Development of drug loaded nanoparticles for tumor targeting. Part 2: enhancement of tumor penetration through receptor mediated transcytosis in 3D tumor models

Supporting Information

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I. Experimental procedures

1.1. Growing cancer spheroids using an optimized hanging drop method

SKOV-3 cancer cells were cultured in a 100 mm cell culture plate at 37^{o}C and 5% CO_2 till 80% confluency. The cells were trypsinized, counted on a cell counter, and centrifuged at 2500 rpm and 4^{o}C for 5 min. Cells were resuspended in serum-containing DMEM containing serum to a concentration of 1000 cells/µl. 20 µl (20000 cells) aliquots of the cell suspension were deposited on the inner surface of a 100 mm petri dish lid. The cover was inverted over the plate containing 10 ml PBS. The plate was incubated at 37^{o}C and 5% CO_2 for 10 days during which the cells accumulated at the bottom of the hanging drop under the effect of gravity causing the formation of disc-like cancer spheroids. To ensure the formation of spherical spheroids, the spheroids were transferred to the bottom of a 24-well plate whose wells are covered with a thin layer of 1% agarose (300 µl) in serum-free DMEM, and containing 0.5 ml serum-containing DMEM. The spheroids were maintained at 37^{o}C and 5% CO_2 for 7 days during which the disc-like spheroids became spherical. NCI/ADR-RES spheroids were grown following the same aforementioned protocol except that RPMI 1640 medium was used instead of DMEM. The three dimensional (3D) morphology of all spheroids was assessed by SEM.

1.2. Preparation of spheroids for SEM imaging

Using a glass pipette, the spheroids were transferred to fine porous holders (Ted Pella, Inc) that are soaked in a container containing 4% glutaraldehyde/PBS and were fixed for 2 hr. The holders were immersed in 0.1 M PBS for 30 min. The spheroids were then dehydrated by placing the holder in serial concentrations of ethanol (25%, 50%, 75%, 95%/water for 30 min each, and then absolute ethanol from a freshly opened bottle for 1 hour). The spheroids were

dried on a critical point dryer, transferred to SEM stubs, and stored under vacuum till time of imaging. The spheroids were then coated with osmium, and imaged on a SEM microscope.

1.3. LIVE/DEAD assay to assess the viability of spheroids

The spheroids were placed on the lid of a cell culture plate, and washed thoroughly with PBS. Each spheroid received 20 µl of LIVE/DEAD reagent containing 2 µM calcein AM and 4 µM ethidium homodimer-1 (EthD1), and incubated for 35 min at room temperature in the dark. The reagents were then removed, and the spheroids were washed with PBS, and transferred to 4-well plates containing serum-containing growth medium. Images were collected immediately (within 30 min) on an Olympus view microscope (FITC channel for calcein AM, Texas red channel for EthD-1).

1.4. Monitoring the uptake of SNPs by SKOV-3 spheroids using flow cytometry

SKOV-3 spheroids were placed on the lid of a 100 mm cell culture plate and washed with PBS. 20 µl of HA-SNP or the equivalent amount of SNP (based on fluorescence) in DMEM was added. PBS (10 ml) was added to the bottom of the cell culture plates to avoid dryness of spheroids during incubation. The spheroids were incubated at 37^{o}C and 5% CO_2 for 6 h. The nanoparticles were then removed and the spheroids were washed with PBS four times. The spheroids were transferred to Eppendorf tubes. Untreated spheroids were used a control. The spheroids were centrifuged and the supernatant was discarded. The spheroids were dispersed using trypsin (200 µl/tube), and serum containing DMEM (800 µl) was then added. The dispersed cells were centrifuged, and washed with serum containing DMEM four times. After the last wash and centrifugation, the cells were dispersed in serum containing DMEM (500 µl), transferred to FACS tubes, and stored on ice till time of measurement. The uptake of nanoparticles was assessed by measuring FITC fluorescence by flow cytometry. For free HA

polymer competition experiments, spheroids were treated with 20 µl of HA in DMEM. The spheroids were incubated in HA polymer at 37^{o}C and 5% CO_2 for 2 h. HA polymer was then removed and 20 µl of HA-SNP or the equivalent SNP (based on fluorescence) in HA/DMEM was added. The spheroids were then treated as indicated above.

