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Methods and electronic supplementary information

Experimental Section

Nanoparticle synthesis and characterization

Materials

All chemicals were used without purification except for styrene, which was passed through a basic aluminum oxide before use. Ammonium hydroxide (28% solution in water, VWR), 2,2⁻ azobis(2-methylbutyronitrile) (V-59, Wako), ferric chloride hexahydrate (FeCl₃·6H₂O, 99%, Acros), ferrous chloride tetrahydrate (FeCl₂·4H₂O, 99%, Merck), *N*-(2,6-diisopropylphenyl)-perylene-3,4-dicarbonacid-imide (PMI, BASF), *n*-hexadecane (99%, Fisher), *n*-octane (99%, Aldrich), oleic acid (58%, Riedel-de Haen), poly-L-lactide (PLLA, Biomer[®]L9000, $M_w = 105,000 \text{ g} \cdot \text{mol}^{-1}$), potassium persulfate (KPS, 99%, Aldrich), sodium *n*-dodecyl sulfate (SDS, 99%, Merck), sodium *p*-styrenesulfonate, (>90%, Aldrich), styrene (>99%, Aldrich). BODIPY dye was synthesized as described elsewhere^[1].

Preparation of oleate-capped iron oxide nanoparticles

The preparation of oleate-capped iron oxide nanoparticles was performed similar to a procedure described in literature.^[2] Briefly, 24.4 g (90 mmol) ferric chloride and 12.0 g (60 mmol) ferrous chloride were dissolved in 100 ml deionized water. 40 ml of a 28% aqueous ammonium hydroxide solution and 4.0 g (14.2 mmol) of oleic acid were added. The reaction mixture was heated to 70 °C for 1 h followed by a further heating to 100 °C at which temperature the mixture was stirred for additional 2 h under constant refilling of evaporating water. The resulting black precipitate was purified several times by magnetic separation and rinsing with deionized water and finally dried under vacuum overnight.

Preparation of dye labeled polystyrene nanoparticles with/without iron oxide nanoparticles

The preparation of the particles was done similar to a recipe described in literature.^[2] Therefore, 1 g of the as synthesized oleate-capped iron oxide nanoparticles was redispersed in 0.5 g *n*octane and 1 mg (2.1 µmol) of BODIPY added. To help redispersion and dissolving, the mixture was sonified for 45 min. In a separate vial, 25 mg of sodium dodecyl sulfate were dissolved in 24 g deionized water, combined with the disperse *n*-octane phase and sonified with a tip sonifier for 3 min (70% amplitude, 10 s pulse, 5 s pause; Branson sonifier 450; Branson, U.S.A.) under ice cooling. For a second emulsion, *n*-hexadecane (20 mg; 0.088 mmol) and styrene (1 g; 9.6 mmol) were mixed with a 0.04_{wt} % aqueous SDS solution. The two phase system was sonified with a tip sonifier for 1 min (10% amplitude, 5 s pulse, 5 s pause) under ice cooling and added to the magnetic miniemulsion. Nitrogen was bubbled through the combined dispersions for 5 min, KPS (25 mg; 0.092 mmol) and sodium styrenesulfonate (30 mg; 0.145 mmol) added and the reaction mixture heated to 80 °C under stirring and kept at this temperature for 6 h. Purification of the magnetic polystyrene particles was performed magnetically.

Polystyrene nanoparticles without iron oxide were synthesized as described here. 6 g of styrene were mixed with 250 mg n-hexadecane, 100 mg V-59 and 2 mg of PMI or BODIPY dye. A solution of 72 mg SDS in 24 g of demineralized water was added and the mixture stirred for 1 h at 1400 rpm followed by sonication for 2 min under ice cooling (½ inch tip, 90%, Branson sonifier W450 Digital). The formed miniemulsion was stirred at 72 °C overnight. Aggregates were removed by centrifugation and excess surfactant by dialysis (MWCO 100,000 membrane).

Preparation of dye labeled PLLA nanoparticles with and without iron oxide

A recipe similar to Urban et al. was used to synthesize iron oxide/PLLA nanoparticles.^[3] Briefly, 300 mg PLLA and 2 mg of PMI were dissolved in 3 g of chloroform. Separately, 150 mg of the oleate-capped iron oxide nanoparticles were dispersed in 8 g chloroform by ultrasonication for 30 min using an ultrasonication bath, mixed with the PLLA solution and sonicated for further 10 min. A solution of 72 mg SDS in 24 g demineralized water were added and the two phase system pre-emulsified for 1 h at 1400 rpm, followed by sonication for 3 min under ice cooling (70%, 10 s pulse, 5 s pause) using a Branson sonifier W450 Digital with ½ inch tip. The resulting miniemulsion was stirred at 300 rpm for 16 h at 40 °C in an open 50 mL flask. Purification from aggregates was done by centrifugation. To remove excess stabilizer, the

dispersion was dialysed (MWCO 14,000 membrane). For the synthesis of dye labeled PLLA particles without iron oxide, the same procedure was followed without the use of iron oxide.

