Supporting information

Nanoparticles based brachytherapy spacers for delivery of localized combined chemo-radiation therapy

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Materials:

Poly(lactic-co-glycolic) acid (M_w 7-17 KDa, acid terminated), Vinyltrimethoxysilane (VTES), (3-Aminopropyl)triethoxysilane (APTES), Dimethyl sulfoxide (DMSO), 1-Butanol, ammonium hydroxide, AOT was purchased from Sigma Aldrich. Tygon tubing (ID 1/32″) was obtained from Fisher scientific. Cyanine 7.5 NHS ester was procured from Lumiprobe Corp. All solvents (hexane, acetone, chloroform, Dichloromethane, ethanol) were used without further purification. Microfuge membrane-filters (NANOSEP 100K OMEGA) are a product of Pall Corporation. The prostate cancer cell line PC3 was obtained from ATCC, VA and cultured according to instructions supplied by the vendor. Unless otherwise mentioned, all cell culture products (nutrient media DMEM, matrigel, trypsin, Penstrep, fetal bovine serum) were obtained from Invitrogen.

Methods:

Synthesis of NIR fluorescent Silica nanoparticles: The synthesis of $^{conCy7.5}$-SNPs was carried out using oil-in-water microemulsion method, following a previously reported protocol with several modifications. (1-2) For conjugating the Cy7.5 fluorophore in the SNPs, Cy7.5-NHS ester was first conjugated to a silane precursor using aminopropyltriethoxy silane. 5 mg Cy 7.5 NHS ester (6.4 μmol), 1.77 mg APTES (8 μmol) and 10 μl neat triethylamine were added to 1 ml of anhydrous DMSO and stirred over night at room temperature under Ar atmosphere. The crude mixture was purified using EtOAc-hexane mixture, dried and resuspended in 1ml of DMSO. For synthesizing the Cy7.5 conjugated ORMOSIL nanoparticles, 10ml of 2.2% (w/v) surfactant AOT solution was prepared in HPLC grade water followed by addition of 600 μl of n-butanol and 50 μl of Cy7.5-silane under vigorous stirring at r.t. After 15 min of stirring 100ul of neat VTES was added and resultant reaction mixture was stirred for another 45 minutes. Finally 10ul of NH_4OH was added and reaction was allowed to stir overnight. The nanoparticles suspension was then dialyzed against distilled water for 48h at room temperature, using a cellulose membrane with a cut-off size of 12-14 kDa. Following dialysis, the nanoparticles were sterile filtered and stored at 4 °C for future use.

Fabrication of INCeRT spacers: For fabricating the spacers, the extracted SNPs from the aqueous mother liquor required a careful assessment, in terms of solvent compatibility with the PLGA polymer, so that both can be dispersed in a single phase without disturbing the integrity of the nanoparticles and the glass transition temperature of the PLGA polymer. Briefly, the dialysed nanoparticles were centrifuged at 11,000 rpm for 30 min at 4^{o}C. Following that, 250 mg of PLGA was dissolved in minimum amount of acetone. The dried nanoparticles pellet was dispersed in minimum amount of acetone via sonication and the slurry obtained was

added to PLGA solution. The entire mixture was mixed thoroughly to get a viscous paste. This paste was transferred to 1ml syringe using a Luer stub adapter (0.5in; 18G) which is attached to a silicon tube (inner diameter 0.8 mm) of desired. The paste was infused at a predetermined flow rate into the silicon tubing using an infusion pump. After infusion the tube was end clipped and dried overnight at 46^{o}C. The dried spacers were cut into 5 mm length and stored at r.t. in dark. For fabricating the free Cy7.5 doped PLGA spacers, same steps were followed expect instead of adding nanoparticles an equivalent concentration of Cy7.5 solution in DMSO was added to the PLGA solution. The Cy7.5 was selected as a model drug to mimic docetaxel because of (a) similar hydrophobicity, (b) similar molecular weights (~782: Cy 7.5 and 807: docetaxel) and (c) Cy7.5 can be easily tracked using the NIR fluorescence in live animals.

Fabrication of INCeRT spacers with Docetaxel: INCeRT spacers doped with docetaxel was prepared in using the same protocol as mentioned above for the Cy 7.5. Briefly, 250mg of PLGA, 25mg of docetaxel was dissolved in minimal volume of acetone and the mixture was infused in the silicone tubing using the same parameters as described above. The dried spacers were cut in 2 mm length and drug concentration in each spacer was measured using high performance liquid chromatography (HPLC). The dried spacer was dissolved in acetone to extract the docetaxel and the sample was spin filtered using centrifugal filtration tubes (100 kDa) to separate the polymer from drug in acetone. The acetone with docetaxel was evaluated for drug content using methanol:water (70:30) solvent system in HPLC. From the standard curve plotted with the free docetaxel the drug concentration in 2 mm spacer was 118 µg.

