Electronic Supplementary Information (ESI)

for

Construction of stable polymeric vesicles based on azobenzene and beta-cyclodextrin grafted poly(glycerol methacrylate)s for potential applications in colon-specific drug delivery

Wen-Xing Gu,[a] Qing-Lan Li,[b] Hongguang Lu,[a] Lei Fang,[c] Qixian Chen,[d] Ying-Wei Yang*[b] and Hui Gao*[a]

[a]School of Chemistry and Chemical Engineering, Tianjin Key Laboratory of Organic Solar Cells and Photochemical Conversion, Tianjin University of Technology, Tianjin 300384, P. R. China

E-mail: hgao@tjut.edu.cn

[b]State Key Laboratory of Inorganic Synthesis and Preparative Chemistry, College of Chemistry, International Joint Research Laboratory of Nano-Micro Architecture Chemistry (NMAC), Jilin University, 2699 Qianjin Street, Changchun 130012, P. R. China

Email: ywyang@jlu.edu.cn, yyang@chem.ucla.edu

[c]Department of Chemistry, Texas A&M University, 3255 TAMU, College Station, TX 77843, USA

[d]Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan.

Table of Contents

1. Materials and methods

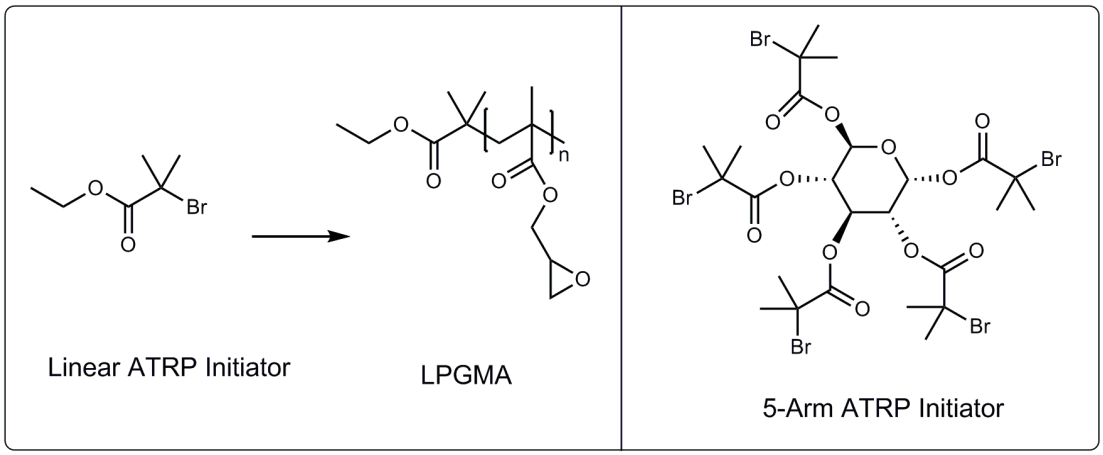
Glycidyl methacrylate (GMA), 2-bromoisobutyryl bromide, bipyridyl and CuBr were purchased from Adamas Reagent Co., Ltd. (Shanghai, China). 3-Bromopropyne was purchased from Heowns Co., Ltd. (Tianjin, China). All the other reagents were obtained from Tianjin Chemical Reagent Co., Ltd. (Tianjin, China). Prior to use, tetrahydrofuran (THF) and ethanediamine (EDA) were dried over sodium, and distilled with benzophenone serving as a dryness indicator.

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a 400 MHz NMR spectrometer (Bruker AV-400). Samples were dissolved in deuterium oxide (D_2O), acetone-d_6, dimethylsulfoxide-d_6 (DMSO-d_6) or deuterated chloroform ($CDCl_3$). Gel permeation chromatography (GPC) measurements were performed at 40 ºC with THF as mobile phase at a flow rate of 0.5 mL/min. The average hydrodynamic diameters (D_h) and polydispersity index (PDI) of the assemblies were measured using dynamic light scattering (DLS) at 25 °C on a Malvern Zetasizer Nano ZS90 instrument. The morphology of vesicles was observed using scanning electron microscopy (SEM) collected on a JEOL JSM 6700F instrument and also using transmission electron microscopy (TEM) on a JEOL JEM-200 CX instrument. The samples were prepared by dropping solutions (ca. 0.5 mg/mL) onto carbon-coated copper grids, stained with phosphotungstic acid (2 wt%), and dried at room temperature for 48 h. Fourier transform infrared (FT-IR) spectra were recorded on a Bruker Vertex 80V spectrometer. Ultraviolet-visible (UV-Vis) spectra were obtained using a Shimadzu UV-2450 instrument. Diffuse reflectance spectroscopy was tested in a UV–Vis diffuse reflectance spectrophotometer (UV-4100, Shimadzu, Japan) equipped with an integrating sphere assembly and using $BaSO_4$ as a reference.

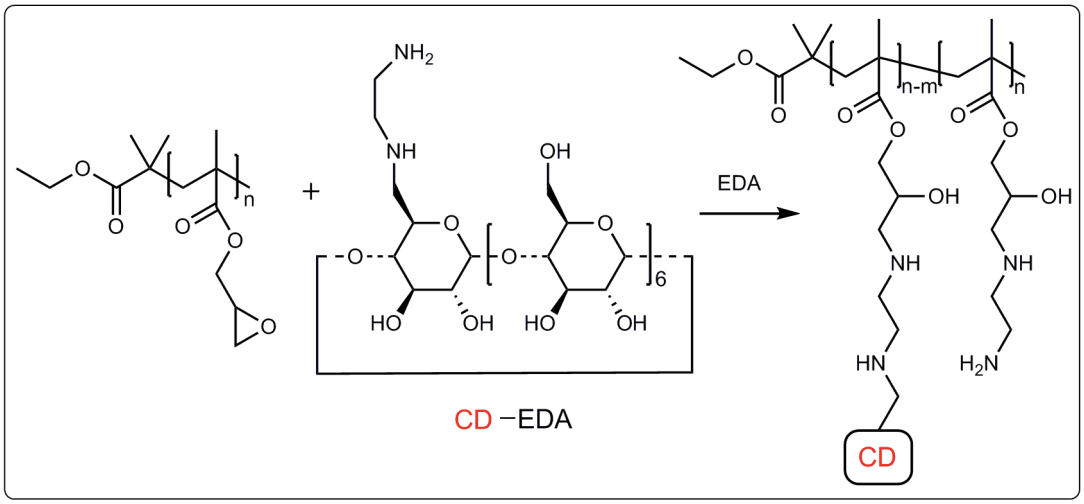
2. Synthetic procedures and characterization

2.1 Synthesis of PGMA and PGMA-CD

Linear and 5-arm poly(glycidyl methacrylate) (PGMA) were synthesized according to the literature[S1] (Scheme S1). β-yclodextrin (CD) grafted PGMA (PGMA-CD) was prepared by the ring-opening reaction of PGMA and mono(6-(ethanediamine)-6-deoxy)-β-CD (CD-EDA) according to our previous reports[S2] (Scheme S2).



Scheme S1. Synthesis of LPGMA and SPGMA from linear and 5-arm initiator, respectively.



Scheme S2. Synthesis of LPGMA-CD.

