

Electronic Supplementary Information (ESI)

Injectable, Remoldable Hydrogels with Thermoresponsiveness, Self-healing and Cytocompatibility Constructed via Orthogonal Assembly of Well-defined Star and Linear Polymers

Dong Yu Zhu,* Zhan Peng Hong, Yan Min Xue, Xin Jie Chen, Lan Yue Zhang, Liang Gao,

Yu Xuan Wang, Chu Fen Yang, Jian Wei Guo*

School of Chemical Engineering and Light Industry, Guangdong University of Technology,

Guangzhou 510006, People's Republic of China

S1. Abbreviations

NIPAAM = N-isopropylacrylamide

PMDETA = N,N,N',N'',N'''-Pentamethyldiethylenetriamine

Me₆TREN = Tris[2-(dimethylamino)ethyl] amine

CuBr = Cuprous bromide

β-CD = β-Cyclodextrin

NaN₃ = Sodium azide

CD-OTs = Mono-6-O-(p-toluenesulfonyl)-β-cyclodextrin

Pβ-CD = Mono-6-deoxy-6-aminopropargyl-β-Cyclodextrin

P-AD = Propargyl 1-adamantanecarboxylate

S2. Materials and Methods

Triethylamine was purchased from Aladdin, and distilled from CaH₂ prior to use. Ethylene glycol was purchased from Aladdin, and distilled from Magnesium sulfate anhydrous before used. Other reagents and materials were purchased from Aladdin and Energy Chemical, and used as received. NIH-3T3 cells were purchased from

American Type Culture Collection (ATCC). Dulbecco's modified Eagle's medium (DMEM, purchased from ATCC) and fetal bovine serum were used in cell culture.

^1H NMR spectra and NOESY spectra were taken on Bruker AVANCE III 400MHz Superconducting Fourier. GPC was carried out using a Waters 2414 refractive index detector at room temperature in tetrahydrofuran (THF) as the eluent at a flow rate of 1.0 mL/min and standard monodisperse polystyrenes for calibration. FT-IR spectroscopy was performed using a Nicolet 6700 FT-IR spectrometer. The thermo-analysis of hydrogels was measured by Differential Scanning Calorimeter on DSC3.

S3. Preparation of hydrogels

A typical procedure for preparation of **[2+4] hydrogel** can be described as follows. **4AD** (1 g, 0.09 mmol, $M_n = 11,000$) and **2CD** (1.45 g, 0.18 mmol, $M_n = 8,000$) were respectively dissolved in 5 ml of distilled water at 4°C. The two solutions were mixed thoroughly, and then allowed to sit at refrigerator (4°C) for 48 h. **[2+4]** hydrogel formed after the mixture was heated at 37°C for several minutes. Following the same method, **[2+6] hydrogel** was prepared with **6AD** and **2CD** molar ratio of 3:1.

S4. Rheological tests of the hydrogels

Rheological tests were performed on MCR301 Rheometer with PP25 flat plate used during all the experiments. Hydrogel samples were placed on the parallel plate and subjected to both dynamic strain sweep test and dynamic temperature sweep test. A) Hydrogel samples were placed between parallel plates with a gap of 1 mm at 27°C or 37°C. The alternate step strain sweep test was then performed at a fixed angular frequency (10 rad/s). Amplitude oscillatory strains were switched from small strain ($\gamma = 0.1\%$, and with 60 s) to subsequent large strain ($\gamma = 500\%$, and with 30 s). B) Mutative Temperature Rheology Test. The hydrogel samples were incubated at 15°C

before testing. The tests were carried out at strain of 1% and angular frequency of 10 rad/s from 15°C to 45°C with a ramping rate of 3 °C/min.

S5. Methods for cytotoxicity assay

Cell culture

NIH 3T3 cells from -80°C freezer were re-suspended in complete growth medium (DMEM supplemented with 10% FBS and 1% penicillin/streptomycin). The NIH 3T3 cells were incubated at 37°C in a humidified atmosphere in the presence of 5% CO₂, and were usable after subculturing 4 times. Cells were trypsinized, and re-seeded in 96-well plates at a density of 5.0×10^4 cells/ml (5.0×10^3 cells/well). Cells were harvested for use after incubated at 37°C for 24 h.

Cytocompatibility assay in vitro

(1) MTT assay

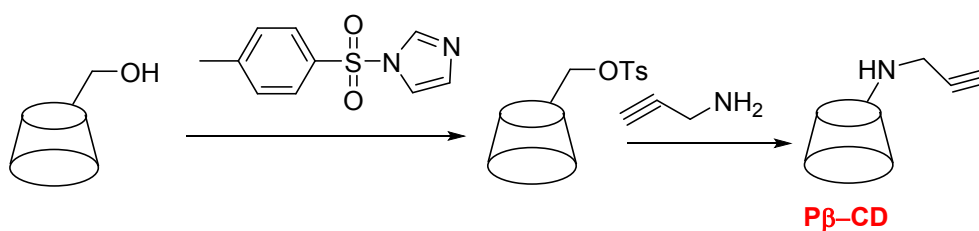
The NIH 3T3 cells were seeded into a 96-well plate at a density of 5.0×10^3 cells/well in 100 µl of complete growth medium at 37°C humidified atmosphere containing 5%CO₂. After 24 h, cells were treated with [2+4] solution at varied concentrations of 25, 12.5, 6.25, 3.125 and 1.5625 mg/mL for 24 h. At the end of treatment, the cell viability was measured using a MTT Cytotoxicity Assay. Procedures were performed according to the manufacturer's instruction. The optical density of each well was measured using the MULTISKAN FC microplate reader at 570 nm. All cell viabilities were calculated according to the formula of Viability = $[(OD_{\text{sample}} - OD_{\text{blank}})/(OD_{\text{control}} - OD_{\text{blank}})] \times 100 \%$, where OD_{sample} 、 OD_{control} 、 OD_{blank} are the optical density of the sample (treated with hydrogel solution), control sample (no treatment with hydrogel solution) and blank sample (no treatment with either hydrogel solution or cells), respectively. All measurements were triplicate.

