Supporting Information for:

Well-defined, Multifunctional Nanostructures of a Paramagnetic Lipid and a Lipopeptide for Macrophage Imaging

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METHODS

Particle synthesis

The synthesis of the carboxyfluorescein-labeled P2A2, palmitoyl-K(palmitoyl) WK(carboxyfluorescein)G(LRKLRKRLLR)$_2$-NH$_2$ (P2fA2) has been described previously.[1]

Nanoparticular formulations were prepared by lipid film hydration. The fluorescent labeled lipopeptide P2fA2 and Gd-DTPA-DSA (gadolinium-diethylene triamine pentaacetic acid-distearyl amide) were dissolved in choloroform/methanol(1/1) in different ratios P2fA2/Gd-DTPA-DSA (see Table 1) and evaporated to dryness by rotary evaporation at 40°C. The obtained film was further dried under nitrogen for 15 min and heated to 65°C. Next, the film was hydrated with hot deionized water at 65°C under constant stirring. Subsequently, all preparations underwent 20 freeze-thaw-cycles until for the most preparations clear solutions were obtained (the formulation with a P2fA2/Gd-DTPA-DSA ratio of 1/9 was turbid at the end of the freeze-thawing cycles).

Table 1. Different formulations, their sizes and techniques applied indicated with 'x'.

P2A2 / Gd-DTPA-DSA	1/0	2/1	1/1	1/2	1/3	1/4	7/40	3/20	1/8	1/9
% P2A2	100	66	50	33	25	20	17.5	15	12.5	10
size in nm (DLS)	7.4	7.7	9.8	10.8	18.6	15.5	34.3	39.1	51.4	338
cryo-TEM	x		x	x	x	x			x	x
NMRD			x	x	x	x			x	x

Cryogenic transmission electron microscopy

Cryogenic transmission electron microscopy (cryo-TEM) was performed on an FEI Tecnai 20, type Sphera TEM instrument equipped with an LaB_6 filament operating at

200 kV. Images were recorded with a bottom-mounted 1k x 1k Gatan CCD camera. A Gatan cryoholder operating at ~ -170 °C was used for the cryo-TEM measurements. The sample vitrification procedure was carried out using an automated vitrification robot (FEI Vitrobot Mark III). TEM grids, R2/2 Quantifoil Jena grids, were purchased from Aurion. The Quantifoil grids were surface plasma treated using a Cressington 208 carbon coater operating at 5 mA for 40 s prior to the vitrification procedure.

Confocal laser scanning microscopy, FACS and MRI of cells

Cell cultivation

Murine macrophage J774A.1 cells (ATTC®, Manassas, VA) were cultured in cell culture in DMEM (Cellgro, Mediatech Inc, Manassas, VA) supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal bovine serum (FBS) (Cellgro, Mediatech Inc, Manassas, VA) at 37 °C in a humidified atmosphere of 5% CO_2.

In vitro cellular uptake of nanoparticles and confocal laser scanning microscopy

Cells were plated on coverslips (diameter 15 mm) positioned in 20 mm plastic culture dishes and cultured for one day. The plates were washed with PBS at pH 7.4 and exposed to 50% or 33% P2fA2-containing nanoparticles for 30 min at 37°C. After washing with PBS, the cells were fixed with 4% paraformaldehyde on ice for 10 minutes. Subsequently, the fixed cells were rinsed three times with PBS and mounted with DAPI containing VectaShield® (1.5 mg/ml DAPI) (Vector Laboratories, Burlingame) on microscopy slides.

Imaging of cellular uptake was performed by using an LSM 510META inverted confocal laser scanning microscope equipped with a Plan-Neofluar 63×/1.4 oil objective (Carl Zeiss, Oberkochen, Germany). Fluorescein-containing P2fA2 was excited at

488 nm and emission was recorded using a BP505-550 bandpass filter. For detection of nuclei, DAPI was excited at 405nm and emission was recorded using a BP420-480 bandpass filter. Image data were captured and analyzed using Zeiss LSM 510 Meta and Image Browser software (Zeiss).

Fluorescence Assisted Cell Sorting (FACS) to determine uptake mechanism

FACS cell sorting was performed on two cell types, i.e. bovine adrenal capillary endothelial cells (BACE) and murine RAW macrophages, that were treated with 100% P2fA2, 50% P2fA2 and 33% P2fA2 particles (peptide concentration 1 μM) at 37 ºC. To demonstrate specificity, the uptake as function of different inhibitory conditions was evaluated as well. These conditions included incubations at 4 ºC and in the presence of different endocytotic inhibitors (cytochalasin D, chlorpromacin and nystatin). Cytochalasin D is used to evaluate actin-dependent processes, while chlorpromacin inhibits clathin-dependent processes and nystatin inhibits caveolae-mediated processes. A FACSCalibur fluorescence-activated cell sorter (BD Biosciences, Franklin Lakes, USA) was used to quantify uptake.

