

Injectable Supramolecular Hydrogels *via* Inclusion Complexation of mPEG-grafted Copolyglutamate with α -Cyclodextrin^{*}

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Abstract A series of monomethoxy poly(ethylene glycol) (mPEG) grafted copolyglutamates (PmPEGs) were synthesized through ring-opening polymerization (ROP) followed by click chemistry. Supramolecular hydrogels based on polymer inclusion complexes (ICs) between PmPEG and α -cyclodextrin (α -CD) were prepared in aqueous solution. The rheological measurements indicated their gelation properties were affected by several factors including the mPEG length, graft density and the sample concentration. These hydrogels displayed thermo-sensitive gel-sol transition under appropriate conditions due to the reversible mPEG and α -CD inclusion complexation. These hydrogels also showed pH-sensitive behavior due to the deprotonate of carboxylic acid side groups. The ICs formation of a channel-type crystalline structure induced gelation mechanism was verified by various techniques. *In vitro* cytotoxicity assays demonstrated that the supramolecular hydrogels are nontoxic and cytocompatible.

Keywords: Supramolecular hydrogel; Inclusion complexation; Copolyglutamate; α -Cyclodextrin.

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INTRODUCTION

Supramolecular hydrogels have recently received increasing attention in biomedical applications, such as tissue engineering^[1], drug delivery^[2,3] and cell encapsulation^[4]. These hydrogels can be obtained through noncovalent interactions including hydrophobic interaction, hydrogen bonding, charge condensation, stereo-complexation, or supramolecular self-assembly^[5–8]. Among them, the hydrogels resulting from the ICs between α -CD and various guests have attracted growing interest in recent years^[9]. α -CD is a cyclic oligosaccharide consisting of 6D-(+)-glucose units linked through α -1,4-linkages^[10,11]. It is soluble in aqueous solution but has a hydrophobic internal cavity for host-guest interaction, leading to forming necklace-like structures which can further aggregate to hydrogels. For example, Harada *et al.* reported the sol-gel transition during ICs formation between high molecular weight PEGs and α -CD in aqueous solutions in 1994^[12]. Tian *et al.* showed that PEGylated poly(organophosphazenes) and α -CD could form biodegradable supramolecular hydrogels with sustained release of BSA^[13]. Ma *et al.* used the PEG grafted heparin and α -CD to prepare bioactive supramolecular hydrogels with controlled dual drug release characteristics^[14].

Compared to traditional polymer hydrogels, polypeptide-based hydrogels have the advantages of inherent biocompatibility and biodegradability^[15–18]. Moreover, the polypeptide-based hydrogels can be modified with

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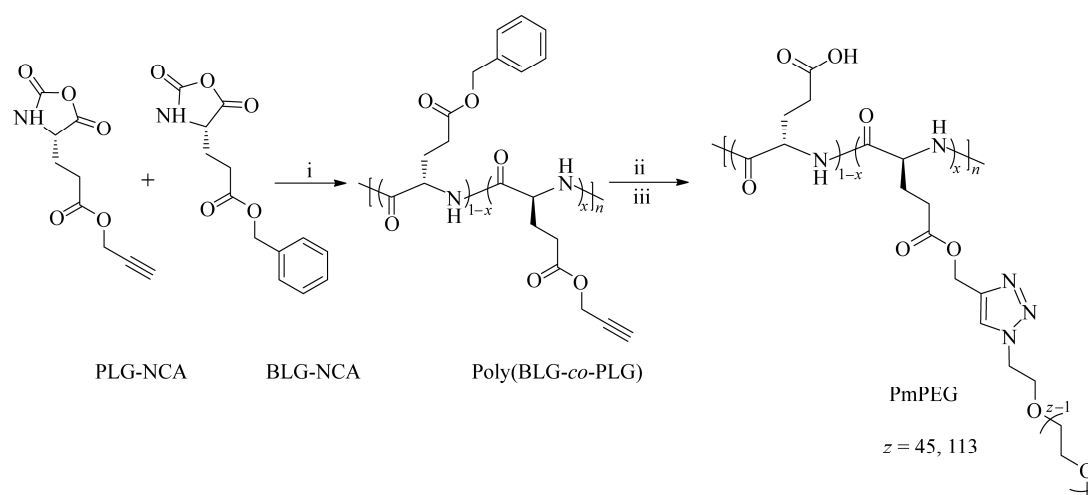
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functional groups in side chains and offer additional factors to tune properties due to their specific secondary structures upon external stimuli. To obtain polypeptides containing different functional groups, “click chemistry” including 1,3-dipolar azide-alkyne cycloaddition reaction, has received much attention because of their high efficiency, mild reaction conditions, functional group tolerance and few byproducts^[19]. For example, Chen *et al.* reported the thermo-sensitive hydrogels based on biofunctionalized PEG-*b*-PPLG copolymers with excellent cytocompatibility through click chemistry^[20]. Recently, our group also synthesized DNA-*g*-PPLG by this method and demonstrated DNA-*g*-PPLG hydrogels can be prepared by hybridization of DNA-*g*-PPLG with a linker-ds-DNA, which contains the complementary “sticky ends” to serve as cross-linkers^[21].