(2) 3D cell culture and live/dead staining assay

NIH 3T3 cells were encapsulated in [2+4] hydrogel under sterile conditions. The [2+4] gel precursor solution was prepared as follows: lyophilized [2+4] gel was dissolved in 2mL cell culture medium with a concentration of 25 mg/ml and stored in the refrigerator (4°C). NIH 3T3 cells were re-suspended in cell culture medium to prepare the cell suspension at a concentration of 10^5 cells/ml. [2+4] gel precursor solution sterilized by sterile 0.2 μm filter and cell suspension were successively added into a 96-well plate (100 μL /well). The mixture in each well was mixed vigorously with the help of a micropipette and the final concentration of [2+4] gel in suspension was 12.5 mg/ml per well, and the final density of NIH 3T3 cells was 5,000 cells/well. The mixture in 96-well plate was incubated at 37 °C with 5% CO_2 for 24 hours or 48 hours. Cell-free [2+4] gel and pure NIH 3T3 cells were also prepared as controls.

Live/dead staining (Molecular Probes) was assessed at 24 h and 48 h. The Live & Dead Viability/Cytotoxicity Assay Kit for Animal Cells was purchased from KeyGEN BioTECH. According to the manufactures' instructions, all samples were incubated in 200 μL of PBS with 1 μM Calcein-AM and 2 μM PI (Propidium iodide) at 37°C for 45 min, followed by washing with 100 μL PBS for 2 times. Representative images were obtained by an inverted fluorescence microscope (Olympus IX71) with live cells in green and dead cells in red.

S6. Synthesis of P β -CD

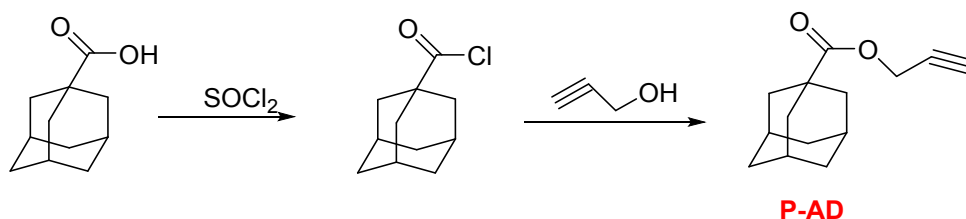


1-(p-Toluenesulfonyl) imidazole (8.9 g, 40 mmol), β -CD (35 g, 30.84 mmol) and 250 mL of distilled water were added into a flask. The mixture was stirred thoroughly for 4 h, forming a white turbid mixture. After adding 50 mL of 20wt% sodium hydroxide solution, the mixture turned into pale yellow liquid. After stirring for another 30 minutes, the system was filtrated to remove unreacted solid. When 2mol/L

HCl solution was added to the filtrate and adjusted the pH to 7~8, precipitate appeared. After filtration, the resultant solid was sequentially washed with deionized water and acetone 3 times. Finally, the product CD-OTs was obtained as white solid after recrystallized twice in hot deionized water, and then dried for 48 hours under vacuum.

CD-OTs (10 g, 7.7 mmol) and 20 mL of propargylamine were added into a reaction flask, the mixture was kept stirring at 50°C for 96 hours in dark under N₂ atmosphere. Followed that, the reaction system was first diluted with a mixture of methanol/water, and then precipitated in plenty of acetone. After repeating the dissolving-precipitation 3 times, the final product P β -CD was obtained. P β -CD: ¹H NMR (400MHz, DMSO-d₆): 5.60~5.89(m, 14H), 4.75~4.81(m, 7H), 4.15~4.62(m, 6H), 3.45~3.72(m, 28H), 3.15~3.47(m, 14H), 2.41(s, 3H), 2.90~3.04(m, 2H), 2.69~2.79(m, 1H). FT-IR (KBr, cm⁻¹): 3354, 2929, 2131, 1705, 1638, 1414, 1366, 1233, 1156, 1028, 946, 859, 756, 706, 580. ESI-MS (m/z): calculated: 1171.4 for C₄₅H₇₃O₃₄N, found 1172.5 for [M+H]⁺.

