Electronic Supplementary Material (ESI) for Biomaterials Science.

Supporting information for

"Efficient Photoacoustic Imaging using Indocyanine Green (ICG) Loaded Functionalized Mesoporous Silica Nanoparticles"

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Supporting Materials

SECTION S1. Calculation of ICG Loading Capacity and Loading efficiency

The loading capacity and efficiency were calculated using the following equations.

(a) $\boldsymbol{Loading\ Capacity}\ (\%) = \frac{Weight(drug)}{Weight(drug)+Weight(particles)} \times 100$

e.g. for NH_2-MSN-ICG $Loading\ Capacity\ (\%)\ = \frac{165\ \mu g}{165+835\ \mu g} \times 100 = 16.5\ \%$

$(\boldsymbol{b})\ \%\ \boldsymbol{Loading\ Efficiency} = \frac{Mass(drug\ loaded\ onto\ the\ particles)}{Mass(Initial\ drug\ added\ from\ stock)} \times 100$

e.g. for LBLMSN-ICG $\%\ Loading\ Efficiency\ = \frac{900\ \mu g}{1000\ \mu g} \times 100 = 90\ \%$

SECTION S2. Cultivation of cell lines (HepG2 and RAW264.7)

Raw 264.7 and HepG2 (P15) cells were cultivated in DMEM (Dulbecco's Modified Eagle Medium) and MEM (Minimal Eagle Medium) respectively. The media were supplemented with 10 % FBS (Fetal Bovine Serum), 1 % Penicillin-Streptomycin (Pen Strep) and 1 % Glutamax (L-glutamate). Cells were split thrice before treatment with MSN particles at a split ratio 1: 4 from cryopreservation state. 100 µL of 50,000 cells mL^{-1} cell suspension was added to each well black clear bottom 96 well plate (four plates in total) using the multichannel pipette to make the concentration 5000 cells per well. Subsequently, 100 µL of media was added into each well of the plates. All four plates were transferred to incubator under sterile conditions for 24 hours culture. Afterwards, treatment was added to each well (quadruplicate) from a 4 mL stock of 200 µg mL^{-1} of each type of MSNs (chemically and physically modified MSNs). Please note prior to adding the sample suspension into the wells equivalent volume was removed from wells to be replaced by treatment volume being added. At 24 hours, 10 % of the well volume i.e. 20 µL of Alamar Blue was added and the plates were further incubated for 4 hours. After 4 hours plates were removed and the absorbance was read using microplate reader at 570 nm 600 nm. Calculations were performed according to the manufacturer's protocol.

SECTION S3. *In-vitro* Photoacoustic Imaging Set-up

The phantom holder is shown in the Figure S1 to load the samples (particles) for photoacoustic signal scan. It can be seen three tubes are fitted into the holder filled with the ICG loaded particles. The phantom holder was filled with tap water above the level of tubing and the transducer was set after turning the B-mode on from the system above 10 mm of the tubing surface.

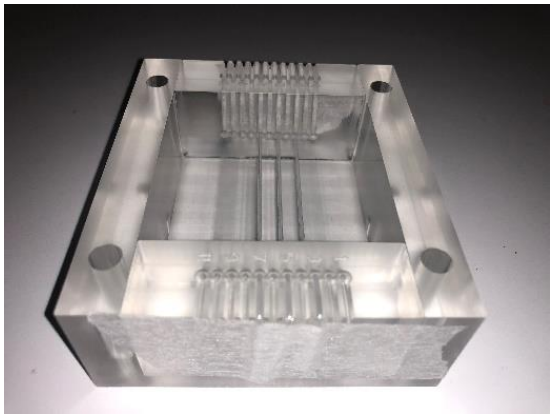

Fig. S1 *In-vitro* PA imaging setup. Custom-made phantom holder fitted with three sample tubes containing ICG loaded particles.

Table S1. Excitation wavelengths for detection of PA signals by MSNs

Samples	**Wavelengths (nm)**
MSN-ICG	680, 796, 805, 894, 924
NH_2-MSN-ICG	680, 796, 810, 894, 924
PO_3-MSN-ICG	680, 700, 796, 894, 924
LB-MSN-ICG	680, 700, 800, 894, 924
LBL-MSN-ICG	680, 718, 760, 924, 965
Free ICG	680, 740, 894, 924
Pristine MSN	680, 718, 760, 924, 965
PBS	680, 730, 752, 886, 924, 935

The wavelengths mentioned next to the type of particles in Table S1 depict the wavelengths, which were unmixed from the final 3D PA intensity signal to subtract any signal coming from oxygenated and deoxygenated haemoglobin.

