Magnetically actuable delivery vehicles

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Magnetically Actuable Delivery Vehicles

by

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PhD thesis

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Abstract

Stimuli-responsive amphiphilic superstructures comprise some of the most successful drug delivery systems in clinical use. Vesicular assemblies in particular, have long been recognized as excellent carrier systems for diagnostic and therapeutic agents due to their potential to encapsulate, transport and release various cargo upon local changes; the latter in response to the microenvironment. Structural incorporation of biocompatible, functional nanomaterials such as superparamagnetic iron oxide nanoparticles can be employed to design universal delivery systems, whose release profiles can be controlled externally by application of a magnetic field. The aim of this work is to establish a theranostic platform by incorporating hydrophobic SPIONs of controlled size and interfacial chemistry into stimuli-responsive amphiphilic superstructures and to demonstrate externally triggered release of a model compound via magnetic actuation. The synthesis of monodisperse nanocrystals and complete exchange of the native hydrophobic dispersant shell for irreversibly grafted nitrocatechol-derived anchors at maximal ligand density is exemplified and shown crucial for controlled capsule assembly. A method for efficient encapsulation of SPION into amphiphile membranes is outlined that allows for direct and quantitative co-self-assembly of bilayer forming amphiphiles with hydrophobic nanoparticles into controlled magnetosomes. The novel method allowed for controlling the vesicle size, lamellarity and the nanoparticle concentration in the membrane with retained vesicle stability. Various capsule systems comprising polymeric superamphiphiles and mixed lipid/superamphiphile blends were investigated and their magnetothermal release efficiencies using alternating magnetic field irradiation to cause local hyperthermia were compared. Mixed membrane capsules were found to combine efficient magnetothermal actuation with the high stability of purely polymeric membrane systems.

Keywords: superparamagnetic iron oxide nanoparticle • stimuli-responsive vesicle • self-assembly • drug delivery • triggered release

Zusammenfassung (Abstract in German)

Supramolekulare Kapseln aus reiz-empfindlichen Trägermaterialien, stellen einige der erfolgreichsten klinischen Auslieferungssysteme für therapeutische Wirkstoffe dar. Vesikel werden seit jeher aufgrund ihres Potentials zur Einkapselung und zur umweltbedingten Freisetzung von therapeutischen und diagnostischen Agenzien als exzellente Trägersysteme geschätzt. Die strukturelle Einbindung von biokompatiblen, funktionellen Nanomaterialien wie etwa superparamagnetischen Eisenoxid-Nanopartikeln kann dazu verwendet werden, universelle Auslieferungssysteme zu kreieren, dessen Freisetzungsprofil durch Anlegen eines selektiven äußeren Reizes, beispielsweise eines magnetischen Feldes, gesteuert werden kann. Das Ziel dieser Arbeit ist die Entwicklung einer solchen theranostischen Plattform sowie die Demonstration einer extern kontrollierten Freisetzung einer Modellsubstanz durch magnetische Anregung. Die Synthese von monodispersen Nanokristallen und der vollständige Austausch der ursprünglichen Ligandenschale ist essentiell für die kontrollierte Herstellung der Kapseln und wird gezeigt. Es wurde eine wirkungsvolle Methode zur Einbettung von magnetischen Nanopartikeln entwickelt, die zur direkten und quantitativen Selborganisation von hydrophoben Eisenoxid-Kristallen und membranformenden Amphiphilen in kontrollierte Magnetosome führt. Das neue Verfahren erlaubt die Steuerung der Vesikelgröße, Lamellarität und der Nanopartikelkonzentration in der Membran bei gleichbleibender Vesikelstabilität. Unterschiedliche Trägersysteme aus makromolekularen Superamphiphilen und gemischten Diblock Copolymer-Lipid Systemen wurden hergestellt und untersucht. Die Wirksamkeit der verschiedenen neuen Kapselsysteme bezüglich induzierter Freigabe durch magnetische Wechselfeld Anregung wurde verglichen. Es wird gezeigt, dass gemischte Membrankapseln eine effiziente magneto-thermische Freisetzung ermöglichen und diese mit der hohen Stabilität der ursprünglich untersuchten reinen Polymerkapseln kombinieren.

Schlüsselwörter: Superparamagnetische Eisenoxid-Nanopartikel • Reiz-sensitive Vesikel • Selbstorganisation • Wirkstoffauslieferung • ausgelöste/gezielte Freisetzung

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1. Introduction

1.1. Nanoscale Encapsulation

In the past therapeutic formulations were limited to solutions, compressed and encapsulated powders in form of tablets and traditional capsules, oils as well as coarse suspensions and emulsions.¹ The physical state of the drug however decisively influences its overall solubility and effectiveness.² Nanoscale properties were found to enhance the performance of therapeutic agents by that vital characteristics like solubilization, distribution profile and release rate can be adjusted and fine-tuned.¹ Nanomedicines generally consist of functional nanoparticles containing the therapeutic agents and are typically suspended in some liquid or solid matrix to give suspensions or gels.

Figure 1: An overview of pharmaceutical nanoscale technologies and their applications (reprinted from Uchegbu et al – *Fundamentals of Pharmaceutical Nanoscience*¹ © (2013) with permission from Springer Science & Business Media New York).

Encapsulation primarily serves to prevent premature degradation by environmental factors but is also extremely valuable for dispersing high-Mw and hydrophobic substances that are poorly soluble and show low availability in biological fluids. Stable encapsulation, shuttling and release are in general highly beneficial as the

applied dosages and thereby costs and side-effects can be minimized while the substances can systematically be deposited and released at the pathological target site. Encapsulation of diverse pharmacologically active agents such as classical prescription drugs, antibiotics, hormones, nutrients but also biopharmaceuticals like peptides, proteins, nucleic acids or even whole genes has been achieved.^{1,3,4} Colloidal carriers can be fabricated from various materials; inorganic capsules, soft matter systems and drug nanoprecipitates / nanocrystals are the most common.² Inorganic capsules provide efficient stabilization but are foremost micron-sized and do not readily release their content in response to minor changes in the microenvironment. Drug nanoprecipitates are solid drug cores in the nanometer range surrounded by a stabilizer. These constitute useful alternatives but suitable formulation procedures are rare and their potential to avoid detection and clearance in vivo is highly uncertain. Nanosized, soft matter systems are most often preferred due to their flexible nature, responsiveness to environmental changes and possibility to engineer for low interaction with biomolecules, which leads to low clearance rates in vivo.^{3,4} Micelles and microemulsion are solely applicable to amphiphilic and hydrophobic cargo. Universal soft matter excipients are amphiphile-drug conjugates and vesicles. Conjugates are suitable when appropriate derivatization sites are available, but they might alter distribution profiles and the therapeutics' effectiveness. Vesicles are capable of incorporating diverse cargo by offering a large aqueous interior volume and membrane area. Such high loading capacity for a multitude of agents accounts for much of their efficiency and popularity as drug delivery vehicles.^{3,4} The compositional and dimensional flexibility furthermore allows to tailor biodistribution and release profiles to specific requirements.^{5,6} Site specific accumulation and uptake patterns can be adjusted through vesicle size and combined with active and passive targeting strategies.^{3,4,6,7} Vesicle dimensions typically influence the in-vivo distribution and govern the internalization pathway. Active targeting invokes conjugation of signaling sequences and targeting ligands that guide the delivery vehicle to its designation, whereas passive accumulation makes use of intrinsic differences in site specific circulation and drainage characteristics. Upon arrival to a designated tissue or compartment, many biological stimuli are conceivable triggers to promote release. Common triggers include temperature aberrations, changes in pH and redox milieu or enzymatic activity.^{3,8–12} The release profile can either be burst-like or continuous slow delivery depending on

vesicular disintegration or switching of membrane permeability. Hence, nanoscale formulation technologies offer immense potential for biomedical and pharmaceutical applications just as for cosmetics and food science. Examples of approved nanodrugs in current use comprise doxil¹³, ambisome¹⁴ and abraxane.¹⁵

The enclosure of a liquid volume and associated compartmentalization is also of great interest to the realization of nanoreactors since it enables to perform complex biosynthetic cascades in this constrained environment.¹⁶ Compartmentalization then specifically functions as a method to protect the biological process from undesired external influences and to retain the different elements and products in the nanocontainer which can subsequently be delivered. Such in-situ approaches will greatly reduce difficulties with drug loading and surface modifications as a small number of internalized modular building blocks will be able to integrate multiple functionalities.

In order to control composition and structure, as well as to obtain the desired release properties and responsiveness, these nanocontainers tend to be made from self-assembled macromolecular components.

1.2. Supramolecular assemblies

Autonomous self-organization of individual building blocks into defined superstructures offers tremendous potential for a manifold of technological applications and constitutes a hallmark of modern research and interdisciplinary science. The guiding principles are omnipresent at various levels of complexity throughout basic science and are particularly popular in molecular catalysis, synthetic biology, biomaterials engineering and nanomedicine because it grants access to remarkably functional and smart soft materials.

The very basics of molecular self-assembly are well established,^{17–20} and wonderfully exemplified by the superstructures formed by amphiphiles in solution. Amphiphiles are bivalent molecules that contain a water-soluble or hydrophilic part and at least one other lipophilic or water-insoluble entity that are covalently linked together. Upon transfer to a polar medium such as an aqueous phase these irreconcilable properties drive the amphiphile to adapt to the prevalent conditions and to arrange into a minimum energy configuration. Typical forces acting on amphiphilic molecules in solution comprise electrostatic interactions and H-bonding associated with the polar headgroups as well as van-der-Waals interactions and the hydrophobic effect which govern non-polar contributions. The hydrophobic effect in particular constitutes a substantial contribution to the total free energy of the system and accounts for much of the amphiphile's tendency to aggregate into well-ordered structures. The above interactions possess different algebraic signs, meaning their contributions can be attractive or repulsive and also differ in their magnitudes and distance dependencies.^{17–19} Their overall interplay, however, is required to yield a total attractive potential to drive molecular self-assembly in solution. The concentration above which the dissolved amphiphiles start to phase-separate into segregated clusters is termed critical aggregation concentration and is accompanied by various physicochemical changes of the solution properties.^{18,20} Such segregation results in a molecular arrangement that extends over distances or length scales that are large compared to distances over which molecules affect one another in a homogeneous liquid. These interfaces distinguish mesoscopic structures, such as micelles, bilayers or microemulsions, from essentially nondescript intermolecular arrangements.²¹ The preferences of molecular building blocks to arrange into complex, ordered superstructures are often illustrated in terms of a dimensionless

critical packing parameter (CPP) (c.f. Scheme 1).^{17–19,22} It provides a sound base for a first-principle design of amphiphilic superstructures in water by that it gives an estimate of the molecular shape of the building blocks and hence on their collective packing preferences.

Scheme 1: Definition of the critical packing parameter (CPP) of an amphiphile in terms of its fundamental geometric factors. Here V is the tail volume, A the head-group area and l the tail length. The CPP defines the molecular shape of the amphiphile and determines its packing preferences into higher superstructures (adapted from Salim et al - *Amphiphilic designer nano-carriers for controlled release: from drug delivery to diagnostics*²² © (2014) with permission from The Royal Society of Chemistry).

The relation of these three parameters defines a critical packing shape. Spontaneous self-assembly and formation of various superstructures based on the CPP is illustrated in Fig 1 [reprinted from ²²]. If for instance, $V \sim A \cdot l$ then the surfactant is fairly cylindrical ($CPP \sim 1$) and it's quite easy to arrange it into planar bilayers. For slightly smaller tail volumes when CPP is around 1/2 to 1, curved bilayers or vesicles result from packing of truncated cones. If the head group is very large and the tail volume is rather small, then $CPP < 1/3$ and the structure formed will be preferentially micellar as for detergents. In between these two cases ($1/3 < CPP < 1/2$) hexagonal packing will favor elongated, cylindrical micelles. At the other extreme with a very bulky tail (V is large) and a small head and/or short tail then $CPP > 1$ and surfactant packing will favor reverse or inverted micelles.

Critical Packing Parameter (V/AI)	Critical Packing Shape	Structures Formed
$< 1/3$	Cone	Spherical micelles
$1/3 - 1/2$	Truncated cone	Cylindrical micelles
$1/2 - 1$	Truncated cone	Flexible bilayers, vesicles
~ 1	Cylinder	Planar bilayers
> 1	Inverted truncated cone or wedge	Inverted micelles

Figure 2: Relations between the critical packing parameter, molecular shape and the resulting assembly structures. Depending on the value of the dimensionless packing parameter various superstructures will form upon aggregation of the individual building blocks at appropriate concentration in aqueous solution (reprinted from Salim et al - *Amphiphilic designer nano-carriers for controlled release: from drug delivery to diagnostics*²² © (2014) with permission from The Royal Society of Chemistry).

Traditional amphiphiles are relatively small molecules, hence their assemblies are rather fragile and of limited mechanical stability. Extending the amphiphilic concept to macromolecular dimensions encompasses superamphiphiles that consist of covalently linked blocks with different chemical compositions. For these, a redefining of the geometric factors is essential since the high-Mw and chemically distinct nature

of the components yields very different degrees of and variable solvation of the blocks. For example, a high-Mw hydrophilic and flexible polymer can occupy many times its volume in water due to its high solvation and thermally induced movement. For polymeric amphiphiles the hydrophilic-hydrophobic balance of the architecture is typically given in terms of the hydrophilic volume fraction f . The latter should preferentially lie within $35 \pm 10\%$ to form curved bilayers or polymersomes.^{23,24} Macromolecules with $f > 45\%$ can be expected to form micelles, whereas polymers with $f < 25\%$ are likely to give inverted microstructures. Superamphiphiles yield highly stable aggregates, such that the formed structures are not necessarily thermodynamically controlled. Kinetically trapped structures pose problems for facile assembly of equilibrium structures, but the additional kinetic barriers to disassembly also confer additional stability to metastable structures.

Vesicular self-assembled superstructures are particularly attractive for biomedical applications because of their ability to shuttle various cargo. Hydrophilic substances can be encapsulated in the large aqueous lumen while hydrophobic and amphiphilic agents can be hosted in the membrane interior or at the interface.^{23,25} Vesicle stability and membrane properties, however, markedly vary with amphiphile species and molecular weight (Mw) due to differing mutual interactions among the constituents. Lipids only exist in a narrow Mw range and are evolutionary optimized for high fluidity and compositional flexibility; polymers have variable Mw and thus adjustable stability and permeability.²³ The dynamics of lipids are largely governed by collective behavior and concerted changes owing to their high lateral mobility, while polymeric assemblies exhibit more dominant intramolecular backbone dynamics because of the much higher effective volume fractions. Increasing polymer Mw enforces membrane toughness and increases the loading capacity for hydrophobic and amphiphilic agents but brings along restrictions for release as permeability depends on Mw of the hydrophobic region of the membrane. Moderate Mw polymers (Mw~1-10kDa) yield thick, bilayer vesicles, while high Mw analogs (Mw~10-100kDa), especially when the constituent polymers exceed the entanglement-Mw, form a continuous membrane region that slows transport and hampers efficient release.²³ The bilayer thickness d is related to the hydrophobic block weight (M_h) by a scaling law of the form $d \sim M_h^{0.55}$.^{23,25} A correlation of membrane properties to amphiphile-Mw is depicted in Figure 2 (reprinted from ²³). Surface elasticities only depend on the interface and therefore on the chemical composition of the headgroup. Typical bending energies for ionic

amphiphiles and ethoxylates are around 1 and $4k_B T$, respectively, causing ionics to be rather flexible and to possess low interfacial tension.¹⁹ Consequently phosphocholine lipids feature remote rupture tensions ($\sim 5\%$ maximum critical strain) and are subject to considerable thermal undulations while polymersomes can withstand 10-fold higher strains (up to $\sim 50\%$ depending on composition and Mw) and are less undulated because of drastically enhanced cohesive energy densities.^{26,27}

Figure 3: A line-up of lipid vs polymeric vesicles (adapted from Ghoroghchian et al - *Near-Infrared-Emissive Polymersomes: Self-Assembled Soft Matter for in Vivo Optical Imaging*²⁸ © (2005) with permission from National Academy of Sciences, USA). The typical arrangement of membrane forming amphiphiles in a vesicle and their corresponding membrane thicknesses are depicted in (a) while (b) illustrates how membrane properties vary with molecular weight of the constituent amphiphile (reprinted from Discher et al – *Polymersomes*²³ © (2006) with permission from Annual Reviews). The inset in (a) shows the supposed lipid packing associated with an unfavorable local bending of the membrane in the vicinity of a nanoparticulate protrusion (reprinted from Amstad et al – *Triggered Release from Liposomes through Magnetic Actuation of Iron Oxide Nanoparticle Containing Membranes*²⁹ © (2011) with permission from The American Chemical Society).

Other properties that correlate with Mw are aggregate stability and exchange rates. Exchange due to solubilized unimers in solution correlates with the critical aggregation concentration (c_{ac}) and is roughly proportional to $c_{aggregate} \sim \exp(-n\epsilon_h/k_B T)$ with characteristic exchanges times of hours for phospholipids.²³ Considerably higher aggregate stabilities and longer timescales are expected for polymers because of the dependence of the monomer's solution energy (ϵ_h) on the number of repeat units (n) or hydrophobic Mw.²³

An improvement of the mechanical properties often comes at the expense of biocompatibility since most synthetic polymers cannot be decomposed and recycled

by the body but offers design of vesicle scaffolds that are long-term antifouling and therefore impart stealth character and prolong in-vivo circulation times by preventing opsonization.³⁰ For lipid based vesicles the incorporation of functional or targeting moieties is limited by the concomitant change in the hydrophilic-hydrophobic balance upon conjugation to the headgroup. In contrast, polymers can be equipped with diverse agents for surface modification at relative ease because their high aggregate stability and block weights make them relatively insensitive to conjugation effects and packing rearrangement. For artificial systems degradability and responsiveness are however issues of central importance that are most often approached via biomimetic compounding.

Self-assembled superstructures thus offer a plethora of possibilities for modern biomaterials engineering to precisely tailor functionality and characteristics to the aspired applications. Recent highlights of successful applications of vesicular carrier systems in biomedicine include magnetic chemotherapy treatment of human adenocarcinoma cells via actively targeted thermoresponsive magnetoliposomes,⁷ passively targeted co-delivery of multiple anti-proliferative drugs to solid tumors in mice and their shrinkage via pH-sensitive, self-porating polymersomes,^{31,32} optical imaging of cancerous tissue in live animals through NIR emissive polymersomes containing multiporphyrin fluorophores,²⁸ nucleic acid and gene delivery to human cell lines^{33–35} as well as simultaneous magnetic resonance imaging and drug delivery.^{36,37}

1.3. Superparamagnetic Iron Oxide Nanoparticles

Iron oxide nanoparticles are of great interest across a wide range of disciplines because of their size-dependent magnetic properties and biocompatibility. Their usage extends from data storage media in information technology to substrates for catalysis to some of the most versatile and safe composite nanomaterials for biomedical applications like targeted drug delivery, magnetic resonance imaging (MRI), hyperthermia treatment, magnetic cell separation or detoxification of biological fluids.^{38–40}

Magnetite is a mixed valance compound that crystallizes in an inverse spinel structure with a lattice constant of $a=8.394\text{\AA}$ comprising eight Fe_3O_4 formula units per unit cell.⁴¹ The 2:1 stoichiometric ratio of ferric to ferrous ions are distributed among tetrahedral (Td) and octahedral (Oh) sites of the cubic closest packed oxygen lattice in the following formal fashion: $[\text{Fe}^{3+}]_{\text{Td}}[\text{Fe}^{3+}\text{Fe}^{2+}]_{\text{Oh}}\text{O}_4$.⁴¹

Figure 4: Model of the bulk crystal structure of magnetite. (a) Overview and (b) schematic drawing of the connectivity and bonding around the Td (green) and Oh (blue) sites in the unit cell. Some atoms on the periphery have been truncated to improve the visibility of the structure (reproduced from Okube et al - *Residual-Density Mapping and Site-Selective Determination of Anomalous Scattering Factors to Examine the Origin of the Fe K Pre-Edge Peak of Magnetite*⁴² © (2012) with permission of the International Union of Crystallography).

Ligand field stabilization in spinels with 3d elements typically favors electronic high spin configurations because oxide ions are weak field ligands. The main electronic interaction occurs among octahedral and tetrahedral spin sites which are antiferromagnetically coupled such that a net magnetic moment of $4\mu_{\text{B}}$ per formula

unit results from surplus ferrous ions.⁴¹ The effective magnetic moment is polarized along the crystallographic [111] direction of the cubic cell which constitutes the anisotropic or easy axis through the bulk lattice.^{41,43} At ambient conditions the electrons associated with octahedral sites are thermally delocalized via polaronic hopping between the t_{2g} orbitals of the conduction band which causes a fractional average charge of +2.5 per octahedral iron. Magnetite's electronic structure above the Verwey transition ($T_V = 124\text{K}$) resembles that of a strongly correlated or spin polarized semiconductor with a band gap of around 0.2-0.4eV.⁴⁴

Particles in the size range of 3-10nm form single magnetic domains and exhibit superparamagnetic behavior due to thermal reordering of the entire crystal volume. Particles exceeding the superparamagnetic limit build up a strong net magnetic moment which leads to emerging ferromagnetic behavior while bulk magnetite and particles above 25-30nm show progressive formation of multiple magnetic domains giving ferrimagnetic crystals.⁴⁵ These size dependent magnetic properties are an essential feature of iron oxides and amongst their biocompatibility account for their popularity and use in technological applications.

Figure 5: Electronic and magnetic interactions among the lattice cations in magnetite. (a) Ligand field schemes and electronic configurations of the coordinated d-metal cations and the resulting spin-polarized semiconductor band-structure of magnetite (reprinted from Stöhr et al *Magnetism - From Fundamentals to Nanoscale Dynamics*; Springer Series in Solid State Science⁴⁶ © (2006) with permission from Springer Science & Business Media New York). (b) The magnetic intersite-coupling in

magnetite resembles that of a collinear ferrimagnet in which the $[\text{Fe}^{3+}]_{\text{Oh}}$ and $[\text{Fe}^{3+}]_{\text{Td}}$ contributions cancel, such that the net magnetic moment results from surplus ferrous ions (reprinted from Pastoriza-Gallego et al - *Study of Viscoelastic Properties of Magnetic Nanofluids: An Insight into Their Internal Structure*⁴⁷ © (2013) with permission from The Royal Society of Chemistry). The magnetic moment is preferentially aligned along the anisotropy axis in the [111] directions of the crystal lattice. (c) Single domain particles exhibit superparamagnetic behavior in the size range from 3-10nm. Larger particles yield emerging ferromagnetic behavior and show progressive formation of multiple domains (reprinted from Pastoriza-Gallego et al - *Study of Viscoelastic Properties of Magnetic Nanofluids: An Insight into Their Internal Structure*⁴⁷ © (2013) with permission from The Royal Society of Chemistry). (d) The magnetic ordering and response of atomic momenta in the presence and absence of a magnetic field is shown on the bottom to illustrate the various forms of magnetism (reprinted from Kolhatkar et al - *Tuning the Magnetic Properties of Nanoparticles*⁴⁸ © (2013) with permission from MDPI).

Superparamagnetic iron oxide nanoparticles (SPIONs) are effectively uniaxially magneto-anisotropic despite their spherical shape.^{40,43,49} For such materials the particle energy in an external magnetic field depends on the orientation of the crystal's anisotropy axis with respect to the field vector which becomes minimal if the two coincide. An alignment of the particle's magnetization direction with respect to the external field is associated with overcoming a rotational energy barrier that depends on composition and particle size. This stored energy can subsequently be dissipated by Néel and Brownian relaxation as heat upon removal of the aligning field. Néel relaxation is a universal property of magnetic materials that depends on core volume and refers to reorientation of the magnetic moment between different easy directions. Brownian relaxation is related to viscous rotation of the entire particle and is thus medium specific and depends on the hydrodynamic volume. The core diameter d_{core} therefore determines the fundamental absorber strength (SAR) and achievable heating rate k_{heat} of SPIONs. Its contribution is preferentially optimized for biomedical applications since it is independent of the local microenvironment and constitutes the lower bound for magnetic heating efficiency, e.g. in cases of restrained particle rotation in viscous matrices like membranes.⁴⁰

Iron oxides are less magnetic than many other high-performance alloys due to lack of heavy metals but can also due to this lack be classified as non-toxic and they are FDA approved for biomedical applications.^{38,39,50} Iron oxides in acid environments dissolve into their constituent ions which are readily absorbed by the body.⁵¹ Iron is generally consumed by hematopoietic tissues in which it is supplied to mitochondria for heme-biosynthesis or stored in cytosolic and lysosomal hemosiderin.^{52,53} An

overload is handled by production of additional non-transferrin bound iron circulating in a chelatable, low molecular weight form.⁵⁴ The latter is weakly complexed to albumin, citrate, amino acids and sugars thus removed from the freely circulating pool and deactivated.^{54,55} Complexed iron accumulates in non-hematopoietic tissues, in particular the liver, endocrine organs, kidneys and heart.⁵⁶ The toxicity of iron oxide nanoparticles is mainly related to uncapped, high energy surface states which catalyse oxidation reactions in a Fenton type way in-vivo thereby inducing cellular stress responses.³⁸ The cytotoxicity of SPIONs, at concentrations of up to 200µg/ml, has been tested via various methods such as the cell-life cycle assay, MTT assay, comet assay, TUNEL assay but no significant toxicity was detected.^{40,57,58}

Among the diverse available methods to synthesize iron oxide nanoparticles non-aqueous, high temperature methods generally give the best results and yield highest quality particles in terms of size distribution, crystallinity, shape and aggregation state.^{38,59,60} Aqueous co-precipitation and sol-gel methods are cheap, easy to functionalize and of high throughput, but suffer from polydisperse size distributions, irregular shapes with unknown surface area, amorphous crystal subpopulations and often uncontrolled aggregation. Microemulsion and hydrothermal technologies ameliorate on size control and crystallinity but are not yet on a par with high temperature organometallic schemes. Likewise aerosol and vapor methods have several intrinsic drawbacks, most notably related to uniformity and surface composition of the particles.

Non-aqueous high temperature approaches are based on the thermal decomposition of an organometallic precursor in high boiling surfactant solution. Surfactant micelles in a non-coordinating solvent capture the organometallic precursor upon injection to form metal-surfactant complexes in solution.⁶¹ A burst nucleation at the supersaturation threshold leads to an instantaneous formation of non-magnetic clusters that start growing collectively above the decomposition temperature of the surface complexes.^{62,63} Separation of nucleation and growth periods is essential to the synthesis of monodisperse particles.⁶²⁻⁶⁴ Such fine tuning of the reaction kinetics and other characteristics like the resulting particle shape and aggregation state of the ferrofluid can be well controlled via the affinity of the ligand. Selective capping of particular crystal planes can favor anisotropic growth while surfactant adsorption in general prevents agglomeration of the synthesized cores.^{61,65} The high temperature

required to decompose metal-surfactant bonds serves to improve crystallinity of the lattice but entails a dense hydrophobic shell with largely unknown decomposition products that need to be exchanged for irreversibly bound ligands to avoid undesired partitioning into the surrounding and to establish a defined interface chemistry.⁶⁶ An intended incorporation into membranes with stringent space conditions favors the application of high temperature methods to optimize the control of the size and shape. However, the pre-existence of a ligand shell on such particles requires advanced methods to assemble and control the composition of a densely grafted shell around the cores that can be used to control the colloidal properties for membrane assembly.

Surface functionalization of SPIONs with biocompatible high-affinity ligands can reduce toxic effects by passivating reactive surface sites and is also vital for stabilizing SPIONs to avoid a change of magnetic characteristics via oxidation or aggregation of the individual cores.^{38,50} Magnetite itself is not long-term stable with respect to oxidation under prevalent conditions but is quite easily transformed to maghemite, a thermodynamically more stable defect structure of reduced magnetic moment (14% per unit volume).⁶⁷ Truncations of the bulk lattice lead to surface relaxation and reconstruction which are typically neglected on macroscopic scales but are striking for nanomaterials since much of their properties are related to the high surface to volume ratio. Notably magnetic materials exhibit spin canting effects that diminish the effective magnetization via formation of a spin disordered surface layer.⁴³ Surface termination and relaxation phenomena are dependent on the exact nature of the lattice truncation and are of particular interest to catalysis since the exposed configurations determine nanoparticle reactivity^{68,69}. For magnetite the lowest-energy and hence most likely terminations occur along the [001] and [111] crystal planes.^{68,69} A protective surface coating not only serves to stabilize the individual cores but also allows for dispersion in various environments.

A multitude of options is available for the surface functionalization of iron oxide nanoparticles.^{50,70–75} Possible monomeric ligands include inorganic anchors like phosphates⁷⁴ and silica⁷⁵ while organic capping agents primarily focus on carboxylic acids^{38,66} and ene-diol ligands.^{50,76} Mere polymeric stabilizers typically adhere via low affinity interactions and hence only physisorb to the particle surface, while monomeric anchors often provide strong chelation and tight binding.⁵⁰ Monomeric high affinity

anchors are a prerequisite for well-defined core-shell nanoparticles displaying a controlled interface, since only through such anchors can unidirectional and irreversible grafting of ligands at high densities be achieved. Anchoring organic ligands to the surface is a delicate balance of complexation strength and electronic structure considerations to grant efficient surface passivation while leaving the magnetic properties largely unaltered.

Figure 6: Surface structures at the magnetite interface. (a) The different surface terminations that can result from possible truncations of the bulk lattice generally determine the surface reactivity (reprinted from Ritter et al - *Fe₃O₄ (111) Surface Structure Determined by LEED Crystallography*⁶⁸ © (1999) with permission from Elsevier Science B.V.). (b) Chelation of under-coordinated surface sites by monomeric, high-affinity catechol ligands restores the bulk coordination pattern and ensures efficient functionalization and passivation of the top layer. The irreversible grafting of such ligand shells to inorganic cores enables the production of well-defined core-shell nanoparticles (reprinted from Chen et al – *Fe₂O₃ Nanoparticle Structures Investigated by X-Ray Absorption Near-Edge Structure, Surface Modifications, and Model Calculations*⁷⁷ © (2002) with permission from The American Chemical Society).

For biomedical applications efficient dispersant capping is not only mandatory to guarantee reproducible manufacturing of magnetic characteristics, stable dispersion, introduction of stealth behavior or functionality, but also to avoid undesired interactions owing to an ill-defined interfacial chemistry. Ligand binding is generally thought to restore the bulk coordination and to passivate surface sites thus protecting the core and reducing surface related defects.^{76,77}

A particularly prominent class for the surface modification of metal oxides comprises bioinspired, mussel adhesives based on catechol due to their broad applicability and excellent adhesion properties even under harsh environmental conditions.^{50,71,78} Nitrocatechol anchors in particular have proven extremely useful in that they exhibit

high affinity and largely retain the magnetic properties while still being easy to functionalize and cost-efficient.^{50,71} The introduction of a nitro group causes a drastic lowering of the pKa of the chelating diol motif via inductive and mesomeric effects, thus increasing complexation strength around physiological conditions.⁷¹ The achieved grafting density is a central parameter that determines steric stabilization, effective particle size and interaction potential with surrounding solutes for a given ligand. In this thesis mainly fatty acid derivatives of nitrodopamine serve as spacers for the creation of dense hydrophobic shells to screen the interactions between individual cores and stabilize them in hydrophobic environments such as the interior of amphiphilic membranes. A densely grafted and stable shell has to be obtained through a suitable high-affinity anchor. The reproducible manufacturing of well-defined core-shell architectures allows for prediction of all colloidal and physicochemical properties and is a prerequisite to clinical approval.

1.4. Magneto-Responsive Vesicles as Theranostic Platforms

Owing to their biocompatibility, therapeutic and diagnostic potential, SPIONs represent a cutting-edge tool for nanomedicine.^{38,40,50} Many of their properties are related to magneto-responsive effects. Notably, iron oxide nanoparticles feature magneto-thermal heating and paramagnetic relaxation enhancement upon actuation with alternating magnetic fields (AMF), which are both of paramount importance for clinical purposes. Pioneering work on biomedical SPIONs was devoted to their capacity for hyperthermia treatment with the goal to reduce side effects of traditional chemo- and radiotherapies and to construction of next generation contrast agents for magnetic resonance imaging (MRI) to provide high-resolution 3D images of soft tissue.⁴⁰ Hyperthermia treatment makes use of intrinsic temperature differences of healthy and cancerous tissue in combination with magneto-thermal heating as a means to locally raise the temperature to harm pathological cells.^{40,79} For sole irradiation with external fields the power deposition pattern however lacks selectivity. An improved spatial control of the energy transfer can be obtained after doping the therapeutic site with a sensitizer.^{40,79} Many nanomaterials can act as strong local converters for external triggers but the combination of SPIONs and magnetic field irradiation proved particularly valuable over other externally applied actuation modalities like microwave, ultrasonic or light based treatments because of the low susceptibility of tissue to magnetic fields and the biological compatibility of iron oxide.^{40,79} For a given sensitizer material, the specific absorption rate (SAR) which determines the field-induced heating of the site is very precisely determined by the volume ratio of the sensitizer material in the tissue.⁴⁰ The prime application of SPION ferrofluids is magnetic resonance imaging and *local* hyperthermia since the required concentration profiles of absorbers at the target site are only seldomly achieved for *regional* heat treatments even via active accumulation strategies like magnetic- or immuno-targeting.⁴⁰ This is mainly related to limitations originating from physiologically acceptable field strengths, accumulation kinetics and dependence of the heating rate on core size and surrounding medium.⁴⁰

The incorporation of low SPION concentrations however turns out to be an invaluable tool for regional applications when merged with thermoresponsive, vesicular drug carriers into magnetosomes that enable to release therapeutics at the pathological site upon application of an external magnetic field trigger.^{29,80,81} The grafting of

functional and antifouling high-Mw dispersants to monodisperse iron oxide cores is still highly researched for diagnostic and industrial applications^{75,82,83} but operational therapeutic approaches have shifted from hyperthermia applications towards the design of stimuli-responsive vesicular delivery vehicles. Self-assembled nanoparticulate systems are attractive alternatives for regional treatments due to their structure and compositional flexibility by which they can serve as efficient carriers for diverse therapeutic cargo while simultaneously being tailorable to specific needs, e.g. via surface modification with targeting ligands or transformation into paramagnetic contrast agents through co-incorporation of magnetic nanoparticles. The implementation of responsiveness to biologically available actuators such as temperature, pH, redox potential or subcellular localization was a major advent improving the performance of such systems and is usually achieved by designing vesicle-forming amphiphiles that break down membrane integrity to trigger burst release or rearrange their packing patterns to switch permeability upon the stimulus.^{8-12,84} Various elaborate systems are available, that, nowadays constitute some of the most successful multipurpose systems in immediate use.¹³⁻¹⁵

A particularly appealing approach towards universal release is to structurally incorporate membrane components that can respond to external field triggers and therefore can cause on-demand release without relying on local environmental conditions.^{29,81,85} As for hyperthermia applications, size-tailored inorganic nanomaterials can serve as a strong local enhancer for selective external field triggers. Coupling of the external field to the sensitizer material supplies energy to the system which is subsequently dissipated as heat upon removal of the field trigger. This magneto-thermal actuation in conjunction with thermoresponsive vesicles can be used to release encapsulated cargo at the therapeutic site.^{29,85-87} Such field-triggered release offers decisive advantages like control over the release profile by switching of the stimulus and delivery in locations where direct changes to the environment are insufficient to cause a significant response.

