Supporting Information

Chondroitin Sulfate Coated Gold Nanoparticles: A New Strategy to Resolve Multidrug Resistance and Thromboinflammation

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Experimental Section

Materials and methods: Chondroitin sulfate-A (CS) sodium salt from bovine trachea and all other reagents and solvents were purchased from Sigma-Aldrich (Sweden) and used as received. Doxorubicin hydrochloride was purchased from Tocris Bioscience, UK. Dialysis membranes used for purification of HA-conjugates were purchased from Spectra Por-3 (MWCO 3500). Monoclonal FITC mouse anti-human-Pgp Ab (clone 17F9) and FITC mouse-IgG_{2b} isotype control (Clone 27-35), was purchased from BD Pharmingen. 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. All the cell lines were obtained from American Type Culture Collection (ATCC)-LGC standards, Sweden. The NMR experiments (δ scale) 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 ^1H. The NMR spectra for all CS conjugates were recorded in D_2O at 293 K. The particle size determination was performed in Zetasizer Nano ZS from Malvern at 25 °C and UV-Vis spectroscopy was performed in Lambda 35 UV-Vis spectrophotometer from PerkinElmer. SEM images of CS coated nanoparticles were recorded using SEM, LEO 1550, Zeiss.

Synthesis of CS-DTPH: CS-DTPH conjugates was synthesized using cabodiimide chemistry with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl) as coupling reagent and HOBt as nucleophilic catalyst. Briefly, CS (300 mg, 0.75 mmol with respect to the disaccharide repeat units) was dissolved in 100 ml of deionized water followed by the addition of 3,3'-dithiobis(propanoic hydrazide)

(DTPH; 17.85 mg, 0.075 mmol) and HOBt (101.3 mg, 0.75 mmol) and stirred at room temperature until the reaction becomes homogeneous (30 min.). Thereafter, pH of the resultant solution was adjusted to 4.7 and solid EDC.HCl (43.13 mg, 0.225 mmol) was added and stirred overnight. The solution was loaded into a dialysis bag (Spectra Por-3, MWCO 3500) and dialyzed against dilute HCl (pH = 3.5) containing 0.1 M NaCl (2 × 2L, 48 h), followed by additional dialysis against deionized water (2 × 2L, 24 h). The solution was lyophilized and 295 mg of CS-DTPH conjugate was obtained. The degree of modification was found to be 13% by ^{1}H NMR and 14.5% by TNBS assay using the excitation wavelength of 502 nm.[1] ^{1}H NMR (400 MHz, D_2O): δ = 1.99 ppm (s, 3 H), 2.82 ppm (br s, 0.57 H), 3.01 ppm (br s, 0.51 H) 3.37 – 4.19 ppm (m, 10 H), and 4.53 (br s, 2 H). CS-DTPH was reduced to CS-SH for ^{1}H NMR studies using dithiothreitol as a reducing agent following reported protocol using different batch of CS-DTPH with higher modification.[2]

Synthesis of CS-Au-NP: CS-Au-NP conjugate was synthesized at 40 °C using CS-DTPH as both a reducing agent and a capping agent. Briefly, $HAuCl_4.3H_2O$ (21.0 mg, 0.053 mmol) was dissolved in 20 mL of deionized water and refluxed for 2 h. CS-DTPH (80.0 mg, 0.16 mmol) was dissolved separately in 40 mL of deionized water and pH of the solution was adjusted to 11.0 using 1 M NaOH. The refluxed gold solution was brought to 40 °C and CS-DTPH solution (pH 11.0) was quickly added under vigorous magnetic stirring. The formation of NP was initiated within 2 h, and the reaction was continued overnight. Formation of CS-Au-NP could be observed by the appearance of a deep purple color. This CS-Au-NP solution was subsequently loaded into a dialysis bag (Spectra Por-6, MWCO 3500) and dialyzed against deionized water (2 × 2L) for 24 h and lyophilized to obtain 88 mg of CS-Au-NP as a purple-violet fluffy material. The λ_{max} for CS-Au-NP was found to be 540 nm using UV spectrophotometer. The ^{1}H NMR analysis of the product showed two sharp triplets indicating the reduction of disulfide bonds and complexation of gold nanoparticle with thiol groups. ^{1}H NMR (400 MHz, D_2O): δ = 1.99 ppm (s, 3 H), 2.79 ppm (t, J = 7.32 Hz, 0.39 H), 3.19 ppm (t, J = 7.32 Hz, 0.42 H), 3.37 – 4.19 ppm (m, 10 H), and 4.57 (br s, 2 H).