Supporting Results

SECTION S4. TEM and DLS of pristine and functionalized MSNs

To measure the hydrodynamic diameter of pristine and functionalized MSNs at 1 mg mL^{-1} concentrations were prepared in milli-Q H_2O and bath sonicated prior to DLS/Zeta potential analysis. Wherever needed, the suspension was further diluted 10 times with water. Total 3 measurements were taken (each measurement with 12 sub runs) and the final value was averaged.

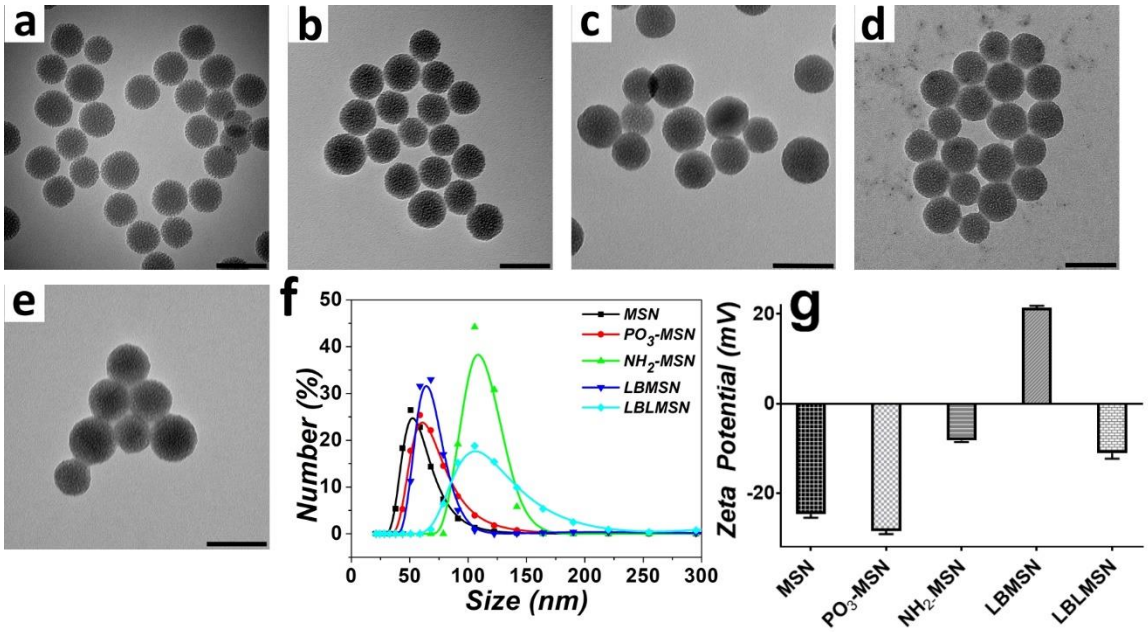

Fig. S2 Transmission electron microscope (TEM) images and DLS data for unloaded MSNs. TEM images of empty (a) MSN particles, (b) PO_3-MSN, (c) NH_2-MSN, (d) LBMSN, (e) LBLMSN, (f) hydrodynamic radius of particles analyzed by suspending in water, NH_2-MSN particles were dissolved in ethanol to avoid aggregation resulting in larger hydrodynamic diameter and (g) Zeta Potential of the particles in (a)-(e) as measured using dynamic light scattering. Scale bar for all the TEM images is 100 nm.

Table S2. Physicochemical properties of unloaded functionalized MSNs measured using BET, DLS, and TGA.

