Supporting information for

Large photoacoustic effect enhancement for ICG confined inside MCM-41 mesoporous silica nanoparticles

Giuseppe Ferrauto,[†] Fabio Carniato,[§] Enza Di Gregorio,[†] Lorenzo Tei,[§ *] Mauro Botta,[§] Silvio Aime[†*]

[†] *Department of Molecular Biotechnology and Health Sciences, University of Turin, Via Nizza 52, 10126 Turin, Italy*

[§] *Dipartimento di Scienze e Innovazione Tecnologica, Università del Piemonte Orientale "Amedeo Avogadro", Viale T. Michel 11, 10121 Alessandria, Italy*

***** *Prof. Lorenzo Tei, Viale T. Michel 11, 10121 Alessandria (IT) TEL* +390131360208*;*

FAX +390131360250 *; lorenzo.tei@uniupo.it*

***** *Prof. Silvio Aime, Via Nizza 52, 10126, Torino (IT) TEL +390116706451;*

FAX 0116706487; silvio.aime@unito.it

Methods:

Synthesis of mesoporous support (MSNs): MSN was synthesized and calcined following the procedure reported in the literature.[1] In detail, tetraethoxysilane (3.5 g) was added to 30 g of a hydrochloric acid solution (pH=0.5) at room temperature, where cetyltrimethylammonium chloride (2.6 g) and a triblock copolymer (Pluronic F127, 2.0 g) were previously dissolved. After stirring for 3 h at room temperature (RT), 3.0 g of 14.7 M ammonia solution were added to the solution. The gel was stirred at RT for 24 h, crystallized at 60°C for 24 h, filtered and then washed with deionised water. The sample was finally calcined under air flow (100 mL min-1) at 600°C for 5 h. The anchoring of NH_2 groups on the silica surface was carried out following this procedure: calcined silica (1.0 g) was firstly treated under vacuum at 250 °C for 2 h to activate the surface of the support, then the material was suspended in toluene (100 mL) under a nitrogen flow and 3-aminopropyltriethoxysilane (40 wt%) was added. The mixture was stirred at 50 °C for 20 h. The suspension was then filtered and the product was washed several times with diethyl ether to remove the unreacted silane. Finally, the sample was dried at 60 °C for 2 h.

Impregnation of ICG in the silica nanoparticles and PEG anchoring: 200 mg of NH_2-MSN were suspended in 10 mL of methanol and stirred for few minutes. 3 mL of ICG solution (0.07 mM, in methanol) were added to the previous suspension and stirred for 8 h at room temperature. The particles were separated by centrifugation, washed with methanol and water (5 mL + 5 mL) and dried at 50°C for 2h. 160 mg of ICG-MSNs were suspended in 5 mL of water. In parallel, 55 mg of PEG_{5000}-COOH, were dissolved in 10 mL of water in the presence of 22 mg of EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and 46 mg of Sulfo-NHS (N-Hydroxysulfosuccinimide). The solution was added to the ICG-MSNs suspension and stirred for 4.30 h at room temperature. Finally, the solid was filtered, washed with 10 mL of water and dried at 50°C for 4 h.

ICG-MSNs containing 1.7 μmol g^{-1} and 3.7 μmol g^{-1} of dye were prepared following the same synthetic strategy, by adding to the NH_2-MSN suspension 1 mL and 3 mL of ICG solution (0.5 mM, in methanol), respectively.

The quantification of ICG loading per particle was calculated assuming the particles to spheres with volume ($4/3\pi r^3$) and considering the density of MSN around 0.8 g/cm^3 (Karen J. Edler, Philip A. Reynolds, John W. White and David Cookson, J. Chem. Soc., Faraday T rans., 1997, 93(1), 199-202).

Tools for MSNs characterization: HRTEM images were collected on a JEOL 3010 High Resolution Transmission Electron Microscope operating at 300 kV. Specimens were prepared by dispersing the sample by sonication in isopropanol and by depositing a few drops of the suspension on carbon-coated grids.

X-Ray diffraction (XRD) patterns were obtained using an ARL XTRA48 diffractometer with Cu Ka radiation (λ = 1.54062 Å).

