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Supporting information

Polymer Brush Coated Upconverting Nanoparticles with Improved Colloidal Stability and Cellular Labeling

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Content

1. Materials

Poly(ethylene glycol) methyl ether methacrylate (PEO_9MEMA, M_n 500, Sigma-Aldrich, Germany) was purified from inhibitors by passing the monomer through a column filled with basic alumina (type 5016A, Fluka, USA). Methacrylic acid (MAA, Sigma-Aldrich, Germany) was distilled under reduced pressure before use. Methanol (MeOH, 99.8%, Sigma-Aldrich, Germany), ethanol (EtOH, 96%, Sigma-Aldrich, Germany), acetone (99%, Sigma-Aldrich, Germany), diethyl ether (Et_2O, 99%, Sigma-Aldrich, Germany), hexane (>99%, Sigma-Aldrich, Germany), 1,4-dioxane (DO, 99.8%, Sigma-Aldrich, Germany), carbon disulfide (CS_2, 99.9%, Sigma-Aldrich, Germany), 1-butanthiol (99%, Sigma-Aldrich, Germany), sodium hydride (NaH, 60% dispersion in mineral oil, Sigma-Aldrich, Germany), iodine (I_2, 99.8%, Sigma-Aldrich, Germany), and 4,4′-azobis(4-cyanovaleric acid) (ACVA, 98%, Fluka, USA), trifluoroacetic acid (TFA, 99%, Alfa Aesar, USA), Yb_2O_3 (99.99+%, Alfa Aesar, USA), Y_2O_3 (99.99+%, Alfa Aesar, USA), Tm_2O_3 (99.99+%, Alfa Aesar, USA), lithium trifluoroacetate (97%, Alfa Aesar, USA), oleylamine (OAm, 70%, Sigma-Aldrich, Germany), 1-octadecene (ODE, 90%, Alfa Aesar, USA) and oleic acid (OA, 90%, Alfa Aesar, USA) were used as received.

2. Instrumental analysis methods

2.1. X-Ray diffraction (XRD) measurements. The crystal phase of upconverting nanoparticles (UCNPs) was confirmed by X-ray powder diffraction (XRD) analysis carried on Bruker D8 Advance (Bruker, Germany) Diffractometer using CuKα radiation.

2.2. Transmission electron microscope (TEM) measurements. Transmission electron microscopy (TEM, Philips Tecnai 12) images were used to determine particle size distribution and morphology of UCNPs.

2.3. Size exclusion chromatography (SEC) measurements. The macromolecular parameters of the synthesized (co)polymers such as number average and weight average molecular weights (M_n and M_w), and dispersity ($Đ = M_w/M_n$) were determined by size exclusion chromatography (SEC). Viscotek TDAmax (Malvern, UK) system equipped with a triple detection array (TDA305), consisting of a differential refractive index detector (RI), light scattering detector (LS) simultaneously measuring the scattered light (laser 3 mW, 670 nm) at two angles – right-angle (90°), and low-angle (7°), and four-capillary bridge viscosity detector (IV), was used. Viscotek (Malvern, UK) columns AGuard (50 × 8.0 mm) and A6000M General Mixed Aq (300 × 8.0 mm), filled with porous polyhydroxymethacrylate, particle size 13 μm, nominal pore size $1.5 \cdot 10^4$ Å, exclusion limit M_w (for Pollulan) < $2 \cdot 10^7$ g/mol, were employed for the separation of hydrophilic polymeric samples. A 350 mM sodium phosphate buffer (pH 9.0) was used as an eluent for analysis at a constant flow rate (0.5 mL/min). The temperature of the column oven and detectors was maintained at 30.0 °C. The diluted polymers solutions with concentration of about 5 mg/mL were prepared by dissolving in SEC eluent and filtered using 0.22 μm polyethersulphone (PES) syringe filters and, subsequently, 100 μL of each sample was injected into SEC. SEC

measurements of polymer samples were triplicated. SEC data were collected and processed using OmniSEC software (Malvern, UK, v. 5.12).

Normalization of the constant values of the SEC detectors (RI, LS, IV) was performed using PEO standards for triple calibration PolyCAL™ TDS-PEO-N (M_w 24 kDa, Malvern, UK) at a concentration of 2.468 mg/mL.

2.4. Nuclear magnetic resonance (NMR) measurements. The NMR experiments were conducted at 22 °C temperature using a Bruker Ascend™ 400 MHz spectrometer (Bruker, Germany). ^{1}H NMR measurements were performed employing a 90° single-pulse sequence for 128 scans with a 5 s recycle delay. ^{1}H NMR spectra of the p(MAA-*stat*-PEO$_9$MEMA) copolymers were recorded in DMSO-d$_6$. The concentration of the samples was 20 mg/mL.

2.5. FTIR measurements. FTIR measurements were performed using Frontier FT-IR spectrometer (Perkin Elmer). Measurement range and resolution were 550-4000 cm^{-1} and 0.5 cm^{-1}, respectively.

2.6. DLS and zeta potential measurements. The hydrodynamic size distributions of UCNPs were measured using the dynamic light scattering (DLS) method. DLS measurements were carried out on a ZetaSizer Nano ZS (Malvern, UK), equipped with a 4 mW He–Ne laser emitting at a wavelength of 633 nm. Measurements were performed at 25 °C and at an angle of 173° using noninvasive backscattering (NIBS) technology. Using NIBS, the particle size detection range was 0.3 nm – 10 μm. The zeta potential values were calculated from the electrophoretic mobility using the Smoluchowski model at 25 °C. The size and zeta potential distribution data were analyzed using ZetaSizer software from Malvern.

2.7. Photoluminescence measurements. The upconversion (UC) luminescence spectra were recorded using the Edinburgh Instruments FLS980 (Edinburgh Instruments, UK) spectrometer, equipped with a double emission monochromator, a cooled (−20 °C) single-photon counting photomultiplier (Hamamatsu R928), and a 1 W continuous wavelength 980 nm laser diode. The emission slit was set to 0.5 nm, the step size was 0.5 nm, and the integration time was 0.2 s with repeat of 10 times. The emission spectra were corrected by a correction file obtained from a tungsten incandescent lamp certified by National Physics Laboratory, UK.

