Supporting Information

# Design and Synthesis of New Sulfur-Containing Hyperbranched Polymer and Theranostic Nanomaterials for Bimodal Imaging and Treatment of Cancer

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## EXPERIMENTAL SECTION

**Materials.** Chemicals were purchased from Sigma-Aldrich, including dimethylformamide, dimethyl sulfoxide, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, *N*-hydroxysuccinimide (NHS), diethylmalonate (DEM), 2-chloroethyl sulfide, taxol and sodium were purchased and used as received. EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) was purchased from Pierce Biotechnology. From Invitrogen, the near infra-red DiI dye and 4', 6-diamidino-2-phenylindole (DAPI) dye were purchased. The folate-receptor-overexpressing human NSCLC cells (lung A549 cells) and folate-receptor negative rat heart cells (H9c2) were purchased from ATCC. Bi-DOTA complex is received from Dr. J. M. Perez's

laboratory. From Spectrum Laboratories, dialysis membranes were obtained. Acetonitrile, potassium carbonate and tetrahydrofuran were purchased from Fisher Scientific.

**Instrumentations.** FT-IR spectra of monomers and polymer were recorded on a PerkinElmer's Spectrum TWO infra-red spectrometer. CARY 50 Bio UV/Vis spectrophotometer was used to record UV/Vis spectra. Fluorescence emission spectra were recorded on a TECAN Infinite M200 PRO multi-detection microplate reader. Bruker's 300 MHz spectrometer was used to record $^{1}H$ and $^{13}C$ NMR spectra of monomers and polymers where TMS was added as a standard internal reference. Shimadzu's SIL-20A instrument was used for Gel permeation chromatography (GPC) experiments, which was equipped with a light scattering precision detector. Malvern's Zetasizer Nano ZS90 instrument was used for the measurement of size and zeta potential of the HBPE-S nanoparticles. Fluorescence microscopic images were taken using Olympus IX73 microscope and Xenogen IVIS optical imaging system was used for the optical imaging of the HBPE-S NPs phantoms using Cy5.5 filter for DiI dye (excitation/emission: 551/580 nm). X-ray images were taken using Concorde Microsystem's X-ray instrument. TECAN Infinite M200 PRO multi-detection microplate reader was used for MTT study.

## 1. Synthesis and characterizations:

**Synthesis of 2-(2-ethyl ethyl sulfide)-malonic acid diethyl ester (3).** The starting material, diethyl malonate **1** (**Scheme 1**, 5.0 g, 31.25 mmol), 2-chloroethyl ethyl sulfide **2** (5.04 g, 40.63 mmol) were taken in a 200 mL flask along with 120 mL of acetonitrile as a solvent, and mixed at room temperature for 5 minutes. Next, we added potassium carbonate (12.84 g, 93.75 mmol) and refluxed for 36 hours. To the final reaction mixture, 100 mL of DI water was added and then ethyl acetate solvent was used for product extraction. The product in ethyl acetate was dried using anhydrous $Na_2SO_4$. The flash column chromatography was used for the purification of resulting product, where 3% ethyl acetate in hexane was used as mobile phase.

Yield: 6.27 g (81%). $^1$H NMR (300 MHz, $CDCl_3$, δ ppm, J Hz): 1.25 (t, 9H, J = 7.2), 2.18 (m, 2H), 2.55 (m, 4H), 3.57 (t, 1H, J = 7.6), 4.21 (q, 4H, J = 7.19). $^{13}$C NMR (75 MHz, $CDCl_3$, δ ppm): 14.08, 15.87, 25.59, 27.64, 30.15, 48.25, 59.84, 172.01. IR ($CHCl_3$): 2956, 1727, 1447, 1389, 1235, 1159, 1017, 814 $cm^{-1}$.

**Synthesis of 2-(2-ethyl ethyl sulfide)-2'-(4-acetoxybutyl)-malonic acid diethyl ester (4).** Compound **3** (5.0 g, 20.16 mmol) and NaH (1.45 g, 60.48 mmol) in dry THF (100 mL) and stirrer at room temperature for 30 min. 4-Bromobutyl acetate (5.89 g, 30.24 mmol) was added drop-wise and continued the reaction at 50 $^o$C for 6 h. The reaction was stopped by adding water and the reaction mixture was extracted using chloroform as organic solvent. The resulting organic layers containing products were dried using $Na_2SO_4$, and then purified using flash column chromatography.

Yield: 5.55 g (76%). $^1$H NMR (300 MHz, $CDCl_3$, δ ppm, J Hz): 1.23 (t, 3H, J = 7.2), 1.28 (m, 8H), 1.64 (m, 2H), 1.91 (m, 2H), 2.03 (s, 3H), 2.15 (m, 2H), 2.40 (m, 2H), 2.55 (q, 2H, J = 7.4), 4.05 (t, 2H, J = 6.4), 4.18 (q, 4H, J = 7.1). $^{13}$C NMR (75 MHz, $CDCl_3$, δ ppm): 14.01, 14.18,

20.64, 20.99, 25.66, 28.73, 32.37, 32.89, 57.31, 61.34, 63.98, 171.13. IR ($CHCl_3$): 2979, 1731, 1451, 1361, 1223, 1141, 1021, 850 $cm^{-1}$.