Analytical methods

Determination of the particle diameter and the standard deviation was done using a NICOMP zetasizer (Agilent Technologies, U.S.A.). The measurement was conducted at 25 °C in a diluted aqueous dispersion at an angle of 90°. Zeta potential measurements were performed with a Malvern Instruments Zeta Nanosizer at a detection angle of 173° in a 10⁻³ M KCl sample dispersion. TEM was performed using a Zeiss EM912 at a working voltage of 80 kV. The sample dispersion was diluted with demineralized water and drop-casted on a 400-mesh carbon-coated copper grid. Thermogravimetric analysis measurements were done with a thermobalance Mettler Toledo TGA/SDTA 851. All measurements were performed at a heating rate of 10 K·min⁻¹ from room temperature to 700°C under nitrogen atmosphere. For molecular mass determination, a PSS SecCurity (Agilent Technologies 1260 Infinity) was used. The dye labeled polymer was freeze dried after synthesis and dissolved in DMF. Size exclusion chromatography was run at a flow rate of 1 mL·min⁻¹ and a column temperature of 30°C. Calibration was done with polystyrene standards purchased from Polymer Standard Service GmbH (PSS). As detectors, a UV S3702 (at 520 nm) and a shodex RI 101 detector were used simultaneously. Hysteresis measurements were performed on a vibrating sample magnetometer. Samples were filled into a gelatine capsules and mounted in a low magnetic moment sample holder.

Cell culture, immunofluorescence and confocal laser scanning microscopy

The experiments were performed with HeLa and Jurkat cells. HeLa cells (DSMZ, Germany) were cultivated in DMEM (Life Technologies, U.S.A.) supplemented with 10% fetal calf serum (Invitrogen, U.S.A.), 100 units penicillin, 100 μ g ml⁻¹ streptomycin and 1 mM pyruvate (all Life technologies) in a humidified incubator with 5% CO₂ at 37°C (Labotec, Germany). Jurkat cells were cultivated in RPMI-1640 supplemented with 10% fetal calf serum (Invitrogen, U.S.A.), 100 units penicillin, 100 μ g ml⁻¹ streptomycin and 1 mM pyruvate. Nanoparticles were added in a concentration of 150 μ g ml⁻¹ for the indicated time. Nanoparticle supernatant was generated by centrifuging 150 μ g ml⁻¹ nanoparticles in medium for 30 min at 20 000 x g. For the detection of lipid droplets, we used guinea pig α -TIP47 (Progen, Germany). The mouse monoclonal α -ADRP

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was obtained from Fitzgerald (U. S. A.). For immunostainings, cells were seeded in Ibidi iTreat u-dishes at 15 000 cells cm⁻². After particle incubation, cells were fixed with 4% paraformaldehyde and 0.025% glutaraldehyde for 20 min (both Sigma Aldrich, U.S.A.). Cell permeabilization was conducted with 0.1% saponin (Sigma Aldrich, U.S.A.) for 10 min at RT. Blocking was performed by the incubation of 3% BSA for 10 min at 37°C. The primary antibodies were incubated for 60 min in 1% BSA at 37 °C. After washing, the respective secondary antibody was applied for 30 min in 1% BSA at 37°C. Subsequently after washing, cells were imaged by confocal laser scanning microscopy. Antibody stained cells were analyzed using a commercial setup (LSM SP5 STED Leica Laser Scanning Confocal microscope, Leica Microsystems, Germany) consisting of an inverse fluorescence microscope DMI 6000 CS equipped with a laser combination and with five detectors operating in a range of 400-800 nm. A HCX PL APO CS 63x/1.4-0.6 oil objective was used. Nanoparticles (pseudo-colored green) were excited with an argon laser (20 mW; λ =488 nm) and detected at 525-540 nm. Secondary antibodies were excited with a 633 nm HeNe laser (pseudo-colored red) and detected at 655-670 nm in a sequential scanning mode. Image analysis was performed with LAS AF software (Leica, Germany).

Feeding of lipid droplets and nanoparticle cargo release

The complexation of fatty acid free BSA (Sigma Aldrich) with oleic acid was performed as stated here. Therefore, 100 mM oleic acid was dissolved in 10 ml of 100 mM NaOH at 50°C. In parallel, 150 mg fatty acid free BSA was dissolved in 1.15 ml PBS under shaking at 50°C. The molar ratio of OA/BSA was set to 6:1 (pH 7.6). For the feeding of lipid droplets, HeLa cells were incubated with 25 μ M OA-BSA overnight. For cargo release experiments, 150 μ g ml⁻¹ nanoparticles were added for 30 min and fluorescence images were acquired using a Olympus XI81 fluorescence microscope.

