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For

A pH-sensitive and biodegradable supramolecular hydrogel constructed from PEGylated polyphosphoester-doxorubicin prodrug and α-cyclodextrin

Fei Li, Jinlin He, Mingzu Zhang and Peihong Ni**

College of Chemistry, Chemical Engineering and Materials Science, Suzhou Key Laboratory of Macromolecular Design and Precision Synthesis, Jiangsu Key Laboratory of Advanced Functional Polymer Design and Application, Soochow University, Suzhou 215123, P. R. China.

*To whom correspondence should be addressed. E-mail: phni@suda.edu.cn (P. H. Ni) and jlhe@suda.edu.cn (J. L. He)

Experimental Section

Materials

Stannous octoate [$Sn(Oct)_2$, 95%, Sigma-Aldrich] was distilled under vacuum before use. Poly(ethylene glycol) monomethyl ether (mPEG-OH, $\overline{M}_n \approx 2000$ g mol^{-1}, PDI = 1.06) and *N, N, N′, N″, N‴*-pentamethyldiethylenetriamine (PMDETA, 98%) were purchased from Sigma-Aldrich and used as received. Pyridinium p-toluenesulfonate (PPTS, 98%, Acros), sodium azide (NaN_3, 98%, Sinopharm Chemical Reagent), 2-chloroethyl vinyl ether (CEVE, 98%, TCI), hydrazine monohydrate (85%, Sinopharm Chemical Reagent), acetic acid (HOAc, A.R., Sinopharm Chemical Reagent), methyl 6-bromohexanoate (Chengdu Aikeda Reagent) and doxorubicin hydrochloride (DOX·HCl, 99%, Beijing Zhongshuo Pharmaceutical Technology Development) were used without further purification. Cuprous bromide (CuBr, 95%, Sinopharm Chemical Reagent) was successively washed three times with glacial acetic acid and acetone, followed by drying under vacuum for 12 h at room temperature. Dichloromethane (CH_2Cl_2, A.R., Sinopharm Chemical Reagent) was refluxed with CaH_2 and distilled before use. Tetrahydrofuran (THF, A.R., Sinopharm Chemical Reagent) was initially dried over KOH for at least 2 days and then refluxed over sodium wire with benzophenone as an indicator until the color turned to purple. Benzyl alcohol (BzOH, A.R., Sinopharm Chemical Reagent) and *N, N*-Dimethylformamide (DMF, A.R., Sinopharm Chemical Reagent) were dried over anhydrous $MgSO_4$ and distilled under vacuum before use. Milli-Q water (18.2 MΩ cm^{-1}) was generated using a water purification system (Simplicity UV, Millipore). mPEG-*a*-N_3,[1] N_3-*hyd*-DOX,[2] and 2-(but-3-yn-1-yloxy)-2-oxo-1, 3, 2-dioxaphospholane (BYP)[3,4] were prepared by the previously reported protocols.

Characterizations

Nuclear magnetic resonance (NMR) spectra were performed on a 400 MHz Bruker NMR spectrometer (INOVA-400, Varian) at 25 °C with $CDCl_3$ or DMSO-d_6 as the solvent and TMS as the internal reference. FT-IR spectra were recorded on a Nicolet 6700 spectrometer using the KBr disk method. The interior structures of the hydrogels were observed by scanning electron microscopy (SEM) using a Quanta 200 FEG electron microscope operating at 15 kV. The mixture of prodrug solution and α-CD solution were added on the surface of a silicon

