Supporting information

Biodegradable fluorescent nanohybrid for photo-driven tumor diagnosis and tumor growth inhibition

Rajendra Prasad[†#], Deepak S. Chauhan[†#], Amit S. Yadav[§#], Janhavi Devrukhkar[†], Barkha Singh[†♠], Mahadeo Gorain[§], Mayur Temgire[Δ], Jayesh Bellare[Δ], Gopal C. Kundu[§], Rohit Srivastava[†*]

[†]Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai, India, [♠]Center for Research in Nano Technology and Science,Indian Institute of Technology Bombay, Powai, Mumbai, India, [§]Laboratory of Tumor Biology, Angiogenesis and Nanomedicine Research, National Center for Cell Science, Pune, India ,[Δ]Department of Chemical engineering, Indian Institute of Technology Bombay, Powai, Mumbai, India

AUTHOR INFORMATION

[#] These authors have contributed equally to the work.

Corresponding Author

*Email: rsrivasta@iitb.ac.in

Experimental details

Synthesis of red emissive NIR responsive carbon nanodots (C-dot). In a round bottom flask, 400 mg of carbon fibers (obtained from mango leaves) were mixed with concentrated and HNO_3 (volume 40 mL) and stirred for 4 h at 40 °C. The above solution was introduced into 40 mL of concentrated H_2SO_4 and further stirred for 24 h at maintained 40 °C temperature. After 24 h the solution turn into dark color that was further diluted with 500 mL DI water. The pH of diluted mixture was fixed to 7.4 using NaOH. After complete mixing the reaction solution was kept at 1-2 °C for overnight to remove the produced salts. The collected supernatant was dialyzed using dialysis bag (molecular weight cut-off 2KD) for 12 h. The obtained carbon nanodots (C-dots) were stored in fridge and used for further characterization and applications.

Preparation of lipid self assembly (liposome) and fluorescent C-dots decorated liposomal nanopitchers (FJNPs). The lipid bilayer assembly viz., liposomes were prepared by the thin film hydration method followed by previously reported method.[10] In brief, a mixture of DSPC lipid and cholesterol with 80: 20 mg ratio was dissolved in chloroform in inert environment. The mixed solvent was evaporated followed by rotary evaporator under mild vacuum at 44 °C from

the above mixture. The obtained thin lipid film was hydrated with a phosphate buffer saline solution (6 mL) by using rotary evaporator rotating (150 rpm) at 50 °C till complete film hydration and a homogeneous dispersion was formed. Prepared multilamellar liposomal suspension was sonicated for 4 cycles with 40 % intensity using probe sonicator (10 minutes with 2 second on/off pulse) to obtain unilamellar liposomes.

To incorporate C-dots with liposomal nanoparticles, 2 mL of small unilamellar liposomes (1 mg/mL) were incubated with 2 mL of synthesized C-dots (0.5 mg/mL) under slow stirring speed viz., 150 rpm at room temperature (37 °C). The above mixture was stirred for overnight and then sonicated for 5 minutes (40 % intensity using probe sonicator with 2 second on/off pulse). After sonication, the obtained formulation was filtered five times by using polycarbonate filter and stored at 6 °C in fridge for further usages. The obtained particles were analyzed through Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) measurement.

Folic acid attachment with FJNPs. The surface functionalization of FJNPs platform was carried out following steps. In the first step, C-dots incorporated FJNPs were modified to have terminal amino functional groups by cystamine dihydrochloride through EDC/NHS activation method. For this, the carboxylic group of FJNPs particles were activated by 50 mM EDC and 20 mM NHS for 3 h at room temperature. On the other hand, FA was also activated by EDC/NHS coupling reaction for 12 h at room temperature. Folic acid (100 mg) was reacted with EDC (26 mg) and NHS (8 mg) in 20 mL of aqueous media at room temperature. After that, amine-functionalized FJNPs (5 mL) particles were added to the activated folic acid solution and allowed to react at room temperature for another 6 h that was protected from light. Finally, the product is collected by mild centrifugation (4000 rpm for 5 minutes) and washed with Milli-Q water**.**

Photothermal transduction test and NIR light responsive degradation of FJNPs. To examine the photothermal response of FJNPs, different concentrations (0.1 to 2.0 mg/mL) of FJNPs in PBS were used. 200 µL of each concentration is added to wells of a 96 well plate. Prior to transduction experiment, the temperature was stabilized at 37 °C in water bath. Then the wells were exposed with 1 W of 808 nm NIR laser source for 1-5 minutes. The time dependent temperature was recorded to understand the thermal response of designed nanohybrid. In

addition, the microscopic measurement was done to evaluate the morphology of NIR light exposed FJNPs nanoparticles (after 5 minutes of light exposure from the 2.0 mg/mL concentration).

Cell viability and cellular uptake study. NIH3T3 normal cells and 4T1 breast cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM Gibco, Carlsbad, CA, USA) supplemented with 10% Fetal Bovine serum and penicillin/ streptomycin, under 5% CO_2 atmosphere at 37 ºC. *In vitro* viability studies were performed over normal cells and cancer cells using 24 h MTT assay. Cells were seeded at density of 2×10^4 cells per well in 96 well plates and incubated for 24 h in 5% CO_2 atmosphere at 37 ºC. After 24 h incubation at 5 % CO_2 and 37 ºC, 100 µl of different concentration (in range of 10 to 500 µg/mL) of C-dots, FJNPs and FJNPs-FA nanoparticles dispersed in media were added into wells. Following 24 h incubation wells were washed off with PBS and 20 µl of MTT dye was added. Formazan crystals formed after 4 h are dissolved by 100 µL of DMSO. Optical absorbance is recorded at 560 nm and 620 nm using microplate reader (Tecan Infinite 200 PRO). Percentage cell viability was calculated in reference to untreated cells (control).

