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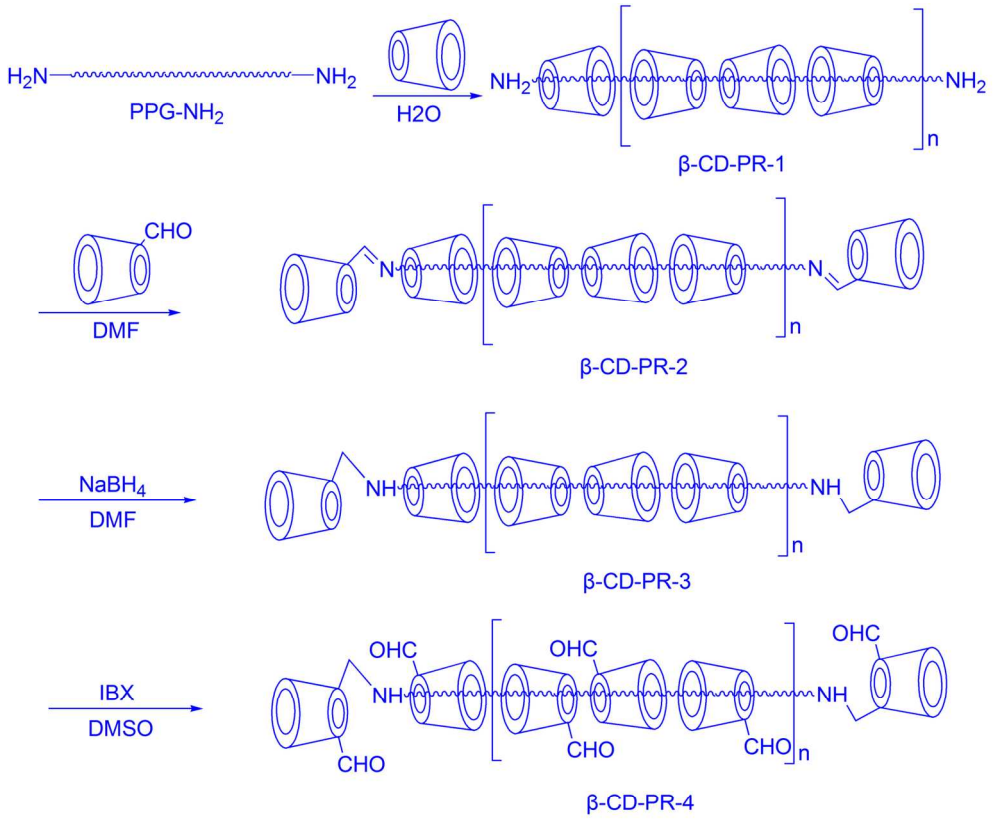

we synthesized CD-capped polyrotaxanes monoaldehydes with appropriate weights which was used as bio-cosslinker with better crosslink efficiency and lower cytotoxicity.

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β-cyclodextrins polyrotaxane monoaldehydes: a novel bio-cosslinker with high biocompatibility

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In this paper, we prepared a multi-functional bio-crosslinker: β-Cyclodextrins (β-CD) Polyrotaxane monoaldehydes (β-CD–PR-4). Compared to conditional crosslinker, such as GA and EDC, this multi-functional bio-crosslinker has better crosslink efficiency and lower cytotoxicity.

Collagen is the most abundant protein and a primary component of extracellular matrices in mammalian connective tissue[1]. It has better biodegradability, biocompatibility, weak antigenicity and low toxicity[2] with broad applications[3]. However, the weak mechanical property, low thermal stability and high biodegradation rate of native collagen are not sufficient for its application in vivo or in vitro[4]. The cross-linking of collagen is a way to improve the defects mentioned above. However physical methods for crosslinking does not result in an adequate degrees of crosslinking although they avoid the potentially cytotoxicity[5]. For chemical crosslink, the most widely used crosslinkers contains Glutaraldehyde (GA), N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) or genipin[6-9]. But these conditional crosslinkers always exhibited evident defect, such as cytotoxicity, insufficient crosslinking effect or some chromogenic reaction. Therefore, it is necessary to develop an alternative crosslinker which showed excellent crosslink efficiency as well as cytocompatibility.

