Supplementary information: Cell type determines the light-induced endosomal escape kinetics of multifunctional mesoporous silica nanoparticles

1 Materials and Methods

1.1 Chemicals

Tetraethylorthosilicate (TEOS, Fluka, >99%), (3-mercaptopropyl)-triethoxysilane (MPTES, Gelest, 95 %), (3-aminopropyl)-triethoxysilane (APTES, Sigma, 98 %), cetyltrimethylammonium chloride (CTAC, Fluka, 25wt % in H_2O), phenyltriethoxysilane (PhTES, Sigma, 98 %), triethanolamine (TEA, Aldrich, 98 %), ATTO633-NHS ester (ATTO-Tec), QSY21 succinimidyl ester (Invitrogen), tetrahydrofuran (THF, Acros, 99.8 %), protoporphyrin IX (Sigma, >95 %), p-phenylenediamine (Sigma, 95 %), triethylamine (Sigma, 99 %), ethyl chloroformate (Fluka, >98 %), ammonium nitrate (Sigma, 99 %), conc. hydrochloric acid (Aldrich, >95 %, 37wt %) 1-maleinimido-3-oxo-7,10,13,16,19,22,25,28-octaoxa-4-azahentriacontan-31-oic acid (mal-dPEG(8)-COOH, Iris Biotech), sulfo-N-hydroxysuccinimide (sulfo-NHS, Aldrich, 98 %), N-(3-dimethylaminopropyl)-N'- ethylcarbodiimide (EDC, Sigma, 97 %), toluene (Aldrich, 99.8 %), ethanol (EtOH, Aldrich, >99.5 %), fluorescein disodium salt dihydrate (Acros, >95 %), L-glutathione (Aldrich, >98 %), 2,2'-dithiopyridine (DTP, Sigma, >99 %), L-cysteine (Cys, Sigma, >97 %), anhydrous N,N-dimethyl-formamide (DMF, Sigma, >99.8 %). All chemicals were used as received without further purification. Doubly distilled water from a Millipore system (Milli-Q Academic A10) was used for all synthesis and purification steps. The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-Dioleoyl-sn-glycero-3-phospho-ethanolamine (DOPE) were purchased from Avanti polar lipids.

1.2 Multifunctional mesoporous core-shell silica nanoparticles

The multifunctional core-shell CMS nanoparticles were synthesized following a previously reported procedure based on a delayed co-condensation approach which employs tetraethylorthosilicate (TEOS) as the silica source, 3-(aminopropyl)-triethoxysilane (APTES) and 3-(mercaptopropyl)-triethoxysilane as functionalized silica precursors, and cetyltrimethylammonium chloride (CTAC) as structure directing agent [3]. The polyalcohol triethanolamine (TEA) slows down the condensation rate of TEOS and leads to a small size distribution and radial growth of CMS. Functionalized CMS with 3-mercaptopropyl moieties in the shells of the particles and 3-aminopropyl groups in the core were synthesized according to this procedure giving the sample CMS_NH_{2in}_SH_{out} after template removal. Post-synthesis modifications of the introduced functionalities are possible due to their reactive nature.

1.3 CMS based detector system

Post-synthesis functionalization of the outer surface of CMS particles involves covering the shell with a short, bifunctional polyethyleneglycol linker (maleimide-dPEG8-COOH, (1-maleimido-3-oxo-7,10,13,16,19,22,25,28-octaoxa-4-azahen-triacontan-31-oic acid), increasing its dispersibility in water. The thiol moiety forms a stable bond with the maleimide functionalization of the PEG-linker in a Michael addition whereas the carboxy-functionality remains accessible for further modification. The amino-modified photosensitizer protoporphyrin-IX (PpIX-NH_2) was synthesized according to a modified literature procedure [9]. This precursor was covalently attached to the carboxy-functions at the particle surface via an N-(3-dimethylaminopropyl-) N'-ethylcarbodiimide (EDC) assisted amidation to give the photosensitizer-modified sample CMS_NH_2in_PEG-$PpIX_{out}$ [11].