For cellular uptake, 4T1 cells were seeded onto 96 well plates with a density of 2×10^4 cells/well and incubated for 24 h in 5% CO_2 atmosphere at 37 ºC and rinsed with PBS before adding nanoparticles. The cells were incubated with 40 µg/mL of FJNPs, FJNPs-FA for 4 h. After 4 h of incubation the treated cells were washed with PBS to remove unbound particles. Later, 4% paraformaldehyde solution was added to the cells and incubated for 10 min and nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI, 1 µg/mL in PBS) the excess DAPI was removed by washing with PBS once. The cover slip was fixed on a drop of 70 % glycerol on glass slide to fix the treated cell. The fluorescence images are taken using fluorescence microscope (Axio observer. Z1).

***In vitro* photostability.** 4T1 breast cancer cells were incubated with 40 µg/mL of folic acid functionalized FJNPs at 37 ºC for 5 h. After 5 h incubation, the treated cells were irradiated with 1 W of 808 nm NIR laser for 5 minutes. Specific tracker for cell nuclei (DAPI, blue) of treated cells and detached C-dots (red) was used to analyze intercellular localization. DAPI can be excited at 335 nm and showed blue fluorescence. C-dots excited by using 488 nm wavelength that showed red fluorescence.

***In vitro* combined therapeutic study.** *In vitro* combined therapy was tested on 4T1 breast cancer cell line. Cancer cells were seeded onto 96 well plates with a density of 2×10^4 cells/well and incubated for 24 h in 5% CO_2 atmosphere at 37 ºC. The cells were incubated with 40 µg/mL of FJNPs and FJNPs-FA for 4 h. After incubation the cells were washed with PBS to get rid of the unbound particles. After washing cells were treated with and without 5 minutes of NIR exposure. Cell viability was determined by the addition of MTT (20 µL, 1 mg/mL dye in sterile PBS). The plate was incubated for an additional 4 h at 37 °C and 5% CO_2, allowing viable cells to convert the blue solution into pink dye. Formazan crystals were dissolved by adding 100 µL DMSO to each well and the absorbance values at 560 nm was collected and cell viability was calculated as a percentage compared to untreated control cells (100 % viability). Additionally, treated 4T1 cancer cells were stained with propidium iodide (PI) dye to visualize the dead cells through fluorescence microscope. Apart from this, ROS production due to FJNPs was analyzed using DCFDA (2′,7′-dichlorofluorescin diacetate) dye staining. Fluorescence (excitation: 495 nm, emission: 520 nm) was recorded after adding DCFDA (10 uM) green dye.

Animal models and tumor growth. The animal study procedures and experimental protocols were approved by Institutional Animal Ethical Committee (IAEC) of National Centre for Cell Science, Pune, India (NCCS, Pune). 8 weeks old female balb/c mice were used for the present study. 1×10^5 4T1 breast cancer cells were injected subcutaneously into the mammary fat pad of balb/c mice and tumor growth evaluated.

***In vivo* localized tumor diagnosis, bio-distribution, photostability, photothermal response and deep tissue visualization of FJNPs-FA.** A single dose (10 mg/kg) of FJNPs-FA particles were subcutaneously injected at tumor site in 4T1 tumor bearing female balb/c mice. The *in vivo* NIR fluorescence images from 1 to 72 h were captured of post-injected animals in the anesthesized condition using In Vivo Imaging System (IVIS) and compared with untreated mice (control). The red emissive intensity from major organs was measure to examine the *in vivo* bio-distribution of post-injected animals at various time points (up to 72 h). Similarly, time dependent ex-vivo images of major organs (heart, kidney, lung, liver and spleen) along with tumor were recorded using IVIS spectrum (Xenogen for NIR fluorescence at 520 nm of excitation) from sacrificed animals. Further, the tumor bearing animals were exposed with 1 W of 808 nm NIR light for 5 minutes. After 5 minutes of light exposure the whole body

photoluminescence was measured by IVIS instrument after 48 h and 72 h of laser irradiation. In addition, the temperature variation from tumor site was recorded using contact less digital thermometer during laser irradiation and results are compared with untreated animals. The good intensity and homogeneous distribution of fluoresces from light exposed area viz., tumor site.

Phototriggered Tumor Growth Inhibition. To examine the *in vivo* phototriggered tumor growth inhibition, the designed FA-FJNPs theranostics was injected at tumor site in 4T1 breast tumor bearing mice (10 mg/kg wt). Three sets of animals groups (4 mice in each group) for treatment were made; (1) untreated 4T1 breast tumor bearing mice, (2) FA-FJNP treated 4T1 breast tumor bearing mice without NIR light irradiation and (3) FA-FJNP treated 4T1 breast tumor bearing mice with 1 W of 808 nm NIR light irradiation for 5 minutes. The treatment sets of individual group were repeated after 2 days time interval using same conditions as mentioned earlier. The animal health, behavior and tumor volume measurement were made on each treatment day, analyzed statistically and represented graphically. Tumor growth was monitored up to two weeks of therapeutic course. After treatment completion, mice were sacrificed and tumors were excised, photographed, weighed and analyzed statistically.

Chemicals. Distearoyl phosphatidylcholine (99 % DSPC) was ordered from Lipoid (Germany) and used without further purification. N-hydroxysuccinimide (NHS), Folic acid (FA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Cystamine dihydrochloride, Cholesterol (CH), 2′,7′-dichlorofluorescin diacetate (DCFDA), Propidium iodide (PI) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Pvt. Ltd., USA. Sulphuric acid (98 % H_2SO_4), Nitric acid (95 % HNO_3), Chloroform (98 %) and Sodium hydroxide (NaOH, 98 %) were purchased from Merck limited, Mumbai, India. Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), Phosphate-Buffered Saline (PBS) and Antibiotic-Antimycotic solution were procured from HiMedia Laboratories Pvt. Ltd., India. All other chemicals were used as reagent grade. Milli-Q grade (Millipore, Bedford, MA, USA) water was used for all experiments. All glasswares were washed with aquaregia and rinsed with double distilled water before using them for synthesis and applications.

