Subcellular co-delivery of two different site-oriented payloads for tumor therapy

*Qingqing Yang [1], Lei Wu[1], Lian Li, Zhou Zhou and Yuan Huang**

Key Laboratory of Drug Targeting and Drug Delivery System, Ministry of Education, West China School of Pharmacy, Sichuan University. No. 17, Block 3, Southern Renmin Road, Chengdu 610041, P.R. China

Corresponding Author

*E-mail: huangyuan0@163.com, Tel.: +86-28-85501617, Fax: +86-28-85501617.

Author Contributions

[1] These authors contributed equally to this work.

Materials

OctaAmmonium polyhedral oligomericsilsesquioxanes (POSS-NH_2) was purchased from Hybrid Plastics (Hattiesburg, USA). N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) was obtained from TCI Chemical Industry Co., Ltd. (Shanghai, China). The following reagents were purchased from Sigma-Aldrich (St. Louis, MO): levulinic acid (LEV), fluorescein isothiocyanate (FITC), dithiothreitol (DTT), 2,4,6-trinitrobenzene-1-sulfonic acid (TNBSA), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). All other reagents and solvents were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China) and used as received.

N-(2-Hydroxypropyl)methacrylamide (HPMA),[1] N-(tert-butoxycarbonyl)-N'-(6-methacrylamido-hexanoyl)-hydrazine (Ma-ah-NHNH-Boc),[2] 3,3'-[4,4'-azobis(4-cyano-4-methyl-1-oxo-butane-4,1- diyl)]bis(thiazolidine-2-thione) (ABIK-TT),[3] N-methacryloyl-aminopropyl-fluorescein-5-isothiocyanate (MA-AP-FITC)[4], 2-(2-pyridyldithio)-ethylamine hydrochloride (PDEA)[5] and the derivative of docetaxel(DTX) with levulinic acid (DTX-LEV)[6] were synthesized according to previous reports.

Synthesis of Pyridyldisulfanyl-Functionalized POSS (POSS-PDS)[6]

POSS-PDS was prepared by the reaction of amino groups of POSS-NH_2 with SPDP as follows: POSS-NH_2 (23 mg, 0.16 mmol amino groups) was dissolved in methanol, and a solution of SPDP (100 mg, 0.32 mmol) and N-ethyldiisopropylamine (10μL) in

2 mL methanol was added. The reaction mixture was subsequently stirred for 2 h at room temperature. POSS-PDS was purified by gel filtration on Sephadex LH-20 column using methanol as eluent.

Synthesis and Characterization of the Thiol-Terminated Semitelechelic HPMA Copolymer (P-SH)[6]

Semitelechelic HPMA copolymer precursor (P-SH) containing *tert*-butoxycarbonyl (Boc)-protected hydrazide groups and copolymer chain terminating with sulfhydryl groups was prepared in three consecutive steps. First, semitelechelic HPMA copolymer terminated in thiazolidine-2-thione (TT) groups (P-TT) was prepared by radical solution polymerization according to the established procedures.[7] Briefly, HPMA (93 mol%), Ma-ah-NHNH-Boc (7 mol%) were dissolved in dimethyl sulfoxide (DMSO) initiated with ABIK-TT (4 wt%). The solution was purged with nitrogen and stirred at 50 °C for 6 h. The copolymer was isolated by precipitation into diethyl ether. Similar procedure was followed to prepare fluorescence labeled semitelechelic polymer precursor (P-TT-FITC), using HPMA (91 mol%), Ma-ah-NHNH-Boc (7 mol%), MA-AP-FITC (2 mol%). Second, the 2-pyridyldisulfanyl (PDS)-terminated semitelechelic HPMA copolymer (P-PDS) was synthesized by the reaction of terminal TT groups of the polymer P-TT with PDEA in N,N-dimethylformamide (DMF) as previously described.[7] Briefly, P-TT (0.048 mmol TT) was dissolved in DMF and a solution of (0.062 mmol) and N-ethyldiisopropylamine (10 μL) in DMF was added. After 3 h of stirring the reaction mixture was diluted with

methanol and purified by gel filtration on a Sephadex LH-20 column using methanol as eluent. Finally, the sulfhydryl group-terminated semitelechelic copolymer precursor (P-SH) was prepared by reduction of chain terminal PDS groups of P-PDS with DTT.[8] Example of the reaction: P-PDS was dissolved in distilled water and excess DTT was added under gentle stirring for 30 min. The resulting P-SH was purified by gel filtration on a Sephadex G-25 column using double distilled water as eluent. The polymer solution was lyophilized to obtain the product P-SH.