Herein, we synthesized a series of PmPEG with different compositions *via* ROP of L -glutamate *N*-carboxyanhydrides (L -BLG-NCA) and γ -propargyl- L -glutamate *N*-carboxyanhydrides (L -PLG-NCA) followed by “click chemistry” between the alkyne groups and azido-substituted mPEG (Scheme 1). Then PmPEG was used to construct supramolecular hydrogels together with α -CD. Moreover, we studied the rheological properties, the thermo-sensitive gel-sol transition and pH-sensitive behaviors of hydrogels. We also examined the cytotoxicity and cytocompatibility of the hydrogels.



Scheme 1 Synthetic routes of mPEG grafted copolypeptides

Reagents and conditions: (i) Hexamethyldisilazane (HMDS), DMF, RT, 72 h. (ii) Azide-terminated mPEG (mPEG-N₃, $M_n = 2$ or 5 kDa), CuSO₄·5H₂O, sodium ascorbate, DMF, 40 °C, 72 h. (iii) TFA, HBr/HAc (33 wt%), RT, 3 h.

EXPERIMENTAL

Materials

Hexane, tetrahydrofuran (THF) and dichloromethane (DCM) were purified firstly by purging with dry nitrogen, followed by passing through activated alumina columns prior to use. Ethyl acetate (EtOAc) was distilled from CaH₂. *N,N*-Dimethylformamide (DMF; anhydrous, 99.8%, packaged under argon in resealable ChemSeal bottles) for NCAs polymerization was obtained from Alfa Aesar. Pyrogen free deionized water (18 MΩ·cm) was obtained from a Millipore Milli-Q BiocelA10 purification unit. Monomethoxy poly(ethylene glycol)s (mPEG, $M_n = 2$ or 5 kDa) were purchased from Aladdin. α -Cyclodextrin (α -CD, 98%, Aladdin) was used without further purification. γ -Benzyl- L -glutamic acid and L -glutamic acid were purchased from GL Biochem (Shanghai) Ltd. Propargyl alcohol was purchased from Sinopharm Chemical Reagent Co., Ltd. and used as obtained. Cell Counting Kit (CCK-8) was purchased from DOJINDO Laboratories. All other chemicals were purchased from commercial suppliers and used without further purification unless otherwise noted. Reaction temperatures were controlled using an IKA temperature modulator.

Supramolecular Hydrogel

The synthesis of random copolypeptides poly(BLG-*co*-PLG) and PmPEG followed reported procedures (Scheme 1)^[22–24]. The PmPEG-based supramolecular hydrogels were prepared by the inclusion complexation of PmPEG with α -CD. Typically, a prescribed amount of PmPEG was dissolved in DI or 0.01 mol/L PBS. The pH value of the solution was adjusted to 3 using 0.1 mol/L HCl aqueous solution, and 0.1 mol/L NaOH aqueous solution was used to adjust the pH value to neutral or alkaline. Then, α -CD aqueous solution (10 wt%) was added at room temperature (RT). The resultant mixture was shaken and homogenized by ultrasonic treatment. The gelation time was estimated by the inverting tube method (ITM) starting from mixing two components. A gel status was confirmed when no flow was observed for at least 30 s after vial inversion. Depending on the sample concentration and the molar ratio of EG: α -CD, the resultant mixtures could be in the form of precipitates, hydrogels, viscous liquids or clear solutions.

Gel-sol Transition

The gel-sol transition temperature ($T_{\text{gel-sol}}$) was measured by ITM with an IKA temperature modulator with a temperature increase of 1 K per step. The sample vials were immersed in a water bath at each temperature for 10 min. The $T_{\text{gel-sol}}$ was defined as the temperature at which the gels became flowing when inverted.

In vitro Cytotoxicity Assay

For *in vitro* cytotoxicity studies, HeLa cells were harvested by trypsinization, and re-suspended in fresh culture medium to a concentration of 5×10^4 cells/mL. The cells were seeded into 96-well flat-bottomed tissue-culture plates at 5000 cells/well, and incubated for 24 h in a humidified atmosphere of 5% CO₂ at 37 °C. The mixture solution of PmPEG and α -CD were diluted with culture medium to obtain a concentration range from 0.002 to 20 mg/mL, and 100 μ L of each solution was added into the individual well of 96-well culture plates. After incubation for 24 h, the cells were subjected to CCK-8 assay. The absorbency of the solution was measured on the Tecan Sunrise Elisa at 450 nm. Results were expressed as percent viability = [A450 (treated cells)–background/A450 (untreated cells)–background] $\times$ 100%.

RESULTS AND DISCUSSION

The PmPEGs were synthesized by the “grafting onto” approach *via* the combination of NCA ROP and “click” reaction. PLG-NCA and BLG-NCA were synthesized following reported procedures (Fig. S1)^[22, 23, 25]. Poly(BLG-*co*-PLG)s were prepared *via* ROP of PLG-NCA and BLG-NCA monomers in DMF using HMDS as initiator. The polymerization was monitored by observing the disappearance of characteristic peaks from NCAs (1853 cm^{−1} and 1785 cm^{−1}) and the appearance of new peaks from the copolypeptides backbone (1655 cm^{−1} and 1550 cm^{−1}) using FTIR spectra (Fig. 1a–ii). Furthermore, both FTIR (Fig. 1a–ii, 2130 cm^{−1}) and ¹H-NMR (Fig. 1b–ii, g, δ = 2.85) verified the presence of the alkyne group in the copolymer. The composition of the copolypeptides was calculated based on the integrated areas at δ = 5.05 of the benzyl –CH₂– group and δ = 4.62 of methylene proton (–CH₂C $\equiv$ CH). The corresponding molecular parameters of poly(BLG-*co*-PLG) are summarized in Table 1. By varying the feeding ratio, we prepared three copolypeptides containing different contents of alkyne groups.