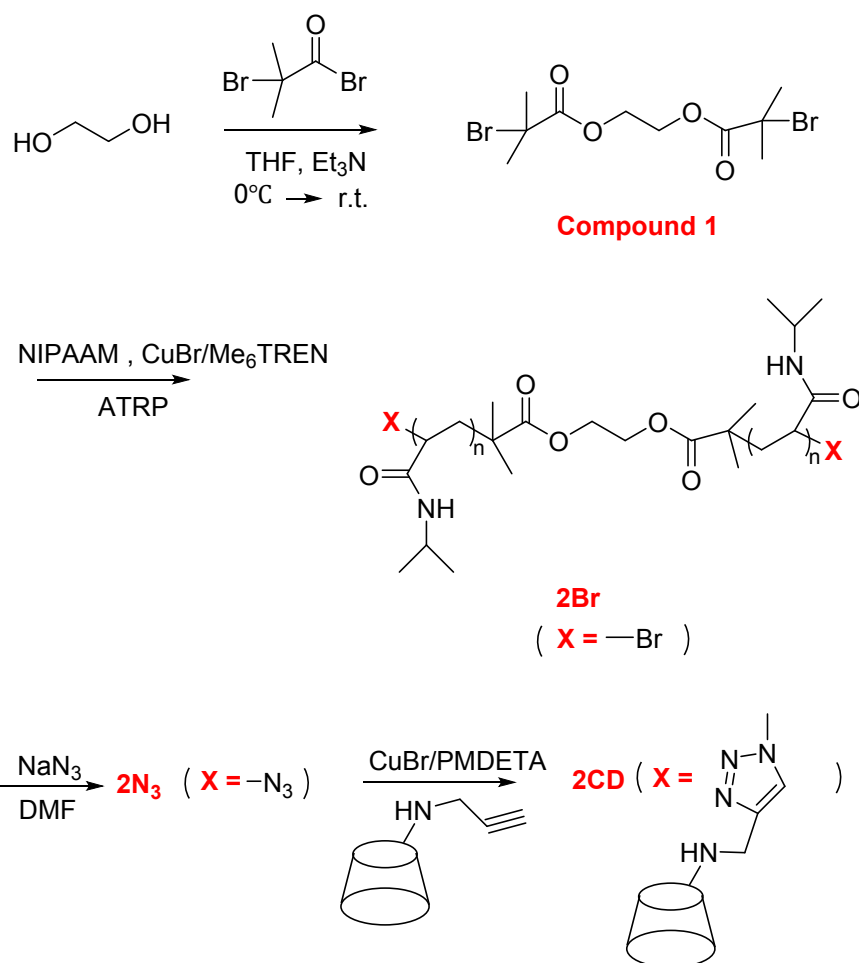
S7. Synthesis of P-AD



1-Adamantanecarboxylic acid (9 g, 50 mmol), 4 drops of dry DMF and 50 mL of dry DCM were mixed. Oxalyl chloride (6.338 g, 50 mmol) diluted with 20 mL of dry DCM, was added dropwise at 0°C. The mixture was allowed to warm up to room temperature and stirred for 12h. After removing the solvent and excess oxalyl chloride by rotary evaporation, a pale yellow solid (1-Adamantanecarbonyl chloride) was obtained.

Dry triethylamine (6.07 g, 60 mmol) and 1-Adamantanecarbonyl chloride (10 g, 50.32 mmol) were dissolved in 100 mL of anhydrous diethyl ether. Then, propargyl alcohol (3.3636 g, 60 mmol) dissolved in 50 mL of dry diethyl ether was added dropwise into the reaction system. The mixture was stirred for 48 h, then filtered. The filtrate was washed sequentially with 10 % HCl, 5 wt% NaHCO₃ and deionized water, each for 3 times, then finally dried using anhydrous MgSO₄ for 12 h. After filtration and concentration by rotary evaporation, the product P-AD was further purified by column chromatography. P-AD: ¹H NMR (400MHz, CDCl₃): 4.64(2H), 2.4(1H), 2.0(3H), 1.9(6H), 1.7(6H). FT-IR (KBr, cm⁻¹): 3308, 2907, 2853, 2129, 1731, 1453, 1365, 1345, 1323, 1269, 1225, 1183, 1157, 1103, 1073, 997.

S8. Synthesis of 2CD



Synthesis of Compound 1. Anhydrous ethylene glycol (2g, 32 mmol), triethylamine (7.20 g, 71 mmol) were first dissolved in 100 mL of dry THF. After the mixture was stirred for 30min at 0°C, α -Bromoisobutyryl bromide (16.33 g, 71 mmol) was added dropwise, then the reaction system was allowed to slowly warm up to room temperature and stirred for another 24h. After removing THF through evaporation, the crude product was re-dissolved in dichloromethane and washed sequentially with 10% HCl, 5 wt% NaHCO₃ and deionized water, each for 3 times, and then dried with anhydrous MgSO₄. The product was further purified by recrystallization from iced ethanol, and vacuum dried overnight. Compound **1**: ¹H NMR (400 MHz, CDCl₃): 4.44 (s, 4H), 1.94 (s, 12H).

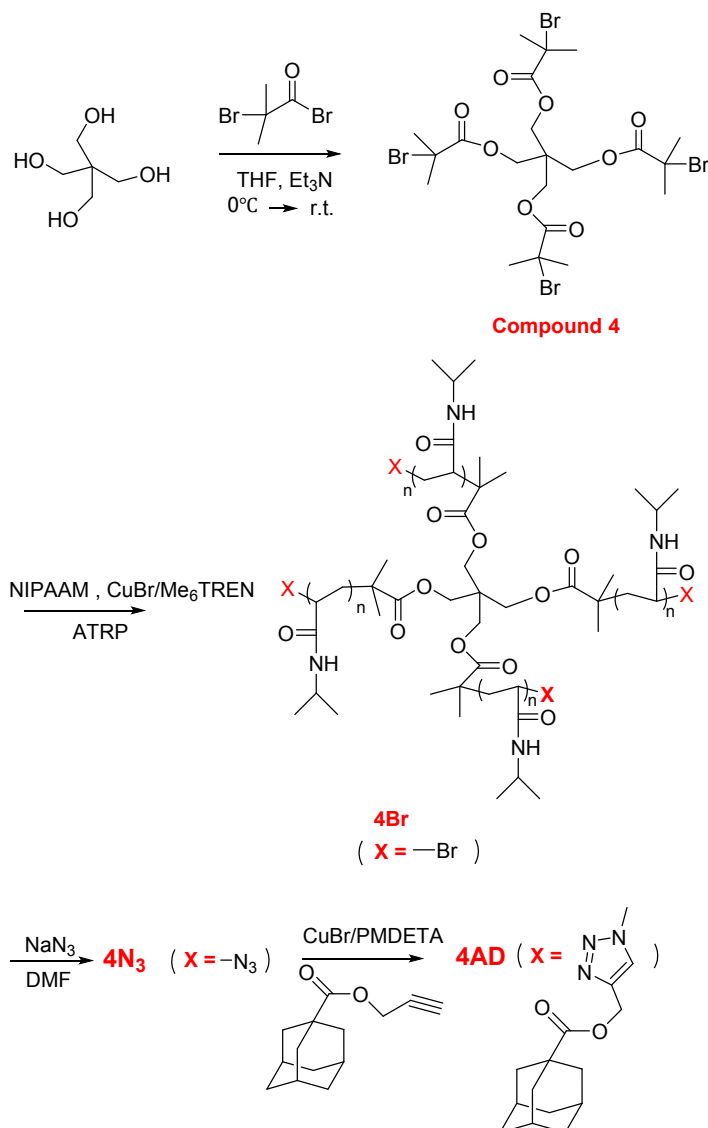
Synthesis of polymer 2Br. Compound **1** (0.258 g, 0.7 mmol), NIPAAM (8 g, 70.7 mmol), and Me₆TREN (0.323 g, 1.4 mmol) were dissolved in 20 mL of a H₂O/DMF (20:80 v/v) mixture. CuBr (0.201 g, 1.4 mmol) was then added under a nitrogen atmosphere. The reaction was continuously stirred at room temperature for 2h. To terminate the reaction, 20 mL of THF was added and the mixture was exposed to air and stirred for another 30min. After purification through column chromatography to remove the catalyst, followed by precipitation from ice diethyl ether, the resultant precipitate was filtered, and dried under vacuum for 24h. $M_{n[NMR]} = 5431$. $M_{n[GPC]} = 5561$. $PDI_{[GPC]} = 1.187$.