N_2 physisorption measurements were carried out at 77 K in the relative pressure range from 1 x 10^{-6} to 1 P/P0 by using a Quantachrome Autosorb1MP/TCD instrument. Prior to the analysis, the samples were outgassed at 100 °C for 3 h (residual pressure lower than 10^{-6} Torr). Specific surface areas were determined using the Brunauer–Emmett–Teller equation, in the relative pressure range from 0.01 to 0.1 P/P0. Pore size distributions were obtained by applying the BJH method and using the desorption branch of the N_2 physisorption isotherm.

Thermogravimetric analyses (TGA/DTG) were performed under oxygen flow (100 ml min-1) with a SETSYS Evolution TGA-DTA/DSC thermobalance, heating the samples from 50 °C to 800 °C at 10 °C min^{-1}.

Infrared spectra of the materials in the form of self-supporting pellets were collected under vacuum conditions (residual pressure $<10^{-5}$ Torr; 1 Torr = 133.33 Pa) using a Bruker Equinox 55 spectrometer equipped with a pyroelectric detector (DTGS type) with a resolution of 4 cm^{-1}.

DLS experiments were performed on a suspension of the particles in PBS solution by using a Zetasizer NanoZS instrument, Malvern, UK, operating at a particle size range from 0.6 nm to 6 mm and equipped with a He-Ne laser (λ=633 nm).

Surface ζ-potential of ICG-MSNs (5 mg/mL) was evaluated in water at 25°C by using a Zetasizer NanoZS instrument, Malvern, UK.

DR-UV-visible spectra were recorded using a Perkin Elmer Lambda 900 spectrometer equipped also with a diffuse reflectance sphere accessory (DR-UV-vis).

Steady-state emission spectra were recorded on a Horiba Jobin-Yvon Model IBH FL-322 Fluorolog 3 spectrometer equipped with a 450 W xenon arc lamp, double-grating excitation and emission monochromators (2.1 nm/mm dispersion; 1200 grooves/mm), and a Hamamatsu Model R928 photomultiplier tube. Emission spectra were corrected for source intensity *(lamp and grating*) and emission spectral response (*detector and grating*) by standard correction curves.

Quantum yields were calculated using the following equation:

$$\phi ICG - MSNs = \frac{I_{ICG-MSNs}}{I_{ICG}} \frac{A_{ICG}}{A_{ICG-MSNs}} \phi ICG$$

where: $\phi ICG - MSNs$ and ϕICG are the fluorescence quantum yields of ICG-MSNs and ICG samples in water at neutral pH; $I_{ICG-MSNs}$ and I_{ICG} are the fluorescence emission intensity and finally $A_{ICG-MSNs}$ and A_{ICG} are the absorbance of the two samples.

ICG-loaded liposomes: ICG-loaded liposomes have been prepared by following the lipidic thin film hydration method.[2] Phospholipids (purchased from Avanti Polar Lipids, Inc, Alabama, USA) were

dissolved in chloroform and evaporated under rotation in a glass balloon for at least 2h ([phospholipids]=20mg/mL, membrane formulation: L-α-phosphatidylcholine/ cholesterol/ DSPE-PEG2000 methoxy 78/20/2 molar ratio; DSPE-PEG2000 = 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[*methoxy*(*polyethylene glycol*)-*2000*]). The film was then hydrated at 55°C with 5% dextrose water solution containing 0.1 mM ICG (pH = 7.3, osmolarity *ca*. 290mOsm/L). The resulting suspension of multilamellar vesicles (MLV) was extruded (Lipex extruder, Northern Lipids Inc., Canada) five times on polycarbonate filters of 400 nm and eight times on filters of 200 nm. The resulting small unilamellar vesicles (SUV) have been extensively purified by means of dialysis against an isotonic buffer solution (3 dialysis cycles, 1.5mL of liposomes against 1.5L of buffer).

Cell cultures

Two cell lines were used for this study, namely TS/A and J774A.1 cells, both ones obtained from American Type Culture Collection (ATCC), Manassas, VA, USA.

Cell culture media (RPMI and DMEM), Fetal Bovine Serum (FBS), penicillin/streptomycin mixture, Trypsin/EDTA, MycoAlert™ Mycoplasma Detection Kit and all other cell culture reagents were purchased from Lonza Sales AG-EuroClone SpA group, Milano (It). All other chemical reagents for cell cultures were purchased from Sigma Chemical Co., St. Louis, MO.