PmPEGs were prepared by conjugating mPEG-N₃ with the pendant alkyne groups of poly(BLG-*co*-PLG) using Cu(I)-catalyzed click chemistry. The products were well verified by FTIR (Fig. 1a–i), ¹H-NMR (Fig. 1b–i), and GPC (Fig. S2a). As illustrated in Fig. 1(b), the resonance peaks from poly(BLG-*co*-PLG) backbone (c) and mPEG side chains (m, k, i, j) were clearly observed. The propargyl peak (g) disappeared completely, and the methylene proton (f) peak at δ = 4.62 shifted downfield to δ = 5.22. The appearance of a new peak at δ = 8.11 (g') was attributed to the characteristic methine proton in the triazole ring, indicating complete conversion of alkyne into triazole rings. Moreover, the disappearance of the methylene peak of the benzyl group verified the successful deprotection. In addition, the disappearance of the absorption band of alkyne group (2130 cm^{−1}) and azido group (2105 cm^{−1}) in the FTIR spectra (Fig. 1a–i) and the dramatic increase of M_n (Fig. S2a) demonstrated the high grafting efficiency. The mPEG graft densities were calculated based on

integral ratios of peaks m to (e + d). The M_n of PmPEG was obtained from the mPEG graft density and DP of the corresponding poly(BLG-co-PLG) (Table 1).

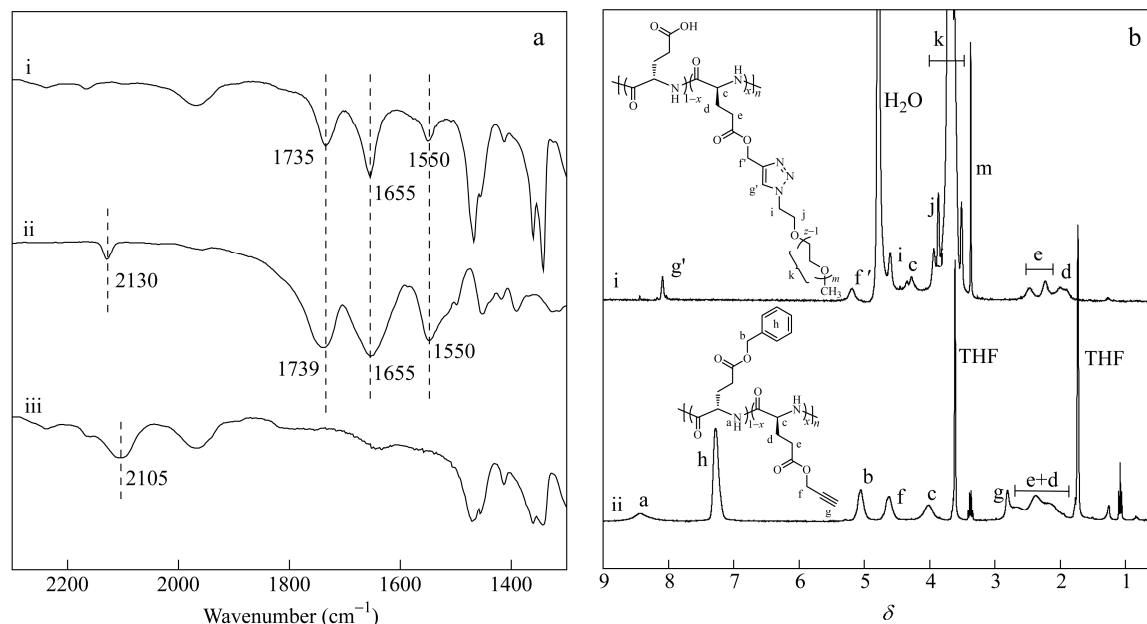


Fig. 1 (a) FTIR spectra of PmPEG_{5k}-0.48 (i), P1 (ii), and mPEG_{5k}-N₃ (iii) in the solid state; (b) ¹H-NMR spectra of PmPEG_{5k}-0.48 (in D₂O) (i) and P1 (in C₄D₈O/CF₃COOH = 6:1) (ii)

Table 1. Molecular parameters of mPEG grafted copolypeptides

Entry ^a	Feed ratio ^b	DP	Poly(BLG-co-PLG) copolypeptides			PmPEG		
			P^c (mol%)	M_n^d (kDa)	PDI ^d	Samples ^e	M_n^f (kDa)	PDI ^d
P1(BP _{0.48}) ₁₁₂	50/50	112	48	21.8	1.20	PmPEG _{2k} -0.48	124.3	1.20
						PmPEG _{5k} -0.48	288.4	1.27
P2(BP _{0.28}) ₈₃	75/25	83	28	17.1	1.08	PmPEG _{2k} -0.28	57.5	1.17
						PmPEG _{5k} -0.28	127.4	1.19
P3(BP _{0.19}) ₈₈	83/17	88	19	18.6	1.09	PmPEG _{2k} -0.19	45.9	1.24
						PmPEG _{5k} -0.19	97.6	1.30

