Electronic Supplementary Material (ESI) for Polymer Chemistry

Cell-penetrating Albumin Conjugates for Enhanced Doxorubicin Delivery

*Qianqian Guo, Huiyuan Wang, Yongxing Zhao, Huixin Wang, Feng Zeng, Haiying Hua, Qin Xu, Yongzhuo Huang**

Experimental methods

1. Synthesis of LMWP-BSA and LMWP-BSA-DOX

Ten milligrams of low molecular weight protamine (LMWP) was dissolved in 500 μl water, and then LMWP was activated using DSS (disuccinimidyl suberate, 16 μM, 500 μl DMSO, ProteoChem, USA). After 4-h reaction, the reactant solution was added to 8 ml of PBS to precipitate the hydrophobic DSS. The filtrate was added to the BSA solution at 1:1 molar ratio, and following 3-h incubation, the resultant LMWP-BSA conjugates were purified by heparin affinity chromatography (HiTrap Heparin HP column, GE Healthcare, USA) based on the interaction of LMWP and heparin. The purified LMWP-BSA was reacted with DSP (dithiobis[succinimidyl propionate], ProteoChem, USA), and then conjugated with DOX via DSP linker. The final LMWP-BSA-DOX was purified using heparin column and then desalted using ultracentrifiltration (Amicon Centrifugal Filters, 10K Millipore, USA).

2. Characterization of LMWP–BSA and LMWP-BSA-DOX

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% acrylamide gel was performed to characterize the LMWP-BSA and LMWP-BSA-DOX conjugates. The protein samples were prepared by mixing loading buffer without thiol-reducing agents. All samples were heated in boiling water for 5 min. An electric potential of 90 V (Bio-rad Mini-PROTEAN Tetra Electrophoresis System) was applied until the bromphenol dye reached the running gel. It was then increased to 110 V, and the protein bands on the gel were visualized with Coomassie Brilliant Blue R-250 staining. The fluorescence spectrum of LMWP-BSA-DOX was scanned using a fluorescence spectrophotometer (Hitachi, Japan).

Differential Differential Scanning Calorimetry (DSC) was performed using Mettler ToledoST System to measure the temperature and heat flow associated the glass transition temperature (Tg) of LMWP-BSA-DOX. It was heated up from 30 to 300 °C at a rate of 10 °C·min^{-1}.

3. Quantitative determination of LMWP-BSA-DOX

The protein content of LMWP-BSA-DOX was determined by the bicinchoninic acid (BCA) assay with BCA Protein Assay Kit (Beyotime, China). The content of DOX in LMWP-BSA-DOX was measured by fluorescence spectrophotometer (Hitachi, Japan) with excitation and emission wavelengths of 485 and 590 nm, respectively.

4. DOX release triggered by reducing agent

LMWP-BSA-DOX was incubated with 10 mM dithiothreitol (DTT) in order to investigate the DOX release ability responding to the reductive environment. After incubation, the sample was ultracentrifuged to remove the cleaving free DOX, and then the fluorescence spectrum of the sample was recorded.

5. In vitro inhibition study

Cytotoxicity of LMWP-BSA-DOX in various cancer cell lines (HeLa, MCF-7 and MCF-7/ADR) was measured using MTT assay. Cells were plated at a density of 5000 cells/well in 96-well plates. After 24h, the cells were exposed to the medium containing serial concentrations of LMWP-BSA-DOX and incubated for 48 h at 37 °C. A volume of 20 μl of the MTT solution (5 mg/ml) was then added to each well, and incubated for another 4 h. Supernatants were discarded, and 200 μl DMSO were added to each well to dissolve the formazan crystals. The optical density of each well was measured using a microplate reader (Multiskan, Thermo Fisher, USA) at a wavelength of 570 nm. The relative cell viability (%) was calculated by $[A]_{test}/[A]_{control}\times 100$.