Modified Cyclodextrins(CD)-based Polyrotaxane supramolecular were proved to be biodegradable and toxicologically acceptable[10-11]. Many researchers[12-16] have prepared CD-based polyrotaxane by theading many CD units onto a polymer chain to acquire the polypeudorotaxane, and subsequently using anthryl, dinitrophenyl, naphthyl, trinitropheenyl trityl groups and various organic molecules as stoppers. However, using these stoppers brings some defects, such as cytotoxicity, indissolvable and the polyrotaxane also lacking of reactive functional groups, which limits its applications. So, we prepared tens of CD-based Polyrotaxane by threading many β-CD units onto a poly(propylene glycol)bis(2-aminopropylether) (PPG-NH_2, MW≈2000) chain and then capped the resultant polyseudorotaxane using β-CD monoaldehydes as stoppers, and then reduction with $NaBH_4$, and the then, it was directly oxidized in high yield to the corresponding monoaldehyde by cyclized 2-iodoxybenzoic acid(IBX) in dimethylsulfoxide(DMSO). And the last we evaluateits cytotoxicity and use it to crosslink collagen.

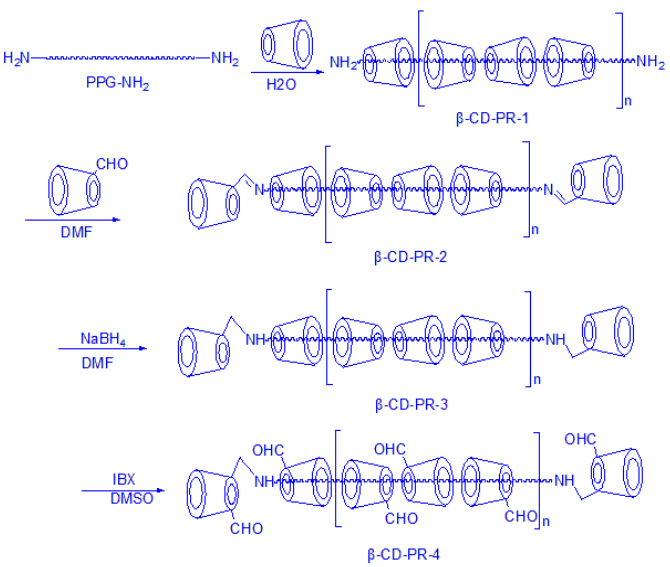

Figure 1. Schematic of synthesis of β-cyclodextrins polyrotaxane monoaldehydes

As shown in Scheme 1, β-CD-based polypseudorotaxane (β-CD–PR-1) and β-CD-based polyrotaxanes (β-CD–PR-2) was synthesized by the known methods[17]. The reduction β-CD-based polyrotaxanes (β-CD–PR-3) was synthesized after the upper reaction. Namely, 50μl acetic acid dropped in the reaction solution and continued to reflux for 2h. After that, a sufficient amount of $NaBH_4$ was added to this reaction mixture, and allowed to react for 24h at room temperature. Then, it dropped into 200ml acetone, stirred for 1 h and stood for 3h. The precipitated products were collected by centrifugation, then washed twice with ethanol and dried under vacuum at 35℃ to get the β-CD–PR-3 complexes. β-CD-based polyrotaxane monoaldehydes (β-CD–PR-4) was synthesized

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according to literature procedure[20]. Briefly, 0.58g β-CD–PR-3 and 0.36g IBX were dissolved in 6ml DMSO. Then magnetic stirred for 24 hour at 25℃ and the reaction mixture was dropped into 200ml acetone, stirred for 1 h and then stood for 3h. After centrifugation, the precipitate dried under vacuum at 30℃. After that it was dissolved in water, stirred for 4h, the precipitate was removed by filtration. At last, lyophilization of the filtrate afforded the desired product.