^a B represents BLG segment, P represents PLG segment, (BP_x)_n: x is the molar content of P, and n is the overall DP of poly(BLG-co-PLG); ^b Feed molar ratio of BLG-NCA/PLG-NCA; ^c Calculated from ¹H-NMR spectra; ^d Determined from GPC/LLS; ^e PmPEG_w-y: w is the mPEG molecular weight, y is mPEG graft density according to [mPEG grafted glutamate residues]/[total glutamate residues] (molar ratio) for each PmPEG chain

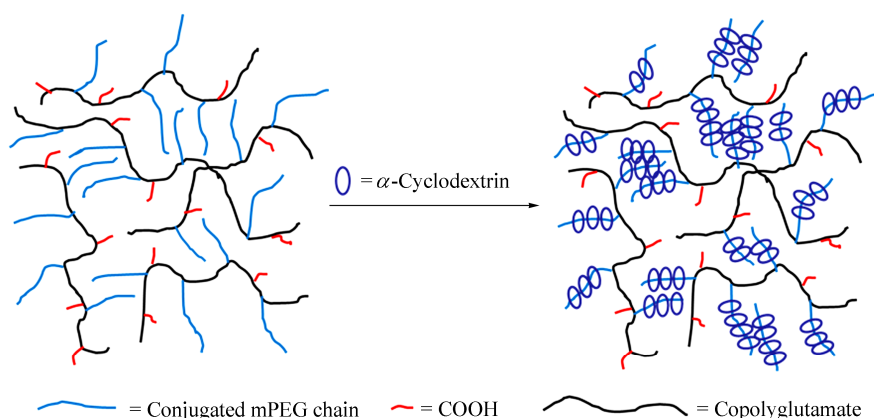
It was reported that low molecular weight PEG could form ICs with α -CD at 2:1 molar ratio between ethylene glycol repeat units and α -CD (EG: α -CD) in aqueous solutions, while PEG of high weight was found to give gels in a wide range of concentrations^[12]. In this work, when the 10 wt% α -CD solution was mixed with PmPEG solution at room temperature, a supramolecular gelation occurred. We found that supramolecular hydrogels were obtained at the ratio of EG: α -CD ranging from 2:1 to 12:1. Here, the EG: α -CD was kept at 6:1 for all the following hydrogels studied. As shown in Fig. S3, after mixing the PmPEGs with α -CD solutions, the mixture firstly became opaque, then gradually became white, and finally formed hydrogels. The gelation time (time_{gel}) ranged from ten minutes to several hours depending on the concentration of PmPEG, the density and length of the mPEG grafts (Table 2). Typically, the time_{gel} decreased with the increase of PmPEG concentration and the mPEG graft density, but increased with increase in the mPEG length. Scheme 2 illustrates the proposed gelation mechanism in aqueous media. In this model, α -CD molecules threaded onto the mPEG grafts *via* host-

guest interaction, and then the α -CDs became hydrophobic during ICs formation due to the hydrogen bonds interaction of the adjacent α -CDs. Finally, the hydrogels formed through the aggregation of ICs as physical cross-linking points arising from the hydrophobic interaction.

Table 2. Preparation of supramolecular hydrogels

Gel precursor	Gel composition ^a			Results ^d		
	EG: α -CD	Copolymer ^b (wt%)	mPEG ^c (wt%)	gels	Time _{gel} ^e (min)	$T_{gel-sol}$ ^e (°C)
PmPEG _{2k} -0.48	6:1	—	1.9	G1	15	50
PmPEG _{5k} -0.48	6:1	2.4	1.9	G2	22	65
	6:1	2.0	—	G3	64	—
PmPEG _{2k} -0.28	6:1	—	1.9	G4	17	49
PmPEG _{5k} -0.28	6:1	2.4	1.9	G5	30	64
	6:1	2.0	—	G6	120	—
PmPEG _{2k} -0.19	6:1	—	1.9	G7	21	46
PmPEG _{5k} -0.19	6:1	2.4	1.9	G8	26	62
	6:1	2.0	—	G9	79	—

^a The concentration of α -CD solution was kept at 10 wt% for all the studies; ^{b,c} The final concentration of PmPEG and mPEG, respectively; ^d Determined by ITM; ^e The time_{gel} and $T_{gel-sol}$ were measured from the hydrogels prepared with DI water



Scheme 2 Schematic illustration of the supramolecular PmPEG/ α -CD hydrogels

All the hydrogels in this work exhibited a gel-sol transition upon temperature increase. As shown in Table 2, the $T_{gel-sol}$ of PmPEG_{5k}/ α -CD hydrogels was higher than that of PmPEG_{2k}/ α -CD. This result could be explained by the formation of longer ICs domains which act as stronger physical cross-links for the hydrogels with higher molecular weight mPEG side chains^[13]. However, the PmPEG/ α -CD system became cloudy mixture at 80 °C. It was inferred that α -CD molecules could not completely detach from mPEG_{5k} or mPEG_{2k} due to chain distortions and kinetics for mPEG with high molecular weight^[26]. In addition, when the temperature decreased to RT, all the opaque hydrogels were obtained again.