3. Synthesis and investigation of ligand-free $LiYF_4$:Tm^{3+}, Yb^{3+} upconverting nanoparticles (UCNPs)

3.1. Preparation of precursors. Yb_2O_3 (0.3125 mmol), Y_2O_3 (0.93125 mmol) and Tm_2O_3 (0.00625 mmol) were mixed with 5 mL TFA and 5 mL of distilled water in a 100 mL three-neck round bottom flask. The mixture was refluxed under vigorous stirring at 80 °C until a previously turbid solution became clear, resulting in rare-earth (Yb^{3+}, Y^{3+} and Tm^{3+}) trifluoroacetate precursors[1,2]. Subsequently, the water and excess of TFA were slowly evaporated at to 60 °C temperature. All precursors were obtained as solid dried materials and were used for the UCNP synthesis without further purification.

3.2. Synthesis procedure of $LiYF_4:Tm^{3+}$, Yb^{3+} UCNPs. Lithium trifluoroacetate (2.5 mmol), 5 mL oleylamine, 20 mL 1-octadecene, and 15 mL oleic acid were mixed together with stoichiometric amounts of dried rare-earth trifluoroacetate precursors. The obtained solution was degassed for 30 min under vacuum at 110 °C with magnetic stirring. Then, the reaction mixture was heated to 330 °C under stirring and a gentle argon flow, and maintained at this temperature for 1 h. Afterwards, the solution was cooled down to room temperature, maintaining the Ar atmosphere and magnetic stirring. The oleate-capped UCNPs were precipitated by addition of ethanol and collected by centrifugation at 7000 RPM for 15 min. A mixture of hexane/ethanol (1/4 v/v) was used to wash the obtained UCNPs twice following the precipitation via centrifugation. Finally, the oleate-capped UCNPs were re-dispersed in hexane for storage and physical characterization.

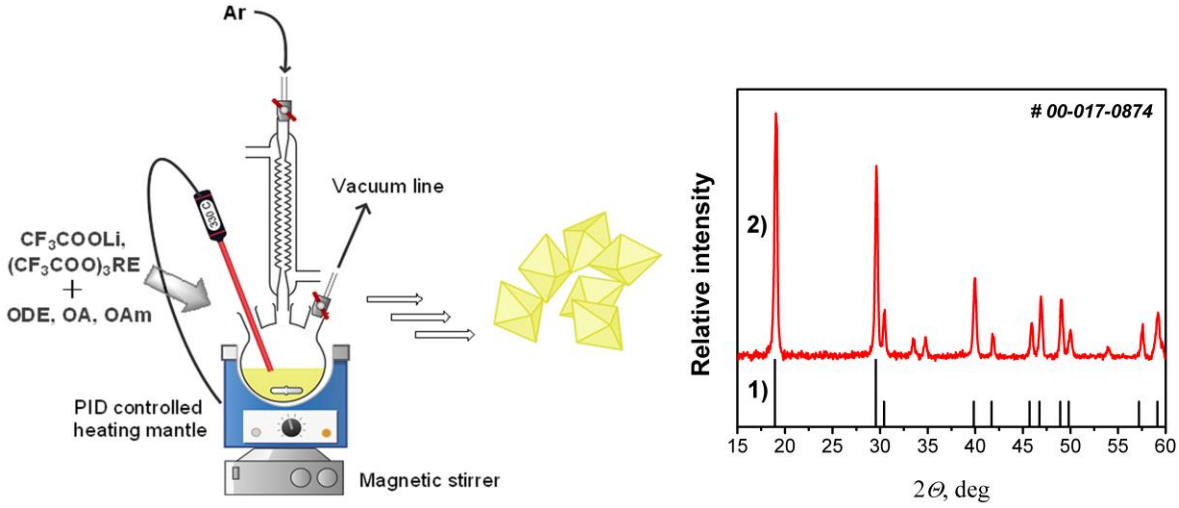

Figure S1. Graphical illustration of UCNPs synthesis and XRD patterns of the obtained $LiYF_4:Tm^{3+}$, Yb^{3+} UCNPs (2) and $LiYF_4$ reference pattern (1).

Graphical illustration of UCNPs synthesis procedure as well as XRD patterns of $LiYF_4:Tm^{3+}$, Yb^{3+} are presented in Figure S1. The X-ray diffraction analysis of the as-synthesized UCNPs indicated that samples are of tetragonal scheelite crystal structure (space group $I4_1/a$ (#88) (PDF ICDD 00-017-0874). No additional peaks from impurity phases were detected in XRD pattern.

3.3. Ligand removal from the $LiYF_4:Tm^{3+}$, Yb^{3+} UCNPs. 50 mg of oleate-capped UCNPs, dispersed in 25 mL of hexane, were mixed together with 25 mL of distilled water (pH 4, adjusted with HCl) and vigorously stirred for 3 h. The aqueous phase containing the UCNPs was isolated from the two phase (aqueous/organic) mixture with a separatory funnel. The UCNPs were precipitated with acetone (1/3 v/v) and collected via centrifugation at 7500 RPM for 30 min. The obtained UCNPs were re-dispersed in 25 mL of distilled water (pH 4) and left under stirring for additional 2 h. Then, UCNPs were centrifuged at 7500 RPM for 30 min with acetone, redispersed in distilled water, and washed twice with a mixture of water/acetone (1/3 v/v). Finally, the ligand-free NPs were re-dispersed in 5 mL of distilled water and stored at 4 °C for further experiments.

3.4. Coating of ligand-free $LiYF_4:Tm^{3+}$, Yb^{3+} UCNPs with brush-type polyelectrolytes. 15 mg of ligand-free UCNPs were fully re-dispersed (under ultrasonic treatment) in 4 mL of slightly acidic aqueous solution (pH 5.0), then 1 mL of aqueous polymer solution (pH 5.0) with concentration of 1.5 mg/mL was added and the obtained mixture was stirred for an additional hour at room temperature.

After treatment of UCNPs with copolymers, the excess of polymer in aqueous solution was removed by two cycles of centrifugation (1000 rpm/15 min) and washing with deionized water. The particles from the last washing cycle were again redispersed in deionized water, freeze-dried and stored in a freezer until further use.