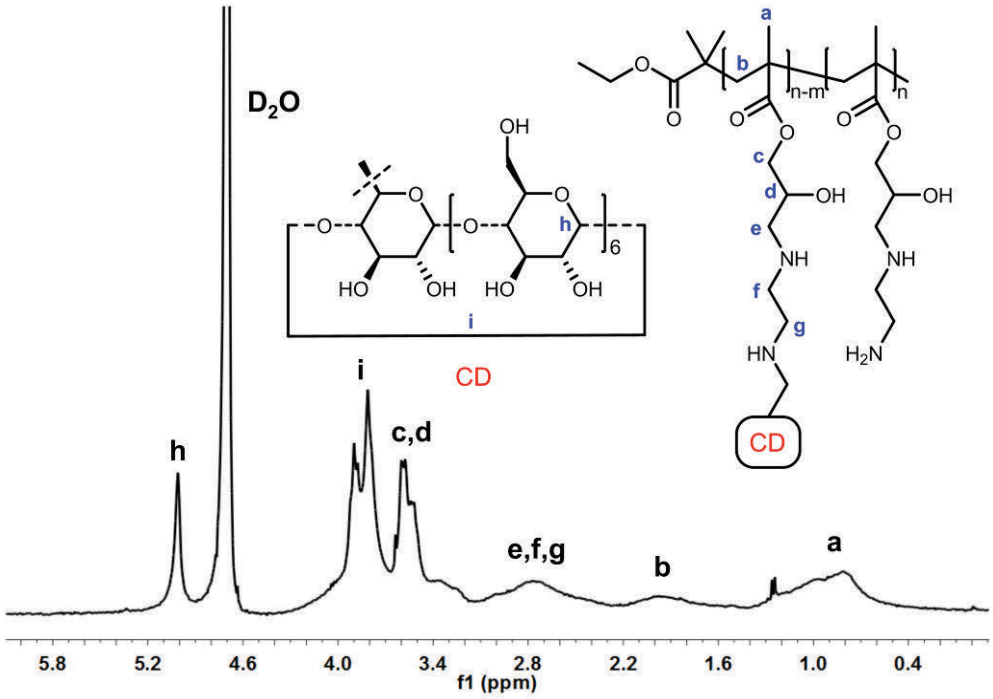
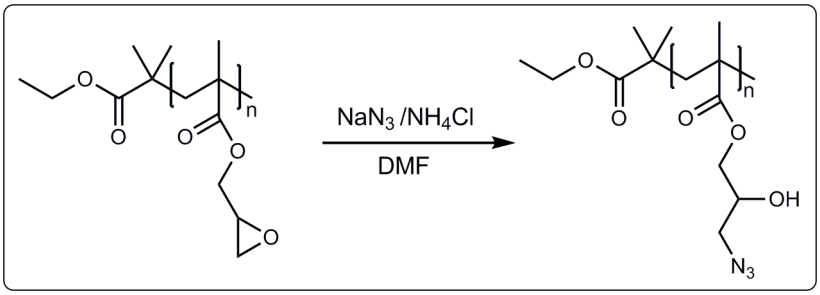


Figure S1. ^{1}H NMR spectra of LPGMA-CD in D_2O ('h' represents -O-C**H**-O-, 'i' represents all protons except 'h' in CDs).

2.2 Synthesis of PGMA-N_3

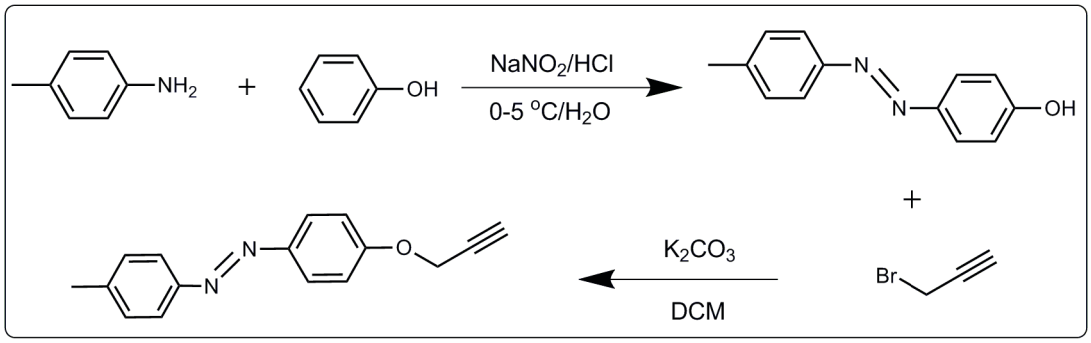
Azido PGMA (PGMA-N_3) was synthesized according to the literature[S3] (Scheme S3). Typically, PGMA (1.0 g, 7.1 mmol) and NH_4Cl (1.90 g, 35.5 mmol) were dissolved in DMF (20 mL), and stirred for 30 min at room temperature. Then NaN_3 (2.31 g, 35.5 mmol) was added, and the mixture was heated at 50 °C for 48 h. After cooling to room temperature, the sediment was removed by suction filtration, and the filtrate was precipitated twice in cold brine solution (200 mL). The resulting precipitate, *i.e.* PGMA-N_3 (2.32 g, yield: 86%) was recovered by suction filtration and dried under vacuum.



Scheme S3. Synthesis of PGMA-N_3.

2.3 Synthesis of 4-methyl-4′-propargyloxy azobenzene (AZO)

4-Methyl-4′-hydroxy azobenzene was synthesized according to the literature[S4] (Scheme S4 and Figure S1). To synthesize 4-methyl-4′-propargyloxy azobenzene (AZO) (Scheme S4), 4-methyl-4′-hydroxy azobenzene (1 g, 4.7 mmol) and K_2CO_3 (0.79 g, 5.7 mmol) were dissolved/suspended in dichloromethane (DCM) (20 mL), and stirred for 30 min at room temperature. Then 3-bromopropyne (1.84 mL, 23.5 mmol) was added dropwise, and the mixture was refluxed at 60 °C for 12 h. After cooling to room temperature, the crude product was passed through a silica gel column with DCM as an eluent. The product (0.96 g, yield: 81%) was obtained after evaporation under reduced pressure.



Scheme S4. Synthesis of 4-methyl-4′-propargyloxy azobenzene (AZO).

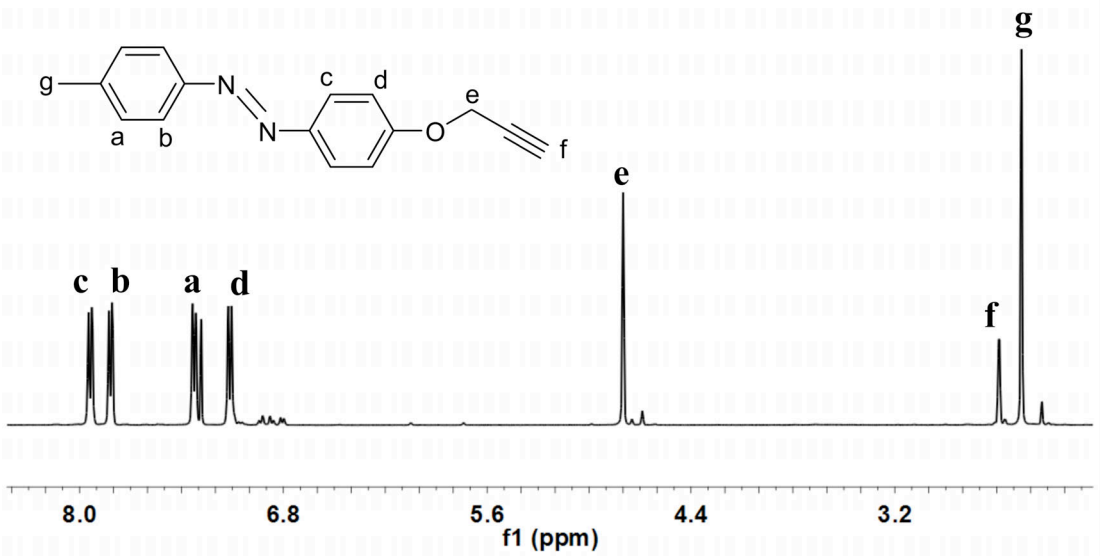
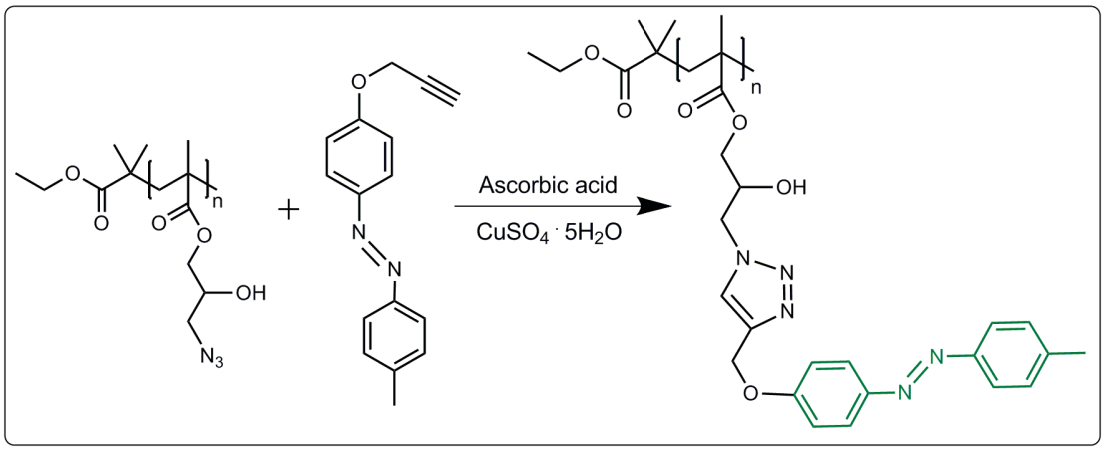


Figure S2. ^{1}H NMR spectrum of AZO in $CDCl_3$.

