Development of drug loaded nanoparticles for tumor targeting. Part 1: Synthesis, characterization, and biological evaluation in 2D cell cultures

Supporting Information

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I. Experimental procedures

1.1. Synthesis of the FITC-doped silica nanoparticles (SNP)

FITC-APTES conjugate was synthesized by reacting FITC (12.3 g, 0.032 mmol) and APTES (70 mg, 0.32 mmol) in ethanol (1 ml) for 18 h at room temperature in the dark to avoid photo-bleaching. A water/oil microemulsion was prepared by stirring a mixture of cyclohexane (7.7 ml), Triton® X- 100 (1.77 g), n-hexanol (1.6 ml), and DI water (0.34 ml) in a 50 ml round bottom flask for 20 minutes. The fluorescent core was formed by polymerizing the FITC-APTES conjugate (100 µl) using 30% NH_4OH (200 µl) and stirring the mixture for 6 h. TEOS (200 µl) was added and the mixture was stirred for 18 h. The surface of the NPs was decorated with amino groups by adding APTES (50 µl) and 30% NH_4OH (50 µl) and stirring the mixture for 6 h. THPMP (20 µl) was added and the mixture was stirred for 18 h. The SNPs were precipitated by adding ethanol (50 ml) and collected by centrifugation. The nanoparticles were repeatedly washed and centrifuged with ethanol (5 x 30 ml) and water (3 x 30 ml). The washing steps were associated with sonication to remove any adsorbed FITC. SNP (45 mg) was produced, and its fluorescence properties assessed by UV-vis and fluorescence spectroscopy.

1.2. Synthesis of HA-SNP

HA (31 kDa) (50 mg) in its acid form, was dissolved in dd water (3ml) by sonication followed by the dropwise addition of acetonitrile (2 ml). NMM (15 µl) was added and the solution was cooled to 4°C in an ice bath. CDMT (15 mg) was added and the mixture was stirred at room temperature for 2 h to activate the carboxylic acid groups on HA. SNP (15 mg) was added and the mixture was stirred at room temperature for 72 h. The pH was then adjusted to 7 using Amberlite H^+. The mixture was filtered and diluted by adding dd water and purified by ultrafiltration (MWCO 100,000) to remove the excess starting material and side products. HA-

SNP (55 mg) was collected with its fluorescence properties assessed by UV-vis and fluorescence spectroscopy.

1.3. Synthesis of DOX-HA-SNP

To a solution of FITC-doped HA-SNP (15 mg) dispersed in dd water (10 ml) was added ADH (75 mg), and the pH was adjusted to 4.5-5 by the addition of 0.1 N aqueous HCl solution. EDCI (2 mg) was added and the solution was stirred at room temperature for 4 h during which the pH was maintained between 4.5 and 5. The pH was then adjusted to 7 using 0.1 N aqueous NaOH solution. The ADH-functionalized HA-coated nanoparticles (ADH-HA-SNP) were collected by centrifugation and washed with dd water (5 times) to remove the undesired reagents. After the final wash, the nanoparticles were resuspended in 0.1 M acetate buffer (pH 6.0, 5 ml) and sonicated for 30 minutes. DOX (3 mg), dissolved in the same acetate buffer (3 ml), was added and the solution was stirred at room temperature for 48 hours in the dark. The nanoparticles were then collected by centrifugation and washed with water (5 times). Each wash was accompanied with sonication to ensure the removal of any adsorbed DOX. The collection of orange-colored nanoparticles suggested the successful conjugation of DOX on the nanoparticles (See Figure 1c in main text). The concentration of the prepared stock solution of DOX-HA-SNP was 0.85 mg-NP/ml. The amount of conjugated DOX was assessed by UV-vis spectroscopy. To eliminate the interference from FITC, equivalent amount (by mass) of FITC-doped HA-SNP was used to collect a baseline that was then subtracted from the DOX-HA-SNP reading. The percentage of DOX on the surface of nanoparticles was calculated to be 0.6% (w/w) as follows:

$A=E\ b\ C_{DOX}$; $0.12= 12500 \times 1 \times C_{DOX}$; $C_{DOX}= 9.6 \times 10^{-6}$ M

<u>In 100 µl stock solution</u>: $m_{DOX}= C \times V \times M= 9.6 \times 10^{-6}$ M $\times$ 0.1 $\times 10^{-3}$ L $\times$ 543.52 mol/L;

m_{DOX}= 0.520 x 10^{-6} g =0.520 x 10^{-3} mg; In 1 ml stock solution: **[DOX]= 5.20 x 10^{-3} mg/ml**

The percentage of DOX (w/w) on NPs: % DOX on SNPs= [(5.09 x 10^{-3})/0.85] * 100; **%DOX (w/w) =0.6%**

1.4. Kinetics of HA-SNP uptake by laser confocal microscopy

SKOV-3 cells (2 x 10^5 cells/well) were cultured in a 4-well chambered plate at 37°C and 5% CO_2 for 24 h. The culture media was removed and the cells were washed with PBS (2 times). HA-SNP nanoparticles (working concentration: HA-SNP: 42 µg/ml; 1 ml) in serum-free DMEM were added. The cells were incubated with the nanoparticles for desired time. The supernatant was removed. The cells were washed twice with PBS, and fixed with 10% formalin (0.5 ml/well) for 15 min. Formalin was removed and the cells were washed twice with PBS. DAPI (300 nM, 300 µl/well) were added, and the cells were incubated for 4-5 min. DAPI solution was removed, and the cells were washed with PBS and dd water. The plate was covered by an aluminum foil and stored at 4°C till imaging time. Images were gathered on an Olympus FluoView 1000 LSM confocal microscope.

1.5. Kinetics of HA-SNP uptake by flow Cytometry

SKOV-3 cells (2 x 10^5 cells/well) were allowed to attach in a 24-well plate overnight at 37°C and 5% CO_2. The cells were washed twice with PBS, and HA-SNP were added (working concentration: HA-SNP: 42 µg/ml; 1 ml) in serum-free DMEM were added. The plate was incubated for desired time at 37°C and 5% CO_2. The cells were then washed with PBS (3 times) and trypsinized with 0.25% trypsin-EDTA (0.5 ml/well). Trypsin was neutralized with serum-containing DMEM (5 times), and the cells were collected by centrifugation (2500 rpm; 4°C).

The cells were resuspended in serum-containing DMEM (300 µl) and transferred to FACS tubes. The cells were stored on ice till the time of FACS analysis.

1.6. Determination of cellular uptake of SNP by flow cytometry

SKOV-3 cells (2 x 10^5 cells/well) were allowed to attach in a 24-well plate overnight at 37^{o}C and 5% CO_2. The cells were washed twice with PBS, and nanoparticles of equivalent fluorescence were added (working concentration: SNP: 27 µg/ml; HA-SNP: 104 µg/ml) in serum-free DMEM were added. The plate was incubated for 18 h at 37^{o}C and 5% CO_2. The cells were then washed with PBS (3 times) and trypsinized with 0.25% trypsin-EDTA (1 ml). Trypsin was neutralized with serum-containing DMEM (5 times), and the cells were collected by centrifugation (2500 rpm; 4^{o}C). The cells were resuspended in serum-containing DMEM (300 µl) and transferred to FACS tubes. The cells were stored on ice till the time of FACS analysis. Propidium iodide (PI) (100 µg/ml, 3.3 µl) was added at the time of analysis.

