub>10k</sub>PRαCD1-DMHZ and (**b**) Plu<sub>9k</sub>PRβCD1-DMHZ (400 MHz, 318 K, DMSO-*d*<sub>6</sub>).

## 2.5. Preparation of PRATs

PRATs were fabricated according to a procedure described in our previous study [23]. Briefly, an atelocollagen solution (25 mg/mL) in a 0.1 M phosphate buffer (pH = 7.0) was poured into a 0.05 M phosphate buffer at 37 °C through an 18 G plastic tube. The obtained atelocollagen thread was crosslinked with PegPR $\alpha$ CD1 or PluPR $\beta$ CD1 via stepwise reductive amination in a 0.1 M borate buffer (pH = 8.5). The crosslinked thread was washed with an aqueous ethanol solution and dried under ambient conditions. The dry thread was fixed to the flexible polypropylene sheet using paper tape for a temporal fixation. Then, the liquid glue was applied to cover the part of the thread 5 mm from the paper tapes. After the liquid was solidified, the thread sample was subjected to tensile testing.

## 2.6. Tensile Testing

A Micro Autograph MST-X HS/HR (SHIMADZU) was employed for the tensile testing. The fabricated thread samples were immersed in a 50 mM phosphate buffer (pH = 7) for 3 min before tensile testing under wet conditions. After the thread samples affixed to the polypropylene sheet were secured onto the measuring apparatus, the frame was severed, initiating the measurement process. The thread was pulled upward under a mist of water

at the rate of 2 mm/min using the top jig connected to the 10 N load cell. The stress–strain curve of the sample was monitored in real time until the thread sample was broken. Statistical significance of the experimental data was assessed by Tukey's test, where p < 0.01 and p < 0.05 were considered significant.

#### 2.7. Analytical Methods and Apparatus

<sup>1</sup>H NMR measurement was performed on an ECS-400 spectrometer (JEOL). The powder PR sample (~5 mg) was dissolved in DMSO-d<sub>6</sub> and employed for the 1D measurement at 293 or 318 K. The <sup>1</sup>H NMR spectrum was obtained using 160 scans with a relaxation delay of 5 s.

Fourier-transform infrared (FT–IR) measurements of the synthesized PRs were performed using an IRAffinity-1S spectrometer (SHIMADZU). Powder PR samples were mixed and milled with potassium bromide (KBr), and the resulting powder mixture was fabricated into a pellet. The pellet was subjected to a transmittance measurement in the infrared region (4000–500 cm<sup>-1</sup>).

A TGA-50 thermogravimetric analyzer (SHIMADZU) was utilized to monitor the thermal decomposition of the samples during thermogravimetric analysis (TGA). The powder sample (~10 mg) was weighed on a platinum pan and placed in the sample chamber. The residual weight was monitored in real time while the sample was heated from 25 to 700 °C at the heating rate of 10 °C/min under N<sub>2</sub> gas flow.