The content of end-chain TT groups in P-TT was determined by UV-vis spectroscopy using ε_{305}=10 700 L mol^{-1} cm^{-1} (methanol). The content of PDS end groups in P-PDS was determined by UV-vis spectroscopy after reaction with DTT.[9] The content of SH groups in P-SH was determined with Ellman's reagent.[10] The content of hydrazide groups in star copolymers was determined by TNBSA assay. The molecular weight and polydispersity index of copolymers were measured based on a HPMA homopolymer calibration using an AKTA Fast Protein Liquid Chromatography (FPLC) system [GE Healthcare Life Sciences; Superose 6 10/300GL analytical column; mobile phase, phosphate buffer (pH 7.4)] equipped with UV and refractive index detectors.

Synthesis of Star Copolymer-Docetaxel Conjugates[6]

POSS-based star copolymers were synthesized by the reaction of thiol groups in P-SH with PDS groups of POSS-PDS as follows: P-SH (10 μmol SH groups) was dissolved

in dimethylsulfoxide (DMSO) and added to a stirring solution of POSS-PDS (8.13 μmol PDS groups) in DMSO under argon atmosphere. After 4 h of agitation, the mixture was diluted with methanol and the products were purified by gel filtration (Sephadex LH-20, methanol).

Star copolymer-DTX conjugates were synthesized after separating the Boc groups from hydrazides of star copolymers using trifluoroacetic acid.[11] Then star copolymers were dissolved in anhydrous methanol and DTX-LEV was added. The reaction was carried out in the dark overnight after addition of acetic acid. The product was purified by gel filtration (Sephadex LH-20, methanol). Cy5.5 labeled conjugates were synthesized by the reaction of Cy5.5-NHS ester with hydrazide groups in star copolymers as previously described.[7]

Linear HPMA copolymer-DTX conjugates (P-DTX) were prepared by radical copolymerization in accordance with previous reports.[12]

Characterization of Star Copolymers and Micelles

The molecular weight (MW) of blank star copolymer was determined by a GPC/HPLC system.[7] The critical micelle concentration (CMC) value of star copolymer-DTX conjugate in distilled water was measured by pyrene fluorescence spectroscopy.[13] The content of FITC in micelles was determined by UV-vis spectrometry using ε_{494}=80 000 L mol^{-1} cm^{-1} (0.1 M borate buffer, pH 9.0). The

conjugation ratio of Cy5.5 to micelles was determined by measuring fluorescence intensity (Ex/Em =676/707nm).

Table S1. Characteristics of Synthesized Semitelechelic HPMA Copolymers

Polymer	Mw (kDa)	Mw/Mn	Reactive group (mmol g^{-1}polymer)	FITC content (wt %)
P-TT	30.1	1.65	TT (0.093)	-
P-TT-FITC	31.3	1.71	TT (0.089)	4.3
P-PDS	30.8	1.78	PDS (0.079)	-
P-PDS-FITC	31.6	1.82	PDS(0.072)	4.2
P-SH	32.2	1.77	SH(0.061)	-
P-SH-FITC	32.7	1.69	SH(0.060)	4.2

The molecular weight (MW) of blank star copolymer was approximately 235 kDa, indicating the formation of well-defined star copolymer which constructed with a POSS core and eight semitelechelic copolymer chains (MW = 32 kDa).

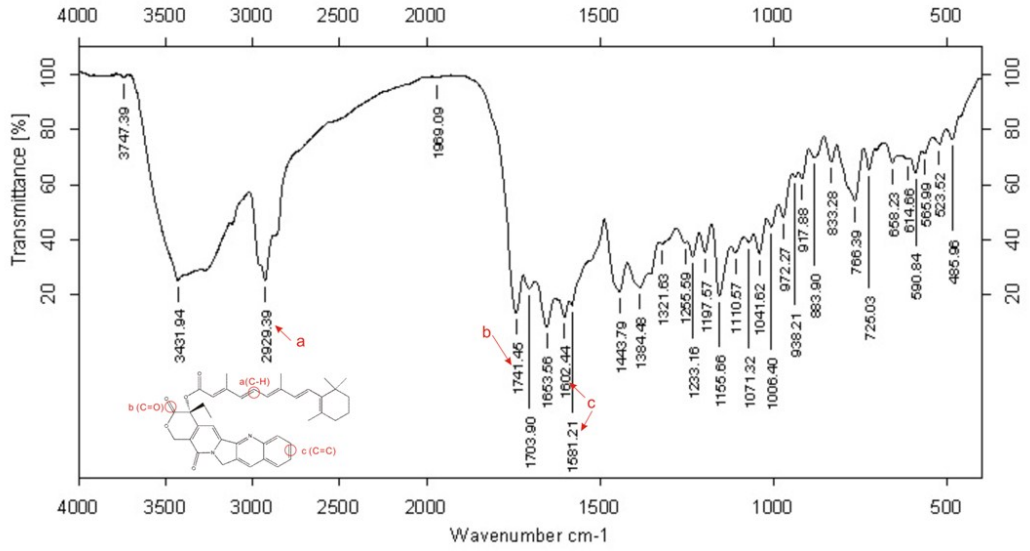

Figure S1 FT-IR spectrum of CPT-RA.