SEM specimen holder, let stand for 5 min, quickly frozen in liquid nitrogen and further freeze-dried in a freeze-drier at -40 °C for 3 days until all the solvent was sublimed. The freeze-dried hydrogel was sputter coated with gold before observation. XRD patterns were recorded on a Rigaku D/max 2500 X-ray powder diffractometer using Cu Kα (1.54 Å) radiation (50 kV, 250 mA). All the samples were scanned from 2θ = 5° to 45° at a speed of 5° min^{-1}. DSC was carried out on a DSC TA-60WS thermal analysis system (Shimadzu, Japan). Samples were first heated from -40 °C to 100 °C at a heating rate of 10 °C min^{-1} under a nitrogen atmosphere, followed by cooling to -40 °C after stopping at 100 °C for 3 min, and finally heating to 180 °C at 10 °C min^{-1}. High performance liquid chromatography (HPLC) (UltiMate 3000, Thermo Fisher Scientific) was equipped with an UltiMate pump, a controller, an autosampler, and a UV-Vis detector conducted at 488 nm. The samples were analyzed on a C18 reverse phase column (4.6 × 100 mm, 5 mm particle size) at 30 °C with acetonitrile-Milli-Q water (v/v, 50/50) as the mobile phase at a flow rate of 1.0 mL min^{-1}. The data was analyzed by the Chromleon 7 software. The number-average molecular weights ($\overline{M}_{n,\,GPC}$) and molecular weight distributions (PDIs) of PBYP and PBYP-*g*-PEG were measured by a GPC instrument (HLC-8320, Tosoh) equipped with a refractive index detector, using two TSKgel Super HM-M columns (6.0 × 150 mm, 3 μm particle size) in series with molecular weights ranging from 1×10^3 - 7×10^5 g mol^{-1}. DMF with 0.01 M LiBr was used as the eluent at a flow rate of 0.60 mL min^{-1} operated at 40 °C. These samples were calibrated with polystyrene standards. The fluorescence spectra were recorded on a spectrofluorometer (Cary Eclipse, Agilent). The excitation was carried out at 480 nm, the emission spectra were recorded at 560 nm, and the slit width was set at 5 nm.

Synthesis of poly(butynyl phospholane) (PBYP)

PBYP was synthesized *via* benzyl alcohol-initiated ROP reaction of BYP monomer with $Sn(Oct)_2$ as the catalyst according to the previously published method.[3,4] Briefly, a 50 mL dry flask containing 10 mL of anhydrous CH_2Cl_2 was charged with $Sn(Oct)_2$ (0.058 g, 0.144 mmol), benzyl alcohol (0.031 g, 0.287 mmol) and BYP (1.6 g, 9.1 mmol), which was then degassed through three exhausting-refilling nitrogen cycles. The mixture was kept stirring at 30 °C for 4 h under a nitrogen atmosphere. The resultant solution was concentrated and precipitated

twice in cold diethyl ether, and the precipitate was dried under vacuum at 25 °C to obtain the viscous product (1.48 g, yield: 92.5%).

The molecular weight ($\overline{M}_{n,NMR}$) of PBYP was calculated according to the ^{1}H NMR analysis by the following equation:

$$\overline{M}_{n,NMR} = n \times 176.02 + 108; \quad n = \frac{A_u}{A_r}$$

In this equation, 176.02 is the molecular weight of BYP monomer, 108 is the molecular weight of the terminal benzyl group and H atom, A_u and A_r are the integral value of the peaks at δ 2.60 and δ 5.20 ppm, respectively. The sample was then designated as $PBYP_{30}$.

Synthesis of PBYP-*g*-PEG

All the magnetic stirring bars and glasswares used in the experiments were dried at 120 °C for 24 h and cooled under vacuum to eliminate the moisture before use. $PBYP_{30}$ (0.174 g, 0.033 mmol), mPEG-*a*-N_3 (0.32 g, 0.16 mmol), CuBr (4.6 mg, 0.033 mmol) and 8 mL of anhydrous DMF were added sequentially in a nitrogen-purged flask, and three exhausting-refilling nitrogen cycles were then taken to degas the solution. Then PMDETA (14.2 μL, 0.066 mmol) was added into the flask by syringe. The mixture was stirred under a nitrogen atmosphere at 30 °C for 5 h. Afterwards, the solution was then exposed to air, followed by dialysis (MWCO 7000) against Mill-Q Water for 2 days to remove copper ions. Finally, the solution was freeze-dried to obtain PBYP-*g*-PEG (0.36 g, yield: 72.1%).