#### 3. Results and Discussion

#### 3.1. Synthesis and Characterization of PegPRaCD1

 $Peg_{10k}PR\alpha CD1$  and  $Peg_{20k}PR\alpha CD1$  were synthesized from the hydroxyl groupterminated Peg via a typical Gabriel amine synthesis process, pseudo-PR formation, terminal amide coupling by adamantane carboxylic acid, and catalytic oxidation of the primary alcohol groups of  $\alpha$ CD (see Figure S1 for the adamantane end-capped PegPR $\alpha$ CD2). The aldehyde groups were selectively introduced as crosslinking sites into the four PRs via catalytic oxidation with TEMPO and PhI(OAc)<sub>2</sub>. Previous research used Dess-Martin Periodinane (DMP) to introduce the aldehyde groups into CDs of PRs, although the DMP potentially produces ketones besides aldehydes [24–30]. Most recently, we have reported that the TEMPO/PhI(OAc)<sub>2</sub> redox couple is more suitable than the DMP for the selective oxidation of hydroxymethyl groups of CDs [23]. To calculate the number of crosslinking sites per PR molecule, aldehyde groups were pre-labeled with DMHZ via reductive amination. Previously, we reported the direct quantification of aldehyde groups as acetal forms by <sup>1</sup>H NMR spectroscopy [23]. However, the limited solubility resulting from the increased molecular weight of PegPR $\alpha$ CD1 led to the development of an alternative method. The representative <sup>1</sup>H NMR spectrum of PegPR $\alpha$ CD1-DMHZ is shown in Figure 2a. A broad spectrum is observed owing to the formation of PegPR $\alpha$ CD1, and its peaks are assigned to the  $\alpha$ CD and Peg units. The N–CH<sub>3</sub> protons (*k*) of DMHZ were detected at 2.54 ppm, while the N–H peak (*j*) likely overlapped with the peaks of the protons at the  $C_{b_r}$ ,  $C_{b'}$ ,  $C_d$ , and  $C_{d'}$  positions. The coverage ratio of 20 mol% was calculated from the ratio between the peaks obtained for the Peg units and the methyl groups. Moreover, the number of DMHZ molecules was consistent with that of aCD species, indicating that one aldehyde group was introduced per  $\alpha$ CD molecule.

#### 3.2. Synthesis and Characterization of PluPR<sub>β</sub>CD1

The *pseudo*-PR formation originates from the complexation between polypropylene glycol (Ppg) units and  $\beta$ CD [46], whereas  $\beta$ CD can move and rotate over the entire polymer units (i.e., Peg and Ppg units) [7,47]. To synthesize Plu<sub>9k</sub>PR $\beta$ CD1 and Plu<sub>15k</sub>PR $\beta$ CD1, we initially attempted to prepare the corresponding *pseudo*-PRs (i.e., PluPR $\beta$ CD3) from Plu–COOH according to a previously developed procedure [46]. Although the earlier study reported the gram-scale preparation of *pseudo*-PR, the corresponding procedure was not reproduced under the same experimental conditions (i.e., a trace amount of *pseudo*-PR was

obtained). Therefore, the preparation conditions were reinvestigated using Plu–OH as a model compound. One gram of Plu–OH was dissolved in a saturated aqueous solution of  $\beta$ CD (56 mL) and stirred at 25 °C for 7 days. A white suspension was obtained during stirring owing to the formation of *pseudo*-PR. The product was collected via centrifugation and freeze-dried. Further addition of  $\beta$ CD to the supernatant enabled the preparation of more *pseudo*-PR. The same procedure was applied for Plu–COOH. In total, approximately 0.7 g of PluPR $\beta$ CD3 was obtained from the initial amount of Plu–COOH. The corresponding <sup>1</sup>H NMR spectra were used to calculate the coverage ratio of  $\beta$ CD with respect to the total number of Peg and Ppg units, which was equal to 6 mol% (Figure S2). PluPR $\beta$ CD2 was synthesized by the terminal end-capping of PluPR $\beta$ CD3 via amide coupling with Trt–NH<sub>2</sub> in anhydrous acetonitrile (Figure S3). Finally, one hydroxymethyl group per  $\beta$ CD in the PRs was converted to an aldehyde group using the TEMPO/PhI(OAc)<sub>2</sub> redox couple. In addition to PegPR $\alpha$ CD1, the aldehyde groups of PluPR $\beta$ CD1 were also successfully quantified using DMHZ (Figure 2b). The coverage ratio of 6 mol% was retained during terminal capping and catalytic oxidation.