2.4 Synthesis of PGMA-Az

Azobenzene modified PGMA (PGMA-Az) was synthesized by click reaction (Scheme S5). Typically, PGMA-N_3 (0.50 g, 3.0 mmol) and AZO (0.75 g, 3.0 mmol) were dissolved in DMF (50 mL) under a nitrogen atmosphere. After 30 min, ascorbic acid (0.53 g, 3.0 mmol) and $CuSO_4$•$5H_2O$ (0.15 g, 0.6 mmol) were added, and the mixture was heated at 50 °C for 12 h. After cooling down to room temperature, the mixture was precipitated twice in cold brine solution (200 mL). The resulting precipitate was collected by filtration, dialyzed against DCM at room temperature for 48 h using a dialysis membrane (cut-off molecular weight 7 kDa), and dried under vacuum to yield 0.95 g (Yield: 76%) of PGMA-Az.



Scheme S5. Synthesis of PGMA-Az

2.5 ^{13}C NMR characterization of PGMA, PGMA-CD and PGMA-Az

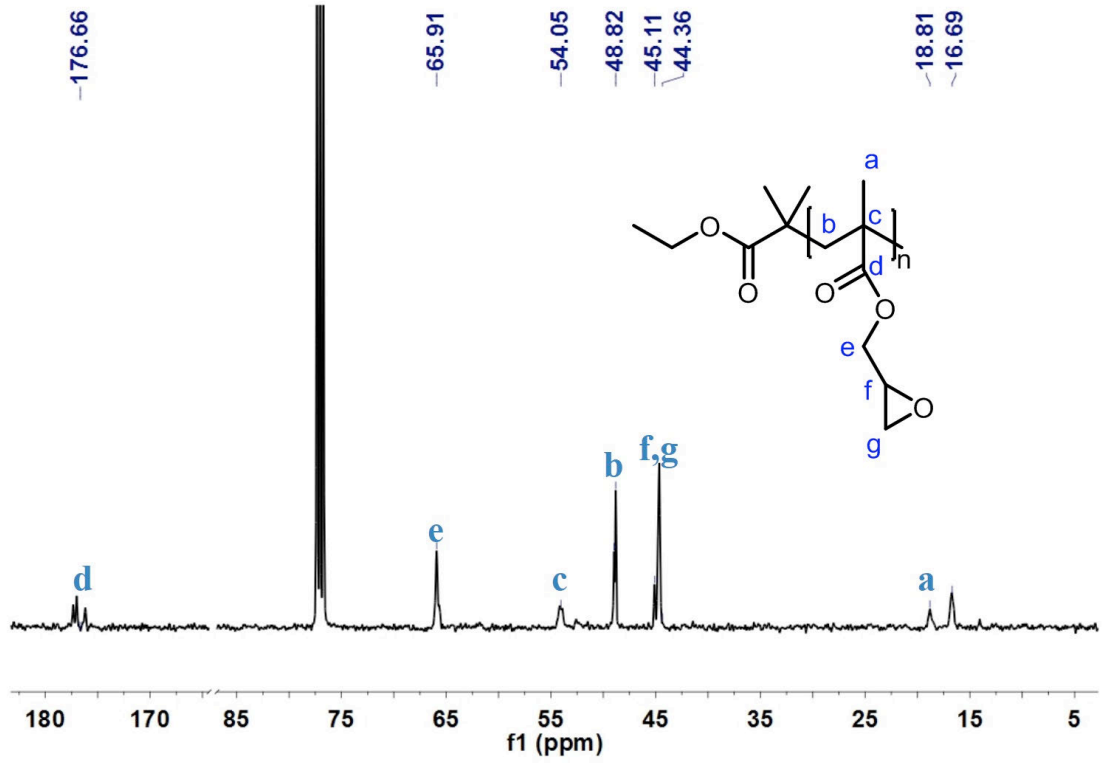


Figure S3. ^{13}C NMR spectrum of PGMA in $CDCl_3$.

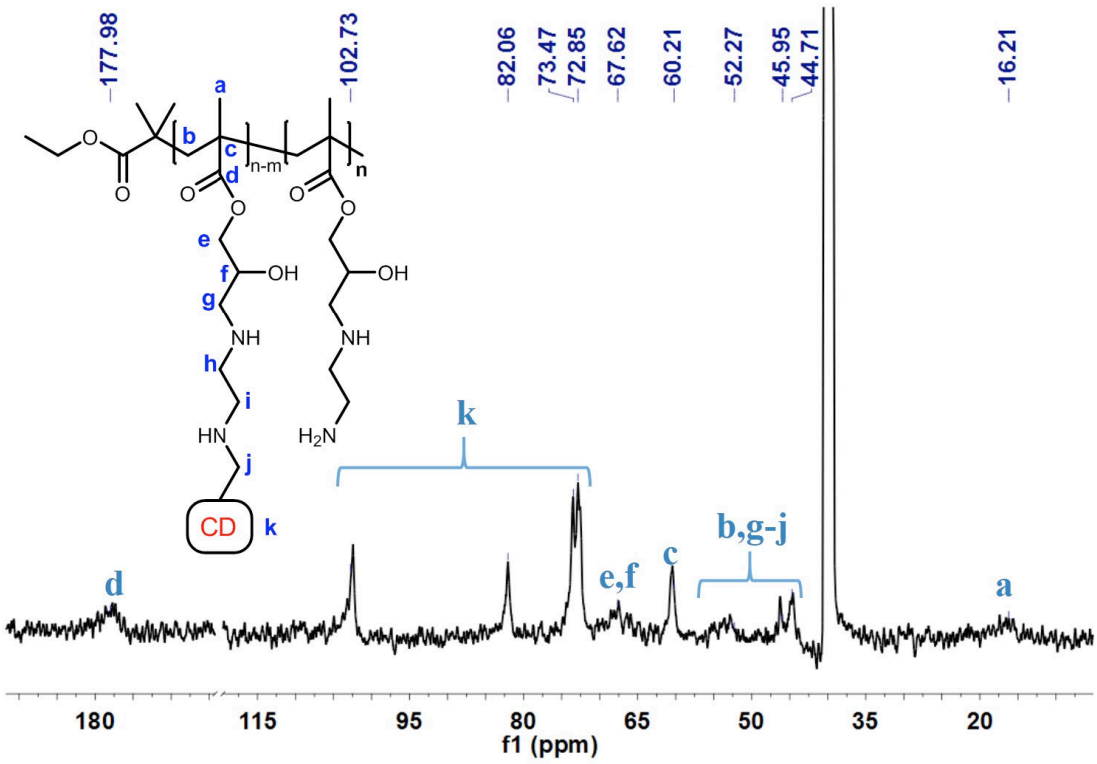


Figure S4. ^{13}C NMR spectrum of PGMA-CD in DMSO-d_6.

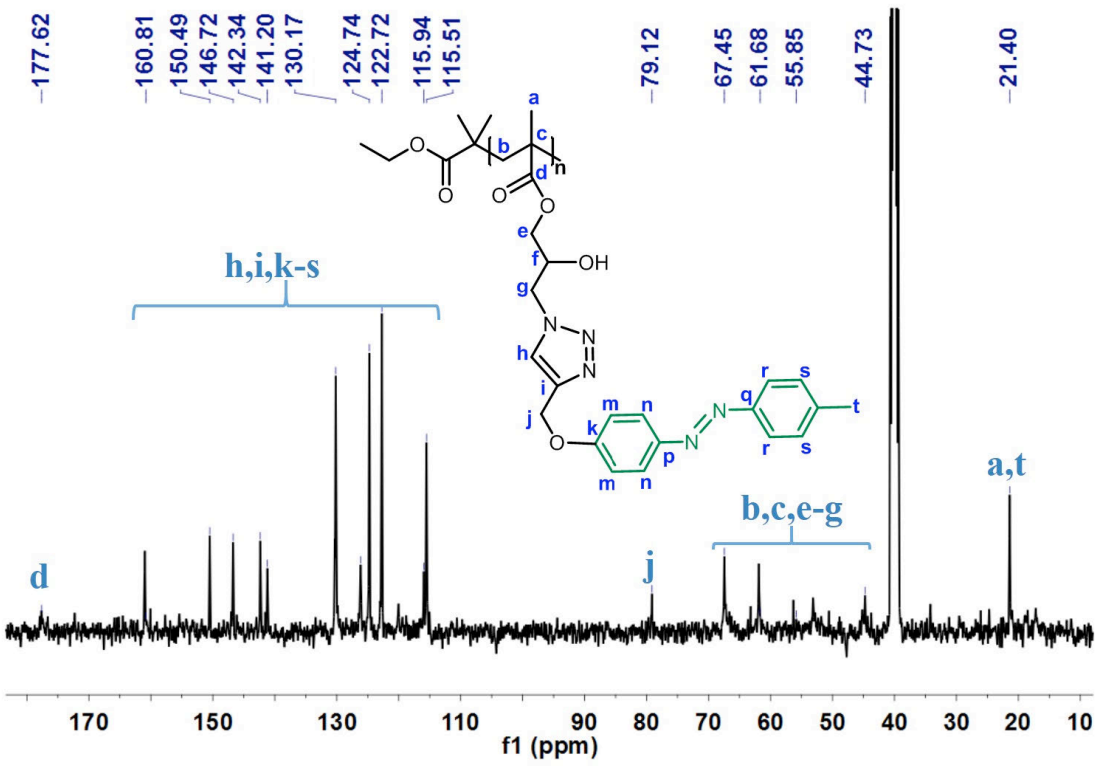


Figure S5. ^{13}C NMR spectrum of PGMA-Az in DMSO-d_6.

