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Supporting Information

Chondroitin Sulfate Derived Theranostic Nanoparticle for Targeted Drug Delivery

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Materials and Methods: Chondroitin sulphate-A (CS-A, 54 kDa from bovine trachea) was purchased from Sigma-Aldrich (Sweden). DOX.HCl was purchased from Tocris Bioscience, UK. Monoclonal anti-CD44-FITC antibody, Fluorescein-5-thiosemicarbazide (FTSC), *N*-hydroxybenzotriazole (HOBt), 1-ethyl-3-(3-dimenthylaminopropyl) carbodiimide hydrochloride (EDC.HCl) was purchased from Sigma-Aldrich (Sweden). ApoTox-Glo™ Triplex Assay, was purchased from Promega. Fetal bovine serum (FBS) was purchased from Hyclone, Perbio Scientific, Sweden. For release experiment, Slide-A-Lyzer® MINI Dialysis Device, 3500 MWCO, 2 ml was used. All other chemicals were purchased from Sigma. All solvents were of analytical grade. Dialysis membranes used for purification were purchased from Spectra Por-6 (MWCO 3500). HCT116 and MCF-7 were obtained from American Type Culture Collection (ATCC)-LGC standards, Sweden.

The NMR experiments (δ scale; J values are in Hz) were carried out on Jeol JNM-ECP Series FT NMR system at a magnetic field strength of 9.4 T, operating at 400 MHz for ^{1}H. D_2O and DMSO-d6 was used as the NMR solvent for analysis. Spectroscopic analyses were carried out on PerkinElmer instruments namely,

Spectrum One AT-FTIR, Lambda 35 UV-Vis spectrophotometer and LS 45 Luminescence Spectrophotometer.

Synthesis of CS FTSC conjugates or CS-NP

CS conjugate with FTSC was synthesized by cabodiimide coupling chemistry as follows. Briefly, 0.5 mmol of CS (with respect to disaccharide units) was dissolved in 60 ml of de-ionized water. Thereafter, FTSC (21 mg, 0.05 mmol) dissolved in 5 ml DMSO was added followed by HOBt (77 mg, 0.5 mmol). The reaction mixture was stirred for 30 min (till it becomes homogeneous) and the pH of the reaction mixture was adjusted to 5.0 by careful addition of 1M NaOH. Finally, EDC.HCl (19.2 mg, 0.1 mmol) was added and stirred overnight. The reaction mixture was loaded into a dialysis bag (Spectra Por-6, MWCO 3500 g/mol) and dialyzed against dilute HCl (pH = 3.5) containing 0.1 M NaCl (4×2L, 48 h), then dialyzed against deionized water (2×2L, 24 h). The solution was lyophilized and fluffy yellow material was obtained in 96% yield. The lyophilized polymer was subsequently washed with methanol to eliminate any traces of free FTSC. Percentage of FTSC conjugation was estimated in water at pH 8.5 by UV measurement using the FTSC extinction coefficient of 78,000 $M^{-1}cm^{-1}$ at 492 nm.

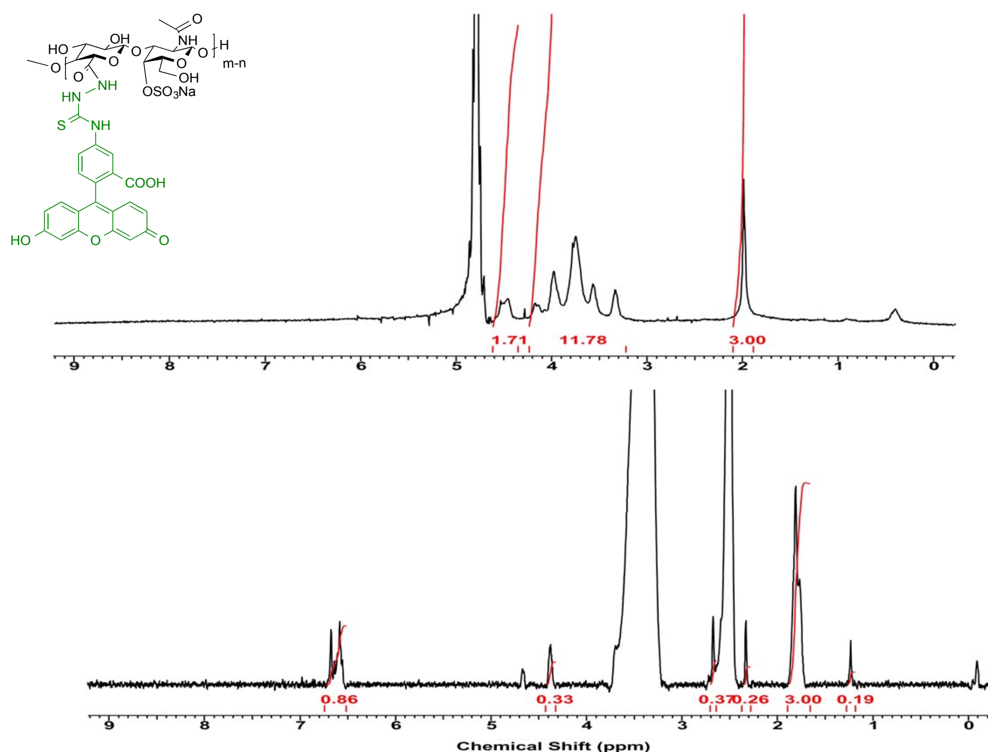

Figure S1 1H NMR of CS-FTSC in a) D_2O and in b) DMSO-d6.