4. Synthesis of anionic brush-type p(MAA-*co*-PEO_9MEMA) polyelectrolytes with different composition

4.1. Synthesis procedure. The p(MMA-*co*-PEO_9MEMA) with two different compositions were synthesized by variation of molar monomer $[MAA]_0$:$[PEO_9MEMA]_0$ ratio in the initial feed, 3:1 and 1:3, respectively. The detailed synthesis description of copolymer with relatively low content of MAA ($[MAA]_0$:$[PEO_9MEMA]_0$ = 1:3) is presented below.

The metacrylic acid (0.301 g, 3.5 mmol), PEO_9MEMA (4.988 g, 10.5 mmol), ACPA (0.0131 g, 0.0467 mmol), CTA (0.0407 g, 0.14 mmol) and 1.4-dioxane was poured into 50 mL round-bottom flask with magnetic stirring. The overall monomer concentration was 15 wt. %. The flask was sealed, purged with N_2 for 20 min and placed into 70 °C thermostat to stir for 24 h. Then the reaction mixture was cooled down to 0 °C temperature and the synthesized copolymer was purified using 3.5 MWCO dialysis tubes. Aqueous p(MAA-*co*-PEO_9MEMA) solution was concentrated using rotary evaporator and separated by freeze-drying, resulting in 4.56 g of viscous yellow oil (86% yield).

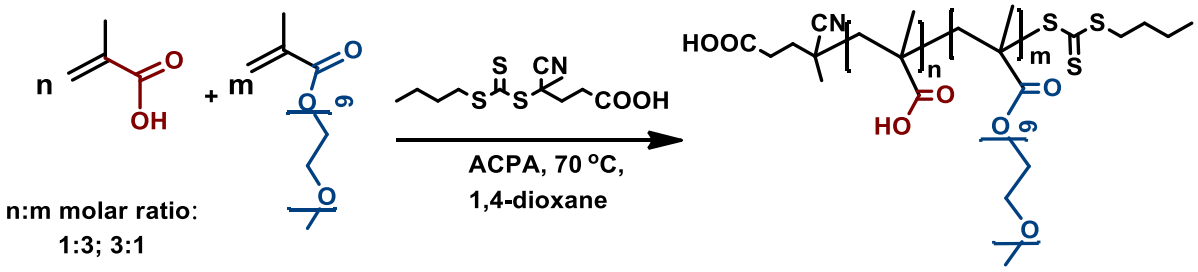

Figure S2. Synthesis scheme of anionic brush-type p(MAA-*co*-PEO_9MEMA) copolymers via RAFT method. The n and m represent the molar ratio of monomer in initial polymerization mixture.

4.2. NMR data and calculation of the exact composition. The exact composition of synthesized polymers was calculated from the recorded ^1H NMR spectra (Figure S3) using eq. S1:

$$F_1 = \frac{\int 4.03}{\int 4.03 + 2 \cdot \int 12.33} \qquad \text{(S1)};$$

where F_1 is the molar fraction of the units of the macromonomer PEO_9MEMA in a copolymer; chemical shifts at 4.03 and 12.33 ppm in ^1H NMR spectra are assigned to oxymethylene group signals of the PEO_9MEMA (2*H*) and -COOH proton (*1H*) of MAA, respectively; and ∫ are integrals of these signals.

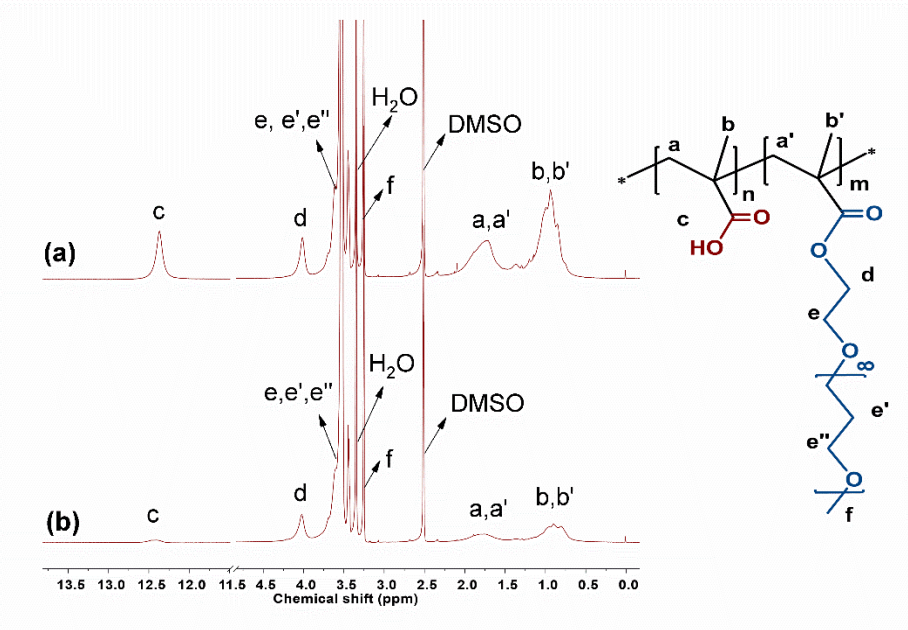

Figure S3. ^1H NMR of p(MAA-*co*-PEO_9MEMA) with two compositions (in DMSO-d^6): consisted of 26.7 mol % (a) and 72.2 mol % (b) of MAA units in composition, respectively.

4.3. Calculation of degree of polymerization. Degree of polymerization (DP) is calculated from molecular weight by eq. S2:

$$DP = \frac{M_n - M_{CTA}}{(M_{PEO_9MEMA} \cdot x + M_{MAA} \cdot y)} \qquad \text{(S2)};$$

where DP is the degree of polymerization; M_n, M_{CTA}, M_{PEO_9MEMA} and M_{MAA} are molecular weights of (co)polymer, RAFT chain transfer agent, PEO_9MEMA and MAA monomers, respectively. The x and y, represent the molar part of PEO_9MEMA and MAA monomeric units in (co)polymer composition.