**Synthesis of 2-(2-ethyl ethyl sulfide)-2'-(4-hydroxybutyl)-malonic acid (5).** Compound **4** (5.0 g, 13.81 mmol) was mixed with 50 mL of methanol solvent and mixed for 5 minutes at room temperature. The aqueous solution of NaOH (1.66 g, 41.44 mmol, 5 mL) was prepared and cooled to room temperature before adding to the reaction flask. This reaction was continued for 7 h at 85 $^oC$. The obtained product was cooled to room temperature before acidifying to pH 5-6 using diluted hydrochloric acid (HCl) solution. The volume of the product mixture was reduced by using rotary evaporator and finally applying higher vacuum. The crude product was diluted with 50 mL of chloroform and bubbled with argon gas at 60 $^oC$ to remove excess HCl. The mixture was brought to neutral pH and then loaded on column chromatography for purification using 30% ethyl acetate in hexane as mobile phase.

Yield: 2.58 g (71%). $^1H$ NMR (300 MHz, $CDCl_3$, δ ppm, J Hz): 1.24 (t, 3H, J = 7.6), 1.31 (m, 2H), 1.58 (m, 2H), 1.93 (m, 2H), 2.21 (m, 2H), 2.42 (m, 2H), 2.48 (q, 2H, J = 7.3), 3.68 (t, 2H, J = 6.5), 5.04 (bs, 1H). $^{13}C$ NMR (75 MHz, $CDCl_3$, δ ppm): 15.22, 21.32, 25.13, 27.52, 30.21, 33.15, 57.26, 63.14, 171.47. IR ($CHCl_3$): 3498, 2903, 1698, 1626, 1459, 1446, 1361, 1187, 1147, 1046, 956, 767, 735, 658 $cm^{-1}$.

**Synthesis of aliphatic hyperbranched polyester polymer containing sulfur pendant (HBPE-S) 6.** The polymerization reaction was performed using monomer **5** and *p*-toluene sulfonic acid as catalyst (100:1 molar ratio). In a typical melt polymerization reaction, 10 mL round bottom flask was used as polymerization vessel. The monomer and catalyst were taken in the flask, high vacuum was applied for 30 minutes for drying and then purged with dry argon gas. The reaction was heated to 150 °C and continued for 2 h. To the resulting melted reaction mixture, high vacuum (0.2 mm/Hg) was applied for 2 h, while the temperature of the

polymerization reaction was kept at 150 °C. The modified solvent precipitation method was used for the purification of HBPE-S polymer. Briefly, the polymer was dissolved in DMF solvent and precipitated in pure MeOH solvent. The pure polymer was collected after centrifugation and dried under reduced pressure to get pure HBPE-S polymer.

Yield: 66%. $^{1}$H NMR (300 MHz, DMSO-$d_6$, δ ppm, J Hz): 1.12 (bs, 3H), 1.25 (bs, 2H), 1.52 (bs, 2H), 1.75 (m, 2H), 1.99 (m, 2H), 2.42 (bs, 2H), 2.53 (bs, 2H), 3.99 (bs, 2H). $^{13}$C NMR (75 MHz, DMSO-$d_6$, δ ppm): 14.93, 15.05, 23.64, 25.27, 28.47, 28.83, 31.65, 32.09, 44.39, 63.86, 174.82. IR (Neat): 2953, 2867, 1726, 1674, 1458, 1436, 1388, 1343, 1240, 1217, 1152, 1093, 1055, 1027, 950, 812, 772, 659 $cm^{-1}$. TGA: 5% weight loss at 235 $^{o}$C.

**Synthesis of cargos-encapsulating polymeric nanoparticles (7): Solvent diffusion method.**[33] Optical dye DiI (6 µL, 5 µg/µL), taxol (6 µL, 2 µg/µL) and Bi-DOTA (6 µL, 30 µg/µL) solutions were mixed in 500 µL of DMF solvent containg 25 mg of HBPE-S polymer and vortexed. This DMF solution of polymer and theranostic agents was slowly added to 5 mL of stirring DI water at 1000 rpm. The resulting cargos encapsulated HBPE-S nanotheranostics (**7**) were purified by two different techniques: 1) PD-10 column and followed by 2) dialysis (MWCO 6-8K) using PBS (pH=7.2) solution.