Characterizations and analysis techniques. Size and morphology of particles were examined by transmission electron microscopy (TEM FEI Tecnai T-20) operating at 200 kV. TEM samples

were prepared by evaporating a droplet of sample onto 200 mesh copper grid. Elemental analysis was done by energy-dispersive X-ray analysis (EDX) and measurements are performed on TEM instrument.

Optical properties of synthesized materials were examined by UV-Vis and photoluminance (PL) spectrophotometer (Jasco V570 and Scinco) using standard quartz cuvette with 1 cm of path length. Fourier transform infrared (FT-IR) spectra were obtained by PerkinElmer FT-IR Spectrum GX instrument. KBr crystals were used as the matrix for preparing the samples. Atomic force microscopy (AFM) images were recorded by using atomic force microscope (PSIA XE- 100) followed by tapping mode. Clean silicon wafers surface were used for analyzing the samples for AFM measurements that were prepared by drop casting method. ESR (electron spin resonance) analysis was performed using ESR spectrometer (JES - FA200). The signals were recorded at room temperature at standard frequency. To check the color property, digital photographs were captured in UV cabinet with the exposure of 365 nm light. Cell-imaging and cellular uptake of materials were examined through fluorescence microscope (Carl Zeiss inverted fluorescence microscope model AXIO OBSERVER.ZI using DAPI (350-430 nm blue) and rhodamine (480-580 nm red) filters). In vivo and ex-vivo images were recorded using IVIS spectrum imaging system (IVIS spectrum Xenogen). NIR light mediated photothermal transduction experiments were performed at ambient conditions using 808 nm continuous wave NIR laser source with 1W/cm^2 power density.

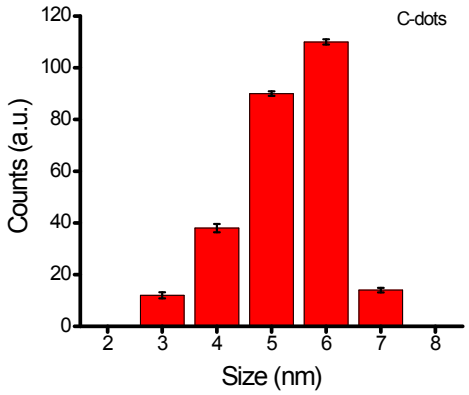


Figure S1. Particle size distribution histogram of carbon dots (C-dots).

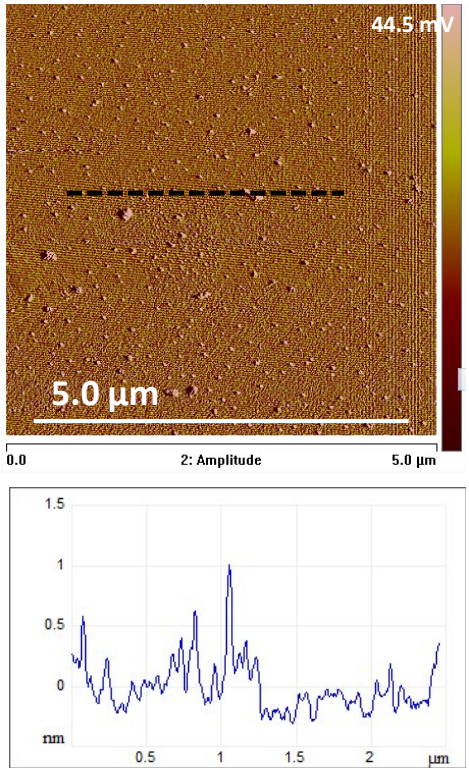


Figure S2. Atomic force microscopy (AFM) image of C-dots with height profile.

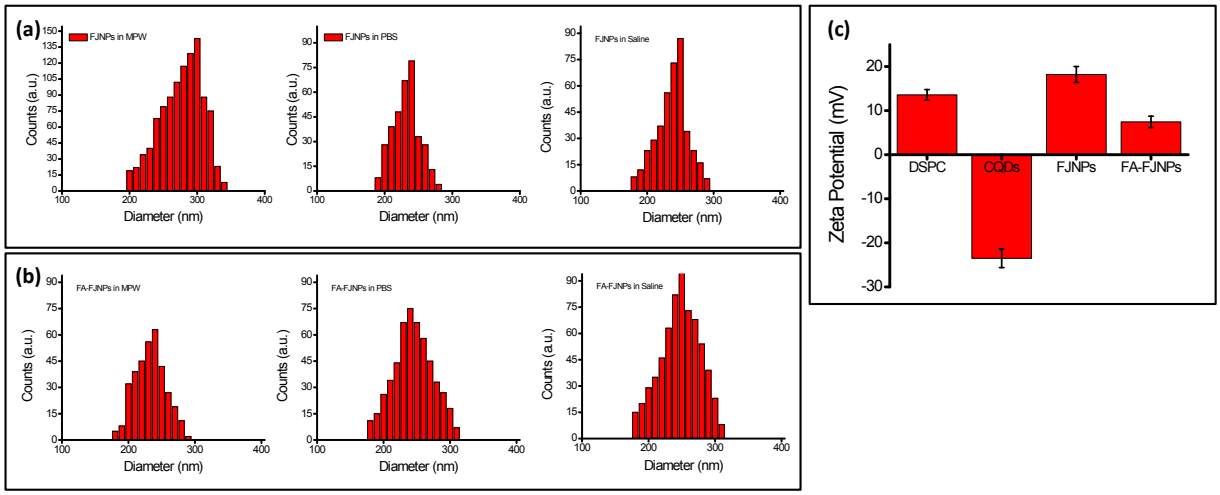


Figure S3. Dynamic Light Scattering (DLS) measurement of (a) synthesized FJNPs particles and (b) folic acid attached FJNPs (FA-FJNPs) nanohybrid and (c) zeta potential measurement of the components of FJNPs nanohybrids.

