Electronic Supplementary Information (ESI)

Superior SWNT dispersion by amino acid based amphiphiles: designing biocompatible cationic nanohybrids

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Materials.

Silica gel of 60-120 mesh, all the amino acids, *n*-hexadecylamine, *n*-hexadecanol, palmitic acid *N*,*N*-dicyclohexylcarbodiimide (DCC), *n*-hexadecanoic acid, 4-*N*,*N*-(dimethylamino)pyridine (DMAP), *N*-hydroxybenzotriazole (HOBT), solvents and all other reagents were procured from SRL, India. Thionyl chloride, iodomethane and sodium hydroxide were purchased from Spectrochem, India. Water used throughout the study was Milli-Q water. Thin layer chromatography was performed on Merck precoated silica gel 60-F_{254} plates. Single walled carbon nanotubes (SWNT, diameter 1.2-1.5 nm diameter), $CDCl_3$ for NMR experiments and Amberlite Ira 900 chloride ion exchange resins were obtained from Aldrich Chemical Co. The LIVE DEAD viability kit (L-3224) for mammalian cells used for cell viability assays was performed procured from Molecular Probes, Invitrogen Chemical Company. All the materials used in the cell culture study such as Dulbecco's Modified Eagles' Medium (DMEM), heat inactivated fetal bovine serum (FBS), trypsin from porcine pancreas and MTT were obtained from Sigma Aldrich Chemical Company. UV-Vis Spectra were taken in Varian Cary 50 Bio spectrophotometer and the UV-Vis-NIR of the conjugates were monitored using Varian Cary 5000 spectrophotometer. Transmission Electron Microscopy (TEM) measurements were performed on JEOL JEM 2010 microscope. Atomic force microscopy (AFM) was performed on

Veeco, model AP0100 microscope in non-contact mode. Probe sonication was done using Omni Sonic Ruptor 250. Bath sonication was performed with a Telsonic Ultrasonics bath sonicator. Sorvall RC 6 and Sorvall RC 90 were used for centrifugation and ultracentrifugation, respectively. Lyophilization was done in Virtis 4KBTXL-75 Freeze Dryer. The zeta potential measurements were carried out in Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK) using a He-Ne laser (λ = 633 nm).

Synthesis of the amino acid based cationic amphiphiles (1a,1b, 2a, 2b,3a,3b,4a,4b):

The amphiphiles were synthesized following the reported protocols.[1] Briefly, Boc-protected L-amino acids were coupled with *n*-hexadecanol (for ester linked surfactants) or *n*-hexadecylamine (for amide linked surfactants) using DCC (1 equivalent) and catalytic amount of DMAP in presence of 1 equivalent of HOBT in dry DCM. Boc-protected amide/ester was then purified through column chromatography using 60-120 mesh silica gel and acetone/hexane as the eluent. Column purified materials were then subjected to deprotection by trifluoroacetic acid (4 equivalent) in dry DCM. After 2h of stirring, solvents were removed on a rotary evaporator and the mixture was taken in ethyl acetate. The EtOAc part was thoroughly washed with 10% aqueous sodium carbonate solution followed by brine to neutrality. The organic part was dried over anhydrous sodium sulphate and concentrated to get the corresponding amines. The primary amines (1 equivalent) thus obtained were quaternized with excess iodomethane using anhydrous potassium carbonate and catalytic amount of 18-crown-6-ether in dry DMF for 2h. The reaction mixture was taken in ethyl acetate and washed with 5% aqueous sodium thiosulphate solution and water. The concentrated ethyl acetate part was then subjected to column chromatography using 60-120 mesh silica gel and methanol/chloroform as the eluent. The column-purified material was crystallized from methanol/diethylether to obtain solid quaternized iodide salt. The

iodide salt thus obtained was subjected to ion exchange on Amberlite Ira-400 chloride ion exchange resin column followed by crystallization from diethylether to get the pure chlorides. Overall yield was ~60-70%.