Transmission electron microscopy: Transmission Electron Microscopy (TEM) images were obtained using a JEOL model JEM- 100CX microscope at an acceleration voltage of 80 kV. The specimens were prepared by drop casting the sample dispersion onto an amorphous carbon coated 300 mesh copper grid, which was placed on a filter paper to absorb the excess solvent.

Dynamic light Scattering: Dynamic light scattering (DLS) measurements were performed by using 90Plus zeta sizer (Brookhaven Inc, NY) for measuring the hydrodynamic diameter of the $^{conCy7.5}$-SNPs in a diluted sample placed in a 3 ml cuvette. Zeta potential measurements were also done using the same instrument.

Optical characterization: The absorption spectra were collected using a Shimadzu model 3101PC UV-Vis-NIR scanning spectrophotometer over a wavelength range from 300 to 800 nm. The samples were measured against water as reference. All samples were used as prepared and loaded into a quartz cell for measurements.

The emission spectra were collected using a Fluoromax 4 Spectrofluorometer (Jobin Yvon; fluorescence spectra). All the samples were dispersed in water and loaded into a quartz cell for measurements.

Release Kinetics Studies: Release of Cy 7.5 and $^{conCy7.5}$-SNPs from spacers was quantified using fluorescence studies. Release studies were carried out in 0.1M Tris-HCl buffer supplemented with 0.01% (w/v) of Tween 80 at physiological pH. As prepared spacers (for both cy7.5-PLGA and Cy7.5-Silica-PLGA spacers) were cut into 5mm each and kept in 3ml buffer with gentle stirring. 1ml of buffer was taken at predetermined time intervals and evaluated for fluorescence emission at 810 nm. A plot of increase in emission intensity with time was made to qualitatively assess the relative profile of Cy7.5/Cy7.5-Silica from spacers.

Spin filtration method was employed to study the release of dye from nanoparticles. (1) Dialysed sample of nanoparticles in which Cy7.5 was covalently conjugated, was subjected to spin filtration using centrifugal

filtration tubes with 100kDa cutoff membrane. All nanoparticles were collected from the membrane and the flow through was checked for percentage of fluorescence as compared to original fluorescence from the sample. The sample was plotted against Cy7.5 dye dispersed in tween-80 solution after 24 hrs incubation time.

Scanning electron microscopy: Fractured spacer surfaces were produced by cooling in liquid nitrogen and fractured with a cooled razor. Fractured and non-fractured spacers were attached to a specimen mount using a conductive carbon adhesive tab and then coated with 10 – 15 nm of carbon using a Denton DV502 vacuum evaporator. Samples were imaged in both SE (secondary electrons) and BSE (back-scattered electrons) mode on a Hitachi S-4800 field emission SEM, at 5 kV. EDS analysis was carried out by selecting several region of interest (ROI) from the spacers and evaluating the elemental composition of the ROI.

Agarose Phantom Studies: Agar phantoms were placed on the imaging platform and trans-illuminated as described above. The movement of free AlexaFluor (AF) 750 dye (Molecular Probes, Life Technologies, Carlsbad, CA), 20 nm nanoparticles, and 250 nm nanoparticles were tested in separate experiments to observe the effect of particle size on diffusion. Free dye and nanoparticles were injected directly into the clear phantom with an insulin syringe. The phantoms were made in 250 mm diameter by 25 mm deep cell culture dishes (Corning Inc., Corning, NY). The phantom material was made with 1.5 grams of Agarose (Mr=-0.10, Acros Organics, Geel, Belgium) and 250 mL of distilled water resulting in 0.6% agar gel. The mixture was boiled for 10 minutes until the powdered agar was dissolved and then poured into the cell culture dish to cool and harden. (3-4) For experiments involving free dye, AF-750 was injected into the agar phantom and images were acquired at 15 minute intervals for a total of 3 hours (N=3). In this case, it was possible to continuously make measurements without removal of the phantom from the stage. Exposure times were 1 ms for intrinsic images (i.e. at the wavelength of the laser) and were between 0.5 and 4s for fluorescence images, depending on the acquisition time point (later times required longer exposures due to dye diffusion and dilution). For experiments involving nanoparticles (30 nm and 200 nm diameter), the phantoms were imaged daily for 15 days after the injection of the nanoparticles (N=3). To prevent drying of the agar, these phantoms were refrigerated between experiments. A wire marker was placed in the upper left hand corner to allow image co-registration between imaging sessions. For nanoparticle formulations, fluorescence images required between 0.5 and 30 s exposures (as above, longer exposures were required for later time points). White light images were taken of the phantoms by back-illumination with the LED ring during each imaging session.

