Supporting Information

Targeted delivery of photosensitizers: efficacy and selectivity issues revelead by multifunctional ORMOSIL nanovectors in cellular systems

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1. Experimental procedures

Instrumentation. Ultrafiltration of the nanoparticles was carried out using a 75 ml Amicon® Ultrafiltration Cell, Millipore® using a cellulose membrane with a cut-off of 10,000 Da.

UV-Vis absorption spectra were measured in water on a Perkin-Elmer Lambda 45 UV-Vis spectrophotometer with 1 cm path length quartz cuvettes.

Fluorescence spectra were measured in water on a Perkin-Elmer LS-50B fluorimeter with 1 cm path length quartz cuvettes. Infrared spectra were recorded on a Nicolet 5700 FT-IR instrument.

NMR spectra in the solution state were recorded on a Bruker Avance (250 MHz ^{1}H frequency) or on a Bruker AC-300 (300 MHz ^{1}H frequency). NMR spectra in the solid state were collected on a Varian 400 equipped with a narrow bore, triple resonance T3 MAS probe spinning 4 mm rotors and operating at ^{1}H, ^{29}Si and ^{13}C frequencies of 400.36, 79.51 and 100.68 MHz, respectively. The nominal temperature of the probe was always set to 298 K. ^{13}C CP-MAS spectra were acquired at 5 kHz MAS with 1200 scans and a repetition delay up to 3 s. The contact time for CP was 2 ms, and an acquisition time of 50 ms was used. ^{29}Si spectra were obtained at 5 kHz MAS with a basic pulse-acquire sequence (2048 scans) and a recycle delay of 60 s. The chemical shifts were referenced externally against the $^{13}CH_2$ resonance observed for adamantane at 38.48 ppm (for ^{13}C) or against the signal of Q8M8 (for ^{29}Si).

The hydrodynamic particle size (Dynamic Light Scattering, DLS) and Z-potential were measured with a Malvern Zetasizer Nano-S equipped with a HeNe laser (633nm) and a Peltier thermostatic system. Measurements were performed at 25 °C in water or PBS buffer at pH 7.0.

Transmission electron microscopy (TEM) was recorded on a Jeol 300PX instrument. One drop of sample was placed on the sample grid and the solvent was allowed to evaporate.

Thermogravimetric analysis (TGA) was run on 5 mg lyophilized nanoparticle samples using a SDT-2960 model TA instrument from 30 to 1,200 °C under a continuous air flow.

Solvents and reagents. Solvents were of analytical reagent grade, laboratory reagent grade or HPLC grade. Water was purified using a Milli-Q® water purification system. High surface area hydrophobic Bio-Beads® SM were obtained from Bio-Rad and used according to instructions of the supplier. Haba-Avidin Test and EZview Red Streptavidin Affinity Gel were obtained by Aldrich and used according to the manufacturer instructions. 2-(4-chlorosulfonylphenyl)ethyltrimethoxy silane (50 % solution in dichloromethane) was obtained by ACROS. 5-(4-Aminophenyl)-10,15,20-triphenyl-porphyrin was purchased by Porphyrins Systems. Cyclo[RGDfK(AcSCH$_2$CO)] and cyclo[RGDfK(AcSCH$_2$CO)] where obtained by Peptides International. 5,10,15,20-tetra(3-hydroxyphenyl)-(2,3-dihydro)porphyrin (mTHPC, temoporfin) was gently provided by Biolitec. Derivatives **1**, **2**, Folate-OSu, Biotin-OSu were prepared as previously reported. All the other reagents were used as received from Sigma-Aldrich.

2. Synthesis of derivatives 3-5

2.1 Synthesis N-[3-(triethoxysilyl)propyl]-N'-[4-(10,15,20-triphenylporphin-5-yl)phenyl]-urea (3).

In a sealed tube, 50 mg (0.08 mmol) of 5-(4-aminophenyl)-10,15,20-triphenylporphyrin and 198 µL (0.8 mmol) of 3-(trimethoxysilyl)propyl isocyanate are dissolved in 6 mL of dry acetonitrile and the reaction mixture is heated at 90°C for 24 hours. The reaction is followed by TLC (eluent: Petroleum Ether/EtOAc = 2:1, Rf = 0.22). The solvent is removed and the solid obtained is redissolved in 2 mL of CH_2Cl_2 and precipitated in 60 mL of n-hexane. The product is recovered by centrifugation at 4,000 g for 20 minutes. The procedure is repeated 3 times providing after drying 52 mg of a violet powder (yield 74%).

^{1}H-NMR (250 MHz, $CDCl_3$): δ -2.78 (s, 2H, N*H*), 0.77 (t, 2H, J = 7.5 Hz, Si*CH_2*), 1.27 (t, 9H, J = 7 Hz, C*H_3*CH_2O), 1.80 (qn, 2H, J = 7.5 Hz, SiCH$_2$C*H_2*), 3.88 (q, 6H, J = 7.5 Hz, CH_3C*H_2*O), 7.73 (m, 11H, Ar*H*), 8.18 (m, 8H, Ar*H*), 8.87 (m, 8H, Ar*H*).

ESI-MS: m/z calcd. for $C_{54}H_{53}N_6O_4Si$ $[M + H]^+$: 877.4; found: 876.9 (100%)

2.2 Synthesis of N-[3-(triethoxysilyl)propyl]-O-[4-(10,15,20-tri(3-hydroxyphenyl)-(2,3-dihydro) porphin-5-yl) phenyl]-carbamate (4).

In a small conical vial, 17 mg (0.025 mmol) of mTHPC, 15.4 µL (0.062 mmol) of 3-(trimethoxysilyl)propyl isocyanate and 8.6 µL (0.062 mmol) of triethylamine are dissolved in 100 µL of dry THF. The reaction is followed by TLC (eluent EtPet: iPrOH = 3:1, Rf $_{mTHPC-Si}$ = 0.3, Rf $_{mTHPC-2Si}$ = 0.4, Rf $_{mTHPC-3Si}$ = 0.5). The solvent is evaporated and the product is used without other purifications.

ESI-MS: m/z calcd. for $C_{54}H_{54}N_5O_8Si$ $[M_{mTHPC-Si} + H]^+$: 928.4; found: 928.7 (100%), $C_{64}H_{75}N_6O_{12}Si_2$ $[M_{mTHPC-2Si} + H]^+$: 1175.5; found: 1175.8 (30%)

UV-vis (toluene): λ_{max} (nm) 420, 518, 545, 601, 654.