Microplate reader release experiments

For PMI release experiments 150 μ g ml⁻¹ of PMI labeled PLLA/magnetite nanoparticles were added to 1) 300 μ l of a 1% DMSO containing water solution, 2) 300 μ l of a 10% FCS solution. 3) 150 μ g ml⁻¹ of PMI labeled PLLA/magnetite nanoparticles were freeze dried and 300 μ l of glyceryl trioleate (Sigma Aldrich) solution was added. The three samples were shortly mixed (1 min) and stored for 45 min at 37 °C. After centrifugation at 20 000 x g for 30 min, the

fluorescence intensity of the supernatant (at an excitation wavelength of 490 nm and an emission wavelength of 530 nm corresponding to maxima of PMI) was measured and compared to the fluorescence intensity in the case of dissolved PMI regularly used for nanoparticle synthesis.

Correlative microscopy

To generate an overlay of a confocal microscopy micrograph with a transmission electron microscopy micrograph, a correlative approach was performed. Therefore, cells incubated with nanoparticles were fixed with 4% PFA for 20 min on ibidi grid-500 to identify the location of the cell. Confocal z-stacks were recorded with a step range of 100 nm. For TEM analysis, the same cell recorded in the cLSM was processed. To add contrast for TEM examination, a 2% solution of OsO₄ in phosphate buffered saline (PBS) was used as staining agent, incubated with the cells for 30 minutes. After two washing steps with PBS, a graded alcohol series was performed to substitute cell water by 2-popanol: the solutions used contained 30%, 50%, 70% and 90% 2propanol in pure water. Each solution was left on the cells for 5 minutes. A saturated solution of uranyl acetate in alcohol was then used for further staining, incubated with the cells for 20 minutes at 37°C. After removal of the staining solution, 100% 2-propanol was added and incubated with the cells for two times 30 minutes. The alcohol was then replaced by a 30:70 EPON 812 : 2-propanol mixture for 15 minutes, followed by a 50:50 mixture for 30 minutes and a 70:30 mixture for one hour. 100% EPON 812 was left on the cells overnight, replaced by new EPON on the next day and left to polymerize at 60°C for 3 days. 60 nm thin sections were cut with a Leica Ultracut UCT equipped with a 35° Diatome diamond knife. Several sections were collected on a single 300 mesh copper grid and visualized using a FEI Tecnai F20 transmission electron microscope operated at an acceleration voltage of 200kV. The generation of micrograph overlays was done by adapting the confocal micrograph to the investigated TEM section

Transmission electron microscopy using high pressure freezing as fixation method

To visualise the PLLA-Fe-PMI nanoparticles at high resolution in cellular environment we applied transmission electron microscopy of HeLa cells treated with 1200 μ g.mL⁻¹ of the particles for 24 h. Before treatment cells were left to grow for 48 h after seeding onto 3 mm Ø sapphire discs at a density of 17.500 cells•cm⁻² in a 24-well plate. At the end of the incubation

period cells were fixed by means of high pressure freezing using a Compact 01 HPF machine (Wohlwend GmbH, Switzerland). Subsequent freeze-substitution was conducted using a Leica EM AFS 2 device (Leica Microsystems, Germany). The substitution medium contained acetone p.a., 0.2% osmium tetroxide, 0.1% uranyl acetate and 5% water and was pre-cooled to – 90°C before samples were added. After freeze-substitution samples were washed twice with acetone p.a. and finally embedded into EPON 812 resin. Ultrathin sectioning of the embedded samples was performed using a Leica Ultracut UCT (Leica Microsystems, Germany) and a diamond knife.Examination of thin sections was conducted using a FEI Tecnai F20 transmission electron microscope (FEI, USA) operated at an acceleration voltage of 200 kV. Bright field images were acquired using a Gatan US1000 slow scan CCD camera (Gatan Inc., USA).

Electroformation of DOPC giant vesicles and release experiments

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) giant vesicles were prepared bv electroformation. 10 mM DOPC (Avanti lipids, U. S. A.) were dissolved in chloroform. 25 µl were introduced dropwise onto indium tin oxide (ITO) coated glass electrodes (5 x 5 cm², 20 nm ITO) and dried under vacuum for 2 h. The two electrodes were clamped together and separated by a PDMS spacer. The gap was filled with 400 µl MilliQ water and the electrodes were connected to an AC volt generator (20 MHz programmable function generator, Series 8200, Kontron Messtechnik, Germany) under the following conditions: 1 V at 10 Hz for 2 h. Vesicles were diluted 1:10 in water and release experiments were started with the addition of 150 μ g ml⁻¹ nanoparticles for 0 to 180 min. The supernatant was centrifuged as stated previously and also added to GUVs for 0 to 180 min. PMI release was tracked on a Olympus XI81 fluorescence microscope.