Because the PmPEG possesses partial carboxylic acid side groups ($pK_a = 4.5$), the hydrogels can be affected by solution pH and ionic strength. We found the secondary structure of PmPEG was nearly 100% α -helix in DI water but they presented random coil conformation in PBS (pH = 7.4) due to electrostatic interaction among carboxylate groups (Fig. S4). When PmPEG_{5k}-0.48 concentration was 2.4 wt% or 1.4 wt%, the hydrogels formed under a broad pH condition ranging from 3 to 8 as shown in Figs. S5(a) and S5(b). When PmPEG_{5k}-0.48 concentration was 1.2 wt%, the hydrogels were obtained at pH = 4.5 or 8 (Fig. S5c). However, only precipitate was obtained in weak acid solution (pH = 3). In that case, all carboxylic acid groups were protonated leading to the destruction of delicate hydrophilic/hydrophobic balance. When PmPEG_{5k}-0.48 concentration was 0.6 wt%, all mixtures remained clear and no hydrogels were observed at the pH values studied (Fig. S5d). Moreover, the

hydrogels also could form in 100 mmol/L NaCl solution, indicating that adding salts does not prevent hydrogel formation.

Rheology measurements were conducted to investigate the effects of the concentration and composition of PmPEG as well as solution pH on hydrogels strength. Figure 2(a) compared the hydrogels with 2.4 wt% or 2.0 wt% PmPEG_{5k}, it was found that the G' values of the hydrogels containing 2.4 wt% PmPEG_{5k} were greater than those of the hydrogels containing 2.0 wt% PmPEG_{5k}. This may be due to the increase of interaction sites of ICs with the increase of the PmPEG_{5k} concentration^[27]. In Fig. 2(b), comparing G1 and G2 (or G4 and G5, G7 and G8) with the same mPEG graft density but different mPEG length, we found the G' values of hydrogels increased with increase of the mPEG length given the same mPEG concentration. The result indicated that longer mPEG chains threaded could result in more stable IC macro-crystalline domains. In addition, comparing G2&G5&G8 (or G1&G4&G7) with the same mPEG length but different mPEG graft density (Fig. 2b), we found the G' values of the hydrogels increased with increase of the mPEG graft density. It was because cross-links