we have done the XRD measurements of β-CD and β-CD–PR-1. The three strong peaks of pure β-CD appear at 10.6°, 12.5°, and 19.5°. It indicates a cage-type crystal structure[18]. The diffractogram of β-CD–PR-1 showed a diffraction pattern different from that of β-CD. The three strong peaks appear at 5.7°, 11.5°, and 17.8°. This constitutes primary evidence that a differen crystal type was formed.

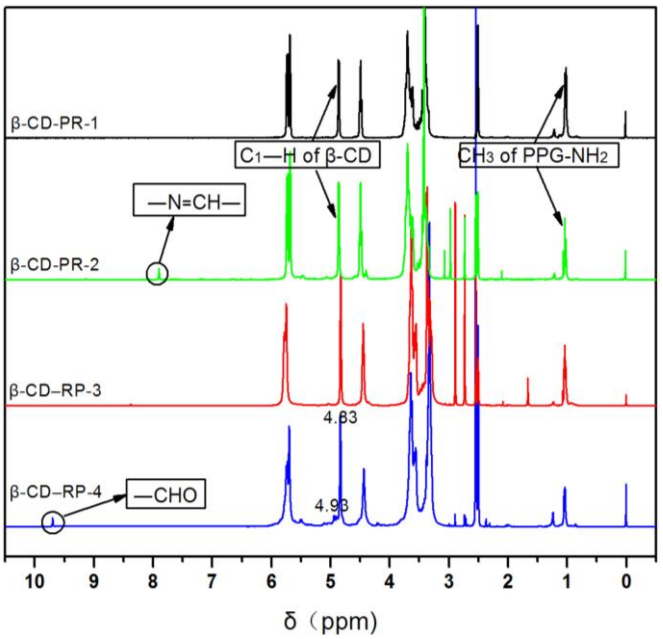

Figure 2. The ^{1}H NMR spectra of the β-CD–RP-1, β-CD–RP-2, β-CD–RP-3 and β-CD–RP-4

The ^{1}H NMR spectra of the β-CD–RP-1, β-CD–RP-2, β-CD–PR-3 and β-CD–PR-4 samples are shown in Fig. 2. In the ^{1}H NMR spectrum, the peaks were identified and were found to belong to both β-CD and PPG-NH_2 molecules. Such as δ(ppm): 1.03-1.05(CH_3 for PPG), 3.56(C_2-H,C_4-H), 3.64(C_3-H,C_5-H,C_6-H), 4.43(C_6-OH),4.83(C_1-H),5.68(C_2-OH,C_3-OH). There are also a shift in some of the CD-protons[19]. The results suggest that the β-CD–PR-1 may be the inclusion complex. Furthermore, a ^{1}H NMR spectrum of β-CD–PR-1 shows that the integral area ratio between methyl protons of PPG-NH2 (a molecule of PPG-NH_2 2000 contains ca. 102 methyl protons, δ =1.03-1.05ppm) and C1-H of β-CD protons (a molecule of β-CD contains 7 C1-H protons, δ= 4.83 ppm) is 7.9:7.0. In the case of β-CD–PR-2, this ratio decreases to 6.8:7.0. According to these ^{1}H NMR data, we can calculate that there are ca. 13 β-CD units in β-CD–PR-1 and ca. 15 β-CD units in β-CD–PR-2. Moreover, the signals assigned to the protons (-CH=N-, δ= 7.95 ppm) is observed in the ^{1}H NMR spectra of β-CD–PR-2. These results clearly indicate that two β-CD cavities are successfully introduced at the amino terminals of β-CD–PR-1. Compared with the ^{1}H NMR spectra of the β-CD–PR-2, in the ^{1}H NMR spectra of the β-CD–PR-3, the signals assigned to the protons (-CH=N-, δ= 7.95 ppm) has disappeared, and a new single is observed at δ 9.70 in the spectra of β-CD–PR-4. It is the characteristic single signal of the formyl proton, so it proved to generate aldehyde groups. Simultaneously, a new signal of an anomeric proton at δ 4.93 appears at the expense of the signal at δ 4.93. And the ratio of these two signals is 1:6, which is the indicative of the monooxidation, and these results are consistent with Jing HU[20] and Cornwell[21]. The molecular weight of β-CD–PR-4 is about 18950.