Sample name	Average size in PBS	Average size in MPW	Average size in Saline
FJNPs	230 ±10 nm **(PDI = 0.720)**	210 ±10 nm **(PDI = 0.468)**	240 ±10 nm **(PDI = 0.870)**
Folic acid attached FJNPs	200 ±10 nm **(PDI = 0.942)**	215 ±10 nm **(PDI = 0.891)**	230 ±10 nm **(PDI = 0.962)**

Table S1. Average particle size obtained from Dynamic Light Scattering (DLS) measurement with polydispersity index (PDI).

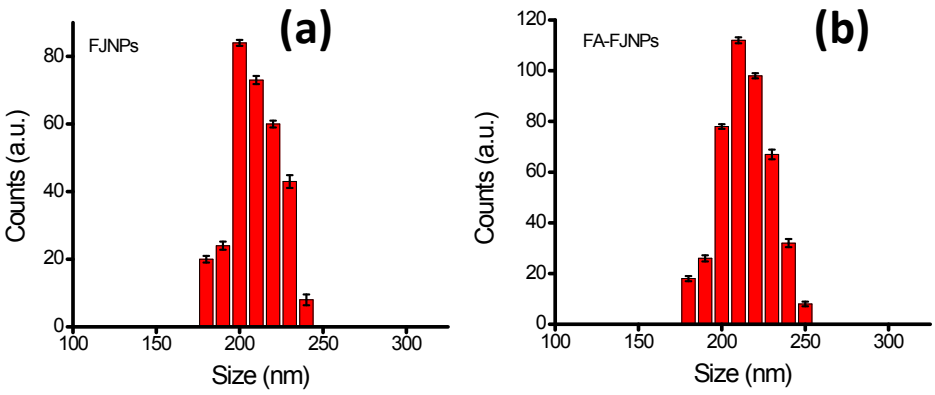


Figure S4. Particle size distribution histogram of FJNPs (a) and folic acid attached FJNPs (b, FA-FJNPs) obtained from TEM images of nanoaprticles.

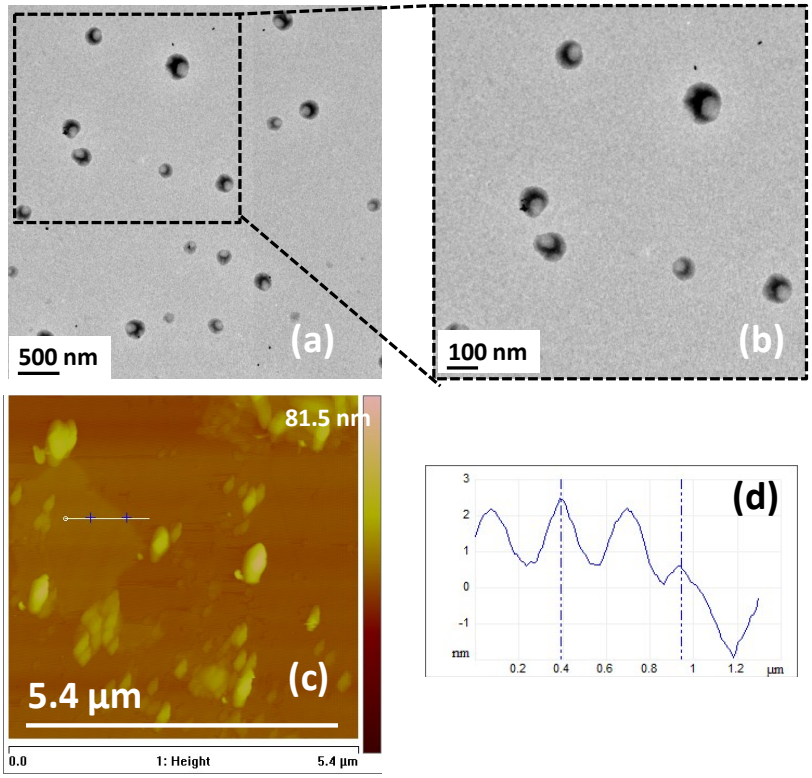


Figure S5. TEM image of (a) carbon dots (C-dots) anchored liposomal spherical nanopitchers (FJNPs), (b) enlarged image of FJNPs and (c, d) atomic force microscopy (AFM) image of FJNPs with height profile.

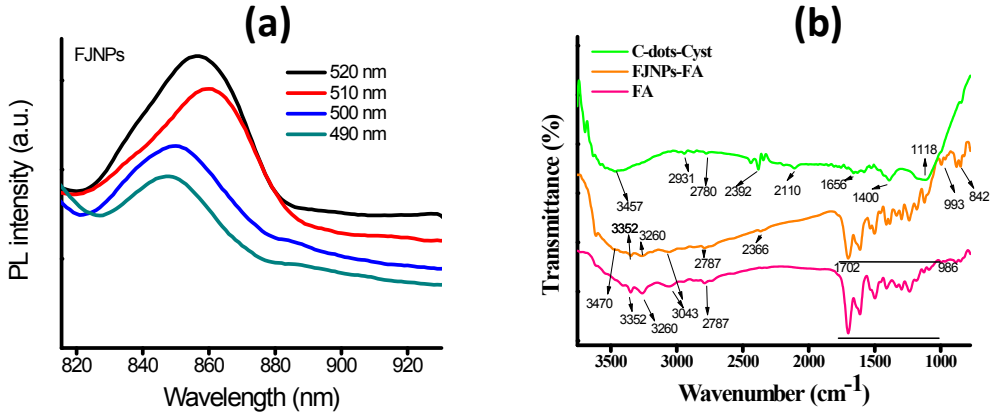


Figure S6. (a) Photoluminance spectra of FJNPs under various excitation wavelengths (490-520 nm) showing that the PL emission of nanohybrid is dependent on the excitation wavelength, and the emission peak is red-shifted with increased excitation wavelength shifted from 490 to 520 nm due to basal surface and edge defects of FJNPs and (b) FTIR spectra of C-dots, FJNPs and FA functionalized FJNPs showing the successful surface functionalization.