**Synthesis of PEGylated and alkynated HBPE-S nanoparticles 8: Water-based EDC chemistry.** First, PEGylations were performed on PNPs **7** (**Figure 2**) using standard EDC/NHS chemistry.[31] Briefly, the carboxylated HBPE-S nanoparticle (**7**, 1.0 mmol) in PBS (pH = 7.20) solution was mixed with solution of EDC (10 mmol) using MES buffer (pH = 6.0) and incubated for 10 seconds before adding NHS (10 mmol) solution. The final reaction mixture was placed on an orbital mixture and incubated for 3 min at room temperature. To the resulting reaction mixture, PEG polymer (HOOC-PEG-$NH_2$, 10 mmol, $M_w$ = 617.17) in water was added drop-wise and continued for 3 h to obtain PEGylated HBPE-S NPs. These propargylated PNPs were

then dialyzed (dialysis bag of MWCO 6-8K) in PBS (pH= 7.2) solution. Next, propargylation reaction was performed on PEGylated HBPE-S NPs using the EDC/NHS carbodiimide chemistry. Next, the PEGylated HBPE-S nanoparticles (1.0 mol) were propargylated using propargylamine and carbodiimide chemistry. Briefly, solutions of EDC (12 mol) and NHS (12 mol) in MES buffer (pH 6.0) were mixed to the PEGylated HBPE-S nanoparticles (1.0 mol) and incubated briefly at room temperature for 3-4 minutes. The propargylamine (12 mol) in DMF solvent was added drop-wise and to the reaction mixture and continued for 3 h in order to obtain propargylated and PEGylated HBPE-S NPs **8**. These propargylated PNPs **8** were purified by dialysis (MWCO 6-8K dialysis bag) in PBS (pH=7.2) solution.

**Synthesis of folate-functionalized HBPE-S NPs 9: "Click" chemisrty.** The triple bond functionalized HBPE-S nanoparticles **8** (0.025 g, 6 x $10^{-3}$ mmol) and the catalyt CuI (0.12 µg, 7 x $10^{-10}$ mmol) in 200 µL of basic sodium bicarbonate buffer (pH = 8.4) were added to an 15 mL eppendorf tube and vortexed for 30 seconds. The previously reported azide-functional folic acid[37] (4 mg, 7 x $10^{-2}$ mmol, **Figure 2,** and the synthesis is described below) was prepared in DMSO solvent and slowly added to the reaction mixture. The reaction was continued for more than 10 h at room temperature. Next, A PD-10 column was used to purify the resulting folate-conjugating HBPE-S nanoparticles (**9**) and then followed by dialysis (MWCO 6-8K) in PBS (pH=7.4) solution. The purified polymeric NPs were stored in refrigerator at 4 $^{o}$C.

**X-ray imaging of Bi-DOTA encapsulating HBPE-S NPs (9).** Different concentrations of Bi-DOTA complex encapsulating HBPE-S NPs (**9**, 1-5 µg/µL) were prepared using solvent diffusion method. These NPs (1 mL) were taken in 1.5 mL eppendorf tubes and housed in the CT scanner probe. The X-ray images were aquired using Concorde Microsystem's X-ray instrument.

**Gel Permeation Chromatography (GPC).** For the determination of weight-average molecular weight of HBPE-S polymer, Gel Permeation Chromatographic (GPC) technique was used. Polystyrene standards were used for the calculation of average molecular weight and HPLC grade DMF was used as mobile phase. The experimentally calculated weight-average molecular weight of HBPE-S polymer **6** was $M_w$ = 38,000, and the polydispersity index was found to be PDI = 1.86.

***In vitro* experiments.**

**Cell culture studies.** The human NSCLC (lung cancer, A549) and healthy rat heart (H9c2 cells, normal tissue) cells were directly purchased from ATCC, and seeded according to the supplier's provided protocols. However, the growth media for A549 cells was prepared using 94% DMEM supplemented with 5% FBS and 1% antibiotic-antimycotic (ABAM) solution, whereas the rat heart cells (H9c2) were cultured in MEM medium containing 10% FBS and 1% ABAM solution. Standard cell culture incubator humidified at 37 $^oC$ under 5% $CO_2$ atmosphere was used to incubate cells.

**Optical imaging: Fluorescence microscopy**. On culture petri-dishes, A549 and H9c2 cells were propagated (approximately, 10,000 cells/dish) and then incubated with various HBPE-S NPs (35 μL, 2.5 mM). The cells were washed using 1X PBS, pH 7.4 and fixed with 4% paraformaldehyde solution following standard protocols. Subsequently, nuclei stained with DAPI (1 mg/mL) solution. These cells were examined using Olympus IX73 fluorescence microscope equipped with a 40X objective.

**Cell viability experiments: MTT assay.** The NSCLC A549 cells and cardiomyocyte H9c2 cells were propagated in a high throughput 96-well plates (2,500 cells/well). Various functional HBPE-S nanoparticles (25 μL, 2.5 mM) were added to these cells and incubated at 37 $^oC$ for 24

h. Every well was cleaned by washing with PBS solution, 1X, pH 7.4 and treated with 25 μL of MTT (3.0 μg/μL) solution for 3 h. Next, the formation of formazan crystals was confirmed by observing purple color which was dissolved using acidic 2-propanol solution (0.1 N HCl). Afterwards, the UV/Vis absorbance spectra were measured at 570 nm (in addition, at 750 nm for background), using TECAN Infinite M200 Pro high throughput microplate reader.