5. Colloidal stability evaluation of UCNPs

Colloidal stability of UCNPs in distilled water, cell culture growth medium (Dulbecco's Modified Eagle Medium (DMEM), Gibco, Waltham, MA, USA) and DMEM supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Gibco, Waltham, MA, USA) were measured for bare (ligand-free), P_9MAA-25 and P_9MAA-75 coated UCNPs. The concentration of UCNPs in different media was 0.1 mg/mL. Short-time (up to 6 hours) stability of UCNPs in different media was evaluated by measuring UC emission intensity (Tm^{3+} emission $^3H_4 \rightarrow {}^3H_6$, 793 nm) as a function of time. Colloidal solutions of UCNPs in different media were first ultrasonicated for 30 s and then transferred to the standard 10 mm light-path quartz spectrofluorometric cuvettes. Cuvettes were placed in a thermostated holder (25 °C) in order to minimize the heating of the solution due to prolonged laser irradiation. In order to evaluate long-term stability, the PL measurements after 1, 2, 3 and 7 days after dilution were performed as well. All measurements were performed as triplicates n=3. Error bars in Fig. 4 a-c of the main text indicates standard deviation.

6. Cultivation of cells. MDA-MB-231 and MCF-7 human adenocarcinoma cell lines were purchased from the American Type Culture Collection and the European Collection of Cell Cultures, respectively, and cultivated for in vitro experiments. For cell culturing DMEM cell growth medium (DMEM, Gibco, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Waltham, MA, USA), 100 U/mL penicillin, 100 mg/mL streptomycin (Biochrom, Berlin, Germany) was used. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

7. Cellular metabolic activity measurements by XTT method. XTT is a method to analyze cellular metabolic activity as a function of redox potential. Viable cells convert XTT to a water-soluble orange colored formazan which is directly quantified with the use of spectrophotometer. XTT was performed according to manufacturer's protocol. For each type of UCNPs 6 wells were used. 6 wells were also left as a control measurement without UCNPs as blank absorbance readings. Optical density at 490 nm was assessed using BioTek 800 TS microplate reader after the incubation with tetrazolium dye was finished. All measurements performed with n=6. Statistical significance of differences between groups was assessed by a two-tailed independent Student's t-test at the 95% confidence level. Significance represented as p-value < 0.05. Error bars in Fig. 4 d (main text) show standard deviation.

8. In vitro imaging of UCNPs in cancer cells. For in vitro imaging, cells were seeded into 8-well chamber slide with removable wells (Lab-Tek, Nunc, Thermo Fisher, Denmark) with a density of $3 \cdot 10^4$ cells per chamber and maintained at 37 °C in a humidified atmosphere containing 5% of CO_2 for 24 hours. Cells were treated with 0.1 mg/mL of UCNPs (for each surface modification) for 24 hours to evaluate UCNPs' uptake dynamics and intracellular localization. Before imaging, cells were covered and fixed with sufficient amount of 4% paraformaldehyde (Sigma-Aldrich, Germany) for 15 min. Nucleus of the cell was stained with 10 μg/mL of Hoechst 33258 (Sigma-Aldrich, Germany) and actin filaments were stained with 165 mM Alexa Fluor 488 phalloidin

(Invitrogen, Thermo Fisher, US). Accumulation of UCNPs in cells was observed with a confocal Nikon Eclipse Te2000-S C1 Plus Laser scanning microscope equipped with continuous wave lasers: 405 nm (Melles Griot, USA), 488 nm (Melles Griot, USA) and 980 nm (Changchun New Industries Optoelectronics Tech. Co., Ltd., China). Both cellular dyes were excited by 404 nm (Hoechst) and 488 nm (Alexa Fluor 488 phalloidin) excitation wavelengths, UCNPs were excited at 980 nm. Emission of nuclear dye was registered in blue (450/17 band pass filter) and actin filaments dye in green (545/45) channel. Emission of UCNPs was registered in blue channel (450/17), although in images red pseudo color was used for better visualization. Cellular imaging was performed using 60 x/1.4 NA oil immersion objective (Nikon, Japan). All images were acquired with the same imaging parameters. 3D cellular imaging was obtained with 1 μm step size. Images were processed with Nikon EZ-C1 Bronze version 3.80 and ImageJ 1.46 software.