1.7. Energy-dependent uptake of HA-SNP

SKOV-3 cells (2 x10^5 cells/plate) were cultured in five 35 mm cell culture plates over night at 37^{o}C and 5% CO_2. The supernatant was removed and the cells were washed twice with PBS. Two plates received SNP (47.5 µg/ml, 1ml) while another two plates received HA-SNP (21.25 µg/ml, 1ml). The fifth plate received serum free-DMEM and was used as a control. One set of SNP and HA-SNP-receiving plates was incubated at 37^{o}C while the other set was incubated at 4^{o}C for 3 h. The nanoparticles were removed and the cells were washed thoroughly with PBS (5 times). The cells were collected using trypsin (0.5 ml/plate) and centrifugation (2500 rpm, 4^{o}C). The cells were washed with serum containing DMEM and centrifuged four times. The cells were resuspended in serum containing DMEM (400 µl), and stored on ice till time of analysis. FITC fluorescence was assessed on a flow cytometer.

1.8. Examining the uptake of HA-SNP in the presence of β-CD

SKOV-3 cells (2 x10^5 cells/plate) were cultured in 24-well cell culture plate over night at 37^{o}C and 5% CO_2. The culture medium was removed and the cells were washed with PBS. Some cells were incubated with β-cylcodextrin (5 mM, 1ml) in serum-free medium for 1 h. Other cells received serum-free medium (1ml). HA-SNP (1.7 mg/ml; 10 µl) was then added to all cells (except negative control well), and the plates were incubated at 37^{o}C for 2 h. The cells were washed with PBS, collected using trypsin (300 µl/well), and washed with serum-containing medium. The cells were finally suspended in serum-containing DMEM, transferred to FACS tubes, and stored on ice till analysis time. Propidium iodide (PI) (10 0 µg/ml, 4 µl/tube) was added right before analysis.

1.9. In vitro release of DOX from DOX-HA-SNP

Equal amounts of lyophilized DOX-HA-SNP (0.45 mg) were suspended in PBS (pH 7.4) or PBS (pH 4.5) to a final volume of 1 ml. At specific time points, the tubes were centrifuged. 100 µl samples of the supernatant were drawn from each tube and transferred to a 96 well black plate (clear bottom). The release of DOX was assessed by measuring the intrinsic fluorescence of DOX on a plate reader (excitation wavelength 483 nm, emission wavelength 580 nm). When the measurement was done, the samples were returned to their respective tubes. To determine the total amount of DOX in the sample, 6N HCl (1.3 ml) was added to the nanoparticles to release the cargo. The amount of DOX that remained was determined by measuring fluorescence of the resulting solution. The fluorescence of unreleased DOX was added to that of the highest measured released DOX. The percentage of DOX released at a given time point is:

% DOX released = [(Fluorescence of released DOX)/Total fluorescence] x 100

1.10. Monitoring the Uptake of DOX and DOX-HA-SNP by confocal microscopy

SKOV-3 cancer cells (2 x 10^5 cells/well) were cultured in a 4-well plate and incubated at 37^{o}C and 5% CO_2 overnight. The supernatant was removed and the cells were washed. DOX-HA-SNP (40 μg-NP/ml; 1 ml) or the equivalent amount of DOX was added to two wells. A third well did not receive NPs or DOX and served as a control. The cells were incubated for 18 h. The supernatants were then removed and the cells were washed with PBS four times, fixed with formalin (0.5 ml/well) for 15 min, and washed again with PBS twice. DAPI (300 nM, 300 μl/PBS) was added to the cells for 5 min, followed by washing the cells with PBS and DI water twice each. The plate was covered with aluminum foil and stored at 4^{o}C in the dark till imaging time. Images were collected on an Olympus view microscope.

