Supplementary Material for

Targeted Therapeutic Nanotubes Influence the Viscoelasticity of Cancer Cells to Overcome Drug Resistance

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1. Synthesis, characterization of CAHA-sSWCNT/PEG-sSWCNT formulation and loading of imaging probes / DOX

Semiconducting (6,5) CoMoCAT SWCNTs (Aldrich, carbon >90 %, ≥77% carbon as sSWCNT) were used with intact optical properties, ideal for photo-thermal and photo-dynamic therapy.\(^1\)\(^2\) sSWCNTs dispersion in cholic acid modified hyaluronic acid (CAHA) solution was performed using a VCX-750 ultrasonic processor (Sonics & Materials, Newtown, CT) probe sonicator, with a weight ratio of 1:2 of sSWCNT to CAHA in distilled water (DW) in a 15-mL Falcon tube.\(^3\) First, pristine SWCNTs was added to DW at 1 mg ml\(^{-1}\) in an eppendorff vial and centrifuged for 5 min at 10, 000 rpm and the supernatant was discarded to remove any carbonaceous materials. To this vial a same volume of CAHA (2 mg ml\(^{-1}\)) was added. The ultrasonic probe (6-mm microtip extension, Sonics & Materials) was immersed into the mixture with a volume ranging from 5 to10 mL. The probe was driven at 50% of the instrument’s maximum amplitude for 90 min at ~ 20 kHz with a 10 sec on / 1 sec off pulse sequence. To avoid heating, the solution was immersed in an ice-bath during sonication. The dispersed SWCNTs were subjected to rigorous centrifugation to separate non-wrapped or loosely wrapped SWCNTs, which got settled at the bottom after repeated centrifugation at 10 000 rpm (Figure S1). The settled nanotube solid was discarded and only the supernatant was collected. The purified SWCNT dispersion was further centrifuged to concentrate the sample. For this the sSWCNT dispersion were aliquated in 1.5-mL Eppendorf vials and centrifuged at 10, 000 rpm for 45 min. Supernatant was discarded and the individually dispersed sSWCNT that settled at the bottom were redispersed in either DW or serum, depending on the applications (Figure S2). The CAHA-sSWCNT dispersions were stable for over a year without noticeable precipitation. For imaging probe loading, the CAHA-sSWCNT and the probes were reacted overnight with EDC activation for covalent coupling of
the probes in a slightly basic condition. Since chemotherapeutic DOX is known to adsorb on the sSWCNT through π stacking along with physical encapsulation in the biopolymer, CAHA-sSWCNT dispersion and DOX were simply mixed to load the drug.\textsuperscript{4,5} CAHA-sSWCNT / DOX were mixed in the ratio of 2:1 (w/w), and the resultant reaction mixture was incubated at different pH values. The CAHA-sSWCNT-DOX mixture was kept in a shaker bath at RT overnight, following which drug-loaded CAHA-sSWCNTs were separated by centrifugation and absorbance of the supernatant was measured at 490 nm. DOX loading was calculated spectrophotometrically using the following formula: \[ \% \text{DOX loading efficiency} = \frac{(A_1 - A_2)}{A_1} \times 100, \]
where \(A_1\) = free DOX absorbance and \(A_2\) = absorbance of supernatant after centrifugation. Percentage (%) DOX loading was estimated as \[ \% \text{DOX loading} = \frac{\text{amount of drug loaded}}{\text{weight of the CAHA-sSWCNTs pellet}} \times 100. \]
Multifunctional sSWCNT nanoformula was purified using molecular weight centrifugation using centricon (100K), to remove any unbound/excess FITC/DOX. CAHA-sSWCNT dispersions and their DOX loading were characterized using Raman, UV-vis absorption, zeta potential and fluorescence spectroscopy for purity, optical and surface property and for fluorophore attachment.

