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

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Author Contributions

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Materials

OctaAmmonium polyhedral oligomeric silsesquioxanes (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-ethylidiisopropylamine (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)**

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. 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. 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 sulphhydryl group-terminated semitelechelic copolymer precursor (P-SH) was prepared by reduction of chain terminal PDS groups of P-PDS with DTT. 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 $\varepsilon_{305}=10700$ 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. The content of SH groups in P-SH was determined with Ellman's reagent. 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**

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. 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.

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

**Characterization of Star Copolymers and Micelles**

The molecular weight (MW) of blank star copolymer was determined by a GPC/HPLC system. The critical micelle concentration (CMC) value of star copolymer-DTX conjugate in distilled water was measured by pyrene fluorescence spectroscopy. 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 = 676/707 nm).
<table>
<thead>
<tr>
<th>Polymer</th>
<th>Mw (kDa)</th>
<th>Mw/Mn</th>
<th>Reactive group (mmol g⁻¹polymer)</th>
<th>FITC content (wt %)</th>
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<tbody>
<tr>
<td>P-TT</td>
<td>30.1</td>
<td>1.65</td>
<td>TT (0.093)</td>
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<tr>
<td>P-TT-FITC</td>
<td>31.3</td>
<td>1.71</td>
<td>TT (0.089)</td>
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<tr>
<td>P-PDS</td>
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<td>1.78</td>
<td>PDS (0.079)</td>
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<tr>
<td>P-PDS-FITC</td>
<td>31.6</td>
<td>1.82</td>
<td>PDS (0.072)</td>
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<tr>
<td>P-SH</td>
<td>32.2</td>
<td>1.77</td>
<td>SH (0.061)</td>
<td>-</td>
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<tr>
<td>P-SH-FITC</td>
<td>32.7</td>
<td>1.69</td>
<td>SH (0.060)</td>
<td>4.2</td>
</tr>
</tbody>
</table>

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.
REFERENCES