9. Evaluation of UCNPs localization in cells

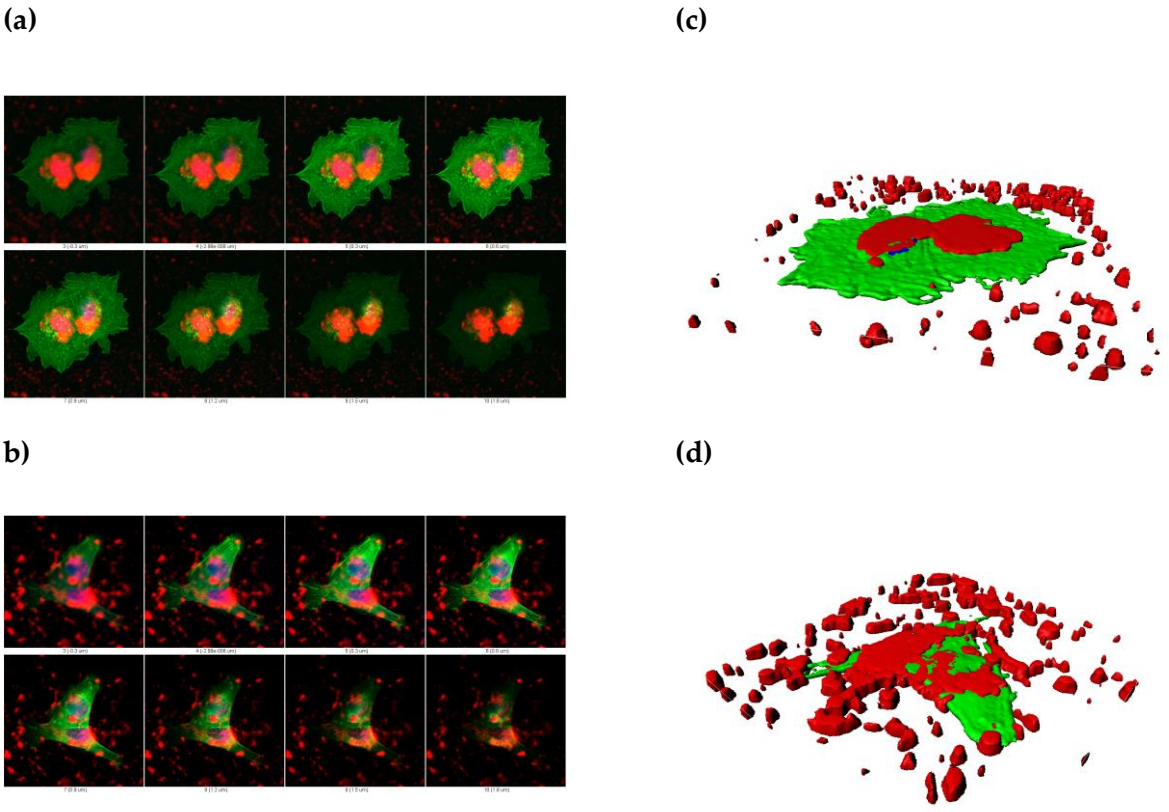

Figure S4. Representative Z-stack confocal microscopy images of **bare UCNPs** in MCF-7 (a) and MDA-MB-231 (b) breast cancer cells at diferent focal planes. Z-stack images were collected at 0.3 μm steps by confocal laser scanning microscopy. Cells were incubated with UCNPs in DMEM **without (w/o) FBS**. UC emission signal, obtained under 980 nm excitation, is represented by the red color. Cell nuclei were stained with Hoechst (blue) (λ_{ex} = 404 nm) and F-actin was stained with Phalloidin-Alexa 488 (green) (λ_{ex} = 488 nm). Images (c) and (d) represents 3D reconstruction of MCF-7 and MDA-MB-231 cells.

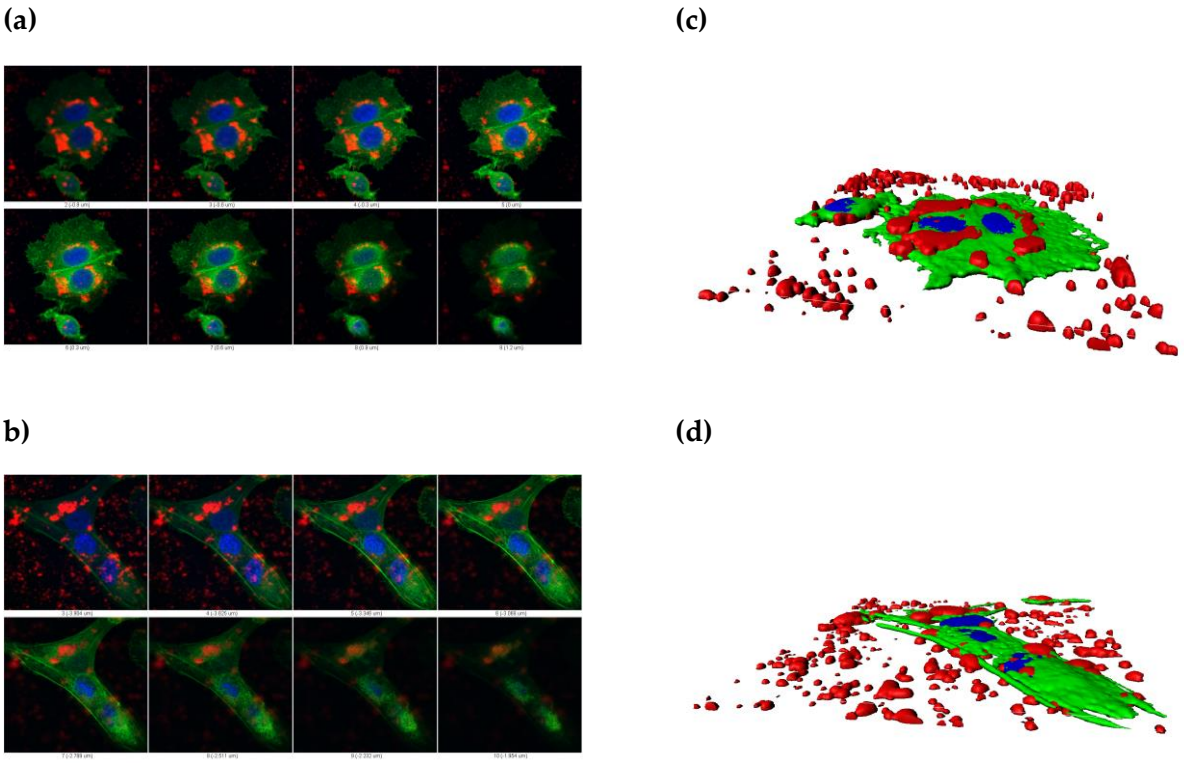

Figure S5. Representative Z-stack confocal microscopy images of **bare UCNPs** in MCF-7 (a) and MDA-MB-231 (b) breast cancer cells at diferent focal planes. Z-stack images were collected at 0.3 μm steps by confocal laser scanning microscopy. Cells were incubated with UCNPs in DMEM **with (w/) FBS**. UC emission signal, obtained under 980 nm excitation, is represented by the red color. Cell nuclei were stained with Hoechst (blue) (λ_{ex} = 404 nm) and F-actin was stained with Phalloidin-Alexa 488 (green) (λ_{ex} = 488 nm). Images (c) and (d) represents 3D reconstruction of MCF-7 and MDA-MB-231 cells.