Figure 7: Magnetically triggered release of cargo from stealth liposomes via external actuation of membrane embedded SPIONs. Irradiation with an alternating magnetic field heats the thermoresponsive amphiphiles in the vicinity of the incorporated SPIONs to above their melting temperature which is accompanied by a change in their packing pattern and vesicle permeability (reprinted from Amstad et al – *Triggered Release from Liposomes through Magnetic Actuation of Iron Oxide Nanoparticle Containing Membranes*²⁹ © (2011) with permission from The American Chemical Society).

Fundamental design principles of triggered, field-induced release from targeted PEG-liposomes have been demonstrated^{7,29,80} just like the combination with simultaneous MRI monitoring.^{36,86,88,89} Most prepared systems however suffer from one or the other shortcoming depending on the preparation method. Co-incorporation of water soluble SPIONs and pharmaceutical agents in the lumen of lipid vesicles was first demonstrated.^{29,90–92} Major drawbacks have been shown for this approach. First, SPIONs that are not properly stabilized interact with the liposome membrane and causes leakage, but properly stabilized SPIONs take up large volume. Second, heating by SPIONs in the lumen to induce a thermal transition requires heating of the entire environment to change the permeability of the membrane due to the high thermal transport of water.²⁹ In contrast, hydrophobic SPIONs embedded in the lipid vesicle's bilayer were shown to be greatly advantageous by directly acting on the capsule wall, thereby allowing for effective release without strong heating of the ambient when actuated by alternating magnetic fields.^{29,85} The drawback here is that the embedding efficiency heavily depends on particle size, i.e. the bending energy of the lipid bilayer around the incorporated particles^{93,94} and that residual impurities from SPION synthesis and coating exchange are likely to partition into the bilayer and thereby affect membrane characteristics. Polydisperse and multilamellar assemblies are generally of limited use for release applications. Standard methods that enforce

size and lamellarity control for liposomes were shown to cause very low SPION loading.²⁹ Although the feasibility of such magnetic carriers for release has been demonstrated,²⁹ to date, control over high loading of monodisperse hydrophobic SPIONs in the membrane and simultaneous control over vesicle architecture has not been achieved. This obstructs the commercialization and efficient formulation of controlled magnetoliposomes.

SPIONs have also been incorporated into various polymeric assemblies.^{86–89,95–98} Recent studies on the co-self-assembly of high molecular weight, nonresponsive poly(acrylic acid-*b*-styrene) block copolymers with SPIONs demonstrated that nanoparticle distribution within polymeric assemblies can be controlled by solvent selection; diverse structures such as micelles, core-shell structures and polymersomes incorporating dense and diffuse layers of SPIONs could be prepared.^{95,96} Magnetically actuated release of cargo from SPION-loaded copolymer vesicles has so far only been demonstrated for poly(trimethylene carbonate-*b*-glutamic acid) with membrane embedded SPIONs^{86,87} and dual responsive nanogel-caged poly(acrylic acid-co-distearin acrylate) with SPIONs encapsulated in the aqueous lumen.⁹⁹ Hydrophobic doxorubicin associated with the membrane of the polymersomes was released by nanoparticle-mediated hyperthermia in these cases. The incorporation of SPIONs in these examples seems to have led to rather undefined structures with aggregated particle clusters, which could explain high passive leakage of the membrane-associated drug.^{86,87,97,99} Also, although local hyperthermia increased the release rate in agreement with predictions, the polymersome membrane for PTMC-*b*-PAA did not undergo a structural phase transition similar to what was demonstrated for the plain polymer vesicles in response to bulk heating¹⁰⁰ or release was primarily due to pH induced disassembly of the outlayered polyelectrolytes.⁹⁹ The slow rate of doxorubicin released from the membrane was therefore only marginally faster than the passive leakage without application of a magnetic field.^{86,87,99} Yet the purely polymer-based vesicle systems presented seem to be insufficiently responsive to magneto-thermal heating, in particular compared to the reported passive release profiles.^{86,87,99}

2. Scope of this work

The aim of this work is to assemble magnetosomes of the highest possible performance for encapsulation and release applications.

A first requirement towards reproducible assembly of multipurpose capsules is the production of magnetic core-shell nanoparticles with highly uniform and precisely controlled physicochemical characteristics. Accurate control of the nanocrystal size distribution and surface chemistry is needed since actuation efficiencies depend on absorber quality, core size and effective concentration. The usage of non-aqueous high-temperature methods is envisioned for this thesis since such approaches provide nanoparticles of highest quality regarding dispersity, aggregation state and crystallinity. Non-aggregated and monodisperse SPIONs are required in order to maintain minimal passive leakage of the assemblies and to grant homogeneous magneto-thermal heating of the membrane. An equally important consideration relates to control over the shell composition and interface chemistry; affinity, grafting density and composition of the shell determine colloidal stability of the dispersed cores and govern their interactions with surrounding amphiphiles in the membrane. The synthesis of such monodisperse and irreversibly grafted core-shell nanoparticles is thus a necessary precondition for the controlled assembly and integrative performance of high-end magnetosomes.

Further, the development of a universal method that attains the goal of controlled, quantitative embedding of these particles into amphiphilic superstructures is needed. Control over the SPION content and nanoparticle distribution is of particular interest to practical applications since susceptibility to magnetically triggered release and magnetic contrast are enhanced in direct proportion to the encapsulated sensitizer fraction. Methods for nanoparticle co-encapsulation that simultaneously allow for control over the vesicle size distribution, lamellarity and loading content of hydrophobic particles on a large scale remain a bottleneck. The main goal here is to develop such a method that not only allows to prepare defined magneto-responsive delivery vehicles but that is also compatible with direct drug loading methods. The release of an entrapped model compound upon irradiation with alternating magnetic fields should be efficient but simultaneously the magnetosomes have to display negligible passive leakage. This concept shall first be tested with the development of a new assembly method for magnetosomes from lipids and optimized

superparamagnetic iron oxide nanoparticles. Magnetoliposomes are limited in particle loading capacity and mechanical stability but constitute a well-researched benchmark for the gauging of different preparation methods. After evaluation, an extension to tough magnetosomes with tunable membrane properties, e.g. diblock copolymer vesicles and hybrid mixed-membrane capsules is intended to improve vesicle stability and actuation efficiency for future applications. A most important aspect for this work is that the superior mechanical and structural properties of superamphiphiles can be used to improve on the magneto-thermal responsive properties demonstrated for liposomes, and that well-defined molecular and nanoparticle building blocks enable controlled co-assembly of advanced responsive superstructures.

3. Publications

3.1. Complete Exchange of the Hydrophobic Dispersant Shell on Monodisperse Superparamagnetic Iron Oxide Nanoparticles

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Motivation

First generation superparamagnetic iron oxide nanoparticles (SPIONs) are typically characterized by polydisperse size distributions and low grafting densities yielding inhomogeneous ensemble properties in terms of magnetic characteristics and interfacial chemistry.^{38,59,101} Facile and inexpensive aqueous co-precipitation yields large quantities of irregular colloids that are stabilized solely by electrostatic repulsion. These particles are easy to surface functionalize but require further size selection processes. More advanced sol-gel strategies improve on crystallinity, shape control and reduce aggregation through ligand capping but still suffer from polydispersity.^{38,67,101} Insufficient size control strongly affects the performance of nanomaterials as confinement and surface effects account for much of their properties.¹⁰¹ Key characteristics for SPION selection towards applicability in magnetically actuated delivery are magnetic moment and surface area which both crucially depend on the particle size distribution. That is, membrane embedding of hydrophobic SPIONs is severely restricted to a few nanometers in core-shell diameter by stringent spatial requirements of the bilayer⁹³ but at the same time such ultrasmall SPIONs only tend to generate modest magnetothermal heating since the usable net moment scales with core volume.^{40,102} Other considerations involve ligand density and binding strength which determine the interfacial chemistry of the particle shell.⁷¹ The latter generally affects aggregation behavior, solubility and environmental interactions.^{50,71} Recent advances in high temperature decomposition approaches of organometallic precursors enabled the production of high quality SPIONs with extremely uniform properties and tunable sizes in the nanometer range that allow for direct biomedical applicability without further size selection.^{60,66} However this strategy requires advanced surface modification strategies that irreversibly replace the capping agent while still leaving the magnetic properties unaltered. Ideal anchors need to be delicately balanced since too low affinity yields colloiddally unstable particles with undefined ligand interactions and too strong complexation results in uncontrolled surface dissolution.^{50,71} Nitrocatechol derived anchors have proven particularly useful in this respect while still being cost-efficient and relatively easy to synthesize.⁷¹

The aim of this work is to demonstrate a strategy that attains the goal of irreversible ligand grafting at maximal density to produce hydrophobic, high-performance SPIONs for biomedical applications in drug delivery. So far research was mainly

focused on high-Mw dispersants that render SPIONs hydrophilic and anti-fouling for stable dispersion in biological media.^{50,75,82,103} For these, incomplete exchange is however foreseen due to the contradicting requirement of anchoring bulky entities at maximal surface coverage.¹⁰³ The effects of mixed dispersant particles with surface capping agents of different binding strengths are largely unknown. For hydrophilic particles with high-affinity polymer-dispersants the effects of mixed ligand shells are not of utmost importance because of superficial shielding by the polymer and insolubility of hydrophobic capping agents in aqueous phases. For hydrophobic SPIONs in immediate contact with amphiphilic superstructures this is however a key issue owing to desorption and partitioning of residual impurities into the hosting assemblies. Residual surfactants are known to interfere with amphiphile packing, alter membrane properties, membrane-drug interactions and to modulate enzyme activity.^{104–106} Moreover there is considerable disparity in the literature on nanoparticle induced effects on membranes which are likely to originate from partitioning of reversibly-bound ligands into membranes.^{85,93,107–112} In this respect it is vital to thoroughly characterize possible remnants of surfactants and to establish procedures for their complete removal.

Contribution:

I designed, conducted, and evaluated all experiments, and co-wrote the manuscript.

Complete Exchange of the Hydrophobic Dispersant Shell on Monodisperse Superparamagnetic Iron Oxide Nanoparticles

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Supporting Information

ABSTRACT: High-temperature synthesized monodisperse superparamagnetic iron oxide nanoparticles are obtained with a strongly bound ligand shell of oleic acid and its decomposition products. Most applications require a stable presentation of a defined surface chemistry; therefore, the native shell has to be completely exchanged for dispersants with irreversible affinity to the nanoparticle surface. We evaluate by attenuated total reflectance–Fourier transform infrared spectroscopy (ATR–FTIR) and thermogravimetric analysis/differential scanning calorimetry (TGA/DSC) the limitations of commonly used approaches. A mechanism and multiple exchange scheme that attains the goal of complete and irreversible ligand replacement on monodisperse nanoparticles of various sizes is presented. The obtained hydrophobic nanoparticles are ideally suited for magnetically controlled drug delivery and membrane applications and for the investigation of fundamental interfacial properties of ultrasmall core–shell architectures.

INTRODUCTION

High-temperature synthesized superparamagnetic iron oxide nanoparticles (SPIONs) are well-defined biomedical materials with homogeneous physical properties in terms of diameter, polydispersity, surface area, and magnetic characteristics. SPIONs have an increasing number of applications, not the least in the biomedical field;^{1–3} they are used as magnetic resonance imaging (MRI) contrast agents in imaging diagnosis⁴ and in magnetic-field-assisted cancer therapy (hyperthermia)⁵ and have emerging use in magnetically addressable drug delivery systems^{6–8} as a result of their inherent low toxicity^{2,3} and suitable size.³ Clinical approval requires reproducible manufacture of highly uniform physicochemical characteristics, which explains the increasing demand for controlled synthesis and improved surface modification approaches.³ While remarkable progress has been achieved in the synthesis of monodisperse SPIONs,^{3,4,9–11} their application requires equally well-defined and stable ligand shells with tailored surface chemistries.^{3,9} Ultrapure interfaces of that kind allow for controlled assembly in amphiphilic superstructures,^{7,12} investigation of nanoparticle–membrane interactions,^{13,14} and internanoparticle interactions in membranes, and they aid in improving the underlying design principles for future generations of drug delivery systems.^{6,7,15} A prerequisite for these studies is exclusion of impurities, such as weakly bound dispersants or residual capping agents, that might obscure nanoparticle-induced effects. Grafting of such shells to

monodisperse SPIONs has thus far received scant attention and remains a bottleneck. The challenge is that a strongly but reversibly bound ligand shell of, e.g., oleic acid (OA) and its decomposition products remains on the surface after high-temperature synthesis of monocrystalline SPIONs.

Attention has been given recently to ligand replacement of OA for hydrophilic dispersants providing steric stabilization to SPIONs, which has demonstrated difficulties in terms of lower achieved grafting density than required for strong colloidal stability.¹⁶ The likely cause is incomplete replacement of OA, as shown by isotope labeling,¹⁷ and the contradicting requirements for exchanging residual OA and densely grafting bulky polar dispersants in its place.

Ligand replacement of hydrophobic dispersants has received even less attention. Although the as-synthesized OA shell on monodisperse SPION is hydrophobic and strongly bound, it has been shown that these ligands dissolve in, e.g., membranes and cause undefined interactions and unstable aggregates.⁸ We elucidate the extent of OA on SPIONs after various purification steps and present a protocol that enables complete ligand replacement and stable hydrophobic shells to be grafted.

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■ EXPERIMENTAL SECTION

Materials. All reagents were purchased from Sigma-Aldrich and used as received (see the Supporting Information for details). To ensure maximal reproducibility, all nanoparticles and ligands originated from the same batches.

Synthesis of Superparamagnetic Iron Oxide Nanoparticles. Monodisperse, spherical SPIONs were synthesized via high-temperature thermal decomposition of an organometallic precursor in a high-boiling surfactant solution following a route adapted from Park et al.¹⁰ Size control was achieved by altering the surfactant to organometallic precursor ratio (see SI 2.1 of the Supporting Information).¹¹

Synthesis of 4-Nitrocatechol-Derived Ligands. 6-nitrodopamine-hemisulfate (NDA-HSO₄) was synthesized according to the literature, with slight modifications (see SI 1.1 of the Supporting Information).¹⁸ *N*-Palmitoyl-6-nitrodopamide (P-NDA) and its alkyl-perdeuterated analogue *N*-(d₃₁-palmitoyl)-6-nitrodopamide (d₃₁-P-NDA) were prepared by COMU-mediated peptide-coupling reactions (see SI 1.2 of the Supporting Information).¹⁹

Surface Modification of Magnetite Nanoparticles. Typically 50 mg of P-NDA (or NDA) was dissolved in 3 mL of dimethylformamide (DMF) under sonication and added to 200 mg of as-synthesized OA-SPIONs dissolved in 6 mL of chloroform (CHCl₃). The SPION–ligand mixture was combined with 9 mL of methanol (MeOH) and sonicated for 3 h under N₂. CHCl₃ was evaporated from the coating mix, and the resulting SPIONs were collected by magnetic precipitation from excess MeOH (40 mL) and purified by 3 rounds of washing and magnetic separation from cold MeOH (20 mL each) to remove excess nitrocatechol ligands.

Purification Methods of Coated Magnetite Nanoparticles. A chart of all investigated purification pathways and their results can be found in SI 3.1 of the Supporting Information.

(a) *As-Synthesized SPIONs.* The crude SPION reaction mixture was purified from reaction solvent and byproducts by five precipitations from minimal toluene (tol) into excess ethanol (EtOH) (300 mL each) and collected by magnetic separation.

(b) *OA (1 mM) in Hot MeOH Pre-extraction.* A total of 200 mg of as-synthesized OA-SPIONs (a) was washed 4 times each with 20 mL of hot (heated with heat gun) 1 mM OA in MeOH and once with pure hot MeOH.

All purification methods on newly capped SPIONs have been performed after cold MeOH washings of the coating mixtures. SPIONs were routinely collected by magnetic separation, unless otherwise stated.

(c) *Hot MeOH Extraction.* A total of 200 mg of OA-SPIONs (a or b) were subjected to the standard coating exchange and subsequently washed 3 times with 30 mL of hot MeOH each.

(d) *Standard Cold MeOH Extraction.* Newly capped SPIONs were purified according to the standard protocol by 3-fold extraction with 20 mL of cold MeOH each.

(e) *Syringe Filtering.* A total of 200 mg of washed, precipitated mixed-dispersant SPIONs were dissolved in 3 mL of CHCl₃ and purified by a single pass through commercially available filter units (0.2 μm cutoff).

(f) *Addition of Surfactants.* Surfactants were added in 100 mg quantities to 200 mg of mixed dispersant SPIONs. The mixes were sonicated in hot ethanol (EtOH) for 5 min. SPIONs were collected by magnetic separation.

(g) *Column Chromatography.* A total of 5 g of SiO₂ 60 was loaded in a 2 × 30 cm column, and 200 mg of mixed dispersant SPIONs dissolved in the mobile phase were eluted with tetrahydrofuran (THF)/MeOH (4:1).

(h) *Ligand-Saturated Column Chromatography.* A total of 200 mg of P-NDA in DMF was run twice over 5 g of SiO₂ 60, and the stationary phase was purged with 2 column volumes of THF/MeOH (4:1) prior to loading of the coated SPIONs.

(i) *Recrystallization from MeOH/*n*-Hexane.* A total of 200 mg mixed dispersant SPIONs was refluxed in 20 mL of a N₂-saturated MeOH/*n*-hexane (1:1) solvent mixture under an inert atmosphere. Recrystallization was repeated 3 times.

(j) *Base (2,6-Lutidine) as Solvent.* A total of 200 mg of as-synthesized SPIONs and P-NDA was dissolved in 5 mL of 2,6-lutidine and sonicated under N₂ for 3 h. Lutidine was evaporated under reduced pressure, and SPIONs were purified with three cold MeOH extractions (20 mL each).

(k) *P-NDA Post-coating.* A total of 200 mg of mixed dispersant SPIONs freed from physisorbed OA [prepared via pre-extraction (a) or cetyltrimethylammoniumbromide (CTAB) addition (f)] was subjected to an additional round of coating with 100 mg of P-NDA in a minimal amount of N₂-saturated 2,6-lutidine at 50 °C for 48 h under an inert atmosphere and magnetic stirring. Lutidine was completely evaporated under reduced pressure, and the post-coating mix was washed 3 times with hot MeOH (30 mL each).

(l) *d₃₁-P-NDA Post-coating.* Pre-extracted OA-SPIONs (b) were ligand-exchanged with d₃₁-P-NDA and purified via hot MeOH extractions as in step c. Post-coating with d₃₁-P-NDA was analogous to step k.

(m) *NDA Post-coating.* Mixed dispersant NDA-HSO₄-coated SPIONs obtained via the standard coating protocol were isolated by evaporating the coating mix to the DMF fraction, adding 10 volumes of *n*-hexane and collecting the SPIONs via magnetic separation. The particles were post-coated by adding 100 mg of NDA-HSO₄ and decocing the dispersion under an inert atmosphere 3 times with each hot *n*-hexane, hot Me₂CO, and hot MeOH (30 mL per wash). NDA-SPIONs were collected by magnetic separation and finally lyophilized from ultrapure water.

Characterization. Transmission Electron Microscopy (TEM). TEM studies were performed on a FEI Tecnai G2 20 transmission electron microscope operating at 120 kV or at 200 kV for high-resolution imaging. Samples were prepared by drop-casting toluene dispersions onto 300-mesh carbon-coated copper grids and evaporating the solvent in air. Size distributions were evaluated using PEBBLES.²⁰

Thermogravimetric Analysis/Differential Scanning Calorimetry (TGA/DSC) Measurements. Thermograms were recorded on a Mettler-Toledo TGA/DSC 1 STAR system in the temperature range of 25–650 °C with a ramp of 10 K/min under 80 mL/min synthetic air gas flow. Typically, 0.5–2 mg of sample was used per run, and the rest of the mass was evaluated at 500 °C by horizontal setting.

¹H and ¹³C Nuclear Magnetic Resonance (NMR) Measurements. ¹H and ¹³C solution spectra were collected on a Bruker DPX operating at 300 MHz using tetramethylsilane (TMS) as an internal standard.

Electrospray Ionization–Mass Spectrometry (ESI–MS) Measurements. Mass spectra were collected using a Q-ToF Ultima ESI (Waters, Milford, MA) mass spectrometer in positive-ion mode. Samples were diluted with NH₄⁺HCOO[−] buffer (65 mM, pH 3) or MeOH to a final concentration of 5–500 μg/mL.

Attenuated Total Reflectance–Fourier Transform Infrared Spectroscopy (ATR–FTIR) Measurements. Mid-infrared (IR) powder spectra of the lyophilized samples were collected using a Bruker Tensor 37 FTIR spectrometer with Bruker Platinum Diamond single-reflection ATR equipment at a resolution of 4 cm^{−1} by averaging 32 scans.

Ultraviolet–Visible (UV–Vis) Measurements. UV–vis absorption spectra were collected at a scan speed of 400 nm/min on a Hitachi UV-2900 spectrophotometer.

■ RESULTS AND DISCUSSION

Figure 1 shows TEM micrographs of the as-synthesized, OA-capped magnetite nanoparticles with narrow size distributions of 3.5 ± 0.4, 5.0 ± 0.4, and 8.3 ± 0.4 nm.

No difference was observed between sonicating and stirring OA-capped SPIONs in P-NDA ligand solution at an elevated temperature to perform the exchange step. Sonication was chosen because of the shorter time of preparation.

A large variety of purification and post-coating methods were tested for their potential to purify P-NDA-coated SPIONs from

Figure 1. TEM of spherical, monodisperse magnetite nanoparticles with narrow size distributions of (a) 3.5 ± 0.4 nm, (b) 5.0 ± 0.4 nm, and (c) 8.3 ± 0.4 nm and (d) high-resolution (HR)-TEM of the 8.3 ± 0.4 nm SPIONs in panel c.

residual OA. Their efficiencies were evaluated by ATR-FTIR and TGA/DSC.

Figure 2 shows the IR spectra of 3.5 nm SPIONs after purification with various methods enumerated as c–m. The purification steps c–m are briefly described with motivations for why they were chosen in the following. Repeated pre-extraction with hot MeOH containing 1 mM OA as a stabilizer (b) to prevent aggregation,²¹ (c) hot MeOH extractions, (d) cold MeOH extractions, and (e) syringe filtering [polytetrafluoroethylene (PTFE)] were used as common, quick, and low-cost methods to remove excess unbound organics and aggregates.¹ In step b, oleate monolayer-covered SPIONs are subjected to a single exchange step, while newly capped SPIONs derived from as-synthesized particles were purified in step c. The addition of surfactants (f), such as cationic cetyltrimethylammoniumbromid (CTAB), was tested because OA forms complexes with countercharged surfactants that might impede re-adsorption to the particle surface and prevent aggregation during the exchange of the coating.²¹ The influence of the charge of the surfactant on the removal of OA was studied by additionally using anionic sodium dodecyl sulfate (SDS) and neutral 1-octanol (OctOH) (see SI 3.4 of the Supporting Information). Column chromatography over SiO₂ with (h) and without (g) P-NDA adsorbed to the stationary phase in THF/MeOH (4:1) was tested as a ubiquitous purification process for surface-active species that could capture OA but leave P-NDA as a result of its lower affinity to SiO₂.²² Recrystallization (i) from MeOH/*n*-hexane (1:1) relies on dissolving the less tightly bound capping agent from the particle surface. The base 2,6-lutidine was similarly tested as solvent (j), which was inspired by the fact that weak hydrogen-bond acceptors significantly speed up the rate of nitrocatechol surface complexation.²³ Lutidine was chosen because it is a sterically hindered, moderate, medium-polar base serving multiple purposes as it acts as a solvent for OA-SPIONs, sufficiently

Figure 2. ATR-FTIR spectra of various 3.5 nm magnetite nanoparticle preparations: (a) as-synthesized SPION containing excess physisorbed OA, (b) purified SPION with a monolayer of chemisorbed oleate, (c–j) mixed dispersant OA/P-NDA-SPION and (l–m) post-coated, pure nitrocatechol-ligand-capped SPIONs. SPIONs were purified by the following methods: (b) pre-extraction in hot MeOH containing 1 mM OA as a stabilizer, (c) hot MeOH extraction, (d) cold MeOH extraction, (e) syringe filtration (PTFE), (f) surfactant addition (CTAB), (g) silica column chromatography (THF/MeOH = 4:1), (h) ligand saturated chromatography, (i) repeated MeOH/*n*-hexane (1:1) recrystallization, (j) 2,6-lutidine as a solvent, (k) post-coated P-NDA-SPION, (l) post-coated d₃₁P-NDA-SPION, (m) post-coated NDA-SPION, and (n) SPION with combusted shell (post-TGA) with 10× scaled inset of residual absorptions. Peaks corresponding to physisorbed and chemisorbed OA are indicated by circles and asterisks, respectively, while crosses depict bands related to nitrocatechol ligands. The post-coated P-NDA (k), d₃₁P-NDA (l), and NDA particles (m) are the only particles that demonstrate an absence of the characteristic OA bands and, therefore, complete ligand replacement.

deprotonates P-NDA, and at the same time, offers the possibility to form ion pairs with OA without complexing the SPION surface strongly. Finally, post-coating strategies (k–m) were devised that emphasize the role of competitive binding to the particle surface; i.e., a high-affinity ligand (in our case, a strongly adsorbed OA-related species) can be displaced by a large excess of other ligands if it has a non-negligible desorption rate (k_{off}). Post-coating with P-NDA (k) and d₃₁P-NDA (l) involved exchange of mixed dispersant particles at 50 °C in 2,6-lutidine containing excess P-NDA for 48 h under an inert atmosphere, while NDA post-coated particles (m) were prepared by selectively desorbing oleate in the presence of excess nitrocatechol ligand, which could adsorb at emerging surface sites.

Association of OA with the primary surfactant layer on the particle surface to quasi-bilayer structures was reported to occur though intercalation of alkyl chains in combination with hydrogen bonding between the headgroups.¹⁵ The presence of physisorbed OA was, therefore, determined from absorptions at 1705 cm⁻¹ in FTIR spectra assigned to the C=O stretch of hydrogen-bonded (dimeric) alkyl carboxylic acids and from other bands characteristic of free acid groups, such as the OH in-plane and out-of-plane bending vibrations at 1430 and 965 cm⁻¹ (see SI 2.2 of the Supporting Information for further assignments).

Oleate complexed to the particle surface was evaluated at 1605, 1520, and 1410 cm⁻¹, corresponding to two asymmetric and one symmetric stretching vibrations of the carboxylate headgroup.²⁴ The respective positions and the split between

$\nu_{as,s}(\text{COO}^-)$ are indicative of the binding mode of the carboxylate headgroup to Fe ions.^{24,25} For perdeuterated samples, oleate exchange was concomitantly monitored by comparing $\nu_{as,s}(\text{CH}_2)$ integral ratios of the newly capped SPIONs to the respective oleate monolayer-covered particles. Fe–O lattice vibrations of magnetite are found at 580 and 385 cm^{-1} corresponding to T_{1u} optical phonon modes of the crystal lattice.^{26,27}

For half of the tested purification methods, there is a substantial amount of unbound or physisorbed OA left (entry 1 in Table 1). Even extracting the coating mix 3 times with cold

complexed oleate. The TOC is strongly reduced and corresponds to the amount that could be expected for a mixed OA/P-NDA monolayer coverage, although an exact grafting density cannot be calculated because the composition of the shell is not known as a result of the remaining complexed OA^- . Interestingly, the improved removal of OA was almost independent of surfactant charge (CTAB, SDS, OctOH; see SI 3.4 of the Supporting Information). Surfactant bands were not detected after the washing steps. CTAB led to least losses during collection of the coated particles.

Residual complexed oleate might be strongly integrated into the shell and possess a low desorption rate. Finding a procedure for complete replacement of the ligand is necessary to ensure uniform SPION shell properties and a stronger binding through nitrocatechol anchors. In a final approach, removal of oleate was, therefore, tested by adding a post-coating step in excess ligand at an elevated temperature. Particles (b or e) were stirred at 50 °C in 2,6-lutidine containing excess P-NDA for 48 h under an inert atmosphere.

FTIR spectra recorded after the post-coating step show markedly decreased absorptions at 1605, 1520, and 1410 cm^{-1} , concomitant with a narrowing and blue shift of the 1645 cm^{-1} band and a slight decrease in the overall $\nu_{as,s}(\text{CH}_2)$ and $\delta_s(\text{CH}_2)$ intensities (see SI 4.3 of the Supporting Information). This is attributed to removal of surface-bound oleate. Complete exchange was additionally verified by post-coating with isotope-labeled $\text{d}_{31}\text{P-NDA}$. Perdeuteration of the alkyl chain leads to a decrease in vibrational frequency and, hence, results in spectral separation of new dispersant and native ligand signals. Noteworthy, the $\nu_{as,s}(\text{CH}_2)$ integral area of the post-coated sample, representing persistent surface-bound oleate, dropped by 22 times compared to its initial value on OA monolayer-covered particles (see SI 4.3 of the Supporting Information). This corresponds to a 96% reduction in the TGA-derived number of initial oleate molecules (see SI 4.2 of the Supporting Information) and roughly 4 remaining oleate molecules per particle. Similarly, OA exchange for NDA- HSO_4 exhibits only marginal CH_2 intensities, which closely correspond to the signal of the pure ligand. Thus, efficient stripping and replacement of the original OA stabilizer is achieved in both post-coating procedures.

The loss of intensities at 1605, 1520, and 1410 cm^{-1} is ascribed to two individual $\nu_{as}(\text{COO}^-)$ and a common $\nu_s(\text{COO}^-)$ mode arising from two different complexation patterns of the carboxylate headgroup to the metal ions on the mixed dispersant particle (see SI 2.2 and SI 4.3 of the Supporting Information).²⁴ A splitting of $\nu_{as}(\text{COO}^-)$ into two separate parts largely accounts for our observations of unequal intensities between the asymmetric and symmetric carboxylate stretching vibrations and their concurrent disappearance upon post-coating. This is reasoned from the fact that $\nu_s(\text{COO}^-)$ is less affected by the coordination pattern than its asymmetric counterpart; therefore, it mostly retains its spectral position and, hence, exhibits twice the intensity of each of the $\nu_{as}(\text{COO}^-)$ modes. The 110 cm^{-1} (1520–1410 cm^{-1}) splitting hints at a bidentate, mononuclear complexation, while the second splitting of 195 cm^{-1} (1605–1410 cm^{-1}) could be due to contributions of either a unidentate or binuclear, bridged OA^- complex.²⁴ Unidentate coordination is rarely found and not supported by our data because we have no indication of an uncomplexed carbonyl around 1710 cm^{-1} . Induced binuclear binding is an appealing explanation for the necessity of a second coating step, in which more strongly bound oleate is

Table 1. TGA Results for Various SPION Preparations

d_{core} (nm)	preparation method	TOC ^a (%, w/w)	$\rho_{\text{graft}}^{\text{a}}$ (nm^{-2})	$\rho_{\text{chain ends}}^{\text{a}}$ (nm^{-2})
3.5	a, d, e, g, h, and j	35–78	nd	nd
3.5	b	28–29	2.2–2.3	0.35–0.37
	c, f, and i	28–30	nd	nd
3.5		38–39	2.6–2.7	0.35–0.4
5	k	32–35	2.8–3.2	0.6–0.7
8.3		22–23	2.8–3.1	1.0–1.1
3.5		26–28	2.2–2.9 ^b	0.9–1.2 ^b
5	m ^b	24–26	2.8–3.5 ^b	1.4–1.8 ^b
8.3		14–15	2.4–2.9 ^b	1.6–1.9 ^b

^aAverage of three measurements evaluated in the temperature interval (100–200 °C to 500 °C). ^bExpanded range of values arises from ambiguous composition of the shell (see SI 4.2 of the Supporting Information).

MeOH was insufficient to remove physisorbed OA (Figure 2d). This is in good agreement with the fact that dissociation of OA dimers is vital for complete removal of physisorbed OA and only occurs above 70 °C (curves b and c in Figure 2).²⁴

Intercalated OA is also present in samples run over the SiO_2 column, despite the fact that the samples passed as single fractions (curves g–h in Figure 2). Filtering either did not succeed in removing associated OA at all [regenerated cellulose (RC)/polyethylene terephthalate (PET)/polyvinylidene difluoride (PVDF) filter material] or introduced additional bands, which originate from dissolvable material of the filter (PTFE/nylon) (Figure 2e and SI 3.3 of the Supporting Information).