TS/A cells, used for the generation of subcutaneous transplantable tumors, derive from a spontaneous mammary adenocarcinoma which arose in a retired breeder BALB/c female.[3] They were grown in RPMI (Roswell Park Memorial Institute) 1064 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were seeded in 75-cm^2 flasks at density of *ca*. 2×10^4 cells/cm^2 in a humidified 5% CO_2 incubator at 37 °C. At confluence, they were detached by adding 1 mL of Trypsin-EDTA solution (0.25 % (w/v) Trypsin- 0.53 mM EDTA).

The second cell line, J774A.1 murine macrophages (obtained from ATCC, Manassas, VA, USA), was used for cell toxicity experiments. They were grown in DMRM (Dulbecco's Modified Eagle Medium) supplemented with 10 % FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in 75-cm^2 flasks at density of ca. 2×10^4 cells/cm^2 in a humidified 5% CO_2 incubator at 37°C. At confluence, they were mechanically detached by using a cell scraper. For in vitro experiments, 1.5 x 10^6 cells were plated in 6 cm Petri dishes overnight and successively incubated for 1h in presence of different amounts of ICG-MSNs of free ICG. After the incubation, cells were extensively washed with fresh PBS, detached and counted by using Tripan blue exclusion assay. Cells viability was evaluated with respect to control unlabelled samples.

All the used cells were negative for mycoplasma as tested by using MycoAlert™ Mycoplasma Detection Kit.

Mouse model

Twelve-week-old female Balb/c mice (Charles River Laboratories, Calco, Italy) were inoculated subcutaneously in the left flank with 0.1 ml of a single suspension containing 0.5×10^6 TS/A murine breast cancer cells. Mice were kept in standard housing with standard rodent chow and water available ad libitum, and a 12 h light/dark cycle. Experiments were performed according to the national regulations and were approved by the local animal experiments ethical committee. For the photoacoustic experiments, mice were anesthetized by intramuscular injection of tiletamine/zolazepam (Zoletil 100; Virbac, Milan, Italy) 20 mg/kg plus xylazine (Rompun; Bayer, Milan, Italy) 5 mg/kg.

In vitro and in vivo photoacoustic experiments

All US and PA images were acquired on a VisualSonics Vevo 2100 LAZR Imaging Station (VisualSonics, Inc., Toronto, Canada) equipped with a LZ250 transducer operating at 21 MHz. For

in vitro PAI, ICG-MSNs suspensions or free ICG at variable concentrations were loaded onto thin-layer plastic capillaries surrounded by 1% agarose gel. A US gel was applied over the region of interest before the image acquisition. Grey scale B-mode US images at high resolution were acquired as anatomical reference images. This was carried out by using a high-frequency ultrasound probe (MS550D, VisualSonics, Canada, broadband frequency: 22 MHz - 55MHz, image axial resolution: 40 um) operating at 40 MHz.

For PA imaging a 21 MHz frequency probes was used. The tunable laser (680-970 nm) energy was continuously monitored and eventually adjusted through laser recalibration and optimization. PA spectra were acquired by investigating 680-970 nm wavelength region, with 2 nm steps (persistence=4). PA images of ICG were acquired by setting wavelength at 810nm (persistence=4).

For in vivo experiments, mice were anesthetized as above reported and hairs were removed over the tumor area using a depilatory cream (the experiments has been repeated in three independent mice and data about one representative mouse are reported in this paper). Tail vein was catheterized for the following i.v. injection of ICG-MSNs. A US gel was applied over the region of interest before the image acquisition. US and PA images were acquired as above reported. PAI spectra and PA images of tumor region were acquired before and after the *i.v.* injection of 10 mg/mL ICG-MSNs.

All PA images were co-registered with grey scale B-mode imaging. ROIs were manually drawn and PA signal intensity measured.