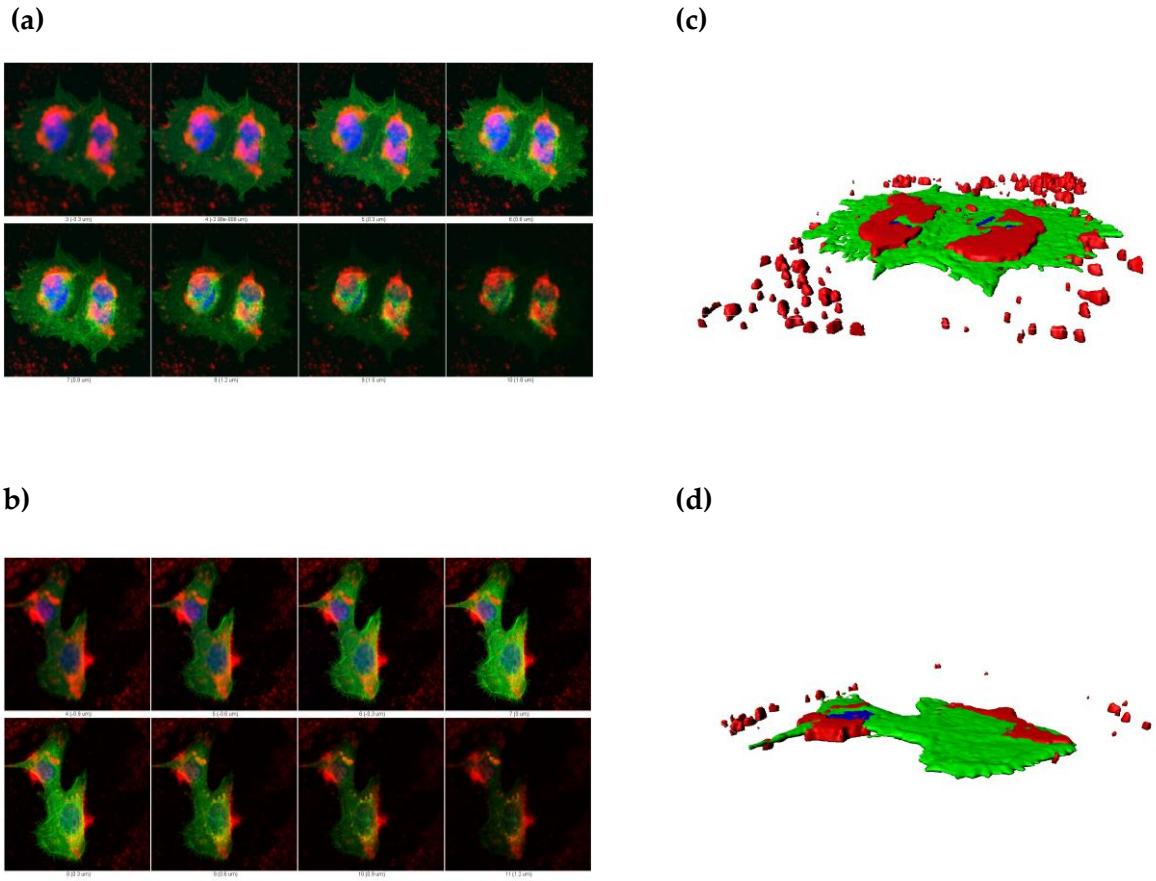

Figure S6. Representative Z-stack confocal microscopy images of **UCNPs modified with P$_9$MAA-25** in MCF-7 (a) and MDA-MB-231 (b) breast cancer cells at diferent focal planes. Z-stack images were collected at 0.3 μm steps by confocal laser scanning microscopy Cells were incubated with UCNPs in DMEM **without (w/o) FBS**. UC emission signal, obtained under 980 nm excitation, is represented by the red color. Cell nuclei were stained with Hoechst (blue) (λ_{ex} = 404 nm) and F-actin was stained with Phalloidin-Alexa 488 (green) (λ_{ex} = 488 nm). Images (c) and (d) represents 3D reconstruction of MCF-7 and MDA-MB-231 cells.

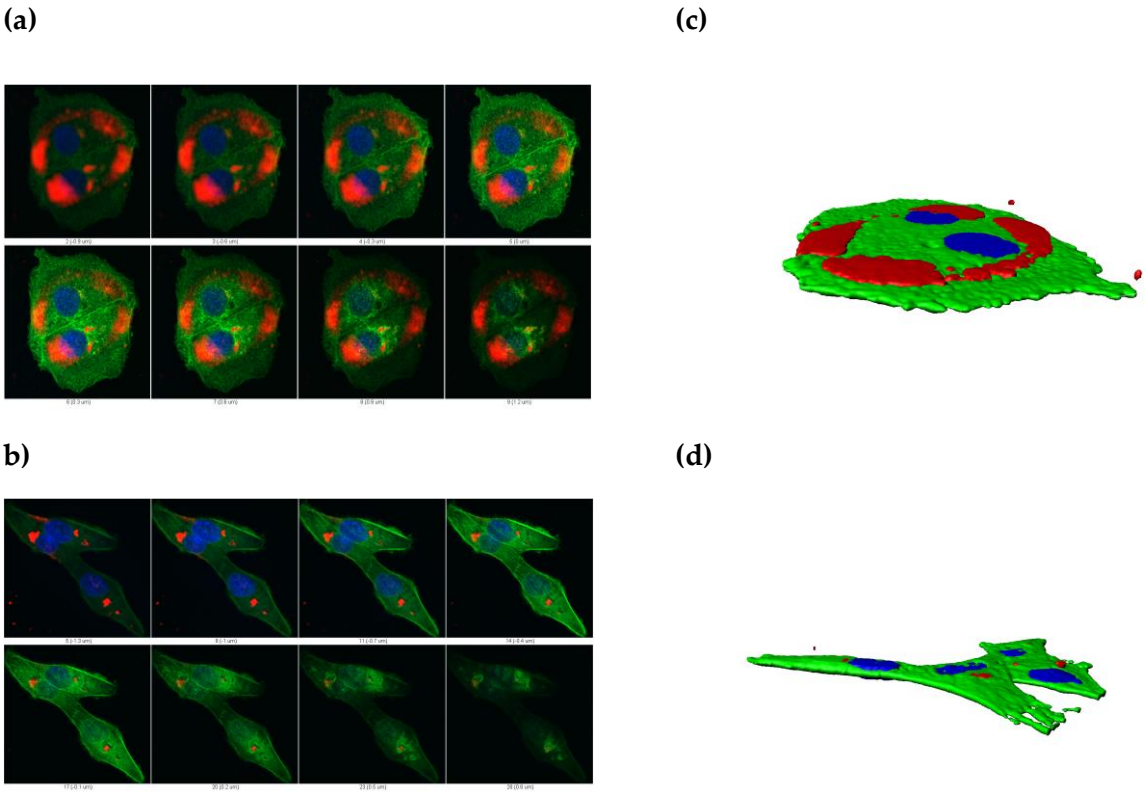

Figure S7. Representative Z-stack confocal microscopy images of **UCNPs modified with P$_9$MAA-25** in MCF-7 (a) and MDA-MB-231 (b) breast cancer cells at diferent focal planes. Z-stack images were collected at 0.3 μm steps by confocal laser scanning microscopy. Cells were incubated with UCNPs in DMEM **with (w/) FBS**. UC emission signal, obtained under 980 nm excitation, is represented by the red color. Cell nuclei were stained with Hoechst (blue) (λ_{ex} = 404 nm) and F-actin was stained with Phalloidin-Alexa 488 (green) (λ_{ex} = 488 nm). Images (c) and (d) represents 3D reconstruction of MCF-7 and MDA-MB-231 cells.