Animal model: Animal experiments were conducted in agreement with all relevant guidelines and regulations set by the XXX University, and with approved institutional protocols by the American Association of Laboratory Animal Care. Six-to-seven week old male athymic nude mice (Hsd:Athymic Nude-Foxn1nu) were procured from Charles River lab, and were housed in a group of five in standard cages with free access to food and water and a 12 hrs light/dark cycle. All animals acclimated to the animal facility for at least 48 hrs before experimentation. All possible parameters that may cause social stress, like group size, type (treated and non treated) etc., among the experimental animals were carefully monitored and avoided. Animals were observed daily for any behavioral abnormalities and weighed weekly.

Sub-Q tumor generation: The xenografted mouse models were generated by subcutaneously injecting PC3 cells at a concentration of $(2-3) \times 10^6$ cells/mouse in the scapular region of 5−6-week-old male athymic nude mice (Hsd:Athymic Nude-Foxn1nu) using a 1 mL Monoject tuberculin syringe. Tumor growth was monitored every 24−48 h until a tumor size of approximately 5 mm in diameter was observed.

In vivo implantation of INCeRT spacers: All spacers were implanted in the mice using clinical brachytherapy applicator needle. The spacers were implanted both intradermally as well inside the Sub-Q tumor. Two different types of spacers were implanted in the mice intradermally on the upper right and left shoulder. The spacer implanted on the right side of the shoulder was a control spacer in which free fluorophore Cy7.5 was incorporated in the PLGA matrix whereas the spacer on the left side was spacer with $^{conCy7.5}$-SNPs doped in PLGA spacers. The control spacer with Cy7.5 contains no nanoparticles. The spacers were also implanted in the right and left hind flank of the mice. Both these spacers are $^{conCy7.5}$-SNPs doped PLGA spacers, the only difference being that the left spacer was implanted intradermally (similar to the upper left shoulder spacer) whereas the right spacer was implanted directly into the tumor. The tiny hole left after the spacer implantation was sealed using animal skin glue to make sure that the tumor internal pressure should not push the spacer out.

INCeRT spacers with Docetaxel implantations in tumor: Two docetaxel loaded INCeRT spacers (2 mm in length) were implanted in mice (n=3) with subcutaneous tumor xenografts (300 mm^3) as described above. The comparison was made with tumored mice (n=3) in which same dose of free docetaxel was injected intravenously. In both the cases, the dose of docetaxel was maintained at 9.44mg/kg body weight. A group of tumored mice (n=4) was used as control mice with no injection/implantation. The tumor volume was measured every alternate day using calipers and plotted against time. The tumor volume was calculated using formula:

$$\text{Tumor volume} = [\ \pi/6 * L * (W^2)\ ]$$

where, L and W is the length and width of the tumor, respectively. Mice were evaluated for weight loss, and any signs so behavioral changes like scruffy appearance, listlessness, and uncoordinated movements.

The in vivo therapy results showed that docetaxel INCeRT spacers were effective in suppressing the tumor growth with no observable behavioral toxicity. The suppression of the tumor volume was higher in mice in which the spacers were implanted when the tumor volume was around 200-300 mm^3. For the mice with higher tumor volume (~700 mm^3), the growth of tumor was minimally affected. A similar response was also observed for the mice injected intravenously with the same dose of free docetaxel, however, all mice in that group lost body weight considerably indicating potential systemic toxicity of the docetaxel. Control mice group with no treatment showed a continuous increase in tumor volume.

In vivo fluorescence imaging: Samples (mice or agar phantoms) were placed on a platform and trans-illuminated with the output a tunable Titanium:Sapphire laser (Mai Tai, XF-1, Spectra-Physics, Inc., Santa Clara, CA). The output wavelength of the laser was set to 740 nm (which matched the absorption spectra of the Cy7.5 dye). The beam was filtered with a 730 nm clean up filter with 30 nm bandpass (730/30 nm, Chroma Technology, Rockingham, VT). The 1 mm diameter beam was expanded 1,500-fold to approximately 150 cm in diameter using a lens pair in simple telescope configuration. The power at the sample was adjusted using a set of neutral density filters and was in the range of 2-15 mW/cm2 for these experiments after the beam was expanded. Samples were also placed on a ground glass diffuser plate to create a relatively homogeneous illumination profile and remove any speckle or interference pattern from the illumination light.