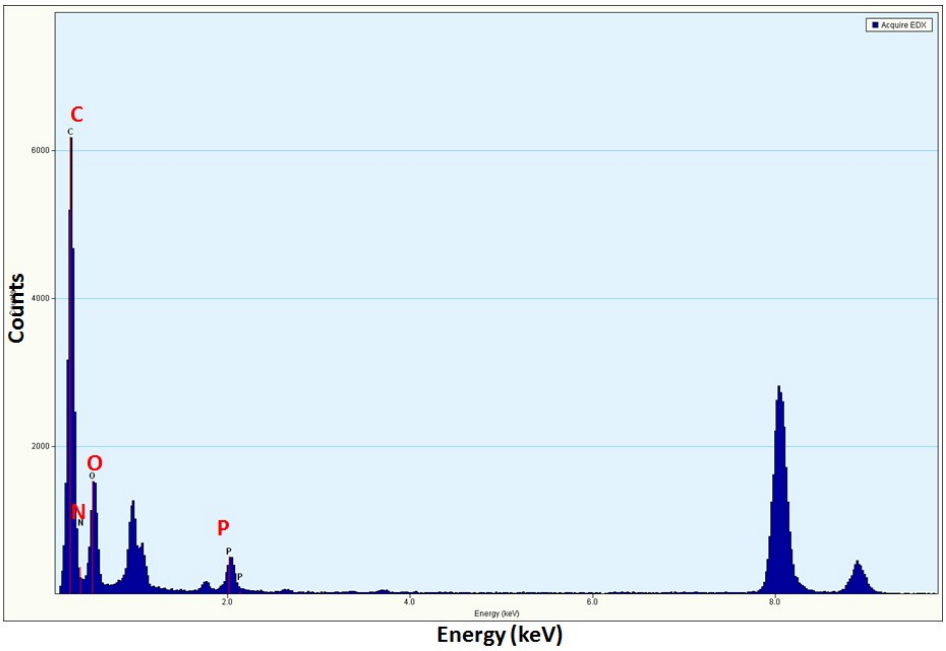


Figure S7. Energy Dispersive X-ray (EDX) elemental analysis of FA functionalized FJNPs.

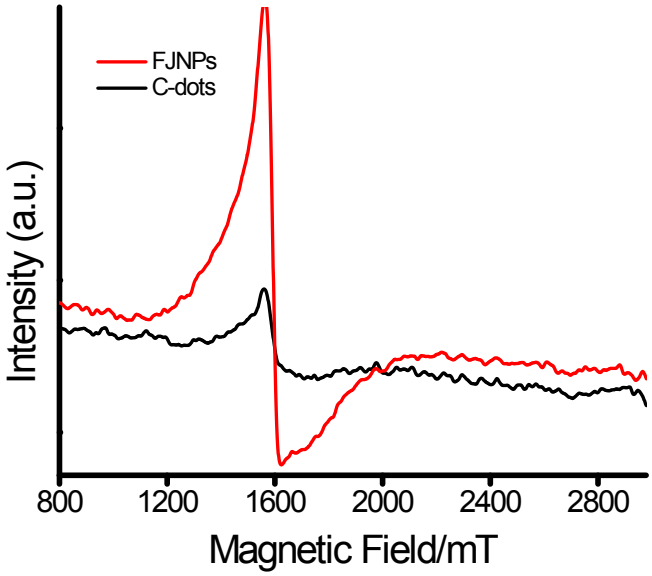


Figure S8. Electron Spin Resonance spectra (ESR) of C-dots and FJNPs nanoparticles at room temperature.

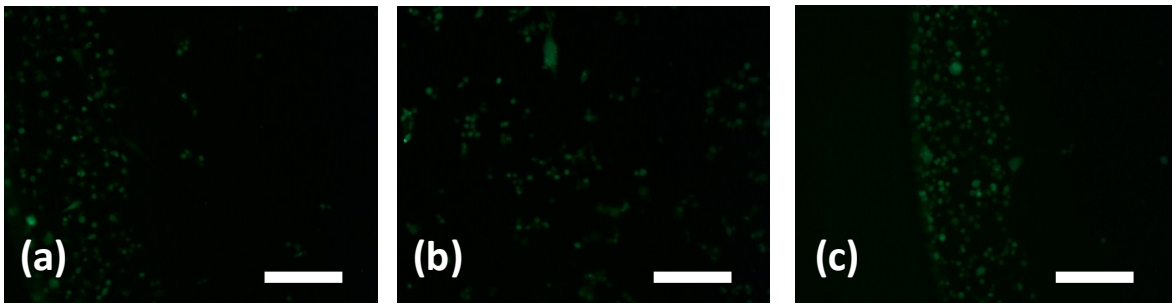


Figure S9. Reactive Oxygen Species (ROS) detection at various time (a) 6 h, (b) 12 h, (c) 24 h staining by green (2′,7′-dichlorofluorescin diacetate, DCFDA) dye in the case of nanohybrid treated cancer cells after 5 minutes of NIR light exposure, scale bar is 250 μm.