Synthesis of the amino acid based anionic amphiphiles (1c, 2c, 3c, 4c):

The amphiphiles were synthesized following the reported protocols.[2] Briefly, methyl ester of the L-amino acids was coupled with C-16 long chain acid chloride in dry chloroform and dry pyridine. The ester protected long chain amides were then purified through column chromatography using 60-120 mesh silica gel and ethyl acetate/hexane as eluent. The column purified products were hydrolyzed using 1N NaOH (1.1 equivalent) in MeOH for 6h with stirring at room temperature. Solvents were removed on a rotary evaporator, and the mixtures were diluted with water and then washed with ether, followed by acidification by 1N HCl to get the corresponding carboxylic acids. The carboxylic acids were extracted in ethyl acetate and washed with brine to neutrality. Sodium salt of the corresponding carboxylic acids were prepared by dissolving the acids in MeOH and to that 1 equivalent 1N NaOH (standardized) was added. After brief stirring, the solvent was evaporated and dried to get the sodium salt. The formation of sodium salt was confirmed from FTIR spectroscopy by the disappearance of the –C=O stretching peak of carboxylic acid ~ 1720-1728 cm^{-1} and also from the improved solubility of the resultant compound in water.

Compound 1a: (Found: C, 71.89; H, 10.92; N, 2.89 Calcd (%) for $C_{28}H_{50}NO_2Cl$: C, 71.84; H, 10.77; N, 2.99.); ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ = 0.91 (3H, t, CH_3), 1.1-1.37 (26H, br, (CH_2)$_{13}$CH$_3$), 1.68-1.72 (2H, m, CH_2(CH$_2$)$_{13}$CH$_3$), 3.09-3.18 (2H, m, CHCH_2(Ph)), 3.68 (9H, s,

NMe_3), 3.93 (2H, t, COOCH_2), 4.68-4.73 (1H, m, NHCOC*H*), 7.28-7.37 (5H, m, Ph), MS (ESI) *m/z* calcd for $C_{28}H_{50}N_2O$ (the 4° ammonium ion, 100%): 432.3836, found 432.4451 [M^+].

Compound 1b: (Found: C, 71.72; H, 11.09; N, 5.92.Calcd (%) for $C_{28}H_{51}N_2OCl$: C, 71.99; H, 11.00; N, 6.00.); ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ = 0.80 (3H, t, CH_3), 1.08-1.18 (28H, br, (CH_2)$_{14}$$CH_3$), 2.82-2.86 (2H, m, COCH(NMe_3)CH_2), 3.15-3.18 (2H, m, NHCH_2), 3.4 (9H, s, NMe_3), 5.63-5.68 (1H, t, NHCOC*H*), 7.18-7.26 (5H, m, Ph), 8.68 (1H, s, CONH); MS (ESI) *m/z* calcd for $C_{28}H_{51}N_2O$ (the 4° ammonium ion, 100%): 431.3996, found 431.2771 [M^+].

Acid form of compound 1c: (Found: C, 74.45; H, 10.19; N, 3.36. Calcd (%) for $C_{25}H_{41}NO_3$: C, 74.40; H, 10.24; N, 3.47.) ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ = 0.88 (3H, t, CH_3), 1.22-1.25 (24H, br, (CH_2)$_{12}$$CH_3$), 1.56-1.66 (2H, d, C$H_2$($CH_2$)$_{12}$$CH_3$), 2.17 (2H, t, C$H_2$($CH_2$)$_{13}$$CH_3$), 3.10-3.27 (2H, m, C$H_2$Ph), 4.85-4.92 (1H, m, NHCOC*H*), 5.87-5.90 (1H, br, CON*H*), 7.15-7.33 (5H, m, Ph); MS (ESI) *m/z* calcd for $C_{25}H_{41}NO_3$: 403.3092, found 426.4451 [M^+ +Na].

Compound 2a: **(**Found: C 71.23, H 10.06, N 5.47. Calcd (%) for $C_{30}H_{51}N_2O_2Cl$: C 71.04, H 10.14, N 5.52.) ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.83 (3H, t, CH_3), 1.06-1.35 (26H, br, (CH_2)$_{13}$$CH_3$), 1.87 (2H, br, C$H_2$($CH_2$)$_{13}$$CH_3$), 3.29 (2H, br, COCH($NMe_3$)C$H_2$), 3.48 (9H, s, NMe_3), 3.73 (2H, br, CH_2(CH_2)$_{14}$$CH_3$), 4.60 (br, 1H, NHCOC*H*), 7.05-7.50 (br, 5H, aromatic protons), 8.02 (1H, br, CONH). MS (ESI): m/z. calcd for $C_{30}H_{51}N_2O_2$ (the 4° ammonium ion, 100%): 471.3945; found: 471.4618 [M^+].