Synthesis of CS-Au-DOX: DOX was loaded to CS-Au-NP by dropwise addition of DOX solution to CS-Au-NP. Briefly, lyophilized CS-Au-NP (50 mg, 0.1 mmol) was

dissolved in 25.0 mL of acetate buffer (pH 5.0) and 5 mg of DOX.HCl (in 2 mL DMSO) was added. The reaction was stirred overnight in dark at room temperature. The reaction mixture was loading into a dialysis bag (Spectra Por-6, MWCO 3500) and dialyzed against deionized water containing 0.1 M NaCl (2 × 2L, 24h) followed by dialyzed against deionized water (2 × 2L, 24h). The solution was lyophilized and 52 mg of CS-Au-DOX was obtained.

Optimization of the synthesis of CS-Au-NP: The molar ratios of gold chloride and CS-DTPH (with respect to disaccharide repeat unit) were optimized to obtain a size below 100 nm. The results are summarized below.

Table S1: Optimization Table

$HAuCl_4$:CS-DTPH	Average size (nm)		PDI[a]
	Before lyophilization	After lyophilization	
1:1	180	178.8	0.40
1:2	142	125	0.301
1:3	80	98.45	0.205

[a]Polydispersity index of the lyophilized product after resuspending in water

Stability studies of CS-Au-NP: The stability of CS-Au-NP was evaluated by UV method by measuring the absorbance at 540 nm at different time point following reported protocol.[3] Briefly, 1 mg of CS-Au-NP was dissolved in 1.0 ml of PBS buffer pH 7.4, PBS buffer pH 5.0 and PBS buffer pH 7.4 containing 20% FBS. Time dependent absorbance was measured at 540 nm by UV/VIS spectroscopy. The CS-Au-NP remained well dispersed and stable under all the conditions tested. Results are summarized in Table S2 in SI.

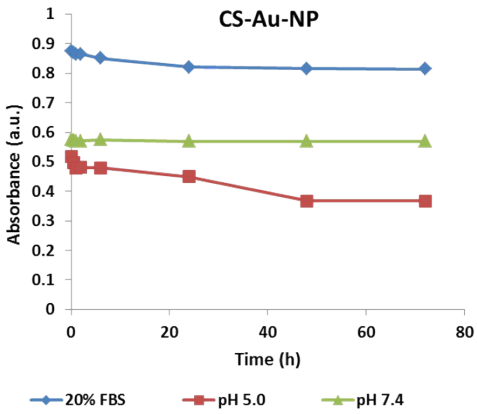

Figure S1. Stability studies of CS-Au-NP under different conditions.

Table S2. Stability studies of CS-Au-NP under different condition

Condition	Avg. Size (nm)	ζ (mV)	PDI	Avg. Size in 24 h (nm)
PBS pH 7.4	61.85	-27.3 ± 0.6	0.319	57.23
PBS pH 5.0	89.63	-23.3 ± 0.283	0.299	77.64
PBS pH 7.4 with 20% FBS	92.34	-20.3 ± 0.4	0.328	91.60

Scanning electron microscopy: For SEM experiments a small portion of CS-Au-NP or CS-Au-DOX suspension in water was placed on a silicon wafer chip and air-dried overnight. Subsequently, it was placed in vacuum at 15 kV. The micrographs were taken by using a Zeiss Scanning Microscope LEO- 1550.

Transmission Electron Microscopy: The transmission electron microscopy (TEM) investigation was carried out using an FEI, TECNAI G^2 F30, S-TWIN microscope operating at 300 kV equipped with a GATAN Orius SC1000B CCD camera.

Determination of DOX loading: As the UV absorbance of DOX overlap with gold signal, the DOX loading was quantified by dissolving a known amount of CS-Au-DOX in acetate buffer pH 4.0 and incubated for 30 min followed by centrifugation at 14000 rpm for 15 min. The supernatant was removed and sample was replenished with fresh acetate buffer and the process was repeated till DOX was undetected in the supernatant. The supernatant was pooled and the final DOX concentration was

estimated using the extinction coefficient of 11,500 $M^{-1}cm^{-1}$ at 480 nm. The percentage of DOX loading was found to be 6.2% by weight.

Dynamic light scattering experiment: The particle size distribution of CS-Au-NP and CS-Au-DOX was carried out using Malvern laser granulometer (Zetasized Nano ZS, Malvern, United Kingdom). Freeze-dried samples were dissolved in deionized water at 1 mg/ml concentration and stirred at room temperature for 30 min. before performing the DLS measurement.