MR imaging

Loosely packed pellets of 1.5 x 10^6 cells were scanned using a 9.4 T MRI system supplied by Bruker Instruments. Cell pellets from cells incubated with media only, 50 μM 33% P2fA2 particles or 50% P2fA2 particles (n=2) were imaged using a spin-echo sequence where the echo time was 10.5 ms, the repetition time 400 ms, the field of view 2.5 x 2.5 cm, the matrix size 96 x 128 and the no. of averages 4. The slice thickness was 0.5 mm and the total scan time 153 seconds.

FIGURES

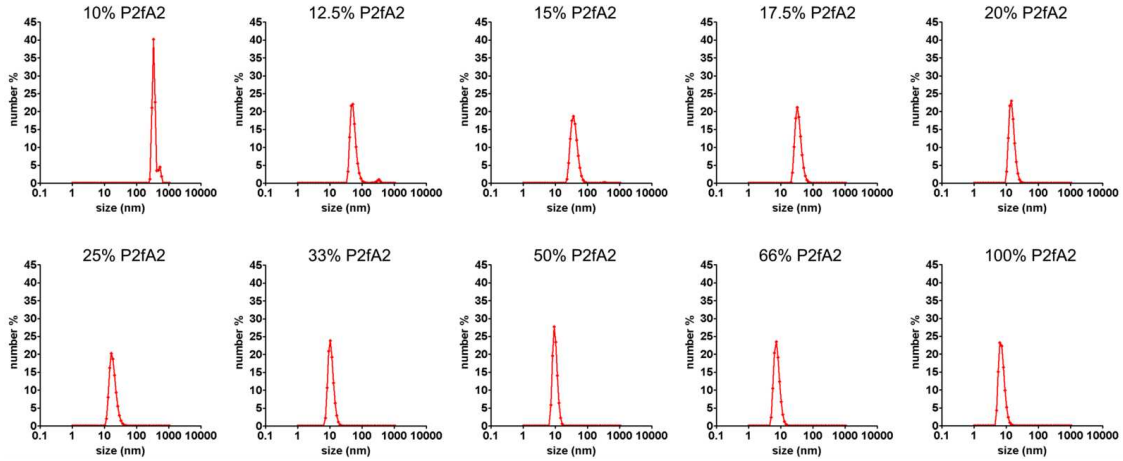

Figure S1. Individual DLS measurements of the different preparations.

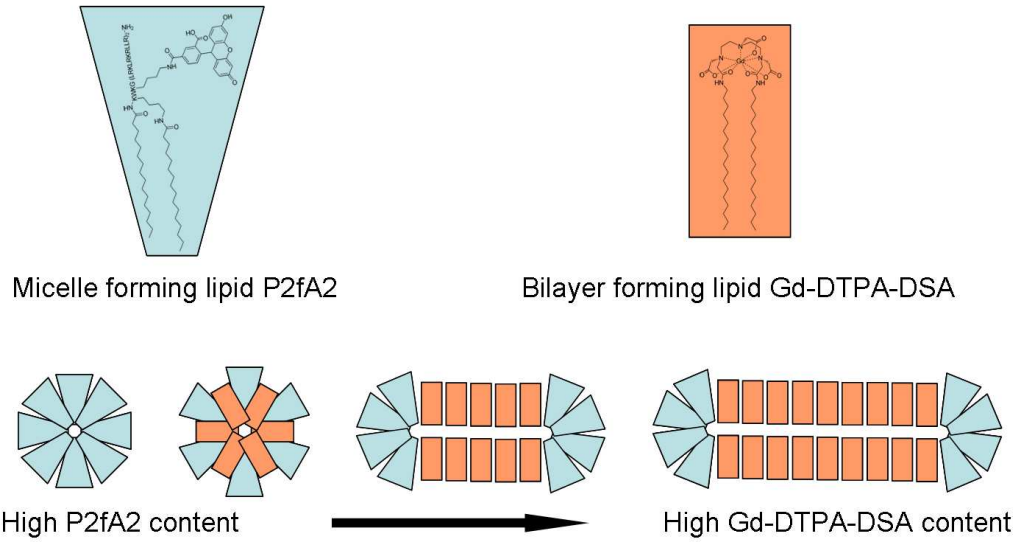

Figure S2. Possible amphiphilic organization of the different morphologies.
TOP: P2fA2 has a structure that favors micelle formation, while Gd-DTPA-DSA has a structure that favors the formation of a bilayer with little curvature.
BOTTOM: At high P2fA2/Gd-DTPA-DSA ratio micelle-like structures will be formed. In case the ratio decreases, the P2fA2 molecules will cluster and form regions with great curvature, while the regions with a high proportion of Gd-DTPA-DSA are relatively straight. Therefore, at lower ratios P2fA2/Gd-DTPA-DSA elongated structures are formed.