2.3 Synthesis of 5,10,15,20-tetra(3-(N-(triethoxysilylpropyl)carbamate)phenyl)-(2,3-dihydro) porphyrin (5).

In a small conical vial, 17 mg (0.025 mmol) of mTHPC, 24.7 μL (0.1 mmol) of 3-(trimethoxysilyl)propyl isocyanate and 13.8 μL (0.1 mmol) of triethylamine are dissolved in 100 μL of dry THF. The reaction is followed by TLC (eluent EtPet: iPrOH = 3:1, $Rf_{mTHPC\text{-}3Si} = 0.5$, $Rf_{mTHPC\text{-}4Si} = 0.6$). The solvent is evaporated and the product is used without other purifications.

ESI-MS: m/z calcd. for $C_{74}H_{96}N_7O_{16}Si_3$ $[M_{mTHPC\text{-}3Si} + H]^+$: 1422.6; found: 1422.7 (100%), $C_{84}H_{117}N_8O_{20}Si_4$ $[M_{mTHPC\text{-}3Si} + H]^+$: 1669.7; found: 1669.8 (20%).

UV-vis (toluene): λ_{max} (nm) 420, 518, 545, 601, 654.

3. **Synthesis and purification of the nanoparticles.**

3.1 Stober nanoparticles

To a thermostated vessel charged with **3** (4 mg, 0.005 mmol) and ethanol (20 mL), 100 μL (0.45 mmol) of TEOS were added under stirring at 25 °C. Subsequently, 1 mL of a 14.8 M aqueous solution of ammonia was added to initiate the polymerization. After 16 hours, the solution was diluted to 80 mL with ethanol and concentrated to the original volume by ultrafiltration (Amicon stirred cell) through a regenerated cellulose membrane (cut-off 10 kDa) under nitrogen pressure (4 bar). The procedure was repeated five times with ethanol and 7 times with Milli-Q water. The resulting solution was filtered through a 0.22 μm cellulose acetate membrane.

3.2 VTES nanoparticles (OS)

Brij 35 (90 mg, 75 μmol, 15 mM) was dissolved under stirring in 5 ml of water at 25°C into a thermostated reaction vessel. Subsequently, 150 μL (1.64 μmol) of *n*-butanol, 25 μL (10 mM DMSO solution) of **3** and VTES (45 mg, 0.24 mmol) were added. The mixture was vigorously stirred for 30 min (very important) and then 10 μL of a 14.8 M aqueous solution of ammonia were added to initiate the polymerization. After 2 hours, the solution was transferred into an Amicon stirred cell and extensively ultrafiltrated with 1.5 L of Milli-Q water, using a regenerated cellulose membrane (cut-off size 10 kD). The nanoparticles suspension was then collected and filtered through a 0.22 μm cellulose acetate filter.

3.2 PEGylated VTES nanoparticles (OS/PEG)

The appropriate amount of Brij 35 (see the following table), **1** (15.2 μmol, 3 mM), *n*-butanol (150 μL, 1.64 μmol) and, when required, **3**, **4** or **5** (10 mM DMSO solution, 10 to 130 μL) were

dissolved under stirring in 5 mL of water into a thermostated reaction vessel. The temperature was adjusted according to the following table and the desired amount of VTES was added:

Np	*Final Size (nm)*	*T (°C)*	*Brij35 (mM)*	*VTES (mM)*
OS/PEG	20	25	15	50
OS/PEG	50	30	5	50
OS/PEG	90	30	5	100

The mixture was vigorously stirred for 30 min (very important) and then 10 μL of a 14.8 M aqueous solution of ammonia were added. The mixture was further stirred for 2 hours at constant temperature. Then 14.3 mg of Bio-Beads® SM per mg of surfactant were added and the suspension was gently stirred for 3 hours. The beads were filtered off with filter paper and the filtrate diluted to 15 mL with Milli-Q water and concentrated to 0.5 mL by ultrafiltration with Amicon Ultra-15 Centrifugal Filter Units (cut-off 10 kDa) at 4,000 g. The procedure (dilution-concentration) was repeated seven times. The resulting solution was diluted to the original volume and filtered through a 0.22 μm cellulose acetate filter.

When required to prepare amine/OS/PEG nanoparticles, part of **1** was replaced with **2 (**0.1 to 30 % in moles) to provide amine groups in the PEG. Some examples are given in the following table.

Np	*Final Size (nm)*	*T (°C)*	*Brij35 (mM)*	***1** (μmol)*	***2** (μmol)*	*VTES (mM)*
OS/PEG **(**1% **2)**	100	30	5	15.0	0.15	100
OS/PEG **(**10% **2)**	100	30	5	13.7	1.5	100
OS/PEG **(**30% **2)**	100	30	5	10.7	4.5	100

Note 1: The amino-PEG derivative **2,** in DMSO solution**,** must be added to the reaction mixture just before the addition of ammonia.

Note 2. 20-nm VTES nanoparticles can be synthesized at 30°C using Tween 80 (33 mg, 25 μmol, 5 mM) in place of Brij 35, in this case *n*-buthanol is not needed.

3.2 Folate and Biotin conjugated nanoparticles

To a preparation of crude (unpurified) amine/OS/PEG (as described in the previous paragraph) Folate-OSu or Biotin-OSu dissolved in DMSO (20 equivalents with respect to the amount of **2** used in the synthesis of the nanoparticles) were added and the mixture was further stirred at 25 °C for 16 h. Then 14.3 mg of Bio-Beads® SM per mg of surfactant were added and the mixture was further gently stirred for 3 hours. The beads were filtered off with filter paper and the filtrate diluted to 15 mL with Milli-Q water and concentrated to 0.5 mL by ultrafiltration with Amicon Ultra-15 Centrifugal Filter Units (cut-off 10 kDa) at 4,000 g. The procedure was repeated seven times for biotin-conjugated nanoparticles and 10 times for folate-conjugate nanoparticles. The resulting solution was diluted to the original volume and filtered through a 0.22 μm cellulose acetate filter.

3.2 RGD and RAD conjugated nanoparticles

Into a thermostated reaction vessel, PBS-10x (0.5 mL) was added to a purified preparation of amine/OS/PEG nanoparticles (5 mL). The pH was adjusted to 7.2, then MBS (20 equivalents with respect to the amount of **2** used for the synthesis of the nanoparticles) was added and the mixture was stirred at 30 °C for 16 h. The mixture was then filtered through a 0.45 μm cellulose acetate filter, the filtrate was diluted to 15 mL with Milli-Q water and concentrated to 0.5 mL by ultrafiltration with an Amicon Ultra-15 Centrifugal Filter Unit (cut-off 10 kDa) at 4,000 g. The procedure was repeated seven times. The resulting solution was diluted to the original volume (5.5 mL) and filtered through a 0.22 μm cellulose acetate membrane. HEPES buffer (5 mM, 0.5mL) was added and the pH was adjusted to 7. Then, the solution was split in three fractions of 2 mL each.