TGA combined with DSC was used to access the total organic content (TOC) associated with the purified SPIONs (Table 1 and SI 3.2 of the Supporting Information). The organic fractions of the samples were evaluated from the rest of the mass of the TGA curve according to Benoit et al.²⁸ The determination of the exact mass ratio of organic ligand to inorganic core is challenging to obtain for fatty acids, because they do not fully combust under an inert atmosphere. Instead, carbonaceous graphitic residues are formed, which cause significant reduction of the inorganic core to wüstite and ferrite above 760 °C.²⁹ Burning in air was reported to only result in moderate residues (mainly CO_x surface complexes) and oxidation of the iron oxide core to hematite at around 520 °C.²⁵ Thus, burning in synthetic air up to 500 °C was used to determine grafting densities of as-synthesized and purified core–shell SPIONs. Samples with an incomplete purification from excess OA yielded unreasonably high TOC values (entry 1 in Table 1); the grafting densities were thus not evaluated.

The purification methods in entry 2 in Table 1, e.g., through the addition of the surfactant, yielded particles that are free of physisorbed OA but still display peaks attributed to surface-

replaced on the surface and agrees better with our model of two different complexation patterns, where the $\nu_s(\text{COO}^-)$ vibrations merge. A slight change in the overall CH_2 intensities upon post-coating presumably originates from alterations in alkyl chain packing caused by different headgroup areas of the capping agents.

It is worth noting that the as-synthesized SPION spectra do not exhibit a pronounced mixture of two different chemisorbed oleate species ($\Delta = 110 \text{ cm}^{-1} \sim 90\%$) nor does washing with hot MeOH during the purification alter the observed 50:50 ratio on the mixed dispersant SPIONs. This highlights the disruption of oleate packing along with a rearrangement of the carboxylate binding induced by intercalation of nitrocatechol ligands. The presence of two oleate species with different complexation strengths has previously been noted as an explanation for differences in precursor reactivity of iron-oleate complexes in nanoparticle synthesis.²⁴ We can further demonstrate that P-NDA only fully displaces both species of surface-bound oleate through competitive binding once excess OA has first been removed, which requires two coating steps (curves k–m in Figure 2). Competitive binding could also explain why partial surface dissolution occurs when no alcohol is added during the coating procedure; this is presumably due to competition of two strongly complexing agents (P-NDA[−] and OA[−]) for surface sites, whereas protonation of the displaced species lowers their affinity.

TGA shows that the grafting density of the pure P-NDA shell is high (Table 1 and SI 4.2 of the Supporting Information). The grafting density was compared for different nitrocatechol-coated SPIONs with size varied from 3.5 to 8.3 nm (Table 1 and SI 4.2 of the Supporting Information) and found to be almost independent of surface curvature and hydrophobic substitution. Slight deviations regarding higher grafting densities for larger particles could originate from relative differences in iron oxide phase content,^{4,30} in line with preferential binding of nitrocatechols to Fe_3O_4 ²³ and/or improved crystallinity of the lattice of the larger nanoparticles. However, the observed trend is within the experimental uncertainty and curvature therefore seems to not affect the grafting density in the case of low-molecular-weight ligands. An approximately constant grafting density suggests that the limiting factor for adsorbing densely packed low-molecular-weight dispersants to the substrate is given by either the number of surface iron sites ($3.5\text{--}5.9 \text{ nm}^{-2}$ depending upon crystal plane and termination)³¹ or the spatial requirements of the nitrocatechol anchor. The latter is substantial as approximated by the molecular areas of palmitic acid ($23 \text{ \AA}^2$) and *N*-stearoyldopamine ($42 \text{ \AA}^2$),³² which suggest a maximum achievable density of 2.4 nm^{-2} . This correlates with the theoretically expected maximum coverage for dopamine (2.4 nm^{-2}) based on an estimate from the molar volume. At similar grafting density, the curvature strongly affects the density of the shell and, thereby, the specific interaction volume with the hydrophobic ligands; the latter increases with decreasing radius, as exemplified by the number of chain ends per outer surface area unit ($\rho_{\text{chain ends}}$ in Table 1 and SI 4.2 of the Supporting Information). Thus, small-sized core-shell SPIONs interact more strongly with surrounding solutes, whereas larger particles feature compact core-shell architectures.

Multistep TGA profiles observed below 400°C have previously been attributed alternatively to vaporization of physisorbed OA,¹⁵ to chemisorbed oleate species with different binding strengths,³³ or to partial cleavage of the capping

agent.²⁹ In our experiments, a significant second step in TGA was only observed when physisorbed OA or other impurities were present (Figure 3 and SI 3.2 of the Supporting

Figure 3. TGA (solid lines) and DSC curves (dashed lines) of representative 3.5 nm SPION preparations measured in synthetic air: as-synthesized OA–SPION containing excess physisorbed oleic acid (black, stars), purified OA–SPION with an oleate monolayer (gray, squares), column-chromatographed SPION with physisorbed impurities (magenta, diamonds), cold MeOH-extracted mixed dispersant SPION containing around 5% w/w physisorbed OA (green, triangles), CTAB-treated mixed dispersant SPION free of physisorbed OA (blue, circles), and spectroscopically clean P-NDA post-coated SPION (red, triangles).

Information). Also, by comparison of the FTIR spectra of the tested particles to the TGA/DSC data, the presence of minor amounts of residual-free OA (around 5% w/w) could not be reliably detected with TGA/DSC (Table 1, Figures 2 and 3, and SI 3.2 of the Supporting Information) using a difference in the decomposition profile as the basis for detection.

The differently chemisorbed oleate species observed in FTIR on mixed ligand SPIONs seem to be too close in their dissociation energies to be resolved by TGA; co-adsorbed P-NDA and chemisorbed OA also give rise to similar curves (see Figure 3). A closer inspection of the DSC curves of the main mass loss shows two distinct heat peaks corresponding to one discernible step in TGA. The two peaks are, however, also observed when only P-NDA is present on the SPIONs, and the relative peak areas vary with the preparation method. The lower *T* peak is increasingly dominant toward more complete exchange of OA for P-NDA.

Only traces of organics were left after heat treatment of the mixed dispersant SPIONs to 500°C , but clearly shifted nanoparticle absorptions became evident at 520 and 430 cm^{-1} (Figure 2n). This is also seen by eye as transformation to a purple inorganic residue. FTIR spectra of these SPIONs showed that all bands attributed to the nitro group have disappeared. Minimal residues of decomposed ligand were detected through bands at 2984 cm^{-1} [$\nu(\text{C}_{\text{sp}^2}\text{--H})$], 2896 cm^{-1} [$\nu(\text{C}_{\text{sp}^3}\text{--H})$], 2651 and $2350\text{--}2200 \text{ cm}^{-1}$ (surface CO_x), $1650\text{--}1400 \text{ cm}^{-1}$ [$\nu(\text{C}=\text{C})$ and $\delta(\text{C}_{\text{sp}^3}\text{--H})$], 1059 cm^{-1} [$\nu(\text{C--O})$], and 906 cm^{-1} [$\delta(\text{C}_{\text{sp}^2}\text{--H})$]. The persistence of minute residual ligand on the particle supports the conclusion that vaporization of loosely associated molecules is the primary cause for multistep profiles in TGA rather than partial ligand decomposition or desorption of diverse chemisorbed agents.

CONCLUSION

We have demonstrated that standard methods to functionalize the surface of hydrophobic, monodisperse SPIONs through ligand replacement result in substantial residual OA. Removal of physisorbed OA requires repeated extraction in boiling solvents to promote dissociation of carboxylic acid dimers or the addition of countercharged surfactants to impede reabsorption via ion pair formation. Even extensive purification of SPIONs prepared by a single-exchange step results in mixed dispersant particles that are characterized by a large remaining fraction of two different chemisorbed oleate species coexisting with P-NDA on the particle surface. Intercalation of nitrocatechol anchors during the initial coating exchange triggers a rearrangement of the carboxylate binding mode, which ultimately results in a mixture of mononuclear, bidentate and binuclear, bridged oleate complexes. Complete ligand exchange to an irreversibly grafted dispersant shell was only achieved after post-coating of already purified, mixed ligand nanoparticle dispersions in high excess of nitrocatechol-derived capping agents. Grafting densities of 2.5–3 molecules/nm² were obtained regardless of SPION size. Thus, we have demonstrated that monodisperse SPIONs can be equipped with an equally monodisperse, irreversibly grafted hydrophobic shell at maximal ligand density that makes them perfectly suited for further structural studies, drug delivery, and membrane applications. In further work, we will report on the highly improved performance of these particles for the creation of magnetically responsive membrane structures.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.5b01833.

Detailed synthetic and purification protocols as well as additional characterization of reagents and samples referred to in the text (PDF)

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Author Contributions

Oliver Bixner designed, conducted, and evaluated all experiments. SPIONs were synthesized by Andrea Lassenberger. Dieter Baurecht and Erik Reimhult provided guidance. The manuscript was written by Oliver Bixner and Erik Reimhult.

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Notes

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Supporting information for

Complete exchange of hydrophobic dispersant shell on monodisperse superparamagnetic iron oxide nanoparticles

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Outline

Experimental details

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SI 4 characterization of pure nitrocatechol derived SPION preparations

SI 4.1 ATR-FTIR of differently sized SPIONs

SI 4.2 calculation of grafting and chain end densities of differently sized SPIONs

SI 4.3 ATR-FTIR comparison upon post-coating

Experimental Details

Reagents

Iron (0) pentacarbonyl 99.99% trace metal basis; dioctylether 99%; oleic acid technical grade 90%; dopamine hydrochloride $\geq 98\%$; sodium nitrite, ACS reagent 97%; sulfuric acid ACS reagent 95-98%; Milli-Q water ($R=18\text{M}\Omega\text{cm}$); palmitic acid $>99\%$; palmitic acid $-d_{31}$ 98 atom % D, 99% (CP); (1-cyano-2-ethoxy-2-oxoethylidenaminoxy)-dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) 97%; 4-methyl-morpholine ReagentPlus 99%; hydrochloric acid ACS reagent 37%; MeOH puriss. $\geq 99.8\%$; EtOH absolute, denaturated with 0.5-1.5 Vol.% 2-butanone and approx. 0.001% Bitrex, $\geq 98\%$; DMF ACS reagent $\geq 99.8\%$; CHCl_3 $\geq 99.5\%$ (containing 100-200 ppm amylenes as stabilizer); EtOAc LC-MS chromasolv $\geq 99.7\%$; n-hexane chromasolv plus for HPLC $\geq 95\%$; Rotilab, PTFE, 0.2 μm , 13mm (Carl Roth); Chromafil PET, PE, 0.2 μm , 15mm (Carl Roth); Millex-GV, PVDF, 0.2 μm , 33mm (Sigma Aldrich); Corning, RC, 0.2 μm , 15mm (Sigma Aldrich); Corning, Nylon, 0.2 μm , 25mm (Sigma Aldrich)

All reagents were purchased from Sigma Aldrich and used as received without further purification.

The employed synthesized magnetite nanoparticles and nitrocatechol ligands originated from the same respective batches.

SI 1 synthesis and spectral characteristics of nitrocatechol ligands

SI 1.1 6-Nitrodopamine-Hemisulfate

6-nitrodopamine-hemisulfate (NDA- HSO_4) was synthesized according to literature with slight modifications^{2,3}. 5g dopamine hydrochloride (26.5mmol DA-HCl) and 7.3g sodium nitrite (4eq. NaNO_2) were dissolved in 150ml ultrapure water and cooled in an ice bath. 25ml of 20%v/v sulfuric acid were added dropwise under vigorous stirring to the cooled solution maintaining a temperature below 10°C. After complete addition the reaction mixture was slowly warmed to room temperature and stirred for 12h. The resulting yellow precipitate was collected by filtration and washed generously with ice-cold water, once with EtOH and Et₂O. NDA- HSO_4 was obtained as a bright yellow powder in 70% yield.

¹H-NMR (d_6 -DMSO, 300MHz, ppm): 7.45 (s, 1H, Ph), 6.62 (s, 1H, Ph), 3.04 (t, 4H, CH₂)

¹³C-NMR and DEPT (d_6 -DMSO, 300MHz, ppm): 151.80 (C), 144.63 (C), 139.47 (C), 125.91 (C), 118.47 (CH), 112.52 (CH), 39.52 (CH₂), 30.97 (CH₂)

ESI-MS ($\text{NH}_4^+\text{COO}^-$, m/z (%)): [M]H⁺ = 199.11 (100), calc. 199.07; electrostat. aggregates: 397.19 (2N;55), 495.16 (2N1C; 50), 693.24 (3N1C; 20), 791.19 (3N2C;10), 891.31 (4N1C; 20), 989.26 (4N2C; 35), 1089.37 (5N1C; 10), 1187.34 (5N2C; 20), 1286.34 (6N1C; 10), 1385.47 (6N2C; 10)

ATR-FTIR (powder, cm⁻¹): 3479 (OH), 3175 (NH_3^+), 3043 (CH), 2946 (CH₂), 1624 (Ph), 1600 (NO_2), 1540 (NH_3^+), 1512 (Ph), 1493 (NO_2)^{NDA-}, 1437 (OH), 1411 (CN), 1376 (CH₂/OH/ HSO_4), 1338 (NO_2), 1311(NO_2)^{NDA-}, 1283 (Ph), 1251 (CO), 1202 (CO/Ph/ HSO_4), 1146 (Ph/ HSO_4), 1068, 1034 (HSO_4), 978, 949 (CH), 906, 882 (CH), 853 (HSO_4), 810 (CH), 753 (OH), 674 (Ph), 621, 603, 557 (NO_2), 451

UV/VIS (MeOH, nm (ε)): 246 (π-π*; 8590), 305 (CT; 5510), 353 (CT; 6575), 425 (CT; 1365)^{NDA-}

SI 1.2 N-Palmityl-6-Nitrodopamine

N-Palmityl-6-nitrodopamine (P-NDA) was synthesized via COMU mediated peptide-coupling reactions^{4,5}.

In brief, 2 g palmitic acid (7.8mmol PA), 3.4 g (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)-dimethylamino-morpholino-carbenium hexafluorophosphate (1 eq. COMU) and 1.7ml N-methylmorpholine (2 eq. NMM) were taken up in 200ml anhydrous DMF and pre-activated at room temperature for 30min under constant N₂ purging before cooling the yellow solution to 0°C.

Meanwhile 2.55 g 6-nitrodopamine hemisulfate (1.1 eq. NDA- HSO_4) and 1.95ml NMM (2.2 eq.) were dissolved in 250ml anhydrous DMF under sonication, purged for 15min with N₂ and cooled in an ice bath.

The activated acid was then dropwise added at 0°C to the NDA solution under inert atmosphere and the mixture was allowed to slowly warm to room temperature. After 3h the mix was cooled to 0°C and the reaction quenched with 3 volumes of 6N HCl. The formed precipitate was collected by suction filtration, washed to neutrality with ultrapure water and short-term air dried. Flash chromatography on silica in CHCl_3 :MeOH=10:1 afforded P-NDA as a light yellow powder in 85% yield.

$^1\text{H-NMR}$ ($\text{d}_6\text{-DMSO}$, 300 MHz, ppm): 7.84 (t, 1H, CONH), 7.47 (s, 1H, Ph), 6.68 (s, 1H, Ph), 3.24 (t, 2H, CH_2), 2.89 (t, 2H CH_2), 2.00 (t, 2H, CH_2), 1.45 (p, 2H, CH_2), 1.23 (m, 24H, CH_2), 0.85 (t, 3H, CH_3)

$^{13}\text{C-NMR}$ and DEPT ($\text{d}_6\text{-DMSO}$, 300MHz, ppm): 173.39 (CONH), 150.66 (C), 143.57 (C), 140.21 (C), 128.16 (C), 118.04 (CH), 112.56 (CH), 39.7 (CH_2), 36.3 (CH_2), 32.8 (CH_2), 31.6 (CH_2), 29.42 (CH_2), 29.26 (CH_2), 29.14 (CH_2), 29.09 (CH_2), 25.56 (CH_2), 22.4 (CH_2), 13.92 (CH_3)

ESI-MS (MeOH, m/z (%)): $[\text{M}]\text{H}^+ = 437.33$ (55), calc. 437.30; $[\text{M}]\text{Na}^+ = 459.31$ (45), calc. 459.28

ATR-FTIR (powder, cm^{-1}): 3526 (OH), 3354 (NH, am), 2918 (CH_2), 2848 (CH_2), 1627 (CO, amI), 1585(NO_2), 1563 (NH, amII), 1484 (Ph), 1463 (CH_2), 1428 (OH), 1400 (CN), 1381 (OH/ CH_3), 1317 (NO_2), 1271 (CO), 1226 (CN, amIII), 1207, 1194 (CH), 1122 (CH), 1046, 1018, 915, 885 (Ph), 812 (Ph), 794, 765, 755, 722, 686, 662, 631, 608, 560, 471, 440

UV/VIS (MeOH, nm (ϵ)): 246 (6810), 305 (3145), 353 (4365), 425 (960)

SI 1.3 N-(d_{31} -Palmityl)-6-Nitrodopamide

N-(d_{31} -palmityl)-6-nitrodopamide ($\text{d}_{31}\text{P-NDA}$) was synthesized via COMU mediated peptide-coupling reactions as in SI 1.2 by using downscaled amounts of all reagents based on 500mg d_{31} -palmitic acid (d_{31}PA).

$^1\text{H-NMR}$ ($\text{d}_6\text{-DMSO}$, 300 MHz, ppm): 7.82 (t, 1H, CONH), 7.47 (s, 1H, Ph), 6.67 (s, 1H, Ph), 3.23 (t, 2H, CH_2), 2.87 (t, 2H CH_2), 1.23 (0.34H, 1.4% CH_2)

ESI-MS (MeOH, m/z (%)): $[\text{M}]\text{H}^+ = 468.54$ (20), calc. 468.50; $[\text{M}]\text{Na}^+ = 490.52$ (80), calc. 490.48

ATR-FTIR (powder, cm^{-1}): 3526 (OH), 3354 (NH, am), 2193 (CD_2), 2089 (CD_2), 1627 (CO, amI), 1585 (NO_2), 1563 (NH, amII), 1484 (Ph), 1428 (OH), 1400 (CN), 1381 (OH), 1317 (NO_2), 1271 (CO), 1226 (CN, amIII), 1207, 1088 (CD_2), 1073 (CD_2), 1046, 1018, 923, 902, 885 (Ph), 812 (Ph), 781 (CD_2), 765, 755, 680, 662, 608, 595, 553, 520, 471, 440

UV/VIS (MeOH, nm (ϵ)): 246 (6810), 305 (3145), 353 (4365), 425 (960)

SI 2 synthesis and characterization of OA-SPIONs

SI 2.1 synthesis protocol and histogram fits

In a typical procedure 1ml of iron pentacarbonyl ($\text{Fe}(\text{CO})_5$) was quickly injected at 100°C into a N_2 -saturated solution of 50ml dioctylether (Oct_2O) containing different amounts of oleic acid (OA), e.g. 3.9ml OA for 3.5nm SPIONs. An equilibration period of 30min was employed to ensure homogenous formation of iron-oleate complexes. The solution was then gradually heated to 290°C with a ramp of 3K/min. The final temperature was held for 1h to obtain the desired particle sizes.

The as-synthesized magnetite nanoparticles were subsequently cooled to room-temperature, precipitated in excess EtOH, collected by magnetic separation and purified by repeated precipitation (toluene into EtOH) / magnetic decantation steps.

Fig S1 PEBBLES¹ histogram fits of the corresponding TEM data for differently sized OA-SPIONs (a) 3.48 ± 0.38 nm, $\text{PDI}=0.0121$, $\text{D}=1.11$; (b) 5.02 ± 0.40 nm, $\text{PDI}=0.0064$, $\text{D}=1.08$; (c) 8.29 ± 0.41 nm, $\text{PDI}=0.0025$, $\text{D}=1.05$; Polydispersity index (PDI) and dispersity (D) are given as $(\sigma/\langle d \rangle)^2$ and $(d(\sigma)/\langle d \rangle)$, respectively.

SI 2.2 table S1 FTIR assignment of OA-SPIONs^{6–10}

major bands

character	wavenumber /cm ⁻¹ (intensity)
$\nu_{as}(\text{CH}_3)$	2956 (m)
$\nu_{as}(\text{CH}_2)$	2917 (s)
$\nu_s(\text{CH}_3)$	2872 (m)
$\nu_s(\text{CH}_2)$	2849 (s)
$\nu(\text{C=O})\text{O-H}_{\text{dimer}}$	1705 (s)
$\nu_{as, \text{bridging}}(\text{COO}^-)$	1605(var)*
$\nu_{as, \text{bident}}(\text{COO}^-)$	1520 (m)
$\delta(\text{CH}_2)$	1463 (s)
$\delta_{\text{ip}}(\text{C=O})\text{-OH}$	1430 (s)
$\nu_s(\text{COO}^-)$	1410 (s, b)
$\delta(\text{CH}_3)$	1380 (s)
$\delta_{\text{oop}}(\text{C=O})\text{-OH}_{\text{dimer}}$	965 (s)
$\gamma(\text{CH}_2) / \delta_{\text{oop}}(\text{C=C})\text{-H}_{\text{cis}}$	720 (s)
$\nu_1(\text{Fe-O})$	580 (s)
$\nu_2(\text{Fe-O})$	385 (s)

* weak for as-synthesized OA-SPIONs, strong for mixed dispersant SPION

minor bands

character	wavenumber /cm ⁻¹ (intensity)
$\nu(\text{O-H})_{\text{ROH}}$	3200-3600 (w, b) [#]
$\nu(\text{C=O})\text{O-H}_{\text{dimer}}$	2500-3300 (w, b)
$\nu(\text{C=C})\text{-H}_{\text{cis}}$	3006 (w)
$\nu(\text{CHO})$	1738 (w) [#]
$\nu(\text{C=C})\text{-H}_{\text{trans}}$	1667 (w) [#]
$\nu(\text{C=C})\text{-H}_{\text{cis}}$	1635 (w)
$\omega(\text{CH}_2)_{\text{prog.}}$	1327 (w)
$\omega(\text{CH}_2)_{\text{prog.}}$	1308 (w)
$\omega(\text{CH}_2)_{\text{prog.}}$	1290 (w)
$\omega(\text{CH}_2)_{\text{prog.}}$	1270 (w)
$\omega(\text{CH}_2)_{\text{prog.}}$	1252 (w)
$\omega(\text{CH}_2)_{\text{prog.}}$	1233 (w)
$\omega(\text{CH}_2)_{\text{prog.}}$	1211 (w)
$\omega(\text{CH}_2)_{\text{prog.}}$	1189 (w)
$\nu_{as}(\text{C-O-C})_{\text{ether}}$	1131 (w) [#]
n.d.	1115 (w) [#]
n.d.	1087 (w) [#]
n.d.	1069 (w) [#]
$\nu(\text{C-O})_{\text{ROH}}$	1050 (w) [#]
n.d.	1016 (w)
$\delta_{\text{oop}}(\text{C=C})\text{-H}_{\text{trans}}$	982 (w) [#]
n.d.	891 (w)

[#] possible thermal decomposition products, rotamers or residual impurities; n.d. – not determined

SI 3 characterization of P-NDA SPIONs

SI 3.1 Purification chart showing an overview of all investigated methods

OA_{phys} refers to physisorbed or intercalated oleic acid

OA_{chem} refers to chemisorbed or surface complexed oleate

SI 3.2 TGA graphs and data chart for different 3.5nm P-NDA SPION purifications

Fig S2 TGA/DSC curves of SPIONs purified by the following methods: hot 1mM OA in MeOH pre-extracted (brown), cold MeOH extracted (purple), syringe filtered (PTFE, green), surfactant treated (CTAB, orange), SiO₂ chromatographed (eluent THF/MeOH=4/1, blue), P-NDA @ SiO₂ chromatographed (eluent THF/MeOH=4/1, red), MeOH/n-hexane (1/1) recrystallized (black), 2,6-lutidine as solvent (light blue)

Table S2 chart of TGA data for various purification methods

prep method	mass loss	step profile	T _{mid} / °C	T _{infl} / °C	OA _f	OA _{sc}	P-NDA	impurity
as-synthesized	-78%	M	316	248	y	y	n	n
pre-extraction	-30%	S	283	271	n	y	y	n
sonicated	-35%	S	284	281	y	y	y	n
filtered PTFE	-43%	M	298	323	n	y	y	y
PVDF	-47%	M	285	246	y	y	y	n
PET	-48%	M	284	323	y	y	y	n
RC	-51%	M	273	235	y	y	y	n
Nylon	-72%	M	270	271	y	y	y	y
surfactant CTAB	-29%	S	283	283	n	y	y	n
SDS	-29%	S	282	278	n	y	y	n
OctOH	-29%	S	281	283	n	y	y	n
chromat. SiO ₂	-47%	M	294	323	y	y	y	y
P-NDA@SiO ₂	-57%	M	293	334	y	y	y	y
MeOH/n-hex boil	-28%	S	280	323	n	y	y	n
2,6-lutidine as solvent	-57%	M	292	248	y	y	y	n
post-coated	-35%	S	282	268	n	n	y	n

M – multistep, S – single step;

OA_f – free or physisorbed oleic acid, OA_{sc} – surface complexed or chemisorbed oleate

SI 3.3 syringe filtering of 3.5nm P-NDA-SPIONs

Fig S3 ATR-FTIR spectra of Polytetrafluoroethylene (PTFE, blue), Polyvinylidene difluoride (PVDF, green), Polyethylene terephthalate (PET, red), Nylon (purple) and regenerated cellulose (RC, black) syringe filtered 3.5nm P-NDA SPIONs. A preparation purified from free oleic acid (grey) is shown as comparison on top. Asterisks mark filter bands and circles denote marker bands of free OA

bands deduced from comparison (RC/PET/PVDF filtrations yielded identical spectra)

RC/PET/PVDF → OA_f: 1705, 1464, 1379, 1090, 1050, 1015, 966, 775, 718

PTFE → filter: 1465, 1377, 1263, 1093, 1048, 1020, 801

Nylon → filter: 3292, 1738, 1642, 1558, 1181

Fig S4 TGA/DSC curves of PTFE (purple), PVDF (blue), PET (green), Nylon (red) and RC (black) syringe filtered 3.5nm P-NDA SPIONs.

SI 3.4 purification of 3.5nm P-NDA SPIONs by surfactant addition

Fig S5 ATR-FTIR spectra of sodium dodecyl sulfate (SDS, blue), cetyltrimethylammonium bromide (CTAB, red) and 1-octanol (OctOH, green) treated 3.5nm P-NDA SPIONs.

Fig S6 TGA/DSC thermograms of CTAB (blue), OctOH (red) and SDS (black) treated 3.5nm P-NDA SPIONs and of the corresponding post-coated preparation (green) for comparison.

SI 4 characterization of pure nitrocatechol derived SPION preparations

SI 4.1 ATR-FTIR of differently sized SPIONs

Fig S7 ATR-FTIR spectra of 3.5nm (red), 5nm (black) and 8.3nm (blue) P-NDA coated SPIONs normalized to the T_{1u} optical phonon band of the Fe_3O_4 lattice.

Fig S8 ATR-FTIR spectra of 3.5nm (red), 5nm (black) and 8.3nm (blue) NDA coated SPIONs normalized to the T_{1u} optical phonon band of the Fe_3O_4 lattice. Peaks at 2300-2400 cm^{-1} are due to uncompensated CO_2 .

SI 4.2 calculation of grafting and chain end densities of differently sized SPIONs

Grafting densities were calculated from the rest masses obtained by horizontal step setting in the temperature interval [100-200°C; 500°C] according to Benoit et al¹¹.

$$\rho^{graft} = \frac{\frac{(\% \frac{W}{W})_{shell}}{(\% \frac{W}{W})_{core}} * \rho_{core} * V_{core} * N_A}{M_{lig} * S_{core}}$$

The larger range of values for NDA-SPIONs arises from consideration of the following species:

$[(NDA)_2SO_4]/2 = 247.1 \text{ g/mol}$ (T_d -distorted SO_4^{2-} at 1105 and 965 cm^{-1})¹² and

$NDA-HSO_4 = 296.1 \text{ g/mol}$ (HSO_4^- bands at 1235, 1165 and 1050 cm^{-1} ; also 1380, 1160 and 860 cm^{-1})¹³

Molecular footprint data (density of chain ends per outer surface area) were calculated by assuming that nitrocatechol dispersants are irreversibly bound to the iron oxide surface which yields a constant total number of adsorbed ligands given by

$$N = S(r) * \rho^{graft} = 4\pi r_{core}^2 * \rho^{graft} = const$$

Since each ligand contributes one chain end, renormalizing N to the outer surface area

$S(r') = 4\pi r'^2$ with $r' = r_{total} = r_{core} + d_{ligand}$ gives the density of chain ends as

$$\rho^{chain\ ends} = \frac{N}{S(r')}$$

Values for d_{ligand} were obtained from literature^{14,15,16}

Fig S9 TGA/DSC thermograms of 3.5nm (red), 5nm (black) and 8.3nm (blue) P-NDA coated SPIONs.

Fig S10 TGA/DSC thermograms of 3.5nm (black), 5nm (red) and 8.3nm (blue) NDA coated SPIONs.

SI 4.3 ATR-FTIR comparison of spectral changes upon post-coating

Fig S11 ATR-FTIR spectra showing bands that significantly decrease upon post-coating. These bands are inferred to result from the displacement of surface complexed OA. Blue – experimental $OA_{sc}/P\text{-NDA-NP}$ spectrum (MeOH pre-extraction), green – calculated $OA_{sc}/P\text{-NDA-NP}$ difference spectrum obtained via spectral subtraction of scaled OA_f , red – experimental $P\text{-NDA-NP}$ spectrum of post-coated sample

OA_f – free or physisorbed oleic acid, OA_{sc} – surface complexed or chemisorbed oleate

Fig S12 ATR-FTIR spectra showing complete exchange for 3.1nm $d_{31}P\text{-NDA}$ post-coated SPIONs (green) as compared to the respective oleate monolayer covered SPIONs (blue). Note that CD_2 and CH_2 oscillator strengths are different so that the total CD_2 intensities seem less than for the corresponding $P\text{-NDA}$ post-coated particles (red). Slightly elevated levels of $\nu(CH_3)$ and $\nu(CO)$ absorptions in the $d_{31}P\text{-NDA}$ post coated sample are likely to

originate from surface bound MeOH. Pure d_{31} P-NDA is shown as reference on the bottom (black) exhibiting faint residual $\nu(\text{CH}_2)$ background absorption due to non-deuterated ethylene units of nitrodopamine and 2% unlabeled alkyl of d_{31} PA. SPION spectra were normalized to the T_{1u} band of the crystal lattice and integrals were compensated for residual CH_2 .

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3.2. Controlled Magnetosomes: Embedding of Magnetic Nanoparticles into Membranes of Monodisperse Lipid Vesicles

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Motivation

Magneto-responsive lipid vesicles based on first generation SPIONs were successfully employed to establish basic concepts and design principles for optimal actuation.^{29,85} An important concept addressed bulk versus membrane magneto-thermal heating of the delivery vehicles.²⁹ It was concluded that bulk heating by hydrophilic SPIONs is ineffective compared to local membrane actuation by embedded, hydrophobic SPIONs because of water's inherent high heat capacity causing fast thermal diffusion. These approaches yet neglect effects of particle size distribution and reversible ligand adsorption on incorporation efficiency and co-self-assembly. Energetic restrictions of SPION membrane embedding usually favor the encapsulation of smaller particles that cause less bending of the membrane leaflets and result in reduced lipid packing mismatch.⁹³ Consequently polydisperse SPIONs are inappropriate for comparing different loading methods because of unknown biasing of the incorporation efficiency towards smaller core sizes and their effective ligand densities. The solid inorganic core influences lateral lipid packing while the more diffuse ligand shell might or might not interact with the surrounding lipids depending on grafting density and particle curvature. Having a well-defined core-shell architecture as presented in the first publication therefore enables us to obtain reproducible information on nanoparticle induced effects upon membrane loading. Besides this fundamental limitation for SPION loading, experimental factors such as preparation method contribute significantly to the maximum achievable loading.^{29,85} Previously exploitations demonstrated preparation methods which admittedly resulted in assemblies that are only of limited use to delivery applications. First, reverse phase evaporation was employed that gives quantitative transfer of particles and lipids, but which yields multilamellar and multivesicular liposomes with fiercely broad size distributions.⁸⁵ Such vesicles are not suited for release applications due to their too large size and heterogeneity, as well as the inefficient release resulting from multilamellar membranes. Alternatively, the focus was on triggered release by producing well controlled large, unilamellar vesicles by rehydration plus extrusion; however, this results in very low SPION loading, leading to weak magnetothermal release, and is a method impossible to scale up for industrial production.²⁹

The aim of this work is to develop a universal method to load monodisperse, hydrophobic SPIONs into lipid vesicles of defined lamellarity and controlled size to produce ready-to-use magnetoliposome formulations with adjustable SPION content.

Contribution:

I co-planned the work, performed the experiments and analysis, and co-wrote the manuscript.

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Controlled magnetosomes: Embedding of magnetic nanoparticles into membranes of monodisperse lipid vesicles

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ABSTRACT

Magnetic nanoparticle-containing capsules have been proposed for many uses, including triggered drug delivery and imaging. Combining superparamagnetic iron oxide nanoparticles (SPIONs) with existing liposome drug delivery technology is an enticing near-future prospect, but it requires efficient methods of synthesis and formulation compatible with pharmaceutical applications. We report a facile way of producing large, unilamellar, and homogeneously sized magnetoliposomes with high content of monodisperse, hydrophobic SPIONs integrated in the lipid membrane by use of a solvent inversion technique. For low lipid concentrations, unilamellar and monodisperse vesicles were obtained that became increasingly multilamellar with higher lipid fraction. Both, the co-self-assembled structure and loading content were significantly influenced by the purity of the nanoparticle shell. SPIONs with homogeneous shells of nitrodopamine-anchored hydrophobic dispersants could be quantitatively loaded up to 20%w/w, while SPIONs also containing residual physisorbed oleic acid exhibited a loading cut-off around 10%w/w. Lipid acyl chain length crucially influenced the formation and resultant stability of the loaded assemblies. The formation of nanoparticle-loaded vesicles is exemplified in different biologically important media, yielding ready-to-use magnetoliposome formulations.