***In vitro* drug release study.** The dynamic dialysis technique was used for the *in vitro* drug release studies at 37 $^{o}$C. In a typical process, a dialysis bag (MWCO 6,000-8,000) was loaded with 100 μL of HBPE-S NPs (**9**, 5 mmol) and esterase enzyme (porcine liver, 20 μL, 1 mM), and dialyzed in a 1 L beaker containing PBS (pH 7.4) solution. With time, as HBPE-S polymer networks degrade, the encapsulating theranostic cargoes released to the external reservoir containing PBS (pH 7.4) solution. The rate of drug released was measured by collecting 1.0 mL of aliquots from the external reservoir at a regular time gap and the fluorescence emission for taxol drug was measured at 370 nm. A standard calibration curve was used for calculating the amount of taxol drug released. The following equation was used to calculate cumulative release and plotted against time.

$$\text{Cumulative release (\%)} = [\text{taxol}]_t \,/\, [\text{taxol}]_{\text{total}} \times 100$$

Where, $[\text{taxol}]_{\text{total}}$ represents total taxol present in the taxol-encapsulating HBPE-S NPs and $[\text{taxol}]_t$ represents at a definite time ‘t’, the amount of taxol drug released.

1. **Time-dependent molecular weight of HBPE-S polymer**

| Heating at 150 °C | High vacuum 0.2 mm/Hg | Molecular weight ($M_w$) | Polydispersity (PD) |
|---|---|---|---|
| 2 h | - | 2,350 | 4.38 |
| 3 h | 1 h | 12,000 | 2.72 |
| 4 h | 2 h | 38,000 | 1.86 |

**Table S1.** Measurement of molecular weight and polydispersity of HBPE-S polymer using gel permeation chromatography.

2. **FT-IR spectra of the functional monomers and HBPE-S polymer**

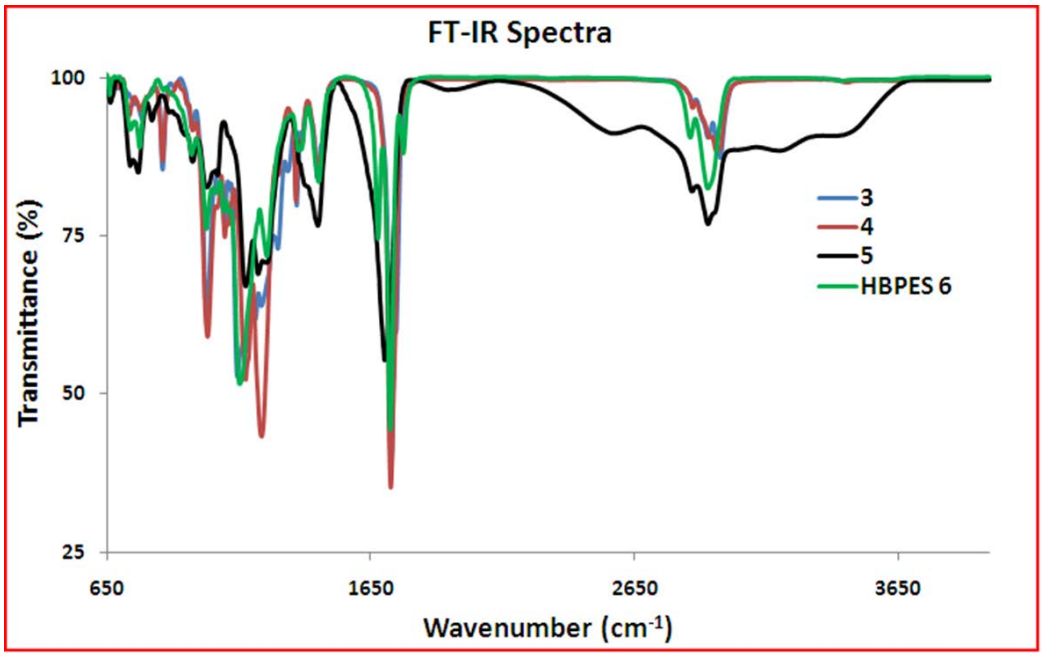


**Figure S1.** FT-IR spectra of monomers (**3**, **4** and **5**) and HBPE-S polymer, **6**. The presence of carbonyl stretching bands both for acid and ester in the polymer's FT-IR spectrum, confirmed for the successful synthesis of HBPE-S polymer.