The molecular weight ($\overline{M}_{n,NMR}$) of PBYP-*g*-PEG were calculated according to the ^{1}H NMR analysis by the following equation:

$$\overline{M}_{n,NMR} = 2110 \times m + 5390; \quad m = \frac{5A_w}{A_q}$$

In this equation, 2110 is the molecular weight of mPEG-*a*-N_3, 5390 is the molecular weight of $PBYP_{30}$ and H atom, A_W and A_q are the integral value of the peaks at δ 7.61 of triazole group and δ 7.38 ppm of benzyl group, respectively. The sample was then designated as $PBYP_{30}$-*g*-5PEG.

Synthesis of PBYP-*g*-PEG-*g*-DOX

Briefly, PBYP-*g*-5PEG (0.1 g, 0.0065 mmol)，N_3-*hyd*-DOX (45.5 mg, 0.065 mmol), CuBr (1

mg, 0.0065 mmol) and 8 mL of anhydrous DMF were added sequentially in a nitrogen-purged flask, and three exhausting-refilling nitrogen cycles were then taken to degas the solution. Then PMDETA (5.68 μL, 0.013 mmol) were added into the flask by a syringe. The mixture was stirred under a nitrogen atmosphere at 30 °C for 5 h. Afterwards, the solution was then exposed to air to terminate the reaction, followed by dialysis (MWCO 7000) against Mill-Q Water for 2 days to remove copper ions. Finally, the solution was freeze-dried to obtain the final product (0.12 g, yield: 82.7%). The DOX content was determined by fluorescence spectroscopy, in which the excitation was carried out at 480 nm, the emission spectra were recorded at 560 nm, and the slit width was set at 5 nm. A series of DOX·HCl solutions in DMF with different concentrations were used as the standards. The sample was then designated as $PBYP_{30}$-*g*-5PEG-*g*-10DOX.

Self-assembly of PBYP-*g*-PEG-*g*-DOX

The morphologies of the self-assembled aggregates from $PBYP_{30}$-*g*-5PEG-*g*-10DOX were observed on a TEM instrument (HT7700, Hitachi) operating at an accelerating voltage of 120 kV. Samples were dissolved directly in Milli-Q water with a concentration of 0.2 mg mL^{-1} or 1.0 mg mL^{-1} and stirred for 2 days. The sample for TEM analysis was prepared by a freeze-drying method.[5] The carbon-coated copper grid was placed on the bottom of a glass cell, which was then immediately inserted into liquid nitrogen. Subsequently, 8 μL of the micellar solution was dropped onto the grid, and the solvent in its frozen solid state was directly removed without melting in a freeze-drier. The morphologies were then imaged on a normal TEM instrument at room temperature.

Preparation of supramolecular hydrogel

The supramolecular gelation could occur under mild conditions without high temperature and the use of chemical cross-linker. 10 mg of $PBYP_{30}$-g-5PEG-*g*-10DOX was dissolved in 0.3 mL Mill-Q water, and 45 mg of α-CD was dissolved in 0.2 mL Mill-Q Water. Both of the solutions were mixed and stirred vigorously, then let stand for 24 h to form the supramolecular hydrogel. The supramolecular hydrogel would be used for SEM, DSC, MTT and drug release analysis after freeze-drying.

Rheological analysis of supramolecular hydrogels

To investigate the gelation kinetic of aqueous $PBYP_{30}$-*g*-5PEG-*g*-10DOX/α-CD system, time sweep test was performed at a constant oscillatory frequency (1.0 Hz) by a RS 6000 rheometer (Thermo Hakke) with parallel plate geometry (20 mm diameter, 0.1 mm gap) at 25 °C. In this case, the sample was placed on the plate immediately after the mixing and the measurement started after standing for 1 min. The viscoelastic parameter was measured as a function of time within the linear viscoelastic region previously determined by a stress scan. In addition, steady rate sweep test was carried out to investigate the shear thinning of resultant hydrogel. In this case, the hydrogel sample was also allowed to consolidate for 24 h before the measurement.