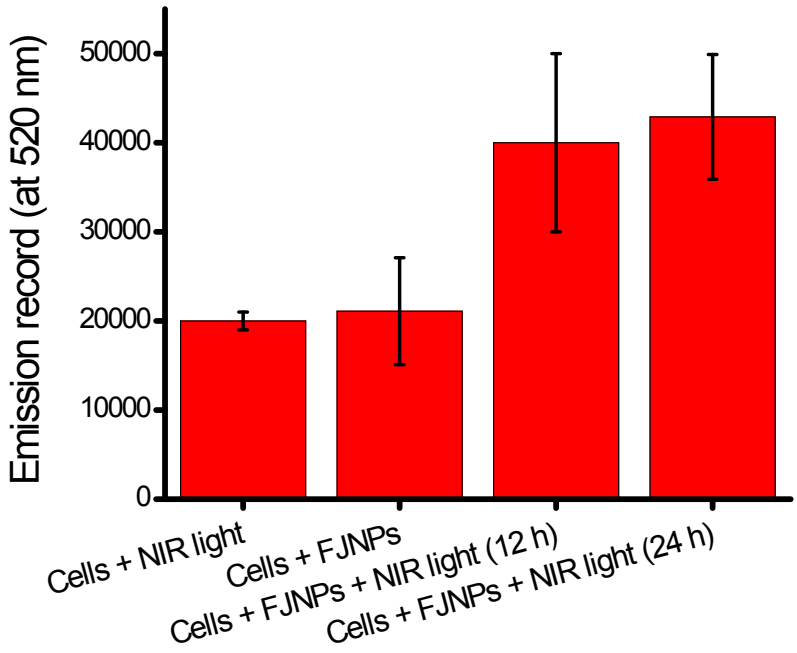


Figure S10. Quantitative analysis of Reactive Oxygen Species (ROS) detection from treated cancer cells staining by green (2′,7′-dichlorofluorescin diacetate, DCFDA) dye.

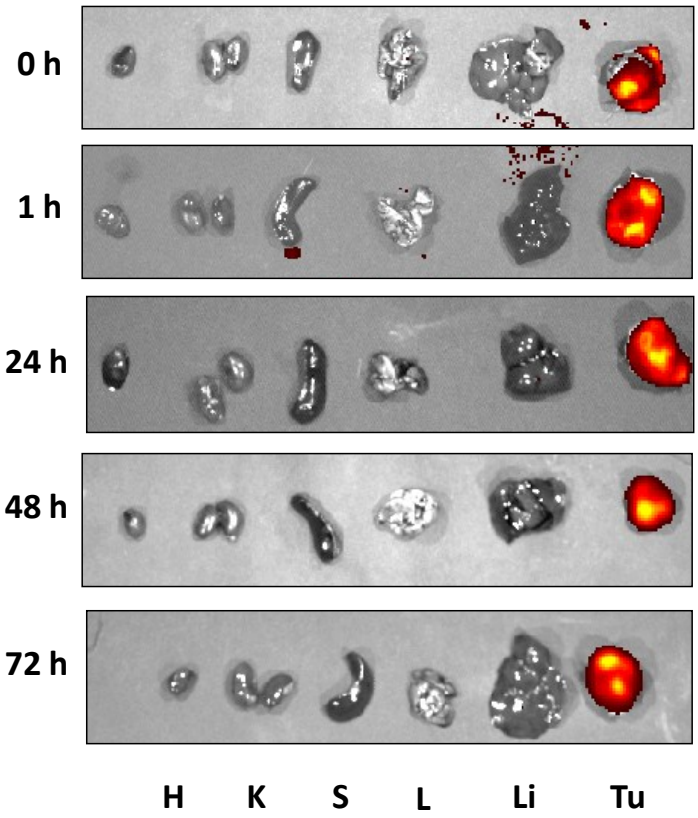


Figure S11. Ex-vivo IVIS images of major organs (heart-H, kidney-K, spleen-S, lung-L, liver-Li) and tumor-Tu at various time points (0-72 h).