DOX release experiment: The assessment of release kinetics of DOX from CS-Au-DOX was performed by dialysis method. Briefly, 3 mg of CS-Au-DOX was dissolved in 1.0 ml of PBS buffer (pH 7.4) or acetate buffer (pH 5.0) containing 2% Tween 20 and transferred to Slide-A-Lyzer® MINI Dialysis Device, (3.5K MWCO, 2 ml). This device was placed in a glass tube with 10 ml buffer, instead of falcon tube to prevent DOX adhesion to plastic surfaces. The samples were protected from light and placed on a shaker (100 rpm) at 37 °C for 72 h and the UV absorbance of the released media at 480 nm was measured at different time points. The release media was placed back to the dialysis chamber after each measurement. The amount of DOX released was analyzed by UV spectroscopy. The percentage of release in each case was plotted using excel software.

Estimation of Pgp expression levels by flow cytometry: Cells in the log phase were seeded onto a culture plate with appropriate cell number and incubated at 37 °C overnight. After 24 h incubation, medium was removed and washed with 1 x PBS, the cells were harvested by trypsin and resuspended in DMEM medium. The cells were then centrifuged at 1000 rpm for 5 min. and was subsequently washed with 3 mL cold PBS containing 10% FBS. Then $5x10^5$ cells per ml were freshly suspended in cold FACS buffer (10% FBS in PBS). To this cell suspension, 2 µL of FITC mouse anti-human-Pgp Ab (BD Pharmingen, clone 17F9) or 2 µL of FITC mouse-IgG_{2b} isotype control (BD Pharmingen, Clone 27-35) was added into each FACS tube and was incubated at 4 °C for 30 min. The cells were washed once with PBS and then re-suspended in FACS buffer. FACS measurement was performed under FITC channel using CyAn ADP Analyzer (Beckman Coulter).

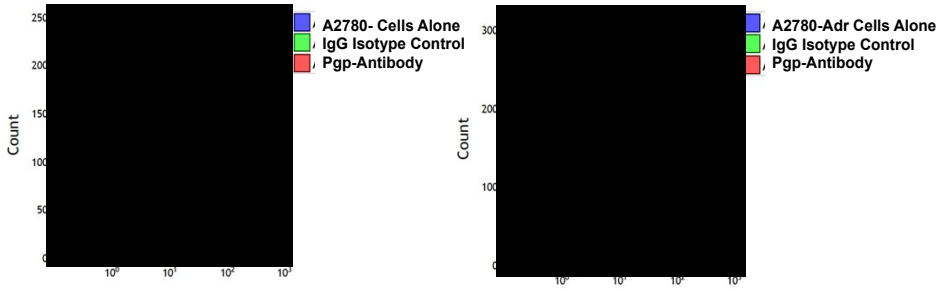

Figure S3. FACS Histogram displaying P-glycoprotein expression levels.

Confocal Microscopy: Monolayers of HCT116 cells were seeded in 8-well chamber plates (2000 cells in 200 µL/well) overnight at 37 °C. After 24 h incubation, cell culture medium (DMEM containing 10% FBS) was replaced with fresh medium containing 25 µg/mL of free DOX or CS-Au-DOX (25 µg/mL DOX equivalent) was added. After 4 h at 37 °C, the medium was removed and washed twice with blocking buffer (PBS, 0.5% FCS). Thereafter, cells were fixed and permeabilized by treating with a 4% (w/v) solution of paraformaldehyde in phosphate buffer for 20 min at 4 °C and washed twice with blocking buffer. Nuclei were stained with DAPI and cells were analyzed by confocal laser scanning microscopy.

Cytotoxicity studies: Cell viability was measured using, ApoTox-Glo™ Triplex Assay kit following manufacturer's protocol. Briefly, HCT116, GP5D, A2780 and A2780-Adr 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 and CS-Au-DOX was prepared in DMSO and cell culture medium (DMEM) respectively. After 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, containing both GF-AFC substrate and bis-AAF-R110 substrate 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. The IC_{50} was estimated by

logarithmic curve fitting of cell viability (%) using Graphpad Prism software against DOX equivalents.

