Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

Supporting Information

Self-assembled, Redox-sensitive, H-shaped Pegylated Methotrexate Conjugates with High Drug-carrying Capability for Intracellular Drug Delivery

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Synthesis of $(CH_3\text{-}O\text{-}CO)_2PEG(CO\text{-}O\text{-}CH_3)_2$.

NH_2-PEG-NH_2 (M_n=2000, 0.5 g, 0.25 mmol) was dissolved in 20 mL of anhydrous methanol, and 5 mL of methyl acrylate was added. Sodium methoxide solution (1-2 drops) was added as a catalyst. The mixture was stirred in darkness at room temperature for 72 h. The solvent and excess methyl acrylate were evaporated with a rotary evaporator. The residue was then taken up in methanol and the solution was dialyzed (MWCO =1000Da) against distilled water to remove the excess sodium methoxide. The resulting product was collected by freeze-drying.

Synthesis of $(cystamine\text{-}CO)_2PEG(CO\text{-}cystamine)_2$.

Cystamine·2HCl was desalinated before use according to the previous work of our group.[1] $(CH_3\text{-}O\text{-}CO)_2PEG(CO\text{-}O\text{-}CH_3)_2$ (0.45 g, 0.19 mmol) was dissolved in 20 mL of anhydrous methanol and then added dropwise to a mixture of cystamine (1.17 g, 7.66 mmol) and sodium methoxide solution (1-2 drops) in 20 ml anhydrous methanol under nitrogen. The reaction was allowed proceed at r.t. for 72 h before the desired $(cystamine\text{-}CO)_2PEG(CO\text{-}cystamine)_2$ was isolated by dialysis and freeze-dried until further use.

Synthesis of $(HO\text{-}Gly\text{-}SS)_2PEG(SS\text{-}Gly\text{-}OH)_2$.

$(HO\text{-}Gly\text{-}SS)_2PEG(SS\text{-}Gly\text{-}OH)_2$ was synthesized using methods similar to that has been previously reported.[2-4] $(cystamine\text{-}CO)_2PEG(CO\text{-}cystamine)_2$ (0.4 g, 0.14 mmol) was

dissolved in anhydrous methanol (20 mL). To the solution was added glycidol (0.05 g, 0.67 mmol). Following reaction overnight at r.t. under nitrogen, $(HO\text{-}Gly\text{-}SS)_2PEG(SS\text{-}Gly\text{-}OH)_2$ was purified by dialysis and freeze-dried.

Polymeric Nanoparticles

$(MTX)_2PEG(MTX)_2$ was allowed to form nanoparticles via membrane-dialysis method. Briefly, copolymer (1 mg) was dissolved in DMF (4 ml). Then the solution was dialyzed against deionized water (2 L) for 48 h using dialysis bags with a MW cut-off = 8-12 kDa. The water was refreshed at every 12 h. Critical micelle concentrations (CMC) for the copolymer was determined using nile red as hydrophobic fluorescence probe. A known amount of nile red in CH_2Cl_2 was added to a series of vials and the CH_2Cl_2 was evaporated. The amount was chosen to give a nile red concentration of 1×10^{-6} mol/L in the final solution. The concentration of the copolymer was varied from 0.95 to 1000 mg/L. The solution was kept at r.t. and equilibrated for 24 h before measurements. Then the fluorescence emission intensity at the wavelengths of 625 nm (excited at 556 nm) was measured using a Hitachi F2500 luminescence spectrometer (Hitachi, Ltd, Hong Kong). The critical micelle formation concentration was obtained as the intersection of the tangents to the two linear portions of the graph of the fluorescence intensity as a function of $(MTX)_2PEG(MTX)_2$ concentration.

Reduction-triggered destabilization of $(MTX)_2PEG(MTX)_2$ nanoparticles

$(MTX)_2PEG(MTX)_2$ nanoparticles dispersed in 0.01 M phosphate-buffered saline, pH 7.4 (PBS), was obtained before test. Sufficient DTT was added to the solution of $(MTX)_2PEG(MTX)_2$ nanoparticles in PBS buffer to achieve a 10 mM DTT reducing environment. At designated time intervals, particle size distribution was determined by

dynamic laser light scattering (DLS) using the Nano-ZS 90 Nanosizer (Malvern Instruments Ltd., Worcestershire, UK).

DTT-mediated in vitro release of MTX

$(MTX)_2PEG(MTX)_2$ (2 mg) was dissolved in DMF (4 mL). MTX-polymer nanoparticles were formed by dialysis (MWCO =3500Da) of the DMF solution against deionized water for 24 h at 37 ^oC. The dialysis medium was changed five times to remove DMF. The whole procedure was performed in the dark. The release profiles of MTX from MTX-polymer micelles was studied at 37 ^oC in two different media respectively, PBS (0.01 M, pH 7.4) with 10 mM DTT and PBS (0.01 M, pH 7.4) only. The solution was placed in a shaking bed at a speed of 150rpm at 37 ºC. At desired time intervals, samples (2 mL) were withdrawn and replaced with an equal volume of fresh media. The amount of MTX released was determined by using fluorescence measurement (excitation at 389 nm).