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1. Introduction

Large unilamellar liposomes (~100–200 nm in diameter) comprise some of the most successful delivery systems in clinical use and are heavily researched for development of new drug delivery systems [1–4]. The advantages of liposomes are manifold [1,5,6]. Foremost, they possess a natural excellent biocompatibility and enable transport of hydrophilic cargo in their large aqueous lumen as well as hydrophobic and amphiphilic drugs in the lipid bilayer [1,7]. The composition and functionality of the lipid membrane can be easily tuned, including addition of charged lipids for transfection [8], PEG-lipids to create so-called stealth liposomes [9,10] with strongly reduced clearance rates in vivo, and tags that provide specific interactions with particular tissues or cells [3,6,10]. An important consideration when applying lipid vesicles for drug delivery is that efficient encapsulation and circulation can lead to inefficient or slow release. Rapid release at the site of action is desired to reach a concentration within the therapeutic range. Destabilizing the lipid membrane to increase its permeability, however, leads to premature drug release during circulation and short shelf-life. The self-assembled nature of lipid membranes offers many possibilities to make the release profile dependent on changes in the environment, thereby utilizing them for stable encapsulation and circulation, but letting a local change in the physical environment increase the release rate at the target [3]. Thermoresponsive liposomes are a well-researched example already in clinical use; they exploit small changes in tissue temperature between the blood stream and cancer tissue to increase the release rate by enhancing membrane permeability through a lipid composition exhibiting the melting phase transition slightly higher but close to the tissue temperature. The small temperature difference and the broad transition range strongly limit this approach. Therefore, externally applied triggers such as light, ultrasound or magnetic fields have captured the imagination to devise new delivery strategies [7,11–15]. The to date perhaps most successful such strategy is to use nanoparticles interacting with an external field to induce a thermal phase transition that increases the permeability of the vesicle membrane [7,14].

Liposomes structurally including biocompatible superparamagnetic iron oxide nanoparticles (SPIONs) are an attractive alternative for such strategies [7,11,14,16]. SPIONs, in contrast to most other magnetic nanoparticles, offer the advantage of being hydrolytically degraded into constituent nontoxic ions [17–19] and are highly compatible with in vivo applications due to the low susceptibility of tissue to magnetic fields [7,17,18]. Additionally, they offer the possibility to simultaneously image and control biodistribution via magnetic field gradients remotely [20], which makes them attractive also as multi-purpose tools for guided drug delivery and bioimaging [21–23]. For all these applications, controlling the size distribution, aggregation state and number of encapsulated magnetic nanoparticles is a key issue since magnetostatic interactions govern the force in magnetophoresis and dictate spin–spin relaxation times which control magnetic resonance imaging contrast and heat dissipation [24,25]. Therefore, high quality nanoparticles [26], ultrapure and stable surface modifications [27] and control over the embedding efficiency and distribution during the assembly process are prerequisites for applications.

To date various preparation methods have been described for producing magnetoliposomes [4,5,28]. Co-incorporation of water soluble SPIONs and pharmaceutical agents in the liposome lumen was first demonstrated [11,29,30]. Major drawbacks have been shown for this approach. First, SPIONs that are not properly stabilized interact with the liposome membrane and cause leakage, but properly stabilized SPIONs take up large volume. Second, heating by SPIONs in the lumen to induce a thermal transition requires

heating of the entire environment to change the permeability of the membrane due to the high thermal conductivity of water [11]. In contrast, hydrophobic SPIONs embedded in the lipid bilayer were shown to directly act on the capsule wall without requiring heating of the surrounding environment for release [11,16]. The drawback however is that the embedding efficiency heavily depends on particle size [31–33]. Small SPIONs can be contained in the membrane but SPIONs >5 nm are thought to lead to micelle formation. However, small SPIONs interact less with magnetic fields due to their lower magnetic moment ($\sim d^3$). It was also demonstrated that the stability and density of the hydrophobic coating of the nanoparticles decisively influences the permeability of the membrane and the stability of the liposomes [33]; excess physisorbed ligands can lead to severe leakage of encapsulated compounds [11,16]. Optimal magnetic liposome preparations therefore aim for high loading of monodisperse SPIONs, as large as can fit in the membrane, to maximize the efficiency of actuation; this requires a dense and stable hydrophobic coating. While the feasibility of such magnetoliposomes for release has been demonstrated [11,16], to date, control over high loading of monodisperse hydrophobic SPIONs in the membrane of liposomes has not been achieved. Previous methods used rehydration and extrusion to produce unilamellar vesicles, which is not compatible with current techniques for liposome drug formulation where carrier liquids with costly drug molecules and lipids are injected to ensure high encapsulation; a similar technique for magnetoliposome assembly compatible with high encapsulation efficiency in a large lumen is therefore desired.

We report such an easy and scalable way using solvent inversion to produce homogeneously sized, large and unilamellar magnetoliposomes with high and tunable content of monodisperse SPIONs embedded in the membrane. The magnetosomes are characterized with respect to their morphology and loading content in various media.

2. Experimental

2.1. Materials

Phospholipids were obtained from Avanti Polar Lipids, USA and used as received.

All chemicals (analytical grade) were purchased from Sigma-Aldrich and used without further purification.

Monodisperse 3.5 nm N-palmitoyl-6-nitrodopamide (P-NDA) capped superparamagnetic iron oxide nanoparticles (SPIONs) were synthesized as reported previously [27]. In brief, 200 mg as-synthesized SPIONs were purified by repeated pre-extraction in hot MeOH containing 1 mM oleic acid as stabilizer before exchanged in a mixture of 150 mg P-NDA in DMF:CHCl₃:MeOH = 6:3:1 for 3 h under nitrogen gas. Newly capped SPIONs were evaporated to the DMF fraction, precipitated by adding excess MeOH and collected via magnetic decantation. The particles were purified by threefold extraction in hot MeOH. Mixed dispersant SPIONs were post-coated with 100 mg P-NDA in minimal 2,6-lutidine for 48 h at 50 °C under inert atmosphere, evaporated to dryness and purified by hot MeOH extractions. SPIONs were lyophilized from THF:H₂O (1:5).

2.2. Vesicle preparation by solvent inversion

The respective amount of high-vacuum dried lipid (usually 5 mg) or respective nanoparticle-lipid mixes were dissolved in 1 ml anhydrous THF and dropwise (approx. 1 drop per second) added into 10 ml aqueous phase (ultrapure water or buffers) under constant magnetic stirring (400 rpm). THF was evaporated for 24 h

under air circulation or N_2 flow. The vesicle suspension was refilled with water or buffer to the original concentration. Where nothing else is specifically stated 1-palmityl-2-oleoyl-sn-glycero-phosphatidyl choline (POPC) was used as lipid.

3. Results and discussion

3.1. Vesicle formation and lamellarity

It is most important to assemble large (~ 100 nm in diameter), monodisperse and unilamellar vesicles to optimize loading and control rapid triggered release. Large unilamellar vesicles composed of 1-palmityl-2-oleoyl-sn-glycero-phosphatidyl choline (POPC) were prepared using solvent inversion. The non-polar, water miscible solvent THF was used as carrier/transfer fluid for the mix of monodisperse N-palmityl-6-nitrodopamide (P-NDA) coated magnetite particles and lipids; the mix is rapidly diluted upon injection into a larger volume of aqueous phase. During the assembly process THF is thought to behave as a co-solvent scaffold for both species followed by progressive dialysis. In this sense THF serves as a fluidizer that provides the system with a combination of solvation and flexibility to rearrange while being slowly forced into the final assembly [34–37]. THF itself is a high vapor pressure solvent and is readily evaporated under continuous nitrogen flow until a homogeneous suspension of lipid vesicles containing SPIONs is achieved. An efficient removal of solvents is especially important with respect to delivery applications as remnants render liposomes leaky [4] and might induce toxicity. The amount of residual THF in our preparations was quantified by NMR to be 0.05% or 50 ppm of its initial value (see Fig. S-1). Such minimal traces of THF retained after 24 h of evaporation are far below any toxic level and suitable for biological and medical applications [4,38].

Attempts to replace THF by other commonly used organic solvents or solvent mixtures like 1,4-dioxane [34,36,37], EtOAc/EtOH [39] or DMF [37], resulted in poor structure of magnetoliposomes and/or insufficient nanoparticle dispersion.

Fig. 1 demonstrates the formation of POPC vesicles at different lipid concentrations for a constant THF:H₂O inversion ratio of 1:10. DLS demonstrates the spontaneous formation of monodisperse liposomes with approximately 100 nm in diameter. The size distribution hardly changed when the lipid concentration was increased from 0.5 to 2 mg/ml (Fig. 1a), but the turbidity increased drastically (Fig. 1b). Measurements of the dependence of the optical density at 436 nm (OD_{436}) on the lipid concentration ([L]) allow for

discrimination between uni- and oligolamellar vesicles [40]. The measured OD_{436} vs [L] curve for vesicle solutions prepared by solvent inversion in Fig. 1b suggests that the observed increase in turbidity with increasing input lipid concentration is mainly related to an increase in lamellarity of the vesicles. The $OD_{436}/[L]$ ratio matches a homogeneous sphere model of the respective diameters at low concentrations while better agreement to optically denser oligolamellar vesicles is obtained for liposomes prepared at higher lipid concentrations [40].

The size distribution, morphology and lamellarity of the liposomes were additionally checked by freeze-fracture/-etching TEM and by trehalose fixation of the preparations. Fig. 2 summarizes the collected data. Lipid suspensions of 0.5 mg/ml exhibited spherical, monodisperse and unilamellar vesicles of uniform morphology. The dispersity obtained from DLS ($PDI = 0.21$) matches commonly used homogenization methods in the same liposome size range such as extrusion ($PDI = 0.14$) through polycarbonate membranes (see Figs. 1 and 2 and S-6). Replicas obtained by freeze-fracture/-etching on liposomes solutions with 2 mg/ml of lipid, frequently revealed multiple layers on the fractured liposome surface (cf. Fig. 2b). The results qualitatively confirmed the results from DLS and OD measurements, by demonstrating similar size and high monodispersity, but an increased frequency of multilamellar membranes for liposomes formed at higher lipid concentration.

3.2. Magnetosome formation in various media

Two common buffer systems were tested: 1x PBS (140 mM NaCl, 10 mM Na_2HPO_4 , pH = 7.4) and 1x TBS (140 mM NaCl, 10 mM Tris, pH = 7.4). PBS is commonly used to mimic intracellular fluids but is particularly incompatible with surface modified iron oxide nanoparticles since phosphate ions can displace dispersants from the particle surface and reduce colloidal stability [41]. TBS is in this respect less challenging but has the same ionic strength.

Typical preparations of SPION-loaded vesicles (0.5 mg/ml POPC; 1:10 inversion; w/w 5%w/w SPION;) exhibited monomodal size-distributions with a scattering maximum slightly above 100 nm in both buffers. Slightly larger average hydrodynamic diameters and broader distributions were observed in PBS than in TBS (see SI 4.3). The cosmotropic or H-bond breaking character of phosphate ions favors the hydrophobic effect and in turn causes enhanced aggregation of lipid acyl chains; this is consistent with the larger assemblies observed in PBS by DLS. The salting out effect also correlates with increasingly polydisperse assemblies at higher

Fig. 1. Effect of POPC concentration on liposome formation via solvent inversion at constant THF:H₂O inversion ratio of 1:10. DLS (a) shows similar size distributions for the vesicles formed in the investigated concentration range (red 0.5 mg/ml, blue 1 mg/ml, black 2 mg/ml lipid), whereas measurements of the optical density (red solid line) (b) demonstrate progressive deviation from reported experimental values for unilamellar, extruded 100 nm POPC vesicles (black dashed line) [40]. The inset shows the respective preparations via solvent inversion exhibiting enhanced turbidity with increasing lipid concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. TEM images of pure POPC liposomes formed via solvent inversion at constant THF:H₂O inversion ratio of 1:10. (a) Pt/C replicas of a 0.5 mg/ml preparation obtained by freeze-fracture/etching TEM give an overview of the morphology and size distribution of the obtained suspension in the native state. (b) Liposomes obtained at 2 mg/ml frequently exhibit multilamellar membranes in freeze-fracture-TEM. (c) Trehalose-fixed preparations of samples formed at 0.5 mg/ml show lipid vesicles which are embedded in a sugar matrix after air drying. This preparation method yields comparable results to Pt/C replicas but is experimentally less demanding to realize. The obtained size distributions (blue – freeze-fracture TEM and red – trehalose fixation) are shown for comparison in (d). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ionic strengths due to poor solubilities of organic solvents in phosphate buffers [42].

3.3. Dependence of vesicle formation on lipid species and temperature

While unsaturated lipids easily assembled into the desired LUVs through solvent inversion from THF in water, formation of saturated lipid vesicles was highly dependent on the chain length of the employed lipid. Saturated lipids such as DMPC, MPPC or DPPC were insoluble in THF at room-temperature and required gentle heating in order to be dissolved prior to inversion into aqueous medium. Saturated lipid samples were assembled by dropwise addition of the warm THF solutions (around T_m of the lipid species) into the stirred, equilibrated aqueous phases immersed in a thermostated water bath ($T = T_m \pm 10^\circ\text{C}$).

For 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC; $T_m = 24^\circ\text{C}$), exhibiting the shortest symmetric acyl chain length that was tested and therefore the lowest melting temperature, no marked differences in size distribution were observed for preparations below (15°C) or above the T_m (37°C). All preparations yielded stable vesicles with rather broad distributions ($\text{PDI} = 0.36\text{--}0.48$) centered around 89 nm (see SI 2.1). In contrast its higher analogue 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; $T_m = 41^\circ\text{C}$) resulted in rather ill-defined micron-sized assemblies that became unstable after removal of THF and precipitated within hours. Initially DPPC formed a clear dispersion in THF-water which became turbid and ultimately lead to flocculation of the sample (cf SI 2.1). We observed that the longer the alkyl chains the less soluble the lipids are in THF and the less defined are the resulting assemblies in terms of size and stability.

A slightly different behavior was observed for lipids with unsymmetric acyl chain lengths. 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (MPPC; $T_m = 35^\circ\text{C}$) preparations formed at $T > T_m$ resulted in size distributions at slightly larger diameters than at $T < T_m$ (cf SI 2.1). Both preparations resulted in vesicles that were stable over weeks.

We mainly attribute the difference in formation behavior among saturated and unsaturated lipids to solvent influence on lipid interdigitation [43,44]. It is generally accepted that a chemical inducer such as organic solvent adsorbs to the interfacial region of the lipid where it causes an increase of the head-group volume of the amphiphile. This in turn alters the head-group tilt causing the formation of unfavourable voids to lamellar phases, which rearrange

to a more stable interdigitated structure [43] in which the lipids adopt a transverse stacked, alternating monolayer pattern. The latter is stabilized by organic solvent capping of the terminal methyl groups which face the aqueous bulk and persist until a lower critical concentration is reached upon which interdigitated structures coalesce into large, vesicular arrangements [43,44]. Saturated phospholipids are prone to interdigitation fusion in aqueous solvent mixtures, notably THF-H₂O, whereas unsaturated lipids are strongly disfavored to interdigitate by virtue of their molecular geometry and lower phase transition temperatures [43,44].

Insensitivity of vesicle quality to temperature is surprising since the lipid phase state is known to have significant influence on interdigitation [43]. Generally the liquid crystalline state, which the lipids are in due to dissolution in THF around their T_m , is highly unfavorable for interdigitation, because disordered acyl chains are more accessible to water. However the steric effect of solvent adsorption to the interface was reported to be dominated at room temperature which also seems to hold in our case [44].

The much higher dispersity achieved for long-chain phosphocholine-lipid assemblies seems to correlate with their overall lower solubility in THF. Quick dilution upon injection triggers rapid loss of co-solvency causing large interdigitated assemblies to be formed for which the rate of fusion is accelerated because reduced curvature provides less steric hindrance to glide fusion of opposing monolayers [43].

Magnetosomes with high T_m and therefore composed of saturated lipids with long fatty acid chains are, however, required for in vivo applications exploiting the difference between body temperature and magnetosome T_m for stable circulation and magnetothermally triggered release. We therefore strived to further refine the vesicle assembly of DPPC by investigating the action of chemical inhibitors of interdigitation such as cholesterol [45], trehalose [46] or DMSO [47]. Admixture of cholesterol at 20%*n/n* readily gave rise to monomodal size distributions centered at 100 nm (Fig. S-4). Yet cholesterol is known to fill up voids in the bilayer and thereby strongly reduce the co-encapsulation of hydrophobic agents such as our SPIONs. Trehalose (1.5 M) yielded a bimodal size distribution of SUVs and a broad distribution of LUVs (Fig. S-4).

Addition of 20%*v/v* DMSO below T_m also resulted in a bimodal size distribution (Fig. SI 4) and low stability. A monomodal size distribution with hydrodynamic diameter of 110 nm and PDI comparable to that of POPC vesicles could be prepared with or without

SPION loading in 20%v/v DMSO at a temperature of 55 °C, which is above T_m (Fig. S-5). Neither DMSO nor elevated temperature on their own resulted in an improvement of DPPC liposome assembly, but a combination of both yielded well-defined vesicles with excellent stability that could be purified from DMSO by overnight dialysis. Thus, this combination yields a general way to form magnetosomes also from long-chain saturated lipids without affecting the co-assembly of SPION into the membrane interior that can be used for all solvent inversion preparation of magnetosomes compared below.

3.4. Comparison of nanoparticle loading methods

Previously, rehydration of SPIONs together with lipids from a dried film followed by extrusion has been used to load SPIONs into membranes of small to large unilamellar vesicles [11]. This benchmark was compared to the solvent inversion method for 3.5 nm in diameter Fe_3O_4 nanoparticles coated with N-palmitoyl-6-nitrodopamine at a grafting density of 2.7/nm² [27]. The DLS data is summarized in Fig. S-6. For comparison, a fixed amount of 5%w/w SPIONs was added to 5 mg lipids and subjected to the respective preparations. The concentration of SPIONs in solution and thus their embedding efficiency can be determined by UV/Vis spectroscopy (see Fig. 3 and S7–8) as nanoparticle absorption dominates the transmitted light spectrum [48].

Pure rehydration gave rise to broad, polydisperse size distributions with a time-dependent loading content of embedded

nanoparticles. SPIONs precipitated over time, which led to a decrease in particle content with time. OD measurements showed that a minimal amount of SPIONs was retained after overnight suspension at room temperature (see S-6).

Subsequent extrusion of rehydrated dispersions through polycarbonate membranes reconfirmed previous observations of significant loss of lipid and nanoparticle material [11]. An almost colorless suspension is obtained, for which the embedding of SPION was pushed below the UV/VIS detection limit (see SI 3). Hydrophilic extrusion filters (track-etched PVP-coated PC, Whatman) performed better than standard polycarbonate membranes (Avanti); however, after 31 passes the amount of incorporated SPIONs was still below detection limit by UV/VIS.

Solvent inversion to 0.5 mg/ml of final lipid concentration yielded markedly colored, clear suspensions with an OD at 350 nm corresponding to the calibrated extinction of 5%w/w addition of SPION (cf. Fig. 3b and SI 3). Such magnetoliposome suspensions were long-term stable. Size distributions of unloaded and SPION-loaded preparations can be found in Figs. 1 and 3, respectively. Thus, the advantage of accurate and high SPION-loading using the solvent inversion methods compared to previous preparation methods is evident.

3.5. Determination of loading content

The influence of the nanoparticle mass fraction on SPION loading and vesicle morphology was investigated at a fixed lipid con-

Fig. 3. Loading content determination of liposome preparations (0.5 mg/ml POPC) with different input weight fractions of spectroscopically clean 3.5 nm P-NDA-SPIONs. (a) DLS size distributions of a 1–10%w/w loading series, (b) the corresponding OD³⁵⁰ quantification curves (note that the offset at zero is due to vesicle scattering) (c) representative TGA graphs of the preparations (from bottom to top: 0% (grey), 1% (black), 5% (red), 10% (green) and 20%w/w (blue) SPION input; the 20% sample is shown for impure SPIONs to illustrate their upper loading limit which is not accessible to UV determination because of higher scattering due to increased polydispersity) and (d) table of loading contents evaluated by UV/VIS and TGA compared to nominal SPION weight percentage. 20%w/w SPION input is split into samples with spectroscopically clean P-NDA coated SPIONs and P-NDA coated SPIONs with residual oleic acid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. TEM micrographs of POPC liposomes loaded with 5%w/w 3.5 nm P-NDA-SPIONs. (a) Overview and (b) magnified vesicles depicting the nanoparticle distribution. Samples were prepared via solvent inversion (THF:H₂O = 1:10) at 0.5 mg/ml lipid and fixed in 1%w/v trehalose by air drying.

centration of 0.5 mg/ml. The SPION concentration was varied from 1 to 20%w/w and prepared by solvent inversion. The lipid-nanoparticle mixture was dissolved in 1 ml THF and added dropwise into 10 ml of Milli-Q water under magnetic stirring at room-temperature. THF was evaporated for 12 h in an open vial under constant magnetic stirring in a well-ventilated area. The as-prepared magnetoliposome suspensions were stable for >3 weeks in plastic cuvettes at both 4 °C and RT.

Fig. 3a shows the DLS curves and Fig. 3b the UV/VIS spectra after evaporation of the organic solvent. The SPION loading content of the LUVs was evaluated by comparing the obtained OD values at 350 nm to those of the calibration curves of pure SPIONs in THF (Figs. S7 and S8). The linear range of the calibration curve was limited to 0.1 mg/ml (20%w/w) SPIONs by strong absorption of the iron oxide cores. As shown in the table in Fig. 3d, excellent agreement was found between the input of SPION and the weight fractions measured by UV/VIS. Precipitation was also not observed for 20%w/w SPION input, which indicates that all SPIONs were loaded into the vesicles and quantitative loading was achieved.

The amount of incorporated SPIONs was cross-evaluated by thermogravimetric analysis (TGA) and resulted in close agreement with the results obtained by UV/VIS (Fig. 3c). Samples were lyophilized after overnight evaporation of THF and taking precautions to exclude any precipitated/non-incorporated particles. The residual inorganic content after thermal decomposition of dry samples between 25–500 °C in synthetic air was analyzed.

TGA could be used also to quantify the loading content of poly-disperse samples, e.g. those obtained at high impure SPION input (see next section) which were inaccessible to UV/VIS determination; TGA is, however, less accurate especially at low inorganic fractions. The incremental steps of increasing nanoparticle concentrations were well reflected by the residual masses at 500 °C although the amount of remaining, non-combusted lipid was substantial (Fig. 3c). Fatty acids, and therefore lipids, do not fully combust under inert atmosphere but yield carbonaceous residue which also causes reduction of the iron oxide core at elevated temperatures [49]. Heating SPIONs in air yields more complete organic combustion but oxidizes magnetite at around 520 °C [50]. Lipid combustion was incomplete even in oxygenic atmosphere at 500 °C [51] with a significant residual mass of 19%w/w found for lipid samples without SPION. The embedded SPION fractions reported by TGA therefore relate to the observed mass excess above the background level of remaining lipid. The deviation from the input concentrations was most pronounced for the lowest

SPION ratio, which we attribute to these errors. Higher SPION input yielded more emphasized multistep TGA profiles (cf. Fig. 3c). Multistep profiles can be caused by the presence of species with different decomposition temperatures or by pronounced interactions of lipids with the nanoparticle shell.

Representative TEM images of the 5%w/w loaded vesicles fixed in 1%w/v trehalose exhibit spherical morphology with a size distribution that agrees well with that obtained by DLS (Fig. 4). The hydrophobic SPIONs are distributed within the observed vesicles rather than dispersed in the background. The employed nanoparticle input of 5%w/w corresponds to 0.3%v/v or 26 SPIONs per 100 nm liposome (see SI 5). It is challenging to determine the number of SPIONs per liposome from TEM images, since the actual size of the vesicles is slightly altered by fixation (partial collapse) and because the observed number of particles depends on the focus, but the obtained micrographs are in general agreement with the expected SPION to lipid ratio. The number of SPIONs per liposome is significantly higher than previously estimated by TGA and SANS for rehydrated and extruded liposomes [11]. The SPIONs do not appear uniformly distributed within the LUV population. Some vesicles exhibit less than the expected number of SPIONs per vesicle while some embed slightly more (cf. Fig. 4), which could be an artefact of the preparation, since other preparations showed higher uniformity (cf. Fig. 6).

3.6. Influence of residual oleic acid from SPION synthesis on assembly behavior

The size distributions of the SPION-lipid assemblies prepared by solvent inversion were significantly influenced by the purity of the SPIONs. We recently described that standard preparation methods for monodisperse hydrophobically coated SPIONs by ligand replacement result in significant fractions of both physisorbed and chemisorbed oleic acid in the sample [27]. The presence of residual impurities was confirmed for magnetoliposome preparations with incompletely purified SPION samples by recording ATR-FTIR spectra of the lyophilized preparations. Free and physisorbed oleic acid shows up for samples prepared with incompletely purified SPIONs at higher input concentrations as a shoulder at 1705 cm⁻¹ (Fig. 5). No such bands were observed in the case of stringently purified P-NDA SPIONs or their magnetoliposome preparations (Fig. 5). An estimate of the oleic acid content of the employed SPIONs was obtained according to Klokkenburg et al. (see SI 4.1) [52]. Evaluation of the relative IR peak intensities yielded 11%w/w physisorbed oleic acid or 29

Fig. 5. (a) DLS and (b) ATR-FTIR of POPC liposomes prepared by solvent inversion and loaded with different contents of 3.5 nm P-NDA-SPIONs purified by standard methods (light color) leading to residual oleic acid in the sample (red – 1%, green – 5%, blue – 10% and magenta 20%w/w SPION input; black – incompletely purified SPIONs with residual OA and grey – pure P-NDA SPIONs are shown for reference). Preparations with clean SPIONs are shown as overlay (dark color). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecules per SPION. This corresponds to a significant mole-fraction of free oleic acid per liposome of around 1.5n/n for a 5%w/w SPION input.

For the same preparation conditions, the addition of incompletely purified hydrophobic SPIONs containing residual physorbed oleic acid initially only slightly shifted the scattering maximum of the formed vesicles to higher diameters than in the unloaded case but gave rise to a bimodal distribution above 5% w/w input (see Fig. 5a). At further increased SPION input the size distributions became increasingly ill-defined with intense polydisperse micron-sized contributions.

This polydispersity with different types of aggregates could explain the quantitatively different OD curves for standard and spectroscopically pure SPIONs, where for the latter only P-NDA could be identified on the particles by IR spectroscopy (Fig. 5b and S-9). A significant increase in OD as compared to unloaded and clean reference samples was observed for impure SPION input above 5–10%w/w. This matches the concentration range above which increasingly polydisperse morphologies were observed in DLS. The appearance of polydisperse structures, including micellar aggregates is reminiscent of recent results reported by Petri-Fink et al. of micellar nanoparticle inclusions in membranes [53].

For spectroscopically pure SPIONs [27] the resulting assemblies showed similar size distributions as for the unloaded case up to >20%w/w SPION input. The PDI for loaded and unloaded preparations were comparable at 0.2. In contrast we observed an upper loading limit for impure SPIONs at around 10%w/w (Fig. 3), which could only be verified by TGA due to the increasingly polydisperse samples at higher concentration that precluded quantification by UV/VIS (cf. SI 4.2). While impure nanoparticles tended to precipitate at input contents approaching 10%w/w, clean SPIONs did not show any visual precipitation in the investigated range (Fig. 3).

Another striking difference was observed in relation to formation of magnetoliposomes in different buffers (cf. SI 4.3). PBS showed a feature-less, polydisperse distribution of large aggregates from 100 to 10,000 nm when SPIONs containing residual OA were employed (Fig. S-11). In contrast, TBS yielded quasi-monodisperse vesicles with a major component around 250 nm, similar to preparations in H₂O. In the case of isotonic NaCl solutions (140 mM) a narrow bimodal DLS distribution with the main populations around 100 nm and 250 nm was found, similar to for TBS. For spectroscopically clean SPIONs overlapping monodisperse size distributions were observed of vesicles slightly larger than 100 nm for PBS and TBS (Fig. S-10). Thus, the destabilizing effect

of the phosphate ions on assembly is higher when there is potential free oleic acid in the liposome membranes.

The demonstration of the strong influence of the partitioning of small amounts of residual oleic acid from the particles to the lipid membrane on the magnetoliposome assembly and stability underscores how important well-defined and characterized starting materials will be for production of, for example, triggered drug delivery liposomes. Accumulation and partitioning of amphiphilic solutes is determined by the molecular structure of the detergent and the phase state of the lipid membrane [54]. Membrane active species such as lysolipids and fatty acids partition to the membrane and have been shown to promote vesicle lysis, leakage, to modulate the lipid phase state, drug-membrane interactions and enzymatic activity, notably the degradation of phospholipids by phospholipase A2 (PLA2) [54–57]. The effects we observe on the assembly can therefore be expected to be extended to low encapsulation stability in biological and medical applications.

3.7. Controlling magnetoliposome size

3.7.1. Low concentration regime – THF:H₂O ratio

A variation of the inversion ratio between 1:5 and 1:20%v/v (THF:H₂O) showed that the obtained size distribution of formed vesicles can be tuned by adjusting the solvent-to-water ratio for lipid concentrations below 1 mg/ml (see SI 6.1). The resulting average size was ~150 nm for high THF-to-H₂O ratio (1:5) and ~90 nm for lower ratios (1:10 and 1:20). Differences in size were also observed for SPION-loaded versus unloaded vesicles when formed at constant inversion ratio of 1:10. Loaded assemblies were slightly larger than their unloaded counterparts with diameters of 110 nm compared to 89 nm.

3.7.2. High concentration regime – post-extrusion

Assemblies formed above a lipid concentration of a few mg/ml were characterized by poor control over size, lamellarity and long-term stability, but formation of concentrated vesicle samples is preferred for applications. Post-extrusion through 100 nm pore-size track-etched polycarbonate membranes after complete evaporation of the organic solvent resulted in unilamellar preparations of controlled size also at high lipid concentrations. Surprisingly this approach allowed producing magnetoliposomes with higher SPION content compared to rehydration and extrusion (see SI 6.2). Loss of nanoparticulate material is presumably minimized by the more similar and homogeneous size and loading of vesicles formed by

Fig. 6. Phase diagram of the prepared nanoparticle-lipid assemblies. The grey region depicts formation of LUVs, the white indicates formation of polydisperse MLVs by only solvent inversion. The shaded region highlights structural changes through association of the vesicles with surfactant remnants from incomplete SPION purification ultimately leading to a loading cut-off around 10%w/w. MLVs with less than 10%w/w loading can be resized to LUVs without significant SPION loss by extrusion.

solvent inversion compared to by rehydration. At high SPION input (>10%w/w) the loss of nanoparticles through extrusion became more pronounced also for vesicles pre-formed by solvent inversion. The loss of SPIONs upon post-extrusion were 8% and 24% of the total 5 and 10%w/w SPION inputs respectively (see SI 6.2).

3.8. Influence of SPION size on assemblies

Differently sized SPIONs (3.5, 4.5 and 8.3 nm) were tested for loading into POPC vesicles by solvent inversion. The SPIONs of different sizes exhibit similar grafting densities but vastly different chain end densities at the outer particle surface due to the increasing free volume at the outer shell for higher particle curvature (decreasing size) [27]. This particle size dependent thinning of the ligand shell yields 3 nm SPIONs with light shells and large interaction volumes for surrounding solutes whereas 8 nm SPIONs show roughly three times higher shell density at the outer surface.

Solvent inversion with all SPION sizes yielded markedly colored suspensions without precipitation. DLS showed comparable size distributions with scattering maxima around 100 nm for all

preparations (Fig. S-7.1). TEM of 4.5 nm SPION-loaded liposomes (Fig. 7) showed homogeneous distribution of nanoparticles among the lipid vesicles, similar as often observed for 3.5 nm SPIONs (cf. Fig. 4). Including the expected thickness of the P-NDA shell, this size is likely at the border of what can be fitted into a lipid bilayer.

Addition of 8.3 nm SPIONs to lipids via solvent inversion resulted in dispersed nanoparticles. However, a closer inspection in TEM of samples with 8.3 nm SPION showed exclusive nanoparticle localization in lipid droplets, i.e. SPION aggregates surrounded by a lipid (mono-)layer [33,53]. These SPION-lipid droplets co-exist with unloaded vesicles (Figs. 7 and S-16). In the literature it is often suggested that micelle formation occurs around single SPIONs too large to fit into a lipid membrane due to unfavorable bending energy. However, in our 8 nm SPION sample we only observed formation of droplets seemingly containing multiple cores, which have strong similarities with the aggregated nanoparticle inclusions in vesicle membranes reported by Petri-Fink et al. [53].

To investigate the fraction of particle aggregates we employed absorption spectroscopy before and after magnetic chromatography of the samples on a homebuilt magnetic column (see SI experimental details) [58,59]. UV/VIS was used to assess the amount of aggregates formed (see SI 7.1). Dilute and non-aggregated SPIONs, for example well dispersed in vesicle membranes, are not possible to retain in such columns.

Samples prepared with 8.3 nm SPIONs lead to almost complete removal of SPIONs during magnetic chromatography even at 5%w/w input (see SI 7.1). Unloaded liposomes with identical size distributions measured by DLS before and after elution from the column could be detected. This behavior clearly correlates with TEM observations of nanoparticle aggregates in lipid droplets.