Synthesis of polymer 2N₃. After dissolving **2Br** (5 g, 0.83 mmol, $M_n=6,000$) in 20 mL of DMF, NaN₃ (0.54 g, 8.3 mmol) was added and the reaction mixture was stirred at 60°C for 48h. The crude product was purified by dialysis (dialysis bag: 3,500Da) against H₂O for 48 h, followed by lyophilization to give the product.

Synthesis of 2CD. 2N₃: P β -CD: PMDETA: CuBr at a molar ratio of 1: 2.2: 2: 2 was added. **2N₃** (3 g, 0.5 mmol, $M_n=6,000$), P β -CD (1.23 g, 1.05 mmol), and PMDETA (0.17 g, 1.0 mmol) were dissolved in 20 mL of DMF and stirred thoroughly. Then CuBr (0.14 g, 1.0 mmol) was quickly added under a nitrogen atmosphere. After the mixture was stirred at 60°C for 48 hours under a nitrogen atmosphere, 20 mL of THF

was added and the mixture was exposed to air under stirring to terminate the reaction. The catalyst was removed by column chromatography using THF as an eluent. The resultant crude product was further purified by dialysis (dialysis bag: 3500Da) against H₂O for 48 hours. The excess water was removed by lyophilization to give the product **2CD** for store.

S9. Synthesis of 4AD



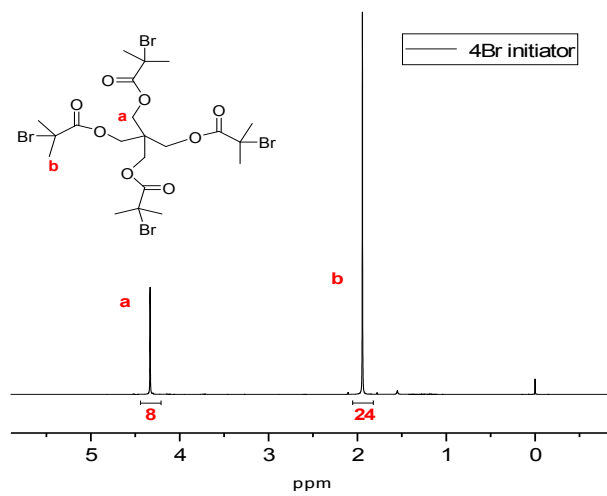
Compound **4** (**4Br Initiator**) was synthesized according to the same method with that of Compound **1**, except that the ethylene glycol was replaced with pentaerythritol. The ^1H NMR spectrum of Compound **4** is shown in Figure S1(a): ^1H NMR (400 MHz, CDCl_3): 4.33 (s, 8H), 1.94 (s, 24H).

4Br was synthesized according to the same method with that of **2Br**. The molar ratio of Compound **4**: NIPAAm: Me_6TREN : CuBr was 1: 400: 4: 4. The ^1H NMR spectrum of **4Br** with marked characteristic Peaks and integrals is shown in Figure S1(b). **a** belongs to the active hydrogen (NH), and Peak **b** represents the specified proton of the Isopropyl (CH) which is overlapped with Peak **c** belonging to the methyl of the initiator. For one molecular, $c=8$, According to the accurate integration by MestReNova as shown in Figure S1(b), $a=b=80$, so we could calculate that the molecular weight of **4Br** is 9781, which is close to the result from GPC (10070). ($M_n [\text{NMR}] = 9781$. $M_n [\text{GPC}] = 10070$. $\text{PDI} [\text{GPC}] = 1.176$).

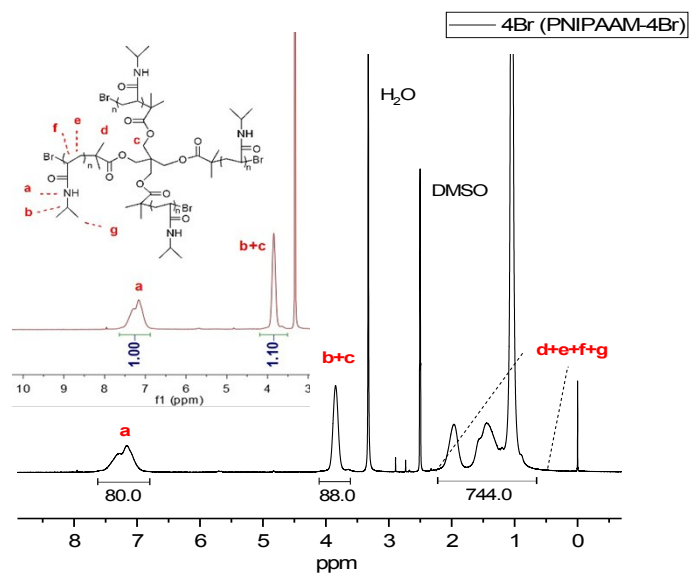
4N₃ was synthesized according to the same synthetic method with that of **2N₃**, with the molar ratio of **4Br** and NaN_3 was 1:20.