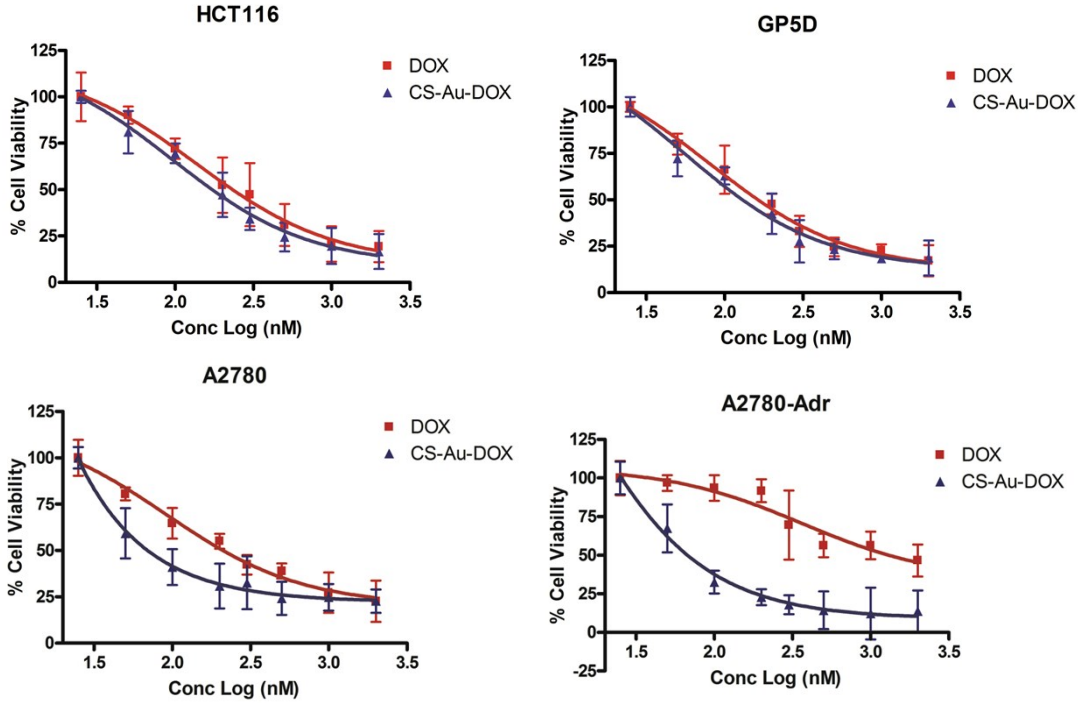

Figure S4. Dose dependent cytotoxicity profile of different cell lines tested.

Hematological evaluation of DOX and CS-Au-DOX in human blood: The toxicity evaluation of DOX and CS-Au-DOX to human platelets and its effect on activation of coagulation factors were performed, using the Chandler loop model as described earlier.[4] In brief, the entire material coming in contact with blood was heparin-coated (Corline Systems AB, Uppsala, Sweden) in advance to ensure that no surface-induced activation takes place. Fresh human non-anticoagulated whole blood samples were obtained from three healthy volunteers who were medication-free for at least 10 days prior to donation. Ethical approval was obtained from the regional ethics committee. Blood was freshly drawn with an 18-G needle connected to silicon tubing allowing the blood to run into collection containers. 1 mL of fresh blood sample was mixed with 60 µM DOX or DOX equivalent of CS-Au-DOX dissolved in PBS 7.4 and enclosed in heparinized tubing coated with Corline® heparin. Equal amount of CS-Au-NP dissolved in PBS 7.4 and identical volume of PBS 7.4 are used as control. The tubing was closed with a Corline® heparinized connector and incubated at 37 °C in heated rocking apparatus for 1 h to simulate blood flow. The blood was subsequently

evacuated from the tubing and poured into cups, where clot formation was registered. The blood was carefully transferred to eppendorf tube with 10 μM EDTA solution (pH 7.4). Platelet count was obtained for each sample using a cell counter (Coulter AcT Diff TM hematology analyzer; Coulter Corporation, Miami, FL, USA). The blood was centrifuged at 2500 x g for 15 min at 4 °C. Plasma samples were collected and stored at -80 °C before analyzing the concentrations of thrombin-antithrombin (TAT) complexes using a commercially available ELISA kit as described previously.[4]

Measurement of blood activation markers: FXIIa-C1INH, FXIa-C1INH, FXIIa-AT, FXIa-AT complexes were measured by sandwich ELISA according to the method of Sanchez et al.[5] Briefly, microtiter plates were coated with either goat anti-human FXII (Enzyme Research Laboratories) or sheep anti-human FXI (The Binding Site, Birmingham, UK). The bound complexes with either biotinylated goat anti-human C1-INH (Enzyme Research Laboratories) or biotinylated rabbit anti-human AT (Dako), followed by HRP-conjugated streptavidin (GE Healthcare). Standards for each assay were prepared by mixing purified FXIIa or FXIa with a molar excess of AT or C1-INH (in the presence of heparin) and followed by measuring residual FXIIa or FXIa activity. These standard solutions were diluted in normal plasma and included in each analysis. The values of the complexes are expressed as nmol/L.

References

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