Compound 2b: (Found: C, 71.28; H, 10.43; N, 8.35. Calcd (%) for $C_{30}H_{52}N_3OCl$: C, 71.18; H, 10.35; N, 8.30.); ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ = 0.83 (3H, t, CH_3), 0.97-0.99 (2H, br, C$H_2$$CH_3$), 1.11-1.32 (24H, br, (CH_2)$_{12}$$CH_2CH_3$), 1.66-1.73 (2H, br, $NHCH_2$CH_2), 2.82-2.91 (2H, m, COCH(NMe_3)CH_2), 3.2-3.24 (2H, m, NHCH_2), 3.31 (9H, s, NMe_3) 5.60 (1H, br, NHCOC*H*), 7.01-7.06 (1H, br, NHC*H*=), 7.29-7.31 (2H, d, =C*H*C*H*=), 7.41 (1H, d, NHC(=C)C*H*), 7.48-

7.51 (1H, d, NHC=CC*H*), 8.02 (1H, br, CONH), 8.39 (1H, s, CNHC); MS (ESI): *m/z* calcd for $C_{30}H_{52}N_3O$ (the 4° ammonium ion, 100%): 470.4110, found 470.3679 [M^+].

Acid form of compound 2c: (Found: C, 73.42; H, 9.47; N, 6.61 Calcd (%) for $C_{27}H_{42}N_2O_3$: C, 73.26; H, 9.56; N, 6.33.); ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ = 0.88 (3H, t, C*H*$_3$), 1.21-1.26 (24H, br, (C*H*$_2$)$_{12}$CH$_3$), 1.51 (2H, br, C*H*$_2$(CH$_2$)$_{12}$CH$_3$), 2.10 (2H, t, C*H*$_2$(CH$_2$)$_{13}$CH$_3$), 3.34-3.37 (2H, m, C*H*$_2$(CH(CO$_2$) NH), 4.91-4.95 (1H, m, NHCOC*H*), 6.06 (1H, br, CONH) 7.01-7.58 (5H, m, aromatic protons), 8.28 (1H, s, aromatic NH); MS (ESI) *m/z* calcd for $C_{27}H_{42}N_2O_3$: 442.3195, found 465.4251 [M^+ +Na].

Compound 3a: (Found: C, 67.31; H, 11.65; N, 3.45. Calcd (%) for $C_{22}H_{46}ClNO_2$: C, 67.40; H, 11.83; N, 3.57.) ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ = 0.88 (3H, t, C*H*$_3$), 1.33 – 1.18 (28H, m, CH$_2$(C*H*$_2$)$_{14}$CH$_3$), 1.75 (3H, m, CHC*H*$_3$), 3.59 (9H, s, NMe$_3$), 4.27 – 4.19 (1H, q, NHCOC*H*), 4.87 (1H, s, CONH); MS (ESI)): *m/z* calcd for m/z $C_{22}H_{46}ClN_2O$ (the 4° ammonium ion, 100%): 356.3523; Found 356.3622 [M^+].

Compound 3b: (Found: C, 67.09; H, 12.31; N, 6.98. Calcd (%) for $C_{22}H_{47}ClN_2O$: C, 67.57; H, 12.11; N, 7.16) ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ = 0.85 (3H, t, CH$_3$), 0.9-1.35 (26H, br, (C*H*$_2$)$_{13}$CH$_3$), 1.54-1.61 (3H, d, CHC*H*$_3$) 1.87 (2H, br, NHCH$_2$C*H*$_2$), 3.13-3.29 (2H, m, NHC*H*$_2$), 3.61 (9H, s, NMe$_3$), 5.43-5.49 (1H, q, NHCOC*H*), 8.65 (1H, s, CONH); MS (ESI): *m/z* calcd for $C_{22}H_{47}N_2O$ (the 4° ammonium ion, 100%): 355.3683, Found 355.2365 [M^+].