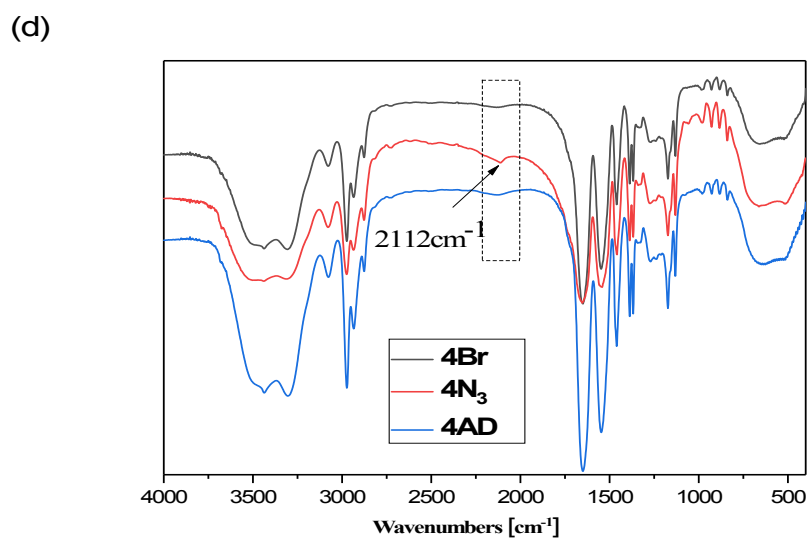
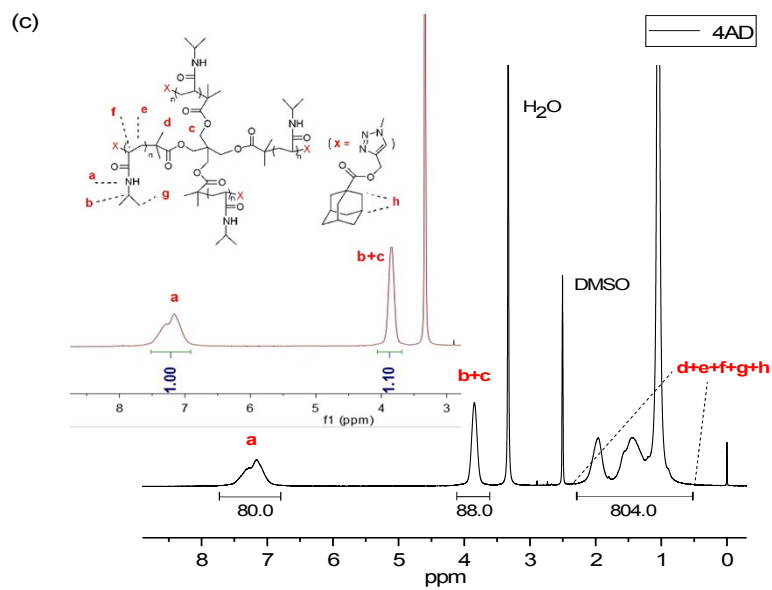
4AD was synthesized according to the same method to that of **2CD**, only that the P β -CD was replaced by P-AD and the molar ratio of **2N₃**: P-AD: PMDETA: CuBr was 1: 4.2: 4: 4. The resultant ^1H NMR spectrum of **4AD** with marked characteristic peaks and integrals is shown in Figure S1(c). Like **4Br**, the peak integral of **a:(b+c)**=80:88. After click-reaction with **P-AD**, the protons of AD group labelled as **h** should be on the ^1H NMR spectrum. However, these peaks are overlapped with those from the mother polymer **4Br** chains in the high field as labelled with **d, e, f, g**, which makes it hard to directly count the number of AD groups. Therefore, we made the whole integration for the multi peaks within the range of 0.5-2.3ppm for the ^1H NMR spectra of both **4Br** and **4AD**. In comparison, we calculated that **h=60** by analyzing the integral difference between **4Br** and **4AD**. (Table 1) The number of AD groups is 4 ($=h/15$, each AD group should have 15 protons). According to the ^1H NMR integral values, we can calculate the molecular weight of **4AD** is 10502, which is generally matched with the data from GPC (10880). $M_n [\text{NMR}] = 10502$. $M_n [\text{GPC}] = 10880$. $\text{PDI} [\text{GPC}] = 1.181$.

(a)



(b)





(e)

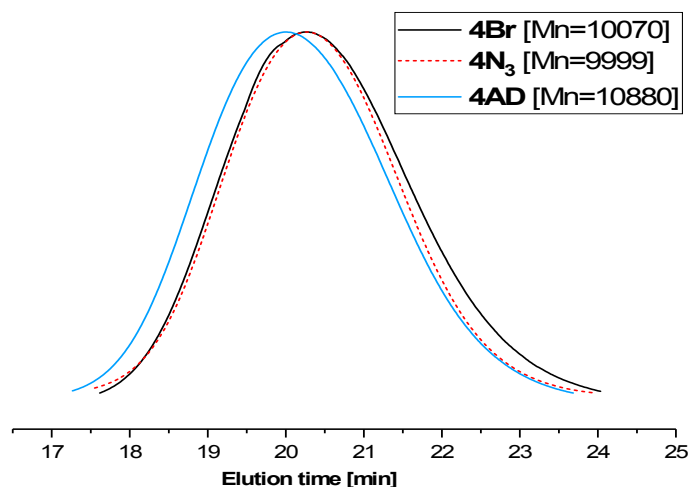


Figure S1 (a) ^1H NMR spectrum (400 MHz, 25°C) of **4Br** initiator (Compound **4**) in CDCl_3 , (b) ^1H NMR spectrum (400 MHz, 25°C) of **4Br** in DMSO, (c) ^1H NMR spectrum (400 MHz, 25°C) of **4AD** in DMSO, (d) FTIR spectra of **4Br**, **4N₃** and **4AD**; In contrast to **4Br** (PNIPAAm-4Br) and **4N₃** (PNIPAAm-4N₃) shows a different peak of 2112 cm^{-1} in the IR spectrum, which demonstrates the terminal group (-Br) of **4Br** was replaced by -N₃, while the peak of 2112 cm^{-1} disappeared in the spectrum of **4AD**, which means the -N₃ groups were further reacted and AD groups were successfully introduced into the end of the polymer chains by azide-alkyne click reaction. (e) GPC curves of **4Br**, **4N₃** and **4AD** in THF. The GPC curves show the changes in the molecular weight of the polymers, which further demonstrates that the polymer ends have been finally linked with AD groups, consistent with our design.

