Supporting Information

Facile Synthesis of Multifunctional Copolymer via a Concurrent RAFT-Enzymatic System for Theranostic Applications

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EXPERIMENTAL SECTION

Materials

2,2,2-Trifluoroethyl methacrylate (TFEMA, 98%, J&K chemical), 6-chlorohexanol (97%, Beijing Ouhe Chem), *p*-hydroxybenzaldehyde (99%, Aladdin Reagents), 3-bromo-1-propanol (99%, J&K chemical), methoxypolyethylene glycol (M_n~350, 99%, Aladdin Reagents), sodium azide (99%, J&K chemical), D-glucosamine hydrochloride (99%, Aladdin Reagents), 4-pentynoic acid (98%, Aladdin Reagents),

dicyclohexylcarbodiimide (DCC, 98%, Beijing OuheChem), copper bromide (99%, Aladdin Reagents), 1,1,4,7,7-Pentamethyldiethylenetriamine (99%, J&K chemical), 2,2'-Azobis-(2,4-dimethylvaleronitrile) (ABVN) (99%, J&K chemical), concanavalin A from *Canavalia ensiformis* (Con A, HEOWNS) were used as received. Immobilized CALB (Novozym 435) was purchased from Beijing Cliscent Science and Technology Co., LTD with enzyme activity of 10800 PLU/g. The RAFT agent 4-Cyano-4-(ethylthiocarbonothioylthio) pentanoic acid (CETPA) was synthesized according to previous literature.[1]

Instruments

Gel permeation chromatography (GPC) analyses of polymers were performed using tetrahydrofuran (THF) or N,N-Dimethylformamide (DMF) as the eluent. The GPC system was a Shimadzu LC-20AD pump system comprising an auto injector, a MZ-Gel SDplus 10.0 μm guard column (50 × 8.0 mm, 10^2 Å) followed by three MZ-Gel SDplus 10.0 μm bead-size columns (10^5, 10^3, and 10^2 Å) and a differential refractive index (dRI) detector. The system was calibrated with narrow molecular weight distribution polystyrene standards ranging from 200 to 10^6 g mol^{-1}.

Nuclear magnetic resonance (NMR) spectroscopy was carried out on a JEOL JNM-ECA400 (400MHz) spectrometer for all samples using tetramethylsilane (TMS) as a reference. Data was reported as follows: chemical shift (δ) measured in ppm downfield from TMS.

Fourier Transform Infrared (FT-IR) spectra were recorded on a PerkinElmer Spectrum 100 FT-IR spectrometer.

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Dynamic light scattering (DLS) was recorded on Brookhaven zetaPlus Zeta Potential Analyzer. The sample was prepared as follow: 1.5 mg polymer-dox conjugate was dissolved in 0.2 mL of THF followed by slow addition of 1.5 mL of deionized water. Then the polymer solution was bubbled using N_2 to remove THF after which the polymer solution was diluted by adding water to final concentration as 0.5 mg/mL for DLS characterization.

Transmission electron microscopy (TEM) images were recorded on a Hitachi-7650B microscope operating at 100 kV and the TEM specimens were prepared by placing a drop of the micelle solution on a carbon-coated copper grid followed by staining by 0.2 wt% phosphotungstic acid solution.

T_1 and T_2 measurement

The ^{19}F relaxation measurements of the polymer were conducted on a 9.4 T Bruker AVANCE III spectrometer (^{19}F at 376.47MHz). T_1 and T_2 experiments used unmodified library pulse sequences for inversion recovery and Car-Purcell-Meiboom-Gill (CPMG) measurements, respectively. In a typical experiment, 16 scans per increment were acquired using a 90^o pulse of 15.9 μs and a relaxation delay of 5 s over a spectral width of 37500 Hz using 64 K data points. For inversion recovery experiments, 16 data slices were collected with an incremented delays of 0.01, 0.025, 0.050, 0.075, 0.100, 0.150, 0.20, 0.30, 0.50, 0.75, 1, 1.5, 2, 2.5, 3, 5 seconds. CPMG experiments involved 12 repetitions incorporating CPMG loops of 0.063, 0.25, 0.5, 1.0, 2.0, 4.1, 8.1, 16.3, 32.6, 65.1, 130.3, 260.5 milliseconds. All data were processed using the Bruker Topspin T_1/T_2 Relaxation Module.