III. Supporting figures and tables

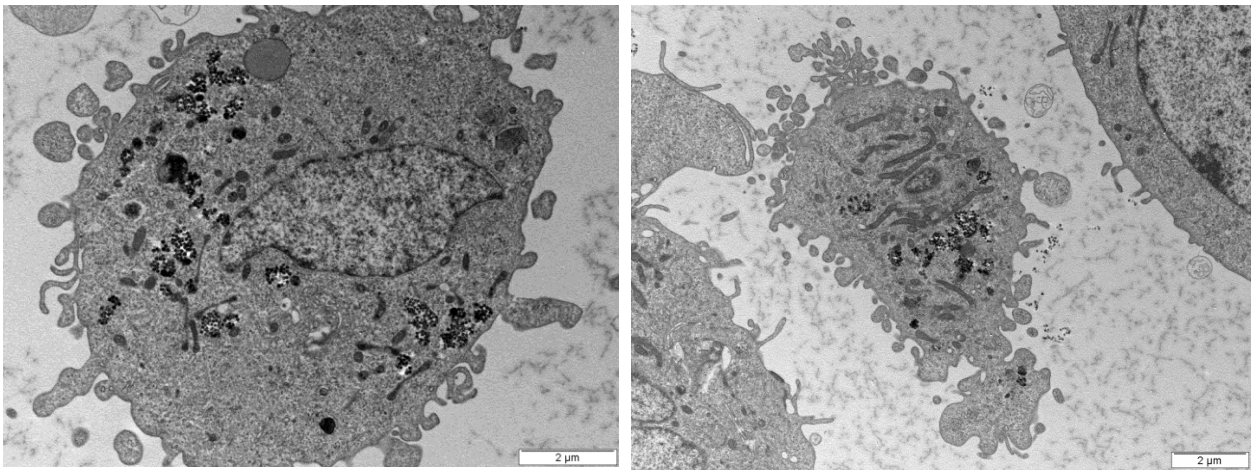

Fig. S1. TEM images of two different SKOV-3 cells incubated with HA-SNP followed by thorough washings. The images clearly indicate that SNPs are localized inside the cells and not on the surface.