***In vitro* drug release**

The DOX-loaded supramolecular hydrogel was prepared by an *in situ* forming method. Briefly, 45 mg of α-CD and 10 mg of $PBYP_{30}$-*g*-$5PEG_5$-*g*-10DOX was individually dissolved in 0.2 and 0.3 mL of Milli-Q water, and the two solutions were mixed thoroughly by stirring for 1 min. Then the mixed solution was divided into 6 pieces in a 1 mL cuvette and stood for 24 h to yield the hydrogel. The cuvette was sealed by a dialysis bag (MWCO 3500) and placed in a test tube with 20 mL of PB (pH 7.4 or pH 5.0) and incubated in a shaking water bath at 37 °C. At the desired time intervals, 5 mL of the released medium was withdrawn for fluorescence analysis and 5 mL of corresponding fresh buffer was added to keep a constant volume. The solution was measured by fluorescence spectroscopy with excitation at 480 nm and emission at 560 nm, and the slit width was set at 5 nm. All the loading and release experiments were carried out in dark.

MTT Assay

The anticancer activity of the supramolecular hydrogel was evaluated by the methyl tetrazolium (MTT) assay using free DOX and $PBYP_{30}$-*g*-5PEG-*g*-10DOX as the controls. 5 mg of freeze-dried hydrogel (containing 0.25 mg DOX) was put into 1 mL of Milli-Q water in a 10 mL centrifuge tube, and then placed in a shaker incubator (37 °C) for two days. After that, the media were filtered with 0.22 μm of sterile filter into a sterile container and stored in a refrigerator at 4 °C before use.

HeLa cells were obtained from American Type Culture Collection (ATCC) and cultured in 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin contained culture medium at 37 °C under a 5% CO_2 atmosphere. The culture media were replaced every three days. HeLa cells were seeded onto a 96-well plate at a density of about 5×10^3 cells per well for 12 h. The sample solutions with different concentrations were then added to the wells and cultured for another 48 h. Afterwards, 25 mL of MTT stock solution (5 mg mL^{-1} in PBS) were added to each well. After incubation for another 4 h, the DMEM medium was removed and the produced purple formazan was dissolved by adding 150 mL of DMSO. The optical density (OD) at 570 nm of each well was measured on a microplate reader (Bio-Rad 680). The absorbance values were normalized to the wells in which cells were not treated with samples. The cell viability was calculated by the equation: $OD_{sample}/OD_{control} \times 100\%$, in which OD_{sample} and $OD_{control}$ are the absorbance values of the testing well (in the presence of samples) and the control well (in the absence of samples), respectively. Data are presented as average values with standard deviations.