Figure 3. shows the results of cytotoxicity test by the addition of β-CD–PR-4 to L929 fibroblasts. While the conditional cross-linker EDC and GA were as control. The cytotoxicity test was according to literature procedure[22-23]. The cytotoxicities of β-CD–PR-4 were not even observed at the concentration of 0.1mg/mL. However, the crosslinker EDC and GA began to show cytotoxicity at the concentration of 10^{-3} mg/mL, and they could kill about 79.3% and 89.7% cells compared to that of β-CD–PR-4 at the concentration of 0.1 mg/mL, respectively. The results show that the β-CD–PR-4 has better compatibility than the conditional crosslinkers.

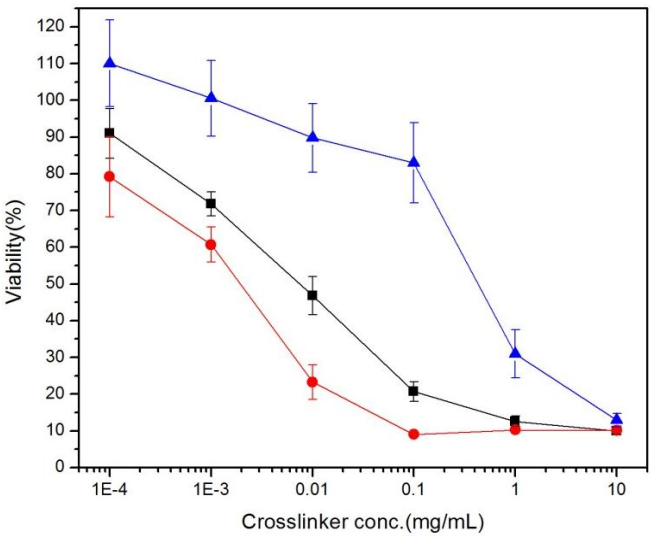

Figure 3. Viability of L929 fibroblasts exposed to various crosslinker at different concentrations: β-CD–PR-4(▲), EDC (■) and GA(●)

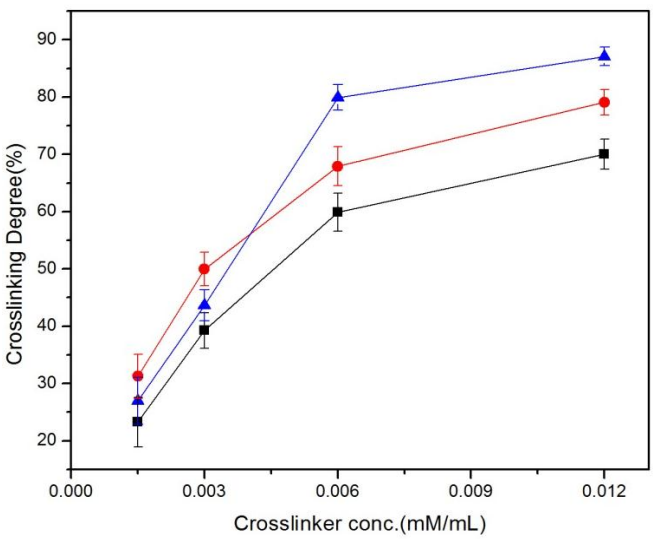

Figure 4. Crosslinking eficiency of different crosslinker concentrations of β-CD–PR-4(▲), EDC (■) and GA(●)