Samples were imaged with a high-sensitivity electron-multiplied charge coupled device camera (EMCCD; iXonEM+855 Andor Technology, Belfast, Northern Ireland) fitted with a 35 m lens (NT54-689, Edmund Optics Inc., Barrington, NJ). The imaging field of view was 150 mm x 150 mm at a working distance of 47 cm. The

camera was fitted with either a 740/40 nm bandpass filter (to record images at the wavelength of the laser as described below) or a 780 nm longpass filter (Chroma) to collect fluorescent light from the sample. The gain of the EMCCD could be set with camera control software on an arbitrary scale from 0 (off) to 250 (arbitrary units). Exposure times and image acquisition was performed using a personal computer running Andor control software.

Data Collection and Image Analysis: Mice were imaged once daily for 16 days after spacers were implanted in the tumor tissue. Three sets of images were acquired in each imaging session as follows. First, white light images were acquired with no filters in place in front of the EMCCD, the laser blocked and a 1 s camera exposure time. Second, images were acquired with the 740 nm filter in place. As discussed below, this "intrinsic" image was used for normalization of the fluorescence image.(3) The laser power at the sample was reduced to 110 $\mu W/cm^2$. The exposure time was set to 5 ms, and the gain on the EMCCD was 0. Third, fluorescence images were acquired with the 780 nm filter. Here the laser power at the sample was 14 mW/cm^2, the camera gain was set to 0 and the exposure time was between 1 and 30 s. Mice were also placed on a black foil mask to avoid saturation of the camera by direct illumination.

Image Processing and Normalization: *Image Processing and Normalization:* To allow direct comparison between imaging days, intrinsic and fluorescence images were first normalized for exposure time and laser intensity so that intensities were in photons/s. Fluorescence images were then normalized by the intrinsic images by pixel-by-pixel division of the 1004 x 1006 image arrays. Other authors have shown previously that this normalization operation improves the accuracy of fluorescence images since it accounts for the laser illumination profile and minor differences in illumination intensity. Normalization also improves quantitative accuracy in the presence of tissue optical property heterogeneities and allows comparisons between tissues of different thicknesses. When using this normalization method the trans-illumination geometry improves imaging depth compared to reflectance imaging which is limited to a few millimeters in depth due to the higher sensitivity on the surface of the tissue. The trans-illumination mode captures minimal excitation light and lower tissue autofluorescence because the light must travel completely through the tissue before being collected. (5)

References:

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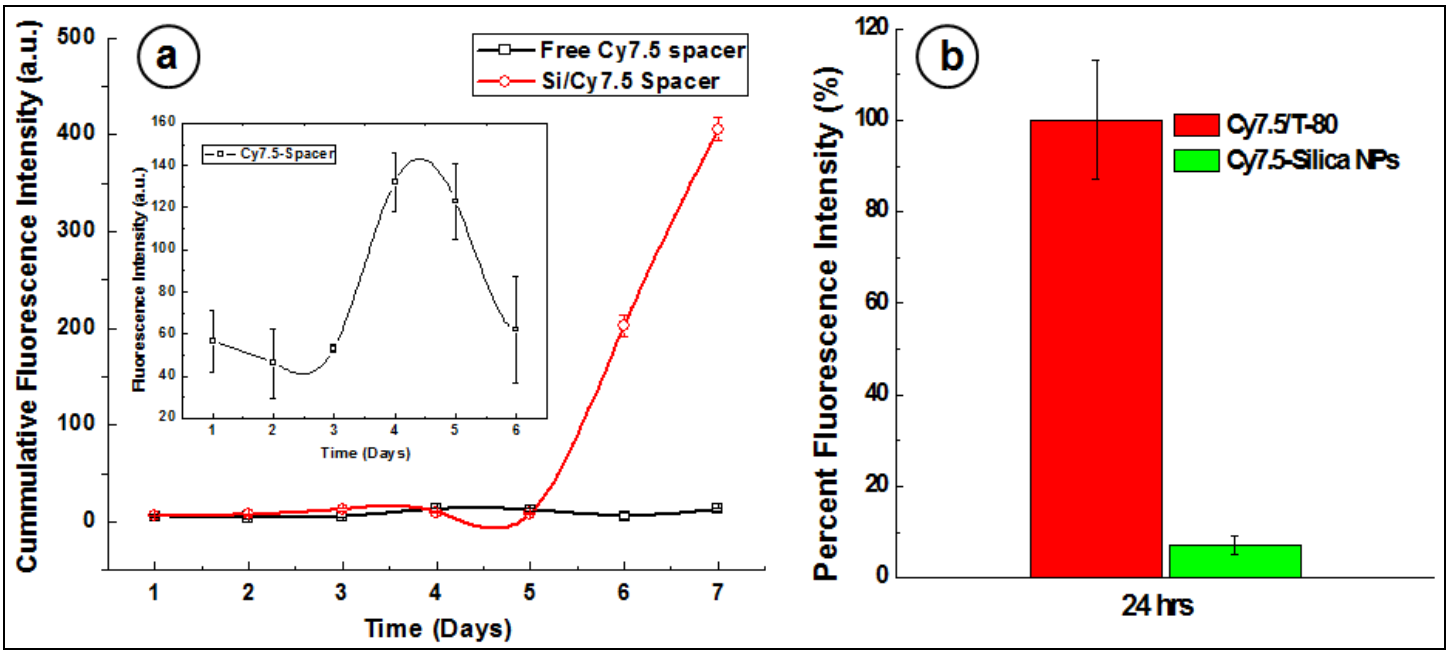
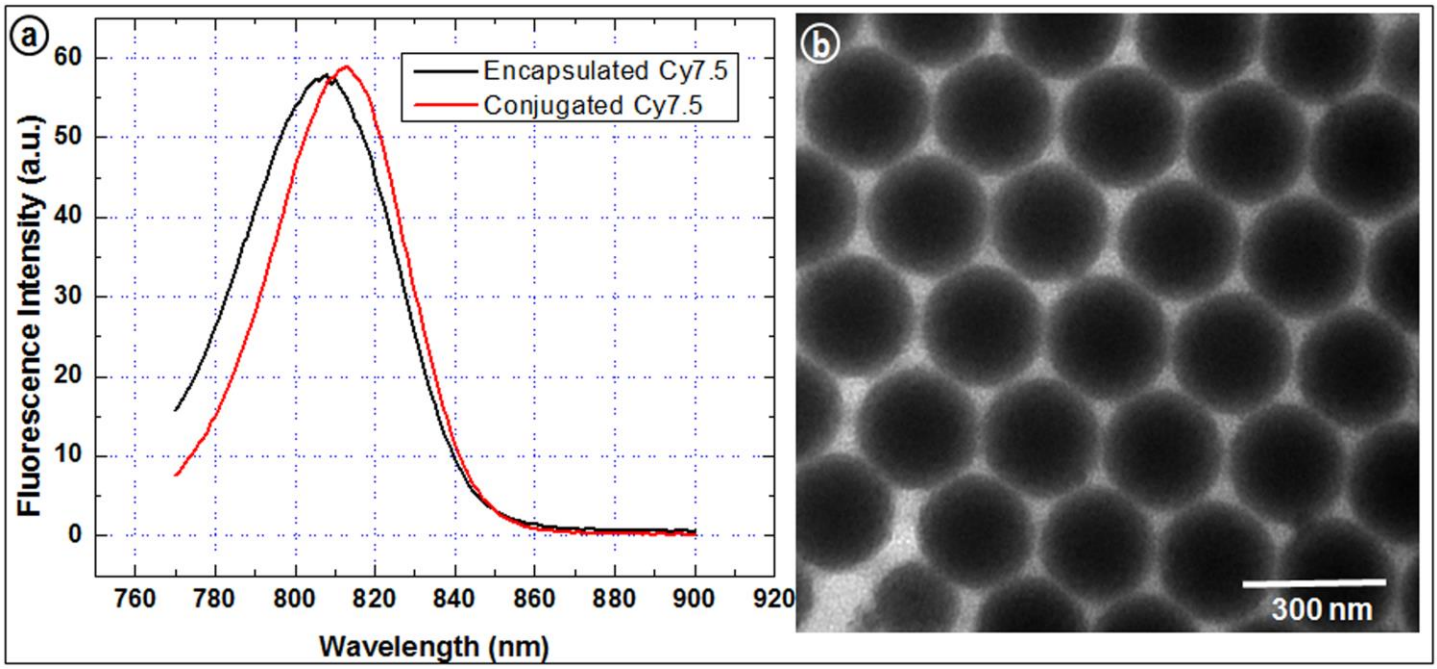
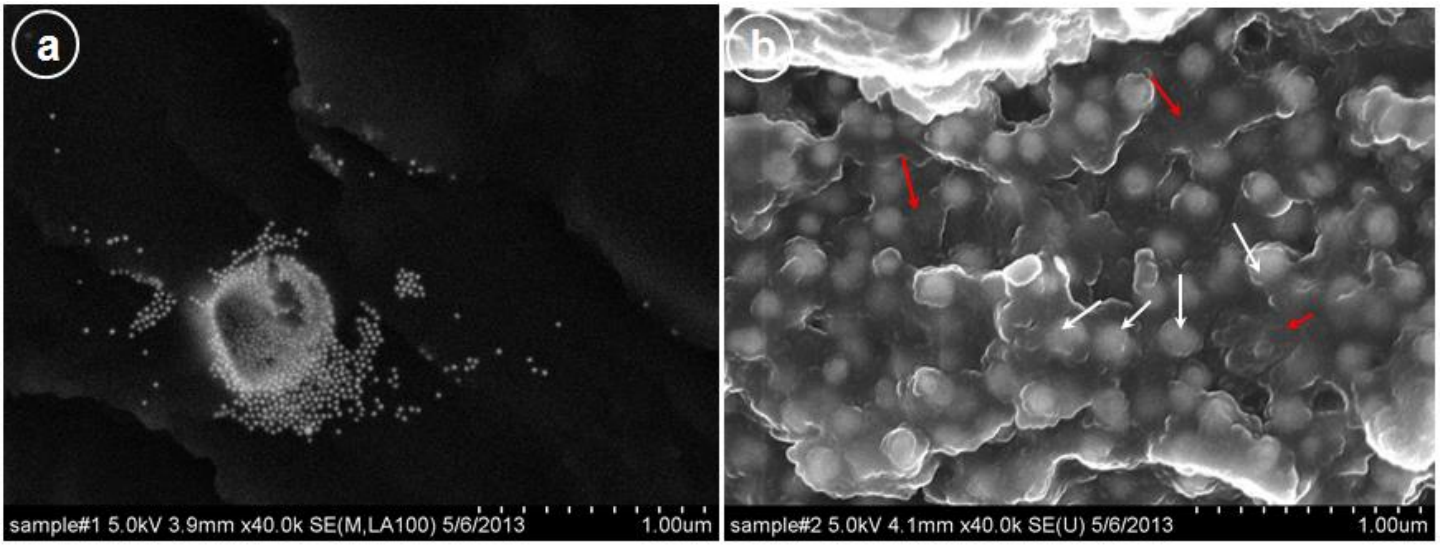
Supplementary Figures:



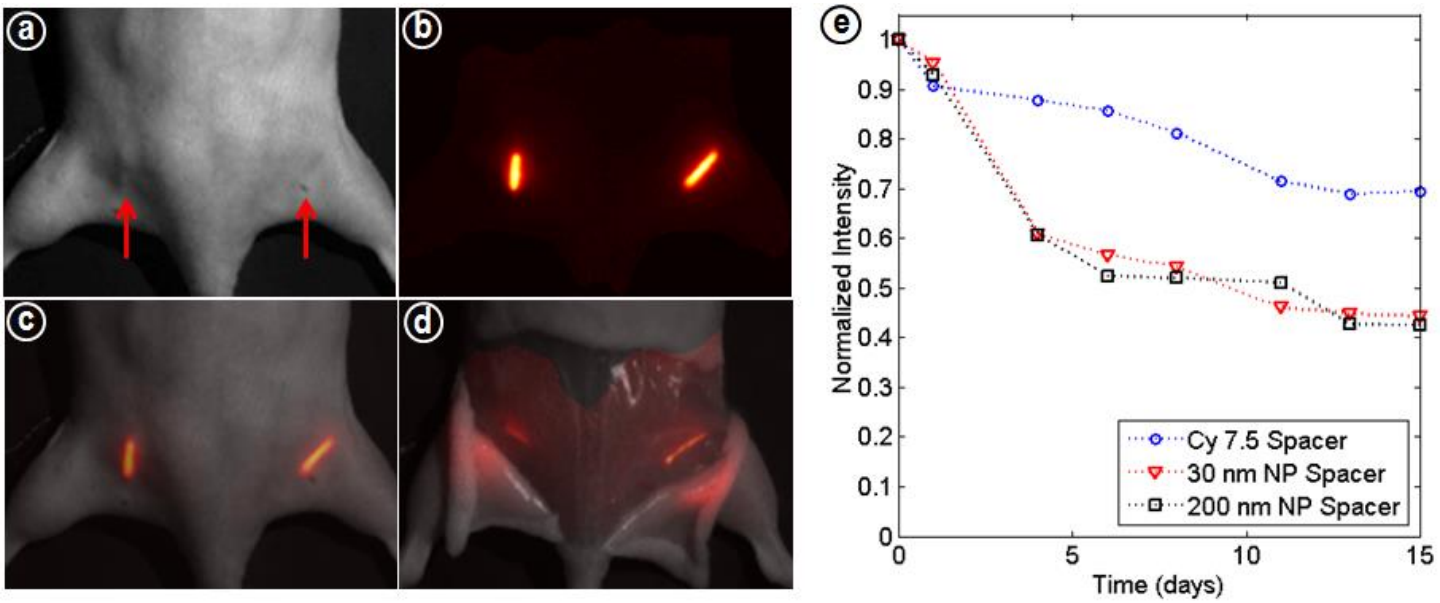
Supplementary Figure S1: (a) Release profile of the Cy7.5 from $^{Cy\ 7.5}$-PLGA spacers (n=4) and Cy7.5 conjugated silica nanoparticles from $^{Cy7.5\text{-}silica}$-PLGA spacers (n=4) in tris-HCl buffer at pH 7.4, (b) Minimal release of Cy7.5 from Cy7.5 conjugated Silica nanoparticles studied using spin filtration method.