Vesicles loaded with 5%w/w of 3.5 nm SPIONs were eluted, indicating magnetoliposomes. Higher SPION fractions (e.g. 20%w/w) did not pass the magnetic column. This either indicates SPION clustering in lipid droplets or that a high SPION-loading in the membrane induced strong magnetic interactions with the column material, which could occur either through aggregation or by the high number of SPION per vesicle. 20%w/w SPION could be accommodated in liposome membranes, since it corresponds to approximately a third of a hexagonally closed packed SPION monolayer (~60%w/w) within 100 nm liposome membranes (see SI 5.3). TEM inspection of samples fixed in trehalose showed loaded liposomes with indications of spherical areas containing nanoparticles. Droplet formation for small core sizes could however not unequivocally be identified in TEM since similar features (high contrast areas) were also observed for samples of lower loading content that easily passed the magnetic column just as for preparations

Fig. 7. TEM images of assemblies from POPC ($c_{\text{lipid}} = 0.5 \text{ mg/ml}$) with 5%w/w SPIONs of different sizes (a) 4.5 nm and (b) 8.3 nm, fixed by trehalose. 4.5 nm SPIONs are incorporated while assemblies with 8.3 nm SPIONs exclusively yielded unloaded lipid vesicles coexisting with nanoparticle loaded lipid droplets.

Fig. 8. Stability of POPC liposomes loaded with 5%w/w 3.5 nm SPIONs stored in water at room temperature (red symbols) or at 4 °C (blue symbols). The hydrodynamic diameter d (intensity weighted average) and polydispersity index PDI of the distributions are indicated by filled and empty squares respectively over the time-course of 1 month. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with large SPIONs (see SI 7.2). Moreover bursting of vesicles and spreading of nanoparticles during transfer to a high vacuum system is commonly observed for fixed vesicles. Additionally, we also could not detect vesicles by DLS in the eluate for high loading contents of small SPIONs, which showed monodisperse LUV by DLS before the column. Thus, all vesicles remained trapped on the magnetic column. The most plausible interpretation is therefore that at high loading of small SPIONs predominantly LUVs are formed with sufficient net inducible magnetic moment to allow facile magnetic extraction of the magnetosomes.

3.9. Magnetosome stability

Magnetoliposomes (5%w/w 3.5 nm SPION with POPC lipids) were stored in PMMA cuvettes at room temperature and at 4 °C under ambient atmosphere. Sample integrity was confirmed at various time intervals using DLS and found to be preserved for at least one month (Fig. 8). The hydrodynamic size (intensity weighted average diameter) varied by less than 5% during storage at both room temperature and at 4 °C while the PDI varied by 0.1 for the narrow distributions (Fig. 8).

4. Conclusion

Controlled and homogeneous interfacial properties are key to monodisperse nanoscale self-assembly. We have demonstrated a new and simplified method to create monodisperse and unilamellar liposomes with P-NDA coated SPIONs dispersed in the membrane. Lipid and SPION suspended in THF are injected into water or buffer and the THF is evaporated; this presents a one-step route to monodisperse magnetosome assembly. This method greatly improves on previous bottle necks of multi-step preparation incompatible with current pharmaceutical formulation technologies and low effective SPION loading [11,14,53].

Quantitative control over encapsulation of 3.5 nm SPIONs with pure P-NDA shells up to at least 20%w/w was confirmed by UV/Vis spectroscopy, TGA and TEM. The 5%w/w loading corresponds to 26 SPIONs per 100 nm vesicle, which is significantly higher than previously reported for other methods. A strong reduction in maximum loading to <10%w/w as well as a strong increase in vesicle polydispersity and lamellarity was observed for monodisperse

SPIONs that had residues of oleic acid within the shell, which is typically observed without extensive purification of the shell. Encapsulation of SPIONs of various sizes highlighted stable suspensions also for large SPIONs using solvent inversion. However, for P-NDA coated nanoparticles exceeding 5–6 nm core size the resulting preparations favored assembly of SPIONs into large aggregated clusters surrounded by a lipid monolayer resembling giant micelles.

In addition to the novel full control over vesicle structure and SPION loading, a major advantage of the solvent inversion method for magnetosome assembly is that it is easily scalable while simultaneously compatible with direct drug encapsulation methods. Liposomes in the ideal 100–200 nm size range which provide a large lumen were directly obtained; the large lumen is important for drug delivery efficiency. Furthermore, the much higher than previously obtained number of SPIONs per vesicle that could be achieved is important for applications. Magnetic contrast and susceptibility to magnetically triggered release is enhanced in direct proportion to the order of magnitude higher loading that was demonstrated.

For biomedical release applications it is important that high- T_m saturated lipids can be assembled into magnetosomes. We demonstrated that careful balancing of interfacial energetics to avoid saturated lipid interdigitation is required to achieve this. For example, DPPC led to poor assembly and low colloidal stability unless formed in the presence of chemical inhibitors of interdigitation. A combination of DMSO plus elevated temperature followed by dialysis allowed for assembling 5%w/w SPION loaded DPPC liposomes of similar quality as their unsaturated counterparts.

Continued work will explore the relationship between lipid composition, nanoparticle loading and drug encapsulation and release efficiency. A higher nanoparticle loading could lead to higher efficiency of triggered release, but also compromise membrane integrity and passive leakage. Future studies will also evaluate solvent inversion as a universal method for controlled assembly of magnetosomes of optimized mixed membrane capsules also including polymeric amphiphiles.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jcis.2015.11.071>.

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Controlled magnetosomes: High-density encapsulation of monodisperse, hydrophobic iron oxide nanoparticles into unilamellar lipid vesicles by solvent inversion

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Outline

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Experimental Details

Reagents

All reagents were purchased from Sigma Aldrich and used as received without further purification.

Ultrapure water (Millipore USA, R=18MΩcm); THF (Chromasolv plus for HPLC, inhibitor free) ≥99%; 1,4-Dioxane (anhydrous) 99.8%; EtOAc (anhydrous) 99.8%; DMF (ACS reagent) ≥99.8%; EtOH (Chromasolv for HPLC, absolute) ≥99.8%; PBS tablets (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C); TBS BioUltra tablets (0.05 M TRIS-HCl buffer ; 0.15 M sodium chloride; pH 7.6 at 25°C)

All employed P-NDA-coated magnetite nanoparticles originated from the same batches.

All lipids were obtained dissolved in Chloroform from Avanti Lipids Inc. and high-vacuum dried for at least 24h before further use.

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) >99%, 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (DMPC) >99%, 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (MPPC) >99%, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) >99%. Where nothing else is stated, POPC was used as lipid.

Measurement conditions

TEM and analysis: TEM studies were performed on a FEI Tecnai G2 20 transmission electron microscope operating at 120 kV or 200 kV for high resolution imaging. Samples were prepared by drop-casting aqueous vesicle dispersions onto 300-mesh carbon-coated copper grids. Size distributions were evaluated using PEBBELS¹.

Dynamic Light Scattering: Hydrodynamic size distributions were measured on a Malvern Zetasizer Nano-ZS (Malvern UK) in Milli-Q water or buffer at 25°C in 173° backscattering mode. Samples were equilibrated for 120s each and the autocorrelation function was obtained by averaging 3 runs. Samples were measured as-prepared without further dilution.

OD measurements: UV-Vis spectra were collected at a scan speed of 400nm/min on a Hitachi UV-2900 spectrophotometer referenced against pure solvent.

TGA/DSC measurements: Thermograms were recorded on a Mettler-Toledo TGA/DSC 1 STAR System in the temperature range 25-650°C with a ramp of 10K/min in synthetic air (O₂). 70µl aluminum oxide crucibles were filled with 0.5-2mg sample and the rest mass was evaluated at 500°C. The mass loss was obtained by placing horizontal steps to the TGA curves.

ATR-FTIR measurements: Mid-IR powder spectra of the lyophilized samples were collected on a single reflection Bruker Platinum Diamond ATR at a resolution of 4cm⁻¹ by averaging 32 scans.

¹H-NMR measurements: ¹H -solution spectra were collected on a Bruker DPX operating at 300MHz in D₂O using 1mg 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as an internal standard.

Sample preparation

Trehalose fixation: An aliquot of 10%w/v trehalose stock solution was added to the vesicle suspension to give a final trehalose concentration of 1-2%w/v. The sample was gently vortexed for 1min before a drop of the sugar-vesicle solution was placed onto a carbon-coated copper grid. The vesicles were allowed to adsorb for 30min before the grid was cautiously washed with a drop of Milli-Q water. Samples were dried for several hours in air before examined in TEM.

Freeze-fracture/-etching: 5µl vesicle suspension was loaded onto a gold specimen holder and shock-frozen by quickly immersing it into dichlorofluoromethane (Freon R22) at -196°C. The fixed sample was mounted onto a sample holder under cryogenic temperatures and transferred to a Balzer BAF400 freeze-etching system. After an initial equilibration period of 10min at -150°C, the sample was slowly warmed to -100°C for fracturing. The sample top was stripped off with a N₂(l)-cooled microtome and the exposed surface etched by sublimation of 40nm ice in high-vacuum (90sec at -100°C and 10⁻⁶ mbar). A 2nm Pt-shadowing was evaporated from a 45° angle followed by a carbon support layer of 20nm. The sample was subsequently warmed to room-temperature and cleaned in 70% H₂SO₄ overnight to digest all organic material. The cleaning medium was exchanged five times for Milli-Q water, the washed replicas loaded onto 300mesh copper grids and dried overnight before imaged in TEM.

NMR determination of residual solvent: Vesicles were prepared by the standard 1:10 solvent inversion procedure to 0.5mg/ml POPC in 10ml D₂O containing 1mg/ml DSS and withdrawing 1ml aliquots right after THF addition and after 24h of evaporation. The size of the formed liposomes was determined to be around 200nm.

Vesicle preparation by rehydration plus extrusion: 5mg POPC mixed with 5%w/w SPIONs in 3ml CHCl₃ were dried on the rotary evaporator and lyophilized in high vacuum for 12h. The dry lipid-nanoparticle film was rehydrated in 1ml Milli-Q water for 2h at 50°C and detached from the flask wall by gentle sonication (3x 30sec). The rehydrated sample was subsequently extruded 31 times through 100nm track-etched polycarbonate (PC) membranes (Avanti Lipids) or PVP-coated PC membranes (Whatman).

Magnetic column separation: A perforated Eppendorf tube was packed with 0.5g of ultrafine steel wool and flushed thrice with ultrapure water. The column was attached to a 1T Nd/Fe/B-magnet and the sample of SPION-loaded lipid vesicles/aggregates was passed through the column. UV/VIS quantification after up-concentration to the initial volume (speed-vac) was used to access the amount of aggregates formed.

SI 1 formation of pure lipid vesicles

SI 1.1 quantification of residual THF

Fig S1 ¹H NMR spectra of POPC in D₂O containing 1mg/ml DSS as reference standard. Liposomes were formed at 0.5mg/ml via 1:10 solvent inversion. (a) NMR spectrum right after dropwise addition of THF at t=0h and (b) after 24h of evaporation. The size of the formed vesicles was around 200nm. DSS signals are found at 2.9ppm (t, 2H, -CH₂SO₃⁻), 1.75ppm (p, 2H, -CH₂-), 0.65ppm (t, 2H, -CH₂SiR₃) and 0ppm (s, 9H, -SiMe₃).

SI 1.2 OD curves of POPC liposomes

Fig S2 OD curves of POPC liposomes formed via 1:10 solvent inversion (THF into water) at 0.5mg/ml (black), 1mg/ml (blue), 1.5mg/ml (green) and 2mg/ml (red) total lipid concentration.

SI 2 formation with saturated lipids

SI 2.1 influence of chain length and temperature

Fig S3 (a) DLS size distributions of DMPC (dashed) and MPPC (full lines) formed at $T < T_m$ (blue) and $T > T_m$ (red) via solvent inversion (0.5mg/ml lipid; THF:H₂O=1:10). (b) shows DLS curves for DPPC assemblies formed via the same conditions and a stability series for 1-20%w/w SPION loaded assemblies at selected times ($t=0$ directly after THF addition, $t=12h$ after evaporation of THF and $t=24h$ after overnight storage at RT).

While the as-prepared DMPC and MPPC assemblies generally were sub-micron sized and stable for a few weeks, DPPC assemblies were distributed over a wide size range and colloiddally unstable. For the latter, precipitation of excess SPIONs occurred during evaporation of the organic solvent accompanied by a significant increase in turbidity of the solutions. Storage at room-temperature led to the formation of flakes and precipitation of the sample.

SI 2.2 chemical inhibitors of interdigitation fusion (DPPC)

Fig S4 DLS size distributions of DPPC liposomes ($c_{lipid}=0.5\text{mg/ml}$; THF:H₂O=1:10) formed in presence of various chemical inhibitors of interdigitation fusion below the lipid T_m . Color coding: blue - 20%*n/n* Chol (cholesterol), red - 55%*w/w* treh (trehalose 1.5M) and black - 20%*v/v* DMSO (dimethylsulfoxide).

SI 2.3 DPPC magnetoliposome formation

Fig S5 DLS scattering curves for DPPC liposomes (red) loaded with 5%w/w SPION exhibiting a similar size distribution as loaded POPC liposomes (black, dash). Samples were prepared via solvent inversion (THF:H₂O=1:10) above the T_m of DPPC ($T=55^\circ\text{C}$) by adding 20%v/v of DMSO to the aqueous phase prior to addition of DPPC in warm THF. After evaporation of THF, the sample was dialysed (Novagen D-tube, 12-14kDa MWCO, RC) for 12h against Milli-Q water to remove residual DMSO.

SI 3 evaluation of SPION loading content and comparison of loading methods

Fig S6 Comparison of loading methods for 5%w/w 3.5nm P-NDA-SPION input (POPC). (a) DLS curves (1:10 dil) of the preparations. The inset shows the following preparations (left to right): first - rehydration (supernatant after 12h resting) at 5mg/ml lipid, second - rehydration plus extrusion through 100nm PVP coated track-etched PC-membranes at 5mg/ml lipid and third - THF-H₂O solvent inversion at 0.5mg/ml. (b) OD curves (1:10 dil) of the preparations. POPC vesicles formed via solvent inversion and 3.5nm P-NDA-SPIONs in MeOH:THF=10:1 are shown for comparison.

Fig S7 OD curves of 3.5nm P-NDA-SPIONs at different concentrations in THF (a) and (b) calibration curves at various wavelengths.

Fig S8 OD curves of POPC vesicles (0.5mg/ml) loaded with 1-10, 15 and 20%w/w (1:1 diluted) 3.5nmP-NDA-SPIONs.

SI 4 vesicle loading with SPIONs containing residual physisorbed oleic acid

SI 4.1 estimation of the amount of physisorbed oleic acid

Calibrated IR extinction coefficients were obtained from literature ² and applied to the $A_{\max}$ band intensities of the ATR-FTIR spectra ($A^{1705}=0.021$ and $A^{588}=0.103$). The peak maxima were slightly red-shifted due to crystal packing effects but good agreement with OA contents determined by TGA was achieved ³.

The evaluated concentrations by this methods applied to our samples gave concentrations of $[OA]=2.53\text{mM}$ and $[Fe_3O_4]=6\text{g/l}$ which translates to 10.5%w/w free OA or 29 molecules per SPION.

This corresponds to a mole-fraction of free oleic acid per liposome of around 1.5% n/n for a 5%w/w SPION input.

SI 4.2 assemblies in Milli-Q water

Fig S9 OD curves of POPC preparations with different weight fractions of 3.5nm P-NDA SPIONs containing residual physisorbed oleic acid (THF:H₂O=10:1; $c_{lipid}=0.5\text{mg/ml}$) The inset shows the following SPION weight fractions: 1, 5, 10, 20% (left to right)

SI 4.3 assemblies in buffers

Fig S10 (a) DLS graphs and (b) OD curves of POPC liposomes ($c_{lipid}=0.5\text{mg/ml}$) in different buffers loaded with 5%wt 3.5nm P-NDA-SPIONs via solvent inversion. 1x PBS (10mM Na₂HPO₄ / 2.7mM KCl / 137mM NaCl) and 1x TBS (50mM Tris / 150mM NaCl).

Fig S11 (a) DLS scattering curves and (b) OD curves of POPC vesicles ($c_{lipid}=0.5\text{mg/ml}$) containing 5%w/w improperly purified SPIONs in PBS (10mM Na_2HPO_4 / 2.7mM KCl / 137mM NaCl), 1x TBS (50mM Tris / 150mM NaCl) and isotonic NaCl (140mM)

SI 5 various calculations

SI 5.1 M_w calculation of core-shell SPIONs ($d=3.5\text{nm}$)

$$\begin{aligned}
 m_{\text{core-shell}} &= m_{\text{core}} + m_{\text{shell}} \\
 m_{\text{core}} &= \rho_{\text{core}} * V_{\text{core}}(r) = \rho_{\text{Fe}_3\text{O}_4} * \frac{4\pi}{3} r_{\text{core}}^3 \\
 m_{\text{shell}} &= \frac{M_{\text{shell}}}{N_A} = \frac{N_{\text{lig}}(r) * M_{\text{lig}}}{N_A} = \frac{4\pi r_{\text{core}}^2 \rho^{\text{graft}} * M_{\text{lig}}}{N_A} \\
 m_{\text{core-shell}} &= 1.91 * 10^{-19} g \\
 M_{\text{core-shell}} &= m_{\text{core-shell}} * N_A \\
 M_{\text{core-shell}} &\sim 1.15 * 10^5 g/mol
 \end{aligned}$$

SI 5.2 M_w calculation of liposomes ($d=100\text{nm}$)

$$\begin{aligned}
 N_{\text{lipids}}(r) &= \frac{4\pi \left(\frac{d}{2}\right)^2 + 4\pi \left(\frac{d}{2} - h\right)^2}{a} \\
 M_{\text{liposome}}(r) &= N_{\text{lipids}}(r) * M_{\text{lipid}} \\
 M_{\text{liposome}}(50\text{nm}) &= 6.08 * 10^7 g/mol \\
 m_{\text{liposome}}(r) &= \frac{M_{\text{liposome}}(r)}{N_A} \\
 m_{\text{liposome}}(50\text{nm}) &= 1.01 * 10^{-16} g
 \end{aligned}$$

SI 5.3 estimation of maximum SPION loading per liposome ($d=100\text{nm}$)

$$\begin{aligned}
 S_{\text{liposome}}(r) &= 4\pi r_{\text{liposome}}^2 \\
 A_{\text{core-shell SPION}} &= \pi r_{\text{total}}^2 \\
 r_{\text{total}} &= r_{\text{core}} + l_{\text{ligand}} \\
 N_{\text{max}}^{\text{SPION}} &= \frac{S_{\text{liposome}}}{A_{\text{core-shell SPION}}} * 0.74 \text{ (hcp - packing)} \\
 N_{\text{max}}^{\text{SPION}} &= 328 \text{ SPIONs / } 100\text{nm liposome}
 \end{aligned}$$

$$\begin{aligned}
 m_{\text{max}}^{\text{SPION}} &= N_{\text{max}}^{\text{SPION}} * m_{\text{core-shell}} \\
 w_{\text{max}}^{\text{SPION}} &= \frac{m_{\text{max}}^{\text{SPION}}}{m_{\text{liposome}}} * 100 = 62\%
 \end{aligned}$$

The calculated %w/w refers to weight-% SPION per lipid to ensure easy comparability to the SPION input values given in the main manuscript.

SI 6 controlling vesicle size

SI 6.1 low concentration regime: THF-H₂O ratio

Fig S12. (a) DLS graphs and (b) OD curves of POPC preparations ($c_{lipid}=0.5\text{mg/ml}$) containing 5%w/w SPION formed at different THF:H₂O ratios.

SI 6.2 high concentration regime: post-extrusion

Fig S13. (a) DLS curves of POPC vesicles formed at 5mg/ml before (dashed lines) and after post-extrusion (solid lines) loaded with 5% (red) and 10% SPION (black). (b) UV/VIS quantification of SPION loss by extrusion. Samples were prepared by solvent inversion (THF:H₂O=1:10) into Milli-Q water and extruded 31 times through 100nm track-etched polycarbonate filters. The loss of SPIONs was evaluated at 350nm by comparing the filter absorption (polycarbonate membrane after extrusion in 1ml THF) to the input SPION absorption (in 1ml THF) at 1:16 dilution. The UV absorption of the plain PC membrane is shown for reference.

SI 7 influence of SPION size on assemblies

SI 7.1 magnetic chromatography

Fig S14 DLS before (straight lines) and after (dashed lines) passing 5%w/w SPION loaded vesicle suspensions (0.5mg/ml lipid) over a magnetic column (dimensions: height x diameter = 3.5cm x 1cm; 0.5g ultrafine steel wool). The slightly altered UV absorption of the 3nm loaded sample post elution is attributed to co-eluted material from the column.

SI 7.2 TEM characterization

Fig S15. (a) Spherical structures of high contrast with associated nanoparticles (red, arrows) were observed for some magnetoliposome preparations at high SPION content. Similar features (red, dashed circles) were however also seen for low loading contents in (b) and in samples where exclusively 8nm lipid droplets were observed (see lower panel). It is likely that the observed features result from trehalose fixation, since such structures were not indicated in other experiments, such as DLS or magnetic chromatography.

Fig S16. TEM images of co-existing empty liposomes and lipid coated SPION aggregates. Similar features (red dashed circles) as in the case of 3.5nm loaded vesicles are sometimes observed in the background.

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3.3. Triggered Release from Thermoresponsive Polymersomes with Superparamagnetic Membranes

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Motivation

Vesicle stability and membrane properties markedly vary with the constituent amphiphile molecular weight.²³ Amphiphilic block copolymers can exist in a broad Mw range and thus their assemblies exhibit highly adjustable stability, fluidity and permeability. Increasing polymer Mw enforces membrane toughness and increases the loading capacity for hydrophobic and amphiphilic agents but brings along restrictions for release as permeability depends on Mw.^{86,87,99} Moderate polymers, approximately in the range of 1-10kDa, yields thick bilayered vesicles, whereas higher Mw analogs, especially when the constituents exceed the entanglement-Mw, form a continuous membrane region that slows transport and thus hampers efficient release.²³ Apart from tailoring membrane characteristics via Mw to the desired attributes, the overall design of the diblock architecture is pivotal to controlled release. A universal carrier system is desired to change its permeability on demand such that the release rate can be externally controlled rather than causing a disintegration and burst release of the whole vesicle.⁸¹

A restraint to the design of temperature sensitive polymeric carriers is the availability of suitable polymers.^{9-12,113} The best researched thermally responsive polymer is poly(N-isopropyl acrylamide) or PNIPAAm which exhibits a thermomorphic transition that can be tuned close to body temperature in water. It is therefore of special interest for biomedical engineering of polymer based delivery systems. Above its lower critical solution temperature (LCST) PNIPAAm rearranges its H-bonding pattern which is accompanied by a change in the polymer's hydration state and is often referred to as a switching of polarity from hydrophilic to hydrophobic.^{9,10} Although collapsed, the PNIPAAm chains are still partially hydrated¹¹⁴ which can be used to alter the vesicle's permeability by changing the packing parameter while still maintaining vesicle integrity.¹¹⁵ Many factors however influence the performance of PNIPAAm based copolymers, e.g. conjugation to a second block (transition temperature, permeability, particle loading, assembly properties), Mw (transition temperature, solubility, actuation efficiency, assembly properties) or polydispersity (transition range, assembly properties).¹¹⁴⁻¹¹⁶

Conceptual work on magnetically triggered release from polymersomes has highlighted that control over the aggregation state of embedded particles is delightfully important even for rather insensitive suuperamphiphilic carriers^{86,87} and

that most purely polymer based carriers presented so far either require an additional stimulus⁹⁹ or more drastic actuation modalities.¹¹⁷

The goal of this publication is to engineer a thermoresponsive diblock copolymer that spontaneously co-self-assembles into magnetopolymersomes with monodisperse SPION embedded in the hydrophobic interior of the superamphiphile membranes, and to show that externally triggered release induced by an alternating magnetic field can be achieved.

Contribution:

I co-planned the work, contributed to the synthesis, performed characterization and experiments except for the release data and co-wrote the manuscript.

Article

Triggered Release from Thermoresponsive Polymersomes with Superparamagnetic Membranes

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Abstract: Magnetic polymersomes were prepared by self-assembly of the amphiphilic block copolymer poly(isoprene-*b*-*N*-isopropylacrylamide) with monodisperse hydrophobic superparamagnetic iron oxide nanoparticles (SPION). The specifically designed thermoresponsive block copolymer allowed for efficient incorporation of the hydrophobic nanoparticles in the membrane core and encapsulation of the water soluble dye calcein in the lumen of the vesicles. Magnetic heating of the embedded SPIONs led to increased bilayer permeability through dehydration of the thermoresponsive PNIPAM block. The entrapped calcein could therefore be released in controlled doses solely through exposure to pulses of an alternating magnetic field. This hybrid SPION-polymersome system demonstrates a possible direction for release applications that merges rational polymersome design with addressed external magnetic field-triggered release through embedded nanomaterials.

Keywords: superparamagnetic iron oxide nanoparticle; thermoresponsive polymer; magneto-polymersome; magneto-thermal release; calcein assay

1. Introduction

Amphiphilic diblock copolymers with hydrophilic volume fractions around 30%–50% v/v spontaneously self-assemble into vesicular spherical structures with bilayer membranes [1–4]. Such polymersomes have received rapidly increased attention since they offer an improved and designable alternative to liposomes for encapsulation and release applications such as delivery of bioactive compounds *in vivo* and *in vitro* [5–9]. A large aqueous lumen can efficiently transport hydrophilic substances; the tailorable amphiphilic membrane can host hydrophobic compounds in the membrane interior of higher molecular weight and more complex composition than lipid membranes can [10]. Improved mechanical stability is readily achieved by amplifying the respective block weights at constant volume ratios without compromising assembly behavior [11]. The high robustness correlates with a drastically lowered permeability and thereby lowered passive leakage of transported cargo. However, high stability and low permeability pose a challenge for release applications that require rapid and efficient release at the target site. Considerable effort has been devoted to the implementation of stimuli responsive vesicles that burst or decompose upon exposure to environmental changes such as temperature, pH or subcellular localization [7–9,12,13]. This is achieved by including stimuli-responsive units into the vesicle membrane that break down the membrane integrity upon the stimulus.

A particularly appealing approach is to include membrane components that respond to external field triggers and therefore can cause release without relying on uncontrolled changes in the

surrounding environment. The external field trigger is typically light or magnetic fields, with which polymers have weak interactions but for which inorganic nanomaterials can serve as strong enhancers [9,14–26]. Field-triggered release offers decisive advantages such as control over the release profile by switching on and off the stimulus and release in locations where direct changes to the environment through e.g. change in pH or heating are not possible due to lack of direct access.

Superparamagnetic iron oxide nanoparticles (SPIONs) actuated by alternating magnetic fields are highly suitable enhancers for biomedical applications due to the low susceptibility of tissue to magnetic fields; they offer the additional advantage of being biocompatible through hydrolytic degradation into constituent ions that can be recycled by the body [27]. Release from thermally responsive PEG-liposomes using embedded SPIONs for magnetic heating has been shown; it was furthermore demonstrated that embedding of the nanoparticles in the membrane interior to directly affect the permeability is greatly advantageous [28,29]. SPIONs have also been incorporated into various polymeric assemblies [15]. Recent studies on the co-self-assembly of high molecular weight (10–22 kDa), nonresponsive PAA-*b*-PS block copolymers with SPIONs demonstrated that nanoparticle distribution within the resulting assemblies can be controlled by solvent selection; diverse structures such as micelles, core-shell structures and polymersomes incorporating dense and diffuse layers of SPIONs could be assembled [16].

The most commonly employed diblock copolymers used for the preparation of thermoresponsive vesicles are poly(*N*-isopropylacrylamide) (PNIPAM) based [30]. Applications that make use of PNIPAM as the hydrophobic block are manifold [8], but they display a release of encapsulated compounds below the lower critical solution temperature (LCST), *i.e.*, the vesicles disassemble upon cooling in hypothermia applications [19–23]. It is, however, desirable to develop a design that achieves release of cargo upon heating, since an increase in temperature can be achieved through actuation of nanoparticles by an alternating magnetic field. PNIPAM partly loses its solubility in water upon heating [31]. PNIPAM is therefore suited to demonstrate release at temperatures above the LCST of the hydrophilic block. Partial dehydration of a hydrophilic PNIPAM block should change the block copolymer packing parameter that determines the integrity and thereby permeability of the polymersome membrane (Scheme 1).

Magnetically actuated release of cargo from SPION-loaded block copolymer vesicles has so far only been demonstrated for poly(trimethylene carbonate-*b*-glutamic acid) [24,25] and poly(acrylic acid-*co*-distearin acrylate) [32]. Hydrophobic doxorubicin associated with the membrane of the polymersomes was released by nanoparticle-mediated hyperthermia in these cases [24–26,32]. The incorporation of SPIONs in these examples seems to have led to rather undefined structures with aggregated particle clusters in the membrane, which could explain high passive leakage of the membrane-associated drug [24,25]. In addition, although local hyperthermia increased the release rate in agreement with predictions, the polymersome membrane did not undergo a structural phase transition similar to what was demonstrated for the more efficient triggered release from liposomes in response to local hyperthermia [24,25,29]. The slow rate of doxorubicin released from the membrane was therefore only marginally faster than the passive leakage without application of a magnetic field.

Our goal with this work is to demonstrate SPION-loaded polymersomes with low membrane permeability that are able to encapsulate hydrophilic compounds in the lumen and release them by magneto-thermal actuation. Our focus here, compared to previous work, is on using the lumen of the vesicles to encapsulate a high concentration of hydrophilic molecules by a magnetic membrane that shows low passive leakage. Importantly, we also strive to improve the release rate and profile over what has been demonstrated for just hyperthermia affecting the hydration and packing of chains in the membrane core; this can be achieved by achieving a larger structural change in the membrane through dehydration of the hydrophilic block. We therefore report on the synthesis and assembly of the vesicle-forming, thermoresponsive di-block-copolymer polyisoprene-block-poly(*N*-isopropylacrylamide) (PI-*b*-PNIPAM) with controlled embedding of SPIONs stabilized with densely and strongly anchored hydrophobic ligands within the polymersome membrane. An important

improvement is the use of nanoparticles with well-defined size, shape and dense hydrophobic ligand shell that is irreversibly grafted; the importance of controlling nanoparticle properties to avoid aggregated nanoparticle-membrane structures and to suppress passive leakage from vesicles has previously been demonstrated for liposomes [29]. In these vesicles, the thermoresponsive polymer PNIPAM serves as the hydrophilic block. Finally, we demonstrate triggered release of calcein encapsulated in the lumen of the polymersomes by magneto-thermal actuation.

2. Results

A PI-*b*-PNIPAM block copolymer (BCP 2) with thermoresponsive volume fractions of 40% v/v was prepared by sequential RAFT polymerization (Scheme 1; for detailed description see Materials and Methods Section and Figures S1–S3). The first step involved polymerization of isoprene at 125 °C to prepare macro RAFT agent **1** with a molecular weight of approximately 1.3 kDa (>90% 1,4-addition). The presence of reactive dithiobenzoate end groups in **1**, which is crucial for the polymerization of the second block, was confirmed by ¹H-NMR resonances at 7.98, 7.51, 7.37 ppm and characteristic UV/VIS absorptions at 295 nm and 500 nm. Macro RAFT agent **1** was subsequently used for the polymerization of *N*-isopropylacrylamide in dioxane where different PNIPAM block sizes could be obtained by adjusting the monomer-to-macro RAFT ratio. The remaining head group was conveniently cleaved by aminolysis and trapped as disulfide to give the final α,ω -heterobifunctional block copolymer **2** after column chromatography. The synthesis of *N*-palmitoyl-6-nitrodopamide coated superparamagnetic iron oxide nanoparticles (P-NDA-SPION) with a core diameter of 3.5 ± 0.4 nm was described previously [33].

Scheme 1. Preparation of thermoresponsive block copolymer by sequential RAFT polymerization, **a** isoprene, AIBN, THF, **b** *N*-isopropylacrylamide, AIBN, dioxane, **c** *S*-methyl methanethiosulfonate, (2-dimethylamino) ethylamine. The schematics show polymersome structure and triggered release. Orange line: hydrophobic block (polyisoprene); blue line: hydrophilic block (poly(*N*-isopropyl acrylamide)); black dots: palmitoyl-nitrodopamide coated iron oxide nanoparticles; red dots: calcein. The triggered release of the calcein is accomplished by an alternating magnetic field that heats the embedded nanoparticles. Increased temperature dehydrates the PNIPAM block and increases polymersome membrane permeability.

Vesicle formation of SPION-loaded BCP **2** depended on experimental conditions such as preparation method, temperature, aqueous phase composition and additional energy input (e.g., sonication). Initial attempts to produce loaded vesicles via standard rehydration in Milli-Q/calcein (5 mg/mL; 0.2 μ m filtered) or phosphate buffered saline (PBS; 10 mM NaHPO₄/150 mM NaCl)/calcein

solution required improvement because of minimal dispersion of the nanoparticle/BCP 2 film into those phases at ambient conditions. Neither gentle temperature variations nor sonication improved on vesicle formation.

Vesicles of BCP 2 ($M_n \sim 2300$ g/mol, $\bar{D} = 1.14$, ϕ (PNIPAM) = 40% v/v) were instead prepared at 1 mg/mL by solvent inversion into ultrapure water and calcein similar to Meier *et al.* for pure polymersomes [34] and Bixner *et al.* for magnetic liposomes [35]. A detailed protocol can be found in the Materials and Methods Section. Typically, BCP 2 (1 mg) and the desired SPION weight fraction (20% w/w) were dissolved in THF (200 μ L) and subsequently added dropwise at room temperature into ultrapure water (2 mL) or calcein solution (5 mg/mL; 0.2 μ m filtered) under magnetic stirring. THF was evaporated at room-temperature under a constant N_2 stream for 3 h and the sample was refilled with Milli-Q to the original concentration. The remaining THF concentration in the sample is after 3 h negligible and far below toxic levels (see Section S3; Figure S4).