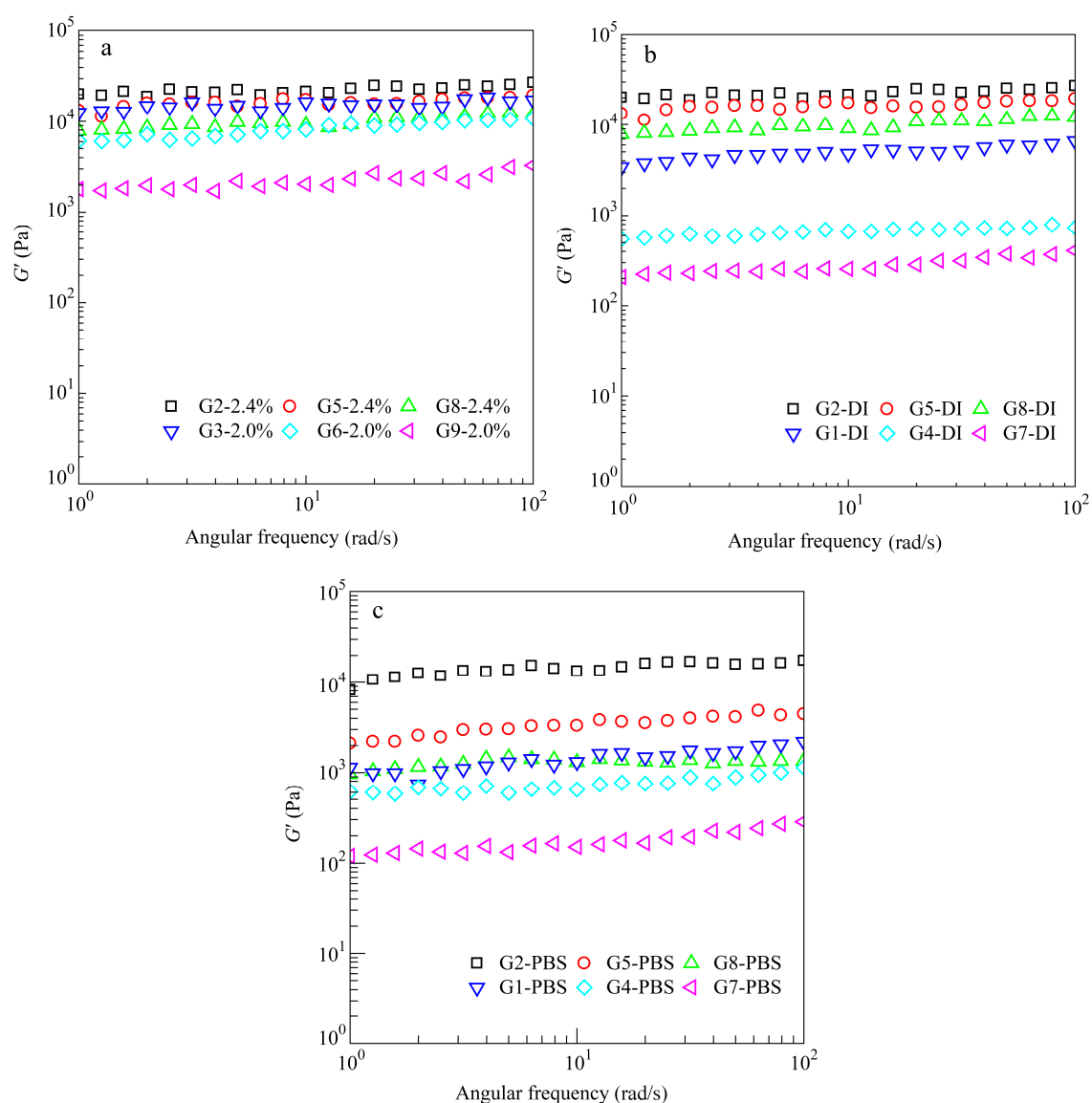


Fig. 2 Frequency sweeps as a function of angular frequency ω for the hydrogels (strain = 0.1%)

formed by ICs of the hydrogels with higher mPEG graft density were denser. Furthermore, when hydrogels were prepared in PBS (pH = 7.4), the carboxylic acid groups are completely deprotonated. In this case, the PmPEG backbone adopted random coil conformation and the PmPEG chain was relatively loose. As a result, the G' values of hydrogels in PBS were lower than those of hydrogels prepared in DI (comparing Figs. 2b and 2c). These results indicated that the hydrogels mechanical strength can be modulated by adjusting the PmPEG concentration, the composition of PmPEG and the solution pH to meet specific application requirements.

To confirm the recovery behavior of the hydrogel system, steady-shear flow test (Fig. S7) and dynamic step strain amplitude sweep (Fig. 3) were performed on the selected hydrogels. First, a strain sweep (Fig. S6) was used to determine the field stress for the hydrogels examined ($\sim 10\%$ strain at yield). In dynamic step strain amplitude measurement, after the hydrogels were subjected to sufficiently large amplitude strain ($\gamma_0 = 100\%$) during 300 s, the G' values were immediately decreased to ~ 5 Pa. Meanwhile, G' dropped below G'' , suggesting that the gel network was broken down. In this condition, the physical cross-links were disrupted due to the hydrogen bond cleavage between α -CD molecules. When the strain was returned to the initial small value ($\gamma_0 = 0.1\%$) again, G' was rapidly recovered to above G'' as the gel network reformed, and the G' values recovered to be about 85% of the original G' for G1 while about 60% for G2 within 400 s. The result was explained longer IC domains as cross-links for mPEG_{5k} chains consumed longer recovery time than those for mPEG_{2k} chains. Such shear-thinning and rapid recovery properties made these hydrogels promising candidates as injectable delivery system.

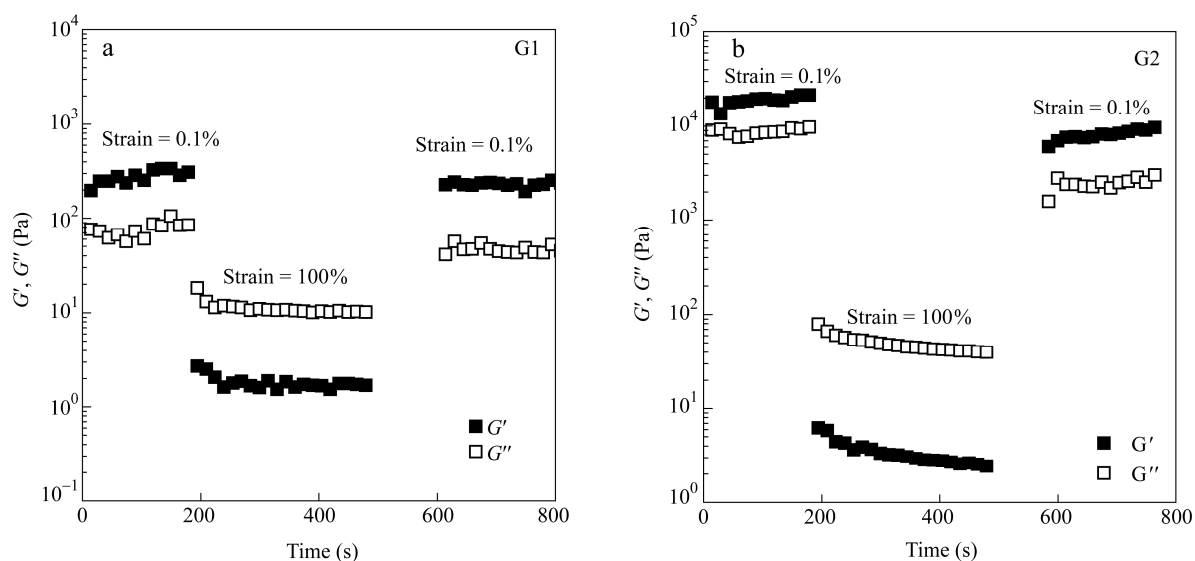


Fig. 3 Dynamic step strain tests of the selected supramolecular hydrogels: (a) G1, (b) G2 (Angular frequency $\omega = 1$ rad/s)