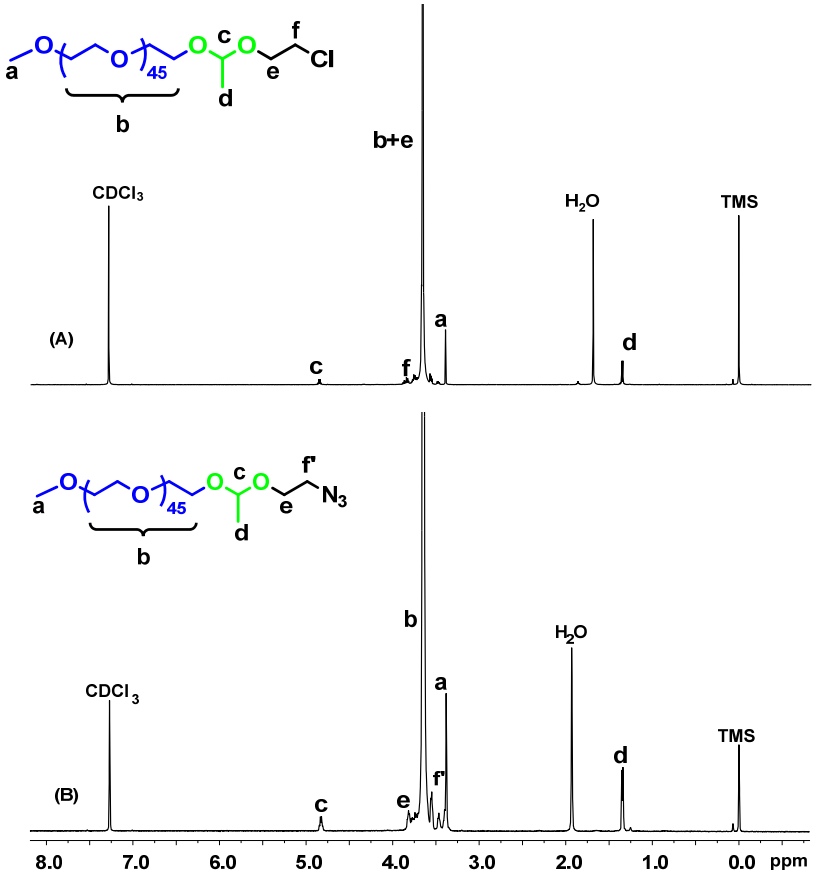


Fig. S1 ^{1}H NMR spectra of (A) mPEG-*a*-Cl and (B) mPEG-*a*-N_3 in $CDCl_3$.

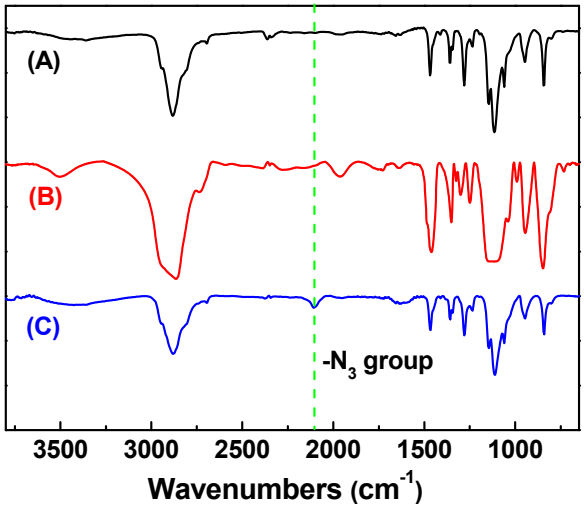
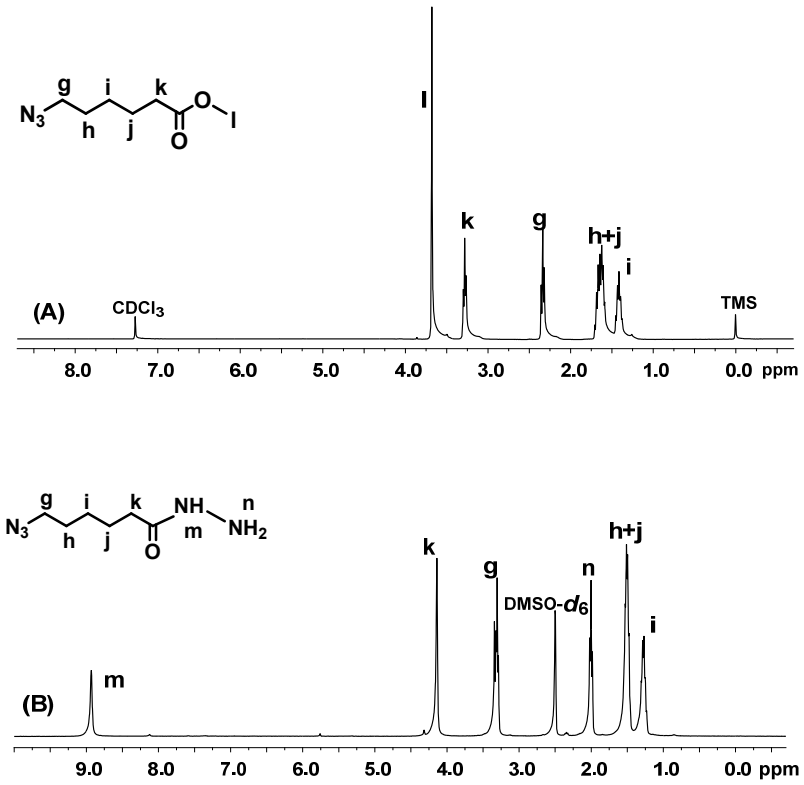