We designed a redox-sensitive fluorimetric dye-quencher system for the CMS core (Figure 2B). In the first step the highly reactive ATTO633-NHS ester was covalently attached to the amino group of cysteine (CysATTO633). Subsequently, CysATTO633 was reacted with the aminopropyl-functionality in an EDC assisted amidation to give the sample CMS_CysATTO633$_{in}$_PEG-$PpIX_{out}$. The thiol group of cysteine was activated with 2,2'-dithiopyridine, and cysteine-modified QSY21 (CysQSY21) was added. This resulted in a disulfide bridged dye-quencher system in the particle core and gave the final sample CMS_CysATTO633-CysQSY21$_{in}$_PEG-$PpIX_{out}$. Detailed information on the synthesis and labeling procedures are given below.

1.3.1 Synthesis of core-shell CMS_NH$_{2in}$_SH$_{out}$

Bi-functional core-shell colloidal mesoporous silica was synthesized following the previously published method [10]. A mixture of TEOS (1.63 g, 9.22 mmol), PhTES (57 mg, 0.24 mmol), APTES (53.1 mg, 0.24 mmol) and TEA (14.3 g, 95.6 mmol) was heated at 90°C for 20 minutes under static conditions in a polypropylene reactor. Then a solution of CTAC (25 % in water, 2.41 ml, 1.83 mmol) in water (21.7 g, 1.21 mol) was preheated to 60°C and added quickly. The reaction mixture was stirred at room temperature for 20 minutes. TEOS (138.2 mg, 0.922 mmol) was added in four equal increments every three minutes. This step was followed by 30 minutes of stirring at room temperature. For the shell functionalization, a mixture of TEOS (19.3 mg, 92.5 μmol) and MPTES (22 mg, 92.5 μmol) was added to the reaction. The resulting mixture was then allowed to stir at room temperature for 12 hours. After the addition of 100 ml ethanol, the CMS particles were collected by centrifugation, re-dispersed in 100 ml of ethanol and extracted according to the procedure described below.

1.3.2 Extraction of CMS_NH$_{2in}$_SH$_{out}$

Extraction of the surfactant from the CMS nanoparticles was performed by heating 250 mg for 45 minutes under reflux at 90°C in a solution containing 2 g ammonium nitrate in 100 ml ethanol, followed by 45 minutes under reflux in a solution of 4 g conc. hydrochloric acid (37 wt%) in 100 ml ethanol. The CMS nanoparticles were separated by centrifugation and washed with ethanol after each extraction step. CMS materials were obtained as colloidal suspensions.

1.3.3 PEGylation of CMS_NH$_{2in}$_SH$_{out}$ with bi-functional Mal-PEG(8)-COOH to CMS_NH$_{2in}$_PEG$_{out}$

An ethanolic suspension containing 1 mg of CMS_NH$_2$_SH$_{out}$ was centrifuged and re-dispersed in 1 ml of water. This washing step was repeated twice. Next, the CMS were suspended in 500 μl of water and 100 μl of a solution of Mal-dPEG(8)-COOH (10 mg/ml) was added under stirring. The reaction mixture was stirred for 48 hours at room temperature. In order to remove the excess of Mal-dPEG(8)-COOH, the particles were washed five times with 1 ml of water to remove unbound PEG. All washings steps were followed by centrifugation. Finally, the sample CMS_NH$_{2in}$_PEG$_{out}$ was re-dispersed in 500 μl ethanol.

1.3.4 Synthesis of protoporphyrin-IX-bis(phenyleneaminoamide) (PpIX-NH$_2$)

Protoporphyrin-IX-bis(phenyleneaminoamide) was prepared according to a modified literature procedure [9, 11]. In a dry and darkened 100 ml three-neck flask, protoporphyrin-IX (100 mg, 0.178 mmol, 1 eq.) was dissolved in 20 ml anhydrous tetrahydrofuran and cooled to 0°C under nitrogen atmosphere. Triethylamine (2.45 ml, 17.7 mmol, 100 eq.) was added with a syringe to the solution. Ethyl chloroformate (1.7 ml, 17.9 mmol, 100 eq.) was subsequently added over a total time of 30 minutes via a dropping funnel. The resulting Mixture 1 was stirred for 2 hours at 0°C. In a second flask, finely powdered p-phenylene diamine (1.95 g, 17.9 mmol, 100 eq.) was dissolved in 10 ml anhydrous THF under nitrogen atmosphere. Mixture 1 was slowly added to Mixture 2 with a syringe. The resulting reaction was stirred for 24 hours at room temperature in the dark. The solvent was removed in vacuo, the resulting precipitate suspended in 15 ml of ice-cold water. The precipitate was filtered off and washed three times with 15 ml of ice-cold water each. PpIX-NH$_2$ was obtained as a brown precipitate (85 mg, 0.114 mmol, 64%).