Cell cytotoxicity

Human liver carcinoma HepG2 cells were generously provided by Cell Center of the Tumor Hospital at Fudan University. Routinely, this cell line was maintained at 37 ºC in a humidified 5% CO_2 atmosphere using DMEM supplemented with 10% fetal bovine serum and 0.1% penicillin-streptomycin. For experiments, HepG2 cells were seeded into 96-well plate at a density of 5000 cells/well. Following an overnight attachment period, the medium in each well was replaced with culture medium containing free MTX dissolved in DMSO or $(MTX)_2PEG(MTX)_2$ prodrug. As a negative control, cells were preincubated with 10 mM GSH-OEt. The preincubation medium was removed after 2 h and cells were washed with prewarmed PBS. Treatment groups with

$(MTX)_2PEG(MTX)_2$ nanoparticles included both cells with and without preexposure to 10 mM GSH-OEt. $(MTX)_2PEG(MTX)_2$ copolymers were tested a concentrations ranging from 31.2×10^{-3} to 1 mg mL^{-1} (contains equivalent MTX of 8.1~260 mg/L). All suspensions were prepared in complete cell culture media. After a 24 h incubation period, the volume in each well was replaced with 200 μL of fresh media and 20 μL of 5 mg mL^{-1} sterile filtered 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution prepared in PBS. The plate was incubated for additional 4 h at 37 ºC, allowing viable cells to metabolically reduce MTT into purple formazan. After addition of 150 μL of dimethyl sulfoxide (DMSO) to each well, the plate was incubated at r.t. for 10 min on a shaking platform before optical density (OD) was measured at λ = 492 nm using a Multiscan MK3 plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Cell viability was calculated according to the following equation:

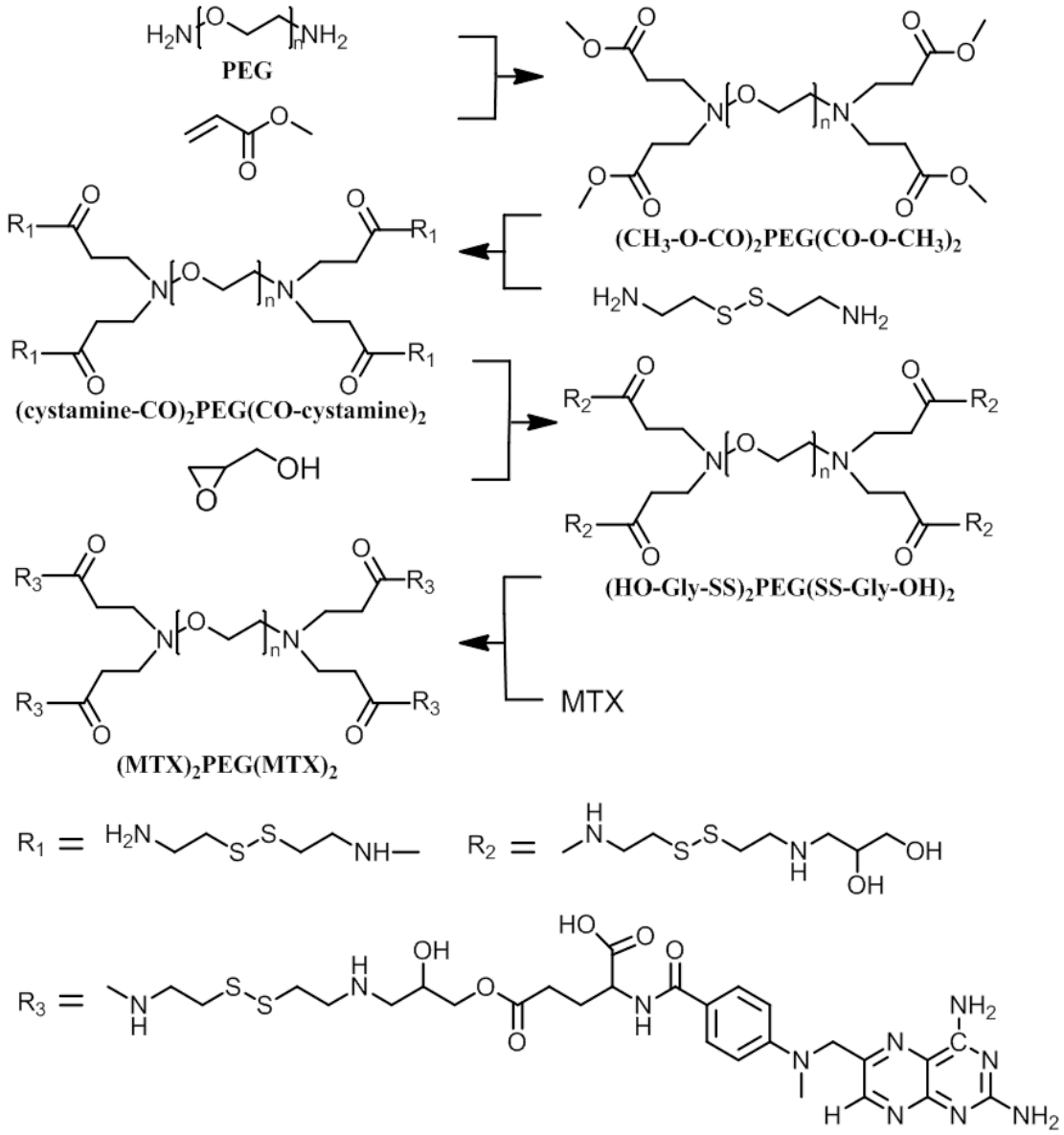
Cell viability (%) = $(OD_{treated} /OD_{control}) \times 100\%$