Acid form of compound 3c (Found: C, 69.58; H, 11.45; N, 4.58. Calcd (%) for $C_{19}H_{37}NO_3$: C, 69.68; H, 11.39; N, 4.28) ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ = 0.88 (3H, t, (CH$_2$)$_{14}$C*H*$_3$), 0.95-1.00 (3H, m, CHC*H*$_3$), 1.24-1.25 (24H, br, CH$_2$(C*H*$_2$)$_{12}$CH$_3$), 1.62-1.64 (2H, br, C*H*$_2$(CH$_2$)$_{12}$CH$_3$), 2.21-2.28 (2H, m, C*H*$_2$(CH$_2$)$_{13}$CH$_3$), 4.56-4.60 (1H, m, NHCOC*H*), 4.54 (1H, s, CONH); MS (ESI): *m/z* calcd for $C_{19}H_{37}NO_3$: 327.2773, Found 350.4127 [M^+ + Na].

Compound 4a (Found: C, 69.46; H, 12.37; N, 3.53.Calcd (%) for $C_{25}H_{52}NO_2Cl$: C, 69.16; H, 12.07; N, 3.23.); ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ = 0.85 (3H, t, $(CH_2)_{13}CH_3$), 0.99-1.08 (6H, m, $CH(CH_2CH_3)CH_3$), 1.23-1.28 (28H, br, $(CH_2)_{13}CH_3$, $CH(CH_2CH_3)CH_3$), 1.51-1.76 (3H, m, $CH(CH_2CH_3)CH_3$, $CH_2(CH_2)_{13}CH_3$), 3.63 (9H, s, NMe_3), 4.12-4.23 (2H, m, $CH_2(CH_2)_{14}CH_3$), 4.45 (1H, s, $CH(COOCH_2(CH_2)_{14}CH_3)$, MS (ESI) *m/z* calcd for $C_{28}H_{51}N_2O$ (the 4° ammonium ion, 100%): 398.3993, found 398.4112 [M^+].

Compound 4b: (Found: C, 69.13; H, 12.02; N, 6.12. Calcd (%) for $C_{25}H_{53}ClN_2O$: C, 69.32; H, 12.33; N, 6.47); ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ = 0.84 (3H, t, CH_3), 0.87-1.02 (6H, m, $CH(CH_3)CH_2CH_3$), 1.13-1.3 (28H, br, $(CH_2)_{13}CH_3$, $CH(CH_3)CH_2CH_3$), 1.41-1.44 (2H, m, $NHCH_2CH_2$), 2.08-2.11 (1H, m, $CH(CH_3)CH_2CH_3$), 3.22-3.3 (2H, m, $NHCH_2$), 3.4 (9H, s, NMe_3), 5.3 (1H, br, NHCOCH), 8.78 (1H, s, CONH); MS (ESI): *m/z* calcd for $C_{25}H_{53}N_2O$ (the 4° ammonium ion, 100%): 397.4158, Found 397.3561 [M^+].

Acid form of compound 4c: (Found: C, 71.69; H, 11.64; N, 3.59.Calcd (%) for $C_{22}H_{43}NO_3$: C, 71.50; H, 11.73; N, 3.79.); ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ = 0.85-0.96 (9H, m, $CH(CH_2CH_3)CH_3$, $(CH_2)_{13}CH_3$), 1.15-1.25 (26H, m, br, $CH_2(CH_2)_{12}CH_3$, $CH(CH_2CH_3)CH_3$), 1.61-1.65 (2H, m, $CH_2(CH_2)_{12}CH_3$), 1.94-1.96(1H, br, $CH(CH_2CH_3)CH_3$), 2.25 (2H, t, $CH_2(CH_2)_{13}CH_3$) 4.58-4.63 (1H, m, NHCOCH), 6.02-6.05(1H, br, NHCOCH) MS (ESI) *m/z* calcd for $C_{22}H_{43}NO_3$ 369.3243 [M^+], found 369.4421 [M^++Na].