7. Analysis of Apoptosis

Apoptotic cells were assessed by fluorescence-activated cell sorting (FACS) analysis. Cells were collected from the culture medium by centrifugation at 1,000 rpm for 3 min and then labeled by annexin V-FITC and propidium iodide (PI) in binding buffer according to the instructions in the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit. Living cells showed negative for both PI and annexin V staining. Cells that showed PI-negative but annexin V-positive staining are at early apoptosis, whereas those showed both PI- and annexin V-positive staining are at late stages of apoptosis. The assay was performed in a FACScan cytofluorometer (Becton Dickinson) with CellQuest (Becton Dickson) software. The fluorescent signals of FITC and PI were measured respectively at 518 nm and 620 nm.

6. *In vivo* antitumor experiments

BALB/c nu/nu female mice were inoculated with Hela cells (5×10^5 cells/mouse) in the back, and randomly divided into three groups (n = 6). When the tumor grew to a size of about 50 mm^3, the mice were received *i.v.* injection of LMWP-BSA-DOX with multiple dosing of 1.5 mg/kg (represented by DOX) every other day (q2d) in a period of 20 days. A group treated with DOX or saline was set as positive or negative control, respectively. The tumor size was measured every other day to evaluate the tumor growth inhibition rate and anticancer efficacy. And at the experimental endpoint, mice were sacrificed and their tumors resected. Organs were collected for histological examination for safety evaluation. The animal experimental procedures were approved by the SIMM Institutional Animal Care and Use Committee.

Supplementary Figures:

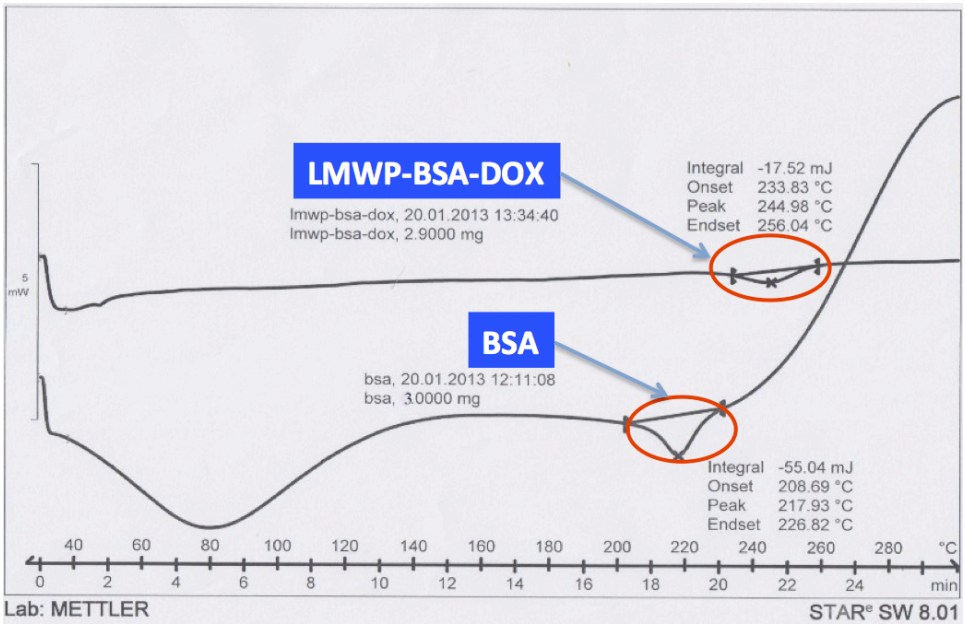

Fig. S1. DSC diagram of the conjugates. The Tg of the LMWP-BSA-DOX shows minor shift toward high temperature, presumably due to the modification of LMWP and DOX, thus increasing the structural rigidity of BSA.

