Supporting Information

Synthesis and Characterization of Cell-Microenvironment-sensitive Leakage-free Gold-shell Nanoparticle with template of Interlayer-Crosslinked Micelle

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Materials and methods: FTIR spectral studies were carried out using a Nicolet/Nexus 670 FTIR spectrometer in the range between 4000 and 500 cm⁻¹, with a resolution of 2 cm⁻¹. All powder samples were compressed into KBr pellets in the FTIR measurements. ¹H NMR spectra were recorded on a Varian Unity 300 MHz spectrometer or a Bruker 400 MHz spectrometer using D₂O or DMSO-d₆ as a solvent according to the polymer solubility. The UV-vis absorption spectra were measured on a UV-vis-NIR spectrophotometer (UV-3150, Shimadzu) spectrometer. The Fluorescence spectra were measured on a spectrofluorophotometer (RF5301PC, Shimadzu). Raman spectroscopy was measured using a Renishaw Invia Raman Spectrometer with excitation wavelength at 785 nm.

The molecular weights of the polymers were analyzed using a gel permeation chromatography (GPC) system consisting of a Waters 1515 pump, an Ultrahydrogel TM 500 column, an Ultrahydrogel TM 250 column, and a Waters 2417 differential refractive index detector with PEG as a standard for calibration. Depending on polymer solubility, a mobile phase of acetic acid (0.3 mol/L)/sodium acetate (0.2 mol/L) (pH 4.5) at a flow rate of 0.6 mL/min or DMF containing LiBr (1 g/L) at a flow rate of 1.0 mL/min was used. TCEP was pre-added into the solutions of mPEG-b-PAsp(MEA)-b-PAsp(DIP) and mPEG-PAsp(MEA)-N₃ to open the disulfide bond completely before the measurements.

Preparation of highly packed interlayer crosslinked micelles with partially hydrated core (HP-ICMs) and DOX encapsulation: After 5 mg of triblock copolymer and 1mg of DOX (if required) were co-dissolved in a mixed solvent of THF and DMSO (1 mL, equivalent volume), TEA was added to adjust the solution pH to 10 and sodium borohydride (10 eq to
copolymers) was added to reduce any existing disulfide of the copolymer for 30 minutes. The above solution was slowly added into a carbonate buffer of pH 10 (Acros) under sonication (60 Sonic Dismembrator, Fisher Scientific), and then the mixture was stirred under bubbling of an oxygen flow for 2 days to form the interlayer crosslinked micelles (ICMs) first. Afterwards, the solution was dialyzed (MWCO: 1 kDa) against PBS of pH 7.4 under bubbling of an oxygen flow to convert the ICMs to HPNs. The HP-ICMs thus prepared were characterized with DLS and TEM. The HP-ICMs solution was lyophilized to obtain the powdery form in order to avoid DOX release before cell culture.

Preparation of pH-tunable leakage-free gold-shell nanoparticles (pH-GSNPs): Firstly, gold seed nanoparticles are firstly prepared on the interlayer of HP-ICMs 1 µL of NaAuCl₄ aqueous solution (1 mg/mL) was added into 1 mL aqueous solution of HP-ICMs (1 mg/mL, pH 7.4 PBS). After the solution was magnetically stirred for 15 min, 1 µL of sodium borohydride (NaBH₄) aqueous solution (50 mM) or 50% NH₂OH aqueous solution was added to further react for 15 min. And then gold nanoshells are then grown around the interlayer of pH-GSNPs. 50 µL of NaAuCl₄ aqueous solution (1 mg/mL) and 50% NH₂OH aqueous solution for preparing gold nanoshell was added into the above solution. Finally, the pH-GSNPs were purified by centrifugation and washed with pH 7.4 PBS.

Size Measurements: The size of nanoparticles was determined using dynamic light scattering (DLS). The solutions of nanoparticles in standard phosphate-citric acid buffers with desired pH values were filtered through a 450 nm syringe filter. Measurements were carried out at room temperature on a 90° Plus/BI-MAS equipment (Brookhaven Instruments Corporation, USA). Data were collected on an autocorrelator with a 90° detection angle of scattered light.

DOX-loading content: The DOX-loading content, defined as the weight percentage of DOX
in the freeze-dried micelle, was quantified by UV-vis analysis using a UV-Vis-NIR Spectrophotometer (UV-3150, Shimadzu, Japan). The dried sample was weighed and re-dissolved in a mixture of chloroform and DMSO (1:1, v/v) before the UV-Vis analysis. The absorbance of DOX at 482.5 nm was measured to determine the DOX concentration in the solution using a pre-established calibration curve.

