

Published in final edited form as:

J Am Chem Soc. 2009 January 21; 131(2): 406–407. doi:10.1021/ja808310u.

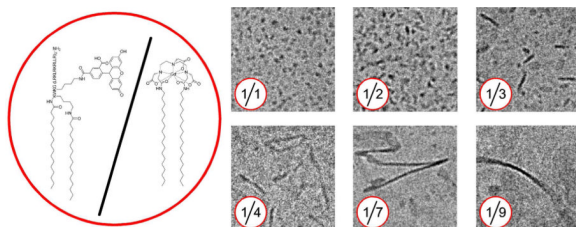
Well-defined, Multifunctional Nanostructures of a Paramagnetic Lipid and a Lipopeptide for Macrophage Imaging

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Abstract



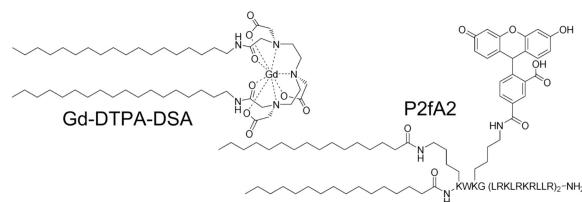
In the field of nanomedicine there is a great demand for technologies that allow the creation of self-assembled structures of which the size and morphology can be accurately controlled. In the current study, we report a nanoparticle platform that is composed of a paramagnetic lipid and a fluorescently labeled lipopeptide. By judiciously controlling the ratio of the aforementioned amphiphilic molecules a variety of well-defined nano-sized supramolecular structures with different sizes and morphologies could be created. The hydrodynamic radii of the different structures were determined by dynamic light scattering. Cryo-TEM revealed the aggregate morphology to vary from small micellar structures to plate-like and even full grown ribbons of which the aspect ratios varied from a diameter of 5–8 nm to structures with a width of up to 25 nm and infinite length. Interestingly, nuclear magnetic resonance dispersion profiling revealed excellent properties for MRI and also showed that the relaxivity of the structures was tunable and morphology dependent. Finally, macrophage cells were treated with two selected nanoparticles and were shown to be avidly taken up.

In conclusion we demonstrate a methodology to create structures that are (1) paramagnetic to enable their detection with MRI, (2) exhibit fluorescent properties, (3) can be tuned to defined sizes and shapes and (4) are efficiently taken up by macrophage cells in vitro.

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Supporting Information Available: Figure S1–S3 Table S1 and detailed experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

In the field of molecular imaging nanoparticulate structures are becoming increasingly important, since they can carry high payloads of contrast generating materials while their surface can be functionalized to improve bio-compatibility or to introduce specificity.^{1,2} In addition to inorganic nanocrystals, such as quantum dots (QDs)³ and iron oxide⁴ or gold nanoparticles,⁵ self-assembled organic structures including liposomes⁶, micelles⁷ and microemulsions⁸ have shown great potential for *in vivo* imaging. In the field of targeted imaging and therapy the pharmacokinetic profile as well as the tissue penetration potential of the nanovehicles is of paramount importance. Hence, there is a great demand for nanostructures of which the final morphology and size can be judiciously controlled. To this aim, we created a variety of well-defined nano-sized supramolecular structures based on two amphiphilic molecules: **Gd-DTPA-DSA**, a Gd³⁺ chelating lipid which exhibits paramagnetic properties for MRI,^{6,9} and **P2fA2** a fluorescein labeled apolipoprotein E derived lipopeptide that has been shown to enhance nanoparticle uptake into rat brain capillary endothelial cells *in vitro*.¹⁰



Here we show how we can control the morphology and size of the imaging probes by varying the ratio of the two amphiphiles and thereby optimizing the molecular relaxivity of the resulting lipidic aggregates. The two most effective morphologies were selected for *in vitro* studies and were demonstrated to be effectively internalized by macrophage cells.

Aqueous dispersions of mixtures of **P2fA2** and **Gd-DTPA-DSA** were prepared (Table 1 Supporting Information) and dynamic light scattering (DLS) revealed that the aggregates had narrow size distributions (Supporting Information Figure S1) and that their mean hydrodynamic diameters increased with decreasing **P2fA2/Gd-DTPA-DSA** ratios (Figure 1). At high **P2fA2** contents of 50 mol% or more small structures with a mean diameter below 10 nm were observed, whereas a transition to larger aggregates (8–15 nm) was observed in case the **P2fA2** content was lower than 33 mol%. Below 20 mol% **P2fA2** the mean hydrodynamic diameter increased to ~60 nm for 12.5 mol% **P2fA2** and to more than 300 nm for a formulation that contained 10 mol% **P2fA2**.