RGD or RAD peptides (1 equivalent with respect to the amount of **2** used in the synthesis of the nanoparticles) were deacetylated in 0.1 mL of an HEPES (17 mM)/$NH_2OH{\cdot}HCl$ (17 mM)/ EDTA

(10 mM) buffer (pH 7.2) for 1 h at room temperature. Then, the resulting RGD/RAD solution (0.1 mL) was added to one of the nanoparticles aliquots (2 mL). The solutions were stirred overnight at RT. The solution was then diluted to 15 mL with Milli-Q water and concentrated to 0.5 mL by ultrafiltration with an Amicon Ultra-15 Centrifugal Filter Unit (cut-off 10 kDa) at 4,000 g. The procedure was repeated seven times. The resulting solution was diluted to the original volume and filtered through a 0.22 μm cellulose acetate filter.

3.3 Cetuximab and Bovine Serum Albumin (BSA) conjugated nanoparticles

For the conjugation of these proteins to the nanoparticle surface we decided to derivatize the PEG-aminogroups with the cross-linker MBS using the same procedures described for the conjugation of RGD and RAD peptides, whereas the proteins (i.e.Cetuximab and BSA) were functionalized by 2-iminothiolane (2-IT) reaction that modified the NH2 of lysine introducing new free sulfhydryl groups. Briefly, the protein functionalization was performed o/n at 4°C, the reaction blocked with glycine 0.2 M for 20 minutes at r.t. and finally the derivatized proteins were purified by gel filtration on PD10 columns (Sephadex G-25M, GE Healthcare) in order to remove the excess of 2-IT. The number of introduced SH groups were calculated using the Ellman's assay; the NP-protein conjugation was performed o/n at 30°C and then a dialysis step was performed for 24hr at r.t. in PBS EDTA 10 mM.

At last nanoconjugates were purified from the unreacted reagent by a first step of centrifugation at 100,000 g for 45 minutes and a second step of size exclusion chromatography on HiLoad 16/60 column (Superdex 75 prep grade, GE Healthcare) using a FPLC apparatus (Biologic Workstation, Bio-Rad). The purified fractions were concentrated by centrifugation.

4. Characterization of the nanoparticles.

4.1 TEM analysis

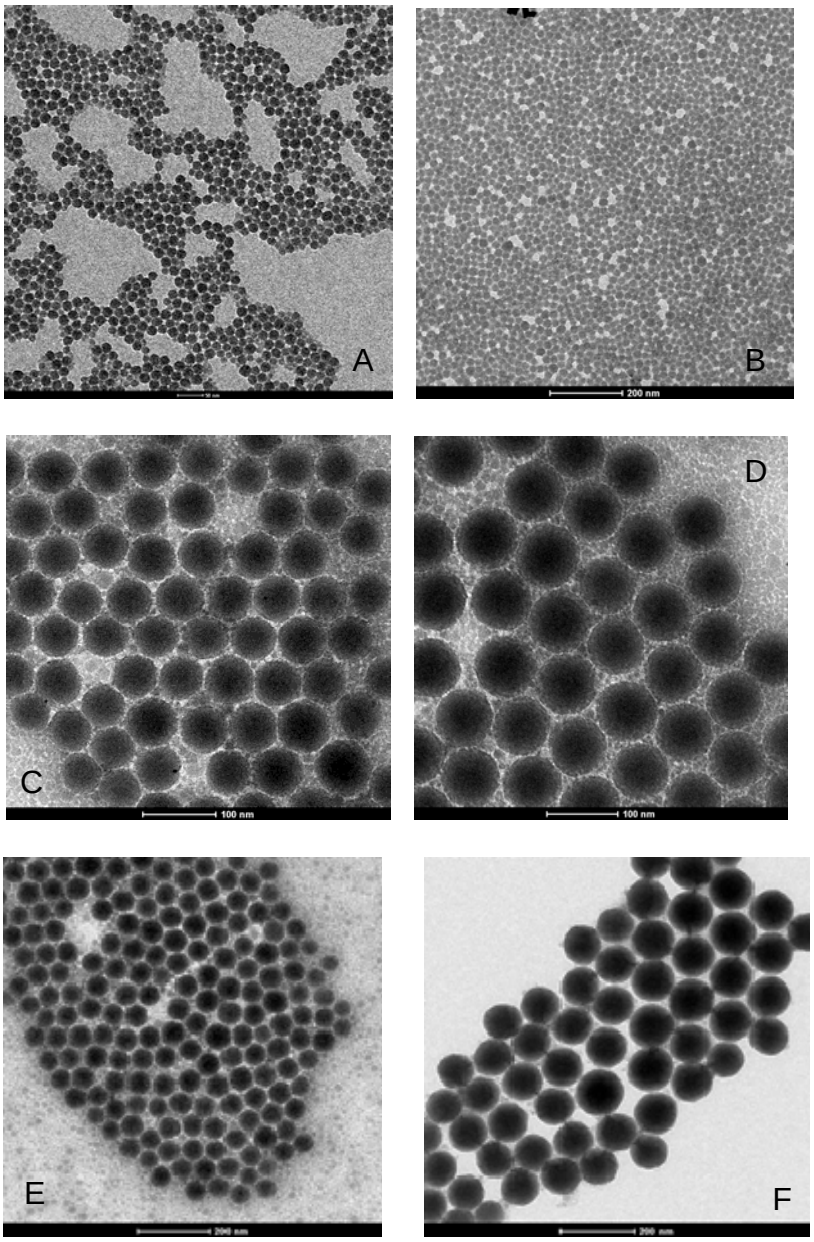

Figure S1. TEM images of **OS/PEG** nanoparticles with different sizes: 20 nm (A,B), 55 nm (C, E), 70 nm (D), 90 nm (F).

4.2 DLS analysis

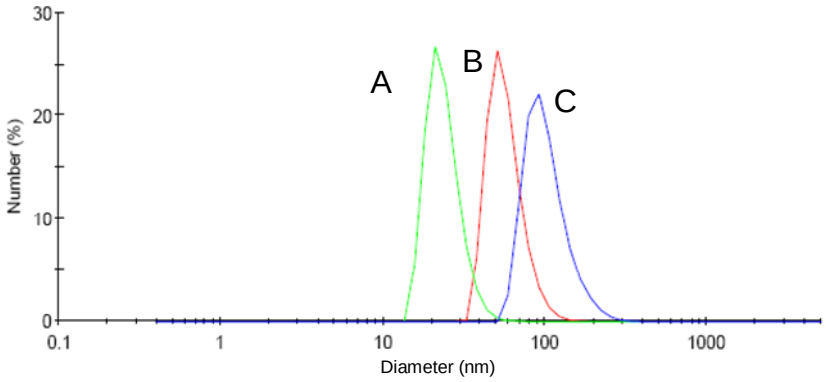

Figure S2. DLS analysis of **OS/PEG** nanoparticles with different sizes: 20 nm (A), 55 nm (B), 90 nm (C).