**3. Zeta potential (overall surface charge) measurement of functional HBPE-S nanoparticles (7, 8 and 9).**

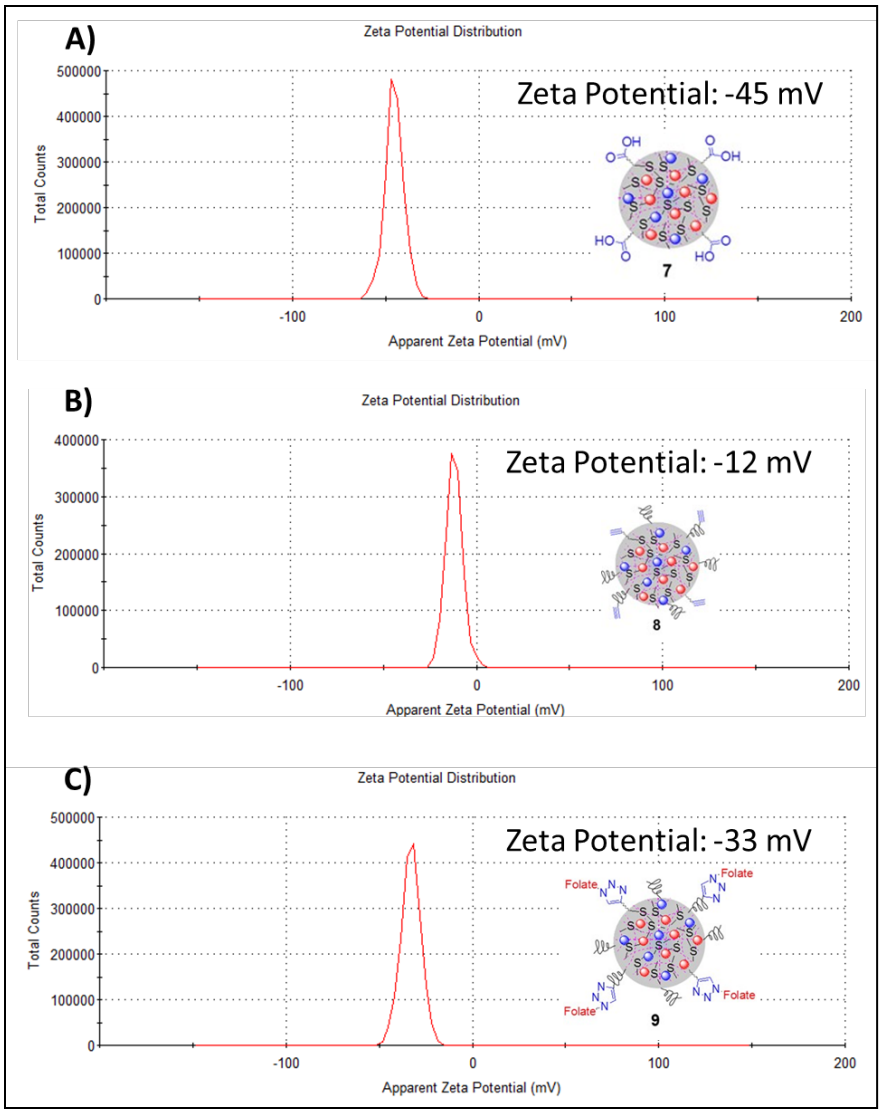


**Figure S2.** Zeta potential measurements for HBPE-S nanoparticles (**7**, **8** and **9**). Different zeta potential values indicated for successful functionalization.

## 4. Stability of functional HBPE-S nanoparticles

| HBPE-S NPs | 7 days | 1 month | 3 months | 6 months |
|---|---|---|---|---|
| **7** | D = 82 ± 2 | D = 83 ± 3 | D = 82 ± 4 | D = 86 ± 4 |
| | $\lambda_{em}$ = 4.8 x $10^4$ | $\lambda_{em}$ = 4.9 x $10^4$ | $\lambda_{em}$ = 4.6 x $10^4$ | $\lambda_{em}$ = 4.2 x $10^4$ |
| **9** | D = 85 ± 1 | D = 85 ± 3 | D = 86 ± 2 | D = 90 ± 2 |
| | $\lambda_{em}$ = 3.7 x $10^4$ | $\lambda_{em}$ = 3.5 x $10^4$ | $\lambda_{em}$ = 3.4 x $10^4$ | $\lambda_{em}$ = 3.2 x $10^4$ |

**Table S2.** Hydrodynamic diameter (DLS, nm) and fluorescence emission at 572 nm (A.U.) of DiI dye encapsulated, carboxylated **7** and folate **9** HBPE-S nanoparticles over the period of time, showed excellent stability of our HBPE-S NPs on PBS (1X, pH 7.4).