**DOX release:** In the direct measurement of DOX fluorescence intensity, solutions of pH-GSNPs in PBS (pH 7.4) were handled by adding GSH (10 mM) and/or decreasing the solution pH to 5.0. All solutions were adjusted to the same pH-GSNPs concentration (0.1 mg/mL) and incubated for 30 min before the intensity of DOX fluorescence emission around 590 nm was measured on a RF5301PC spectrophotometer (Shimadzu). To quantitatively determine the release rate of DOX into the pH-GSNPs solutions, freeze-dried samples (15 mg each) were re-suspended in 5 mL of phosphate-citric acid buffer (pH 7.4 or 5.0) and then transferred into dialysis bags (MWCO: 14 kDa). The bags were placed into the same buffered solutions (50 mL). Release study was performed at 37 °C in an incubator shaker (ZHWY-200B, Shanghai Zhicheng, China). At selected time intervals, 5 mL of solution outside the dialysis bag was removed for UV-vis analysis and replaced with the same volume of fresh buffer solution. DOX concentration was calculated based on the absorbance intensity of DOX at 482.5 nm. The cumulative amount of released drug was calculated, and the percentages of drug released from micelles were plotted against time.

**Confocal laser scanning microscopy (CLSM):** The intracellular distribution of DOX was determined using CLSM. SKOV-3 cells were seeded in petri dishes at a density of 1×10⁶ cells per dish and incubated for 2 h and 6 h with PBS solutions containing DOX-loaded pH-GSNPs at a DOX dose of 10 µg per dish. Cells were washed three times with PBS, and cell nuclei and lysosome were stained with Hoechst 33342 and Lysotracker Green (Beyotime Biotech,
China) respectively in order to identify the drug location inside cells. Cells were observed on a FluoView FV1000 microscope (OLYMPUS, Japan). DOX, Hoechst 33342 and Lysotracker Green were excited at 485, 352 and 504 nm, respectively. The emission wavelengths of doxorubicin, Hoechst 33342 and Lysotracker Green are 595, 455 and 511 nm, respectively.

Transmission Electron Microscopy (TEM): Transmission electron microscopy (TEM) was performed using a Hitachi model H-7650 TEM operated at 80 kV. Samples were prepared by drying a drop (10 µL, 1 mg/mL) of the sample solution on a copper grid coated with amorphous carbon. For the negative staining of samples, a small drop of uranyl acetate solution (2 wt % in water) was added to the copper grid, and then blotted with a filter paper after 1 min. The grid was finally dried overnight in a desiccator before TEM observation.

Synthesis of propargylamine (PA)-terminated prepolymer PA-PAsp(DIP). Propargylamine-terminated poly(β-benzyl L-aspartate) (PA-PBLA) was first synthesized by ring-opening polymerization of BLA-NCA using PA as an initiator. In the glove box free of water and oxygen, 55 µL of propargylamine (0.8 mmol, 0.803 mL) was dissolved in 50 mL of dry dichloromethane, and 5 g of BLA-NCA was dissolved in 5 mL of DMF. The two solutions were then mixed, sealed off under argon and stirred for 72 h at 35 °C. The solution was concentrated by rotary evaporation, precipitated into cold ether, filtrated, and vacuum-dried to get PA-PBLA. Next, 2.92 g of PA-PBLA (1.26 mmol) and 1.1 mL of N,N-diisopropylethylendiamine (6.33 mmol, about 5 eq) were dissolved in 8 mL of DMF, and then the solution was stirred for 24 h at 35 °C. After the reaction, the solution was dialyzed (MWCO: 1 kDa) against methanol for 48 h, distilled using rotary evaporation, and finally lyophilized to get the pH-sensitive prepolymer PA-PAsp(DIP) (Mn=3.3 kDa) for click reaction (Figures S1).
Synthesis of azide-terminated prepolymer mPEG-PBLA-N₃. Dioblock copolymer of Methoxy-PEG (mPEG) and poly(β-benzyl L-aspartate) (PBLA), i.e. mPEG-PBLA, was synthesized by ring-opening polymerization of BLA-NCA using the same method as preparing PA-PBLA instead that mPEG-NH₂ (Mₙ= 2kDa) replaced PA as an initiator. In the second step, 0.91 g of mPEG-PBLA (0.3 mmol) was dissolved in 15 mL of chloroform containing triethylamine (1.5 mmol, ~5 eq) and bromoacetyl bromide (0.9 mmol, ~3 eq), and then reacted for 24 h at room temperature under argon protection. Afterwards, the solution was precipitated into cold ether, filtrated and vacuum-dried to get 0.9 g of bromoacetyl group-terminated mPEG2k-PBLA5k-COCH₂Br (0.29 mmol). This prepolymer was then dissolved in 10 mL of DMF containing 0.94 g of sodium azide (14.5 mmol, ~10 eq), followed by stirring the solution for 24 h at room temperature. After the reaction, the solution was dialyzed (MWCO: 3.5kDa) against distilled water for 48 h and lyophilized to get mPEG-PBLA-N₃ for click reaction (Figures S1).