Note: Size: Number mean; PDI: Polydispersity Index; ζ: Zeta potential

Sample	**BET**			**DLS**			**% Weight Loss (TGA)**
	Pore Volume (cm^3/g)	Pore size (A°)	Surface Area (m^2/g)	Size (nm)	PDI	ζ (mV)	
MSN	0.61	27.18	442.39	56.8 ± 4.06	0.31 ± 0.07	-24.54 ± 0.81	0.42
PO_3-MSN	0.24	28.52	221.24	64.4 ± 1.10	0.30 ± 0.01	-28.63 ± 1.00	3.89
NH_2-MSN	0.55	76.38	165.67	110.8 ± 3.68	0.65 ± 0.05	-8.30 ± 0.31	9.85
LBMSN	-	-	-	65.3 ± 14.83	0.43 ± 0.08	21.33 ± 0.51	-
LBLMSN	-	-	-	112.6 ± 15.58	0.79 ± 0.12	-11.98± 0.90	-

Note: Size: Number mean; PDI: Polydispersity Index; ζ: Zeta potential

SECTION S5. BET Nitrogen Adsorption Surface Area Analysis

The MSNs (pristine, PO_3-MSN, NH_2-MSN) were weighed around 70-100 mg and put for degassing prior to Nitrogen sorption analysis to remove any moisture or air bubbles from the pores. The samples were degassed at 70-80 °C for 24 hours and the weights before and after degassing were recorded. After degassing the samples were subjected to Sorption analysis and the reports were extracted.

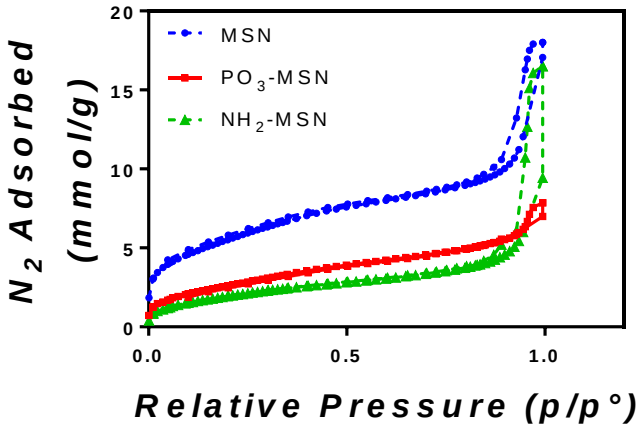

Fig. S3 N_2-BET plot for chemically functionalized MSNs**.** Nitrogen physisorption Analysis of Pristine MSNs (Blue), PO_3-MSNs (Red) and NH_2-MSNs (Green). The particles exhibit type-4 hysteresis loop and retain the porous architecture after chemical modifications.

SECTION S6. Thermogravimetry Analysis (TGA) of pristine and chemically functionalized MSNs.

TGA of pristine particles and chemically functionalized MSNs was performed to calculate the % mass grafting onto the MSNs. 5 mg of each particle was weighed into the alumina crucibles and subjected to TGA.

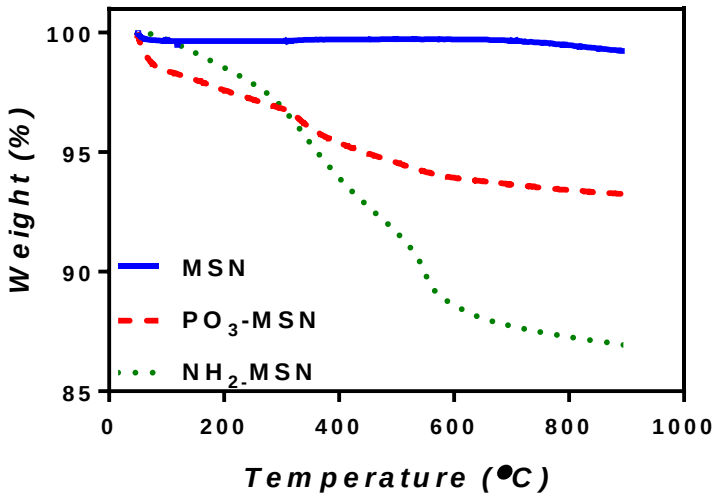

Fig. S4 Thermogravimetry Analysis (TGA) of chemically functionalized MSNs. TGA plot to calculate % mass grafting onto pristine MSNs. The method comprised of a temperature range from 50 °C to 900 °C with a heat rate of 10 degrees per minute.

SECTION S7. Fourier Transformed Infrared spectroscopy (FTIR) of pristine and functionalized MSNs with and without ICG

The FTIR analyses were performed on Perkin Elmer FTIR Spectrometer. A pinch of sample was used and the force gauge was set to 50. Number of scans were set to 254 and resolution was set to 16 (Figure S5 and S6).