Preparation of the calibration plot of SWNT dispersion in water:

The calibration curve was obtained by plotting the absorbance vs concentration of SWNT in the dispersed state in water. To determine the quantity of nanotubes in dispersed state, SWNT (4 mg) was added to an aqueous solution (16 mL) of SDBS (2.5mg/mL) to achieve an initial

concentration of SWNT in the aqueous mixture, 0.25 mg/mL. This aqueous suspension was tip sonicated for 10 min (at 40% power output) followed by bath sonication for 2h and tip sonication again for 10 min to prepare the aqueous dispersion of SWNT. Then the dispersion was centrifuged at 2300 g for 90 min. The supernatant was kept aside and used later. While the residue was washed well (3 times) so that it does not contain any trace of SDBS. Finally, the residue was lyophilized to remove the residual water. The residue was then weighed which was found to be 0.24 mg. Therefore, the total amount of the dispersed SWNT in the supernatant was 0.16 mg i.e., the concentration of SWNT in dispersion was 0.1 mg/mL. For the determination of the absorbance, the optical density of this supernatant was measured at 550 nm through progressive dilution. The dilution was achieved by using SDBS solution (2.5mg/mL) to ensure that similar surfactant concentration is maintained while a successive dilution is obtained for the SWNT dispersions. The absorbance values were plotted as a function of quantity of SWNT in dispersed state. In every case, background correction was performed with the aqueous solution of the respective surfactants in the absence of SWNT. The entire process was repeated for 5 times.

Quantification of the SWNT percent dispersions for the amino acid based amphiphiles: SWNT (1mg) was added to the aqueous solution (4 mL) of the respective surfactants (2.5 mg/mL). Similar sonication and centrifugation was done as mentioned above to prepare the aqueous dispersion of SWNT. The amount of the dispersed SWNT in the supernatant was calculated from the observed absorbance value at 550 nm that was fitted in the above said absorbance vs concentration linear plot equation (Fig. 3b inset in the manuscript). The experimental errors were in the range of 3-5 % in triplicate experiments.

Sample preparation for UV-Vis-NIR, zeta potential, AFM and TEM: The supernatant obtained after the centrifugation was utilized for the all the studies. In case of the UV-Vis-NIR, a

background correction was performed with the aqueous solution of the respective surfactants. For AFM, a drop of the supernatant was cast on a freshly cleaved mica surface and the samples were air-dried overnight before imaging. In a similar fashion, for TEM images a drop of the supernatant was placed on a 300-mesh Cu-coated TEM grid and dried under vacuum for 4 h before taking the image. The AFM image of SWNT dispersions with CTAB is given in Fig. S2. Also the AFM images of **3a**-SWNT dispersion are given in Fig. 4a (in the manuscript) and Fig. S2. Fig. S5 shows the additional TEM image of the SWNT dispersions with **3a**.

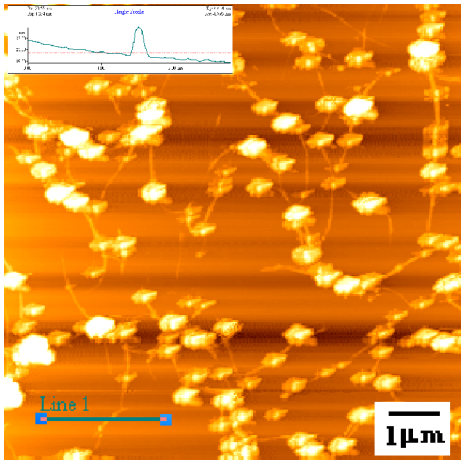

Fig. S1 The AFM image of SWNT-**3a** hybrids.

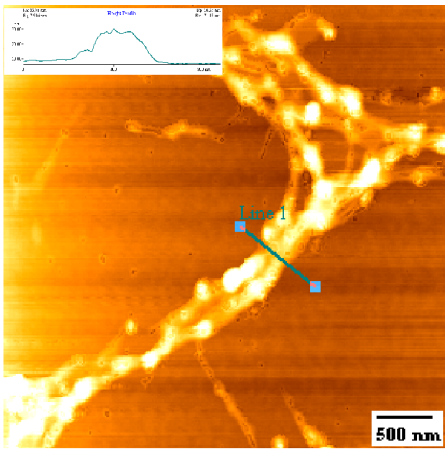

Fig. S2 The AFM image of SWNT-CTAB hybrids.