Dynamic light scattering (DLS) showed structures with a broad distribution of hydrodynamic sizes of 0.1–1 μ m for the turbid as-prepared suspension (Figure 1A). TEM of the same sample showed spherical hollow structures with a size distribution similar to the one obtained by DLS, further supporting successful formation of polydisperse block copolymer vesicles (Figure 1B). Figure 1A also shows the results of temperature-dependent DLS in the range of 25–75 $^{\circ}$ C in 5 $^{\circ}$ C steps. During temperature cycling, the initial broad distribution sharpened at 30 $^{\circ}$ C to a maximum centered at 250 nm. In the range from 35 to 70 $^{\circ}$ C the hydrodynamic diameters only shifted slightly to approximately 200 nm but steadily increased in intensity to ultimately settle at seven-fold of the initial value at 50 $^{\circ}$ C. No further change in size distribution up to 70 $^{\circ}$ C was observed. This result demonstrates the thermoresponsiveness of BCP 2 vesicles with a transition temperature range of 35–50 $^{\circ}$ C; this is higher than the typical literature value of 32 $^{\circ}$ C, but an increased LCST and even suppression of the collapse of the coil is expected for low molecular weight PNIPAM in an amphiphilic environment [36].

Figure 1. (A) Temperature-dependent DLS size distributions at 25–70 $^{\circ}$ C in 5 $^{\circ}$ C steps of the crude PI-*b*-PNIPAM assemblies at 1 mg/mL prepared by THF solvent inversion into Milli-Q water; (B) TEM after trehalose fixation of the sample shows spherical objects with a similar size distribution as obtained from room-temperature DLS. The lower contrast of the vesicular structures is because water in the lumen of the vesicles is not replaced by trehalose.

Multilamellar large vesicles are of limited use for release applications. Standard methods to enforce unilamellarity and decrease vesicle size are sonication and extrusion, which have been successfully applied to polymersomes [36]. Sonication at constant $T = 20$ $^{\circ}$ C led to polymer and nanoparticle precipitation. Extrusion through track-etched polycarbonate membranes caused loss of hydrophobic SPIONs and some polymer but did not lead to precipitation. The measured DLS curves and OD³⁵⁰ values (Figure 2) of the extruded preparations matched the expected changes

based on similar preparations of liposomes, for which the lamellarity is known to be reduced. The solution became clearer, which indicates a reduction in size but primarily a lower fraction of multilamellar vesicles. Multilamellarity is well-known to more strongly increase scattering in the optical range than size. The similar hydrodynamic sizes measured in DLS before and after extrusion also strongly indicate that the major change in polymersome morphology is a reduction in lamellarity. We therefore used extrusion (10 $\times$, 100 nm polycarbonate membranes) to create monodisperse unilamellar thermoresponsive polymersomes encapsulating calcein in the lumen, while retaining a high SPION content.

Figure 2. (A) DLS size distribution and (B) TEM micrograph of calcein-loaded, extruded PI-*b*-PNIPAM polymersomes at 1 mg/mL with 20% w/w 3.5 nm hydrophobic SPION input. Samples were prepared by THF solvent inversion into 5 mg/mL calcein solution to form polydisperse, large polymersomes and subsequent extrusion through 100 nm track-etched polycarbonate membranes after evaporation of the organic solvent. A high SPION content is seen from the high contrast of most vesicles and the cores are directly visualized in the inset (see also Figure S5); (C) Optical density curves of extruded PI-*b*-PNIPAM vesicles without nanoparticles (red), SPION loaded polymersomes before (black) and after (blue) homogenization by 10 passes through 100 nm track-etched polycarbonate membranes. The inset shows a digital image of the preparations before and after extrusion; (D) TGA curves (20–650 °C) of BCP 2 and BCP 2 extruded with 20% w/w P-NDA-coated iron oxide nanoparticles. Iron oxide content (taking inorganic residue of BCP into account) is estimated to be ~9% w/w, which is significantly higher than described for liposome [28].

DLS size distributions (159 ± 66 nm) and TEM of extruded SPION-loaded vesicles with encapsulated calcein are shown in Figure 2A,B. The orange-brown suspensions after extrusion were clear as expected for predominantly unilamellar vesicles. More SPION than polymer are lost in the extrusion and for an initial input of 20% w/w 3.5 nm SPION we determined an incorporated weight fraction of around 10% w/w by TGA (rest mass after thermal decomposition relative to total organic

content) and UV/VIS spectroscopy (characteristic wavelength at 350 nm) (see Figure 2C,D). A detailed description of the determination of the nanoparticle loading content is provided in the supporting information (Sections S4.2 and S4.3).

The fluorescent dye calcein was encapsulated at self-quenching concentrations. Calcein is water-soluble and serves as an easily quantifiable model compound to investigate passive and magneto-thermally triggered release through the polymersome membrane. Samples were purified from excess dye by size exclusion chromatography over a Superdex 75 FPLC column (GE Life Sciences, Austria) in ultrapure water and fractionated according to UV absorption and refractive index. The purification reduced the sample concentration from the original 2 mg/mL to 0.5 mg/mL. The encapsulated calcein thereby remains at self-quenching concentration while the external calcein is removed.

Release of encapsulated calcein to the bulk phase was quantified by recording the increase in fluorescence intensity as function of time and membrane actuation on the bulk sample stored in a cuvette. The change in fluorescence intensity was obtained after subtraction of background fluorescence and normalizing to the total fluorescence after disruption of the vesicles by Triton X-100 (Sigma Aldrich, Austria). Magneto-thermal release was triggered by applying an alternating magnetic field (AMF) of variable duration and intensity. The resulting relative increase in fluorescence was compared to the passive release in absence of an alternating magnetic field. The fluorescence resulting from triggered release of calcein from PI-*b*-PNIPAM polymersomes with 3.5 nm SPIONs incorporated in the membrane is shown in Figure 3. The AMF causes heat to dissipate locally from the magnetic cores due to Néel relaxation [27,28]. It was found that only a long pulse duration of 10 min led to significant release. Application of one pulse of 8 min duration triggered only 3% release of entrapped calcein whereas application of one 10 min pulse triggered release of 25% of encapsulated calcein as shown in Figure 3A. Similar release was achieved for 8 min pulses only after four repetitions.

Figure 3. (A) Release kinetics of calcein encapsulated in 3.5 nm hydrophobic SPION-loaded PI-*b*-PNIPAM polymersomes. The samples were actuated with 10 min AMF pulses followed by a 5 min cool-down period. 50% release was achieved in less than 1.5 h (black line—squares, left axis). The passive leakage measured at 24 °C during the same period was negligible (red line—circles, right axis); (B) The hydrodynamic size distribution of the polymersomes measured before (blue) and after (red) actuation by AMF is almost unchanged, indicating increased permeability without destruction of the vesicles.

Figure 3A shows that the magneto-thermally triggered release plateaued close to 50% of the encapsulated calcein set free. The passive release during the period leading to actuated release is negligible (Figure 3A; see Figure S6 for long-term passive release). However, after 5 h storage the passive release reached close to 20% with a linear release profile. The relatively high passive leakage

over long time scales might be caused by per-methylation of the hydrophobic core material which was shown to render liposomes more permeable.

The PI-*b*-PNIPAM polymersomes showed reversible decrease in hydrodynamic size upon increased temperature rather than disintegration of the whole vesicles. This behavior was independent of the upper temperature (35 °C, 45 °C or 55 °C; Figures S7 and S8). In addition, for extruded vesicles, no significant change in scattering intensity or size was observed after reversible heating (Figure 3B). We therefore attribute reversible, thermally induced vesicle shrinking to a reversible partial dehydration of the interfacial PNIPAM corona that changes the membrane integrity but does not alter the vesicle topology. Thus, permeability could be increased without disassembly of the vesicles similar to what has been observed previously for polymersomes and liposomes [24,25,29].

3. Discussion

The control over permeability of the vesicles in this study is obtained through magneto-thermal actuation, by which an alternating magnetic field locally produces heat through its interaction with the superparamagnetic iron oxide nanoparticles embedded in the polymersome membranes. We will first discuss the principle behind the observed actuation in more detail and then compare it to a few related earlier studies on the use of magnetic hyperthermia directly on the membrane to control release from vesicles.

Magnetic relaxation and therefore local heat generation through superparamagnetic iron oxide nanoparticles can occur primarily via Néel and Brownian relaxation [37]. Néel relaxation is a universal property of magnetic materials that depends on particle core volume and refers to reorientations of the magnetic moment between different easy directions of the crystal lattice while Brownian relaxation is related to viscous rotation of the entire particle and is thus medium specific and dependent on the hydrodynamic volume. Dissipation in a restrained and viscous microenvironment like a lipid or polymer membrane is thought to preferentially proceed via Néel dissipation. Smaller particles, however, show faster spin reorientation dynamics and therefore feature faster heating rates than larger cores because the height of the rotational energy barrier is mainly associated with particle size and material anisotropy but they are also less efficient absorbers of energy from the magnetic field. The optimum size for hyperthermia of superparamagnetic nanoparticles is expected to be larger than the 3.5 nm Fe₃O₄ core diameter used in our vesicles. However, our attempts to embed P-NDA capped SPIONs with 8 nm cores into PI-*b*-PNIPAM block copolymer vesicles were unsuccessful. Presumably, the unfavorably high effective extension of the PI block required to encapsulate the core-shell particle in the hydrophobic core of the membrane prevented incorporation of larger particles. A redesign of the block-copolymer to higher molecular weights of both blocks could remedy this drawback. The frequency and strength of the alternating magnetic field was not optimized in our experiments. A lower field strength and a frequency tuned to the maximum of the specific absorption rate function of the SPION could be used to minimize bulk heating while maximizing the local heat generation at the cores, and therefore result in more specific and effective release.

The heat generated by the magnetic actuation of the core-shell nanoparticles diffuses to the surrounding liquid. An increased temperature exceeding the LCST of the PNIPAM corona, comprising the outer part of the polymersome membrane, leads to its partial dehydration. The resulting change in amphiphile packing parameter of the block copolymer affects membrane integrity; this is the expected cause of the increased membrane permeability that is observed in our release experiments.

This pulse length required to achieve substantial release of calcein from the polymersomes is significantly longer than required for release from liposomes with T_m comparable to the LCST of the PI-*b*-PNIPAM and with similar nanoparticles incorporated in the membrane using the same magnetic field actuation [29]. As comparison, DPPC ($T_m = 41$ °C) liposomes with 4% w/w loading of 3.5 nm SPION released 90% of encapsulated calcein after two 4-min pulses (unpublished data). For liposomes, the release has been demonstrated to be due to a change in membrane permeability by direct heating of the membrane by the nanoparticles without requiring bulk heating [29]. The long pulse duration

necessary for triggered release from the PI-*b*-PNIPAM vesicles indicates that a thermally induced structural transition through purely local heating of the PNIPAM is not likely to have been achieved. This is further supported by the fact that the bulk temperature at the end of the AMF pulse application exceeds the temperature required for thermal transition of the polymer (*cf.* Figure 1 and Figure S6).

A maximum of 50% of the encapsulated calcein was released through magneto-thermal actuation, which given the release mechanism and the nanoscopic dimensions of the vesicles indicates that only half of the vesicles released their cargo. Since the preparation method strongly favors formation of unilamellar vesicles, as demonstrated by the OD measurements, it is likely that an inhomogeneous distribution of SPIONs between different polymersomes is the main reason for that only half of the encapsulated calcein could be released. The lower contrast of some of the small polymersomes observed in TEM (Figure 2B) could indicate low SPION loading in small vesicles and that high particle loading is required for efficient release. This, though, indicates that local actuation due to nanoparticles plays an important role in the triggered release in addition to the global temperature increase. The relative contribution of the two effects requires further experiments in which the structure and properties of the polymersome membrane are varied.

If the release rate achieved for calcein is compared to the rates previously demonstrated for membrane-associated doxorubicin purely through magnetic hyperthermia [24,25], the triggered release rates achieved here are at least twice as high and the total fraction of released compound is also higher, while the passive release is negligible and significantly lower over these time-scales than in those previous studies. This observation holds over a time-scale of a few hours, which is the time-scale foreseen for a circulating drug carrier, and which would allow for rapid release of a controlled dose.

Similar to for magnetically actuated liposomes, we observe that the release could be dosed by application of multiple pulses, realizing a major advantage of field-triggered release. Although the release behavior of our polymeric vesicles parallels that of liposome analogues during magneto-thermal actuation we note that there are fundamental differences in the underlying mechanism due to different intermolecular interactions among the constituent amphiphiles. Lipid membrane dynamics are governed by collective behavior such as lateral mobility, while polymeric assemblies are characterized by higher aggregate stability and some degree of kinetic trapping that depends on molecular weight [4]. Moreover, for magnetopolymersomes with a thermoresponsive corona, sufficient liberated heat from the SPIONs in the membrane interior needs to reach the peripheral region in order to trigger the rearrangement of the H-bonding pattern of the PNIPAM backbone; this is inherently difficult to achieve due to the high thermal conductivity of water. Although the synthesized BCP 2 is below the entanglement molecular weight of the individual blocks ($M_e(\text{PI}) \sim 6$ kDa and $M_e(\text{PNIPAM}) \sim 20$ kDa) and thus expected to undergo Rouse diffusion, actuation primarily proceeds intramolecularly through local chain dehydration of the hydrophilic block and concomitant changes in the packing parameter [4]. In the case of liposomes with membrane-embedded SPION, the dissipated heat is released in the direct vicinity of the thermoresponsive site. The lipids undergo a phase transition due to chain melting or rotational isomerizations of the alkyl chains that alters the packing pattern of the membrane forming amphiphiles. At the phase-boundary of co-existing phases close to T_m , defects exist that increase membrane permeability greatly compared to single-phase membranes above and below T_m . These differences between liposomes and polymersomes make it more difficult to engineer controlled, local, triggered release through magnetically induced hyperthermia for polymersomes than for liposomes, but the generally greater versatility of physical and chemical properties of block co-polymers compared to lipids makes further optimization worthwhile.

Although our aim was to investigate the feasibility of using thermoresponsive polymersomes with well-organized SPION inserted into the membrane for magneto-thermal actuation, its primary application might be in the biomedical field. Biomedical application requires that issues of stability in biological environment and toxicology are investigated, which lie beyond the scope of this work. However, in brief, the efficient loading of SPION into the polymersome membranes is not expected to lead to toxicity due to the general low toxicity of iron oxides leading to that they are FDA approved

for biomedical applications [38] Namely, iron oxides dissolve into their constituent ions in acid environments such as the lysosome, and the ions are readily absorbed by the body [39].

As is shown by the thermal tests of the polymersome stability and the magneto-thermal release tests, the vesicles are stable at body temperature and the major structural transition takes place at higher temperatures. Applicability for biological applications of the presented non-optimized model system could therefore be expected. However, it is also indicated that the passive release of the PNIPAM-stabilized polymersomes might be faster at 37 °C and another hydrophilic block with higher LCST should preferably be chosen for applications. For *in vivo* application, the potential toxicity of PNIPAM also has to be considered, and favors the exchange for another thermoresponsive polymer block. A low toxicity has however previously been demonstrated for polymersome delivery systems [40] and a cell-type dependent cytotoxicity for PNIPAM [41].

4. Materials and Methods

4.1. Synthesis

4.1.1. Polyisoprene MacroRAFT Agent (1): HOOC-PI(1300)-DTB

Polyisoprene macroRAFT agent (1) was prepared as in reference [42] with slight modifications. RAFT agent (81 mg, 0.29 mmol) and AIBN (24 mg, 0.146 mmol) were weighed into a thick walled glass tube. N₂-saturated anhydrous THF (5.5 mL) and isoprene (6 mL, 59.9 mmol) were added and the resulting mixture was sealed under inert atmosphere. The glass tube was placed in a preheated oil bath ($T = 125\text{ }^{\circ}\text{C}$) and polymerized for 2 h. The tube was then allowed to cool down to room temperature and the content was concentrated in vacuo. The resulting red-pinkish viscous oil was taken up in minimal DCM and precipitated in methanol. Compound **1** was collected by centrifugation (5000 rpm/10 min/rt), washed with methanol and dried in vacuo. Yield: 225 mg (5.5%). The macro RAFT agent was dissolved in N₂-saturated, anhydrous dioxane at a concentration of 75 mg/mL and stored at $-20\text{ }^{\circ}\text{C}$ until further use.

¹H-NMR (300 MHz, CDCl₃, δ): 7.98 (d, 2H, $J = 7.5\text{ Hz}$, Ph), 7.51 (t, 1H, $J = 7.3\text{ Hz}$, Ph), 7.37 (t, 2H, $J = 7.4\text{ Hz}$, Ph), 5.76 (1H, 1,2-PI), 5.12 (1H, 1,4-PI), 4.90 (2H, 1,2-PI), 4.69 (2H, 3,4-PI), 4.01 (t, 2H, $J = 7.9\text{ Hz}$, CH₂-S-C(S)), 1.5–2.3 (CH₂, CH₃ PI).

UV/VIS (1,4-dioxane, λ_{abs} , nm): 280 (Ph-), 296 (C=S), 334 (sh), 500 (Ph(C=S)S). The PI-block of the macro-RAFT agent was evaluated according to references [18,43] and displayed the following microstructure: 90% 1,4-addition (*cis/trans* $\sim 2/1$), 5% (1,2-addition) and 5% (3,4-addition).

4.1.2. RAFT Terminated Diblock-Copolymer: HOOC-PI(1300)-*b*-PNIPAM(1000)-DTB

The thermoresponsive PNIPAM blocks were prepared similar to Shan *et al.* [44] Macro-RAFT agent **1** (1.33 mL, 75 mg/mL) was added to a solution of *N*-isopropylacrylamide (NIPAM, 1.52 g, 13.4 mmol) and AIBN (0.82 mg, 0.005 mmol) in anhydrous dioxane (6.4 mL) to yield a (M)/(macroRAFT agent) ratio of 167/1. After purging the solution with nitrogen for 20 min, the flask was immersed into a preheated oil bath (70 °C) for 20 h. After cooling down, the flask was attached to a high vacuum system to remove dioxane and sublimate residual monomer. The crude residue was washed with hot water several times and subsequently freeze-dried.

4.1.3. Disulfide Terminated Diblock-Copolymer (BCP **2**): HOOC-PI(1300)-*b*-PNIPAM(1000)-SSMe (**2**)

Cleavage of the DTB head group was conducted according to a modified procedure of Roth *et al.* [45]. The light orange polymer was dissolved in anhydrous THF (4 mL) and mixed with *S*-methyl methanethiosulfonate (188 μL , 2.25 mmol). After purging the resulting solution with nitrogen, (2-dimethylamino) ethylamine was dropwise added via a syringe (110 μL , 1 mmol). Discoloration to a faint-yellow solution is indicative of dithioester removal and was observed within 3 h. To assure complete conversion, the reaction was allowed to stir overnight. The solution was concentrated and

the residue was washed with water and methanol. After drying, the crude product was purified via silica gel column chromatography. First, residual polyisoprene was eluted using DCM/MeOH 100/1, then block copolymer **2** was obtained using DCM/MeOH 6/1 as eluent. Yield: 38 mg (21%).

$^1\text{H-NMR}$ (300 MHz, CDCl_3 , δ): 6.90 (1H, NH, PNIPAM), 5.76 (1H, 1,2-PI), 5.12 (1H, 1,4-PI), 4.90 (2H, 1,2-PI), 4.69 (2H, 3,4-PI), 4.00 (1H, s, $\text{CH}(\text{CH}_3)_2$ PNIPAM), 0.8–2.2 (CH_2 , CH_3 PI, CH_2 , CH, PNIPAM), calculated from the M_n (MALDI-TOF MS) the block copolymer composition is $\text{PI}_{17}\text{-}b\text{-PNIPAM}_{8.5}$.

$^{13}\text{C-NMR}$ (75 MHz, CDCl_3 , δ): 174.6 (C=O, PNIPAM), 135.1 (1,4 C=C, PI), 125.0 (1,4 C=C, *cis*, PI), 124.2 (1,4 C=C, *trans*, PI), 111.2 (1,2 and 3,4 C=C, PI), 41.6 (CH-CO, PNIPAM), 39.8 (CH_2 , PI), 38.5 (CH_2 , PNIPAM), 32.0 (CH_2 , PI), 29.7 (CH_2 , PI), 28.3 (PNIPAM), 26.7 (CH_2 , PI), 23.5 (CH_3 , PNIPAM), 22.5 (CH_3 , 1,4-*cis*, PI), 16.0 (CH_3 , 1,4-*trans*, PI).

MALDI-TOF MS (DHB, no salt added) M_n : 2337 g/mol, polydispersity: 1.14. For (M)/(Macro RAFT agent): 167/1 a BCP with 40 vol % PNIPAM was obtained.

ATR-FTIR (powder, cm^{-1}): 3600–3200 (b, –OH), 3300 (NH, amA), 3070 ($=\text{CH}_2$, 3,4-PI), 2966 (CH_3), 2924 (CH_2), 2874 (CH_3), 2854 (CH_2), 2234 (CN), 1715 ((C=O)OH), 1642 (C=O, amI + C=C, 3,4 & 1,2 PI), 1540 (NH, amII), 1453 (CH_3 , PNIPAM), 1383 (CH_3 , t-1,4-*trans* PI + PNIPAM), 1368 (CH_3 , PNIPAM), 1264 (NH, amIII), 1172, 1130 (C–C, c-1,4-*cis* PI), 1098, 1027 ($=\text{C}-\text{CH}_3$, c-1,4-*cis* PI), 1004 (C–C, 3,4-PI), 909 ($=\text{CH}_2$, 1,2-PI), 886 ($=\text{CH}_2$, 3,4-PI), 840 ($-\text{CH}=\text{CH}-$, c,t-1,4-*cis,trans* PI), 690 (NH, amV), 510.

UV/VIS (MeCN, λ_{abs} , nm): 208 (CONH), 272 (sh, –SSMe)).

4.2. Vesicle Formation and Release Study

4.2.1. Solvent Inversion

Multilamellar vesicles (MLVs) were formed by a protocol modified from Bixner *et al.* [35]. Typically, 4 mg block copolymer were mixed with the respective weight percentage of hydrophobic SPIONs and dissolved in 200 μL THF. The mixture was dropwise added into 2 mL aqueous medium (buffer or ultrapure water) containing 5 mg/mL calcein (0.2 μm filtered) under magnetic stirring. The solvent was evaporated at room temperature under a constant N_2 stream for 3 h and while adding Milli-Q to keep the original total volume. The as-prepared vesicle suspension was extruded 10-times through 100 nm track-etched polycarbonate membranes in a hand-held extruder (Avanti) to increase the encapsulation efficiency and improve lamellarity.

4.2.2. Release Assays

Removal of non-encapsulated dye and free nanoparticles from the extruded samples was performed on a Bio Logic DuoFlow chromatography system equipped with a UV-detector (Bio-Rad, Austria), a Knauer Smartline RI 2300 detector (Bio-Rad, Austria) and a Bio Logic BioFrac collector (Bio-Rad, Austria). In detail, the samples (2 mL, 2 mg/mL) were purified by passing over a FPLC-column (length $\times$ diameter: 60 cm $\times$ 3 cm, stationary phase: Superdex 75, (GE Life Sciences, Austria) in Milli-Q water with a flow rate of 0.75 mL/min. Fractions of 2 mL containing the desired sample (usually 4 fractions) were identified by UV and RI detection. The sample concentration decreased to 0.5 mg/mL by the purification process.

4.2.3. Magnetic Actuation

The as-prepared sample was filled in a PMMA cuvette which was placed in an Ambrell Easy Heat LI magnetic heater, with a current of 438.9 A and a frequency of 228 kHz, coil dimension (height $\times$ outer diameter $\times$ coil thickness $\times$ number of turns = 37 mm $\times$ 37 mm $\times$ 2 mm $\times$ 6). The field strength in the center of the coil where the sample is placed is with these parameters estimated to be ~ 95 mT. Magnetic actuation was performed in 8 min or 10 min cycles, with a delay of 5 min between the cycles for recording of the released amount of calcein via fluorescence spectroscopy.

4.2.4. Fluorescence Measurements

Fluorescence spectra were collected with a PerkinElmer LS 55 luminescence spectrometer (PerkinElmer, Austria) at an excitation wavelength of 495 nm and an emission wavelength of 515 nm with a scan speed of 100 nm/min and a slit width of 2.5 nm. In some cases, the sample was diluted further in order to be within the optimal working range of the photo detector. Release of calcein was calculated according to the formula:

$$\text{Release\%} = \frac{I_i - I_{AMF}/I_{PL}}{I_i - I_{tot}} \quad (1)$$

where I_i is the initial fluorescence intensity measured immediately after column purification, I_{AMF} is the fluorescence intensity measured after the sample was subjected to individual AMF treatments and I_{PL} is the fluorescence intensity measured at different times without applying any AMF in order to calculate passive leakage. I_{tot} is the total fluorescence intensity measured after complete lysis of the vesicles by addition of Triton X100 (10% v/v of 20% Triton in MQ water).

4.3. Methods

4.3.1. TEM

TEM studies were performed on a FEI Tecnai G2 20 transmission electron microscope (FEI, Austria) operating at 160 kV. Samples were prepared by dropping aqueous vesicle dispersions containing 1%–3% w/w (+)-D-trehalose onto 300-mesh carbon-coated copper grids and subsequently air drying the samples for a few hours.

4.3.2. Dynamic Light Scattering

Hydrodynamic diameters, aggregation points and temperature cycling experiments were measured on a Malvern Zetasizer Nano-ZS (Malvern Instruments, Germany) in Milli-Q water at variable temperature in 173° backscattering mode. Samples were equilibrated for 120 s each and the autocorrelation function was obtained by averaging 3 runs. Samples were measured at 100 µg/mL.

4.3.3. TGA/DSC Measurements

Thermograms were recorded on a Mettler-Toledo TGA/DSC 1 STAR System (Mettler Toledo GmbH, Vienna, Austria) in the temperature range 25–650 °C with a ramp of 10 K/min under 80 mL/min synthetic air gas flow. The mass loss was evaluated by horizontal step setting.

4.3.4. ¹H- and ¹³C-NMR Measurements

¹H and ¹³C-solution spectra were collected on a Bruker AV III 300 spectrometer (Bruker Austria GmbH, Vienna, Austria). Chemical shifts were recorded in ppm and referenced to residual protonated solvent (CDCl₃: 7.26 ppm (¹H), 77.0 ppm (¹³C)).

4.3.5. MALDI-TOF-MS Measurements

Mass spectra were collected using a Bruker Autoflex (Bruker, Austria) speed in reflector positive mode (laser power: 20%, number of shots: 30,000). Matrix: DHB (20 mg/mL) in THF was mixed with sample and dropped on a sample holder. Calibration was performed with Peptide Calibration Standard II from Bruker (Bruker, Austria).

4.3.6. ATR-FTIR Measurements

Mid-IR powder spectra of the lyophilized samples were collected using a Bruker Tensor 37 FTIR spectrometer with a Bruker Platinum Diamond single reflection ATR equipment (Bruker, Austria) at a resolution of 4 cm^{−1} by averaging 32 scans.

4.3.7. UV-Vis Measurements

UV-Vis absorption spectra were collected at a scan speed of 400 nm/min on a Hitachi UV-2900 spectrophotometer (Hitachi Power Tools, Austria).

5. Conclusions

In summary, we have demonstrated synthesis and assembly of PI-*b*-PNIPAM block copolymersomes into which well-stabilized hydrophobic core-shell SPIONs can be incorporated. The size and stability of the assemblies are suitable for nanoscale encapsulation and release applications. A hydrophilic compound could be released by magnetic heating that induced a reversible structural change in the polymersome membrane. The release was controlled but not as efficiently as previous demonstrations of similar release strategies using liposomes; this could possibly be improved by optimizing the structure using a higher molecular weight block copolymer for which the hydrophilic block undergoes a more drastic volumetric change upon dehydration than is the case for short PNIPAM blocks. A correspondingly higher MW hydrophobic block also allows for incorporation of large SPIONs that provide more efficient heating. Compared to previously demonstrated release of membrane associated compounds from polymersomes using magnetically induced hyperthermia, the release rate of our hydrophilic model compound was higher and the passive release over relevant time scales significantly lower. Further development of the introduced structure and mechanism for magneto-thermal release from polymersomes here could result in a delivery system optimized for advanced, dosed release in, for example, the biomedical field.

Supplementary Materials: The following are available online at www.mdpi.com/1996-1944/9/1/29/s1.

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Supplementary Materials: Triggered Release from Thermoresponsive Polymersomes with Superparamagnetic Membranes

Oliver Bixner, Steffen Kurzhals, Mudassar Virk and Erik Reimhult

1. Reagents

All chemicals were purchased from Sigma-Aldrich (Austria) and used without further purification except otherwise noted.

4-cyano-4-(phenylcarbonothioylthio)pentanoic acid > 97%; isoprene 99% (contains < 1000 ppm *p*-tert-butylcatechol as inhibitor); *N*-isopropylacrylamide 97%; 2,2'-azobis(2-methylpropionitrile) 98%; *S*-methyl methanethiosulfonate 97%; *N,N*-dimethylethylenediamine 95%; Milli-Q water ($R = 18 \text{ M}\Omega\text{-cm}$); methanol anhydrous $\geq 99.8\%$; acetone chromasolv for HPLC $\geq 99.9\%$; dichloromethane anhydrous $\geq 99.8\%$ (contains 50–150 ppm amylene as stabilizer); chloroform $\geq 99.5\%$ (containing 100–200 ppm amylenes as stabilizer); tetrahydrofuran chromasolv plus for HPLC $\geq 99.9\%$ (inhibitor-free); 1,4-dioxane ACS reagent $\geq 99.0\%$; toluene anhydrous 99.8%; *n*-hexane anhydrous 95%; aluminum oxide activated, basic, Brockmann I (150 mesh); (+)-D-trehalose dihydrate from corn starch > 99%.

N-isopropylacrylamide (NIPAM) was recrystallized from hexane/toluene: 1/1 v/v. 2,2'-azobis(2-methylpropionitrile) (AIBN) was recrystallized from methanol. Isoprene was purified by passing through a column of basic alumina.

2. Spectroscopic Analysis

Figures S1–S3 show additional characterization of the synthesized block copolymer BCP 2, demonstrating purity of the product.

Figure S1. ¹H-NMR spectrum of BCP 2 in CDCl₃.

Figure S2. MALDI-TOF mass spectrum of BCP 2, DHB, no salt added, 1000–5000 Da, reflector positive mode.

Figure S3. ATR-FTIR spectrum of lyophilized BCP 2.

3. Characterization of Residual THF

THF remaining at high concentration in the sample could affect stability over time and lead to toxicity in biological applications. However, THF is a high vapor pressure solvent and is readily evaporated under continuous nitrogen flow until a homogeneous suspension of vesicles containing SPIONs is achieved. The amount of residual THF for the preparation of liposomes through the same protocol for solvent inversion was previously quantified by us by NMR to be 0.05 % or 50 ppm of its initial value (Figure S4) [1]. Also after 3 h under continuous nitrogen evaporation it is negligible. Such minimal traces of THF retained are far below any toxic level and suitable for biological and medical applications [2].

Figure S4. ^1H NMR spectra (300 MHz) of POPC in D_2O containing 1 mg/mL DSS as reference standard. Liposomes were formed at 0.5 mg/mL via 1:10 solvent inversion. (A) NMR spectrum right after dropwise addition of THF at $t = 0$ h and (B) after 24 h of evaporation. Residual THF was quantified by integration of the resonances at $\delta = 3.74$ (CH_2O) and 1.87 ppm (CH_2).

4. Determination of the Iron Oxide Nanoparticle Loading

4.1. Additional TEM of SPION-Loaded Polymersomes

Figure S5 shows additional TEM micrographs of calcein-loaded, extruded PI-*b*-PNIPAM polymersomes with 20% w/w 3.5 nm hydrophobic SPION input.

Figure S5. TEM micrographs of calcein-loaded, extruded PI-*b*-PNIPAM polymersomes at 1 mg/mL with 20% w/w 3.5 nm hydrophobic SPION input. Samples were prepared by THF solvent inversion into 5 mg/mL calcein solution to form polydisperse, large polymersomes and subsequent extrusion through 100nm track-etched polycarbonate membranes after evaporation of the organic solvent.

4.2. Thermogravimetric Analysis

For TGA determination of the effective SPION content of the polymersome membranes the lyophilized samples were burnt under oxidative conditions (synthetic air) to yield near complete combustion. Yet a considerable residue (~11% w/w) remained even in the case of polymersomes containing no SPIONs. The reported final SPION loading content therefore refers to the non-combusted material at 650 °C in excess of the residue for samples not containing nanoparticles, which amounts to approximately 9% w/w for extruded SPION loaded samples.

4.3. Optical Density Determination

Optical density (OD) values at 350 nm (OD^{350}) were used for spectroscopic quantification of the SPION embedding efficiency. The OD^{350} values were obtained by dilution of the respective suspensions to match the amide absorptions at 208 nm. Background spectra of the plain extruded PI-*b*-PNIPAM vesicles were recorded to account for vesicular scattering. The OD^{350} value of the initial SPION loaded suspension was assigned to the input SPION weight fraction (20%) and the final loading content was determined by evaluating the OD^{350} decrease upon extrusion. In this way we estimate an effective loading content of around 9% w/w which agrees with the results obtained by TGA. The slight difference between the two methods is likely to be caused by remote mass loading and use of augmenting relations in TGA while the choice of evaluation wavelength and variations in lamellarity cause deviations in UV/VIS.

5. Characterization of SPION Loaded, Extruded Assemblies of BCP 2

Figure S6A shows the increase in bulk temperature resulting from magnetic heating and Figure S6B shows the long-term passive release of calcein at room temperature when no actuation is applied. Figures S7 and S8 show the effect of temperature on polymersome size from bulk heating and from localized heating by application of an alternating magnetic field.