S10. Synthesis of 6AD

Compound **7** (**6Br** initiator) was synthesized following the same synthetic method with that of Compound **1**, except that ethylene glycol was replaced with dipentaerythritol. The ^1H NMR spectrum of Compound **7** is shown in Figure S2(a): ^1H NMR (400MHz, CDCl_3): 4.22(s, 12H), 3.53(s, 4H), 1.87(s, 36H).

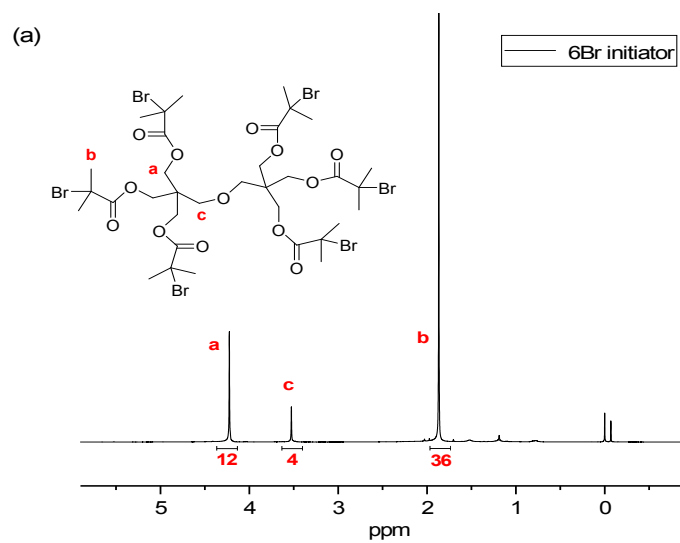
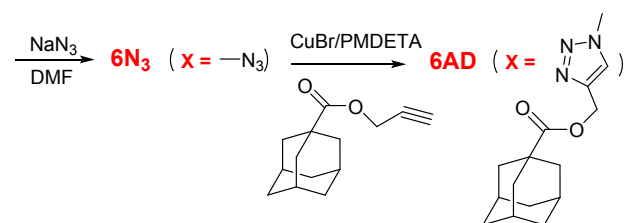
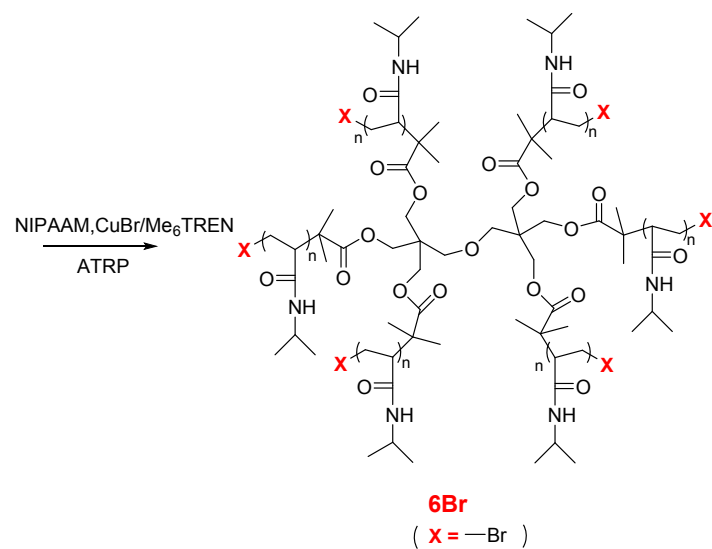
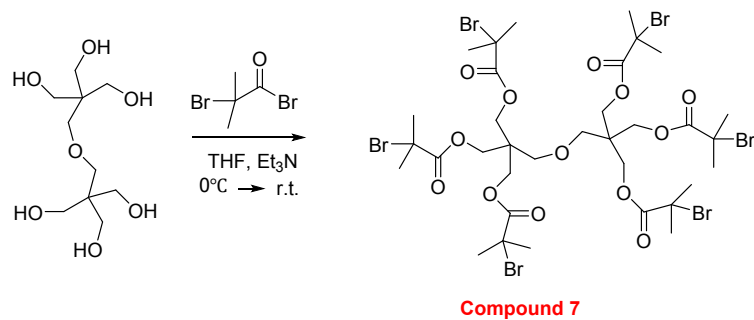
6Br (Polymer **8**) was synthesized following the same method with that of polymer **2**, while the molar ratio of Compound **7**: NIPAAm: Me₆TREN: CuBr was 1: 600: 6: 6. The ^1H NMR spectrum of **6Br** with marked characteristic Peaks and integrals is shown in Figure S2 (b). **a** belongs to the active hydrogen (NH), and Peak **b** represents the specified proton of the Isopropyl (CH) which is overlapped with Peak **c** belonging to the methyl of the initiator. For one molecular, **c** = 12, According to the accurate integration by MestReNova as