## 3.3. FT-IR Measurements

Previous FT-IR studies produced peaks originating from Peg, Ppg, and CDs [48–51]. Comparative measurements with different coverage ratios enabled the assignment of characteristic peaks. Figure 3a shows a representative FT-IR spectrum of PegPR $\alpha$ CD2. The peaks at 1032 and 1152 cm<sup>-1</sup> correspond to the C–O and C–O–C stretching vibrations of  $\alpha$ CD, respectively. A new absorption band is observed at 1103 cm<sup>-1</sup> because of the overlap between the  $\alpha$ CD peaks and C–O stretching mode of Peg at 1082 cm<sup>-1</sup> (Figure 3b–d). The peak at 2887  $\text{cm}^{-1}$  is an overlap of the stretching vibrations of Peg C–H and free OH groups of  $\alpha$ CD. In contrast, the wide absorption peak at 3385 cm<sup>-1</sup> indicates the presence of the OH groups of  $\alpha$ CD in the inter- and/or intramolecular hydrogen bonds. The bending vibration of  $\alpha$ CD–C–H is observed at 1639 cm<sup>-1</sup>, while that of Peg is detected at 1360 cm<sup>-1</sup>. The PluPR $\beta$ CD2 spectrum is similar to that of PegPR $\alpha$ CD2; however, its  $\beta$ CD peaks are relatively small owing to the lower coverage ratio (Figure 3c). The absorption peaks of Ppg almost completely overlap with those of the Pegs because of their similar vibration frequencies. The absorption peak of the stopper molecules (i.e., trityl groups) likely overlaps with that of the C–H bending vibration of  $\beta$ CD at 1649 cm<sup>-1</sup>. Interestingly, although aldehyde groups were successfully labeled with DMHZ for <sup>1</sup>H NMR measurements, their characteristic peaks are absent from the FT-IR spectra (Figure 3b,d). Instead, the peaks originating from the presence of the free OH groups of CDs (located at 2911 and  $2934 \text{ cm}^{-1}$  in Figures 3b and 3d, respectively) decreased after catalytic oxidation, confirming the production of acetal species from aldehyde and OH groups in the solid state [23]. Meanwhile, we cannot exclude the possibility of the hydration of aldehyde groups by residual water. The acetal formation also caused noticeable changes in CD absorption in the wavenumber range of  $1250-1000 \text{ cm}^{-1}$ .

## 3.4. TGA Measurements

TGA was also performed to characterize PegPRαCD1 and PluPRβCD1 (Figure 4). The weight losses observed for Peg–NH<sub>2</sub> are consistent with those of a previously reported TGA profile [52], thus validating the utilized experimental conditions (Figure 4a). αCD exhibited a weight loss due to the loss of moisture by 120 °C and decomposition of glucose units by 370 °C [53]. In comparison, the oxidation of one hydroxymethyl group of αCD did not cause a significant change in the TGA profile (i.e., αCD–CHO). The *pseudo*-PR PegPRαCD3 containing 20 mol% αCD produced weight losses arising from the thermal decomposition of both Peg–NH<sub>2</sub> and αCD [50]. The weight loss of PegPRαCD2 was initiated at 200 °C owing to the decomposition of the stopper moiety [54], while its loss temperatures were lower than those of PegPRαCD3 above 320 °C. The weight loss of PegPRαCD1 started at 250 °C owing to the decomposition of polyacetal structures (i.e., inter- and/or intramolecular crosslinking) and hydrated aldehydes in the solid state [23].