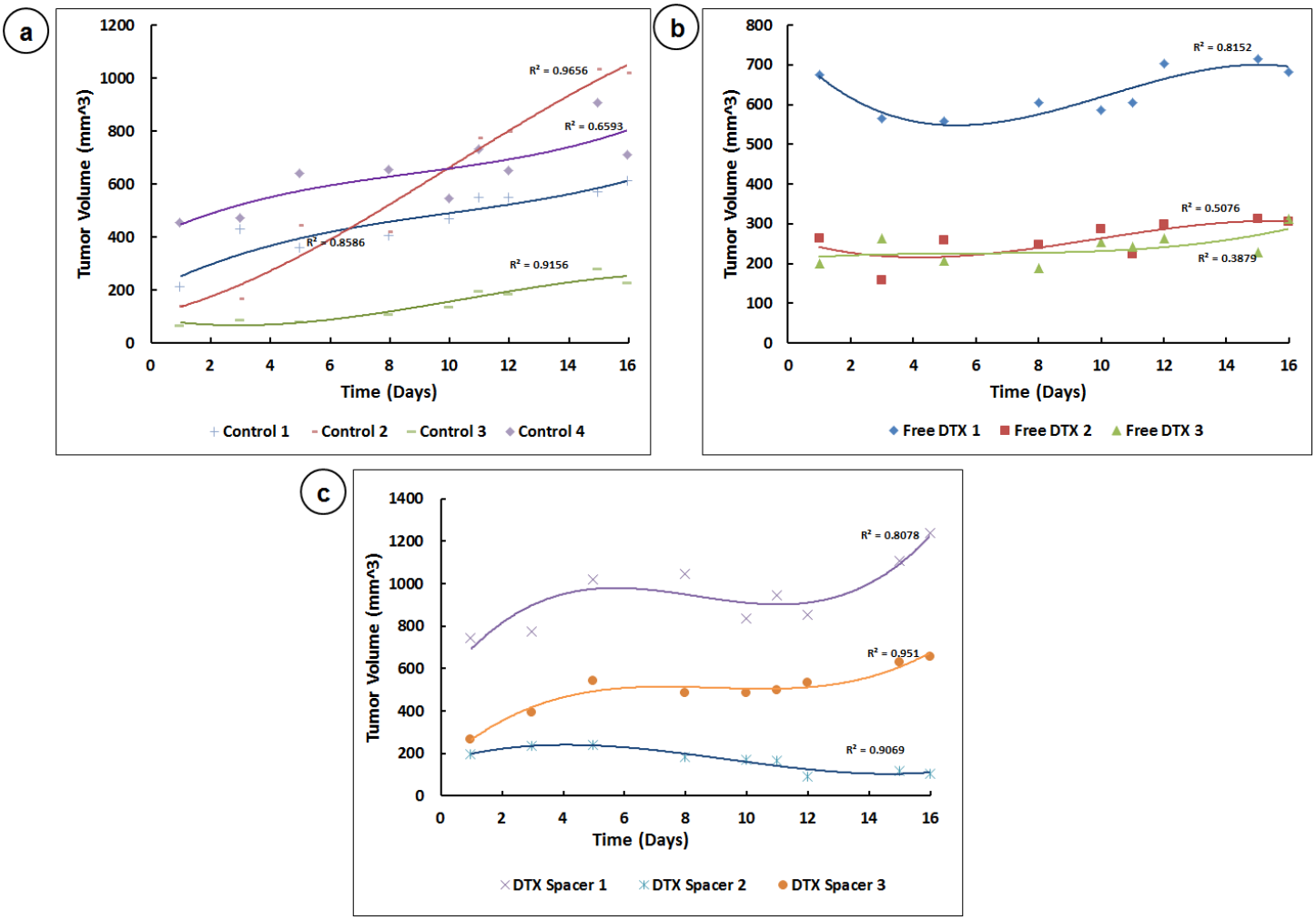
Supplementary Figure S2: Characterization of SNPs labelled with Cy7.5 by (a) Photoluminescence studies and (b) representative TEM image.