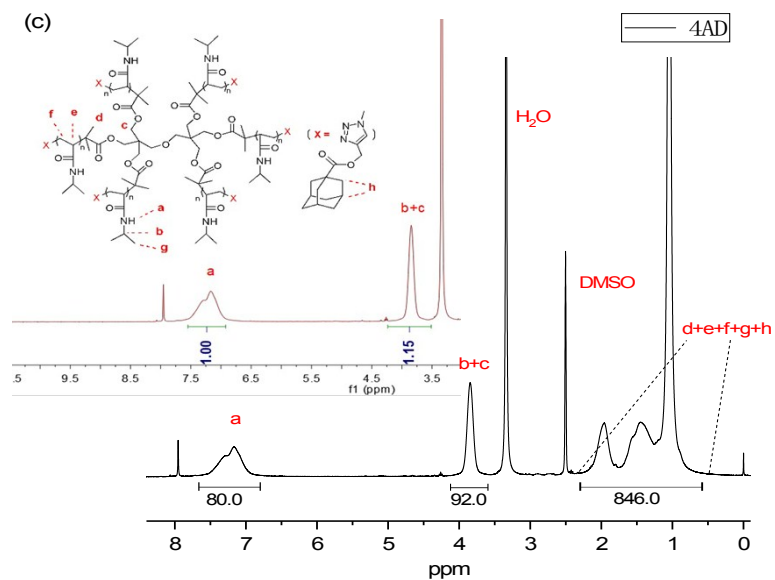
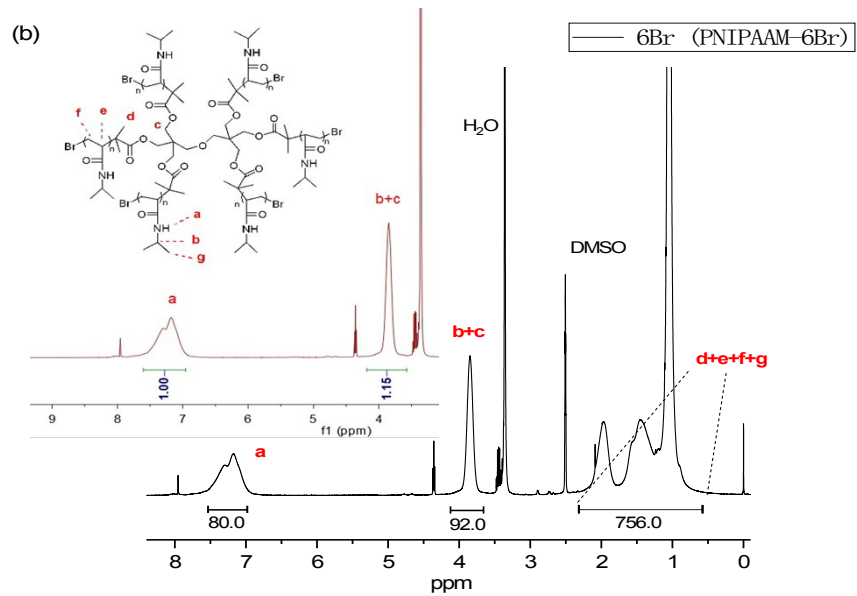
shown in Figure S2 (b), $a=b=80$, and the integral of Peak “d+e+f+g” is 856, so we could calculate that the molecular weight of **6Br** is 10201, which is close to the result from GPC (10875). $M_{n[NMR]} = 10201$. $M_{n[GPC]} = 10875$. $PDI_{[GPC]} = 1.186$.

6N₃ (Polymer **9**) was synthesized following the same method with that of polymer **3**, while the molar ratio of polymer **8** and NaN₃ was 1:30.

6AD was synthesized following the same synthetic method of **4AD**, while the molar ratio of polymer **9**: P-Ad: PMDETA: CuBr was 1: 6.3: 6: 6.

The resultant ¹H NMR spectrum of **6AD** with marked characteristic peaks and integrals is shown in Figure S2(c). Like **4AD**, we calculated that $h=90$ by analyzing the integral difference between **6Br** and **6AD**. (Table 1). Hence, the number of AD groups should be 6 ($=h/15$, each AD group should have 15 protons). According to the ¹H NMR integral values, we can calculate the molecular weight of **6AD** is 11284, which is generally matched with the data from GPC (11745). $M_{n[NMR]} = 11284$, $M_{n[GPC]} = 11745$, $PDI_{[GPC]} = 1.175$.