2.6 Synthesis of PGMA-OH and PGMA-EDA

Poly(glycerol methacrylate) (PGMA-OH) and amino PGMA-OH (PGMA-EDA) were synthesized. Briefly, PGMA (0.4 mM) was dissolved in 1-methyl-2-pyrrolidinone (NMP, 72 mL) at 60 °C in a 250 mL three-necked round-bottomed flask, and then deionized water (25 mL) was injected. The mixture was stirred at 120 °C for 4 h. The mixture was then cooled down, dialyzed against deionized water for 48 h, and freeze-dried to yield PGMA-OH (65%). To synthesize PGMA-EDA, PGMA (0.4 mM) was dissolved in EDA (50 mL) in a 100 mL round-bottomed flask. The mixture was stirred at 120 °C for 4 h. The mixture was then cooled down, dialyzed against deionized water for 48 h, and freeze-dried (yield, 80%).

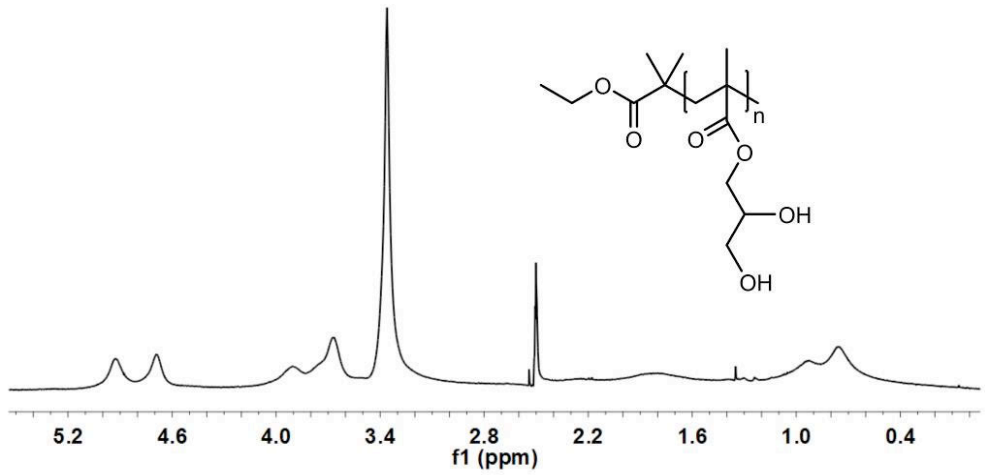


Figure S6. ^{1}H NMR spectrum of PGMA-OH in DMSO-d_6.

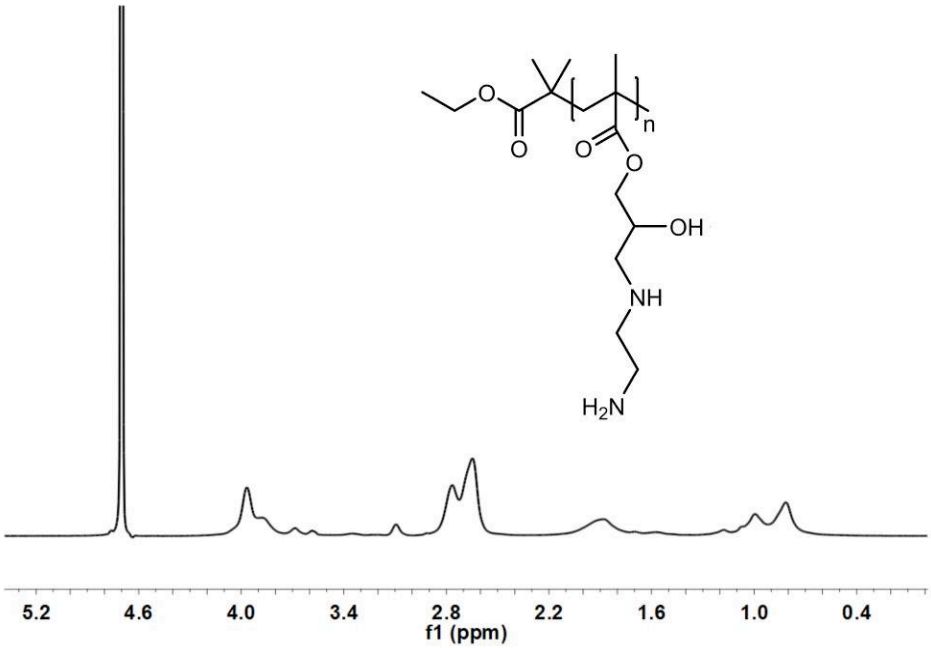


Figure S7. ^{1}H NMR spectrum of PGMA-EDA in D_2O.

2.7 Synthesis of PEG2000-HDI

PEG-based polyurethanes (PEG2000-HDI) were synthesized. Briefly, PEG2000 (1.995 g, 1.995 mmol) was dissolved in 1,2-dichloroethane (DCE) and heated at 110 °C. Residual water was removed by azeotropic distillation. After cooling to 70 °C, HDI (0.369 g, 2.1945 mmol) was added to this solution. Dibutyltindilaurate (0.5 wt%, 14 mL) in dried DCE was added to the solution as a catalyst. The mixture was stirred at 70 °C for 8 h under dry nitrogen. The resulting polyurethane was poured into Et_2O (200 mL). The precipitate was washed 3 times with Et_2O, and dried for 24 h at 40 °C under vacuum.

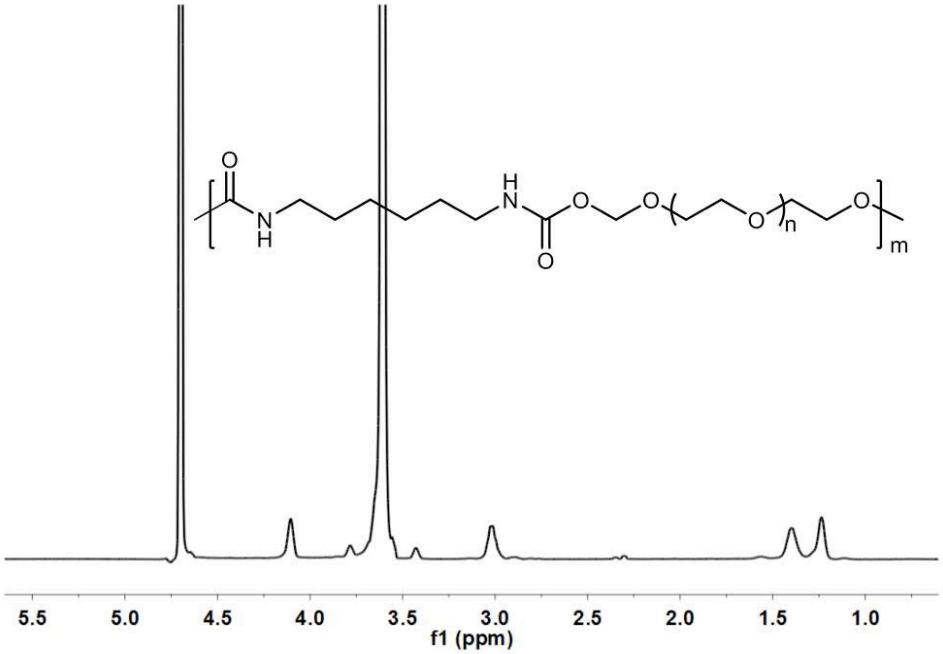


Figure S8. ^{1}H NMR spectrum of PEG2000-HDI in D_2O.