Supplementary Results:

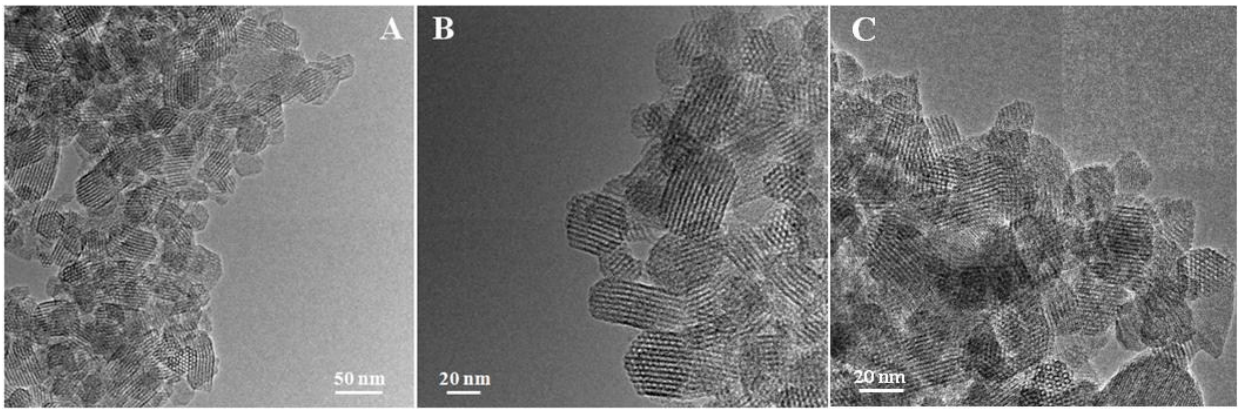

Figure S1. HR-TEM micrographs at low (A) and high magnifications (B) of MSNs nanoparticles and of ICG-MSNs nanoparticles (C).

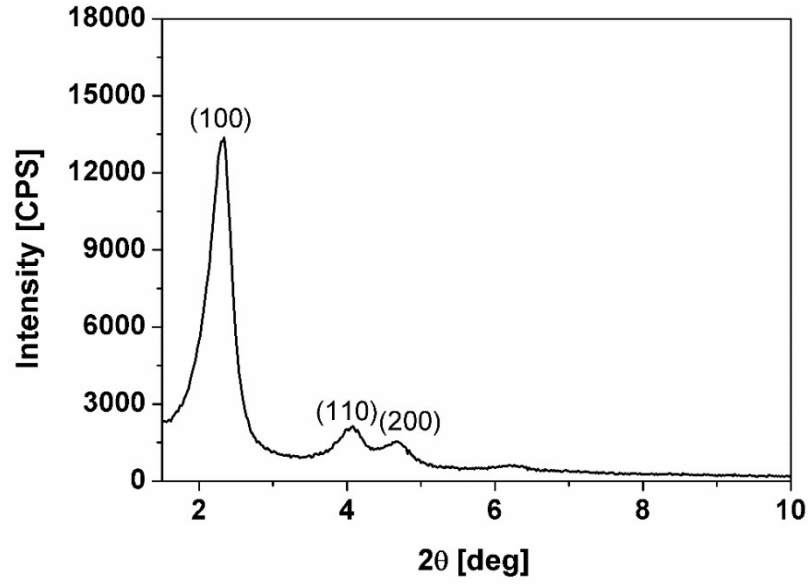

Figure S2. X-Ray patter of MSNs nanoparticles.

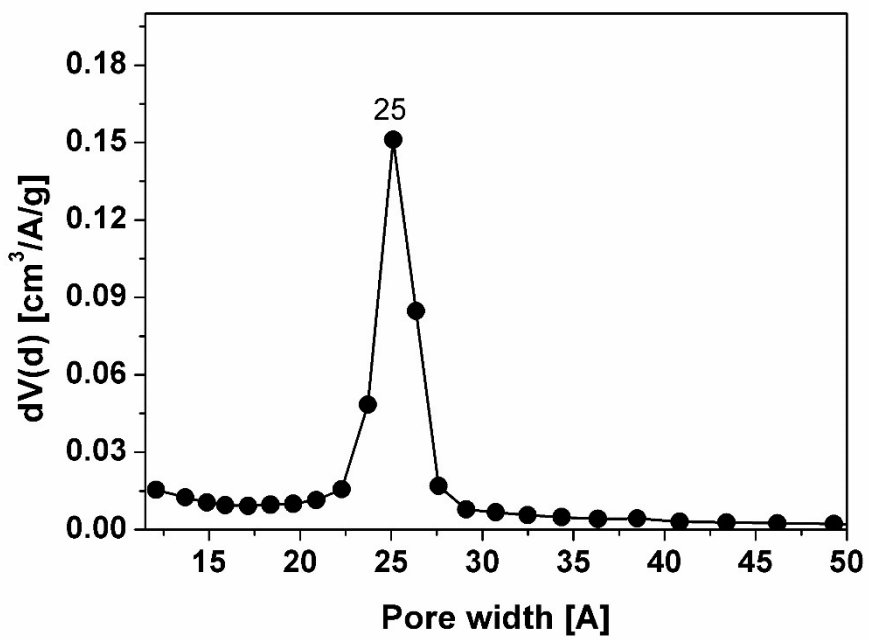

Figure S3. Pore-diameter distribution of nanosized MSNs.