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MRI imaging experiment

For MR imaging, a phantom was constructed comprising two compartments, containing ^{19}F polymer and a proton rich imaging region, respectively. The polymer compartment consisted of a screw cap Eppendorf container (ID ~ 8 mm) which was filled with **Polymer 2** aqueous solution (300 mL, 10 mg/mL). The container was placed inside an ID ~ 25 mm Falcon tube filled with a H_2O/(Gd/DTPA) (0.2 wt%) solution as ^{1}H imaging compartment. MR Imaging experiments were performed in a 9.4 T Bruker BioSpec 94/20 Avance III micro-imaging system (Bruker, Ettlingen, Germany), equipped with BGA-12S HP gradients with maximum strength 660 mT/m and slew rate 4570 Tm/s. A custom built Dual Frequency $^{1}H/^{19}F$ TxRx surface coil was used for RF-transmission and signal reception (DOTY Inc.), consisting of a butterfly coil (OD ~ 35mm) tuned to ^{1}H @ 400 MHz and single loop coil tuned to ^{19}F@ 376.3 MHz (OD ~ 20mm).

^{1}H images were acquired using a Fast Low Angle Shot Sequence (FLASS) in coronal orientation with the following parameters: TE = 3.3 ms, TR = 100 ms, FOV = 30×30 mm, Imaging Matrix = 128×128, Resolution = 230×230 mm, 3 slices, FA = 40 deg, zero filling = 1.34, acquisition time = 2 min.

^{19}F images were acquired in the same slice orientation and positioning with adapted a Fast Low Angle Shot Sequence (FLASS) after manual frequency, reference pulse and receiver gain adjustments. For ^{19}F imaging the following parameters were used: TE = 2.7 ms, TR = 100 ms, FOV = 20×20 mm, Imaging Matrix = 32×32, Resolution = 625×625 mm, single slice acquisition, FA = 40 deg, acquisition time = 14 h.

Turbidity assay of the affinity between Polymer 2 and Con A

400 μL of Con A solution (0.5 mg/mL, 10 mM PBS buffer, pH 7.4) was put into 0.6 mL quartz cuvette, the absorption at 420 nm was set as zero, and 100 μL of **Polymer 2** (1.0 mg/mL, 10 mM PBS buffer, pH 7.4) was added into the cuvette. Absorption at 420 nm was collected every 1 s for 20 min, then 50 μL of glucose aqueous solution (2.0 mg/mL) was added into the cuvette, the absorbance at 420 nm was recorded every 1 s for another 10 min.

In vitro release of dox

18 mg **Polymer 3** was dissolved in 0.5 mL of DMSO followed by slow addition of 5 mL of deionized water. The obtained solution was divided into 2 equal portions which were placed into two dialysis bags separately (MWCO~3500). The dialysis bag was put into 30 mL of PBS buffer with pH of 7.4 or 5.5 respectively. The release was conducted at 37 ^{o}C in a shake bed. Samples (0.5 mL per time) were withdrawn at designed time interval and subjected for UV-vis (480 nm) analysis to determine the amount of dox.