4.3 UV-Vis and Fluorescence analysis

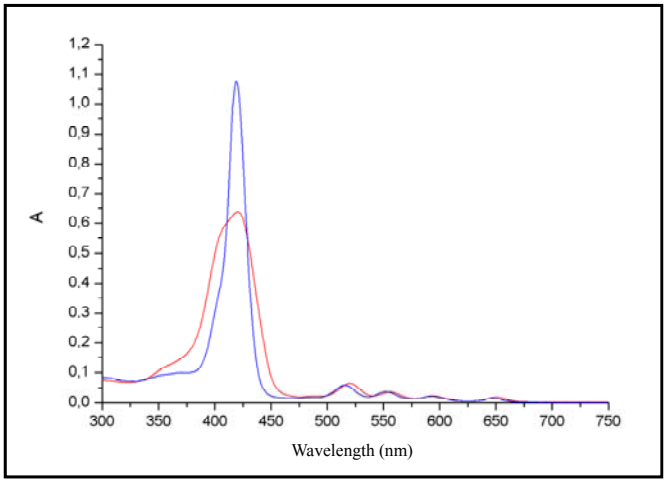

Figure S3. UV-Vis spectrum of 20-nm diameter Stober (red) and OS (blue) nanoparticles doped with dye **3** (water, 25 °C). See Table 1, entries 2 and 3.

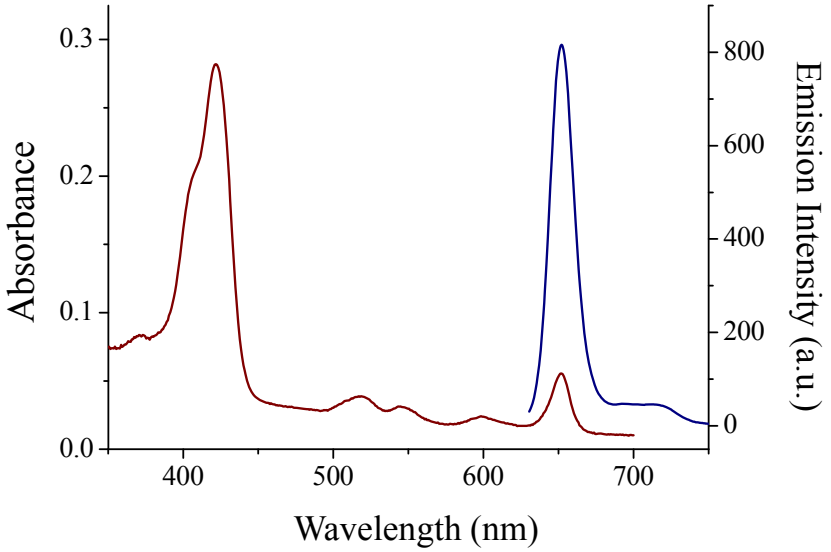

Figure S4. UV-Vis absorption (purple) and emission (blue) spectrum of 90-nm diameter OS/PEG nanoparticles doped with dye **4** (water, 25 °C). See Table 1, entry 7.

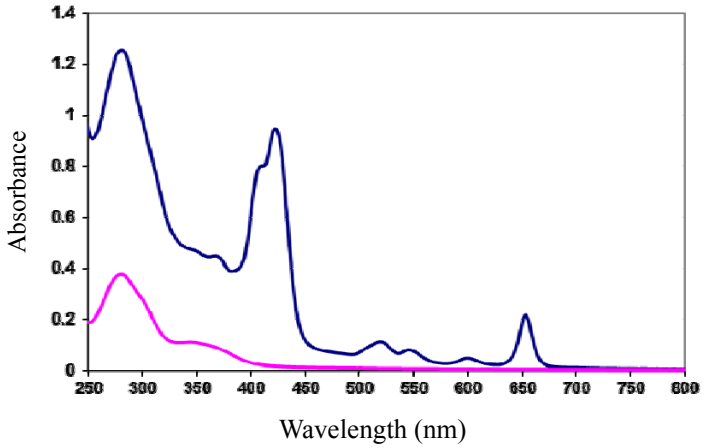

Figure S5. UV-Vis spectrum of 90-nm diameter amine/OS/PEG nanoparticles doped with dye 4 and targeted with folic acid (blue, water, 25 °C and of folic acid (magenta, water, 25 °C).

4.4 TGA analysis

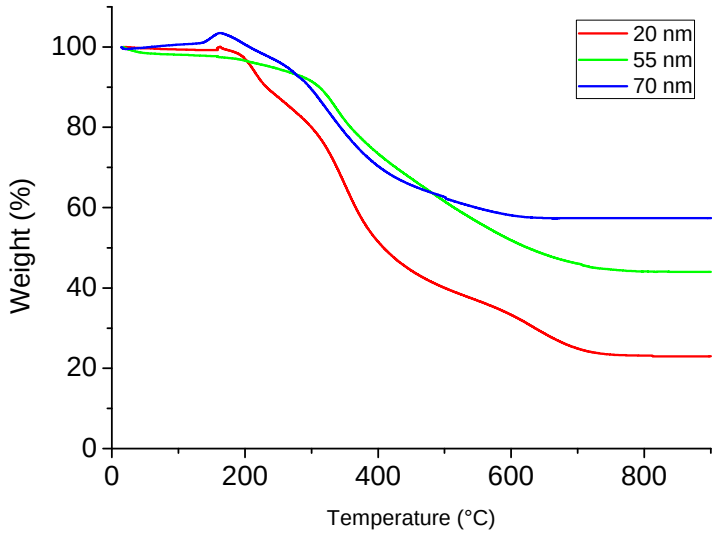

Figure S6. Thermogravimetric (TGA) profiles in air of OS/PEG nanoparticles of different sizes: red: 20 nm diameter; green: 55 nm diameter; blue: 70 nm diameter.

Weight fraction of the ormosil cores was calculated by assuming that the residue at 900° is silica (SiO_2) and adding the corresponding weight of the vinyl groups. Weight of a single ormosil core was calculated from the particles diameter using an estimated density of 1.5 g/cm^3, as vinyltriethoxysilane ormosil nanoparticles are considered (see ref. 9) to be less dense than pure silica ones (2.0 g/cm^3). PEG surface density is then easily calculated on the basis of geometrical considerations.