2.8 Characterization of polymers

The average molecular weights of PGMA, the corresponding PGMA-CD and PGMA-Az were showed in Table S1. The typical GPC traces were shown in Fig. S9. The conjugate ratio of CD to the side chain of PGMA was ca. 13-15% calculated using phenol-sulphuric acid assay. The method consists of adding a fixed volume (0.1 ml) of liquefied phenol (80% w/w) to the PGMA-CD solution followed by 5 ml of concentrated sulphuric acid (98%). A brownish-yellow colour develops immediately and its absorbance is measured at 490 nm. The concentration of CD is determined from a standard curve for CD (Fig. S10).

Table S1. GPC data of PGMAs and labeling of the polymers

PGMA	Mn (kDa)[a]	Mw/Mn[b]	PGMA-CD	PGMA-N_3	PGMA-Az
L10	10	1.32	L10CD	L10-N_3	L10Az
L20	20	1.45	L20CD	---	---
S10	10	1.15	S10CD	S10-N_3	S10Az
S19	19	1.42	S19CD	---	---

Note: [a] and [b] were measured by GPC. **L** and **S** represent linear and star-shaped PGMA, respectively, **10/ 20/ 10/ 19** represent the Mn of PGMA.

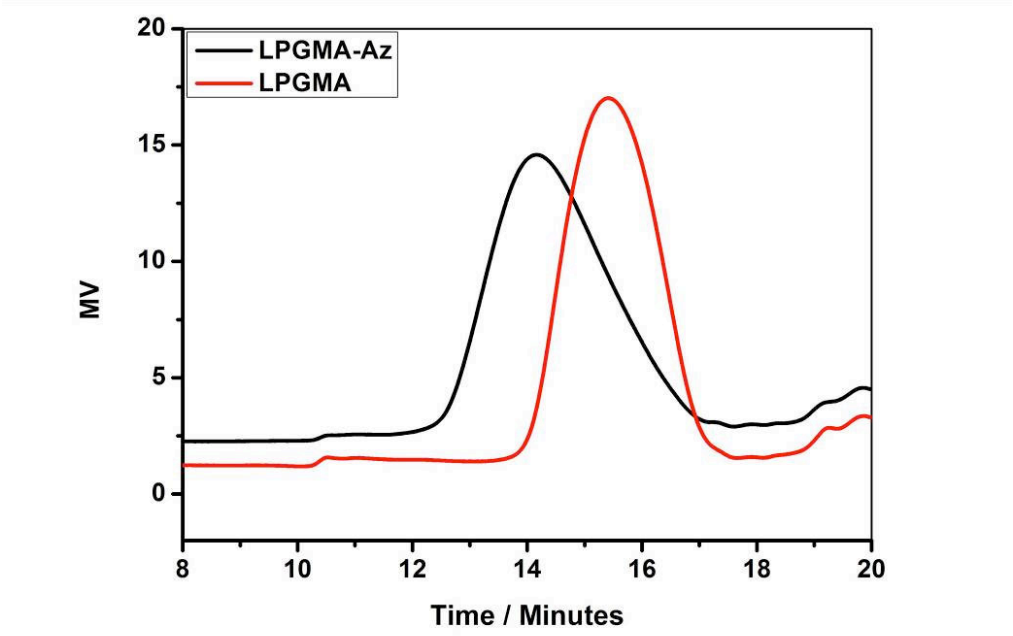


Figure S9. GPC traces of LPGMA and LPGMA-Az in THF.

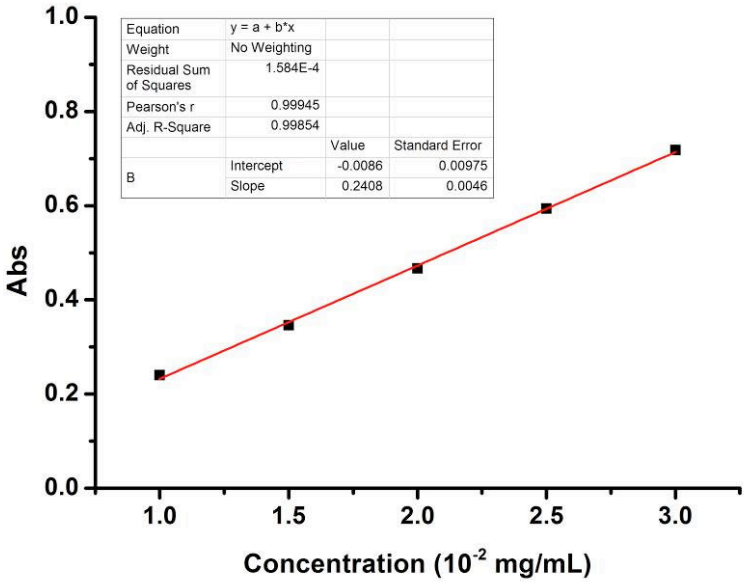


Figure S10. The standard curve of CD solution done by using phenol-sulphuric acid assay.

The chemical structures of the polymers were characterized by ^{1}H NMR (Fig. S11). The peaks at 3.17, 2.78 and 2.57 ppm (peak "**e**") belonging to the protons of epoxy units in L10 migrate to low field at 4.74 and 3.49 ppm, which is due to the electron receptor effect of azido units. At the same time, the peak at 4.79 ppm (peak "**g**") corresponding to hydroxyl groups appears after the introduction of azido units, demonstrating that L10-N_3 was synthesized successfully.[S3] In Fig. S11(iii), the characteristic peak of triazoles at 8.12 ppm (peak "**f**") was observed, demonstrating the success of "click" reaction between azido and alkynyl groups. The conjugate ratio of Az was ca. 100% calculated from the integral area ratio of peak "**d**" and "**f**" in Fig. S11(iii).

The introduction of azido groups, azobenzene and CD units was also confirmed by FT-IR analysis. As can be seen from Fig. S12 (ii), the characteristic peaks of epoxy units in L10 at 908 cm^{-1} and 848 cm^{-1} disappear completely,[S3] while a strong peak at 2104 cm^{-1} and 1350 cm^{-1} corresponding to the azido groups in L10-N_3 is observed, demonstrating that L10-N_3 was

successfully synthesized. In the spectrum of L10Az (Fig. S12 (iii)), the peak at 2104 cm^{-1} cannot be observed. While the peaks at 839 cm^{-1} corresponding to the characteristic peaks of *p*-disubstitution of benzene appeared, demonstrating that Az was grafted on L10-N_3 successfully. The peak at 3500 cm^{-1} is observed and peaks of epoxy units at 908 cm^{-1} and 848 cm^{-1} disappeared in Fig. S12 (iv), demonstrating that L10CD was successfully synthesized.

As shown in Fig. S13, L10Az was UV-irradiated at λ = 365 nm. The absorption peak at 350 nm decreases remarkably with the irradiation time, and the absorption peak around 440 nm increases slightly, which assigned to π-π^* and n-π^* transitions of the azobenzene unit, respectively.[S5] The change of the absorption bands induced by UV-irradiation is indicative of the photo-isomerization of azobenzene from *trans* to *cis*.