Cytotoxicity of polymer

The cell viability of **Polymer 2** on HeLa cells was evaluated by cell counting kit-8 (CCK-8) assay. Briefly, HeLa cells were seeded in 96-well microplates at a density of 5×10^4 cells per mL in 110 μL of DMEM (or 1640 for L929 cells) culture media. After 24 h of cell attachment, the culture media was removed and the cells were further treated with different concentrations of **Polymer 2** solutions. After incubation for 24 h, the solution was removed from microplates and the cells were washed with PBS

buffer for 3 times. Then 10 μL of CCK-8 dye and 100 μL of DMEM cell culture media were added to each well and then the cells were incubated for another 2 h at 37 ^{o}C. Plates were then analysed with a microplate reader (VictorⅢ, Perkin-Elmer). Measurements of Formazan dye absorbance were carried out at 450 nm with a reference wavelength of 620 nm. The values were proportional to the number of live cells. The percent reduction of CCK-8 dye was compared to controls (cells not exposed to polymers), which represented 100% CCK-8 reduction. Three replicate wells were used per microplate and the experiment was repeated three times. Cell survival was expressed as absorbance relative to that of polymer-free controls. Results are presented as mean standard deviation (SD). The cytotoxicity of free dox and **Polymer 3** against HeLa cells (or L929 cells) was evaluated with the similar procedure.

Confocal imaging

Prior to the experiment, HeLa cells were seeded in 35 mm cell culture dishes. The cells were treated with an aqueous solution of Polymer 3 with the final dox concentration of 5 μg/mL. The cells were cultured at 37 ^{o}C under a humidified atmosphere containing 5% CO_2 for 4 h and 24 h, respectively. Cell imaging was obtained using a a confocal laser scanning microscope (CLSM) Zeiss 710 3-channel (Zeiss, Germany) with an excitation wavelength of 488 nm and emission was collected between 565 and 630 nm

Synthesis of 3-azido-1-propanol

3-Bromo-1-propanol (3.0 g, 0.021 mol), sodium azide (2.1 g, 0.032 mol), sodium

iodide (0.05 g) were dissolved in 50 mL of deionized water and reacted at 80 °C for 24 hours. After cooled to room temperature, ethyl acetate was added to the reaction for extraction. The organic phase was collected and dried by $MgSO_4$. After removing ethyl acetate by rotary evaporation, the product was obtained as a clear and colorless liquid (yield: ~75%).

^{1}H NMR (400 MHz, $CDCl_3$)/ppm: 3.71 (t, 2H, J = 6.8 Hz, HOC**H**$_2$CH$_2$), 3.42 (t, 2H, J = 6.8 Hz, N$_3$C**H**$_2$CH$_2$), 1.80 (m, 2H, J = 6.8 Hz, CH$_2$C**H**$_2$CH$_2$).

^{13}C NMR (100 MHz, $CDCl_3$)/ppm: 60.0, 48.5, 31.3.

FT-IR: ν_{max}/cm^{-1} 3345, 2946, 2882, 2089, 1455, 1258, 1046, 955, 901.

ESI-MS (M+Na$^+$): expected (observed) 124.0481 (124.0488).

Synthesis of 4-((6-hydroxyhexyl)oxy)benzaldehyde

6-Chlorohexan (5.0 g, 0.033 mol), *p*-hydroxybenzaldehyde (4.04 g, 0.033 mol), potassium carbonate (9.1 g, 0.066 mol), sodium iodide (0.1 g) were dissolved in 15 mL of DMF and reacted at 100 °C for 24 hours. After cooled to room temperature, 200 mL of deionized water was added to the reaction followed by extraction using ethyl acetate (50 mL). The organic phase was collected and dried by $MgSO_4$. After removing ethyl acetate by rotary evaporation, the product was purified by column chromatography using mixed eluent (petroleum ether:ethyl acetate = 4:1) as a yellowish solid (yield: ~60%).

^{1}H NMR (400 MHz, $CDCl_3$)/ppm: 9.86 (s, 1H, C**H**O), 7.80 (d, 2H, J = 8.0 Hz, Ar), 6.96 (d, 2H, J = 8.0 Hz, Ar), 4.03 (t, 2H, J = 8.0 Hz, ArOC**H**$_2$CH$_2$), 3.65 (t, 2H, J = 8.0 Hz, HOC**H**$_2$CH$_2$), 1.80 (m, 2H, ArOCH$_2$C**H**$_2$), 1.28-1.67 (m, 6H,

$HOCH_2C\mathbf{H}_2C\mathbf{H}_2C\mathbf{H}_2CH_2$).