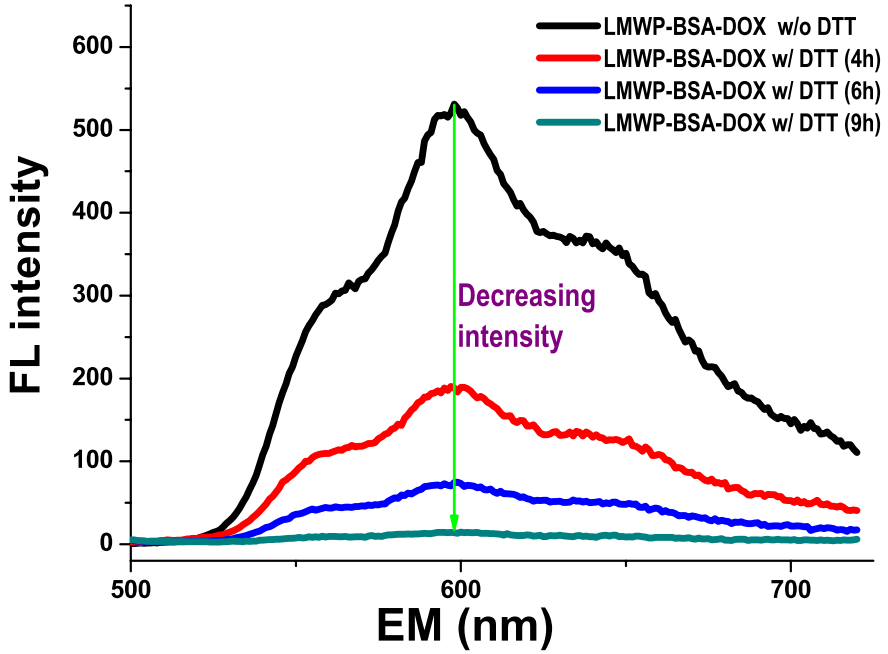

Fig. S2. The decreasing fluorescence intensity due to the cleavage and removal of DOX in the presence of DTT at varying time points.

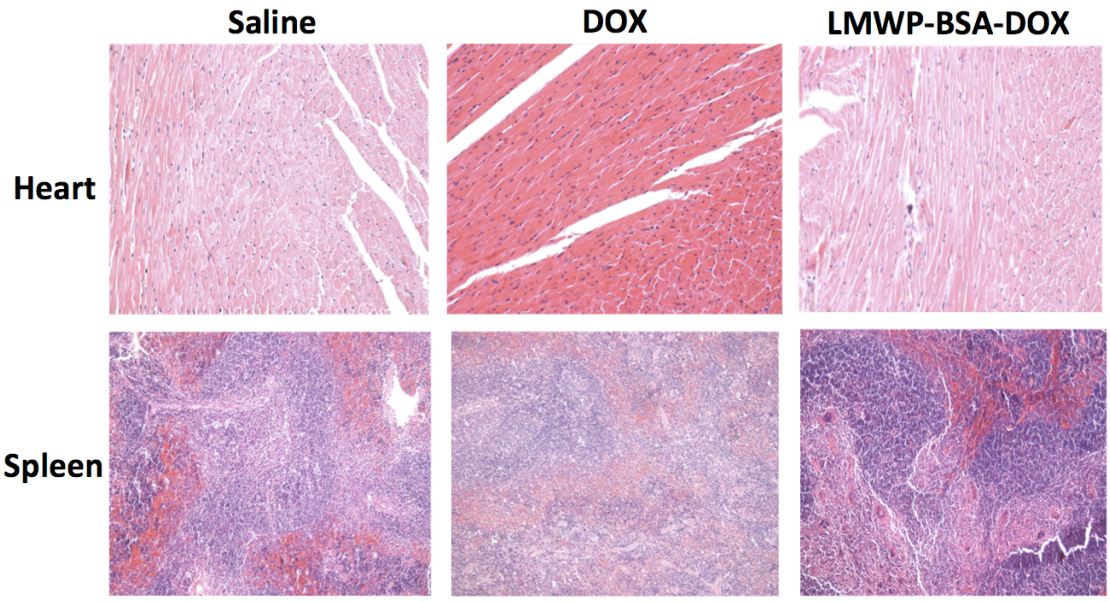

Fig. S3. No appreciable change and side toxic effect displaying in the heart and spleen.