1.3.5 Attachment of PpIX-NH$_2$ to CMS_NH$_{2in}$_PEG$_{out}$ to the sample CMS_NH$_{2in}$_PEG-PpIX$_{out}$

1 mg of CMS_NH$_{2in}$_PEG$_{out}$ was re-suspended in 1 ml THF before PpIX-NH$_2$ (2 mg, 2.7 μmol) was added. The mixture was stirred for 5 minutes. Subsequently, EDC (3.5 μl, 20 μmol) and sulfo-N-hydroxysuccinimide (4.3 mg, 20 μmol) were added and the reaction was stirred for 14 hours in the dark. The sample was washed five times with 1 ml of THF to remove unbound PpIX-NH$_2$ and three times with a total amount of 3 ml water.

1.3.6 Fluorescence labeling procedure for the amino group of L-cysteine (CysATTO633)

L-Cysteine (Cys, 1 mg, 8.25 μmol) was dissolved in 100 μl of freshly prepared 0.1 M sodium bicarbonate buffer (pH 8.3) to which 10 μl (26.7 nmol) of ATTO633-NHS (1 mg dissolved in 500 μl anhydrous DMF) was added. The large excess of Cys is intended to minimize the amount of remaining free dye, since no purification of the product was performed. The resulting mixture was stirred for two hours in the dark to give CysATTO633.

1.3.7 Attachment of Cys-ATTO633 to the core of CMS_NH_{2in}_PEG-PpIX$_{out}$

A solution of 1 mg Cys-ATTO633 in water was added to a colloidal suspension of 1 mg CMS_NH_{2in}_PEG-PpIX$_{out}$ in water. The reaction mixture was stirred for 5 minutes in the dark before EDC (3.5 μl, 20 μmol) and sulfo-NHS (4.3 mg, 20 μmol) were added. The reaction was stirred for 14 hours in the dark. The sample was washed two times with 1 ml water and then five times with 1 ml ethanol each to remove unbound dye.

1.3.8 Quencher QSY21 labeling procedure for the amino group of L-cysteine (CysQSY21)

L-Cysteine (Cys, 1 mg, 8.25 μmol) was dissolved in 100 μl of freshly prepared 0.1 M sodium bicarbonate buffer (pH 8.3). Then, 21.8 μl (26.7 nmol) of a solution of QSY21-NHS (1mg/ml in anhydrous DMF) was added. The large excess of Cys is intended to minimize the amount of remaining free dye, since no purification of the product was performed. The resulting mixture was stirred for 2 hours in the dark to give CysQSY21.

1.3.9 Attachment of Cys-QSY21 via a disulfide bond to the Cys-ATTO633 moiety in the core of CMS_NH_{2in}_PEG-PpIX$_{out}$

150 μl of an ethanolic suspension of the sample CMS_NH_{2in}_PEG-PpIX$_{out}$ containing 1 mg particles was adjusted to the total volume of 1 ml by adding 850 μl absolute ethanol, to which 2,2'-Dithiopyridine (DTP, 1 mg, 4.5 μmol) was added. The sample was stirred for 15 minutes in the dark. Three washing steps with 1 ml ethanol each were followed by subsequent centrifugation. The DTP-activated CMS were re-suspended in 500 μl water and a solution containing 1 mg CysQSY21 in 100 μl water was added. The reaction mixture was stirred 14 hours in the dark. Unbound CysQSY21 was removed by five washing steps with 1 ml ethanol each.

1.4 CMS NP characterization

1.4.1 Dynamic Light Scattering

Dynamic light scattering (DLS) measurements were performed on a Malvern Zetasizer-Nano instrument equipped with a 4 mW He-Ne laser (633 nm) and an avalanche photodiode detector. The hydrodynamic radius of the particles was determined by dynamic light scattering in ethanol suspension. For this purpose, 100 μl of an ethanolic suspension of the particles (ca. 10 mg/ml)

was diluted with 3 ml of ethanol prior to the measurement. The data for the samples CMS_NH$_{2in}$_SH$_{out}$, CMS_NH$_{2in}$_PEG$_{out}$ and CMS_NH$_{2in}$_PEG-PpIX$_{out}$ can be seen in 1.