Synthesis of mPEG-PAsp(MEA)-PAsp(DIP) by click and aminolysis reactions. In the glove box free of water and oxygen, 0.3 mmol of mPEG-PBLA-N₃ and 0.36 mmol of PA-PAsp(DIP) (1.2 eq) were dissolved in 10 mL of DMF containing the catalyst system CuBr/PMDETA (both 1 eq to PA) for click reaction, sealed off under argon, and stirred for 48 h at 40 °C. After the reaction, the excessive PA-PAsp(DIP) was eliminated by dialysis against methanol (MWCO: 7 kDa). The methanol solution was then precipitated into cold ether, filtrated, and vacuum-dried to get the triblock prepolymer mPEG-PBLA-PAsp(DIP), which subsequently underwent the aminolysis reaction to remove the β-benzyl protection group and meanwhile to introduce the cysteamine group to the middle block. In brief, 0.8 g of mPEG-PBLA-PAsp(DIP) (0.16 mmol) was dissolved in 4 mL of DMSO containing MEA·HCl (2 eq) and TEA (3 eq), stirred for 12 h at 35°C, dialyzed against methanol and rotary evaporated. To remove copper ion, the as-synthesized copolymer (0.5 g) and copper
ion-complexing ligand \(N,N',N'',N''\)-pentamethyldiethylenetriamine(5eq to the copolymer) were dissolved in 4 mL of DMF. The solution was stirred for 24 h, dialyzed against methanol, and rotary evaporated. The procedure was repeated three times in order to completely remove the copper ion from the final product. \(^1\)H NMR resonance peaks at 0.85-1.26 ppm (s, \(-\text{CH(CH}_3\text{)}_2\), a), 2.75 ppm (m, \(-\text{NCH}_2\text{CH}_3\text{SH}\), c), 2.97 ppm (m, \(-\text{CH}_2\text{CON}-\), d), 3.19-3.26 ppm (m, \(-\text{CONHCH}_2\text{CH}_2\text{N}-\), e; m, \(-\text{NCH}_2\text{CH}_2\text{SH}\), g), 3.33-3.56 ppm (s, \(-\text{OCH}_2\text{CH}_2\), b; m, \(-\text{CONHCH}_2\text{CH}_2\text{N}-\), f); \(^{13}\)CNMR resonance peaks at 18.99 ppm (-\text{CH(CH}_3\text{)}_2, a), 36.39 ppm(-\text{NCH}_2\text{CH}_2\text{SH}, c), 41.10 ppm (-\text{CH}_2\text{CON}-, d), 58.50 ppm (-\text{CONHCH}_2\text{CH}_2\text{N}-, e; -\text{NCH}_2\text{CH}_2\text{SH}, g), 69.34 ppm (-\text{OCH}_2\text{CH}_2, b; -\text{CONHCH}_2\text{CH}_2\text{N}-, f).
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\text{mPEG-PBLA} \xrightarrow{(i) \text{Br} \rightarrow \text{NaN}_3} \text{mPEG-PBLA-N}_3
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\text{PA-PBLA} \xrightarrow{\text{DIP}} \text{PA-PAsp(DIP)}
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\text{mPEG-PBLA-N}_3 \xrightarrow{(i) \text{CuBr/PMDETA, DMF}} \text{PA-PAsp(DIP)} \xrightarrow{(ii) \text{MEA-HCl/TEA, DMSO}} \text{mPEG-b-PAsp(MEA)-b-PAsp(DIP)}
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*Fig. S1.* Synthetic scheme of mPEG-b-PAsp(MEA)-b-PAsp(DIP).
Fig. S2. Scan electron microscopy (SEM) images of highly packed interlayer-crosslinked micelles (HP-ICMs) from the triblock copolymer, mPEG-b-PAsp(MEA)-b-PAsp(DIP) at different conditions. (A) at pH7.4; (B) at pH7.4 and adding 10 mM glutathione (GSH); (C) at pH5.0. The key feature of the HP-ICMs, i.e. the reduction and pH dual sensitivity, was confirmed in SEM measurement (Fig. S2, supporting information). For the HP-ICMs at pH 7.4, SEM observations obtain the small polymeric nanoparticles of legible images of high magnification (59.4 nm by DLS). When GSH was added to the polymeric nanoparticles solution at pH 7.4, and then the highly cross-linked interlayer was destructed, the nanoparticles size got larger than only at pH 7.4 (500 nm ~ 1 µm of swollen Micelles vs. 50 ~ 60 nm of HP-ICMs, described in Figure S2, supporting information). In the meanwhile, when adjusted to pH5.0 from pH7.4, the highly cross-linked interlayer still existed, and the partially hydrated core transformed into the totally hydrophilic cavity (1 µm of nanocages).

Fig. 3S. Transmission electron microscopy (TEM) images of pH-tunable leakage-free gold-shell nanoparticles (pH-GSNPs) by using a reduction agent NH$_2$OH, not stained with uranyl acetate, at pH 7.4. 1µL NaAuCl$_4$ volume with concentration of 1 mg/mL at pH 7.4 PBS for gold seed nanoparticles, and continue to add NaAuCl$_4$ for gold nanoshells: 50 µL (A), 100 µL (B), 200 µL (C), and 400 µL (D).