PEGylated sSWCNT were synthesized following our previously published protocol.\textsuperscript{2} sSWCNT were coated with phospholipid polyethylene glycol amine (LP) (1,2-distearoyl-sn-glycero-3-phosphoethanolmine-N-[amino(polyethylene glycol)-2000] (ammonium salt) from Avanti Polar Lipids, Inc., AL) in aqueous medium. sSWCNTs (1 mg) were added to an aqueous solution of LP (1 mg ml\(^{-1}\)) and sonication was carried out with the help of an tip sonicator (Qsonica, CT) for 3 h under cold condition. This process resulted in aqueous dispersed PLPEG-sSWCNT. To remove any uncoated sSWCNT, the PEGylated sSWCNT were centrifuged for 2 h at 10,000 rpm. After the centrifugation the bare uncoated nanotubes settled at the bottom of the vial. The
supernatant was recovered, and unreacted PEG was removed using a 5K MW centricon filter and stored for later use. DOX loading was done similar to what is described above for CAHA-sSWCNT-DOX formulation (data not shown).

2. Serum stability using photon correlation spectroscopy and biocompatibility

We introduce a distinctive way of looking into the serum stability of sSWCNT dispersions using photon correlation spectroscopy (PCS), also known as dynamic light scattering (DLS). Routine PCS protocol involves measuring the hydrodynamic size of the nano-dispersions at room temperature in aqueous or serum solutions. However for biological applications serum stability of the nano-dispersions under physiological conditions is important.\(^7\) For this we set up the PCS instrument to operate at physiological temperature (37 °C), which can acquire the size distribution continuously without the need of taking the dispersion sample back and forth. The PCS cuvette was filled with serum containing sSWCNT dispersions (Figure S6) and measured the hydrodynamic size distribution for over a period of time, taking measurements every hour. The data obtained shows that the nanotube dispersions are quite stable in serum containing media with negligible fluctuations in the size distribution over a period of 50 h, which was also reflected in our cell viability assay (Figure S7).

3. Photothermal analysis of CAHA-sSWCNT formulations

sSWCNT are known to absorb near infrared light (NIR, \(\lambda = 650-900\) nm) efficiently at their M11 band of the metallic SWCNTs and the S22 band of the sSWCNT. The NIR laser can penetrate deep into the tissue and is relatively harmless since minimal light is absorbed by hemoglobin (<650 nm) and water (>900 nm).\(^1\) sSWCNT formulations were assessed for the photothermal properties. For this a NIR 808 laser probe was shone on different concentrations of
sSWCNT dispersions at power of 1 W/cm$^2$ for 2.5 min (Figure S8). Irradiation of the nanotubes showed quick temperature increase then exponential decrease after the laser was turned off.

4. CD44 expression levels

CD44 expression levels in various cancer and fibroblast cells were analyzed with flow cytometer. As expected, SCC7 showed low CD44 expression and NIH3T3 cells are CD44 negative. Both DOX sensitive OVCAR8 and DOX resistant OVCAR8/ADR ovarian cancer cell lines showed very high levels of CD44 expression. In addition, CD44 positive cells showed prominent while CD44 negative cells had almost negligible FITC green fluorescence, confirming CD44-specific targeting of CAHA-sSWCNT.

5. Computational analysis of DOX uptake in DOX sensitive and resistant cells.

Tracking chemotherapeutics in a live dynamic cellular environment is very challenging, as the cells keep migrating and also the nucleus boundaries are not very well defined. To meet these challenges, we have developed a semi-automated method to track cell nuclei in recorded live microscopy images. DOX uptake in ovarian cancer cells was carried out using live confocal fluorescence imaging and with the help of a novel data analysis algorithm. The image processing to extract the average intensity follows the steps indicated in Figure S11. First, each frame is segmented by intensity threshold resulting in a binary image. Multiple erosion and dilation operations on this binary image give candidate regions for cluster of cells. These candidates are filtered based on expected size and location, which are obtained from the previous frame. External contours of selected regions are extracted. The points on this contour are resampled such that the distance between two neighbors is not less than 5 pixels, but no more than 10 pixels. At the same time smoothness and continuity of the contour are also checked. Next all the
selected contours are combined to result in a binary mask image, which can be applied to the
drug intensity channel. This composite mask is used to compute the average intensity inside the
cluster of cells. Finally, a low-pass filter removes high frequency noise due to variation in cell
image intensity.