4.5 Avidin tests

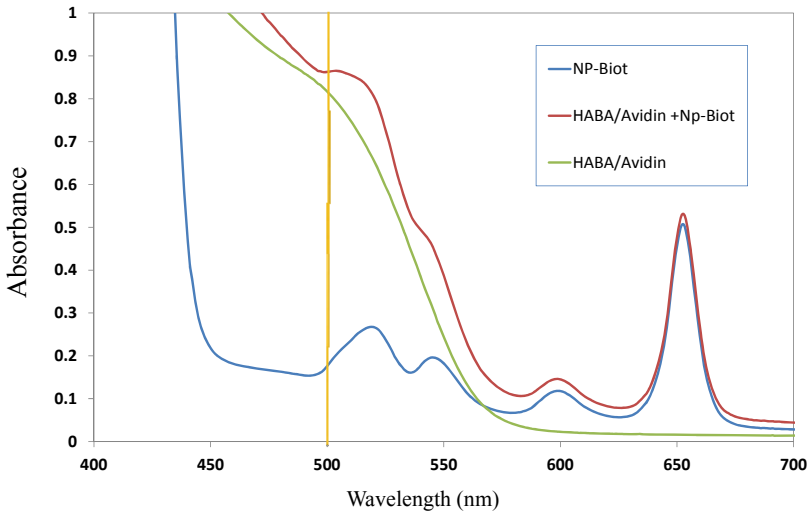

Figure S7. HABA/AVIDIN test on 90-nm diameter amine/OS/PEG nanoparticles doped with dye **4** and targeted with biotin. The test is based on the displacement of the HABA dye bound to avidin by biotin. The displacement causes a decrease in the absorption of the HABA dye at 500 nm that allow to measure the amount of biotin present in the sample analyzed.

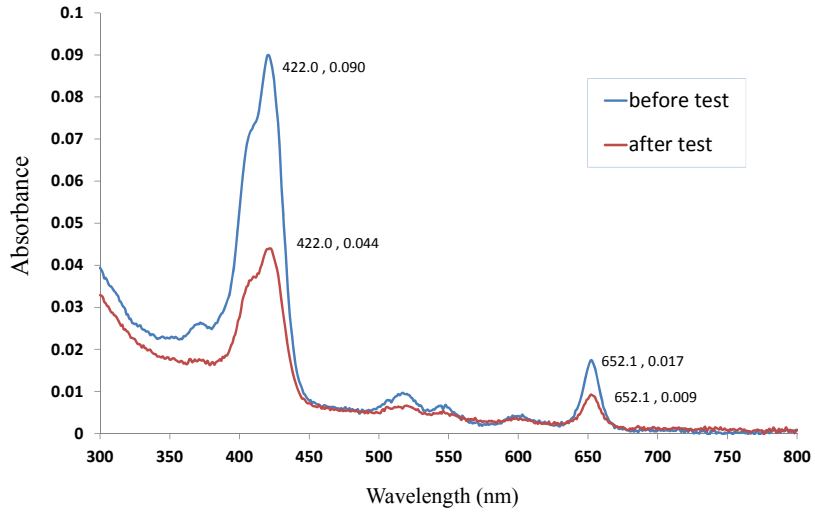

Figure S8. EZview^{TM} Red Streptavidin Affinity Gel test on 90-nm diameter amine/OS/PEG nanoparticles doped with dye **4** and targeted with biotin. The test is based on streptavidin coated agarose beads, the nanoparticles are incubated with the beads for 1 hour and the beads are then removed, the difference in the absorbance before and after the beads incubation confirm the conjugation of the particles with the biotin.

4.6 Western blot analysis of Cetuximab-conjugated nanoparticles

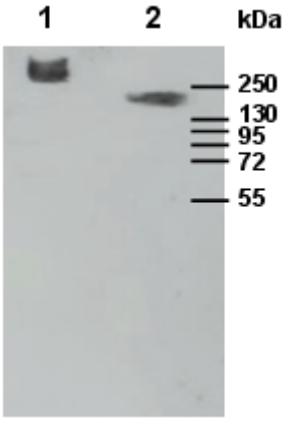

Figure S9. Western blot analysis to confirm the antibody conjugation to the nanoparticles (NPs). Cetuximab-conjugated NPs (lane 1) and Cetuximab (lane 2) were separated by SDS-PAGE under non reducing condition and after the transfer onto a membrane of nitrocellulose the antibodies were stained using an anti-human antibody conjugated to HRP. Chemiluminescent signals, developed using ECL substrate, were detected by photographic films.

5. Photophysical studies

5.1 Absorbance and Fluorescence Measurements

Absorbance spectra were measured using a Perkin-Elmer (Beaconsfield, UK) Lambda 25 UV/Vis spectrometer with 1 cm pathlength quartz cuvettes. Fluorescence spectra were measured using a Perkin-Elmer LS50-B spectrofluorimeter equipped with a bifurcated fiber-optic probe.

5.2 Fluorescence Lifetime Measurements

Fluorescence lifetimes were measured using time correlated single photon counting (TCSPC). Dilute solutions were prepared with the dye concentration at 0.5 μM and placed in a 1 cm pathlength quartz cuvette. A pulsed laser diode module (90 picosecond pulse duration) was used to excite the samples at 405 nm at a 5MHz repetition rate (EPL-405, Edinburgh Instruments Ltd., UK). The fluorescence was detected using a fast multialkali photomultiplier module (model H5773-04, Hamamatusu Photonics K.K., Japan) via a longpass filter (OG510, Schott, UK) and a monochromator (model M300, Bentham Instrument Ltd, UK). A Lyot depolarizer (Thorlabs Ltd, UK) was incorporated to minimise any polarisation anisotropy artefacts. TCSPC was carried out using a PC-mounted TCSPC board (TimeHarp, Picoquant GmbH, Germany) and lifetimes were derived using Fluofit software (PicoQuant GmbH, Germany). The Instrument Response Function (IRF) was obtained from a non-fluorescent scattering Ludox® solution (Sigmal-Aldrich, Gillingham, UK). Optimum fitting with minimisation of the residuals was confirmed using a Chi-squared value $\chi 2 < 1.4$.