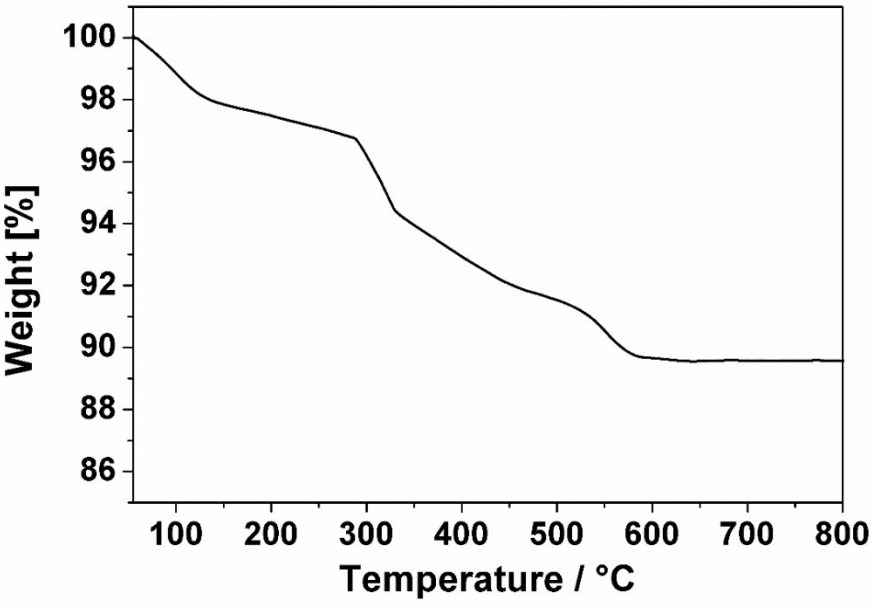

Figure S4. Thermogravimetric analysis performed under oxygen flow of NH_2-MSNs nanoparticles.

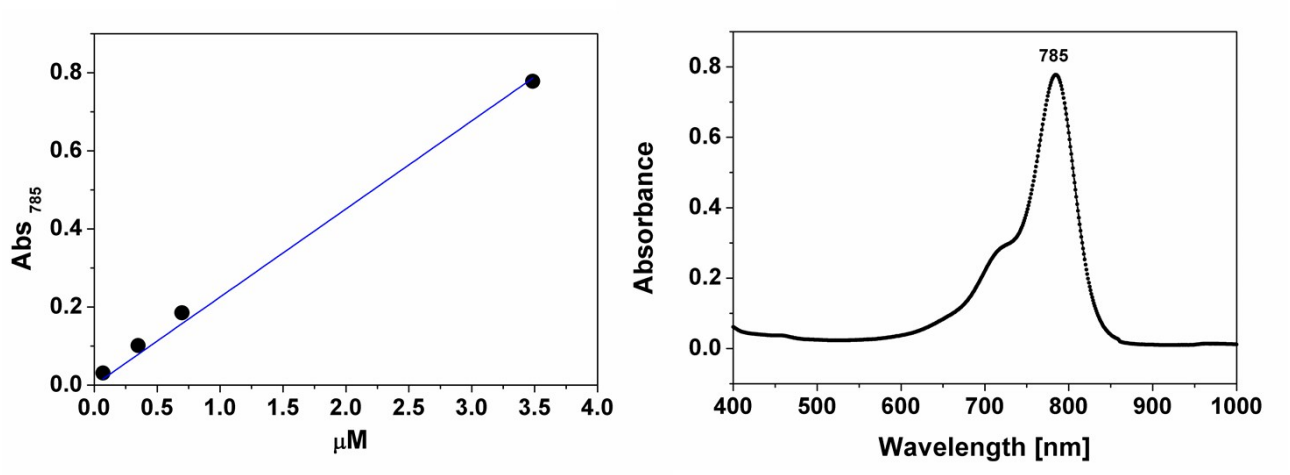

Figure S5. Left: calibration curve obtained by UV-Visible spectra (λabs = 785 nm) of ICG in methanol solution. Right: UV-Visible spectrum of ICG in methanol solvent.