Matlab algorithm to track and mask the DOX in live ovarian cancer cells (Figure S12) dynamic
environment is provided below.

**Cell Tracking**

function

[area,intensity]=cleantrackingdata(dirname_parent,dirname_output,dirname_mask,img,pts,img2)

filenameM=[dirname_parent,dirname_mask,'
ImgM'];

filenameO=[dirname_parent,dirname_output,'
ImgO'];

n=size(pts,1);

se=strel('disk',4);

mm=max(img2(:));

mn=min(img2(:));

cr=[linspace(0,1,256)',zeros(256,1),zeros(256,1)];

fig=figure;

set(fig,'DoubleBuffer','on');

area=zeros(n,1);
intensity=zeros(n,1);

for i=1:n

    pt=[pts(i,1:2:end)',pts(i,2:2:end)'

    id=pt(:,1)<0 & pt(:,2)<0;

    pt(id,:)=[];

    pt=[pt;pt(1,:)];

    BW = roipoly(img(:,:,i), pt(:,1), pt(:,2));

    BW = imdilate(imerode(BW,se),se);

    L=bwlabel(BW);

    prop=regionprops(L,'Area');

    [m,id]=max([prop.Area]);

    BW2=(L==id);

    BW2=imdilate(BW2,se);

    prop=regionprops(L,'Area');

    imgm=img(:,:,i);

    imgm(~BW2)=0;

    img2m=img2(:,:,i);
img2m(~BW2)=0;

area(i)=prop(1).Area;

intensity(i)=sum(img2m(:));

a1=sc(imgm,'gray');

a2=sc(mat2gray(img2m,double([mn,mm])),cr);

imwrite(0.5*a1+.5*a2, sprintf('%s%04d.bmp',filenameO,i));

imwrite(BW2, sprintf('%s%04d.bmp',filenameM,i));

if (mod(i-1,10)==0) i, end

end

Cell Masking

function [area,intensity]=combinemasks(dirname_parent, dirname_output, dirname_mask, img,img2)

filenameO1=[dirname_parent,dirname_mask,'1\ImgM'];

filenameO2=[dirname_parent,dirname_mask,'2\ImgM'];

filenameO3=[dirname_parent,dirname_mask,'3\ImgM'];

filenameOC=[dirname_parent,dirname_output,'\ImgO'];

filenameOM=[dirname_parent,dirname_mask,'\ImgM'];

n=size(img,3);
area=zeros(1,n);

intensity=zeros(1,n);

mm=max(img2(:));

mn=min(img2(:));

cr=[linspace(0,1,256)',zeros(256,1),zeros(256,1)];

for i=1:n

    im1=imread(sprintf('%s%04d.bmp',filenameO1,i));

    im2=imread(sprintf('%s%04d.bmp',filenameO2,i));

    im3=imread(sprintf('%s%04d.bmp',filenameO3,i));

    BW = or(or(im1,im2),im3);

    imwrite(BW,sprintf('%s%04d.bmp',filenameOM,i));

    L=bwlabel(BW);

    prop=regionprops(L,'Area');

    imgm=img(:,:,i);

    imgm(~BW)=0;

    imgm(~BW)=0;