5.3 Singlet Oxygen Measurements

The singlet oxygen phosphorescence at 1270 nm was detected using time-resolved photon counting from air-equilibrated, deuterated aqueous or methanolic solutions in quartz cuvettes. For detection in the near-IR, a thermoelectrically cooled photomultiplier (model H10330-45, Hamamatsu

Photonics Ltd, UK) was used, and emission was collected via a series of lenses from the cuvette in combination with a long-pass and a band-pass filter centered at 1270 nm (BK Interferenzoptik Electronik, Germany). The solutions were excited using a 532 mn Nd:YAG laser (Lumanova GmbH, Germany) with the beam axis aligned orthogonally to the collection optics. The laser was pulsed at a repetition rate of 3 kHz and a pulse length of 3 ns, giving a mean power of 8 mW, and a fast photodiode (1 ns rise time, Becker-Hickl, Berlin, Germany) was used to synchronise the laser pulse with the photon counting detection system. A series of neutral density filters was used to attenuate the laser power. The photon counting equipment consisted of a PC-mounted multiscaler board (model MSA-300, Becker-Hickl, Germany) and a pre-amplifier (Becker-Hickl, Germany) which gave a resolution of 5 ns per channel. Time-resolved phosphorescence measurements were accumulated by the multiscaler board. The traces were analysed using FluoFit software (PicoQuant GmbH, Germany) to extract the singlet oxygen decay lifetime. To calculate the quantum yield, Rose Bengal was used as the standard which has singlet oxygen yields of 0.76 in D2O and 0.79 in MeOD (R. W. Redmond, and J. N. Gamlin, “A compilation of singlet oxygen yields from biologically relevant molecules” *Photochem. Photobiol.* 1999, **70**, 391-475). The concentrations were adjusted to give the same absorbance at 532 nm, and to analyse the time-resolved data we used the standard zero-intercept method.

6. Protein binding studies

6.1 Nanoparticles incubation with human plasma

Venous blood was taken from healthy volunteers and immediately anticoagulated with 3.8% trisodium citrate (9 vol blood + 1 vol citrate). Plateled poor plasma (PPP) was obtained by centrifuging blood at 2,000 g for 10 min, without use of a brake. Plasma aliquots were frozen in liquid nitrogen and stored at -20°C until time of use. Before use, plasma was thawed and centrifuged at 16000 g at 4°C for 20 min, to eliminate any precipitate. Then, it was diluted in PBS EDTA 1 mM pH 7.4 at a final concentration of 24% (v/v), filtered through a 0.22 µM filter and incubated with PBS (ctrl) and 2 mg of Ormosil or Ludox nanoparticles for 1 hour at 37°C under gentle stirring. Then, nanoparticles were recovered by ultracentrifuging at 100,000 g for 1 h at 4°C, using a XL-70 Ultracentrifuge (Beckman), and washed twice with PBS EDTA 1 mM pH 7.4.

6.2 Identification of plasma proteins adsorbed to nanoparticles surface

The pellet of nanoparticles was dissolved in 150 µl of non-reducing sample buffer (50 mM Tris HCl pH 6.8, 2% SDS, 0.06% bromophenol blue, 6% glycerol) plus protease inhibitors cocktail (Sigma Aldrich), heated at 95°C for 5 min and loaded on a 12% (v/v) SDS-PAGE. After protein staining with colloidal Comassie G-250, bands of interest were excised and analyzed by mass spectrometry.

7. Biological studies

7.1 Cell culture and incubation experiments

A549 (human lung carcinoma cells), CCD-34Lu (human normal lung fibroblasts), KB (folate receptor positive cells derived via HeLa contamination), HeLa (human malignant cervical cells expressing low amounts of EGF receptor), A431 (human epidermoid carcinoma cells, expressing high amounts of EGF receptor) and HUVEC (human umbilical vein endothelial cells, overexpressing $\alpha_v\beta_3$ integrin) cell lines were obtained from American Type Culture Collection (ATCC, Rockville, USA). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 90% of humidity. A549 were cultured in F-12K medium containing 2 mM L-glutamine and 2.5 g/L sodium bicarbonate; CCD-34Lu, HeLa and A431 were cultured in Dulbecco Modified Eagle's Medium (DMEM) containing 3.7 g/L sodium bicarbonate, 4.5 g/L glucose and supplemented with 0.1 mM MEM non essential amino acids and 0.02 M HEPES while KB were cultured in Modified Eagle's Medium (MEM). All the media were supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Invitrogen, NY, USA) and antibiotics (38 units/mL streptomycin and 100 units/mL penicillin G (Sigma-Aldrich, St Louis, MO, USA). HUVEC cells were cultured in Medium-200 supplemented with antibiotics and with the Low Serum Supplement Kit (Invitrogen) having a final FBS concentration of 2%. To obtain stabilized EGFR-expressing HeLa cells (HeLa EGFR +), $3x10^6$ cells were transfected with 30 μg of pBABE-EGFR plasmid (Addgene) by means of electroporation treatment (250 V, 950 μF) in 0.45 cm electroporation cuvettes (Gene Pulser, Bio-Rad); after two days, transfected cells were diluted and selected in 2 μg/ml puromycin-containing medium (Sigma).

In all the experiments, the cells (A549, CCD-34Lu, KB, A431, HeLa and HeLa EGFR +) were seeded and maintained for 24 h in culture medium supplemented with 10% FBS (complete medium) before starting the treatment; then, the medium was replaced with fresh medium containing 3%

FBS, in which free mTHPC or nanoparticle suspensions were freshly diluted. For HUVEC cells no additional serum was used nor for the seed nor for the treatments.

7.2 Cellular uptake of mTHPC in standard solvent or loaded in ORMOSIL/PEG nanoparticles

A549 and CCD-34Lu cells (10^5) were seeded in 2 mL of complete medium in 35 mm diameter tissue culture dishes. After 24 h, the cells were incubated with increasing concentrations (0.25-1.75 µM) of mTHPC, delivered by standard solvent (Foscan®, ethanol/poly(ethylene glycol) 400/water; 20:30:50, by vol.) or by ORMOSIL/PEG nanoparticles in the form of dye **4**, (Table 1). After 24 h of incubation, the cells were washed twice with 2 mL of versene, detached with 500 µL of trypsin (Invitrogen) that was neutralized with the addition of 200 µL of FBS. Cells were centrifuged and resuspended in versene before measuring mTHPC fluorescence by flow cytometry with a BD FACSCanto™ II (Becton Dickinson, San Jose, California, USA). The blue laser at 488 nm was used as excitation source and wavelengths longer than 670 nm (PerCP channel) were used for the detection of the mTHPC fluorescence. Ten thousand events/sample were acquired and analyzed with the FACSDiva Software.

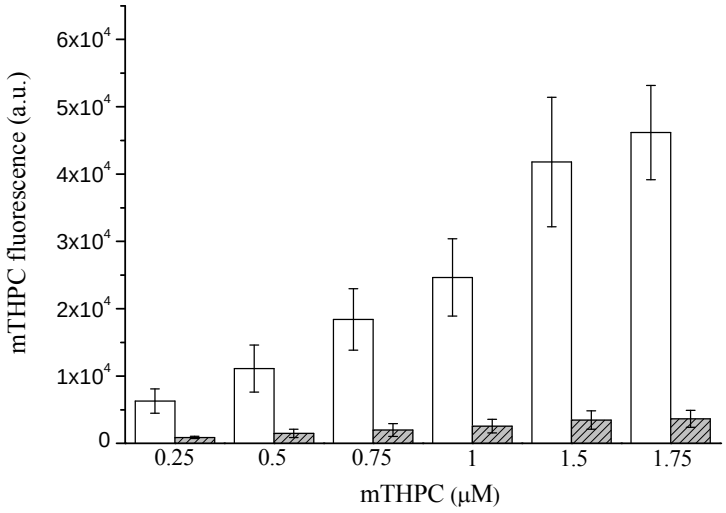

Figure S10. Flow cytometry measurements of mTHPC and derivative **4** uptake in CCD-34Lu normal cells incubated for 24 h with increasing concentration of free mTHPC (white) or **4**-doped PEG-ORMOSIL nanoparticles (grey).