Characterization of the Inclusion Complexations

We have shown the hydrogel formation based on PmPEG and α -CD. The ICs between PEG side chains and α -CD were further characterized. PmPEG_{5k}-0.48 is given as an example. As illustrated in Fig. 4(a), the decomposition point of PmPEG_{5k}-0.48 IC ($T_d = 282$ °C) was relatively higher than pure α -CD ($T_d = 276$ °C), implying that formation of IC could stabilize α -CD^[13]. DSC trace (Fig. 4b) revealed that melting peak of mPEG in PmPEG_{5k}-0.48 is at around 60 °C. However, for PmPEG_{5k}-0.48 IC, no melting peak was observed, and pure α -CD showed no melting peaks in the temperature range. These results suggested the successful formation of ICs, which completely inhibited crystallization of mPEG chains. Such results were also consistent with previous studies on ICs of polymers and α -CD^[28–30].

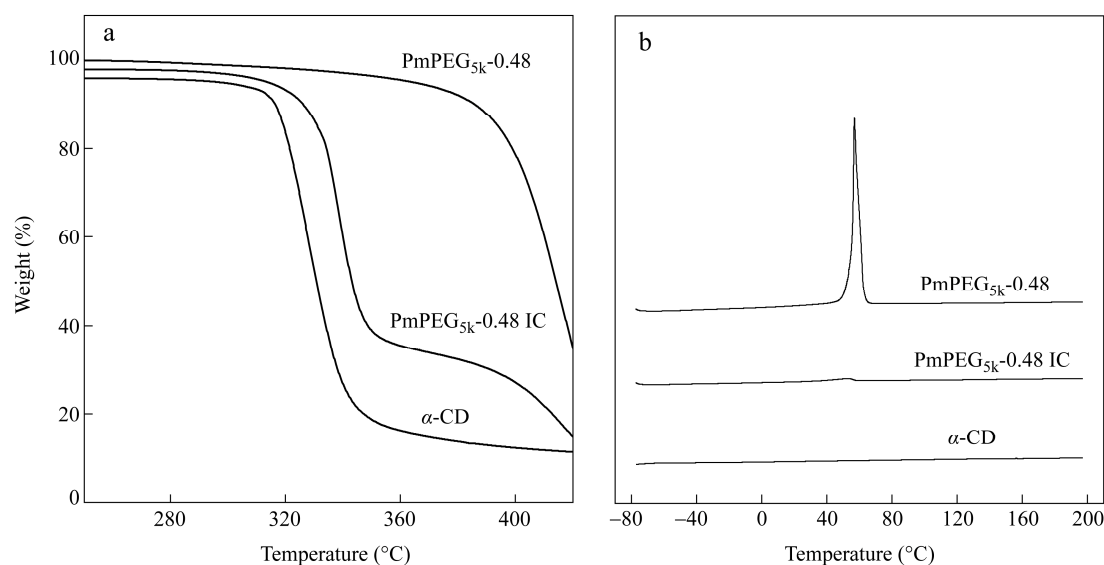


Fig. 4 Thermal characterizations of pure PmPEG_{5k}-0.48, freeze-dried PmPEG_{5k}-0.48/ α -CD IC, and α -CD: (a) TGA and (b) DSC

The successful IC formation was also confirmed by the ^{13}C CP/MAS NMR spectra. Pure α -CD assumes a less symmetrical conformation when mPEG chains were not threaded in the cavity. In this case, multiple resolved C1 and C4 resonances of the glucose residues at $\delta = \sim 98$ and 80 were observed in Fig. 5(a)^[31, 32]. When mPEG_{5k} grafts were threaded into the internal cavity of the α -CD to crystallize in a channel structure, each carbon of the glucose unit can be observed in a broad single peak while C1 and C4 in pure α -CD with the peaks at $\delta = \sim 98$ and 80 disappeared. This result indicated that α -CD adopted a symmetrical conformation and each glucose unit of α -CD was in a similar environment^[26, 32].