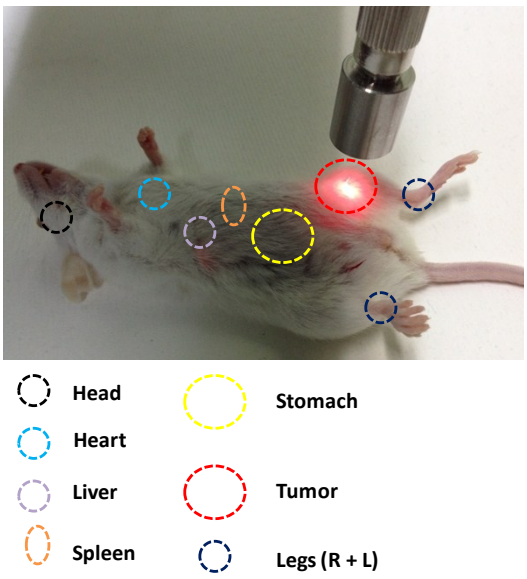
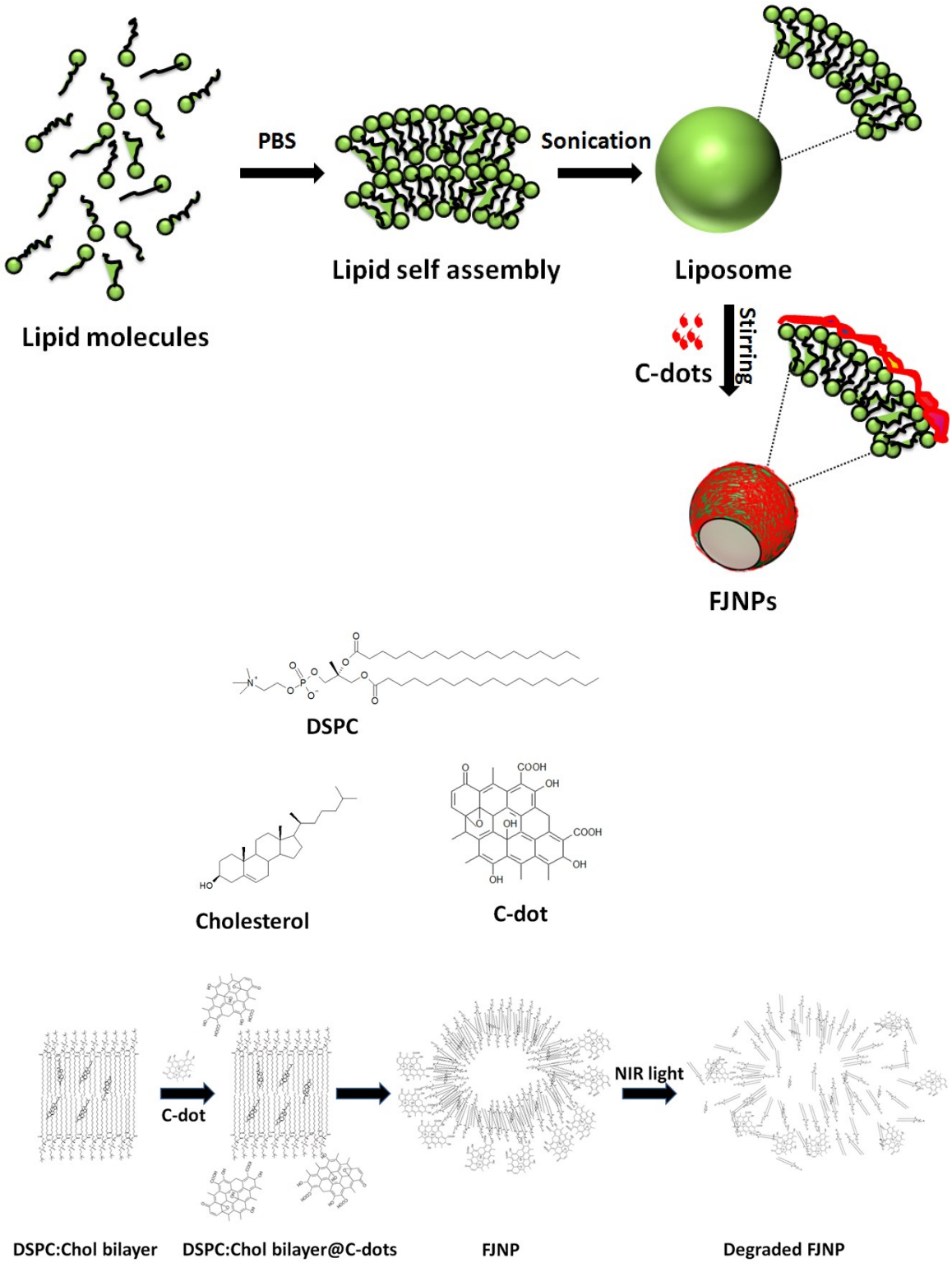
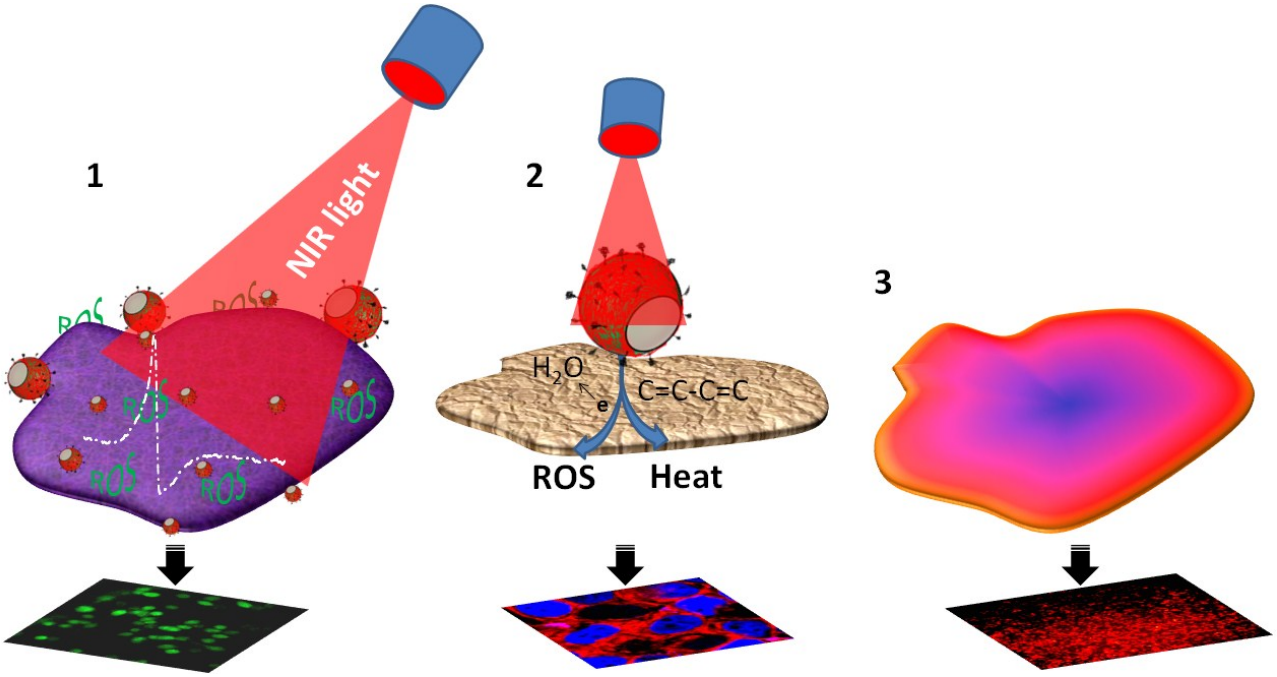


Figure S12. Temperature measurement spots on post-injected mice body during 5 minutes of NIR exposure.