7.3 Specific uptake of mTHPC (dye 4) loaded in targeted ORMOSIL/PEG nanoparticles

Folate-targeted nanoparticles. 10^5 KB cells were seeded in 2 mL of folate-deficient RPMI medium and 24 h after the seeding were incubated for 5 h with increasing concentration (0.005-1 μM) of mTHPC (dye 4, Table 1) in folate-targeted (0.1 or 1% folate) or un-targeted (0% folate) ORMOSIL/PEG nanoparticles. After incubation time, the cells were washed, detached from the plates and analyzed by flow cytometry to measure mTHPC fluorescence as described above. Competition experiments with 1 mM of free folic acid (Sigma-Aldrich) were carried out incubating the cells for 1 h prior the addition of NPs in order to saturate folate receptors present on KB cell surface.

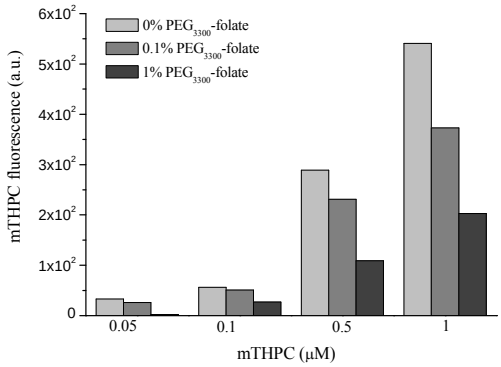

Figure S11. Flow cytometry measurements of mTHPC derivative **4** uptake in folate receptor positive KB cells incubated for 5 h with increasing concentration of **4**-doped PEG-ORMOSIL nanoparticles conjugated with folate in different amounts (light grey: 0%, grey: 0.1%, dark grey 1% PEG_{3300}-folate/PEG_{2000}).

RGD-targeted nanoparticles. 50,000 HUVEC cells were seeded in 500 μl of M-200 medium in 24-well plates. After 24 h, the cells were incubated with 0.1 or 1 μM mTHPC (dye 4, Table 1) loaded in RGD-NPs or RAD-NPs for 3 or 24 h. After incubation, the cells were washed twice with 500 μl of versene, detached with 200 μL of trypsin and neutralized with 100 μL of FBS before flow cytometry analysis (see text).

Cetuximab-targeted nanoparticles. To analyze the cellular uptake 100,000 A431 cells were seeded in 500 µl of DMEM medium in 24-well plates. After 24 h, the cells were incubated with 1 µM mTHPC (dye 4, Table 1) loaded in Cetuximab-NPs, NPs-alone or BSA-NPs for 90 minutes at 37°C; finally, the cells were washed twice with 500 µl of versene, detached with 200 µL of trypsin and neutralized with 100 µL of FBS before flow cytometry analysis. For competition experiments Cetuximab and BSA-NPs were mixed with a molar excess of free Cetuximab before the incubation with the cells. In order to investigate if the uptake is dose-dependent, A431 cells were incubated with increasing amounts of mTHPC (dye 4, Table 1) loaded on Cetuximab-NPs or BSA-NPs for 90 minutes at 37°C and then analyzed by flow cytometry. Finally to evaluate the serum stability, Cetuximab-NPs or BSA-NPs were incubated for 90 minutes at 37°C with increasing concentrations of serum (i.e. ranging from 10% to 100%) before to assess their functionality in uptake experiments on A431 cells.

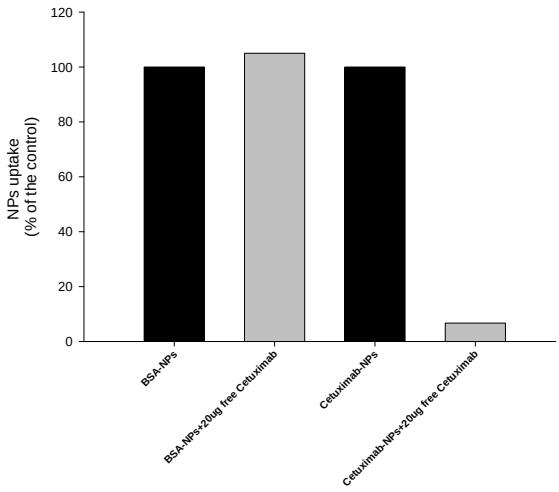

Figure S12. Displacement of the uptake of Cetuximab or BSA-4 doped PEG-ORMOSIL on A431 cells by free Cetuximab. Live adherent cells were incubated with a fixed NP concentration (concentration of 0.25 uM in dye 4) and with or without an excess of free Cetuximab (20 ug of antibody) for 90 minutes at 37°C. After washings, detachment from the well and a further washing step cells were analyzed by flow cytometry measuring the dye 4 fluorescence (APC channel).

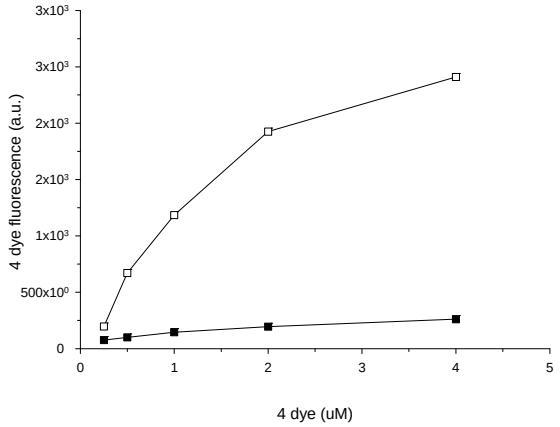

Figure S13. Differential uptake of Cetuximab-NPs (□) and BSA-NPs (■) on A431 cells. Live adherent cells were incubated with increasing concentrations (concentration ranging from 0.25 to 4 uM in dye 4) of 4-doped PEG-ORMOSIL for 90 minutes at 37°C. After washings, detachment from the well and a further washing step cells were analyzed by flow cytometry measuring the dye 4 fluorescence (APC channel).