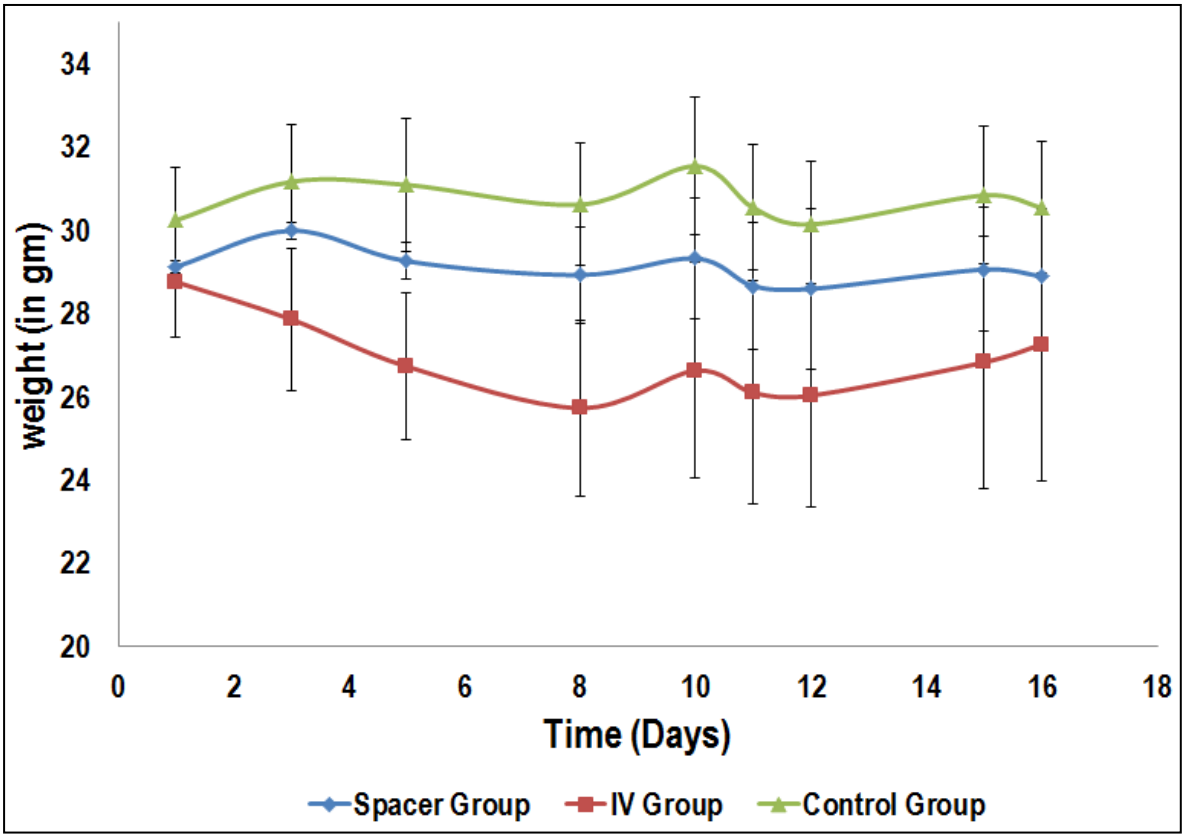
Supplementary Figure S3: SEM images of the PLGA spacer doped with electron dense gold nanoparticles to confirm the technique to visualize nanoparticles embedded in the PLGA matrix at (a) lower and (b) higher magnifications.



Supplementary Figure S4: In vivo image sequence of Cy 7.5 spacers. (a) White light image, (b) fluorescence image, (c) normalized image overlaid on (a), and (d) normalized residue image overlaid on (a). (e) Normalized intensity profile of the Cy7.5-spacer, 30 nm and 200 nm Cy7.5-SNPs-spacers implanted on the hind flank of the mice (n=6).



Supplementary Figure S5: Tumor regression studies as measured as change in tumor volume measured over a period of 16 days in prostate cancer subcutaneous tumor xenogarft mice model. (a) Control mice with no injections (n=4); (b) Free docetaxel injected intravenously (n=3) and (c) docetaxel INCeRT spacers implanted intratumorly (n=3). The docetaxel dose was 9.4mg/kg body weight in both the intravenous injection as well as in docetaxel INCeRT spacers implantation.



Supplementary Figure S6: Change in bodyweight of mice treated with control-no injection (green curve), free docetaxel injected intravenously (red curve) and docetaxel INCeRT spacers implanted intratumorly (blue curve) in prostate cancer subcutaneous tumor xenograft mice model.