Scheme S1. Schematic is showing the formation of spherical FJNPs through C-dots incorporation in lipid self assembly viz., liposome under the ultrasonication process in the aqueous solution.



Scheme S2. Cartoon is showing the generation of (1) Reactive Oxygen Species (ROS) detection from treated cancer cells staining by green (2′,7′-dichlorofluorescin diacetate, DCFDA) dye, (2) heat production and cell uptake under NIR light exposure and (3) cancer cell ablation after NIR light irradiation.

S. No.	Temperature spot	Temperature (°C) in case of without NIR exposure	Temperature (°C) in case of with NIR exposure
1	Tumor	39.3	57
2	Heart	38	44
3	Liver	39	45.4
4	Spleen	40.1	41
5	Stomach	38.6	39
6	Head	39	39
7	Legs (R + L)	39 + 38.2	38.3 + 38.8

Table S2. Temperature measurement in post-injected mice with and without 5 minutes of NIR exposure using contact less temperature sensor.

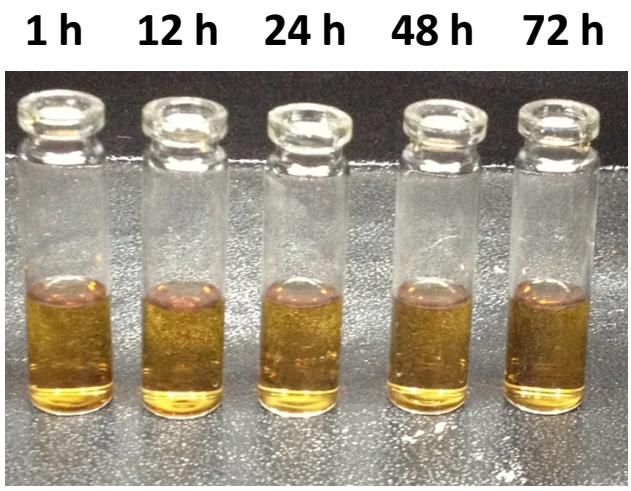


Figure S13. Digital photographs of C-dots showing time dependent water dispersibility.

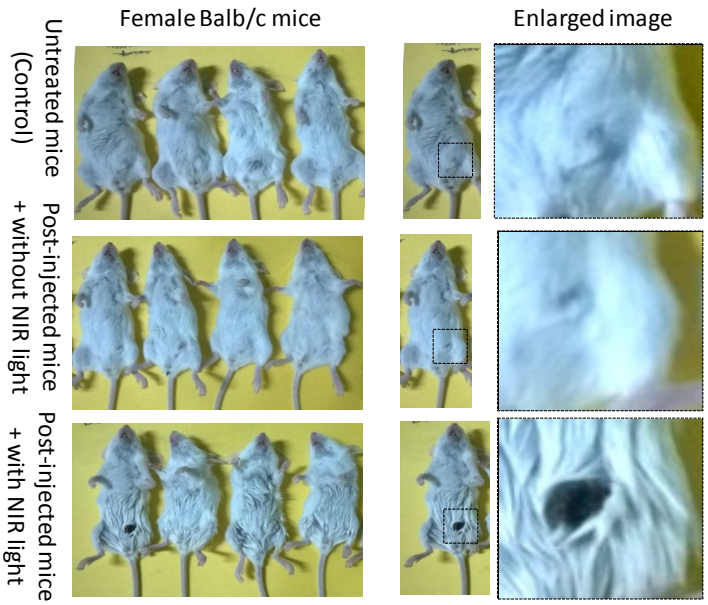


Figure S14. Digital photographs of treated animals showing significant observation of NIR light triggered localized therapy and observation of eschars.

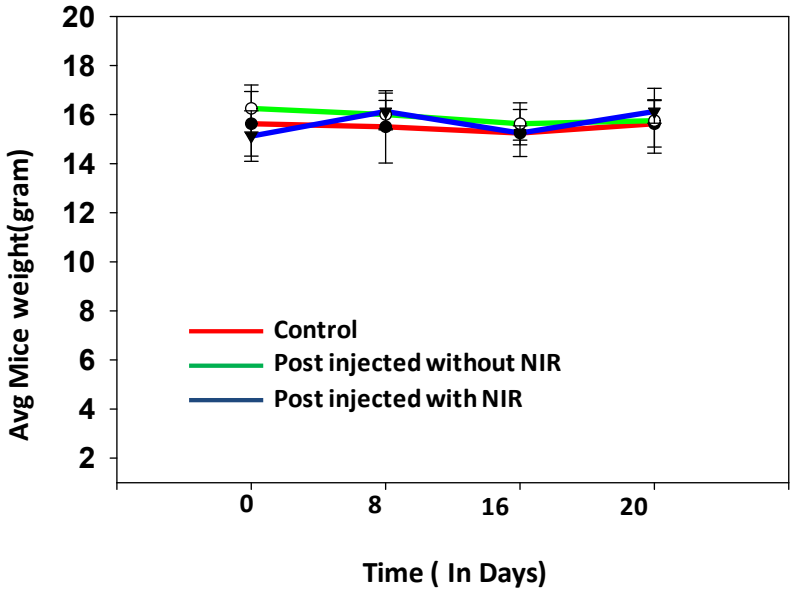


Figure S15. Time dependent body weight measurement of treated animals (with and without NIR irradiation after post-injection of designed nanohybrid) and comparison with untreated animals (control group of mice).