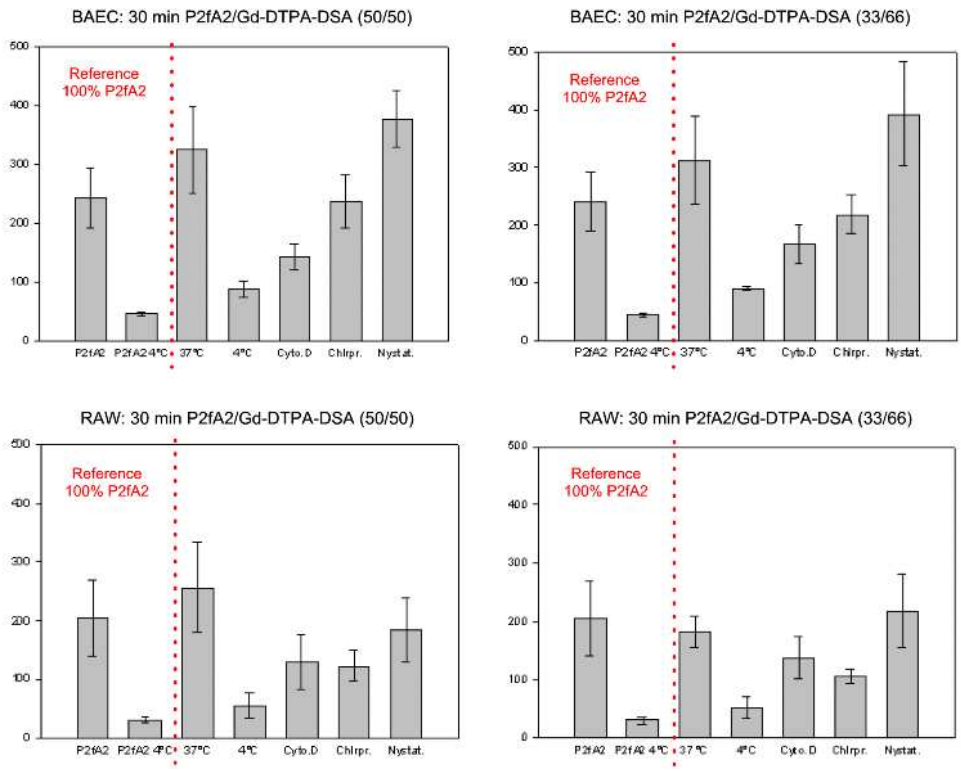

Figure S3. FACS determined uptake of 50/50, 33/66 P2fA2/Gd-DTPA-DSA particles in endothelial cells (BAEC) and macrophages (RAW). Incubations of pure P2fA2 particles at 37 ºC and 4 ºC served as a reference.

Reduced uptake of the different micelles at 4 °C points to endocytosis. Uptake of P2fA2/Gd-DTPA 50/50 and 33/66 particles in BAEC and RAW cells follows the same uptake pattern, but the uptake modes of the nanoparticles into RAW and BACE are different. Cytochalasin D, chlorpromacin and nystatin are inhibitors of different endocytotic pathways. Cytochalasin D is used to evaluate actin-dependent processes, while chlorpromacin inhibits clathin-dependent processes and nystatin inhibits caveolae-mediated processes. The uptake into RAW cells indicates the contribution of a clathin-dependent process (chlorpromacin reduces the uptake), but non-actin involving processes play also a role since cytochalasin D did not completely reduce the uptake. Nytatin has no influence which points out that the process is not caveolae-mediated. Furthermore, the uptake into BAEC is also not caveolae-mediated. The contribution of clathrin-mediated endocytosis is low, but incubation with cytochalasin D resulted in a significantly reduced uptake.

Reference

(1) Keller, S.; Sauer, I.; Strauss, H.; Gast, K.; Dathe, M.; Bienert, M. *Angew.Chem.Int.Ed Engl.* **2005**, *44*, 5252-5255.