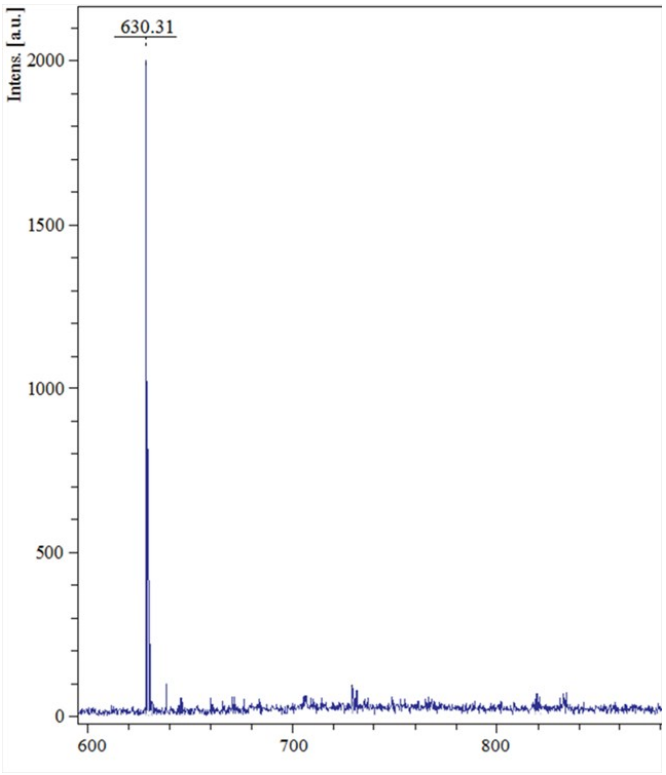

Figure S2. MALDI-TOF MS spectrum of CPT-RA.

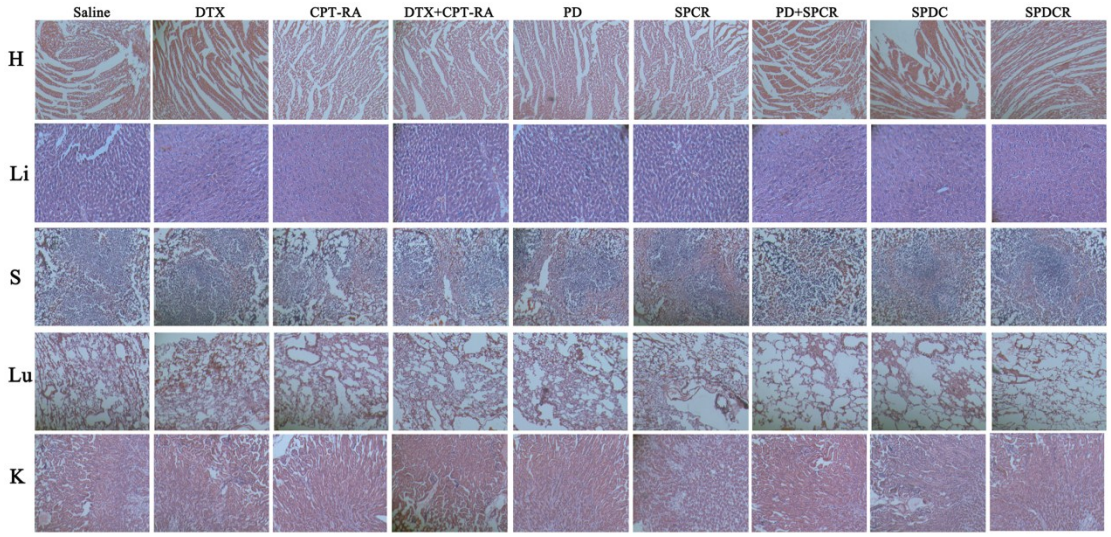

Figure S3. Histological evaluation of major organs (heart, liver, spleen, lung, and kidney) from mice bearing stroma-rich prostate xenograft tumor using hematoxylin and eosin (H&E) staining after treatment with either saline, DTX, CPT-RA, DTX+CPT-RA, PD, SPCR, PD+SPCR, SPDC or SPDCR.

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