Preparation of DOX loaded nanoparticles (NPs)

DOX was loaded on the CS nanocarriers by nanoprecipitation method. Briefly, 100 mg CS-NP was dissolved in 25 ml of PBS of pH 7.4. Thereafter, 5 mg of DOX.HCl (in 1 ml of DMSO) was added dropwise to the reaction mixture in 30 min. The pH was adjusted again to 7.4 and the reaction mixture was stirred overnight. Thereafter, the solution was loaded into a dialysis bag (Spectra Por-6, MWCO 3500) and dialyzed against 0.1 M NaCl (2×2 L, 24 h), followed by dialysis against deionized water (2×2 L, 24 h). This solution was lyophilized yielding 96 mg of yellow fluffy product.

In-vitro release experiment

The amount of DOX loading and rate of DOX release from CS-NP was performed by dialysis method, and release medium was analyzed by UV/VIS spectroscopy at 485

nm (Lambda 35 UV/VIS spectrometer, Perkin Elmer). Briefly, 10 mg of the sample was dissolved in 1 ml of PBS buffer (pH 7.4) and transferred to Slide-A-Lyzer® MINI Dialysis Device, 3.5K MWCO, 2 ml on a glass tube with 18 ml buffer. The samples were placed on a shaker (at 100 rpm) at RT and the UV absorbance of the released media was measured at various time points at 485 nm. The release medium was placed back into the MINI dialysis device after each measurement. The percentage of release in each case was plotted using excel software. The release experiment was carried out in duplicates. The samples were protected from light throughout the release experiment to prevent DOX degradation.

The percentage of drug loading was estimated by measuring the UV absorbance of the final release media using the extinction coefficient of 11,500 $M^{-1}cm^{-1}$ at 485 nm. The drug loading efficiency was estimated according to the following equation

$$\%\text{Drug loading efficiency} = 100 - \frac{[C]Total\ DOX\ loaded - [C]DOX\ released\ at\ time\ T}{[C]Total\ DOX\ loaded} \times 100$$

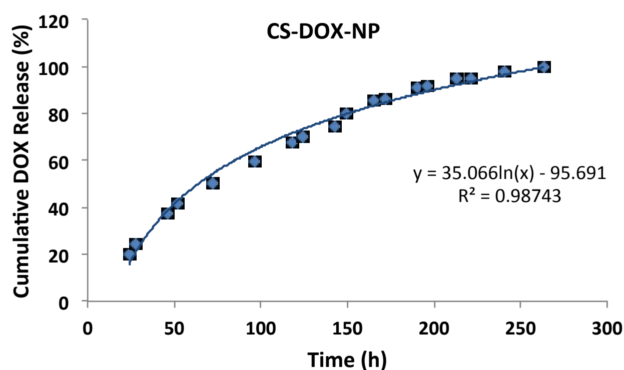

Figure S2. In vitro cumulative release profile of CS-DOX-NP in PBS 7.4 (for 14 days).

Particle size measurement: The particle size distribution was carried out using Malvern laser granulometer (Zetasized Nano ZS, Malvern, United Kingdom). Freeze-

dried CS-NP were dissolved in PBS 7.4 pH at 1 mg/ml concentration and stirred at room temperature for 3 h before performing the DLS measurement.

Cell culture experiments

MCF-7 and HCT116 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Fetal bovine serum (FBS) (Gibicol) and 1% antibiotics (10.000 U penicillin and 10 mg/ml streptomycin, Sigma). Cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

Quantification of CD44 receptors

HCT116 and MCF-7 cells were detached by addition of 1 ml 0.25% trypsin and incubated at 37 °C for 5 min. After detachment, 5 ml complete DMEM was added and cells were centrifuged at 1000 rpm, 5 min and washed with cold PBS. Thereafter, 5×10^6 cells were collected and incubated with 200 µl monoclonal anti-CD44-FITC antibody at the concentration of 2 µg/ml. Cells were washed with cold PBS thrice and re-suspended in FACS buffer. 10,000 cells were analyzed by Flow cytometer BD LSR II (BD Biosciences) with 488 nm excitation laser and fluorescence detection in the PE channel. The data was analyzed using FlowJo (flow cytometry analysis software). All the experiments were performed in triplicates.

Cell viability analysis

Cell viability was measured using, ApoTox-Glo™ Triplex Assay kit following manufacturer's protocol. Briefly, HCT116, and MCF-7 cells were seeded in 384-well BD Falcon™ black microplates (1000 cells in 50 µL/well) using automated Biomek FX pipetting workstation and incubated at 37 °C for 24 h for cell attachment. Stock

solution of DOX (in DMSO) and CS-DOX-NP (in cell culture medium (DMEM)) were prepared separately. After an overnight cell attachment, different volume of stock solution was added to each well using non-contact acoustic dispenser (Echo 555), to obtain a gradient concentration ranging from 25 nM to 2 μM and incubated for additional 48 h at 37 °C. After 48 h, 20 μL of medium was treated with 5μL of cell viability assay reagent, and incubated for 30 min at 37 °C. Fluorescence values were recorded at two wavelength sets: 400Ex/505Em (viability) and 485Ex/520Em (cytotoxicity) using Envision Multilabel Plate Reader and the cell viability were obtained as a percentage of the untreated control (100% cell viability). All the cell experiments were performed in triplicate.

Confocal microscopy and image analysis of cellular localization: Cellular localization of CS-NP was analyzed using confocal microscopy. Cells were cultured on tissue-culture treated glass chamber slides (BD Biosciences) at a concentration of 100,000 cells/2 ml and allowed to attach overnight before replacing the medium with fresh medium containing 1 mg/ml of CS-NP. Images were made of the glass plates with cover slips after 4 h at 63x magnification with a Zeiss LSM 510 META confocal microscope, using excitation at 488 nm and emission at 530 nm.