## 5. Diameter of the PEGylated PNPs:

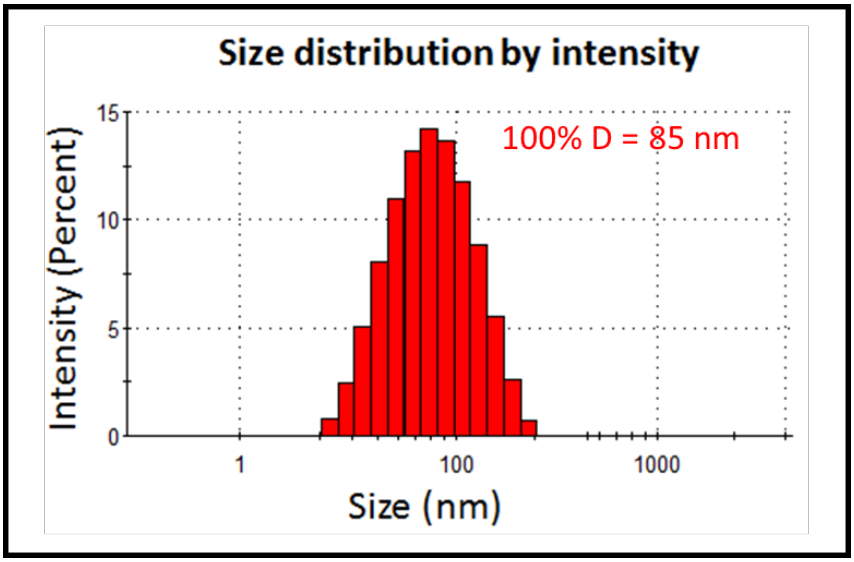


**Figure S3.** The overall diameter of the PEGylated PNPs **7** using DLS method.

## 6. Spectrophotometric characterization of taxol-encapsulating PNPs (9)

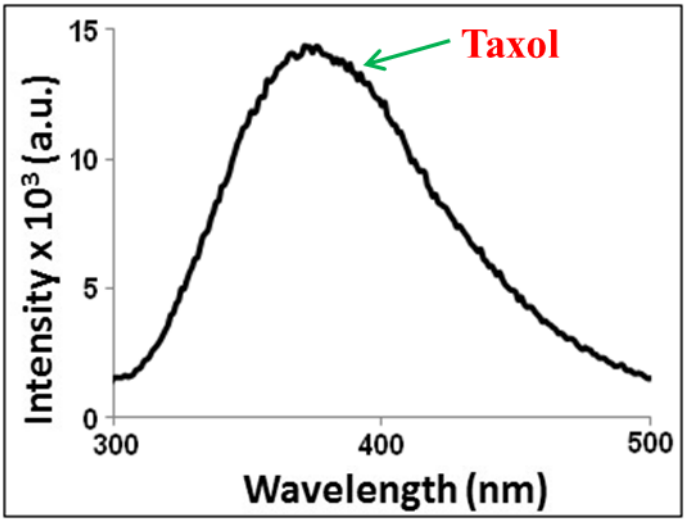


**Figure S4.** Fluorescence emission spectrum confirming for the effective encapsulation of taxol in PNPs **9** (taxol: $\lambda_{em}$ at 370 nm)

## 7. X-ray imaging of Bi-DOTA encapsulating HBPE-S, HBPE nanoparticle phantoms and that of clinically approved Omnipaque standard solutions.

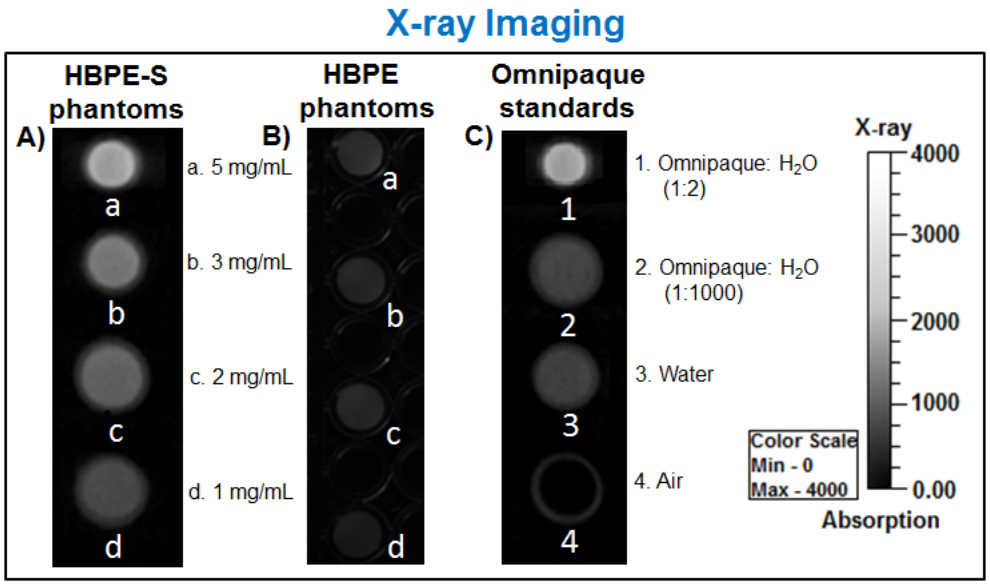


**Figure S5.** X-ray imaging of functional **A**) HBPE-S nanoparticles (**9**) phantoms and **B**) HBPE NPs phantoms. **C**) Different concentrations of Omnipaque, the clinically approved X-ray contrast agent, water and air were used as standards for comparing X-ray contrasts obtained from HBPE-S and HBPE nanoparticle phantoms.