In the temperature range above 350 °C, the TGA curves of PegPR $\alpha$ CD2 and PegPR $\alpha$ CD1 are very similar. Meanwhile, Plu-COOH exhibits a clear two-step weight loss (Figure 4b). Because the significant thermal decomposition of Peg begins at around 350 °C (Figure 4a), the initial weight loss that starts at 200 °C results from the thermal decomposition of the Ppg units of Plu–COOH. The weight losses of  $\beta$ CD and  $\beta$ CD–CHO assigned to the loss of moisture (~110  $^{\circ}$ C) and decomposition of glucose units (~310  $^{\circ}$ C) were consistent with those observed in the previous studies [23,53]. Owing to the relatively low coverage ratio of PluPR<sub>β</sub>CD3, the decomposition of Ppg units after the loss of moisture is clearly observed between 200 and 310 °C. PluPRβCD2 exhibits a gradual decrease in the residual weight by 200 °C caused by the loss of moisture and thermal decomposition of trityl groups [55]. Similar to PegPR $\alpha$ CD1, the weight loss of PluPR $\beta$ CD1, which begins at 250 °C, indicates the decomposition of polyacetal structures and Ppg units. However, unlike the other samples, no significant residual weight changes are observed for this compound in the temperature range above 350 °C. It is noteworthy that the introduction of aldehyde groups produced a similar trend in the TGA profiles, while the residual weights at the inflection points (i.e., 275 and 350 °C) strongly depended on the CD molar content.



**Figure 3.** FT–IR spectra of (a) PegPR $\alpha$ CD2, (b) PegPR $\alpha$ CD1, (c) PluPR $\beta$ CD2, and (d) PluPR $\beta$ CD1.



**Figure 4.** TGA curves: (a) Peg–NH<sub>2</sub>,  $\alpha$ CD,  $\alpha$ CD–CHO, PegPR $\alpha$ CD3, PegPR $\alpha$ CD2, and PegPR $\alpha$ CD1; (b) Plu–COOH,  $\beta$ CD,  $\beta$ CD–CHO, PluPR $\beta$ CD3, PluPR $\beta$ CD2, and PluPR $\beta$ CD1. The samples were heated to 700 °C at a heating rate of 10 °C/min.

#### 3.5. Tensile Testing of PRATs

The successful characterization of the synthesized polymers led to an investigation of the effect of PRs on the mechanical properties of the PRATs fabricated via reductive amination. The obtained threads contained wrinkles along the thread direction owing to the alignment of atelocollagen molecules [23], which were not previously observed for other types of soft materials based on collagen and atelocollagen (e.g., gels and membranes). Molecular alignment is potentially advantageous for increasing the strength and flexibility of PRATs. The results of tensile testing obtained under humidified conditions are presented in Figure 5 (Figure 5a shows a representative photograph of the utilized tensile testing setup). Crosslinking of atelocollagen thread (AtCol) by  $Peg_{10k}PR\alpha CD1$  and  $Peg_{20k}PR\alpha CD1$ increased both the fracture stress and strain as compared with AtCol alone (Figure 5b,c,f), leading to a 5-fold toughness increase (Figure 5d). The Young's modulus calculated from the initial linear region of the stress–strain curve increased by a factor of 4 (Figure 5e). The obtained mechanical parameters reflected the entropic elasticity derived from the slide-ring characteristics of PegPRaCD1 [7,56]. However, the molecular weight of Peg did not significantly affect the mechanical properties of the synthesized PRATs. Previously, Takeoka et al. examined the extensibility of N-isopropylacrylamide-based polymer gels crosslinked by PRs with the same coverage ratio but different molecular weights of the axile polymer (i.e., Peg) [57]. Both the fracture stress and strain were enhanced by increasing the molecular weight of the PRs from 20,000 to 100,000, which indicated that the increased effective range of  $\alpha$ CD resulting from the higher molecular weight significantly affected the gel mechanical properties. A similar effect of molecular weight was reported for atelocollagen hydrogels post-crosslinked with carboxymethyl PRs [58]. Note that molecular weight exerted a stronger effect on the fracture stress and strain than coverage ratio in the previous studies. The major difference between the PRATs and previously reported PR-crosslinked gels is the nature of matrix molecules. The matrix molecules were randomly oriented in the gels, whereas the atelocollagen molecules of the PRATs were tightly aligned along the thread direction [23]. Therefore, we tentatively concluded that the effects of the PRs depended on the alignment of the molecules to be crosslinked. We have found that  $Plu_{9k}PR\beta CD1$  and  $Plu_{15k}PR\beta CD1$  significantly enhance the mechanical properties of the PRATs.  $Plu_{9k}PR\beta CD1$  and  $Plu_{15k}PR\beta CD1$  further increased the fracture stress by approximately 2.3 times as compared with that of PegPR $\alpha$ CD1, maintaining the fracture strain (Figure 5b). Consequently, the toughness was further increased by a factor of 1.6 (no significant differences between  $Plu_{9k}PR\beta CD1$  and  $Plu_{15k}PR\beta CD1$  are observed in Figure 5c,d), while the Young's modulus and fracture strain were maintained constant (Figure 5e,f). We found consistency in that molecular weight showed a negligible effect on the mechanical properties of the PRATs. In addition to the molecular alignment of atelocollagens, the observed effect of PluPRBCD1 was discussed based on the PR characteristics. One of the possible reasons for this phenomenon is the modified property nature of Peg units upon the application of an external force [59]. Ito et al. observed the crystallization of