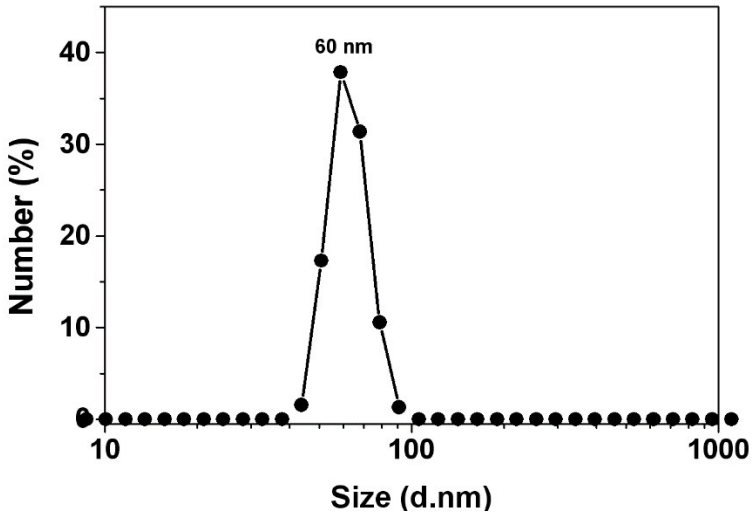

Figure S6. DLS analysis of pegylated NPs in PBS suspension (5 mg in 1 mL of phosphate buffer).

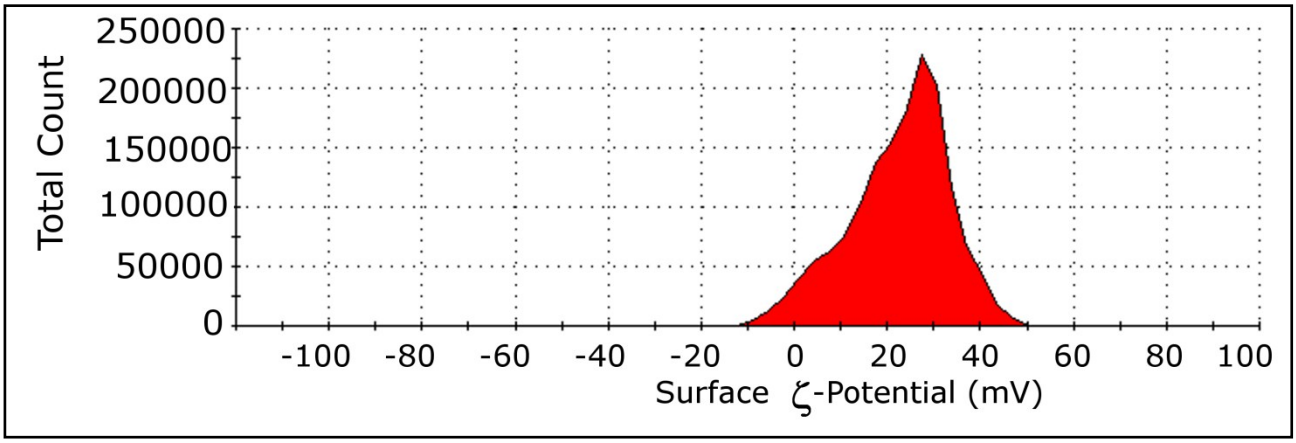

Figure S7. Surface ζ-potential of pegylated NPs in water suspension (5 mg in 1 mL of water, 25°C).