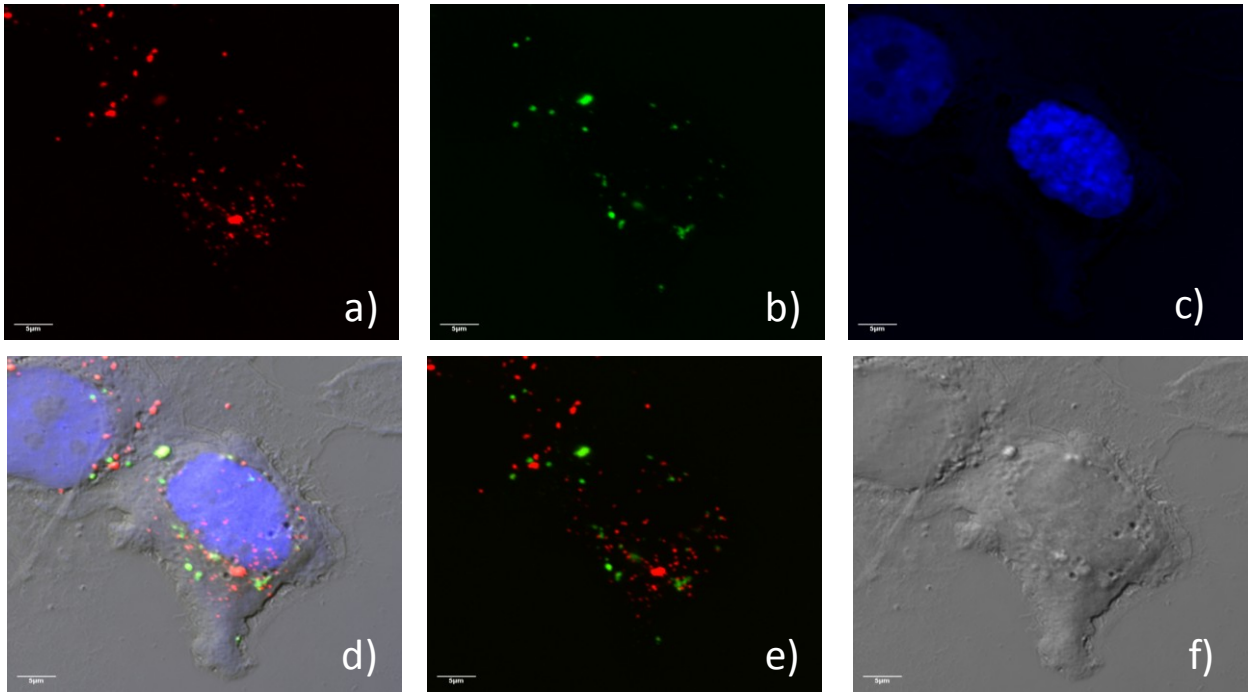

Fig. S2. Confocal images of SKOV-3 cells in the two particle assay. a, RITC channel; b, FITC channel; c, DAPI channel showing location of the nucleus; d, overlay of RITC, FITC, DAPI channels and DIC images; e, overlay of RITC and FITC channels; f, DIC image. The scale bars are 5 µm.

Time (h)	0	12	24	48
Transmembrane resistance (Ω x cm^{-1})				
SNP	117	111	116	114
HA-SNP	121	118	124	122

Table S1. TEER measurements of the MCC at various time points of the transcytosis assay. No significant changes in the transendothelial electrical resistance of the MCC were observed, indicating that the addition of SNPs or HA-SNPs did not affect the junction dynamics of the cells.

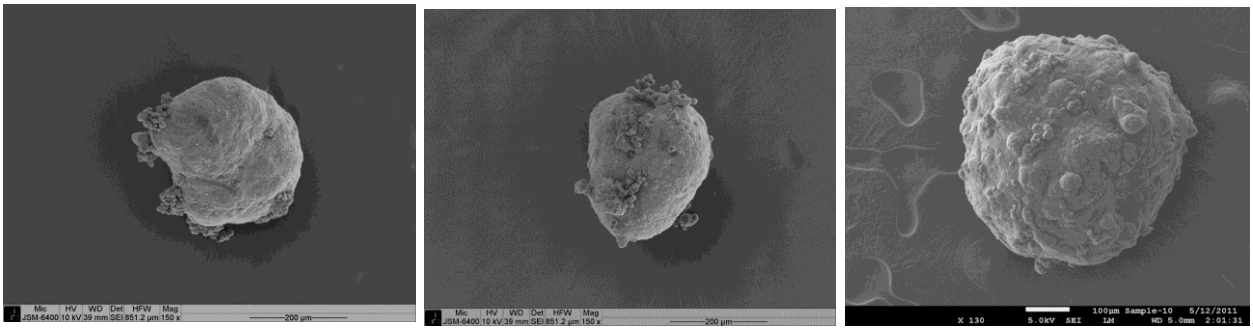

Fig. S3. Representative examples that show the reproducibility of the optimized method to generate fully grown, spherical and compact SKOV-3 spheroids.