Fig. S2 FT-IR spectra of (A) mPEG-OH, (B) mPEG-*α*-Cl and (C) mPEG-*α*-N_3.



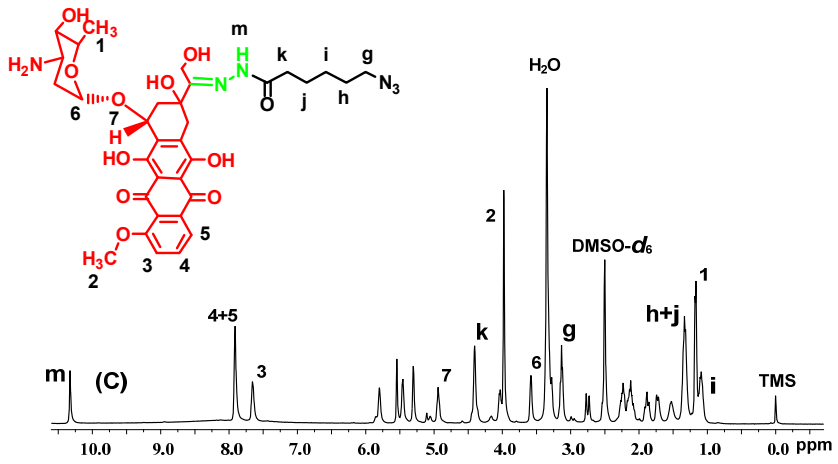


Fig. S3 ^{1}H NMR spectra of (A) methyl 6-azidohexanoate in $CDCl_3$, (B) 6-azidohexanehydrazide and (C) N_3-*hyd*-DOX in DMSO-d_6.

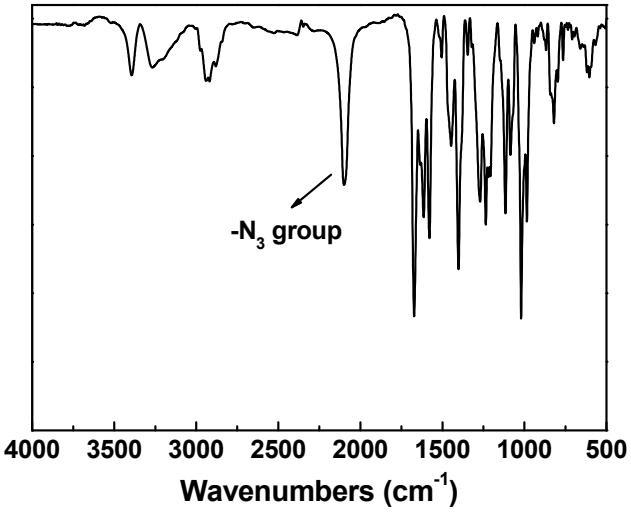
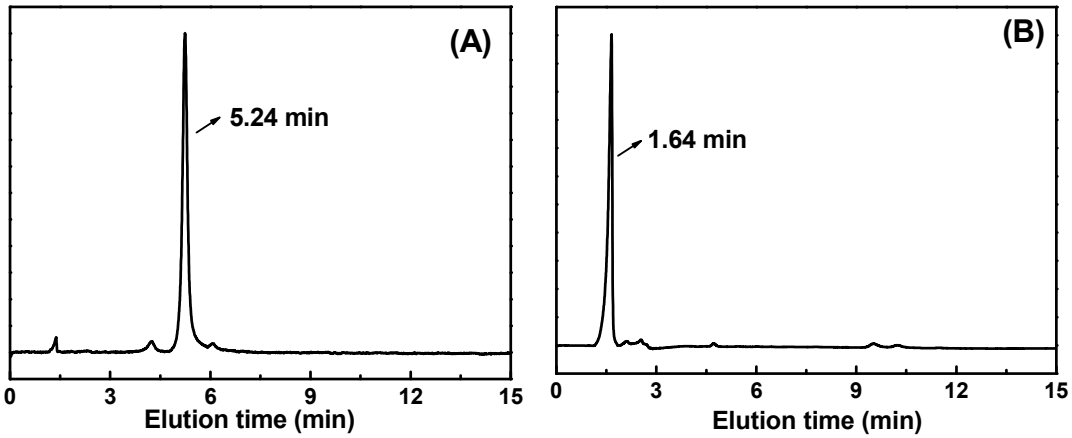