Average bundle diameter analysis from AFM images: Using about 5 images for surfactant **3a**, **1a** and CTAB the bundle sizes were measured. A statistical analysis of the bundle diameter was done by plotting histograms. The histogram corresponding to **3a** and **1a** are given in Fig. S3 and S4.[3] In case of CTAB, the presence large bundles were evident from the AFM image (Fig. S2).

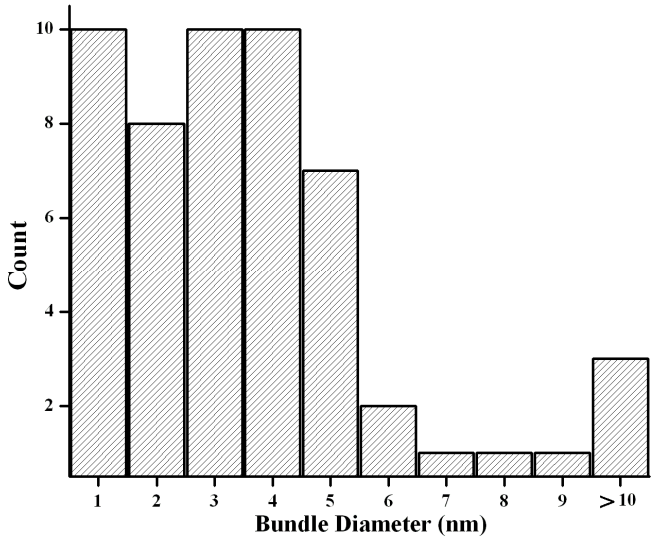

Fig. S3 The histogram obtained from AFM images of **3a**.

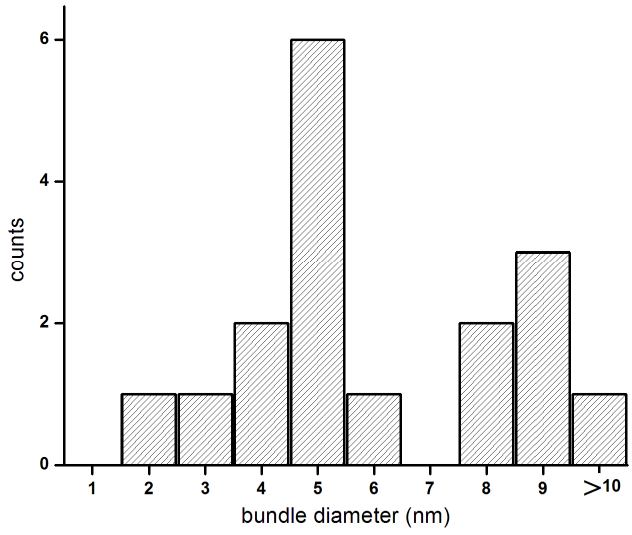

Fig. S4 The histogram obtained from AFM images of **1a**.

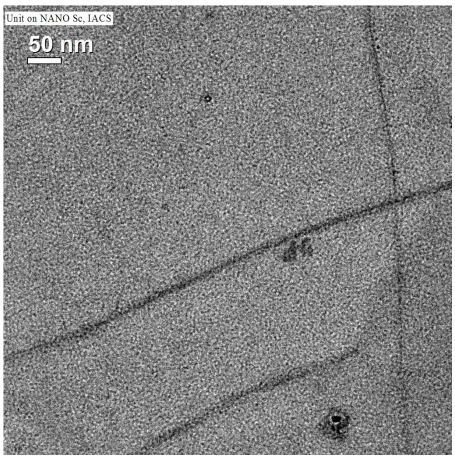

Fig. S5 The TEM image of SWNT-**3a** hybrids.