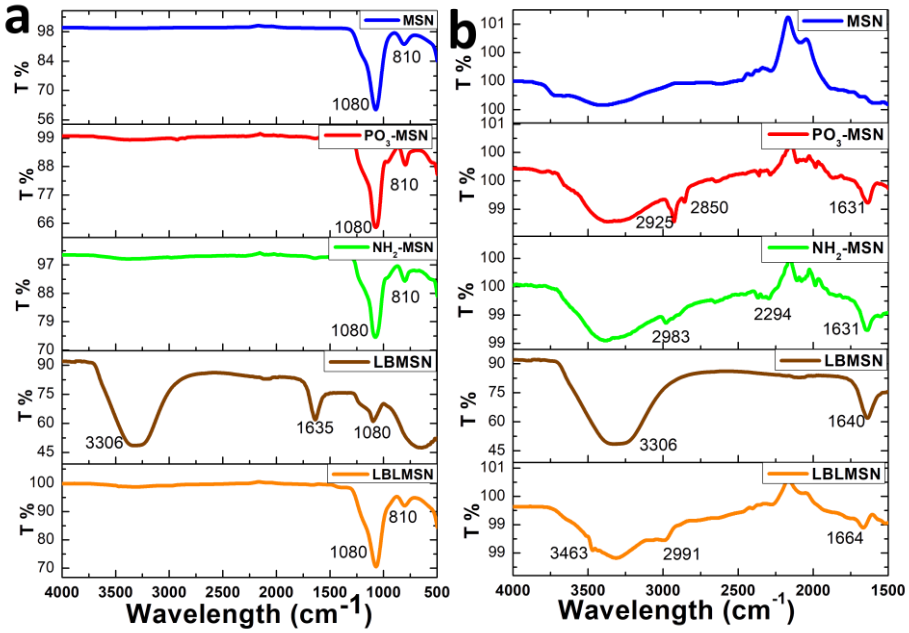

Fig. S5 Fourier Transform infrared (FTIR) spectroscopy of empty functionalized MSNs. FTIR Spectra of Blank MSNs (without ICG) (a). MSN, PO_3-MSN, NH_2-MSN, LBMSN and LBLMSN for range 500 cm^{-1} to 4000 cm^{-1}. (b). IR spectra for the respective particles for a range 1500 cm^{-1} to 4000 cm^{-1}.

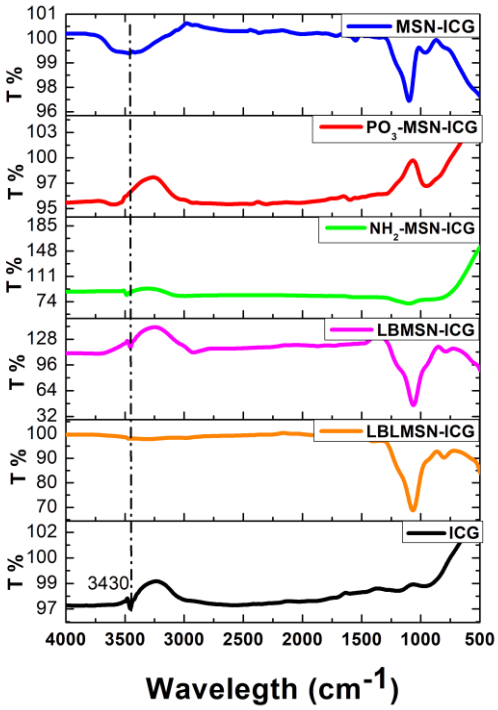

Fig. S6 Fourier Transform infrared (FTIR) spectroscopy of ICG loaded functionalized MSNs. FTIR Spectra of ICG Loaded MSNs and free ICG for a wavelength range 500 cm^{-1} to 4000 cm^{-1}.