8. Label-free real-time assessment of nanoformula-cancer cell interactions using QCM-D

A typical QCM-D setup with output is shown in Figure 5 as a valuable tool of acoustic wave (mass) sensing. The change in frequency ($\Delta F$) and dissipation ($\Delta D$) are recorded simultaneously at the order of overtone, $n = 5$, for a confluent monolayer of drug sensitive and resistant ovarian cancer cells to which CAHA or CAHA-SWCNT had been added at physiological temperature. The penetration depth ($\delta$) of the acoustic shear wave of the QCM-D decays exponentially into the liquid as follows,

$$\delta = \left( \frac{f}{\pi pf} \right)^{1/2}$$
where $\eta$ is the viscosity of the liquid, $\rho$ is the density of the liquid, and $f$ is the frequency of the acoustic wave.\textsuperscript{8} Previous studies has reported that at this overtone ($n = 5$), the acoustic shear wave has a penetration depth of approximately $\sim 100$–$150$ nm from the surface of the $5$-MHz crystal sensor. This depth is coincident with the height of the basal region of the cell layer.\textsuperscript{9} Before introducing the nano-dispersions, the QCMD resonators were analyzed for baseline calibration (Figure S13). QCMD plots for CAHA and CAHA-sSWCNTs treated OVCAR8 and OVCAR8/ADR cells are described in Figure 5. The frequency and dissipation amplitude difference at 2 min after OVCAR8/ADR cells exposed to free DOX is plotted in Figure S14.

9. Apoptosis analysis of drug resistant ovarian cancer cells.

DOX resistant OVCAR8/ADR cells were treated with CAHA-sSWCNT-DOX and irradiated using NIR 808 nm laser. Confocal imaging of cells stained with live cell (Calcein AM) and dead cells (TUNEL) assays were carried out in order evaluate the toxic behavior of the nanoformula (Figure S15-S16). Detailed explanation of imaging, irradiation and staining are given in figure legends.