^{13}C NMR (100 MHz, $CDCl_3$)/ppm: 180.0, 163.8, 132.1, 129.4, 114.8, 68.3, 62.9, 32.7, 29.1, 25.9, 25.6.

FT-IR: ν_{max}/cm^{-1} 3378, 2934, 2859, 2740, 1683, 1598, 1575, 1509, 1471, 1427, 1394, 1312, 1254, 1215, 1157, 1110, 1057, 1007, 831.

ESI-MS ($M+Na^+$): expected (observed) 245.1148 (245.1150).

Synthesis of alkyne modified glucosamine

4-Pentynoic acid (0.372g, 0.38 mmol) and DCC (0.86 g, 4.2 mmol) were mixed in 20 mL of THF and stirred for 30 minutes at room temperature. D-glucosamine hydrochloride (0.80 g, 3.6 mmol) and triethylamine (0.37 g, 3.6 mmol) were dissolved in 20 mL of methanol. Then, the 4-pentynoic acid/DCC mixture was added to D-glucosamine/triethylamine methanol solution. The reaction was carried out at room temperature for 24 hours. The mixture was filtrated to remove white solid and subjected for column chromatography purification using dichloromethane/methanol (4:1) as eluent. The product was obtained as a white solid (yield: ~54%).

^1H NMR (400 MHz, DMSO-d_6)/ppm: 7.66 (s, 1H, N**H**), 6.37 (s, 1H, C**H**), 4.49-4.31 (m, 4H, C**H** on the glucose), 3.64-3.21 (m, 5H, C**H** on the glucose), 2.70 (s, 1H, $C\mathbf{H}{\equiv}CHCH_2$), 2.28 (m,4H, $C\mathbf{H}_2C\mathbf{H}_2$).

^{13}C NMR (100 MHz, DMSO-d_6)/ppm: 170.7, 91.2, 84.0, 71.9, 71.6, 71.2, 70.4, 61.2, 55.0, 34.2, 14.3.

FT-IR: ν_{max}/cm^{-1} 3290, 2921, 2850, 1645, 1548, 1419, 1364, 1313, 1282, 1146, 1121, 1081, 1063, 1023, 978, 910.

ESI-MS ($M+Na^+$): expected (observed) 282.0948 (282.0952).

One-pot synthesis of multifunctional copolymer (Polymer 1)

TFEMA (1.36 g, 8.1 mmol), 3-azido-1-propanol (0.222 g, 2.0 mmol), 4-((6-hydroxyhexyl)oxy)benzaldehyde (0.50 g, 2.0 mmol), mPEG (0.78 g, 2.0 mmol), triethylamine (0.60 g, 6.0 mmol), CETPA (19.8 mg, 0.075 mmol), ABVN (4.0 mg, 0.024 mmol), toluene (6.0 mL) and Novozym435 (0.5 g) were introduced in a schlenk tube equipped with a magnetic stir bar and purged by nitrogen flow for 20 minutes. The reaction mixture was put into an oil bath maintained at 50 ^oC. Samples were withdrawn periodically for ^1H NMR and GPC analyses for conversion and molecular weight determination, respectively. At the end of the polymerization, the polymerization was quenched by ice-water bath and the reaction mixture was centrifuged to remove enzyme. After removing toluene by rotary evaporation, the crude was re-dissolved in THF, and the polymer was purified via precipitation from THF to hexane for three times, and then dried under vacuum for further characterization. The ratio of corresponding functional groups in the obtained polymer was calculated according to the ^1H NMR integrals:

$$\oint {}^{19}F : \oint N_3 : \oint CHO : \oint PEG = I^{4.48} : (I^{4.18\sim3.71} - I^{7.05}*2 - I^{3.18}*2/3) : I^{7.05} : (I^{3.18}*2/3).$$