## 8. Diameter of the protein corona-coated PNPs:

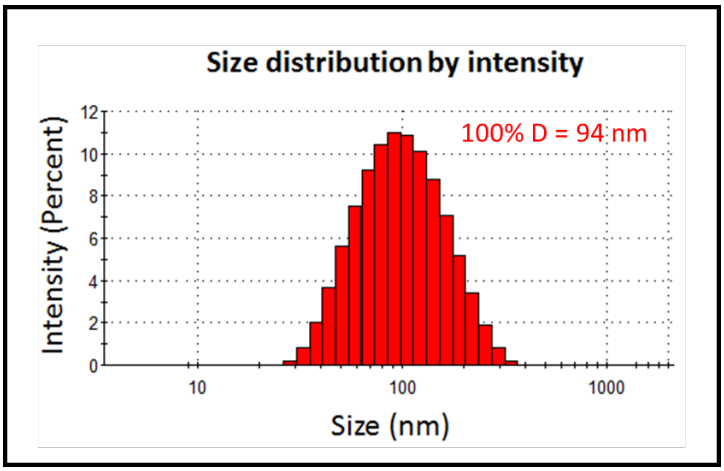


**Figure S6.** The overall diameter of the protein corona-coated PNPs was found to be 94 nm.

## 9. Drug release study using *in vivo* mimicking PNPs.

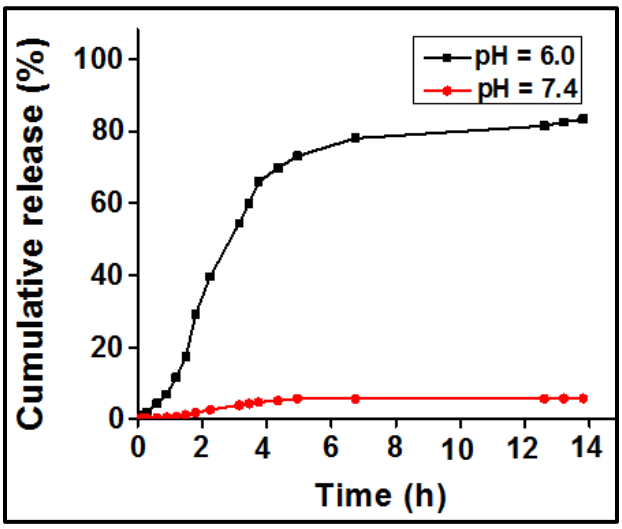


**Figure S7.** Evaluation of drug release profiles for taxol encapsulating protein corona-coated HBPE-S PNPs using dialysis method at pH = 6.0, at 37 $^{o}$C. As expected, a slower rate of taxol release was observed when compared with PNPs with no prior incubation with plasma protein (**Figure 4B**). No significant release of drug was found in physiological pH (red line).

**10. Cytotoxicity experiments with serum-starved A549 cells using protein corona-coated PNPs.**

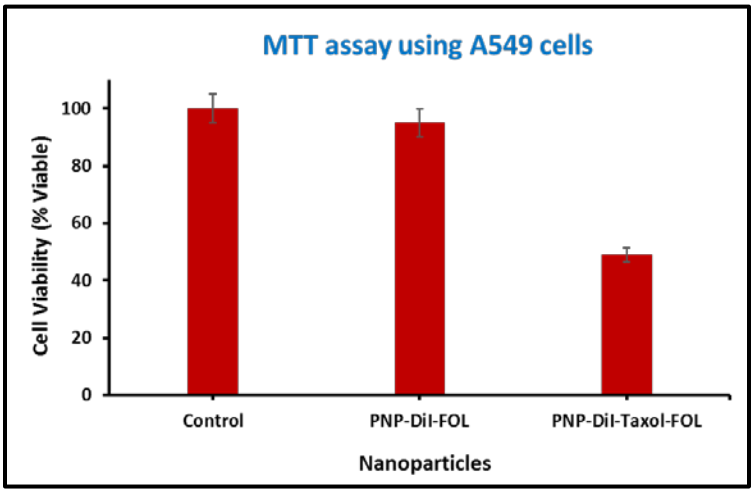


**Figure S8.** Cytotoxicity of protein corona-coated PNPs with serum starved A549 cells using MTT assay. Functional HBPE-S PNPs (PNP-DiI-Taxol-Fol) showed more than 55% of cell death after 24 h of incubation. Minimal cytotoxicity was observed for PNPs with no taxol (PNP-DiI-Fol). Control: 1X PBS (pH = 7.4) buffer was used to treat control cells. Standard error was calculated from the mean of three measurements.