10. Biodistribution of sSWCNTs using Raman spectroscopy. sSWCNT injected i.v. into mouse bearing OVCAR8/ADR tumor xenografts was assessed following previously established standard protocol.\textsuperscript{10, 11} First Raman intensity vs. sSWCNTs concentration calibration curve for CAHA-sSWCNTs solution of various concentrations was obtained using Raman spectroscopy at a laser excitation of 785 nm wavelength. Raman intensity of sSWCNTs from water or tissue lysate was obtained by integrating the G-band peak area (data not shown). The tumor, primary organs (liver, spleen, kidney, lung, heart) along with other organs were lyophilized and sonicated in appropriate lysis buffer (1% SDS, 1% Triton X-100, 10 mM EDTA, 10 mM DTT,
40 mM Tris acetate) and heated for ~70 °C for 2 h to obtain homogenous suspensions for sSWCNT detection to determine the % Injected Dose per gram (%ID/g). Raman spectra of the suspensions were acquired with a Raman instrument from BWTek Inc.
Figure S1. Schematic of semiconducting single-walled carbon nanotubes (sSWCNT) dispersion in cholic acid derivatized hyaluronic acid biopolymer (CAHA) along with actual photos. CoMoCAT sSWCNT were used with intact optical properties, ideal for photothermal therapy. sSWCNT dispersion in CAHA and removal of undispersed / loosely dispersed SWCNTs are illustrated.
Figure S2. UV-Vis spectrum of individually dispersed CAHA-sSWCNT with strong absorption peaks at 568 and 996 nm indicating semiconducting (6,5) SWCNTs. The purified CAHA-sSWCNT were found to be well dispersed in water, buffer, and cell media without any sign of aggregation over time.
Figure S3. UV absorption spectra of CAHA-sSWCNT Absorption spectra of CAHA-sSWCNT with serial dilution.
Figure S4. zeta potential analysis of CAHA-sSWCNT-FITC. CAHA-sSWCNT-FITC has a zeta potential of around -32 mV.
Figure S5. Serum stability of the CAHA-sSWCNT nanoDDS was tested under a physiologically relevant setup. A photon correlation spectroscope was set to 37 °C before the introduction of the sample. Hydrodynamic size acquisition was set to take readings every hour. Known amount of sSWCNT formula was then added to cell media containing 10 % FBS and the solution placed in the cuvette holder with the cuvette being capped. It is extremely important to make sure that there is no air bubble in the solution, which can otherwise interfere with the acquisition, giving inconsistent data.
Figure S6. Hydrodynamic size analysis of CAHA-sSWCNT and PEG-sSWCNT using dynamic light scattering (DLS) in serum medium at 37 °C. Hydrodynamic particle sizes were collected for 24 h at 1 h interval. Both formulas showed similar serum stability under the physiologically simulated condition. CAHA-sSWCNT showed narrower monodispersed dispersion.
Figure S7. Viability of OVCAR8/ADR drug resistant cells. OVCAR8/ADR cells were treated with CAHA, sSWCNT or CAHA-sSWCNT (by weight) to assess the toxicity of individual component of the nanotube formulation. Non-wrapped sSWCNT but not CAHA or CAHA-sSWCNT are cytotoxic.
Figure S8. Photothermal effect of CAHA-sSWCNT. CAHA-sSWCNT aqueous dispersions of different concentrations (5-100 µg mL$^{-1}$) were irradiated by 808 nm laser (1 W/cm$^2$) for 2 min. A rapid temperature increase was found followed by exponential decrease after the laser was turned off.
Figure S9. Thermal images of CAHA-sSWCNT dispersion in serum and blood. Images show that there is significant temperature change in serum and blood under NIR 808 nm laser exposure (1 W/cm² for 90 s).
Figure S10. UV-Vis absorption spectra of CAHA-sSWCNT-DOX before and after dialysis at acidic pH. (a) CAHA-sSWCNT-DOX at neutral pH; (b) CAHA-sSWCNT without DOX loading; (c) CAHA-sSWCNT-DOX after dialysis at acidic pH (~5.5). Most of the DOX molecules were released under acidic pH, which is also found inside of cancer cells.⁴
Figure S11. Flow diagram showing the sequential flow of the computational algorithm to assess dynamic drug uptake in live cells.
Figure S12. Computational algorithm helps track and mask ovarian cancer cells treated with DOX loaded CAHA-SWCNTs. The mask changes with the cell movement. A typical snapshot from live video is shown. The amount of DOX (red fluorescence) from the cells changes with time (0-120 min). Since the cells are in a dynamic environment, the cell mask tracking the DOX uptake within the cells also changes with time.
Figure S13. QCMD baseline frequency and dissipation plots of four Au-coated quartz-crystal resonators, each with a monolayer of ovarian cancer cells cultured on top: (a-b) drug sensitive cells, (c-d) drug resistant cells. This step is important, as variations from resonator to resonator can affect the final outcome of the data being obtained, leading to inconclusive and ambiguous conclusion.
Figure S14. Frequency and dissipation of DOX on drug resistant cancer cells. Frequency and dissipation amplitude difference at 2 min post-treatment is shown for various concentrations of free DOX treated OVCAR8/ADR cancer cells (n=3 sensor-cell layers). Increase in frequency with increase in DOX concentration shows uptake of DOX, while relative dissipation changes are indicative of drug transporters.
**Figure S15.** Confocal images of CAHA-sSWCNT-DOX treated OVCAR8/ADR cells for apoptosis staining. **a** Free DOX showed little to no staining, **b** DOX co-delivered with sSWCNT at the same time showed TUNEL staining and **c** DOX loaded SWCNTs treated cells showed very high intensity of TUNEL staining. **d** Not all the cells treated with co-delivered DOX and sSWCNT showed TUNEL staining while **e** DOX loaded SWCNTs exerted maximum damage to the drug resistant cancer cells.
Figure S16. Confocal z-stack images of CAHA-sSWCNT-DOX treated OVCAR8/ADR cells for live cell staining. a-b Cells alone and free DOX treated ADR cells showed maximum staining for live cells. c-d Minimal staining was observed in cells treated with either nanoformula or DOX co-delivered with sSWCNT.
Figure S17. SCID Mice treated with nanoDDS followed by PTT. After laser exposure the skin in the tumor region turned brownish.
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