Fig. S4 FT-IR spectrum of N_3-*hyd*-DOX.



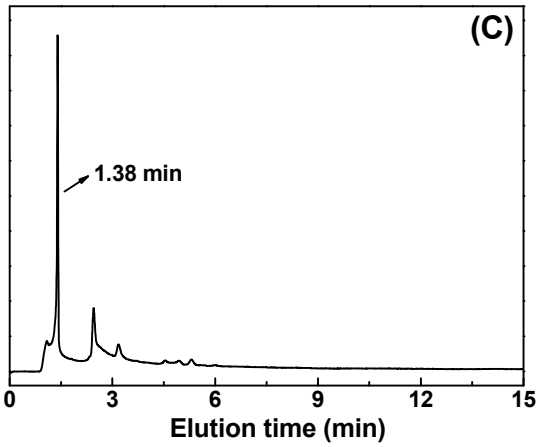


Fig. S5 HPLC elugrams of (A) DOX, (B) N_3-*hyd*-DOX and (C) $PBYP_{30}$-*g*-5PEG-*g*-10DOX, which were performed with acetonitrile-water (50/50, v/v) as the mobile phase at 30 °C at a flow rate of 1.0 mL min^{-1}.

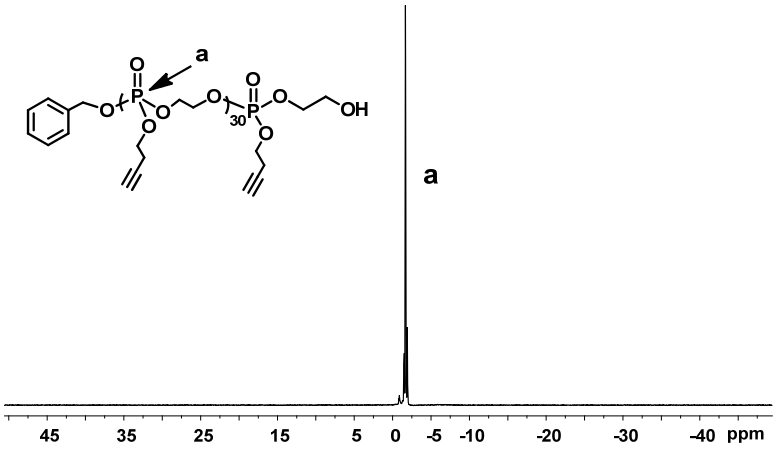


Fig. S6 ^{31}P NMR spectrum of $PBYP_{30}$ in $CDCl_3$.