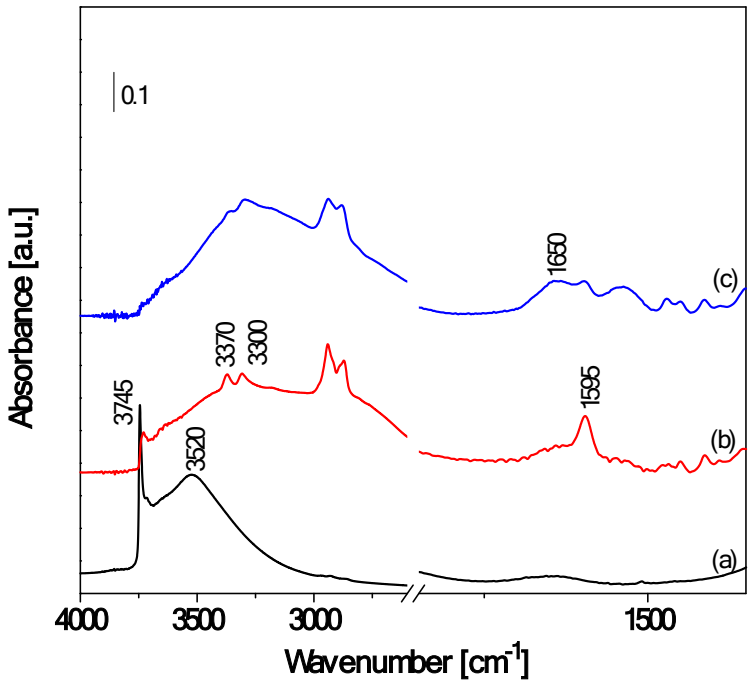

Figure S8. FT-IR spectra collected under vacuum of MSN (a, black curve), MSN-NH_2 (b, red curve) and the pegylated ICG-MSNs nanoparticles (c, blue curve). The IR spectrum of MSN sample showed two main absorptions at 3745 cm^{-1} and 3520 cm^{-1}, assigned to the stretching modes of isolated and vicinal silanol groups, respectively. A decrease of the bands attributed to the silanols grous and the presence of absorptions at 3370 and 3300 cm^{-1} and 1595 cm^{-1}, assigned to the asymmetric and symmetric stretching and bending modes of NH_2 groups, respectively, were observed for MSN-NH_2. Finally, the IR spectrum of the pegylated NPs showed, along with the bands typical of the NH_2 groups, a new absorption at 1650 cm^{-1}, which was attributed to the stretching mode of the amide groups formed by the attachment of PEG to the surface.

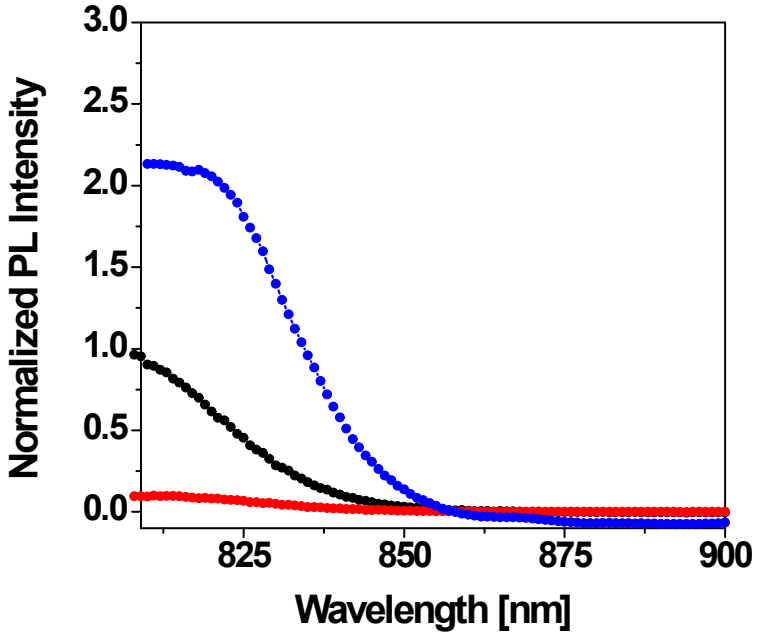

Figure S9. Photoluminescence normalized spectra of pegylated ICG-MSNs (red curve), ICG-liposomes (blue curve) and ICG (black) in aqueous solution, under excitation at 780 nm. The concentration of ICG for all samples was 1.70 μM.