Figure S6. (A) Bulk temperatures upon magnetic heating for SPION-loaded samples (blue) and ultrapure water (green) as reference; (B) Passive leakage of the vesicles evaluated over a 5 h time course at room temperature (24 °C) shows linear release of encapsulated calcein. Over a time period equivalent to that of the complete magnetic actuation sequence there is no release observed.

Figure S7. DLS size distributions of SPION-loaded polymersomes before (A) and after (B) AMF heating.

Figure S8. DLS size-distributions of the thermoresponsive polymersomes during temperature jumps (A) 25 °C–35 °C–25 °C; (B) 25 °C–45 °C–25 °C and (C) 25 °C–55 °C–25 °C exhibiting reversible size changes of the vesicles without signs of disintegration.

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3.4. Magnetically Actuated Thermally Responsive Hybrid Vesicles of Lipids and Superamphiphiles

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Motivation

Lipid-based carrier systems offer excellent biocompatibility, high compositional versatility and distinguished response to magnetic actuation. A drawback for practical purposes is however their short half-life and mechanical sensitivity.²³ Clearance kinetics needs to be controlled by adding PEGylated additives that prevent opsonization and enzymatic degradation.³⁰ Synthetic high-Mw amphiphilic block copolymer analogs can form tough, antifouling vesicles that are entirely composed of protein-repellant materials and that are relatively insensitive to conjugation effects.^{26,27,30} Although polymersomes enjoy a great popularity as mimics of lipid vesicles for drug delivery their mechanical robustness and colloidal stability come at the price of complicating the dosed release of entrapped cargo. In particular, purely polymer-based vesicles incorporating magnetic nanoparticles suffer from inefficient magnetic actuation^{86,87,99} and exhibit low targeting efficiencies due to a statistical positioning of the active ligand within the polymer-coil.¹¹⁸

Vesicles of mixed amphiphilic block copolymer and lipid membranes are designated as a means to create composite systems that combine the stability and anti-fouling properties of macromolecular superamphiphiles with the magnetic response characteristics of lipids for efficient and easy actuation. Tuning the vesicle properties via admixing of a functional lipid species would experimentally be extremely appealing and straight-forward to realize since it avoids laborious derivatization and purification schemes while reducing the system's cytotoxicity. We speculate that these blends as well allow to adjust the effective density of the scaffold polymer, hence in-vivo vesicle stability, and to tune the exposure and presentation of conjugated biologically active motifs for targeting applications.¹¹⁹ As such, magnetolipopolymersomes are exploited as ideal candidates for merging facile magnetic actuation and immediate antigen presentation of lipid vesicles with the mechanic robustness, stealth and antifouling properties of synthetic superamphiphilic vesicles.

Contribution:

I co-planned the work, prepared all samples, evaluated most experiments and co-wrote the manuscript.

Magnetically Actuated Thermally Responsive Hybrid Vesicles of Lipids and Superamphiphiles

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ABSTRACT

Hybrid vesicles of lipids and superamphiphile block copolymers could combine the biological and functional versatility of lipid drug delivery systems with the mechanical stability and robustness of polymersomes. We investigate the structure and physical characteristics important for thermal actuation of vesicle-forming blends of saturated phospholipid, poly(butadiene-*b*-ethylene oxide) and thermoresponsive poly(isoprene-*b*-N-isopropylacryl amide) which are additionally loaded with hydrophobic superparamagnetic nanoparticles in the vesicle membrane. Lipopolymersomes are shown to be the most efficient option to trigger release of encapsulated compounds from superamphiphile vesicles by application of an external magnetic field causing local hyperthermia. This superiority is shown to depend on the formation of lipid domains in the hybrid vesicle membrane and the efficient loading of hydrophobic nanoparticles.

Introduction

Traditional lipid-based carrier systems have long been appreciated as excellent delivery vehicles for therapeutic agents because of their inherent biocompatibility, compositional flexibility and potential to shuttle diverse cargo. They are since long established in clinical use.^{1,2} A tentative drawback of liposomes is their short *in-vivo* half-life and mechanical instability.³ PEGylated lipids that prevent opsonization and enzymatic degradation are added to liposomes to increase their circulation time,⁴ but the maximal fraction of PEGylated lipids is limited to a few mole-percent ($\leq 10\%$) as the large and heavily hydrated polymer coil conjugated to the headgroup changes the packing preference of the modified lipid.^{3,4} Synthetic, high- M_w , amphiphilic block copolymer analogs on the other hand can form tough, antifouling vesicles with tunable membrane properties.^{3–6} Polymersomes have therefore received significant attention for their potential to mimic lipid vesicles for drug delivery, but with improved stability *in-vitro* and *in-vivo*. A tunable membrane structure makes it possible to increase the loading of hydrophobic and amphiphilic components as well as to tailor environmental responsiveness and other useful properties.^{7–10} However the mechanical robustness and colloidal stability of polymersomes come at the price of complicating the dosed release of entrapped cargo.^{11–14} An elegant approach to achieve external control over the release profile is through structural incorporation of functional nanomaterials; these can act as efficient sensitizers for externally controlled electromagnetic fields that actuate the vesicle membrane, e.g. through local heating.^{15–17} One such system with emerging clinical applications is the incorporation of FDA-approved, biocompatible, superparamagnetic iron oxide nanoparticles (SPIONs) in combination with magneto-thermal actuation.^{16,18–20} Purely polymer-based vesicles incorporating magnetic nanoparticles still suffer from inefficient magnetic actuation and/or exhibit high passive release.^{11–14} However, recent work by e.g. Weitz and coworkers on micrometer-sized polymer capsules showed that effective release can be achieved in such systems using plasmonic ablation when only a fraction of the polymersome membrane is thermoresponsive.²¹ The large size of these capsules, their microfluidic production and the optical field actuation are however not suited to biotechnological and biomedical applications. Vesicles with mixed amphiphilic block copolymer and lipid membranes were initially proposed as a means to create composite systems of reduced toxicity with adjustable texture and membrane characteristics.^{22–27} These blends not only allow for adjusting the effective density

of the scaffold polymer and hence *in-vivo* vesicle stability but also for tuning the exposure and presentation of conjugated biologically active motifs for targeting applications.^{25,28} We hypothesize that such hybrid vesicles incorporating a fraction of thermoresponsive lipid membranes in a highly robust polymersome also can confer the property of responsiveness and magneto-thermally actuated release to polymersomes that retain their desired properties; thus, a more robust and versatile hybrid vesicle systems can be created and applied for release applications. Therefore, we present an approach to magneto-responsive superstructures comprising blends of the densely PEGylated, vesicle-forming scaffold polymer PBD(1200)-b-PEO(600) with high- T_m lipids in combination with membrane embedded, monodisperse SPIONs. These partly thermoresponsive hybrid vesicles are compared to PBD-b-PEO polymersomes incorporating a thermoresponsive PNIPAM-based block copolymer. A detailed investigation of the incorporation of nanoparticles in the PBD-b-PEO block copolymer scaffold was performed as well as an investigation of the hybrid vesicle structure and thermoresponsive properties. This data is used to interpret the magneto-thermal release efficiencies for an entrapped water-soluble model compound.

Results

Assembly of mixed lipid and superamphiphile vesicles

Generally, the mixing behavior of different membrane forming species depends on amphiphile characteristics, their weight fractions, respective molecular weights (M_w) and the extent of phase separation.^{22–24,26,27} We evaluated the phase partitioning of the mixed membrane capsules of different sizes using confocal microscopy and differential scanning calorimetry (DSC). The findings for micron-sized giant unilamellar vesicles (GUVs) formed by electrosweeling and nanoscale large unilamellar vesicles (LUVs) formed by solvent inversion and extrusion are summarized in Fig 1.

Saturated phospholipids were found to form lipid domains in hybrid lipopolymersomes upon electrosweeling in aqueous media (see Fig. 1B). This is in good agreement with recent literature reports.²² Domain formation was observed regardless of whether a fluorescent label covalently conjugated to the polymer or if a fluorescently labeled lipid probe that partitions into the polymer phase were imaged (see Fig 1B and SI 1); this suggests that phase separation into lipid rich areas is not an artefact of the probe induced by illumination as seen for some lipid systems.²⁹ The lipid distribution observed by confocal microscopy was found to vary within the vesicle population and in particular with vesicle size (see Fig. 1A). For small GUV diameters the patches seemingly became more singular whereas patterns of dispersed and isolated domains were spotted in large lipopolymersome GUVs. This might be influenced by that small GUVs approach the resolution limit of the confocal microscope. However raft formation on the nanoscale has been reported using super-resolution techniques.³⁰ Differential scanning calorimetry of hydrated vesicles suspensions was used to investigate the mixing behavior of the two amphiphile species on different lengths scales.^{31,32} The main melting transition of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) lipids is characterized by a sharp and symmetric high enthalpy peak located at 41°C. During this main transition the lipid membrane changes from a relatively ordered crystalline-like gel state to a disordered fluid-like state which typically involves a high level of cooperativity that accounts for the sharpness of transition peak. This transition is due to the concerted melting of the hydrocarbon chains associated with conformational changes from the all-trans configuration to a state with an increased frequency of rotamers. Upon formation of the lipid diblock copolymer blend the main melting transition temperature shifted slightly and significantly broadened (see Fig. 1C). A shift of the melting transition is expected upon formation of a mixture with a second component whose T_m differs from that of the lipid. Generally, the more homogeneous the mixture is, the more the distinct transitions of the individual components will merge into a single one of the blend that is located in between the original ones. The position of the new T_m and its deviation from that of the ideal mixture then constitutes a measure of the extent of phase separation.³³ For the investigated blends the main melting transition temperature (T_m) of the admixed DPPC only slightly shifted within a range of 3°C. For the GUV preparation seen in Fig 1A the T_m of DPPC was raised by 2°C to 43°C. The shift was accompanied by a nearly 100-fold decrease in intensity and 7-fold broadening at the FWHM. The thermograms of polydisperse GUVs were found to be similar to those of the nanoscale vesicles formed purely by solvent inversion. The significant broadening and decrease in the peak value of the main lipid transition can be attributed to inhomogeneity of the sample such as irregular domain sizes and lipid content per hybrid vesicle.^{31,32} The thermograms of polydisperse GUVs were found to be similar to those of the large vesicles formed purely by solvent inversion. The small shift in lipid chain melting temperature from its original value is indicative of a phase separated state in which lipid-lipid contacts are retained. The block copolymer does not have a transition temperature in the measured range. The vesicle size distribution is typically reflected in the DSC thermograms and influences the location and profile of the transition peak.^{31,32} Local curvature affects the cooperativity of the main transition by altering the packing of the hydrocarbon chains causing lower T_m and broader transitions for increasing curvature.³⁴ For SUV preparations a decrease in lipid T_m was reported due to this size dependence.³⁴ The lipopolymersome suspensions prepared via solvent inversion were inherently bimodal in DLS (see Fig. S-3) but their overall DSC peak profile closely resembled that of the phase separated GUVs. The observed small difference in melting temperature between GUV and LUV preparations could therefore be related to preferential distribution of lipids into smaller vesicles. This shift of the T_m is usually not directly observed in heating curves of small lipid vesicles because these tend to fuse upon heating. An observation

for lipopolymerosomes however seems plausible since polymeric vesicles are not expected to fuse due to their high aggregate stability.³ Impurities such as residual surfactant or solvent can as well lower the T_m of the preparations;³⁵ this is commonly known as van't Hoff's law of melting point depression in eutectics. The extent of lowering varies with the source of impurity and preparation method,^{31,32} and can e.g. be due to stabilization of interdigitated lipid structures. Such effects seem unlikely since we observe an increase in melting temperature in Fig 1C upon formation of the blends for all preparation methods. Moreover NMR quantification of similarly prepared samples showed that the residual solvent content is far below the required threshold for THF-induced interdigitation.^{35,36} A mixing of lipids with a higher T_m component such as PBD-b-PEO on the contrary will raise the T_m of the resulting blend,³³ as observed for the GUV and solvent inversion preparations. A similar curve profile in terms of broadening and intensity plus the characteristic increase in melting temperature was obtained both for the GUVs formed by electrosweeling and the corresponding nanoscale suspension prepared via solvent inversion (Fig 1C). It is likely that domain boundary effects leading to different membrane tensions and the polydispersity of lipid domains in the vesicle membranes lead to the shift in T_m and broadening of the transition. The similarity between DSC curves of GUVs and nanoscale vesicles also strongly indicates that lipids are separated into lipid domains within polymerosomes and not predominantly demixed into pure liposomes. Upon variation of the admixed lipid weight fraction no particular trend could be observed in the DSC traces (see Fig. S-2). This suggests that segregation into lipid rich domains is not markedly influenced by the employed weight fraction within the principal miscibility range of the two components. At high lipid fraction a miscibility gap was reported for compositionally similar diblock copolymers with higher M_w ;²³ the mixing behavior is governed by interfacial line tension such that a mismatch in M_w enforces a demixing of the system.²³ Further, we investigated a system of mixed diblock copolymers having the thermoresponsive polymer PNIPAM as the hydrophilic block. Optical micrographs of PBD(1200)-b-PEO(600) giant polymerosomes containing 30-50% w/w PI(1300)-b-PNIPAM(1000) produced by electrosweeling revealed homogeneous fluorescence distribution throughout the entire GUV population (cf. Fig. 1B). No phase separation or segregation into enriched domains could be detected on optical length scales for mixed polymer vesicles regardless of size. The result was independent of which block copolymer carried the fluorophore. The PI-b-PNIPAM system was specifically designed to give similar volume fractions and overall M_w as the scaffold polymer. The hydrophobic block is made from a rather fluid polymer with low glass transition temperature (T_g) and comparable texture while the thermoresponsive, hydrophilic corona consists of well hydrated PNIPAM. The shell compatibility of the two hydrophilic moieties has already been demonstrated by measurements of the melting temperature depression in binary PNIPAM-PEO mixtures³⁷ and by the formation of di- and triblock copolymer vesicles composed of PNIPAM-PEO and PI-PNIPAM-PEO.^{38,39} The mixing behavior of polymer blends with similar hydrophobic block compositions has previously been found to be homogeneous.^{40,41} This is in good agreement with our finding of no identifiable phase separation of the scaffold and thermoresponsive block copolymers in GUVs. GUVs are not suitable for delivery applications and therefore assembly for the investigation of magnetothermal release was exclusively performed on LUVs. Sample preparation by solvent inversion indicated vesicle formation for the nanoscale assemblies that paralleled that of the GUV preparation. The individual diblock copolymers assembled into vesicles with a sharp, bimodal size distribution for PBD-b-PEO or a broad, featureless one for PI-b-PNIPAM.¹⁴ Vesicles formed at 2mg/ml via solvent inversion frequently showed multilamellar and multivesicular assemblies both in optical microscopy and in TEM. The blend of the two block copolymers assembled into a monomodal population of 150nm in hydrodynamic diameter (see Fig. S-3). The formation of a single diblock copolymer LUV population with hydrodynamic diameters in between those of the individual components is a strong indication of uniform mixing as observed microscopically for the polymer GUVs. Lipopolymerosomes showed a bimodal size distribution which got biased towards the SUV population upon increasing the lipid fraction (see Fig. S-3). The observation of a bimodal vesicle size distribution for the mixture of two molecular species could be an indication of the individual preference of each species to form vesicles of a certain curvature. Lipid partitioning and distribution is known to depend on vesicle curvature.⁴² Theoretically, the higher curvature of smaller vesicles favors an asymmetric amphiphile distribution in which sterically less demanding species are incorporated into the inner leaflet, which leads to the hypothesis that SUVs are richer in lipids. Further clues that underpin this hypothesis are presented in the magnetic release section. It should be remarked again that the DSC results show that a complete demixing to two pure composition vesicle populations is not taking place, but the observed broad transition does not rule out populations which are differently rich in lipid. Temperature-dependent DLS of these preparations showed that the mixed polymer vesicles decreased in diameter upon heating to temperatures similar to the LCST of PNIPAM at 34°C, but did not aggregate (see Fig 1D and S-3). The pure PI-b-PNIPAM vesicles showed a decrease in size in the same range. This indicates that PI-b-PNIPAM mixes homogeneously with PBD-b-PEO during solvent inversion and collapses upon heating within blended polymerosomes. As expected, lipid melting was not reflected in a noticeable change of vesicle size at the T_m of neither pure liposomes nor hybrid vesicles of DPPC mixed in PBD-b-PEO (see Fig. S-3).

Dispersed SPION assembly in polymerosome membranes

Efficient magnetically triggered and thermally actuated release requires the incorporation of magnetic nanoparticles in the vesicle.^{16,17} The co-self-assembly behavior of the scaffold diblock copolymer PBD(1200)-b-PEO(600) with monodisperse

hydrophobic SPIONs was therefore first investigated and optimized. PBD(1200)-b-PEO(600) itself is not stimuli-responsive but can be used to efficiently host hydrophobic SPIONs at high density in the membrane interior and to impart mechanical and *in-vivo* stability to mixed and hybrid polymersome capsules as described above. SPION localization within the bilayer has been shown to be required for direct magneto-thermal actuation of membrane permeability.^{14,17,35} Assembling SPIONs at high density in the membrane without adversely affecting other desired properties for release applications such as unilamellarity and monodispersity is challenging. Accordingly, a method that allows for direct control over the spatial distribution and embedding efficiency of SPIONs as well as over vesicle size and lamellarity has to be found. We employed solvent inversion from THF into water (1 : 10), as it has been shown to readily assemble controlled polymersomes and nanoparticle-containing liposomes with high efficiency.^{35,43,44} In brief, 0.5-1mg of PBD(1200)-b-PEO(600) and the desired weight fraction of 3.5nm N-palmityl-6-nitrodopamide capped SPIONs (P-NDA-SPION) were dissolved in 100 μ l THF and dropwise added to a magnetically stirred solution of ultrapure water. The solvent was subsequently evaporated under a gentle stream of nitrogen gas for several hours. No visual precipitation of iron oxide nanoparticles occurred, indicating quantitative incorporation into the polymeric bilayer. This is further supported by determinations of the optical density (OD) at 350nm of the suspensions (see Fig. S-4). The incremental weight percentage of SPIONs is reflected as an increase in OD. TEM micrographs of the assembled structures at 0.5mg/ml polymer concentration mainly exhibited unilamellar polymersomes whereas preparations formed at concentrations $\geq$ 2mg/ml frequently exhibited multilamellar vesicles (see Fig S-5). Fig 1 displays representative TEM images of the SPION distribution in PBD-b-PEO vesicles formed at polymer concentrations of 1mg/ml.

The vesicle size distribution observed in transmission electron microscopy images correlates well with those obtained by DLS (see Fig. S-5). The preparations appeared bimodal by both methods with the main scattering peak in DLS intensity weighted distributions at around 150nm and a minor fraction at 20nm. The latter could indicate the presence of micellar structures or very small vesicular structures (cf. Fig. 1A); these smaller structures did not seem to contain as many SPIONs as larger structures in TEM. Ultrathin-sections of the loaded polymersomes clearly depict unilamellar vesicles with SPIONs exclusively localized in the membrane interior (Fig. 1B), as expected for hydrophobic, nanoparticles. Trehalose fixed vesicles exhibit a homogeneous distribution profile of monodisperse SPIONs through the entire vesicle membrane (Fig. 1B inset). Densely packed SPIONs were observed in thin sections with focus on the membrane wall where the projection might over-emphasize density (Fig. 1B and 1A inset). Whether the particles are individually well dispersed in the membrane or found close-packed in rafts at high content of SPION in the membrane is important. Magnetostatic interparticle coupling at close packing affects the Néel relaxation of field-induced magnetization, which is the predominant relaxation mechanism in viscous microenvironments. The rate of magnetic dissipation is directly related to the SPION core volume and readily translates into the achievable heating rate, thereby constituting a crucial parameter of efficient membrane actuation.

Magnetically triggered thermal release from nanoparticle loaded superamphiphile vesicles

For quantification of magnetically triggered release, the fluorescent dye calcein was encapsulated at self-quenching concentrations into the aqueous lumen of the SPION-loaded, mixed membrane capsules. Calcein is a water-soluble dye that serves as an easily quantifiable model compound to investigate passive and magneto-thermally triggered release through the membrane by the increase in fluorescence when it is diluted after permeation through the membrane. Magneto-thermal release was triggered by applying an alternating magnetic field (AMF) of variable duration and intensity. The resulting relative increase in fluorescence was compared to the passive release at room temperature in absence of an alternating magnetic field. For all release assays we employed monodisperse 3.5nm N-palmityl-nitrodopamide capped magnetite nanoparticles with a grafting density of 2.7molecules/nm².⁴⁵ Nanoscale, mixed amphiphile capsules were formed via solvent inversion (THF to calcein solution 1 : 10, 3mg/ml calcein in Milli-Q water). Due to the high fraction of multilamellarity of polymersomes and the different size distributions for polymersomes and hybrid lipid-polymersomes an additional step of homogenization is required to assure comparability and reproducibility of the relative release from the different capsule systems. Therefore, all samples were sonicated for 30min and subsequently extruded through 100nm polycarbonate membranes in a hand-held extruder prior to release measurements to improve sample homogeneity and calcein encapsulation. Release was therefore measured on a monomodal extruded suspension with an average diameter of 150nm after size exclusion chromatography. An alternating magnetic field ($I=438.9$ A, $f=228$ kHz, coil dimension : height x outer diameter x coil thickness x number of turns = 37mm x 37mm x 2mm x 6, $B=94.7$ mT) applied for different pulse lengths was used to investigate triggered thermal release actuated by the magnetic nanoparticles at biomedically relevant frequency and field strength. As expected, the very stable vesicles of the non-thermoreponsive PBD(1200)-b-PEO(600) superamphiphile were not susceptible to magneto-thermal actuation. Only a negligible release of $\leq 10\%$ in 3h could be obtained for 30%w/w SPION loaded PBD(1200)-b-PEO(600) vesicles when actuated even at extremely long magnetic pulse durations of 40min (see Fig 3A). Such long pulse lengths resulted in significant increase in bulk sample temperature to approximately body temperature (Fig. S-6). The same rate of release was observed without actuation, demonstrating that neither temperature increase nor local actuation led to the observed release. In contrast, magnetoliposomes made of lipids that undergo a phase transition above ambient bulk temperature demonstrate controlled

release upon short-term irradiation with alternating magnetic fields due to direct heating of the lipid membrane in which the particles are incorporated.^{17,46} Polymersomes with a thermoresponsive hydrophilic block of PNIPAM also were shown to allow for limited magnetothermal release at long magnetic actuation times, where bulk heating contributed to the release.¹⁴ Both these systems are less stable than PBD(1200)-b-PEO(600) vesicles, but we hypothesized that the blended and hybrid vesicles that incorporate a thermoresponsive component could combine stability with efficient release.

The released percentages of encapsulated calcein from hybrid and blended magnetosomes with 3.5nm SPIONs incorporated in the membrane resulting from exposure to AMF pulses are shown in Figures 3A and 3B. Figure 3B shows that the magneto-thermally triggered release from blended PBD-b-PEO / PI-b-PNIPAM vesicles is comparable to the passive release even when bulk temperatures of 40°C were reached for 30min AMF pulses. No passive release could be detected at low particle loading of 5%w/w and both actuated and non-actuated samples showed zero release within the 90min time-span of the experiment. For a 30%w/w SPION loading we observed 10% release of the encapsulated content after 90min regardless of application of the alternating magnetic field. Incomplete and inefficient magnetic release for pure PI(1300)-b-PNIPAM(1000) vesicles with a LCST temperature of 35-50°C was previously reported and attributed to the high thermal conductivity of water and therefore inefficient heat transport from the bilayer embedded particles to the thermoresponsive PNIPAM corona.¹⁴ Due to the homogeneous texture of the blended polymer vesicles the increase in bulk temperature induced by the magnetic heating to 40°C was presumably not sufficient to trigger release. We note that the passive release is strongly increased by high particle loading, but it is negligible for pure PI-b-PNIPAM vesicles and its blends with PBD-b-PEO at low nanoparticle loading. The finding that SPION content influences passive leakage in polymersomes was reported earlier.¹¹⁻¹³ However, these reports employed polydisperse SPIONs and SPIONs with dispersants that can easily desorb and partition into the bilayer and thereby alter membrane permeability. For our monodisperse and irreversibly surface modified SPIONs the passive release predominantly observed at high particle loading is likely due to engulfment of small SPION aggregates. At 30%w/w of SPION the loading is close to that estimated for a close-packed monolayer. Small clusters also appeared in TEM micrographs slightly below this concentration (see Fig 2B). A direct influence of individually dispersed SPION is less likely as evidenced by the negligible passive release observed at low and intermediate SPION fraction and the lower sensitivity to membrane deformation of superamphiphilic membranes compared to lipids stemming from the nearly doubled hydrophobic membrane thickness of the PBD(1200)-b-PEO(600) polymer.^{47,48} Release assays for lipopolymerosomes prepared with 30%w/w DPPC are shown in Figure 3A. Unilamellar lipopolymerosomes formed by solvent inversion and then homogenized through extrusion demonstrated magneto-thermally triggered release up to 50% of the encapsulated calcein (Fig. 3A (black solid line)), after which the release rate significantly decreased. Quite long pulse durations of 40min were needed to trigger a significant release from these lipid blended capsules, but release could be achieved at SPION loadings of just 5% and at bulk temperatures significantly lower than the lipid T_m . Higher SPION loading and actuation times did not markedly improve on the release efficiency. The observation that the release strongly abates at 50% of the encapsulated calcein suggests an inhomogeneous distribution of either lipids or SPIONs among the vesicles, with 50% of the vesicles being deficient in one of the two components required for triggered release. Given that we showed that SPIONs can efficiently be loaded at high concentration by solvent inversion into both liposomes and PBD-b-PEO polymersomes,^{14,35} the most likely reason for the reduced fraction of released cargo is an inhomogeneous distribution of lipid between vesicles. As described above, this could originate from an inhomogeneous lipid distribution among the SUV and LUV during solvent inversion. The selection of LUVs through column separation for the release experiments implies that this method might result in polymersomes with much lower lipid fraction and prevalence of lipid domains. We have previously shown that solvent inversion to assemble amphiphile vesicles is a delicate balance of interfacial energies.³⁵ Especially the assembly of saturated, long chain phosphocholine lipids is susceptible to slight adjustments of the solvent composition. Adding components to the blend that suppress interdigitation fusion and affect domain size and shape (such as cholesterol^{22,35}) could suppress the formation of a bimodal vesicle population displaying non-uniform release. Indeed, addition of 15%w/w cholesterol to 30%w/w DPPC lipopolymerosomes clearly suppressed the formation of SUVs (Fig. S-7). The effects of such ternary mixtures on SPION loading and drug co-incorporation however remain unclear at the moment and, although promising, optimization of such conditions will be the task of future work. Alternatively, lipopolymerosomes can be formed through direct rehydration of lipid, block copolymer and SPION, followed by extrusion to form a unilamellar and monodisperse vesicle population. With this method a high SPION content cannot be reached, since a large fraction of the nominally added SPION weight fraction is lost as aggregates during extrusion,^{17,35} but a bimodal distribution of vesicles is avoided. We prepared such lipopolymerosomes to test the hypothesis that lipid selection to SUVs rather than the distribution of SPION led to low release efficiency and total released amount. Lipopolymerosomes formed by rehydration plus extrusion showed much improved release efficiencies (Fig. 3A (blue and red lines)), i.e. release was achieved for much shorter pulse durations and a higher release rate was achieved than for vesicles formed by solvent inversion. The release rate for rehydrated lipopolymerosomes marginally depended on AMF pulse length. A short pulse length is desirable since bulk heating through induced eddy currents in the solution can be minimized; this is also reduced by the selected field frequency and power.⁴⁹ During the 90min period of actuation resulting in 50% release for the lipopolymerosomes formed by solvent inversion only

a negligible passive release of $\leq 3\%$ was recorded. The passive leakage for the faster actively releasing lipopolymerosomes formed via rehydration plus extrusion did not exceed 10% over 3h and was also $\leq 5\%$ over the time to achieve 50% actuated release. Taken together, the comparisons of both the actuated and the passive release demonstrate that the lipids and lipid domains were inhomogeneously distributed between large and small vesicles for samples prepared by solvent inversion. It also demonstrates that the effective lipid fraction can be used to tune release rate, efficiency and possible total release amount. To effect the release, SPIONs have to locally produce heat that causes a thermal transition in neighboring lipid membranes such that the membrane permeability changes. Global heating of lipopolymerosomes was reported to cause the lipid domains to homogeneously disperse into the polymer matrix.^{22,27} Such behavior would however compromise the permeability switching function of phase-separated lipid patches and lead to a significant reduction in release. In our case, the release profile was observed to be at most weakly dependent on the pulse length used and global temperature, which emphasizes that membrane actuation primarily proceeds via local SPION heating. It is possible that SPIONs are distributed in both lipid and polymer domains, but the higher capacity to load SPION into the polymer membrane and the achieved low passive release also at high SPION loading suggests that SPION preferentially localize to the polymer membrane and actuates at the edge of the lipid domains.

Conclusion

We have demonstrated the successful formation of unilamellar, polymer vesicles composed of PBD(1200)-b-PEO(600) and embedding of monodisperse, hydrophobic SPION at high density in the membrane. Individually dispersed SPIONs were found in the membrane which could be filled to close to monolayer density without strong cluster or multilayer formation. Release of hydrophilic compounds encapsulated in the stable vesicles required the incorporation of thermally responsive domains in the vesicle membrane. Release was only achieved for saturated lipids that phase separated into domains, but not for the thermoresponsive block copolymer PI(1300)-b-PNIPAM(1000) which mixed homogeneously. The lipopolymerosomes showed efficient release but with the high stability and low passive release of PBD(1200)-b-PEO(600) at a particle loading of 5%w/w that is higher than shown for liposomes. Higher particle loading increased passive release. At least half the loaded content could be efficiently released for lipopolymerosomes. The remaining entrapped and possibly slowly releasing fraction was attributed to an inhomogeneous distribution of lipids among vesicles of different size during solvent inversion, which could be suppressed by further optimization of the membrane composition. A higher lipid fraction resulted in more efficient release, but also higher passive release. In sum, the high polymersome stability could be conferred to vesicles that displayed magneto-thermal release similar to what has been reported for liposomes. Our proof-of-concept of magnetically actuated hybrid vesicles offers a plethora of possibilities to tune the release profile via tailoring of the lipid window domain size and composition, as well as potentially independent control of SPION size and distribution within robust polymersome membranes. Magnetolipopolymerosomes thereby combine the opportunities of facile magnetic actuation of lipid vesicles with the mechanic robustness, stealth and antifouling properties of synthetic superamphiphile vesicles.

Methods

Materials

The synthesis of monodisperse, N-palmitoyl-6-nitrodopamide coated superparamagnetic iron oxide nanoparticles (P-NDA-SPION) with a core diameter of 3.5 ± 0.4 nm was described previously.⁴⁵ Poly(isoprene-b-N-isopropylacrylamide), PI(1300)-b-PNIPAM(1000) ($M_n = 2300$ g/mol, $D = 1.14$) with a LCST in the range of 35-50°C was prepared by sequential RAFT polymerization.¹⁴ Poly(butadiene-b-ethylene oxide), PBD(1200)-b-PEO(600) ($M_w = 1800$ g/mol, $D = 1.17$) was obtained from Polymer Source Inc. and used as received. For fluorescence imaging, an aliquot of the respective polymer was conjugated to a dansyl tag. For PI-b-PNIPAM the fluorescent label was bound via an asymmetric disulfide during the cleavage of the RAFT head group,⁵⁰ whereas for PBD-b-PEO standard procedures for sulfonate ester conjugation⁵¹ were employed (see synthesis section in the supplementary information for details on the preparations and characterization). Lipids and lipid probes 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl ammonium salt (DOPE-RhoB) were purchased from Avanti Lipids Inc. and used without further purification.

Sample preparation

Solvent inversion: Large unilamellar vesicles (LUVs) were formed as described previously with modifications.³⁵ 1-5mg block copolymers were mixed with the respective weight percentage of hydrophobic SPIONs and dissolved in 200 μ l warm THF. The mixture was dropwise added into 2ml ultrapure water containing 5mg/ml calcein (0.2 μ m filtered) under magnetic stirring. The solvent was evaporated at room-temperature under a constant N_2 stream for 6h and continuously refilled with Milli-Q to its initial level. To increase the encapsulation efficiency and improve lamellarity, the as-prepared vesicle suspension was sonicated

for 30min and subsequently extruded 10-times through 100nm track-etched polycarbonate membranes in a hand-held extruder (Avanti).

Rehydration plus Extrusion: Large unilamellar vesicles (LUVs) were formed similar to literature reports.¹⁷ 4mg block copolymers were mixed with the respective weight percentages of lipid and SPION. The mixture was taken up in 2ml chloroform and sonicated for 5min before the solvent was removed on a rotary evaporator at T=50°C. The resulting homogeneous film was dried in high vacuum for 6h before the film was swollen for 1h in 5mg/ml calcein solution (0.2µm filtered) at 50°C. The sample was sonicated several times for 5min during the swelling period to disperse adherent material. The suspension was sequentially extruded 31 times at room-temperature through 100nm polycarbonate filters using a hand-held extruder (Avestin, Mannheim, Germany).

Electroformation: Giant unilamellar vesicles (GUVs) were formed by electrosweeling of the dried blends in 250mM sucrose solution at 50°C for lipopolymerosomes or at room-temperature for polymer blends using a Nanion electroformation stage at 15Hz and 3V AC.²⁴ The samples (containing 0.5-2% fluorescent labels) were mixed at the desired weight fractions and taken up in chloroform at 5mg/ml total polymer concentration before 50µl of the suspension was spread on conducting indium tin oxide (ITO) coated glass slides. The solvent was slowly evaporated in a gentle stream of nitrogen before the slides were dried in high-vacuum for several hours prior to electroformation. For imaging the vesicle suspension was diluted with isoosmolar glucose and GUVs were allowed to settle for 10min prior to imaging.