In line with the DLS measurements cryo-TEM revealed aggregates of which the size increased with decreasing **P2fA2** concentration (Figure 1). At **P2fA2** concentrations ≥ 50 mol% small micellar structures with diameters of 5–8 nm were observed. By decreasing the **P2fA2** concentration plate-like morphologies appeared with a constant thickness of 5–8 nm of which the aspect ratios increased, from by 10×15 –25 nm (33 mol% **P2fA2**) to 10 – 15×150 nm (25 mol% **P2fA2**) and up to 10 – 15×250 nm (20 mol% **P2fA2**). In the preparations that contained 10–12.5 mol% **P2fA2** fully grown ribbons were present of up to 25 nm in width and with infinite lengths.

Previously, in a study by Johnsson and Edwards it was demonstrated that by carefully mixing regular phospholipids and poly(ethylene glycol) (PEG) conjugated phospholipids a variety of structures, ranging from vesicular, discoidal and micellar, could be created.¹¹ The morphological changes were ascribed to the differences in geometry of the amphiphiles used. In our study **Gd-DTPA-DSA** tends to form bilayers with little curvature, while **P2fA2** acts like a strong detergent and curves such that it forms micelles. Therefore aggregates of mixtures of such molecules can have morphologies that are more spherical, plate-like and

even long elongated structures (Supporting Information Figure S2), depending on the ratio of both components.

To assess the applicability of the different nanostructures for magnetic resonance imaging and to characterize their paramagnetic properties nuclear magnetic resonance dispersion profiles (NMRD-profiles) were obtained (Figure 2). Typical so-called macromolecular profiles with maximum intensities around 20 to 30 MHz were observed. These indicate a diminished mobility of the Gd^{3+} chelates in the lipidic aggregates which causes their tumbling rate τ_R to decrease. This result is typical for supramolecular and macromolecular contrast agents¹² at these moderate, but clinically relevant field strengths and is not observed for low molecular weight Gd^{3+} chelates. Moreover, these results further support the incorporation of **Gd-DTPA-DSA** in the lipidic aggregates. Our approach allowed the formation of structures with tunable ionic relaxivities, varying between 13 to 18 $\text{mM}^{-1}\text{s}^{-1}$ at 20 MHz (inset Figure 2), which is 3 to 5 times higher than commercially available Gd-DTPA at this field strength. Interestingly, the highest ionic relaxivity of 18 $\text{mM}^{-1}\text{s}^{-1}$ was observed for the formulation containing 33% **P2fA2**, the preparation with predominately small plate-like aggregates.

In general such differences in relaxivity are largely determined by the variations in the interplay between the rotation correlation time (τ_R) and the exchange of water molecules coordinated to a single Gd^{3+} chelate. At high **P2fA2** concentrations, *i.e.* at low local concentrations of **Gd-DTPA-DSA**, it is unlikely that the water exchange rate is the limiting factor. Therefore the observed sub-optimal relaxivity at this composition is most likely due to the high τ_R , related to the small aggregate dimensions. Although the aggregate size increases with decreasing **P2fA2** concentrations, a reduction in the relaxivity is observed upon further lowering of the **P2fA2** content past the optimum value of 33 mol%. In this regime, the reduction in ionic relaxivity cannot be related to τ_R , as this parameter is expected to decrease further with the increasing dimensions of the aggregates. Rather, at the concomitant higher Gd-DTPA-DSA concentrations, the water exchange will become a limiting factor and the amount of water that is relaxed per Gd^{3+} chelate will be limiting the ionic relaxivity. Clearly at 33 mol% we find the morphology that optimally benefits from a low rotation correlation time associated with the aggregate dimensions and a Gd^{3+} surface concentration that allows a high enough water exchange rate.