III. Supporting figures and tables

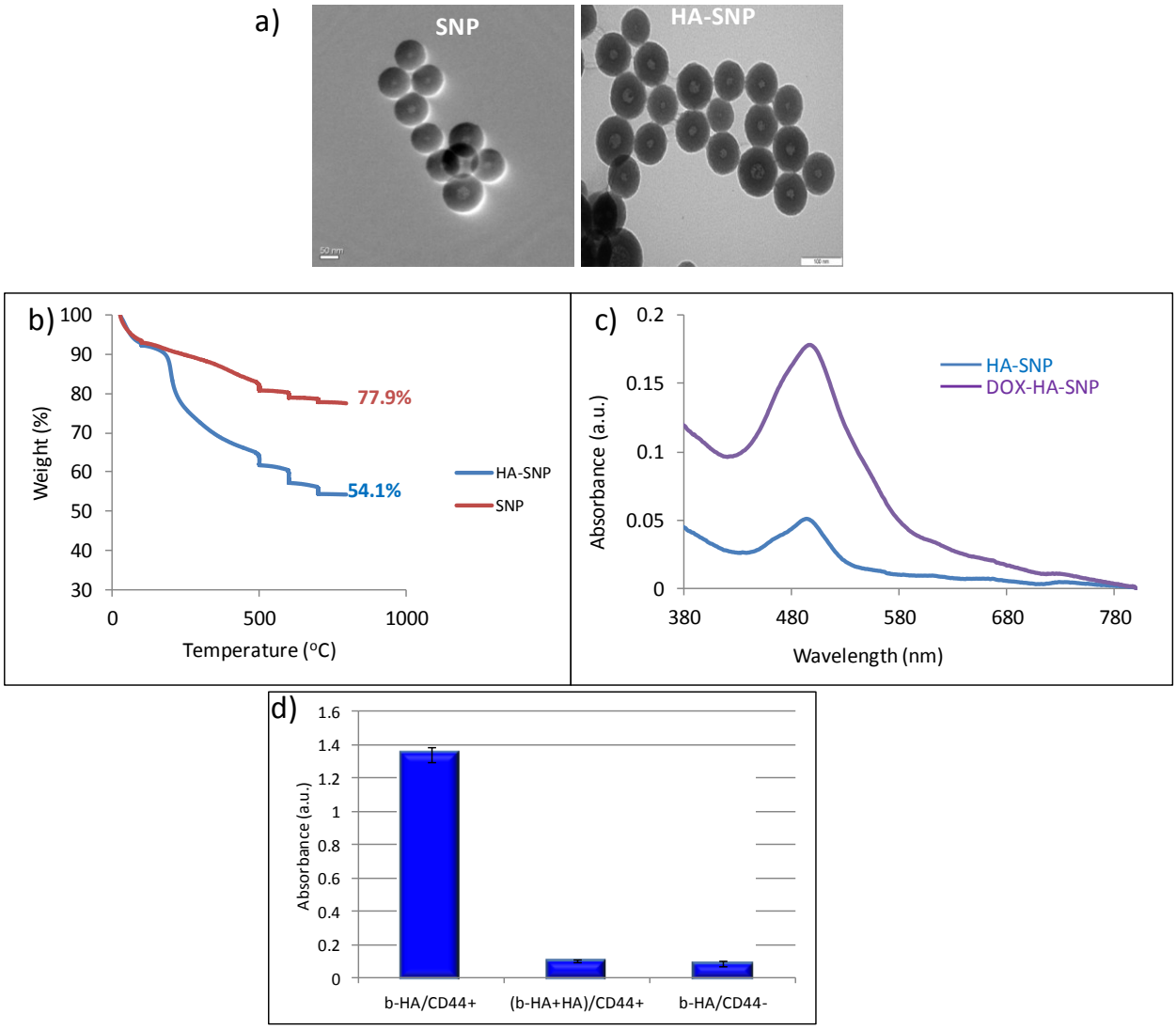

Fig. S1. Characterization of SNPs; (a) TEM of SNP (scale bar 50 nm) and HA-SNP (scale bar 100 nm); (b) TGA supporting the successful immobilization of HA on the surface of SNP. The percentage of HA coating on SNP was 31%. (c) UV-vis spectra of DOX-HA-SNP and HA-SNP at the same NP concentration. (d-e) Competitive ELISA assay demonstrating the HA polymer retained its intrinsic binding to CD44 after conjugation to SNP. (d) HA (31 kDa) polymer competed with biotinylated HA (b-HA) for binding with CD44. Maximum absorbance was obtained when b-HA was added to CD44 positive wells (b-HA/CD44+). However, minimal signal was collected in the absence of CD44 (b-HA/CD44-). When HA polymer was added with b-HA, the binding of the latter dropped dramatically (b-HA+HA/CD44+).