Peg units during the tensile testing of PR-crosslinked materials by lowering the coverage ratio (~2 mol%). Because PluPR $\beta$ CD1 also contains wide uncovered Peg units, an external force applied to the PRATs may similarly induce the crystallization of Peg units. However, we cannot exclude the possibility that the ring size affects the slide-ring properties of the PRs. The increased ring size from  $\alpha$ CD to  $\beta$ CD can promote solvent uptake into the CD inner cavity. The interactions between the OH groups of CDs, oxygen atoms in Peg units, and solvent molecules (i.e., water) facilitate the formation of hydrogen bonds, leading to molecular friction that prolongs the stress relaxation time [50,60]. These two factors, in addition to the alignment of atelocollagen molecules, may contribute to the unique mechanical properties of the produced PRATs. It should be noted that the increased fracture stress but maintained fracture strain provided by PluPR $\beta$ CD1 had not been previously observed for PR-crosslinked materials. Further experimental investigations combined with theoretical studies regarding the unprecedented results should clarify the role of PRs as the characteristic components of the PRATs.



**Figure 5.** Tensile testing of AtCol alone and PRATs. (**a**) Representative photograph of the tensile testing setup. (**b**) Representative stress–strain curves. Bar graphs of the (**c**) fracture stress, (**d**) toughness, (**e**) Young's modulus, and (**f**) fracture strain. Fracture stress was calculated by dividing the fracture test force (N) by the cross-sectional area (ca. 0.03 mm<sup>2</sup>). Young's modulus was determined from the slope of the initial linear region (~6% strain) of the stress–strain curve. \* *p* < 0.01, \*\* *p* < 0.05 (Tukey's test).

# 4. Conclusions

In this study, we investigated the effects of the molecular weight and coverage ratio of PRs on the mechanical properties of the produced PRATs. A novel method was established for the syntheses of PRs with relatively low coverage ratios. The obtained PRs were successfully characterized by <sup>1</sup>H NMR, FT–IR, and TGA. Interestingly, the results of tensile testing demonstrated that coverage ratio was the main factor affecting the mechanical properties of the PRATs, whereas molecular weight produced a negligible effect on these properties. It is noteworthy that the effects of PRs on the PRAT properties potentially depend on the alignment of atelocollagen molecules. Although further investigations are required to support our conclusions and to further improve the mechanical properties for practical use in humans, the results of this study offer insights into the fundamental properties and applications of PR-reinforced biomaterials.

**Supplementary Materials:** The following supporting information can be downloaded from: https: //www.mdpi.com/article/10.3390/polym15153325/s1, <sup>1</sup>H NMR spectra. Figure S1: <sup>1</sup>H NMR spectrum of PegPR $\alpha$ CD2. Figure S2: <sup>1</sup>H NMR spectrum of PluPR $\beta$ CD3. Figure S3: <sup>1</sup>H NMR spectrum of PluPR $\beta$ CD2.

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