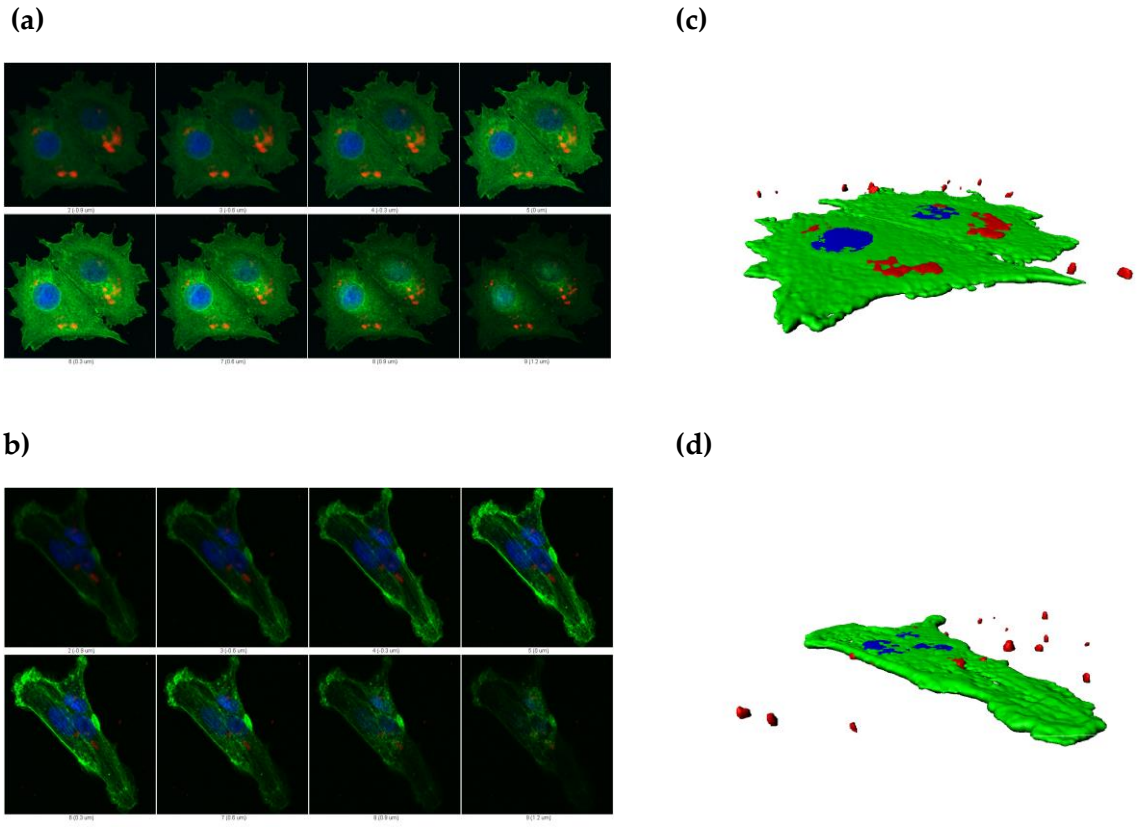

Figure S8. Representative Z-stack confocal microscopy images of **UCNPs modified with P_9MAA-75** in MCF-7 (a) and MDA-MB-231 (b) breast cancer cells at diferent focal planes. Z-stack images were collected at 0.3 μm steps by confocal laser scanning microscopy. Cells were incubated with UCNPs in DMEM **without (w/o) FBS**. UC emission signal, obtained under 980 nm excitation, is represented by the red color. Cell nuclei were stained with Hoechst (blue) (λ_{ex} = 404 nm) and F-actin was stained with Phalloidin-Alexa 488 (green) (λ_{ex} = 488 nm). Images (c) and (d) represents 3D reconstruction of MCF-7 and MDA-MB-231 cells.