Release Assays: Excess calcein, nanoparticles and small assemblies were removed by size exclusion chromatography. For purification of the vesicles a Bio Logic Duo Flow chromatography system, equipped with a UV-detector, a Knauer Smartline RI 2300 detector and a Bio Logic BioFrac collector was used. Samples (2mL, 2mg/ml, homogenized) were purified by passing over a FPLC-column (length x diameter: 60cm x 3cm, stationary phase: Superdex 75) in Milli-Q water with a flow rate of 0.75mL/min. Fractions of 2mL containing the desired sample (usually 4 fractions) were identified by UV and RI detection. The sample's concentration decreased to 0.5mg/mL by purification.

Measurement conditions

TEM and analysis: TEM studies were performed on a FEI Tecnai G2 20 transmission electron microscope operating at 160kV. Samples were prepared by dropping aqueous vesicle dispersions containing 2%w/v (+)-D-trehalose onto 300-mesh carbon-coated copper grids and subsequently air dried for a few hours.

Confocal microscopy: Imaging was conducted using a DeltaVision microscope (Applied Precision Inc., Issaquah, WA, USA) fitted with a PLAPON 60XO/1.42 NA oil-immersion objective (Olympus, Tokyo, Japan), and DAPI, TRITC, FITC, and CY5 Semrock filters (New York, NY, USA). Images were processed using ImageJ freeware and DeltaVision software (Softworx 4.1.0, Applied Precision, Inc., Issaquah, WA, USA).

Dynamic Light Scattering: Hydrodynamic diameters and temperature cycling experiments were recorded on a Malvern Zetasizer Nano-ZS (Malvern UK) in Milli-Q water at variable temperature in 173° backscattering mode. Samples were equilibrated for 120sec. each and the autocorrelation function was obtained by averaging 3 runs.

DSC measurements: Thermograms were recorded on a MicroCal VP-Capillary DSC System (Malvern, UK) in the temperature range 5-80°C with a scan rate of 1K/min in high feedback mode. Data points were fitted to non-two-state equilibrium-unfolding models by the Lavenberg/Marquardt (LM) non-linear least square method.

OD measurements: Optical density curves were collected at a scan speed of 400nm/min on a Hitachi UV-2900 spectrophotometer.

Magnetic Actuation: A PMMA cuvette with the sample was placed in an Ambrell Easy Heat LI magnetic heater with coil dimensions of height x outer diameter x coil thickness x number of turns = 37mm x 37mm x 2mm x 6 at I=438.9A, f=228kHz and B=94.7mT. Magnetic actuation was performed in 5-40 min cycles, with a delay of 5 min between the cycles for monitoring the release via fluorescence spectroscopy.

Release - fluorescence measurements: Release of the encapsulated calcein to the bulk phase was quantified by recording the increase in sample fluorescence intensity as function of time and magnetic field actuation of the composite membrane. Fluorescence spectra were collected with a PerkinElmer LS55 luminescence spectrometer at an excitation wavelength of 495nm and an emission wavelength of 515nm with a scan speed of 400nm/min and a slit width of 2.5nm. In some cases the sample was diluted further in order to be within the optimal working range of the photo detector. Release of calcein was calculated according to the following formula,

$$Release\% = \frac{I_i - I_{AMF/PL}}{I_i - I_{tot}}$$

where I_i is the initial fluorescence intensity measured immediately after column purification, I_{AMF} is the fluorescence intensity measured after the sample was subjected to individual AMF treatments and I_{PL} is the fluorescence intensity measured at different times without applying any AMF in order to calculate passive leakage. I_{tot} is the total fluorescence intensity measured after complete lysis of the vesicles by addition of Triton X100.

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Author contributions statement

OB, MN and ER planned the work. OB, GLB, MV, SK, AS, AM and NG conducted and evaluated the experiments. SRK and MN provided guidance. OB and ER wrote the manuscript. All authors reviewed the manuscript.

Additional information

To include, in this order: **Accession codes** (where applicable); **Competing financial interests** The authors declare no competing financial interests.

The corresponding author is responsible for submitting a [competing financial interests statement](#) on behalf of all authors of the paper. This statement must be included in the submitted article file.

Figure 1. Fig. 1 Confocal microscopy images of PBD(1200)-b-PEO(600) GUVs blended with (A) 30% w/w DPPC (0.5% n/n DOPE-RhoB) and (B) 30% w/w PI(1300)-b-PNIPAM(1000) (1% n/n PI-b-PNIPAM-Dns). Lipopolymerosomes (A) exhibit phase-separated lipid-rich domains (dark areas) whereas the mixed block copolymerosomes (B) appear homogeneously mixed on a similar length scale. (C) DSC of the lipopolymerosomes of various sizes (GUVs – 100nm-100 μm ; solv. inv. – 20 and 150nm; DPPC – 110nm, C_p scaled) show a slight shift in the lipid T_m accompanied by a significant broadening of the transition indicating that polydisperse phase separated domains are also present for nanoscale assemblies. (D) Temperature dependent DLS of mixed diblock copolymer vesicles depict that the thermally induced change in hydrodynamic diameter occurs at similar, but possibly slightly higher temperatures than for the pure PI(1300)-b-PNIPAM(1000) vesicles.

Figure 2. Fig. 2 TEM micrographs of SPION-loaded PBD-b-PEO vesicles formed via solvent inversion at 1mg/ml. (A) shows an overview of pure vesicles after trehalose fixation while the inset depicts a zoom of the bilayer region with embedded 3.5nm SPIONs (20%w/w). Ultrathin sections in (B) of the preparations show SPION localization exclusively in the peripheral membrane region of the sliced sample. The inset in (B) shows a trehalose fixed 2D projection of a post-extruded polymer vesicle exhibiting a homogeneous distribution of particles throughout the part of the polymersome in focus.

Figure 3. Fig. 3 Release kinetics of encapsulated calcein from (A) lipopolyversomes (30% w/w DPPC) and pure PBD-b-PEO vesicles loaded with 3.5nm 5% w/w SPIONs. Lipopolyversomes prepared with solvent inversion method and actuated for 40min pulses (black solid lines) and their passive release (black dotted). Lipopolyversomes prepared with rehydration plus extrusion method, actuated for 20min pulses (blue solid) and 10min pulses (red solid) and their passive release (blue dotted). Pure PBD-b-PEO vesicles loaded with 30% w/w SPION were actuated for with 40min pulses (green solid) and their passive leakage (green dotted). (B) Blended polymer vesicles (30% w/w PI-b-PNIPAM) loaded with 5% w/w (red) and 20% w/w (blue) SPION. Solid lines show release upon actuation for 30min long pulses and dotted lines show passive leakages.

Supporting Information

Magnetically Actuated Thermally Responsive Vesicles of Lipids and Superamphiphiles

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Reagents

All chemicals were purchased from Sigma-Aldrich and used without further purification except otherwise noted.

4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid >97%; Isoprene 99% (contains <1000 ppm *p*-tert-butylcatechol as inhibitor); *N*-Isopropylacrylamide 97%; 2,2'-Azobis(2-methylpropionitrile) 98%; *S*-Methyl methanethiosulfonate 97%; *N,N*-Dimethylethylenediamine 95%; 5-(Dimethylamino)naphthalene-1-sulfonyl chloride BioReagent, powder and chunks ≥99% (HPLC); 1,4-Diazabicyclo[2.2.2]octane Reagent Plus ≥99%; Cysteamine hydrochloride ≥99% (titration); 2,2'-Dithiopyridine ≥99% (GC); Ethanolamine ACS reagent ≥99.0%; Triethylamine ≥99%; Milli-Q water (R=18MΩcm); Methanol anhydrous ≥99.8%; Acetone Chromasolv for HPLC ≥99.9%; Dichloromethane anhydrous ≥99.8% (contains 50-150 ppm amylene as stabilizer); Chloroform ≥99.5% (containing 100-200 ppm amylenes as stabilizer); Tetrahydrofuran Chromasolv Plus for HPLC ≥99.9% (inhibitor-free); 1,4-Dioxane ACS reagent ≥99.0%; Diethylether ACS reagent, anhydrous, ≥99.0% (contains 1 ppm BHT as inhibitor); Toluene anhydrous 99.8%; *n*-Hexane anhydrous 95%; Aluminium oxide activated, basic, Brockmann I (150 mesh); (+)-D-Trehalose dihydrate from corn starch >99%.

SI 1 Synthesis

SI 1.1 Poly(isoprene(1300)-*b*-isopropylacrylamide(1000))-dithioethyl-dansylsulfonamide: PI-*b*-PNIPAM-Dns

PI(1300)-*b*-PNIPAM(1000) was synthesized by sequential RAFT polymerization as in Bixner et al.¹ Fluorescent labeling was achieved in situ during DTB head group cleavage by the following modification: MeSSO₂Me (MTS) was replaced by 2.25mmol of ((2-dithiopyridyl)-ethyl-dansylsulfonamide) for trapping of the nascent thiol. After stirring the reaction overnight, the solution was concentrated and the residue was washed with hot water and MeOH. The crude product was purified via silica gel column chromatography. First, excess dye and residual polyisoprene were eluted in MeCN and DCM/MeOH 100/1, then the fluorescently labeled block copolymer was obtained using DCM/MeOH 6/1 as eluent. (degree of functionalization: 30%)

¹H-NMR (300 MHz, CDCl₃, δ): 9.39 (0.3H, PhH Dns), 8.89 (0.3H, PhH Dns), 8.66 (0.3H, PhH Dns), 8.40 (0.3H, PhH Dns), 7.89 (0.3H, -SO₂NH- Dns), 7.77 (0.3H, PhH Dns), 6.90 (b, -CONH-, PNIPAM), 5.76 (1H, CH₂=CH- 1,2-PI), 5.12 (15H, -CH=C(CH₃)- 1,4-PI), 4.90 (2H, CH₂=CH- 1,2-PI), 4.69 (2H, CH₂=C(CH₃)- 3,4-PI), 4.00 (8.5H, CH(CH₃)₂ PNIPAM), 3.45 (0.6H, -SO₂NHCH₂-), 2.91 (1.8H, Me₂N-), 0.8-2.4 (199H, CH₂, CH₃ PI, CH₂, CH, PNIPAM)

ATR-FTIR (powder, cm⁻¹): 3600-3200 (b, OH & NH), 3300 (NH, amA), 3070 (=CH₂, 3,4-PI), 2966 (CH₃), 2924 (CH₂), 2874 (CH₃), 2854 (CH₂), 1715 ((C=O)OH), 1642 (C=O, amI & C=C, 3,4 & 1,2 PI), 1540 (NH, amII), 1453 (CH₃, PNIPAM), 1383 (CH₃, 1,4-*t* PI & PNIPAM), 1368 (CH₃, PNIPAM), 1316 (SO₂NH) 1264 (NH, amIII), 1172, 1140 (C-C, 1,4-*c* PI & SO₂NH), 1098, 1027 (=C-CH₃, 1,4-*c* PI), 1004 (C-C, 3,4-PI), 909 (=CH₂, 1,2-PI), 886 (=CH₂, 3,4-PI), 840 (-CH=CH-, 1,4-*c,t* PI), 690 (NH, amV)

UV/VIS (MeCN, λ_{abs}, nm): 208 (CONH), 260 sh (Dns), 340 sh (Dns)

Fluorescence (MeCN, λ_{em}, nm): 510 (λ_{exc} = 340)

SI 1.2 (2-dithiopyridyl)-ethyl-dansylsulfonamide: DnsEtSSPy

Initially (2-dithiopyridyl)-cysteamine hydrochloride was synthesized as in literature.² In brief, 2.5g 2,2'-dithiopyridine (Py₂S₂, 11.3mmol) was dissolved in 15ml anhydrous MeOH and purged with nitrogen gas for 15min. Then 300mg cysteamine hydrochloride (HSEtNH₃⁺Cl⁻, 2.6mmol) were slowly added over 15min at room temperature and the resulting solution was left stirring for 8h under inert atmosphere. The crude reaction mixture was poured into 200ml Et₂O and allowed to crystallize for 15-30min. After decantation the residue was purified by two successive precipitations from MeOH (10ml) into Et₂O (100ml). Filtration of the product and washing with Et₂O yielded (2-dithiopyridine)-cysteamine hydrochloride (PySSEtNH₃⁺Cl⁻) as a white solid in 85-90% yield.

100mg dried PySSEtNH₃⁺Cl⁻ (0.45mmol) was dissolved in anhydrous acetonitrile (MeCN, 15ml) by sonicating for 10min with 75μl triethylamine (TEA, 1eq). 120mg dansyl chloride (Dns-Cl, 0.45mmol) in 10ml MeCN (anhydr.) was added dropwise and the solution was purged with N₂-gas for 10min. The reaction solution was sealed with a rubber septum and another 75μl TEA were dropwise added via a syringe. The mixture was reacted for 3-4h, evaporated to dryness, redispersed in dichloromethane (DCM, 50ml) and extracted thrice against ultrapure water, brine and dried over Na₂SO₄. ((2-dithiopyridyl)-ethyl-dansylsulfonamide) was obtained after rotary evaporation as a yellow-greenish viscous solid in 92% yield.

¹H-NMR (300 MHz, CDCl₃, δ): 8.65 (d, 1H, Py), 8.58 (d, 1H, Dns), 8.45 (d, 1H, Dns), 8.27 (d, 1H, Dns), 7.83 (t, 1H, SO₂NH), 7.59 (t, 1H, Py), 7.50 (t, 1H, Dns), 7.49 (t, 1H, Dns), 7.39 (d, 1H, Py), 7.21 (d, 1H, Dns), 7.17 (t, 1H, Py), 3.30 (q, 2H, -SO₂NHCH₂-), 2.91 (s, 6H, Me₂N-), 2.78 (t, 2H, -, -CH₂SSPy)

ATR-FTIR (powder, cm⁻¹): 3286 (b, NH), 3079 (C=CH), 3052 (C=CH), 2985 (CH₃), 2939 (CH₂), 2867 (CH₃), 2832 (CH₂), 2786 (NCH₃), 1612 (C=C), 1573 (C=C), 1503, 1477 (CH₂ & CH₃), 1450 (Ar), 1416, 1354 (Ar), 1314 (SO₂NH), 1231 (Ar), 1201 (ArH), 1159 (ArH), 1139 (CH₃), 1113 (SO₂NH), 1073 (C=C), 1062, 1045 (NCH₃), 1014 (NCH₃), 988 (ArH), 944 (ArH), 886 (ArH), 838 (ArH), 787 (Ar), 759 (Ar), 736 (Ar), 716 (ArH), 682 (Ar), 621 (CN), 568 (CS), 535, 498 (SO₂NH), 472

UV/VIS (MeCN, λ_{abs}, nm): 218 (Ph), 246 (Dns), 287 (PySS), 344 (Dns)

Fluorescence (MeCN, λ_{em}, nm): 515 (λ_{exc} = 340)

SI 1.3 Poly(butadiene(1200)-*b*-ethyleneoxide(600))-O-(dansyl sulfonate ester): PBD-*b*-PEO-Dns

PBD-*b*-PEO-Dns was synthesized similar to literature reports on preparations of dansyl sulfonate esters with some modification.³ Typically, 100mg poly(butadiene(1200)-block-ethyleneoxide(600)) (PBD-*b*-PEO; 0.055mmol) and 15mg dansyl chloride (Dns-Cl; 0.085mmol, 1.5eq) were dissolved in 10ml N₂-saturated, anhydrous acetone (Me₂CO) under sonication. A solution of 7mg 1,4-diazabicyclo[2.2.2]octane (DABCO; 0.085mmol, 1.5eq) in 5ml Me₂CO was added dropwise at room temperature and the reaction mixture was sonicated at 45°C for 20h under inert atmosphere. After cooling to room temperature, the formed precipitate was removed by filtration and the mixture was purified by silica gel chromatography. First impurities and side products were removed in CHCl₃/EA mixtures before the fluorescently labeled target compound was eluted with CHCl₃:MeOH=10:1. After evaporation and drying in-vacuo PBD-*b*-PEO-Dns was obtained as a yellowish, viscous residue in 95% yield (degree of functionalization: 50 %).

¹H-NMR (300 MHz, CDCl₃, δ): 9.51 (d, 0.5H, PhH Dns), 8.86 (d, 0.5H, PhH Dns), 8.45 (d, 0.5H, PhH Dns), 7.92 (t, 0.5H, PhH Dns), 7.84 (d&t, 1H, PhH Dns), 5.45 (22H, CH₂=CH- 1,2-PBD, -CH=CH- 1,4-PBD), 4.94 (34H, CH₂=CH- 1,2-PBD), 4.19 (t, 1H, -CH₂SO₃-), 3.64 (60H, -CH₂CH₂O- PEO), 3.45 (s, 3H, Me₂N-), 2.3-1.85 (m, 30H, -CH₂CH- 1,2-PBD, -CH₂-C=C 1,4-PBD), 1.5-1.0 (40H, -CH₂CH- 1,2-PBD, alkyl-CH₂), 0.82 (t, 3H, alkyl-CH₃)

ATR-FTIR (powder, cm⁻¹): 3074 (=CH PBD), 2972 (CH₂ PBD), 2915 (CH₂ PBD), 2868 (CH₂ PEO), 2845 (CH₂ PBD), 1640 (C=C PBD), 1574 (Ph), 1514 (Ph), 1452 (CH₂ PBD & PEO), 1418 (CH₂ PBD), 1347 (CH₂ PEO & S=O), 1295 (CH₂ PEO), 1244 (CH₂ PEO), 1176 (S=O), 1143 (C-C & C-O PEO), 1107 (C-C & C-O PEO), 1040 (C-C & C-O PEO), 993 (=CH t-1,4-PBD), 963 (CH₂ PEO), 948 (S-O), 907 (=CH 1,2-PBD), 842 (CH₂ PEO), 794 (S-O), 673 (c-1,4-PBD), 627 (PhR), 575 (PhR)

UV/VIS (MeCN, λ_{abs}, nm): 215 (Ph), 254 (Dns), 343 (Dns)

Fluorescence (MeCN, λ_{em}, nm): 535 (λ_{exc} = 340)

SI 2 Characterization of hybrid vesicles

SI 2.1 Confocal images of hybrid vesicles

Figure S-1. Confocal microcopy images of PBD(1200)-*b*-PEO(600) GUVs blended with (A) 30%w/w PI(1300)-*b*-PNIPAM(1000) and (B) 30%w/w DPPC. Samples were prepared by electroformation at 3V AC and 15Hz in 250mM sucrose at 50°C for 120min and stained via addition of covalently labeled PBD(1200)-*b*-PEO(600)-Dns (2%w/w).

SI 2.2 DSC traces of lipopolyversomes vesicles with different DPPC weight fractions

Figure S-2. DSC traces of PBD(1200)-*b*-PEO(600) vesicles blended with (A) varying weight fractions of DPPC and (B) raw versus fitted data for a 30%w/w DPPC blend of various sizes (blue – GUVs, black – solvent inversion, red – pure DPPC reference 1/100 scaled).

SI 2.3 Temperature-dependent DLS of lipid and thermoresponsive block copolymer blend vesicles

Figure S-3. T-dependent intensity weighted DLS distributions of (A) 30%w/w PI(1300)-b-PNIPAM(1000) and (B) 30% DPPC blended PBD(1200)-b-PEO(600) vesicles from room temperature to 75°C.

SI 3 Characterization of scaffold vesicles

SI 3.1 Optical density curves of PBD(1200)-b-PEO(600) vesicles loaded with different weight fractions of SPION

Figure S-4. OD curves of pure (red) and SPION loaded assemblies (10%wt – green, 20%wt – blue, 30% – black) formed via solvent inversion at 1mg/ml. The samples were diluted to the linear range for collection of the OD curves and back-compensated for the dilution factor in the calibration.

SI 3.2 PBD(1200)-b-PEO(600) vesicles formed at high polymer concentration

Figure S-5. DLS size distribution (left) and TEM images (right) of polymersomes formed at 2mg/ml via solvent inversion. Multiple lamellae are clearly visible in the ultrathin-sections after positive staining of the PBD-block with OsO_4 .

SI 3.3 Magneto-thermal heating effect on bulk temperature

Figure S-6. Bulk temperatures measured for different AMF pulse durations (red -10min, green - 20min, magenta – 30min and blue - 40min).

SI 3.4 DLS of ternary hybrid vesicles

Figure S-7. Intensity weighted DLS distributions of lipopolyversomes formed via solvent inversion at 1mg/ml containing 30%w/w DPPC (red) and upon co-addition of 15%w/w cholesterol (green).

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4. Conclusion

In this work, various aspects on the formation and performance of nanoparticle loaded delivery systems were investigated. Particular emphasis was put on the controlled direct co-self-assembly of monodisperse core-shell nanoparticles with various amphiphile species into responsive superstructures to give ready-to-use magnetosome suspensions in the ideal 100-200nm size range. The performance and limitations of lipid carriers, polymeric vesicles and mixed membrane capsules with respect to externally field triggered release of encapsulated cargo were evaluated.

The synthesis of monodisperse, oleic acid-capped SPIONs with controlled size on the nanometer scale was achieved by high temperature decomposition of an organometallic precursor in hot surfactant solution. A particular challenge to membrane applications relates to the complete removal of bound stabilizers and unknown decomposition products. It was demonstrated that standard methods to functionalize the surface of hydrophobic, monodisperse SPIONs through ligand replacement result in substantial residual oleate complexes on the surface of the SPIONs. A method was described that attains the goal of complete exchange of the native shell for nitrocatechol derivatives at maximal ligand density. This was a crucial step towards a controlled co-self-assembly into magnetically responsive membrane structures.

A new and simplified method to formulate monodisperse and unilamellar liposomes with ultrasmall, monodisperse P-NDA coated SPIONs dispersed in the membrane was established. Lipid and SPION suspended in tetrahydrofuran are injected into water or buffer and the transfer solvent is completely evaporated. This approach presents a residue-free, one-step route to controlled magnetosomes. Quantitative control over vesicle lamellarity and encapsulation of 3.5nm SPIONs with pure shells up to at least 20%w/w was confirmed. The achieved particle contents and the homogeneity of the vesicle suspensions are significantly higher than previously reported for other preparation methods. A strong reduction in maximum loading accompanied by a drastic increase in vesicle polydispersity was observed for monodisperse SPIONs that had residues of oleic acid within the shell from standard purification methods. Encapsulation of spectroscopically clean SPIONs with core sizes exceeding the lipid bilayer thickness resulted in formation of giant micellar structures in which SPION aggregates were surrounded by a lipid monolayer. This

illustrates the importance of controlling both interface properties and overall SPION size for the preparation of stable and controlled magnetosomes. In addition to the novel full control over vesicle structure and SPION loading, a major advantage of the solvent inversion method for magnetosome assembly is that it is easily scalable while simultaneously compatible with direct drug encapsulation methods. Liposomes in the ideal 100-200nm size range which provide a large lumen were directly obtained; the large lumen is important for drug delivery efficiency. Furthermore, the much higher than previously obtained number of SPIONs per vesicle that could be achieved is important for performance. Magnetic contrast and susceptibility to magnetically triggered release is enhanced in direct proportion to the order of magnitude higher loading that was demonstrated.

The concept of controlled co-self-assembly via solvent inversion was successfully extended to thermoresponsive superamphiphiles into which SPIONs were incorporated. The block-copolymer was synthesized via sequential RAFT polymerization and tailored in MW and composition to give a hydrophilic weight fraction of 40%. This enables the formation of vesicles with a thermal transition slightly above body temperature and with low non-specific interactions with biological fluids. The design of the polymeric carrier system was specifically configured to switch membrane permeability upon magneto-thermal heating by use of PNIPAM as the hydrophilic block. The release of encapsulated calcein was controlled but moderate compared to demonstrations of similar release strategies using liposomes; noteworthy, the long pulse duration necessary to achieve cargo discharge lead to a significant rise in bulk temperature to 38-40°C which is within the transition range of the polymer. It is thus plausible that most of the triggered release for PI-b-PNIPAM vesicles is due to bulk heating through the combined effect of Joule heating of the bulk and the heating induced by SPIONs, but not through local SPION-heating alone. A likely cause is that the heat transfer from membrane-embedded SPIONs to the hydrated, thermoresponsive corona is ineffective due to the high thermal conductivity of water which quickly dissipates the generated heat. Hence, the incorporation of ultrasmall, membrane-embedded SPIONs was insufficient to adequately perturb robust superamphiphilic membranes but passive release of the vesicles could be strongly reduced as compared to other presented systems by employment of monodisperse nanoparticles with a controlled aggregation state.

In a final approach, the potential of mixed membrane capsules containing amphiphile blends was investigated. PBD(1200)-b-PEO(600) was employed as densely pegylated scaffold polymer as a means to enhance particle loading and to augment the resulting hybrid vesicles with superior in-vivo stability. For mixed polymer capsules of PBD-b-PEO/PI-b-PNIPAM homogeneous mixtures were formed on optical and nanoscopic length scales, which resulted in a drastic lowering of the achievable release. At high particle loading contents the observed release was mainly due to passive leakage, presumably because of the engulfment of small SPION aggregates at high weight fractions. PBD-b-PEO/DPPC mixtures assembled into vesicles with interspersed lipid domains which were suggested to also be present for nanoscale assemblies. These segregated lipid windows were shown to be functional and to exhibit lipid-like magnetic release characteristics during AMF actuation. Magnetolipopolymerosomes were shown to combine the opportunities of facile magnetic actuation of lipid vesicles with the mechanic robustness, stealth and antifouling properties of synthetic superamphiphilic vesicles. The potential applicability of such systems goes far beyond any commercially available lipid based drug delivery system.

In summary it was shown that monodisperse, ultrasmall, hydrophobic iron oxide nanoparticles with an irreversibly grafted shell can efficiently be loaded into membranes of responsive superstructures via solvent inversion. The co-self-assembly process is however sensitive to residual impurities and the employed particle sizes. AMF actuation was quantitative for magnetoliposomes for which the thermoresponsive units are in immediate contact with the embedded sensitizers. For polymersomes equipped with a thermally sensitive corona, the same particles proved to only moderately actuate superamphiphilic membranes. Hybrid amphiphile systems with mixed membranes of lipids and polymers seemed most promising for clinical applications and commercialization in that this combination provided high in-vivo stability, good biocompatibility and efficient magnetic release.

5. Outlook

5.1 Magnetoliposomes

For liposomal carriers the achievable particle loading of hydrophobic SPIONs is severely limited by the unzipping of the bilayer around the embedded particles which is associated with the creation of unfavorable voids and local packing mismatches of the membrane-forming amphiphiles. Expanding the available space in the membrane interior seems natural and would be highly beneficial for increasing the incorporation efficiency of SPIONs, for decreasing passive leakage and for enhancing assembly stability. Glycerophospholipids however only exist in a rather narrow Mw range with typical acyl chain lengths of 12-20 hydrocarbons. Thus the available space is restricted to a few nm, generally 3-5nm. Naturally occurring phospholipids with saturated chain lengths exceeding those of common lipids can be commercially obtained but exhibit impractically high melting temperatures and unfavorably rigid membranes. It is therefore proposed to investigate the possibility to tune magnetoliposome characteristics via the affinity of the surface coating. Ultrasmall SPIONs were shown to exhibit chain-end densities that are comparable to those of glycerophospholipids and therefore in principle would offer the possibility to interact with surrounding lipids. Tailoring the affinity of the surface coating might be achieved via variation of the fatty acid substituents for nitrodopamine anchors and/or via the introduction of a second short spacer to nitroDOPA-ligands that additionally modulate the overall grafting density. Envisioned fatty acid moieties to affect the interaction with surrounding lipids primarily include sterols that are known to easily intercalate into phospholipid membranes.

Continued work should also address the optimization of assembly conditions for complex multicomponent lipid mixtures and explore the relationship between system composition, nanoparticle loading, drug encapsulation and release efficiency. A higher nanoparticle loading might lead to higher efficiency of triggered release, but also compromise membrane integrity and passive leakage. The co-incorporation of additives such as cholesterol to adjust membrane permeability might interfere with drug and nanoparticle loadings since sterols are known to fill up voids in the membrane and to modulate lipid packing. Pre-tests have shown that admixture of common additives lead to quite different outcomes for saturated and unsaturated lipid

species. In particular PEGylated lipids which are of utmost importance to practical applicability lead to the appearance of a micellar subpopulation in DLS, presumably because THF is a selective solvent for PEG while long chain phospholipids are quite insoluble at ambient temperature. Further homogenization steps like extrusion, sonication or freeze-thaw cycles could be considered for smoothing the additive distribution.

5.2 Thermoresponsive Polymersomes

The release from thermoresponsive polymersomes could possibly be improved by optimizing the structure using a higher MW block copolymer for which the hydrophilic block undergoes a more drastic volumetric change upon dehydration than is the case for oligomeric PNIPAM blocks. A correspondingly higher MW hydrophobic block will also allow for incorporation of large SPIONs that provide more efficient heating. The possibility to embed large SPIONs will be a key improvement towards efficient actuation of thermoresponsive polymersomes. Investigations of the local heating capacity of SPIONs by Riedinger showed that 15nm cores yielded a local temperature rise of about 45°C close to the particle surface which decayed exponentially with distance, while no local heating could be detected within the sensitivity of the method for particles smaller than 6nm.¹⁰² Large SPIONs will also facilitate actuation by providing higher absorber strengths than the currently used ultrasmall SPIONs and thus less undesired bulk heating.

In addition, optimization of the vesicle characteristics for practical applications should be undertaken. This would include a lifting of the LCST above body temperature to maintain stable encapsulation under physiological conditions but to set the transition range to within the temperature rise that can be accessed by the incorporated SPIONs. The latter is unfortunately a complex issue due to its dependence on diblock composition, SPION size, distance from the core and heat transport properties. Also a reduction of the passive leakage is required for long term applications. This might be achieved by a replacement of the hydrophobic block for one with more favorable characteristics. An interesting alternative that could be considered is the usage of hydrophobic blocks that exhibit UCST behavior. These would minimize the heat transport issue without requiring detailed knowledge on the heating properties of differently sized SPIONs.

5.3 Hybrid Vesicles

For lipopolymerosomes the next logical step should be to establish methods to investigate lipid domain formation in nanoscale vesicles. These are required for a more thorough characterization of patch sizes and homogeneity throughout the formed vesicle population. One could utilize monodisperse SPIONs of intermediate core size that do not fit the lipid bilayer regions but into polymeric membranes as a means to depict phase separation on the nanoscale. Alternatively selective staining agents such as OsO_4 or freeze-fracture/freeze-etching replicas of the hybrid vesicles might shed some light onto lipid segregation. Thereafter possible methods to regulate domain sizes in hybrid vesicles could be addressed, as for instance thermal quenching at different cooling rates or via addition of agents that preferentially influence lipid distribution like cholesterol.¹²⁰ Controlling the lipid distribution could be employed to tune the release profile via tailoring of the lipid window size and its composition.

Investigating the influence of various presentation strategies on targeting efficiencies and vesicle stability would as well be vital for further in-vitro and in-vivo studies. One could envision various presentation schemes where the signaling tag is conjugated to one amphiphile at a time and then either tune lipid domain size to improve the accessibility of saturated lipid patches or adjust the PEG density via variation of the weight fraction with unsaturated lipids that intersperse homogeneously.

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7. Abbreviations

Å	Ångstrom
a	Lattice Constant
A	Headgroup Area
AMF	Alternating Magnetic Field
ATR-FTIR	Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy
c	Concentration
cac	Critical Aggregation Concentration
CHCl ₃ (CDCl ₃)	Chloroform (deuterated)
COMET	Single Cell Gel Electrophoresis Assay
COMU	(1-Cyano-2-ethoxy-2-oxoethylidenaminoxy)dimethylamino-morpholino-carbenium hexafluorophosphate
CPP	Critical Packing Parameter
d	Membrane Thickness
d _{core}	Core Diameter
d _h	Hydrodynamic Diameter
DCM	Dichloromethane
DLS	Dynamic Light Scattering
DMF	<i>N,N</i> -Dimethylformamide
DMPC	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphocholine
(d ₆ -)DMSO	(deuterated) Dimethylsulfoxide
DOEPC/DOPC+	1,2-dioleoyl- <i>sn</i> -glycero-3-ethylphosphocholine (chloride salt)
DPPC	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine
DSC	Differential Scanning Calorimetry
ε _h	Solution Energy

eV	Electron Volt
ESI-MS	Electrospray Ionization Mass Spectrometry
f	Hydrophilic Volume Fraction
ff-TEM	Freeze-fracture /-etching TEM
k_B	Boltzmann Constant
l	Alkyl Chain Length
M_h	Molecular Weight of the Hydrophobic Block
MALDI	Matrix Assisted Laser Desorption/Ionization
(d_4 -)MeOH	(deuterated) Methanol
MRI	Magnetic Resonance Imaging
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
M_w	Molecular Weight
n	Number of Repeat Units
nm	Nanometer
NDA	6-Nitrodopamine
NMM	<i>N</i> -Methyl-Morpholine
NMR	Nuclear Magnetic Resonance
O_h	Octahedral Site
OD	Optical Density
pH	Acidity
pKa	Acid Dissociation Constant
PBD	Poly(butadiene)
PBS	Phosphate Buffered Saline
PdI	Polydispersity Index
PEG or PEO	Poly(ethylene glycol) or Poly(ethylene oxide)
PI	Poly(isoprene)

PNIPAM	Poly(<i>N</i> -isopropyl acrylamide)
P-NDA	<i>N</i> -Palmityl-6-Nitrodopamide
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
ρ	Density
ρ^{graft}	Grafting Density
SAR	Specific Absorption Rate
SPION	Superparamagnetic Iron Oxide Nanoparticle
T_d	Tetrahedral Site
T	Temperature
TEM	Transmission Electron Microscopy
TGA	Thermogravimetric Analysis
THF	Tetrahydrofuran
TBS	Tris(hydroxymethyl)-aminomethan Buffered Saline
TUNEL	Terminal deoxynucleotidyl mediated dUTP nick end labeling
UV/VIS	Ultraviolet/Visible Absorption Spectroscopy
V	Alkyl Chain Volume

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