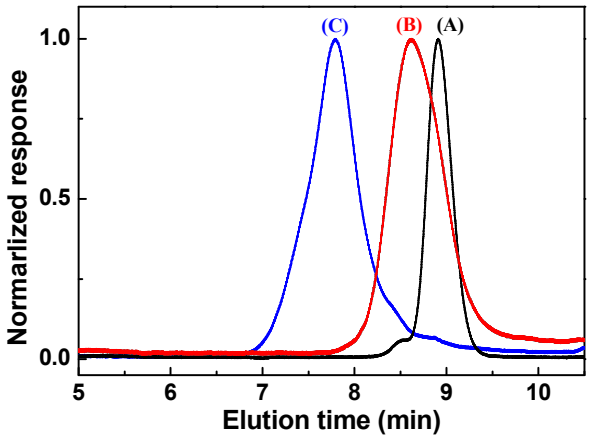


Fig. S7 GPC curves of (A) mPEG-*a*-N_3, (B) $PBYP_{30}$, and (C) $PBYP_{30}$-*g*-5PEG.

Table S1 The characterization data of various polymers.

Samples	$\overline{M}_{n,\,NMR}$[a]	$\overline{M}_{n,\,GPC}$[b]	PDI[b]
mPEG-*a*-N_3	2110	4380	1.06
$PBYP_{30}$	5390	6100	1.23
$PBYP_{30}$-*g*-5PEG	15940	26280	1.14

[a] Calculated on the basis of ^{1}H NMR analysis;

[b] Determined by GPC using DMF with 0.01 mol L^{-1} LiBr as the eluent, and polystyrene as the standards.

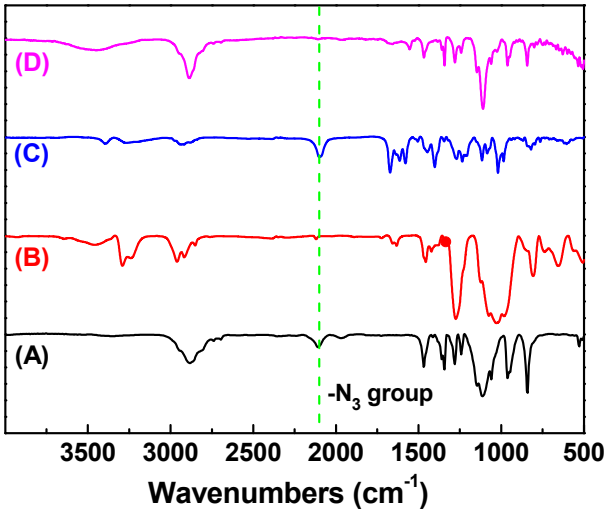


Fig. S8 FT-IR spectra of (A) mPEG-*a*-N_3, (B) $PBYP_{30}$, (C) N_3-*hyd*-DOX and (D) $PBYP_{30}$-*g*-5PEG-*g*-10DOX.

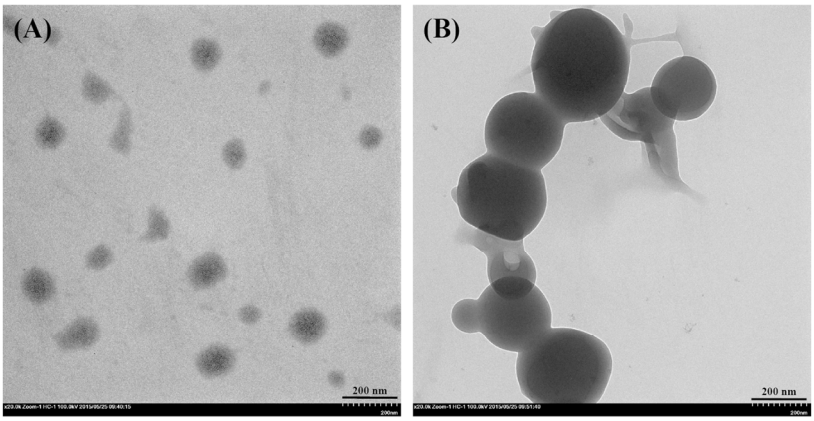


Fig. S9 TEM image of aggregates self-assembled from $PBYP_{30}$-*g*-5PEG-*g*-10DOX in the aqueous solution at the concentrations of (A) 0.2 mg mL^{-1} and (B) 1.0 mg mL^{-1}, respectively.

References

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