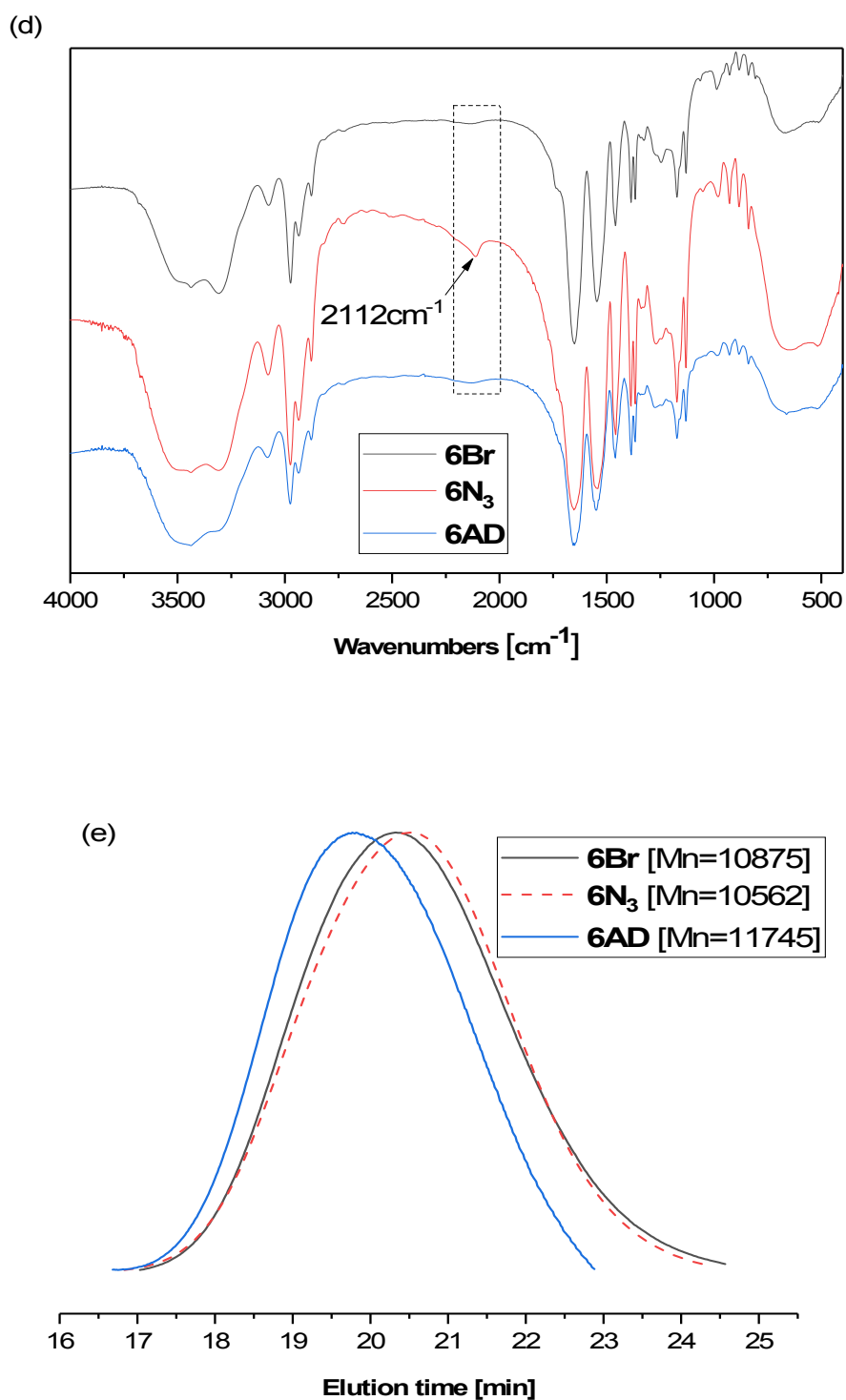


Figure S2 (a) ¹H NMR spectrum (400 MHz, 25°C) of **6Br** initiator (Compound 7) in CDCl₃, (b) ¹H NMR spectrum (400 MHz, 25°C) of **6Br** in DMSO, (c) ¹H NMR spectrum (400 MHz, 25°C) of **6AD** in DMSO, (d) FTIR of **6Br**, **6N₃**, and **6AD**. Compared to **6Br**, **6N₃** shows a new peak at 2112 cm⁻¹ in the FTIR spectrum, which demonstrates the terminal group (-Br) of PNIPAAm-6Br was replaced by -N₃, while the peak at 2112cm⁻¹ disappeared in the spectrum of **6AD**, which means the -N₃ groups were

further reacted with P-AD by azide-alkyne click chemistry. (c) GPC curves of **6Br**, **6N₃** and **6AD** in THF. The GPC curves show the variation of molecular weight of the polymers with different ends, further demonstrating that the AD groups have been successfully linked into the polymer ends.

Table 1. The ¹H NMR integral value and calculation results for AD groups of 4Br, 4AD, 6Br and 6AD

Result\Name	4Br	4AD	6Br	6AD
a:(b+c)	1.00 : 1.10	1.00 : 1.11	1.00 : 1.15	1.00 : 1.16
integral value of Peak a	80	80	80	80
integral value of Peak "d+e+f+g(+h)"	744	804	756	846
number of AD group (=h/15)	-	4	-	6
Mn (¹ H NMR)	9781	10502	10201	11284
Mn (GPC)	10070	10880	10875	11745

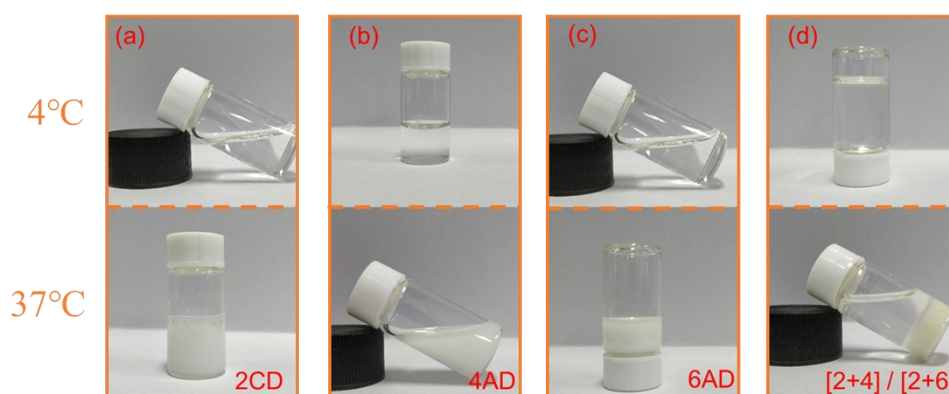


Figure S3 (a) 20 wt% **2CD** aqueous solution at 4°C was transparent colorless, while it immediately turned into opaque suspension when the temperature increased to 37°C (exhibited phase transition); no gel formed by heating the single polymer unit of **2CD** aqueous solution at 37°C. (b) 20 wt% **4AD** aqueous solution at 4°C was transparent colorless, while it immediately turned into opaque suspension when the temperature increased to 37°C (exhibited phase transition); also, no gel formed by heating the single polymer unit of **4AD** aqueous solution at 37°C. (c) 20 wt% **6AD** aqueous solution at 4°C was transparent colorless, while it immediately turned into opaque suspension when the temperature increased to 37°C (exhibited phase transition); and also, no gel formed by heating the single polymer unit of **6AD** aqueous solution at 37°C. (d) 20 wt% **2CD** aqueous solution and 20 wt% **4AD** (or **6AD**) aqueous solution at a molar ratio of 2:1 (or 3:1) were fully mixed at 4°C, which is shown transparent colorless solution, Gel was formed when the mixture was maintained at 37°C for several minutes. It demonstrates that gel formation is based on the self-assembly of β-CD and AD.