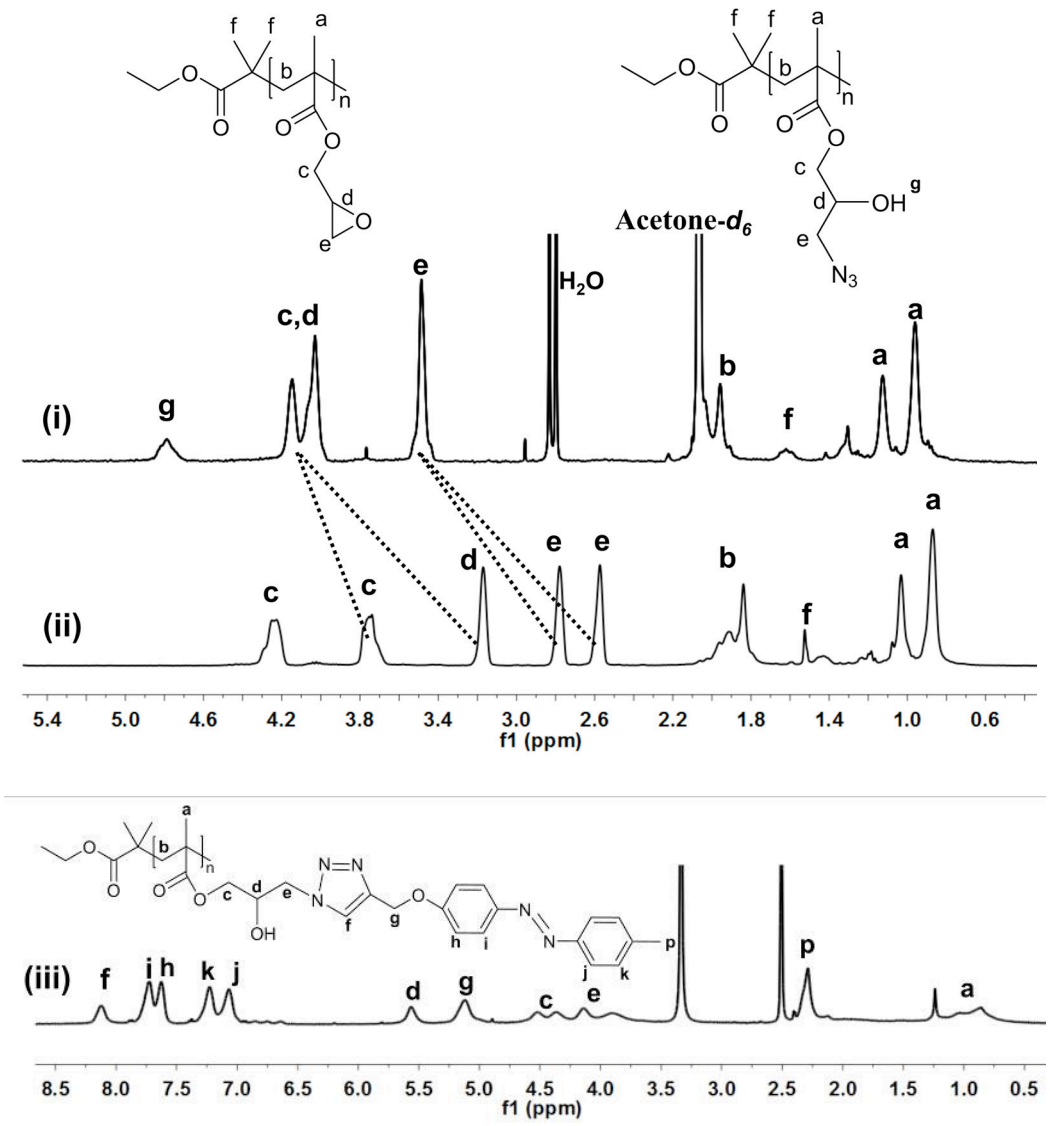


Figure S11. ^{1}H NMR spectra of L10-N_3 in acetone-d_6 (i), L10 in $CDCl_3$ (ii) and L10Az in DMSO-d_6 (iii).

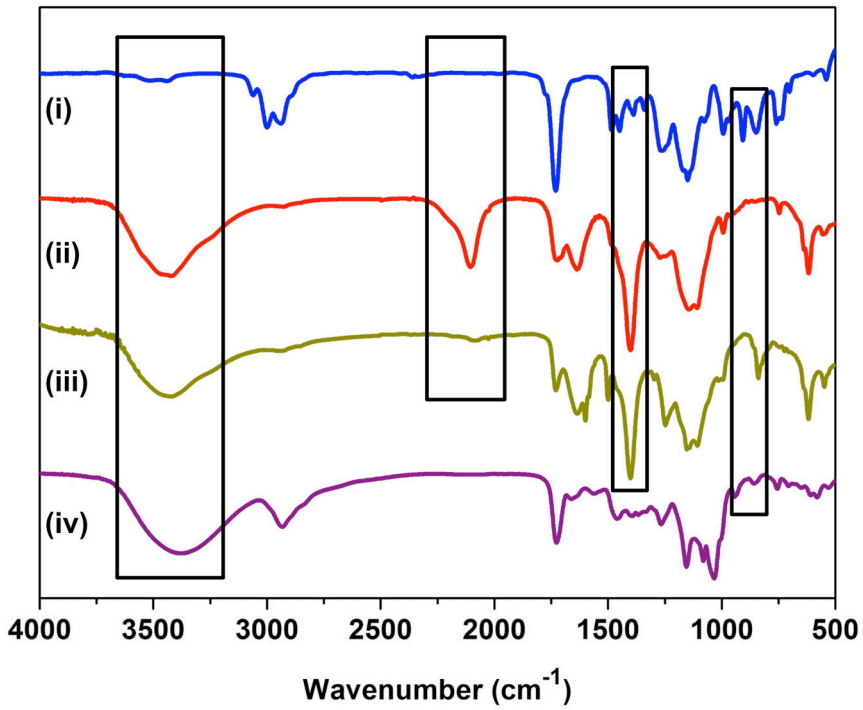


Figure S12. FT-IR spectra of L10 (i), L10-N_3 (ii), L10Az (iii) and L10CD (iv).

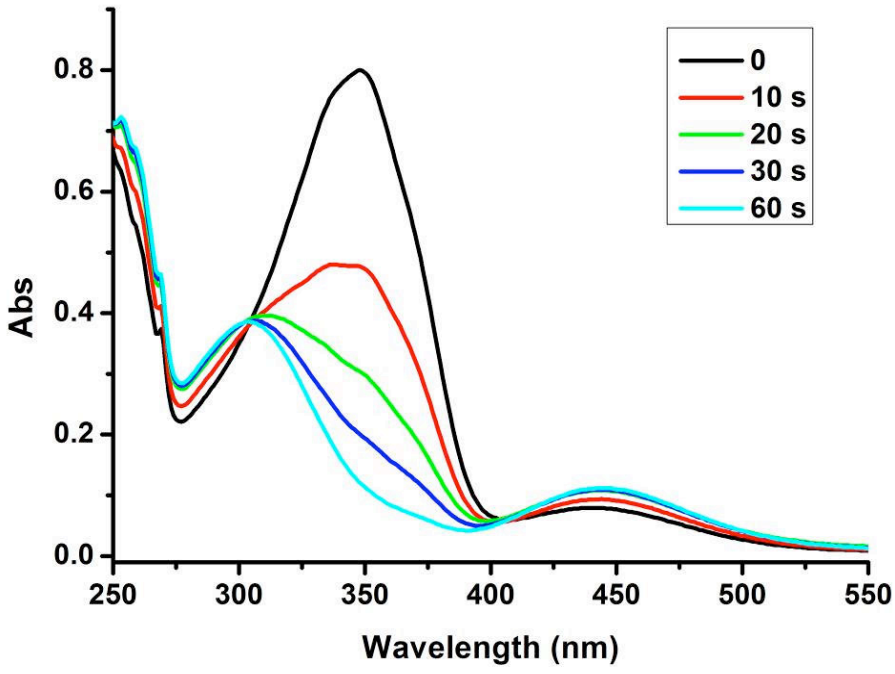
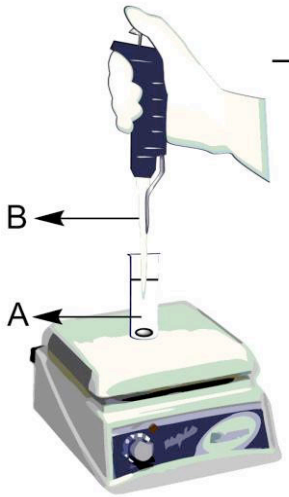


Figure S13. Time-resolved UV-Vis spectra of L10Az under photo irradiation (λ = 365 nm, 8W).

3. Construction and characterization of polymeric assemblies

Polymeric vesicles were prepared by a "dissolve-dialysis" method with different Az/CD molar ratio. Typically, L10Az (14 μmol of Az group) dissolved in 100 μL of THF (or DMSO) was added dropwise into L10CD (14 μmol of CD) in aqueous solution (5 mL) and stirred for 1 h. Then the mixture solution was dialyzed against distilled water at room temperature for 12 h using a dialysis membrane (cut-off molecular weight 7 kDa).