Section S8. *In-vitro* biocompatibility of functionalized MSNs

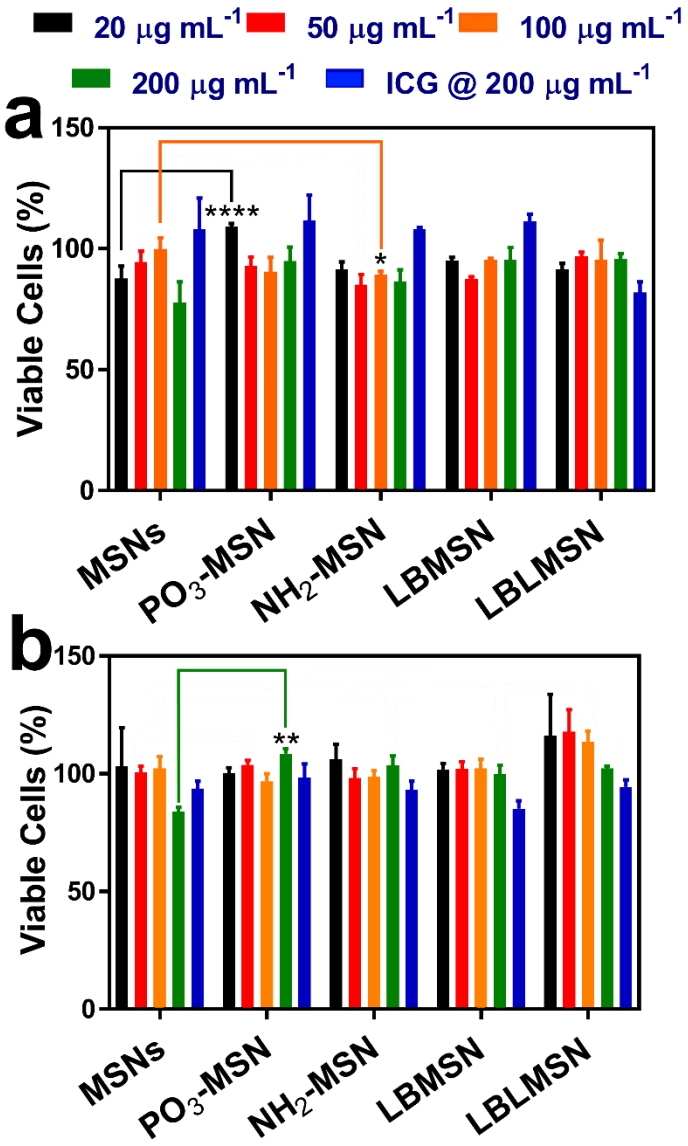

Fig. S7 Cell Viability studies. % cell viability of unloaded MSNs, PO_3-MSN, NH_2-MSN, LBMSN, LBLMSN in concentrations of 20 μg mL^{-1}, 50 μg mL^{-1}, 100 μg mL^{-1}, 200 μg mL^{-1}, free ICG and ICG loaded particles in a concentration of 200 μg mL^{-1} for 24 hours. (a) HepG2 cells and the corresponding p values are MSNs vs PO_3-MSN at 20 μg mL^{-1} ****p <0.0001 and MSN vs NH_2-MSN at 100 μg mL^{-1} ***p=0.0423. (b) RAW-264.7 cells and the corresponding p values are MSNs vs PO_3-MSN at 200 μg mL^{-1} **p= 0.0055. The data are represented as Mean ± S.D. n = 3 and analyzed by non-parametric one-way ANOVA, post-hoc Dunn's test.

SECTION S9. *Ex vivo* Photoacoustic Imaging Videos of Brain Tissues injected with ICG and ICG loaded functionalized MSNs.

The freshly euthanized mice were skull opened and the brains were taken out and fixed onto the petri dishes. The fixed organs were then injected with ICG, MSN, PO_3-MSN, NH_2-MSN, LBMSN, LBLMSN (injection volume, 10 µL) and the 3D scan was recorded as videos.

Description of the supporting videos.

Video S1. Brain injected with 10 µL of pure ICG solution (200 µg mL^{-1}).

Video S2. Brain injected with 10 µL of MSN-ICG (1 mg mL^{-1}).

Video S3. Brain injected with 10 µL of PO_3-MSN-ICG (1 mg mL^{-1}).

Video S4. Brain injected with 10 µL of NH_2-MSN-ICG (1 mg mL^{-1}).

Video S5. Brain injected with 10 µL of LBMSN-ICG (1 mg mL^{-1}).

Video S6. Brain injected with 10 µL of LBLMSN-ICG (1 mg mL^{-1}).