Media stability of the dispersion:

For the investigation of the media stability of the SWNT dispersions, it is important to remove the excess dispersing agent, which is present in addition to the optimum amount required for the stable dispersion.[4] This was done by ultracentrifugation of the SWNT dispersion at 307500 g for 30 mins. This resulted in the formation of a pellet of the monodispersed nano-hybrids. Water was added to the pellet, sonicated and ultracentrifugation was performed again. The supernatant was discarded to ensure complete removal of excess surfactants. The residue was then taken in minimal quantity of water and added to 10% FBS media-solution. The stability of the dispersion in the media was followed by UV-vis-NIR study (Fig. 5a in manuscript).

Cytotoxicity MTT assay:

The biocompatibility of the surfactant-SWNT hybrid was done by microculture MTT reduction assay as reported. This assay is derived from the reduction of a soluble tetrazolium salt by mitochondrial dehydrogenase of the viable tumor cells to an insoluble colored formazan product. The amount of formazan product formed can be measured spectrophotometrically after dissolution of the dye in DMSO. The enzyme activity and the amount of the formazan produced is proportional to the number of alive cells. The decrease in absorbance value can be attributed to

the killing of the cells or inhibition of the cell proliferation by the nanostructures. HepG2 cells were seeded at a density 20,000 cells per well in a 96-well microtiter plate for 18-24 h before the assay. The media stable aqueous-SWNT dispersion was taken as the stock solution and the concentration of the SWNT was calculated from the calibration curve. Sequential dilutions of these stock solutions were done during the experiment to vary the concentrations of the conjugate (10 to 100 μg/mL) in the microtiter plate. The HepG2 cells were incubated with SWNT-**1a** and SWNT-**3a** hybrids for 3 h at 37 °C under 5% CO_2. Then, 10 μL MTT stock solution (5 mg/mL) in phosphate buffer saline was added to the above mixture and the cells were further incubated for another 4 h. The precipitated formazan was dissolved thoroughly in DMSO and absorbance at 570 nm was measured using BioTek® Elisa Reader. The number of surviving cells were expressed as percent viability = [A_{570}(treated cells)-background/ A_{570}(untreated cells)-background] × 100.

Fluorescence Microscopic Study

The LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells was used to examine cell viability under a fluorescence microscope. The kit contains a mixture of two nucleic acid binding strains, specifically referred to as Calcein AM (Component A) and Ethidium homodimer-1 (Component B). Acetomethoxy derivative of Calcein (Calcein AM) has the ability to pass through the cell membrane. After its transportation into the cell the esterase enzyme present in live cells removes the acetoxy group. This form of the compound then intercalates with the DNA and this results in an enhancement of fluorescence and bright green colour can be observed. But this binding occurs in the presence of the esterase enzyme, which is present only in live cells. But the Ethidium Homodimer can only pass through damaged cell membranes thus it gets incorporated only into dead cells. It does not require any enzyme and shows red

fluorescence upon binding with DNA in the dead cells. The kit was stored at –20 ºC in dark, which is taken out and thawed at room temperature just prior to assay. 4 μL of the supplied 2 mM EthD-1 stock solution (Component B) was added to 2 mL of sterile, tissue culture–grade D-PBS and the mixture was vortexed to ensure thorough mixing. This gave an approximately 4 μM EthD-1 solution. 1 μL of the supplied 4 mM calcein AM stock solution (Component A) was then added to the 2 mL EthD-1 solution and vortexed. The resulting approximately 2 M calcein AM and 4 μM EthD-1 working solution was then added directly (500 μL) to HepG2 cells treated with the stock solution and incubated for 3 h. After incubation with the fluorescent dye, the cells were observed under the Olympus IX51 inverted microscope using an excitation filter of BP460-495 nm and a band absorbance filter covering wavelength below 505 nm. The bright green colour (Fig. 5c) resulting from the enhanced fluorescence of DNA intercalated calcein indicated the presence of viable cells. But when the images were taken using the excitation filter BP530-550 and a band absorbance filter covering wavelength below 570 nm, negligible red fluorescence were observed which further confirmed the abundance of live cells (Fig. S6).

Fig. S6 Fluorescence micrograph of the HepG2 cells after treating with SWNT-**3a** dispersions for 3 h in presence of LIVE/DEAD viability/cytotoxicity kit. The excitation filter BP530-550 and a band absorbance filter covering wavelength below 570 nm were used.

References:

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