A (water)	B (THF)	Result
PGMA-CD	PGMA-Az	good
PGMA-CD	AZO	poor
PGMA-CD	Blank	poor
CD	PGMA-Az	poor
Blank	PGMA-Az	poor
PGMA-EDA	PGMA-Az	good
PGMA-OH	PGMA-Az	good
PEG2000	PGMA-Az	poor
PEG2000-HDI	PGMA-Az	good

Figure S14. Construction of polymeric assemblies from different components.[S6-S8]

Table S2. Characteristics of assemblies with Az/CD molar ratio 1:1

Polymeric assemblies	D_h (nm)	PDI	Zeta Potential (mV)
L10Az/L10CD	220±10	0.18±0.03	+32±4
L10Az/L20CD	210±6	0.14±0.02	+30±4
L10Az/S10CD	200±7	0.12±0.01	+31±4
L10Az/S19CD	200±9	0.13±0.02	+28±5
S10Az/S10CD	190±6	0.14±0.02	+31±3
S10Az/S19CD	220±12	0.16±0.02	+29±5
RhB@L10Az/L10CD	230±15	0.15±0.02	+33±4
RhB@S10Az/S10CD	230±12	0.12±0.01	+35±4

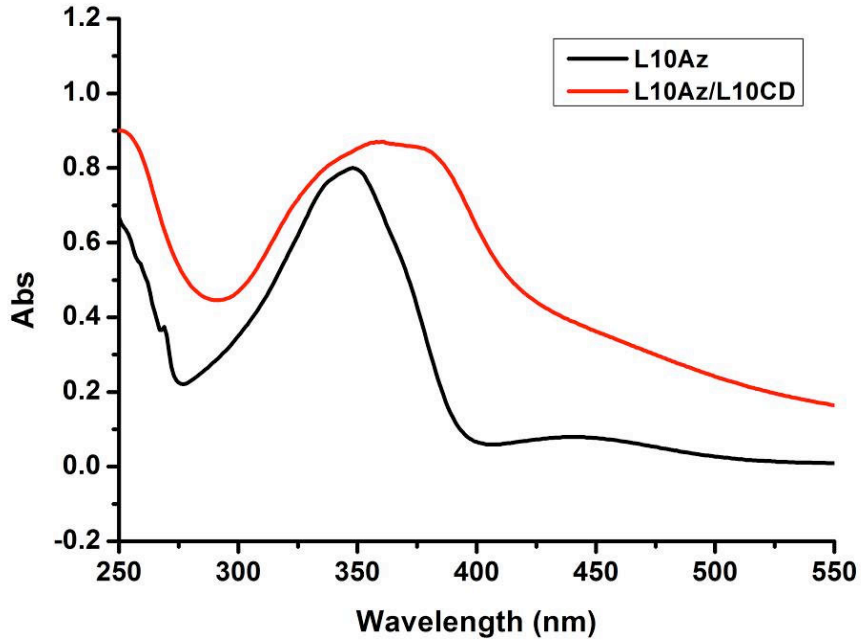


Figure S15. UV-Vis spectra of L10Az and L10Az/L10CD polymeric vesicles.

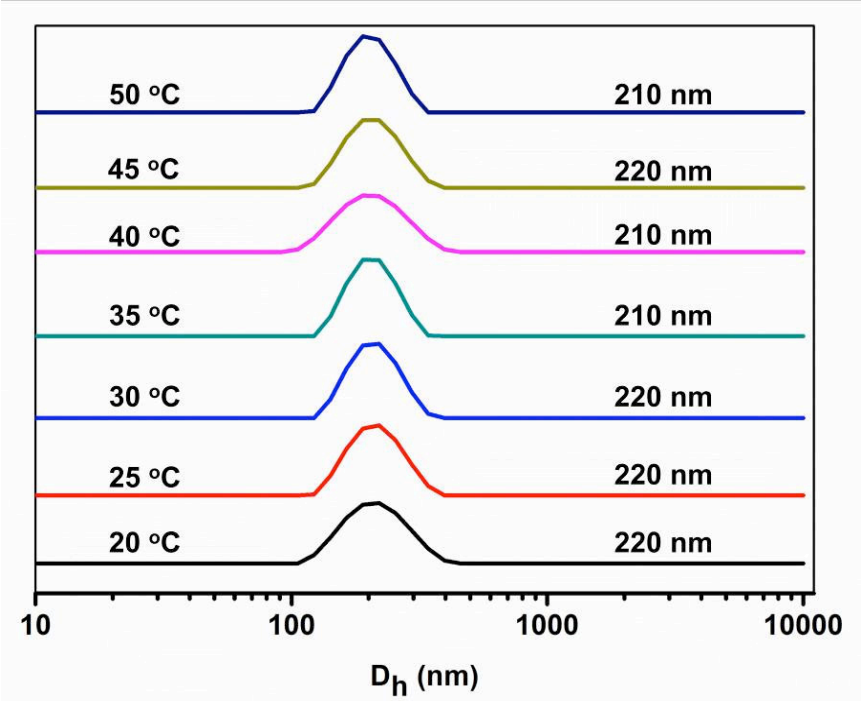


Figure S16. Temperature-variable DLS of L10Az/L10CD vesicles.

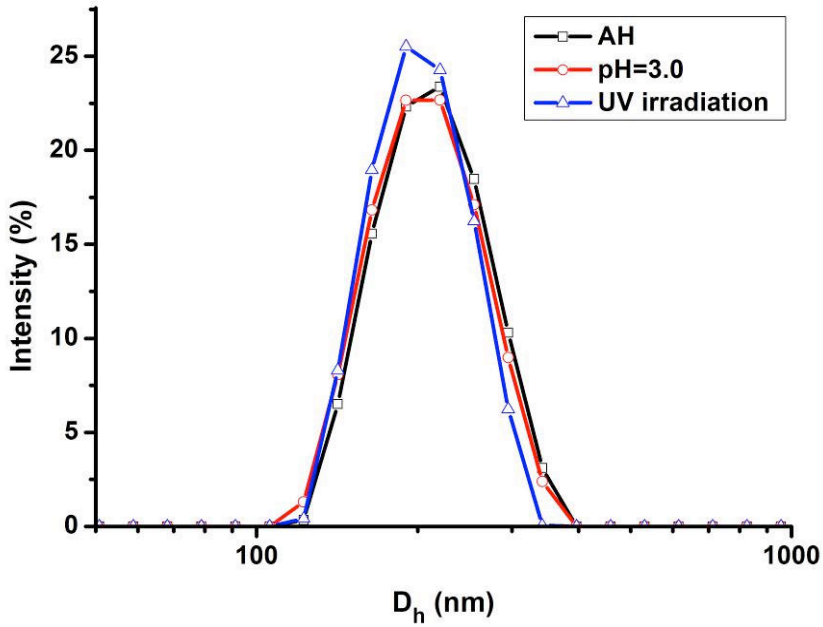


Figure S17. Size distribution of L10Az/L10CD vesicles with UV-light irradiation (λ = 365 nm) (blue), adjusting pH to 3.0 (red) and upon addition of a competitive binding agent AH (black).

4. Disassembly of polymeric assemblies

As showing in Figure S18, sample **A** was the initial polymeric vesicles solution, and freeze-dried to yield yellow powder ①. To get sample **B**, $Na_2S_2O_4$ solution (155 mM, PBS, pH=7.4) was added into polymeric assemblies and stirred at 37 ^oC for 36 h. Sample **B** was dialyzed against deionized water for 48 h, and centrifuged to obtain precipitate ②.

As showing in Figure S19, ^1H NMR spectra of ① appeared to be the same as that of PGMA-CD, suggesting that the vesicle surface was mainly composed of PGMA-CD.

In the presence of $Na_2S_2O_4$, the N=N bonds of Az groups can be reduced so that the polymeric vesicles disassembled. As can be seen from Fig. S20, the characteristic absorption peaks of Az groups disappeared in spectra of ②, demonstrating that the N=N bonds were reduced and polymeric vesicles disassembled.

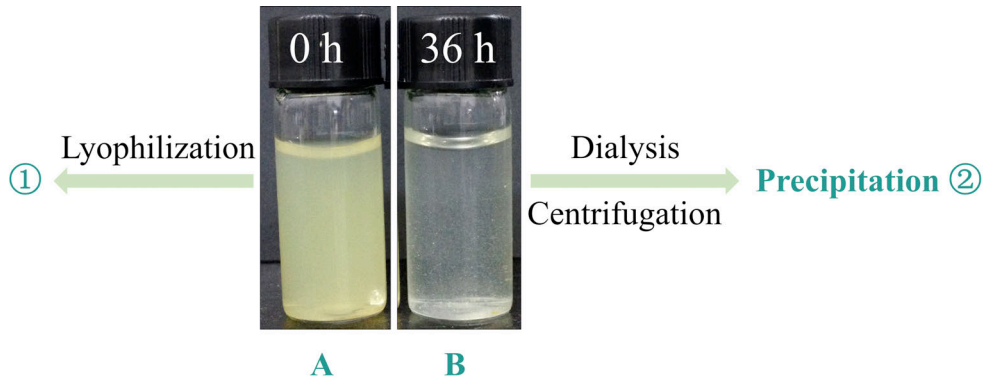


Figure S18. Disassembly of polymeric assemblies.