1.4.2   Nitrogen Sorption

Nitrogen sorption measurements were performed on a Quantachrome Instruments NOVA 4000e. All three samples (15 mg each) were heated to 393 K for 12 h *in vacuo* to outgas the samples before nitrogen sorption was measured at 77 K. For calculations of pore sizes and volumes a non-local density functional theory (NLDFT) equilibrium model of nitrogen on silica was used. The Brunauer-Emmett-Teller (BET) model was applied to evaluate the surface areas. The isotherm and pore size distribution (PSD) can be found in Figure 2 of the main text.

1.4.3   Thermogravimetric analysis

Thermogravimetric analysis of the bulk samples CMS_NH$_{2in}$_SH$_{out}$, CMS_NH$_{2in}$_PEG$_{out}$ and CMS_NH$_{2in}$_PEG-PpIX$_{out}$ was performed on a Netzsch STA 440 C TG/DSC with a heating rate of 10 K/min in a stream of synthetic air of about 25 ml/min. The resulting data can be found in Figure 2.

1.4.4   Fluorescence spectroscopy of adsorbed Fluorescein in the CMS_NH$_{2in}$_PEG-PpIX$_{out}$ sample

In order to investigate the functionality of the lipid bilayer and to prove the accessibility of the mesopores, we studied these CMS particles with our custom-made fluorescence release system. The two compartments of the cuvette are separated by a dialysis membrane (Molecular weight cut-off 16.000 kDa), preventing the nanoparticles from diffusing but allowing small dye molecules to pass into the large compartment which is monitored by fluorescence spectroscopy. We used the unsealed sample CMS_NH$_{-2in}$_PEG-PpIX$_{out}$ as a reference. Laser activation with 405 nm leads to singlet oxygen generation and to a rupture of the SLB, respectively. Adsorbed fluorescein can diffuse from the mesopores whereas the non-activated sample shows no release. The obtained release curves of fluorescein can be seen in Figure 3.

1.4.5   Transmission electron microscopy (TEM)

The template extracted sample CMS_NH$_{2in}$_SH$_{out}$ was investigated with a FEI Titan 80-300 operating at 300 kV with a high-angle annular dark field detector. A droplet of the diluted CMS solution in ethanol absolute was

dried on a carbon-coated copper grid. Figure 2A of the main text shows a TEM micrograph of CMS_NH$_{2in}$_SH$_{out}$.

1.5 Lipids

We chose DOPC and DOPE based on both the number of double bonds with which the singlet oxygen produced on PpIX activation can react [7]. Additionally, both lipids have been previously reported to form stable, fluid bilayers on the CMS core in their pure form [1, 6]. CMS particles were covered with SLBs comprised of either pure DOPC (DOPC@CMS) or pure DOPE (DOPE@CMS) lipid membranes labelled with 1 mol % Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (TR-DHPE).

1.6 Supported lipid bilayer (SLB) deposition on CMS particles through solvent exchange

SLBs were deposited on CMS particles as previously described [5]. Briefly, CMS NPs in solution are centrifuged at 13000 rpm for five minutes. The supernatant is discarded and the particles are redispersed in a 2.5 mg/ml lipid solution in 40% vol ethanol:60% vol water; this solution was subsequently titrated with water until a 95% vol water content is reached. The solution is allowed to equilibrate for 15 minutes, in the process forming the SLB on individual particles [5, 2]; the NPs are separated from excess lipid by centrifugation, and subsequently resuspended in a smaller volume comprised of 50% vol complete cell culture medium:50% vol water solution. In this alcohol-free environment, the SLB is fully stabilized. The aggregation of NPs in this solution is minimized by brief sonication followed by filtration.