where $OD_{control}$ was obtained in the absence of polymer and $OD_{treated}$ was obtained in the presence of polymer.

Confocal laser scanning microscopy observation of $(MTX)_2PEG(MTX)_2$ micelle treated HepG2 cells

HepG2 cells were seeded in a 6-well plate at a density of 1×10^5 cells/well with complete medium. The cells were incubated for 24 h in a humidified atmosphere with 5% CO_2 at 37 ºC. Cells were washed by PBS and incubated at 37 ºC for 24 h with $(MTX)_2PEG(MTX)_2$ micelles in complete DMEM. The cells were then washed with PBS twice and fixed with 4% paraformaldehyde. The slides were mounted and observed with a confocal laser scanning microscope (Olympus, FV300, IX71, Tokyo, Japan)

equipment.



Scheme S1. Synthesis route of $(MTX)_2PEG(MTX)_2$.

Table S1. Properties of MTX-PEG conjugate.

Disulfide-linked copolymer (Theoretical Molecular weight)	**M_n [a]**	**M_n [b]**	**Number of MTX in each unit ([a]/[b])**
MTX-PEG(2K)-MTX (4962)	5076	5326	3.80/4.21
MTX-PEG(4K)-MTX (6962)	7007	7288	3.64/4.13

[a]: Determined by ^{1}H NMR [b]: Determined by MALDI-TOF

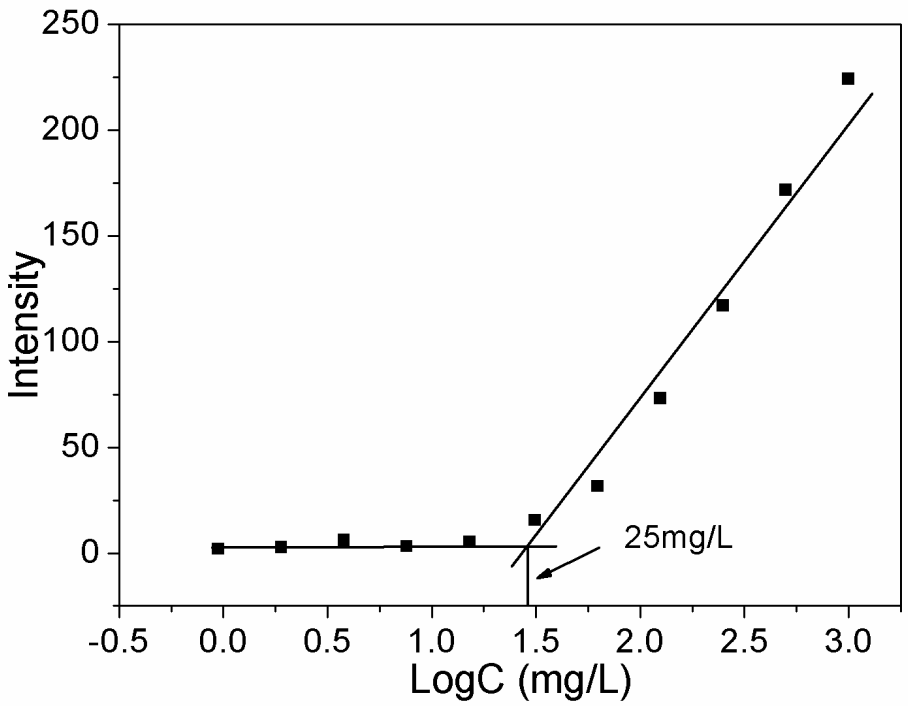


Fig. S1. CMC of MTX-PEG(4K)-MTX measured by fluorescence spectroscopy.

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