HA loading calculation based on TGA data

SNP has 77.9% by weight the inorganic core and 22.1% the organic coating from the TGA weight loss. Upon HA immobilization, the weight of the total organic coating increased to 45.9%. Solving equation $(0.221+x)/(0.221+0.779+x) = 0.459$ gave x a value of 0.44. Thus, the weight of HA on HA-SNP was $0.440/(0.587+0.413+0.44)*100\% = 31\%$.

Sample	Hydrodynamic radius		ζ-potential (mV)	% Weight loss (TGA)
	Z-Average Size (nm)	PDI		
SNP	88	0.173	-40.4 ± 8.0	22.1
HA-SNP	112	0.218	-52.0 ± 4.6	45.9
DOX-HA-SNP	124	0.333	-46.4 ± 7.7	ND

Table S1. Summary of the hydrodynamic radii, polydispersity indices (PDI), zeta potentials, and TGA data for SNP, HA-SNP, and DOX-HA-SNP. ND: not determined. The TGA data was not collected for DOX-HA-SNP due to the small weight change from DOX immobilization (0.6% w-DOX/w-NP by UV-vis).

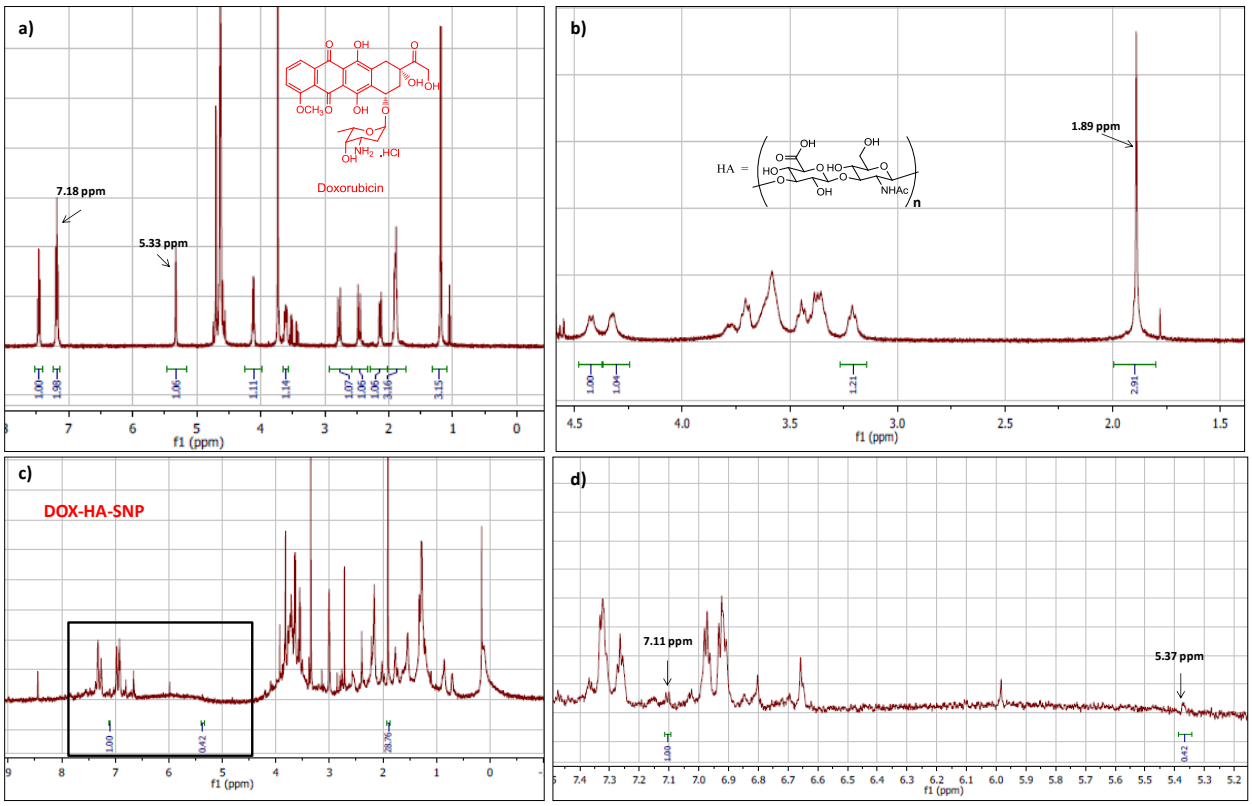

Fig. S2. NMR spectra of free DOX (a), free HA polymer (b), and DOX-HA-SNP (c). The inset box in (c) is expanded in spectrum (d) to show the characteristic peaks of DOX at 7.11 ppm and 5.37 pm (see spectrum (a) for comparison). Note that the anomeric protons from HA, shown at 4.3 ppm and 4.45 ppm in spectrum (b) did not show up in the spectrum of DOX-HA-SNP due to the suppression of the water peak. The NMR spectra were used to confirm the successful conjugation of DOX onto NPs. An accurate quantification of DOX loading on NPs was assessed from UV-vis absorbance spectra which indicated 0.6% loading (w-DOX/w-NP).

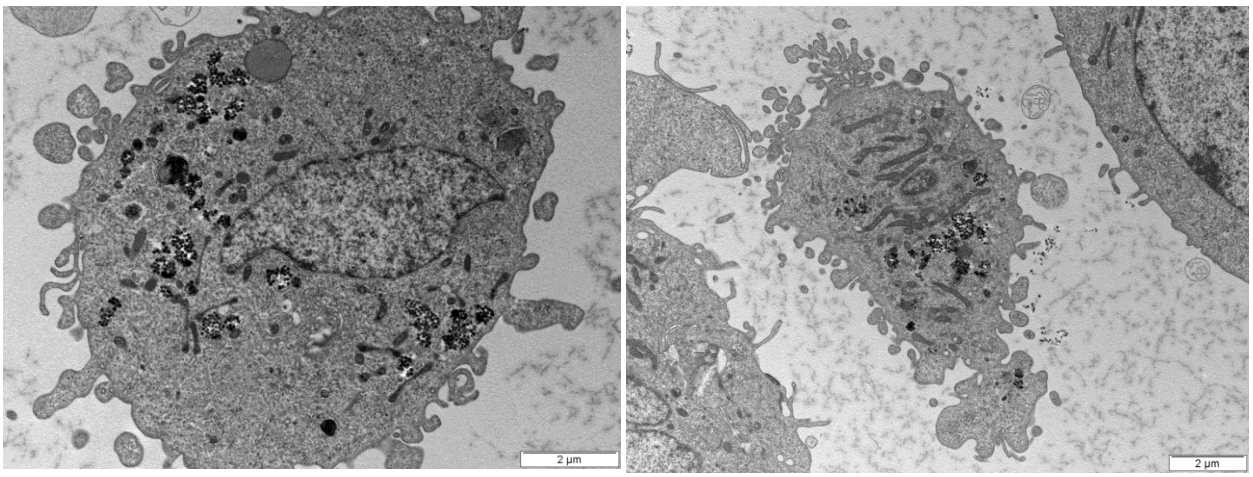

Fig. S3. TEM images of two different SKOV-3 cells incubated with HA-SNP followed by thorough washings. The images clearly indicate that SNPs are localized inside the cells and not on the surface.