**11. Cytotoxicity experiments with cardiomyocytes.**

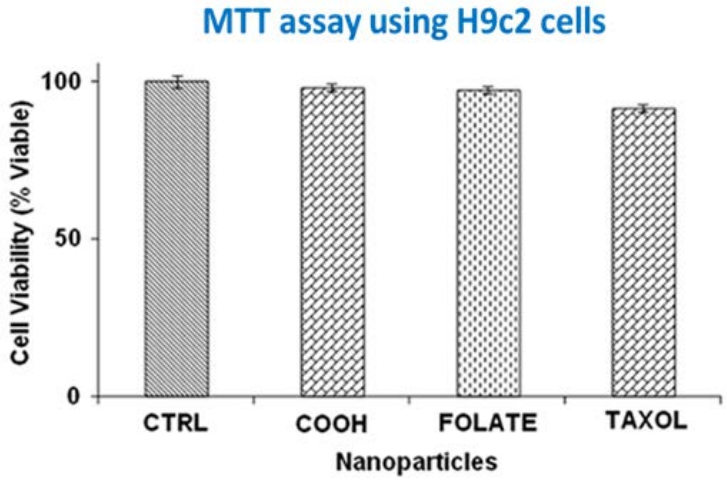


**Figure S9.** Assessment of cytotoxicity of functional HBPE-S nanoparticles after 24 h of incubation using MTT assay. Functional HBPE-S PNPs (TAXOL, **9**) showed minimal cytotoxicity for H9c2 cells due to the lack of folate receptor over-expression and thereby, no effective internalizations. CTRL: 1X PBS (pH = 7.4) buffer was used to treat control cells. Standard error was calculated from the mean of four measurements.

**12. Competition assay: folate saturation.**

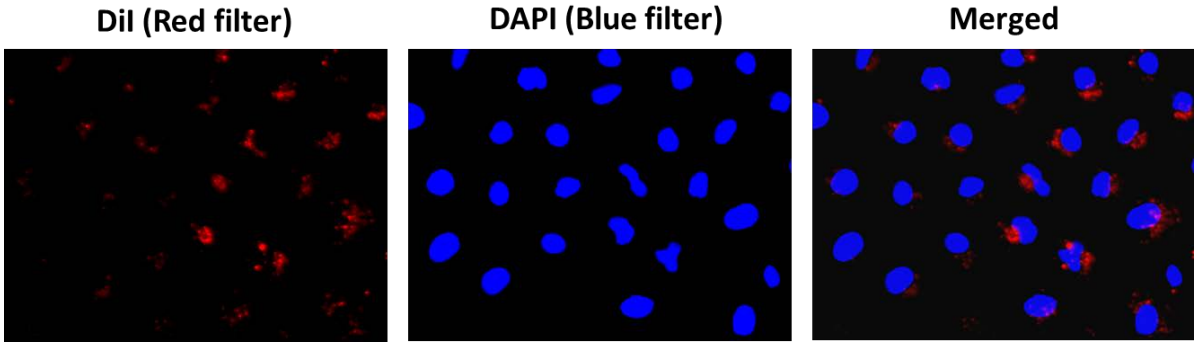


**Figure S10.** Minimal internalization of our folate NPs (**9**) was visualized when NSCLC A549 cells (FR +) were pre-incubated with folic acid in excess. These results further indicated for the targeted receptor-mediated internalizations of our folate conjugating NPs.

**13. *In vitro* cellular uptake of folate conjugating HBPE-S NPs in rat cardiomyocyte (H9c2) cells.**

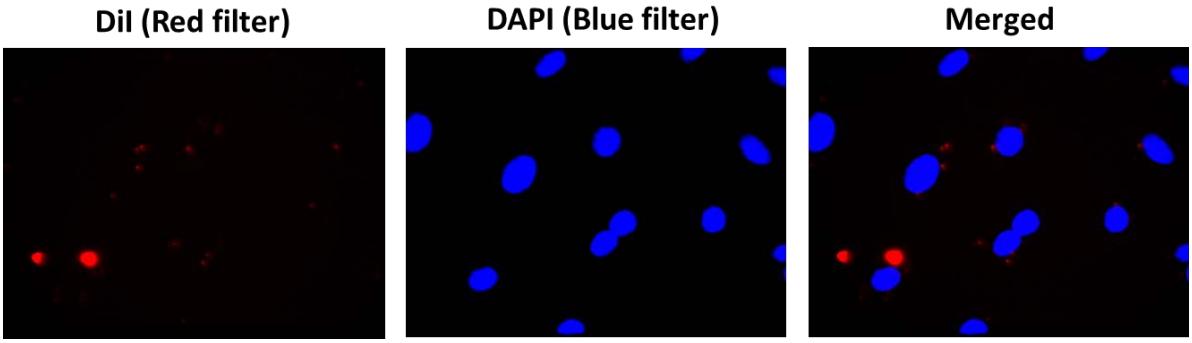


**Figure S11.** No effective internalization was observed in folate-receptor negative H9c2 cells, when incubated with folate conjugating HBPE-S NPs (**9**). This result further corroborated for the folate-receptor mediated internalization and cancer targeting capability of our HBPE-S nanoparticles.