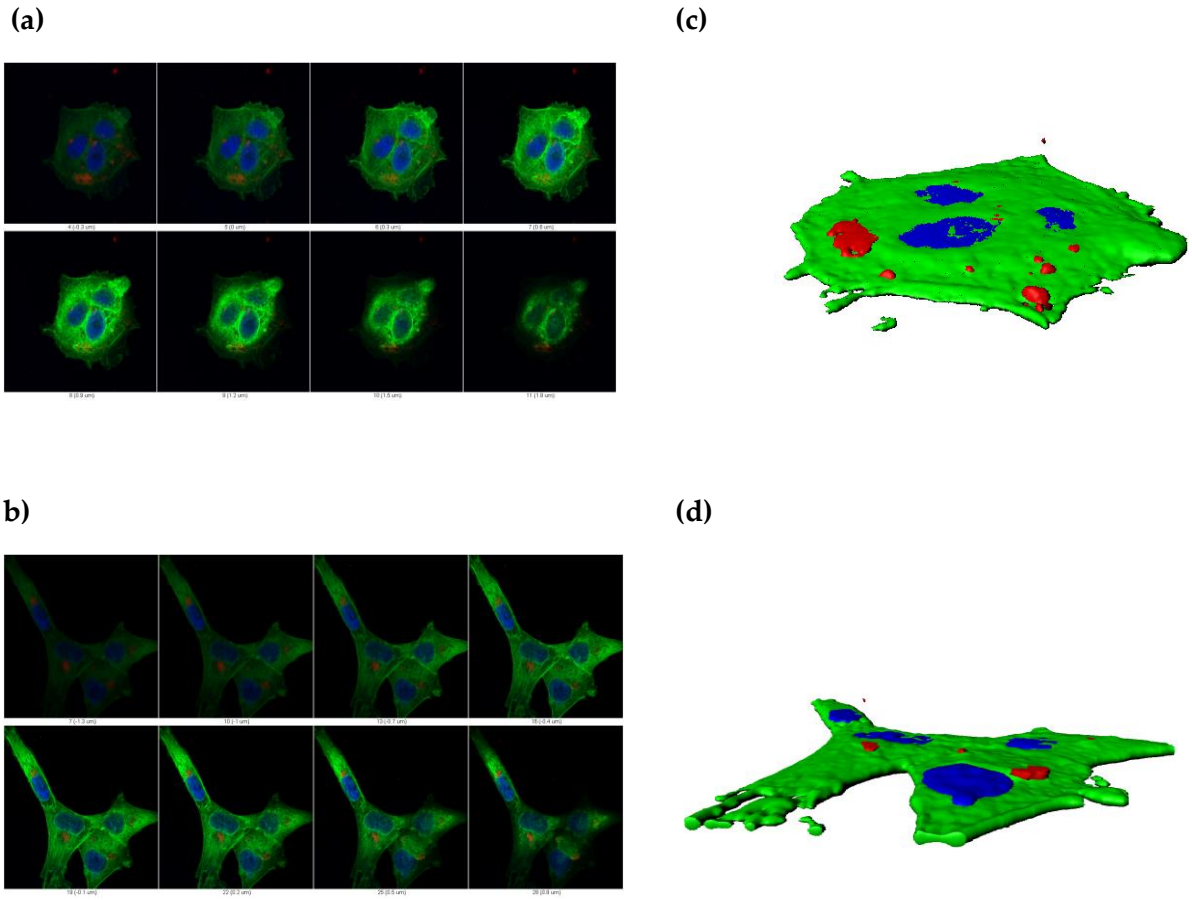

Figure S9. Representative Z-stack confocal microscopy images of **UCNPs modified with P$_9$MAA-75** in MCF-7 (a) and MDA-MB-231 (b) breast cancer cells at diferent focal planes. Z-stack images were collected at 0.3 μm steps by confocal laser scanning microscopy. Cells were incubated with UCNPs in DMEM **with (w/) FBS**. UC emission signal, obtained under 980 nm excitation, is represented by the red color. Cell nuclei were stained with Hoechst (blue) (λ_{ex} = 404 nm) and F-actin was stained with Phalloidin-Alexa 488 (green) (λ_{ex} = 488 nm). Images (c) and (d) represents 3D reconstruction of MCF-7 and MDA-MB-231 cells.

10. Intracellular localization of UCNPs using Lysotracker

MCF-7 and MDA-MB-231 cells in complete DMEM medium were treated with 0.1 mg/mL UCNPs for 24 h and for 2h with 75 nM of lysosome dye (LysoTracker Deep Red, Invitrogen, USA). Confocal fluorescence images were obtained separately in each fluorescence channel and composed to one image afterwards. Pearson's correlation coefficient (PCC) was calculated from 5 images for each sample.

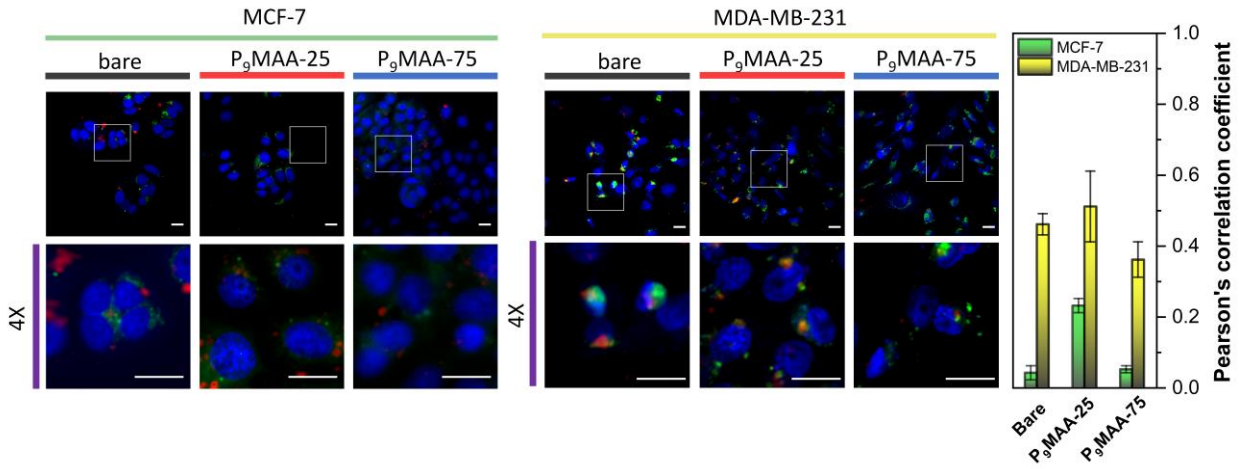

Figure S10. Confocal fluorescence images of UCNPs' localization in MCF-7 and MDA-MB-231 cells after 24 hours of incubation in DMEM supplemented with FBS Fluorescence of Lysotracker in images is shown in green color. Red color codes emission of UCNPs inside the cells, blue – nucleus of the cells. Cell images are shown in a wide-field and 4x zoom view. Scale bars in all images are 20 μm. Analysis of Pearson's correlation coefficient of colocalization between bare, P9MAA-25 or P9MAA-75 coated UCNPs and lysosomes is presented on the right hand side of the figure.

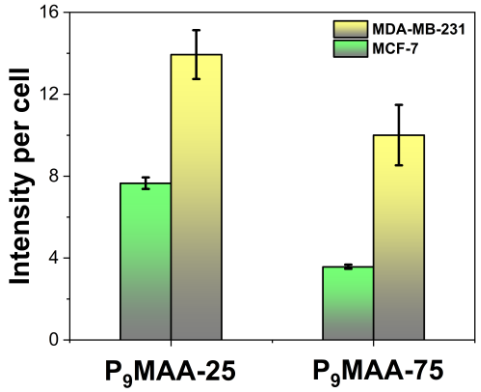

Figure S11. Accumulation of UCNPs coated with different polymers in cells after 24 h incubation in full media, assessed by the average upconversion emission intensity per cell.

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