Based on these results we set out to test these **P2fA2** containing nanostructures for their suitability as MR imaging probes for extravascular targets, such as macrophages. Since small nanoparticles are more likely to escape from the circulation and cross the diseased and permeable endothelium, the two formulations with a high relaxivity and small size, *i.e.* those with 33 mol% (small platelets) and 50 mol% (micelles) **P2fA2** were selected for further *in vitro* experiments with cultured mouse macrophage cells (J774A1). The incorporation of **P2fA2** and **Gd-DTPA-DSA** allowed the visualization of nanoparticle uptake using fluorescence confocal laser scanning microscopy and MRI, respectively (Figure 2). The CLSM images show nuclei in blue (DAPI) and **P2fA2** in green, merged with bright field images of the macrophage cells. While control cells revealed no nanoparticle-associated fluorescence or MRI contrast (Figure 2B), significant amounts of intracellular fluorescence as well as MRI signal enhancement were observed after 30 min of incubation for cells that were incubated with 50% (Figure 2C) and 33% (Figure 2D) **P2fA2** nanoparticles. In addition, we performed quantitative uptake experiments under inhibitory conditions (Supporting Information Figure S3). The results revealed the particle uptake to be specific and further confirmed their multifunctional character.

In summary we have demonstrated that by carefully controlling the ratio of two functional amphiphiles **Gd-DTPA-DSA** and **P2fA2**, it was possible to create a variety of well-defined

supramolecular structures, containing both fluorescein and Gd³⁺ chelates. Moreover, we showed that by changing the morphology we could optimize the relaxivity of the imaging probes, since NMRD-profiling disclosed excellent and tunable MRI properties for the different formulations, especially at clinically relevant field strengths.

We envision the application of these contrast agents for pathologies in which macrophage inflammation plays a key role, such as atherosclerosis, rheumatoid arthritis, or cancer.¹³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Partial support was provided by NIH/NHLBI R01 HL71021, NIH/ NHLBI R01 HL78667 (ZAF) and the DFG (DA 324/5-2 and FG 463, E.L.). David P. Cormode and Heike Nikolenko are gratefully acknowledged for their input.

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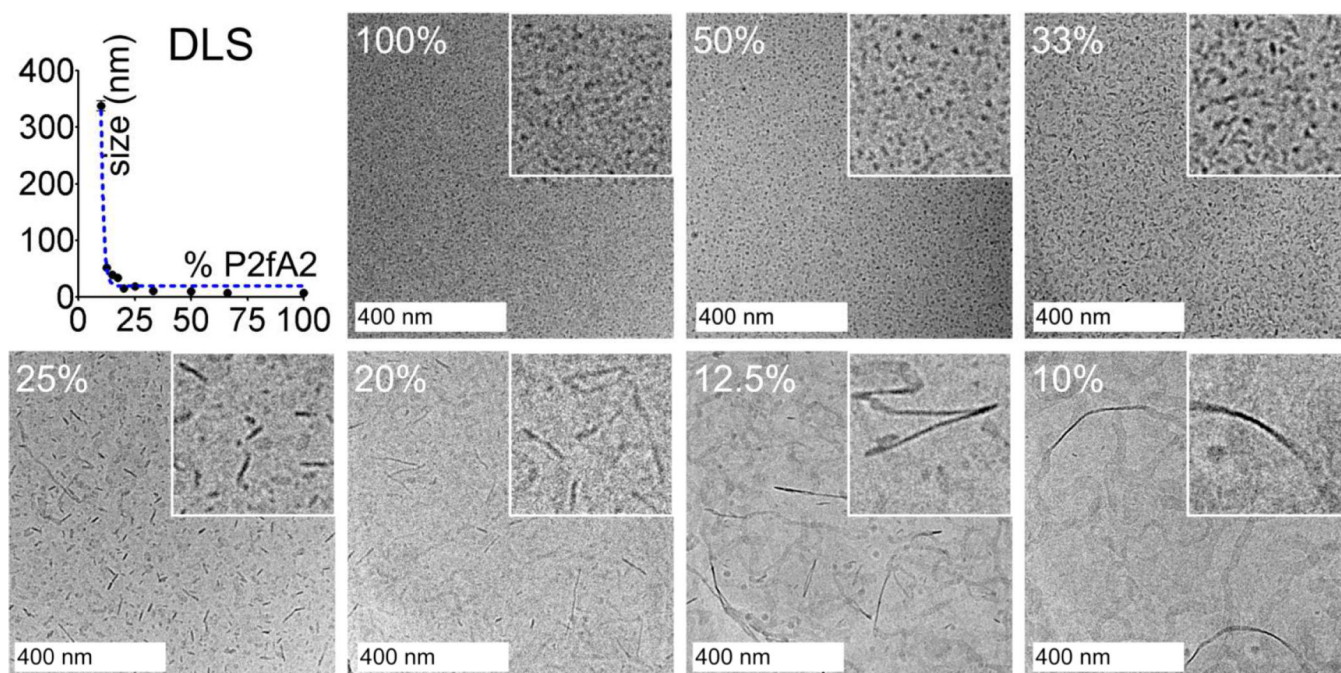