1.7 Uptake experiments

DOPC@CMS particles were tested on non-phagocytic cell lines whose interactions with NPs were of interest: Beas2B, a human normal bronchial epithelium cell line; Huh7, a human hepatocarcinoma cell line; Renca, a transformed murine renal carcinoma cell line; and 3T3, a mouse embryonic fibroblast cell line. Cells were plated to a density of 0.75 x 10^4 cells/cm^2 on 8-well ibiTreat-coated Ibidi chambers 3 - 4 days before the start of the experiment. Cells were subsequently incubated with 50 μg/cm^2 DOPC@CMS over two-hour intervals up to eight hours and washed with PBS five times post-incubation. External fluorescence was additionally quenched with a wash

using 0.4 % w/w trypan blue [8, 4]. Cells were then fixed with 2% formalde-hyde in Leibovitz medium, reported to reduce cellular fluorescence [12], sup-plemented with 10% FCS for a total of two hours. Internalized NPs were visualized using fluorescence microscopy at 40x magnification, and recorded at an exposure time of 1 s and a density power of 0.12 W/cm^2. For each setup, eight random viewfields were chosen. Experiments were performed in triplicate. The relative number of particles taken up by each cell was approx-imated by taking the mean gray values across the area occupied by the cell. Analysis was done semi-automatically in ImageJ using an in-house program [13]. Control experiments were performed using 30 nm, fluorescein labeled Latex NPs (Sigma Aldrich).

1.8   Lysis experiments

For in vitro lysis experiments, cells were incubated for 12 hours with DOPC@CMS or DOPE@CMS functionalized with PpIX and ATTO633-QSY21 to ensure completion of uptake. Lysis experiments were performed on 3T3 and Renca cells, which appear to exhibit the highest and lowest uptake characteristics for DOPC@CMS particles. AlexaFluor Dextran 488 (MW=10 kDa) was used as a fluid-phase marker for endocytosis. Following incubation, cells were washed with PBS five times post-incubation, or until most of the fluorescence from the AlexaFluor Dextran was removed; washing with 0.4 % w/w trypan blue was performed to quench any remaining external fluorescence. PpIX was activated by a one- or two- minute exposure to a 405 nm laser with a den-sity power of 0.12 W/cm^2 prior to imaging. Fluorescence images were taken every second with an exposure time of 200 ms over a period of two minutes per viewfield. NP and endosome fluorescence were monitored using the fol-lowing excitation/emission filter sets: 488 nm/525 nm (AlexaFluor dextran); 560 nm/645 nm (Texas Red); and 640 nm/690 nm (ATTO633) (Chroma Technology). A negative control composed of DOPC@CMS or DOPE@CMS functionalized with ATTO633-QSY21, but not PpIX, was used in parallel with all setups. Single endosome lysis events were detected using the same ImageJ plugin described previously. Briefly, images are segmented based on the fluid-phase marker channel (Figure 4). Discontinuities that occur in both the red and green channels were recorded as an endosome lysis event, and the time distribution of these events was collected for the different experimental setups. Apart from lysis times, the endosome area and NP colocalization intensity, based on the mean gray values in the red channel, are recorded. Background correction based on the substraction of the 640 nm/690 nm im-age from that obtained from the 560 nm/645 nm to prevent any influence of ATTO633 fluorescence from affecting readings of colocalization intensity.
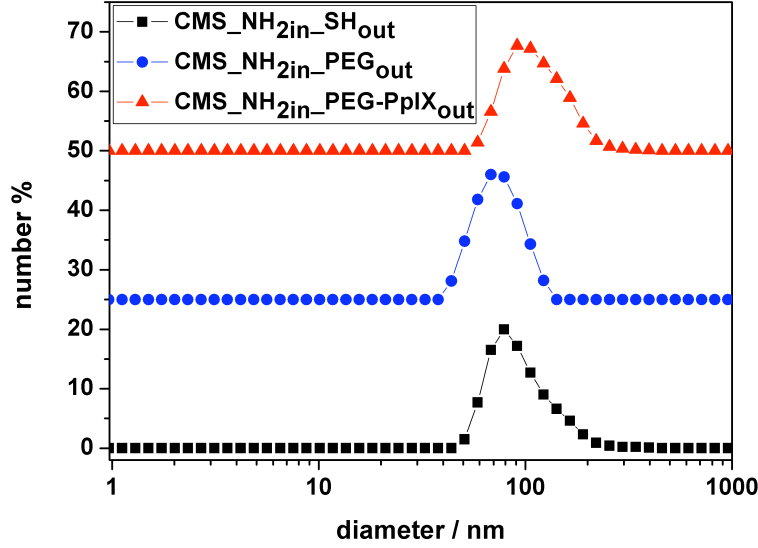
Figure S 1: Dynamic light scattering data for the samples $CMS\_NH_{2in}\_SH_{out}$ (black), $CMS\_NH_{2in}\_PEG_{out}$ (blue) and $CMS\_NH_{2in}\_PEG\text{-}PpIX_{out}$ (red). To emphasize the difference between the data, curves for $CMS\_NH_{2in}\_PEG_{out}$ and $CMS\_NH_{2in}\_PEG\text{-}PpIX_{out}$ were shifted along the y-axis by a value of 25 each.
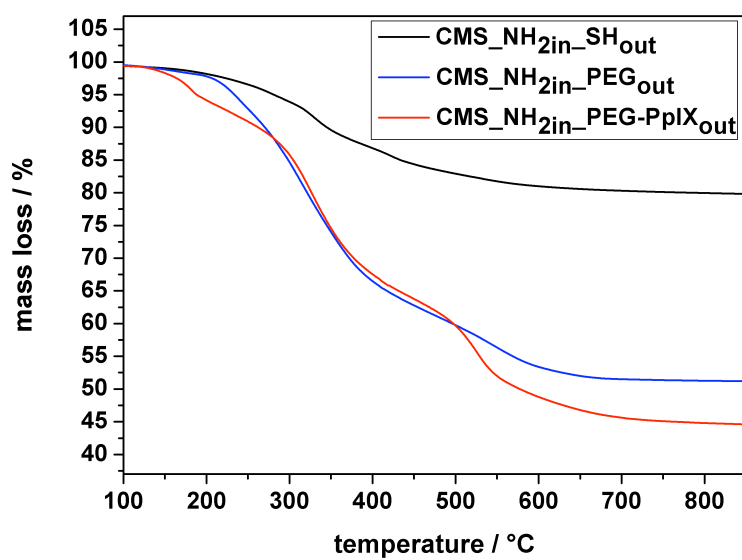
1.9 Supplementary figures