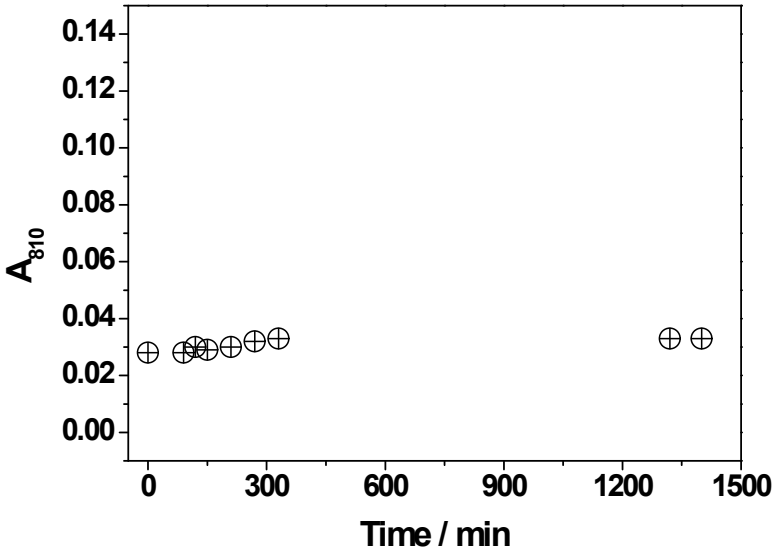

Figure S10. Stability of ICG-MSNs in physiological media (PBS). The suspension stability was evaluated by monitoring the absorbance maximum over the time.

Table S1. *Absorption molar extinction coefficient (ε)* and PAI signal of ICG-MSNs with a different amount of encapsulated ICG.

[ICG] in the MSNs preparation	Absorption molar extinction coefficient (ε) ($M^{-1}cm^{-1}$) in water at 780 nm	Relative PAI signal
0.8 µmol g^{-1}	18000	+370 %
1.7 µmol g^{-1}	3800	-94 %
3.7 µmol g^{-1}	2200	Not detectable
Free ICG as reference	80000	1

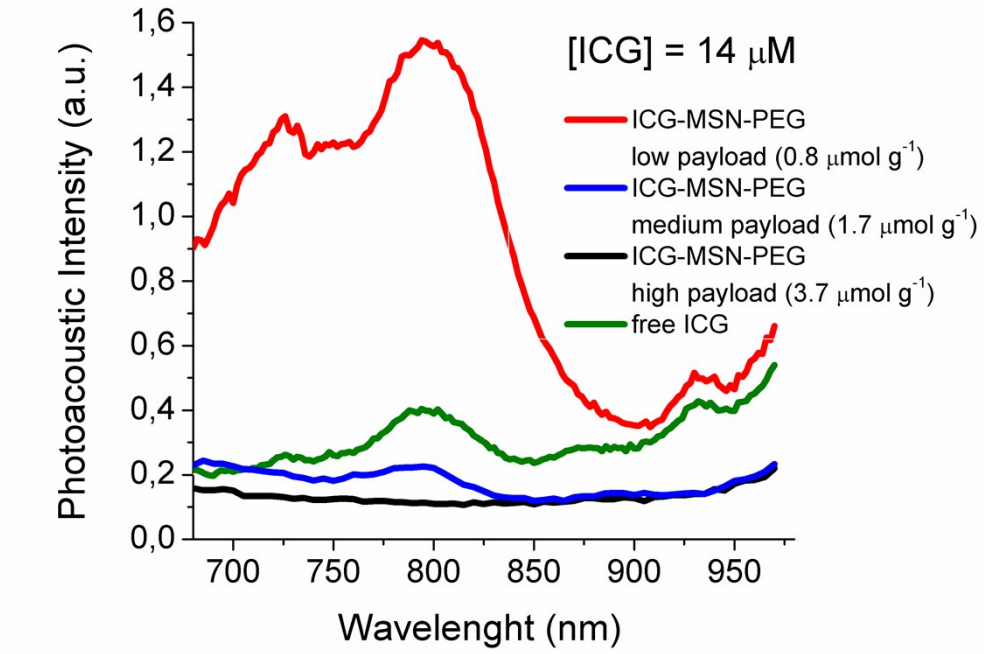

Figure S11. PAI spectra of ICG-MSNs with different amount of encapsulated ICG. All spectra were registered at an ICG concentration = 14 µM.

Supplementary references:

1. Suzuki,K.; Ikari, K.;Imai, H. J. Am. Chem. Soc., 2004, 126, 462–463.
2. Torchilin, V.P.; Weissig, V. Oxford University Press, New York, 2003, 3-27.
3. Nanni, P.; de Giovanni, C.; Lollini, PL.; Nicoletti, G.; Prodi, G. Clin Exp metastasis, 1983, 1, 373-380.