Figure 1. Dynamic light scattering (top, left) and cryo-TEM images of different **P2fA2/Gd-DTPA-DSA** preparations. The insets show a close up of the cryo-TEM images and represent 200 by 200 nm. The percentages refer to the amount of **P2fA2** in the formulations.

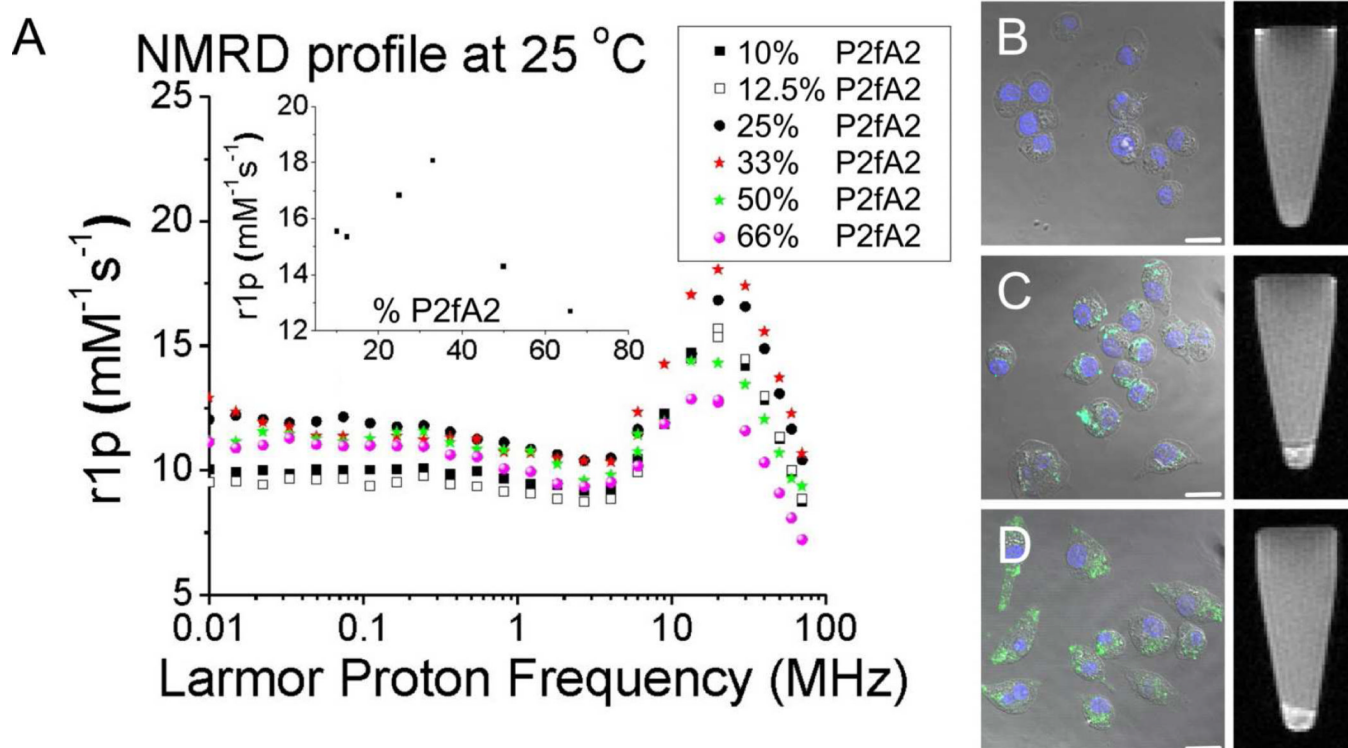


Figure 2.
 (A) NMRD-profiles of the different structures. The inset displays the relaxivity as function of % **P2fA2** at 20 MHz. Confocal microscopy (left) and MRI of loosely packed cell pellets (right) of macrophage cells that were (B) left untreated, incubated with 33% (C) and (D) 50% **P2fA2** nanoparticles. Scale bar: 20 μm .