Figure S 2: Thermogravimetric analysis curves for the samples CMS_NH$_{2in}$_SH$_{out}$ (black), CMS_NH$_{2in}$_PEG$_{out}$ (blue) and CMS_NH$_{2in}$_PEG-PpIX$_{out}$ (red).
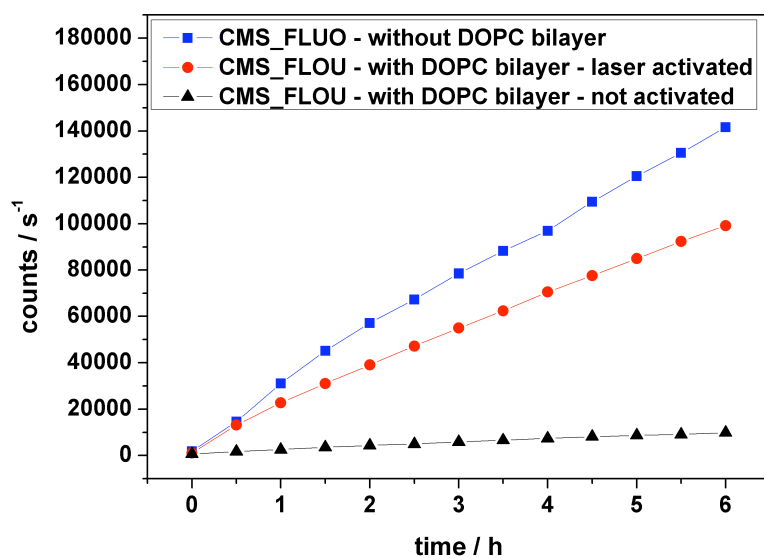
Figure S 3: Fluorescein release curves for the samples CMS_NH$_{2in}$_PEG-PpIX$_{out}$ (blue), laser-activated CMS_NH$_{2in}$_SH$_{out}$_DOPC (red) and tightly capped, non-activated CMS_NH$_{2in}$_SH$_{out}$_DOPC (black).
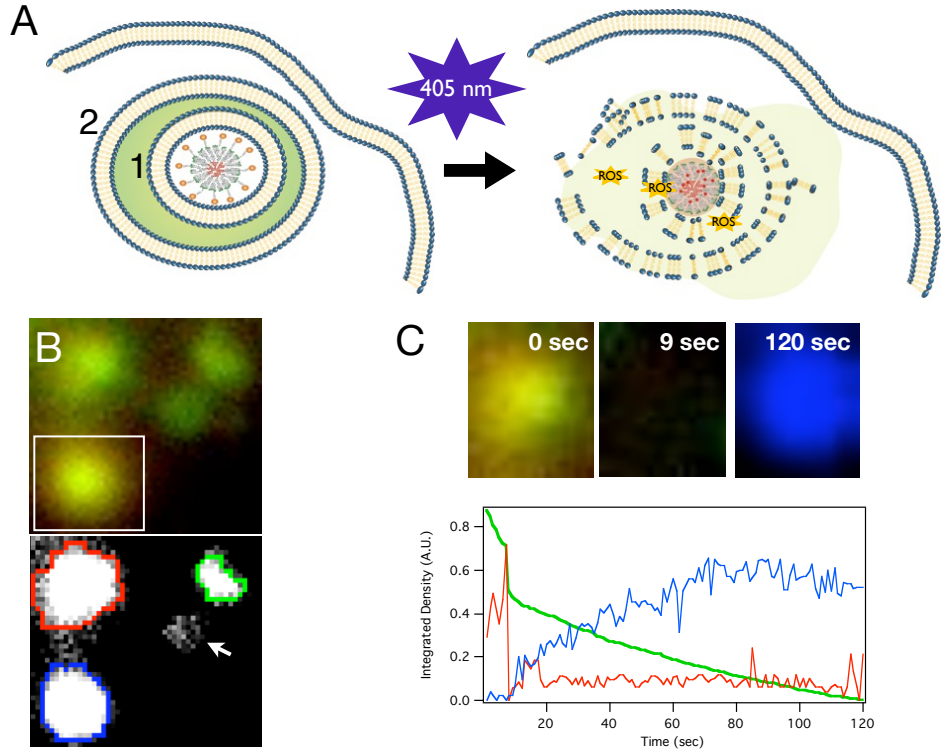
Figure S 4: Single-endosome lysis time evaluation. Individual endosomes (A) are identified using automatic segmentation [13] performed on the green channel, which is able to identify three out of four endosomes; the arrow indicates an endosome that was not properly segmented, and that had to be manually added. Colors represent automatically-defined ROIs, and not RGB channels (B). ATTO633 fluorescence is artificially colored blue. For each endosome, the lysis time is determined through the occurrence of discontinuities in the time course in the red and green channels, as well as an increase in fluorescence in the long red channel (C).