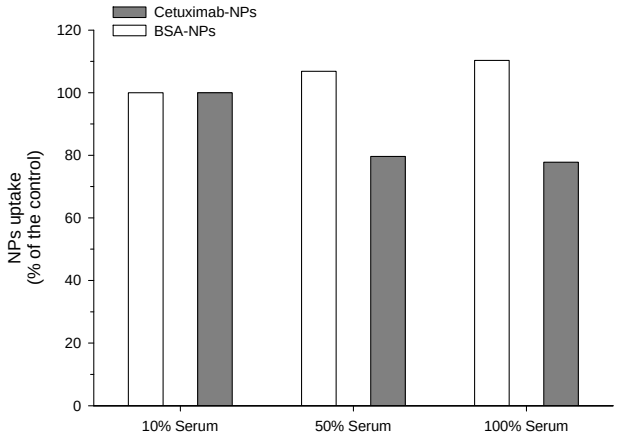

Figure S14. Serum stability of Cetuximab-NPs and BSA-NPs. After a pre-incubation of NP batches in serum at 37°C for 90 minutes, the NP functionality was assessed measuring the uptake on A431 cells. Briefly, serum-treated NPs were incubated on A431 cells for 90 minutes at 37°C; after washings, detachment from the well and a further washing step cells were analyzed by flow cytometry measuring the dye 4 fluorescence (APC channel).

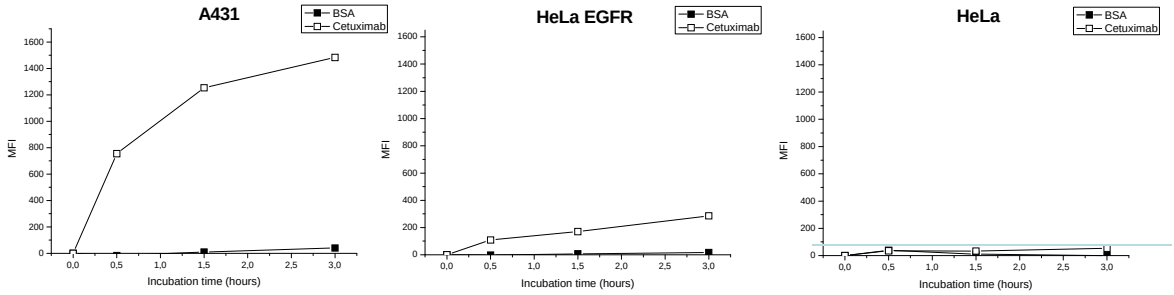

Figure S15. Time-dependant uptake of Cetuximab- and BSA- conjugated nanoparticles with different cell lines. EGFR positive A431 cells, EGFR over-expressing HeLa and parental HeLa, having reduced EGFR levels, were incubated at 37°C for different times (up to 3 hours) with a concentration of Cetuximab-NPs corresponding to 1 μM mTHPC. Cells were then washed and directly analyzed by FACS.

7.4 Measurement of EGFR down regulation induced by Cetuximab-conjugates NPs.

The day before the experiment $1x10^5$ A431, HeLa and HeLa EGFR + cells were seeded on 24 wells plastic plates (Falcon). The day of the experiment, cells were incubated for two hours with different concentrations of Cetuximab or BSA-conjugated NPs (up to 50 μg/ml) at 37°C, then they were washed, trypsinized and stained with a FITC-conjugated anti-EGFR antibody (Abcam) for 30 minutes at 4°C. After washing, cells were analyzed by FACS, acquiring 10,000 events for each sample.

7.5 Confocal analysis of A431, HeLa and HeLa cells incubated with Cetuximab-conjugated NPs. The day before the experiment, cells ($1x10^5$) were seeded on cover glasses; after 24 hours they were treated for two hours at 37°C with an amount of nanoparticles corresponding to 1 μM mTHPC, washed, fixed with 2% paraformaldehyde (Sigma) in PBS for 20 minutes at room temperature, permeabilized with 0.2% Triton X-100 (Sigma) for 10 minutes at 4°; cells were then washed and saturated with 1% BSA in PBS for 1 hour at room temperature; Cetuximab conjugated

to NPs was then stained with a FITC-conjugated anti-IgG antibody (Millipore) for 1 hour at 4°C. After three washings coverslips were mounted in mounting medium (KPL) and were analyzed with a confocal microscope (SP2 Leica), using 488 nm and 630 nm excitation sources. Images have been acquired with the different fluorescence filters, representative pictures were collected as Tiff files and processed with standard imaging programs (Photoshop and Image J).

7.6 Photokilling experiments.

$8x10^3$ A431, HeLa and HeLa EGFR cells were seeded on 96 wells plastic plates (Falcon) and after 24 hours were treated with different amount of Cetuximab- or BSA-conjugated NPs for 6 hours at 37°C. Cells were then washed in PBS and irradiated or not with a red light (Waldmann Meidizintechnik PDT 1200, 60 mW/cm^2) for 10 minutes (36 J/cm^2) at room temperature. Fresh culture medium was added and after 24 hour cell viability was assessed by MTS assay (Promega), following manufacturer instructions.

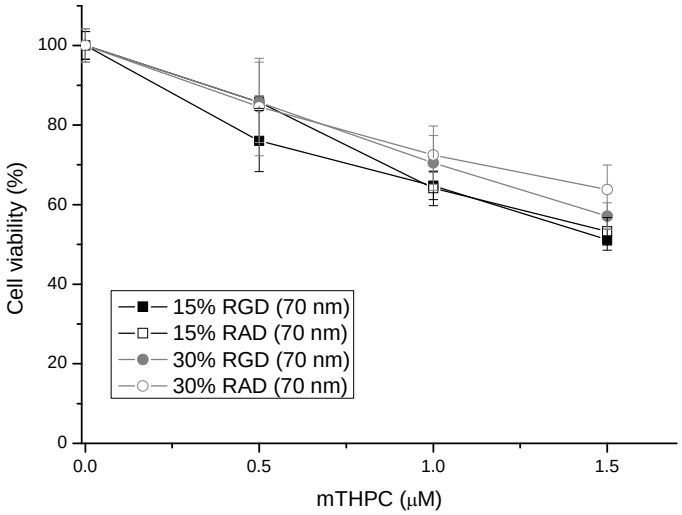

Figure S16. Phototoxicity in HUVEC cells after exposure to different preparations of RGD- or RAD conjugated 4-doped PEG-ORMOSIL nanoparticles. Cells were irradiated with 2.4 J/cm^2 of red light (Waldmann, 600-700 nm, 12 mW/cm^2) after 24 h incubation with increasing doses of mTHPC in RGD targeted NPs or RAD conjugated NPs as untargeted control. Cell viability was measured 24 h post-irradiation by MTS assay and expressed as mean percentage ± S.D. with respect to not incubated but irradiated, control cells.