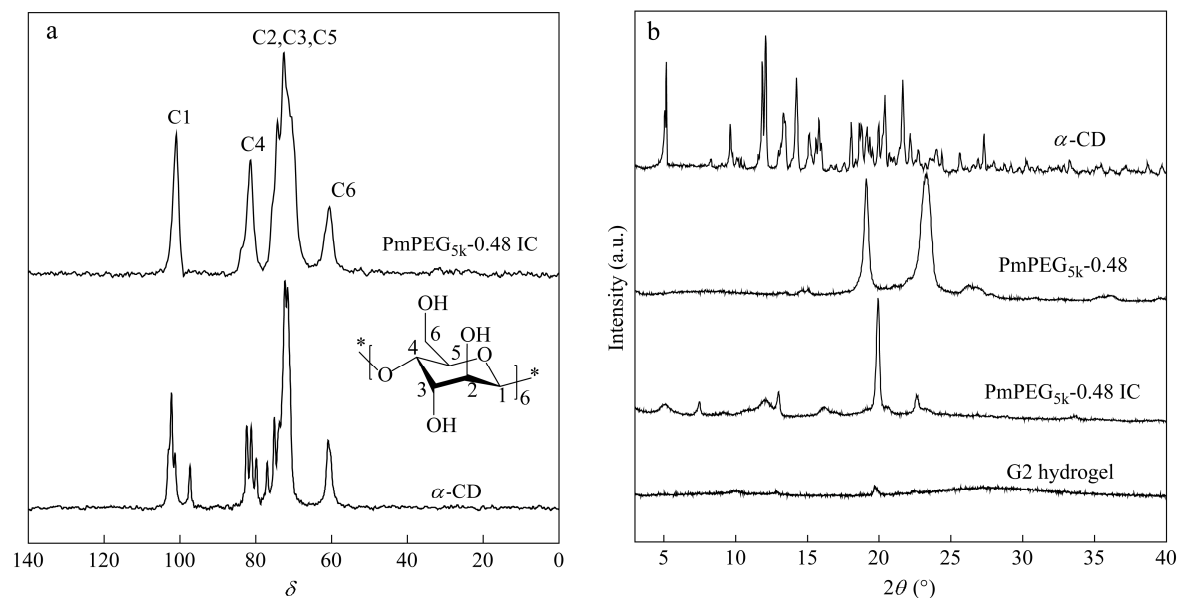


Fig. 5 (a) ^{13}C CP/MAS NMR spectra of freeze-dried PmPEG_{5k}-0.48/ α -CD IC and pure α -CD; (b) XRD patterns of pure α -CD, PmPEG_{5k}-0.48, PmPEG_{5k}-0.48 IC, and G2-DI hydrogel

XRD was applied to characterize the crystalline structure of the ICs between PmPEG and α -CD. As shown in Fig. 5(b), two characteristic diffraction peaks at $2\theta = 20.2^\circ$ ($d = 0.444$ nm) and 22.6° ($d = 0.396$ nm) assigned to the 210 and 300 reflections from the hexagonal lattice were observed in the freeze-dried or hydrated PmPEG_{5k}-0.48/ α -CD IC, although there were broader and weak peaks resulting from some disorder in the macro-crystalline aggregations. In contrast, the α -CD was characteristic of multiple diffraction peaks, and the PmPEG_{5k}-0.48 was characteristic of two main strong peaks at 19.10° and 23.28° . XRD results showed that these ICs had a channel-type crystalline structure (necklace-like) similar to those of ICs of α -CD with valeric acid or octanol, which have been reported to have extended column structure^[31, 33].

In vitro Cytotoxicity Study

The cytotoxicity of PmPEG_{5k}-0.48/ α -CD hydrogels was assessed by CCK-8 viability assay against HeLa cells at various concentrations ranging from 0.002 to 20 mg/mL. At a PmPEG_{5k}-0.48 concentration of 20 mg/mL, the solution formed a hydrogel in the culture dish. As shown in Fig. 6, the viabilities of the HeLa cells treated with sample were higher than 85% at all concentrations, indicating that the PmPEG_{5k}-0.48/ α -CD system was relatively nontoxic to the cells and possessed excellent cytocompatibility, and showed great potential in the biomedical applications.

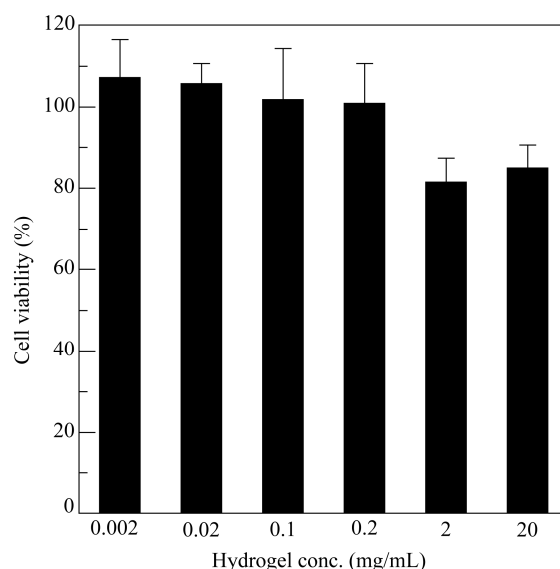


Fig. 6 Proliferation/survival of HeLa cells after treatment with the G2 hydrogel for 24 h assessed by CCK-8 assay

CONCLUSIONS

Novel supramolecular hydrogels were prepared from ICs formation between PmPEG and α -CD in aqueous solution. In this system, the aggregation of ICs created hydrophobic micro-domains, which served as physical cross-links. The hydrogels displayed thermo-responsive gel-sol transition because of the mPEG grafts and pH-sensitive behavior coming from carboxylic acid groups. The gelation time and mechanical strength can be modulated by adjusting the PmPEG concentration, the PmPEG composition and the solution pH. The rheological measurements of the hydrogels showed partially shear-thinning and rapid recovery properties. Moreover, the ICs formation was demonstrated by several techniques. The *in vitro* cytotoxicity assays showed the supramolecular hydrogels were nontoxic and cytocompatible. As a result, these new biocompatible polypeptide-based hydrogels are promising as injectable material in biomedical applications.

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