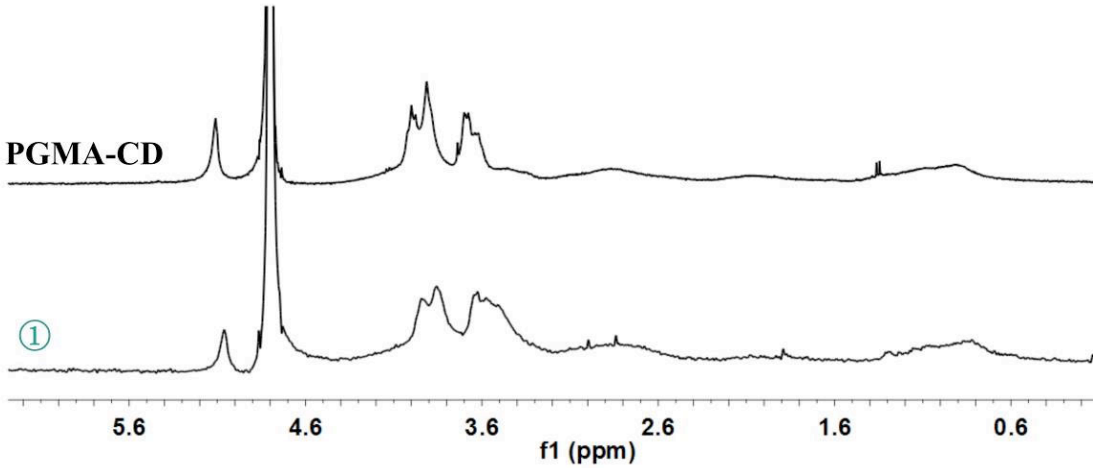


Figure S19. ^{1}H NMR spectra of ① and PGMA-CD in D_2O.

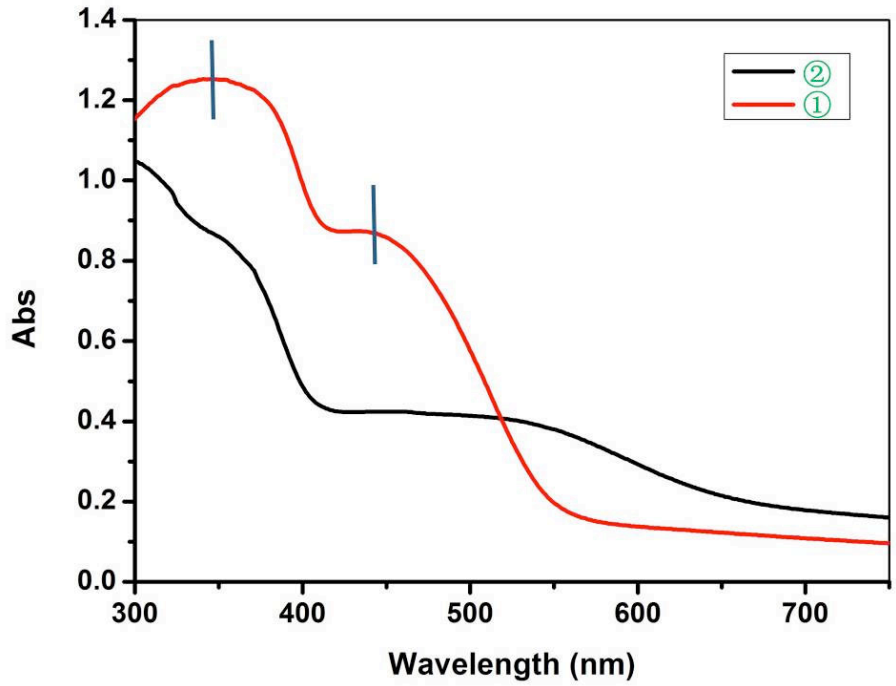


Figure S20. UV-Vis diffuse reflection spectra of ① and ②.

5. Encapsulation and release of cargo

5.1 Encapsulation and release of rhodamine B (RhB)

RhB was encapsulated concomitantly in the progress of self-assembly of polymeric assemblies. Typically, L10CD (14 μmol of CD) and RhB (1 mg)

were dissolved in H_2O (5 mL). Then L10Az (14 μmol of Az group) dissolved in DMSO (100 μL) was added dropwise and stirred for 1 h, followed by dialyzing against distilled water at room temperature for 24 h using a dialysis membrane (cut-off molecular weight 7 kDa). To study the released kinetics of RhB, 155 mM $Na_2S_2O_4$ solution (PBS, pH 7.4) was carefully added into RhB-loaded polymeric vesicles solution (3 mL) and stirring for 1 h. And then the mixture was transferred to dialysis membranes (cut-off molecular weight 7 kDa) immersed in beakers (PBS, pH 7.4, 20 mL). At pre-decided intervals, samples of 5 mL were withdrawn for UV detection, and 5 mL of fresh buffer solution or $Na_2S_2O_4$-contained PBS solution were then immediately added to the release medium. The cumulative RhB release was calculated as:

$$\text{Cumulative Cargo Release (\%)} = \frac{M_t}{M_\infty} \times 100\%$$

where M_t and M_∞ represent the amount of RhB released from vesicles at time t and infinity, respectively.

5.2 Encapsulation and release of bovine serum albumin (BSA)

BSA was also encapsulated concomitantly in the progress of self-assembly of polymeric assemblies. Typically, L10CD (14 μmol of CD) and BSA (5 mg) were dissolved in H_2O (5 mL). Then HCl solution (0.05 M) was added dropwise until the aqueous solution become clarified. Then L10Az (14 μmol of Az group) dissolved in DMSO (100 μL) was added dropwise and stirred for 1 h, followed by dialyzing against distilled water at room temperature for 24 h using a dialysis membrane (cut-off molecular weight 7 kDa). To study the released kinetics of BSA, 3 mL of $Na_2S_2O_4$ solution (155 mM, PBS, pH 7.4) was carefully added into BSA-loaded polymeric vesicles solution (3 mL) and stirring for 1 h. The released tubes were kept in a thermostatic water bath at 37 °C and shaken at 120 rpm. At pre-decided intervals, samples of 1 mL were withdrawn for UV detection, and 1 mL of fresh buffer solution or

$Na_2S_2O_4$-contained PBS solution were then immediately added to the release medium. The released BSA concentration was determined by coomassie blue method. Typically, samples were centrifuged at 12000 rpm (4 °C) for 5 min. The supernatant was transferred and 2 mL of coomassie blue solution was added. The UV-visible absorbance was recorded at 595 nm. The cumulative BSA release was calculated as:

$$\text{Cumulative Cargo Release}\,(\%) = \frac{M_t}{M_\infty} \times 100\%$$

where M_t and M_∞ represent the amount of BSA released from vesicles at time t and infinity, respectively.

6. Cell viability assays

Mouse fibroblasts cells (L929) are pre-cultured for 18 h in 96-well plate (100 μL/well) at a density of 5000 cells per well in RPMI-1640 medium containing bovine serum (10%). The polymeric assemblies were freeze-dried, and PBS (pH 7.4) was added to obtain polymeric vesicles with concentrations of 200, 400, 600, 800, 1000 g/mL. Polymeric assemblies (10 μL) were added to the medium and incubated at 37 °C for 24 h, followed by being replaced with 100 μL of RPMI-1640. CCK-8 solution (10 μL/well) was added to 96-well plates, incubated for another 4 h, and analysed at 450 nm using a microplate reader (Model 680, Bio-Rad). The cell viability was calculated from the following equation:

$$\text{Cell Viability}\,(\%) = \frac{OD_{sample}}{OD_{control}} \times 100\%$$

where OD_{sample} and $OD_{control}$ represent the OD value of the well treated with samples and PBS buffer solution, respectively.

7. References

S1. H. Gao, M. C. Jones, P. Tewari, M. R. Chen, R. E. Prudhomme, and J. C. Leroux, *J. Polym. Sci.: Part A: Polym. Chem.*, 2007, **45**, 2425.

S2. W.-X. Gu, Y.-W. Yang, J. Wen, H. Lu, and H. Gao, *Polym. Chem.*, 2014, **5**, 6344.

S3. P. Zhao, Y. Yan, X. Feng, L. Liu, C. Wang, and Y. Chen, *Polymer*, 2012, **53**, 1992.

S4. Q. Zhang and C. G. Bazuin, *Macromolecules*, 2009, **42**, 4775.

S5. Y. Takashima, S. Hatanaka, M. Otsubo, M. Nakahata, T. Kakuta,A. Hashidzume, H. Yamaguchi, and A. Harada, *Nature Commun.*, 2012, **3**, 1270.

S6. X. Lu, H. Gao, C. Li, Y.-W. Yang, Y. Wang, Y. Fan, G. Wu, and J. Ma, *Int. J. Pharm.*, 2012, **423**, 195.

S7. W. Gu, Y. Ma, C. Zhu, B. Chen, J. Ma, and H. Gao, *Eur. J. Pharm. Sci.*, 2012, **47**, 556.

S8. X. Du, N. Song, Y.-W. Yang, G. Wu, J. Ma and H. Gao, *Polym. Chem.*, 2014, **5**, 5300.