Supplementary Figures

Supplementary Table 1 shows the investigated nanoparticles of this study.

Supplementary Table 1: Characterization of nanoparticles.

Particle	Polymer	Functionalization	Surfactant	Diameter (nm)	Dye	ζ-Potential (mV)
PS-COOH	PS	СООН	SDS	73	PMI	
PS-NH2- Bodipy	PS	NH2	CTMACL	60	Bodipy II	
PLLA-Fe- PMI	PLLA	OH + magnetite	SDS	126	PMI	
PLLA-PMI	PLLA	ОН	SDS	122	PMI	-47
SPIOPSN- Bodipy	PS	Sulfonate + magnetite	SDS	126	Bodipy II	-62

PS-COOH-PMI, a polystyrene nanoparticle as well as PLLA-OH-PMI, another PLLA nanoparticle shows kiss-and-run and releases PMI into lipid droplets (Supplementary Figure 1). In the case of PS-NH2-Bodipy, a polystyrene nanoparticle with covalently-bound Bodipy, a lack of lipid droplet staining was observed. Here, a more endocytotic like distribution pattern can be seen.

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Supplementary Figure 1: Investigations on additional nanoparticles, not mentioned in the main text. (A) PS-COOH-PMI and (B) PLLA-OH-PMI nanoparticles show a fast accumulation of PMI inside lipid droplets as identified by their high diffractive index. Both nanoparticles are loaded with PMI. (C) Polystyrene nanoparticles (PS-NH2-Bodipy) lack the staining of lipid droplets. Arrows depict lipid droplets as identified in the middle, while no green signal is seen at the tip of the arrows on the left (Bodipy channel only) or in the overlay (right picture). Instead they show an endolysosomal distribution after 20h of incubation.

To exclude the unlikely uptake of polystyrene nanoparticles into lipid droplets, we performed a correlative approach of cLSM and TEM (Supplementary Figure 2). An overlay of the cLSM and the TEM micrograph was generated. We have selected a cell with two nuclei for a more precise adjustment of the micrographs. Areas of nanoparticle-membrane interaction (Box 2) as well as areas of fluorescent lipid droplets (Box 1) are visible.



Supplementary Figure 2: Correlative approach of cLSM and TEM. (A) TEM micrograph. (B) Confocal micrograph. (C) Magnification of two areas of interest. (1) Lack of PLLA-Fe-PMI nanoparticles (green) in the direct area of lipid droplets after 30 min of incubation as demonstrated by the lack of electron dense (magnetic subparticles)/white material (PLLA) (2) PLLA-Fe-PMI (contrast of electron dense material (magnetite) with electron permeable material (PLLA), arrows) nanoparticles on the membrane coinciding with the fluorescence in the CLSM picture (red=cell mask deep red) (bar=10 μ m).

No intracellular localized nanoparticles could be detected by TEM studies after a short time (30 min). After 24h of incubation investigation of the organelles that transport PLLA-Fe-PMI nanoparticles reveal an association with macropinocytotic structures and endolysosomal vesicles. Nanoparticles were not found in lipid droplets.



Supplementary figure 2: PLLA-Fe-PMI nanoparticles (1200 μ g ml⁻¹) during uptake and endocytosis after 24h of exposure (cryo-TEM). Nanoparticles were not found in lipid droplets, but only in endosomes like on the figure on the left side (arrow) (bar 1 μ m).

SPIOPSN-Bodipy nanoparticles were tested on their kiss-and-run abilities revealing a short interaction with the membrane after detaching from it (white arrows, Supplementary Figure 3).

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Supplementary Figure 3: SPIOPSN showing the kiss-and-run mechanism lacking the staining of the membrane (white arrows). Note that the vesicle had to be marked here by the circle as it was not stained as with the non-covalently bound dye PMI.

- [1] I. Nikiforow, J. r. Adams, A. M. König, A. Langhoff, K. Pohl, A. Turshatov, D. Johannsmann, Langmuir 2010, 26, 13162-13167.
- [2] M. B. Bannwarth, S. W. Kazer, S. Ulrich, G. Glasser, D. Crespy, K. Landfester, *Angewandte Chemie International Edition* **2013**, *52*, 10107-10111.
- [3] M. Urban, A. Musyanovych, K. Landfester, *Macromol. Chem. Phys.* 2009, 210, 961-970.