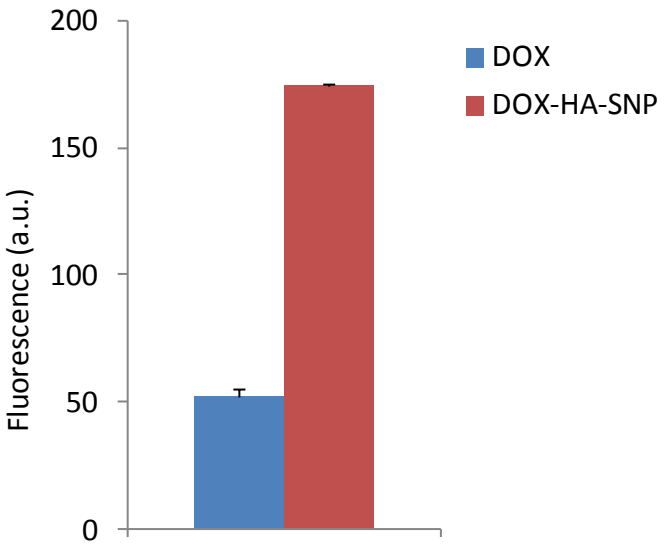

Fig. S4. Fluorescence intensity of DOX (excitation 483 nm, emission 580 nm) in the bottom wells of SKOV-3 MCC 24 hours after adding DOX or DOX-HA-SNP to the chamber above the MCC. DOX-HA-SNP significantly enhanced the ability of DOX to penetrate through the MCC.

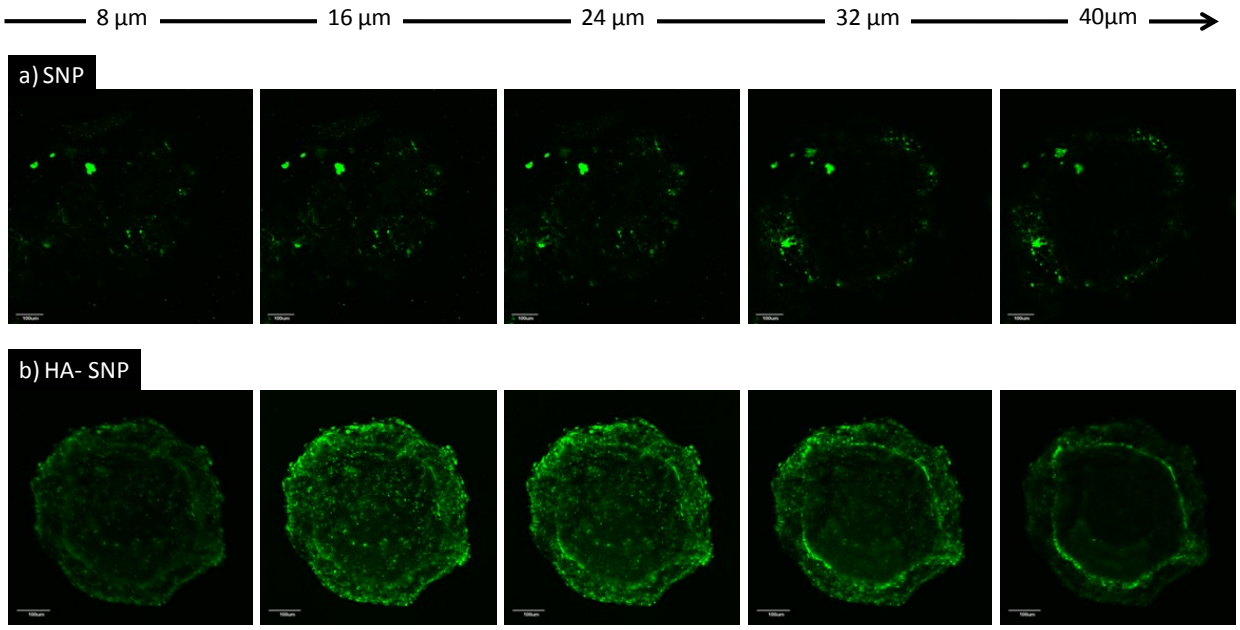

Fig. S5. Z-stake confocal images of NCI/ADR-RES spheroids incubated with a) SNPs and b) HA-SNPs after 18 hour incubation. HA-SNPs penetrated much deeper into the spheroid compared to the SNPs. The scale bar is 100 µm.