Synthesis of glucose polymer (Polymer 2) through CuAAc reaction

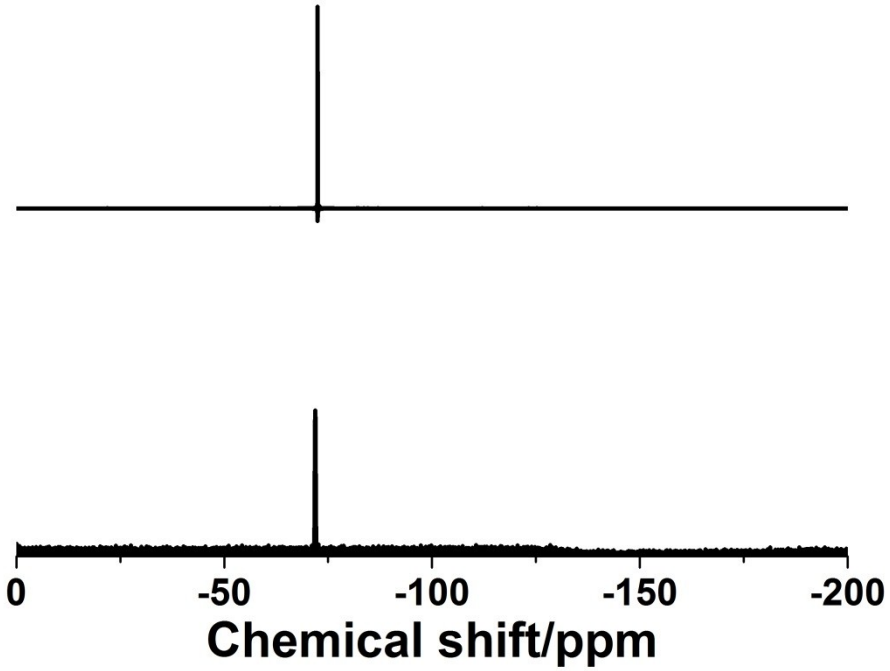
Polymer 1 (400 mg, containing 0.45 mmol N_3), alkyne glucosamine (140 mg, 0.55 mmol) and PMDETA (95 mg, 0.55 mmol) were dissolved in methanol/THF (5 mL/5 mL) in schlenk tube A and bubbled with nitrogen for 20 minutes. CuBr (33 mg, 0.23 mmol) was placed in schlenk tube B equipped with a magnetic stirring bar and purged

by nitrogen for 20 minutes. The solution in schlenk tube A was carefully introduced to tube B via a metal cannula under nitrogen atmosphere. Then the reaction was kept at 25 ^{o}C for 24 hours. Afterwards, the organic solvents were removed under vacuum and the residue was dissolved in THF. The polymer solution was passed through a neutral aluminium oxide column to remove metal salt, then concentrated and precipitated in diethyl ether. The polymer was collected by centrifugation as a white solid and dried under vacuum.

Synthesis of polymer-dox conjugate through imine chemistry (Polymer 3)

Polymer 2 (200 mg, containing 0.18 mmol CHO), doxorubicin chloride (100 mg, 0.36 mmol) and triethylamine (40 mg, 0.36 mmol) were dissolved in 20 mL DMSO and reacted at 30 ^{o}C for 48 hours. Afterwards the reaction mixture was dialyzed against THF to remove free doxorubicin (MWCO: 3500). The dialysis solvent was changed every 12 hours until no doxorubicin in THF was detected by UV-vis. The solution in dialysis bag was collected. After removing ~ 50% THF, the polymer was precipitated in diethyl ether and dried under vacuum to yield a red solid.

Supporting data



SFig. 1 ^{19}F NMR spectrum (DMSO-d6) of TFEMA (top) and **Polymer 2** (bottom).

References

(1) Tao, L.; Liu, J. Q.; Davis, T. P. *Biomacromolecules* **2009**, *10*, 2847-2851.