1.10 Supplementary tables

Sample	Activation Time (min)	Mean	Width
DOPC@CMS, 3T3	2	17.4 ± 0.9	17.0 ± 1.5
DOPC@CMS, 3T3	1	23.7 ± 1.8	17.2 ± 2.9
DOPE@CMS, 3T3	2	5.3 ± 0.3	5.2 ± 0.4
DOPE@CMS, 3T3	1	15.4 ± 1.6	24.0 ± 2.7
DOPC@CMS, Renca-LacZ	2	25.9 ± 7.3	39.2 ± 15.6
DOPC@CMS, Renca-LacZ	1	77.7 ± 1.9	13.6 ± 2.96
DOPE@CMS, Renca-LacZ	2	31.07 ± 5.3	28.0 ± 10.1
DOPE@CMS, Renca-LacZ	1	49.8 ± 5.0	35.0 ± 15.2

Table S 1: Selected gauss fit parameters of endosomal escape data for DOPC@CMS and DOPE@CMS NPs in 3T3 and Renca-LacZ cultures

Sample	Mean	Width
DOPC@CMS, 3T3	675.7 ± 0.3	17.2 ± 0.2
DOPC@CMS, Renca-LacZ	644.5 ± 1.7	41.7 ± 2.4
DOPC@CMS, Huh7	673.9 ± 1.2	20.9 ± 1.1
DOPC@CMS, Beas2B	726.9 ± 9.4	132.5 ± 14.3
Latex, 3T3	1314.1 ± 29.9	227.8 ± 43.3
Latex, Renca-LacZ	1603.5 ± 19.3	1065.7 ± 31.6
Latex, Huh7	613.4 ± 69.4	1314.5 ± 102.0

Table S 2: Selected gauss fit parameters of cell-type specific NP uptake profiles at eight hours post-incubation

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