The ninhydrin[24] assay was used to estimate the crosslinking efficiency of β-CD–PR-4 to collagen, and the results were shown in Fig. 4. The crosslinking index of collagen

increased with the increase of the crosslinkers concentrating. For β-CD–PR-4, the maximun crosslinking degree is around 87.1% at the reactive functional groups concentration of 0.012 mM/mL, which was higher than that of EDC and GA, whose crosslinking degree is 69.3% and 79.0%, respectively, at the same concentration. The high crosslinking ability of β-CD–PR-4 to collagen should be attributed to strong binding, as β-CD–PR-4 had both covalent and non-covalent interactions with collagen. Hence, aldehydic functionality in β-CD–PR-4 covalently crosslinked with amino groups of collagen and the hydroxyl groups could involve in hydrogen bonding interaction that brings significant increase in thermal and enzymatic stability[25].

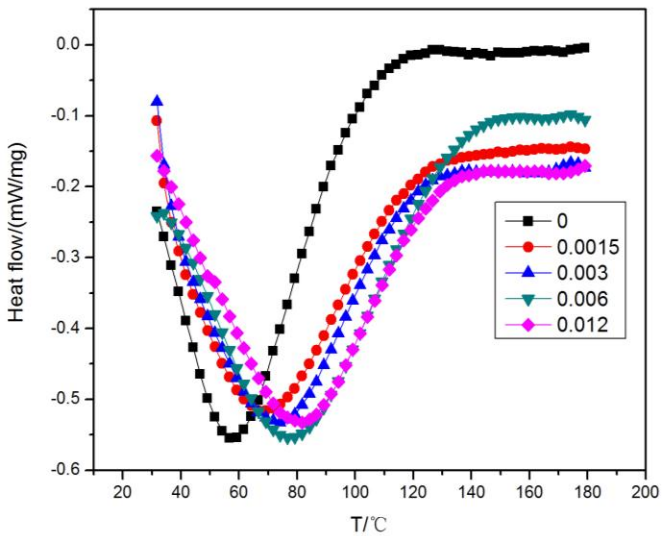

Figure 5. DSC thermagraphs of pure collagen and different concentrations (mM/mL) of β-CD–PR-4 crosslinked collagen

After cross-link, we tested the denaturation temperature of the collagen with DSC, and the results were shown in Fig. 5. The thermal stability of the collagen increased with increasing in β-CD-PR-4 concentration. The denaturation temperature of un-crosslinked collagen was about 58.0 ℃, while that of β-CD-PR-4 treated collagen would increase with the increase of β-CD-PR-4 concentration. After cross-linked with 0.012 mM/mL β-CD-PR-4, the denaturation temperature of collagen would increase by 23.6 ℃. The increased in thermal stability could be related to the increasing in the number of crosslinking, as they decreased the entropy of transiton[26] which would lead the collagen gel to be stiffer. In addition, the special and rigid structure of β-CD-PR-4 could also improve the thermal stability of the collagen effectively.

In summary, we had successfully prepared a multi-functional bio-crosslinker: β-Cyclodextrins (β-CD) Polyrotaxane monoaldehydes (β-CD–PR-4) in a convenient and efficient method. On the basis of this method, we could synthesis CD-capped polyrotaxanes monoaldehydes or multi-aldehydes with appropriate molecular weights by changing the reaction conditions or selectinng various polymer chains. It exhibited low cytotoxicity to L929 cells and could cross-link the collagen, and also had no chromogenic reaction. The maximun crosslinking degree is around 87.1%, which was much higher than that of EDC and GA at the same concentration. In addtion, it could improve the thermal stability of the collagen effectively. Our results revealed the application of β-CD–PR-4 as a multi-functional bio-crosslinker. It also has the potential to be applied in the field of molecular machines, tissue engineering scaffolds, human biological sensors, especially used as drug controlled release carrier materials.

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