Review

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Magnetoliposomes: opportunities and challenges

Abstract: Combining liposomes with magnetic nanoparticles is an intriguing approach to create multifunctional vesicles for medical applications, which range from controlled drug delivery vehicles to diagnostic imaging enhancers. Over the past decade, significant effort has been invested in developing such hybrids – widely known as magnetoliposomes – and has led to numerous new concepts. This review provides an overview on of the current state of the art in this field. The concept of magnetic fluid hyperthermia and stimuli-responsive nanoparticles for drug delivery is briefly recapitulated. The materials needed for these hybrids are addressed as well. The three typically followed approaches to associate magnetic nanoparticles to the liposomes are described and discussed more in detail. The final chapters are dedicated to the analytical methods used to characterize these hybrids and to theoretical considerations relevant for bilayer-embedded nanoparticles.

Keywords: electron microscopy; magnetic nanoparticles; magnetoliposome; membrane energetics; stimuli-responsive.

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Introduction

A nanoparticle (NP) is defined as a material with all three external dimensions in the nanoscale (ISO/TS: 27687:2008), i.e., below 100 nm. The current choice of available NPs is colossal and ranges from relatively simple single NPs to highly complex surface derivatized NPs or NP assemblies. All engineered NPs have one common link: Their chemical, physical and biological properties can differ considerably from the bulk material properties. For example, iron oxides – such as maghemite (γ-Fe_2O_3) and magnetite (Fe_3O_4) – lose their permanent magnetization below a certain size, which is typically below 20 nm (1). At this point, iron oxide NPs possess only one magnetic domain, and consequently exhibit superparamagnetic behavior at temperatures above the so-called blocking temperature (2). Nowadays, magnetic NPs are found in a rapidly increasing number of applications, including catalysis (3), sensing (4), and filtration (5). In nanomedicine, superparamagnetic iron oxide NPs (SPIONs) have gained wide acceptance in diagnosis and are used for contrast enhancement in magnetic resonance imaging (MRI) (6), (stem) cell tracking and labeling (7) or magnetic separation technologies (e.g., rapid DNA sequencing) and ultrasensitive diagnostic assays (8).

The benefits of magnetic NPs for therapeutic purposes are indisputable, and magnetic targeting for drug or gene delivery and magnetic fluid hyperthermia (MFH) are arguably the two most important potential therapeutic applications. In particular, SPIONs are promising because of their outstanding magnetic behavior (9), their biocompatibility (10), and the large amount of information available on these materials. However, the process of converting basic research into clinical nanomedicine settings and commercially sustainable products is long and complicated (11), and the acceptance and integration of nanotechnologies particularly into nanomedicine are very challenging.

Magnetic fluid hyperthermia – a brief recapitulation

Magnetic fluid hyperthermia (MFH) was first proposed by Gilchrist and colleagues (12). In short, it involves the injection of SPIONs directly into a specific tissue or organ (e.g., lymph nodes) and the subsequent exposure to an alternating magnetic field (AMF) to heat the region in question up to 45 to 47°C. Temperatures so far above the physiological

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norm can lead to widespread necrosis, coagulation or – depending on the temperature – even carbonization (13). This technique is mostly used as a complementary therapy to radiation or chemotherapy with the motivation to render cells of a tumor more sensitive to the principal treatment (14). The method is fundamentally linked to the nanosize of the magnetic particles, which – when exposed to an AMF – dissipate heat through relaxation losses. Typically, the heating potential of magnetic NPs depends on the material itself, its concentration and size (distribution) (15). Energy dissipation occurs either through the physical rotation of the NP in the fluid (Brownian relaxation) or by the rotation of the atomic magnetic moments within the particle itself (Néel relaxation) (16). According to the theoretical model for the volumetric energy dissipation rate developed by Rosensweig (9), the energy dissipation rate (i.e., heating potential) increases with the applied AMF (i.e., its amplitude and frequency). However, it has been shown that a strong AMF can lead to non-specific heating due to eddy currents.

In recent years, significant effort was dedicated to optimize the magnetic materials (15). This development is, however, related to the applied magnetic field, and many reports (17, 18) have investigated the effects of AMF on healthy tissues in order to elucidate the maximum magnetic field strength at a given frequency applicable to humans (15). Currently, magnetic field conditions are chosen to be compliant with what has been approved in Europe for MFH. For example, for treatment of glioblastoma multiforme (MagForce, Berlin, Germany) magnetic field frequencies in the order of 100–200 kHz at around 20 mT are typically chosen. In addition to the parameters related to the magnetic field, i.e., alternating field amplitude and frequency, the surrounding medium, type of magnetic material, and particle crystallinity play a crucial role. As demonstrated theoretically and experimentally by Hergt and colleagues (19), adequate mean particle size and narrow particle size distribution are extremely important requirements for efficient heating. Moreover, in order to successfully annihilate cancer cells, it is imperative that sufficient heat is locally administered to account for the losses to the surrounding tissue. This point has been addressed by theoreticians and experimentalists with controversial results (20, 21).

Nanomaterials for drug delivery

The ability to directly deliver drugs to relevant cell types, and possibly to specific intracellular organelles, is essential (22) for optimally exploiting the potential of any drug delivery vehicle. To achieve this, many nanomaterials have been highlighted as favorable modalities, and the majority of the currently available formulations comprise "soft" NPs (e.g., organic polymers and liposomes) (23). Liposomes are artificial vesicles consisting of a phospholipid bilayer and have been promoted for many years as future drug delivery vehicles. The contributions of numerous researchers over five decades have led to significant advances in the field, and liposomes are perhaps the first nanocarriers which have succeeded in translating from bench to bedside (24), Doxil/Caelix being the most prominent example.

Historically, classical or first-generation drug delivery nanocarriers comprise a container, (e.g., a liposome) and an active principle (i.e., the drug molecule). Second-generation nanocarriers were developed to target their therapeutic site via antibodies and other biomolecules. Third-generation nanocarriers are designed to fulfill more complex functions, such as time-controlled deployment of active vesicles across different biological barriers and different subcellular targets.

In analogy to liposome development, inorganic NPs are nowadays promoted as potential drug delivery vehicles, but despite important progress, many of the presently investigated delivery systems are far from meeting the required needs. Further careful design is thus imperative (25). In this category, biocompatible SPIONs (10) are conceived as beneficial, alternative targeting tools compared to other NPs, as they are easily synthesized and surface-functionalized (26). Due to their advantageous magnetic properties (9), SPIONs can be used for magnetic targeting, which relies on the delivery of magnetic NPs to the desired target area through the application of a magnetic field gradient (27). Following successful targeting, the SPIONs remain within the desired region for optimal therapeutic treatment. Then they are subsequently released and excreted. Recently, such a concept was aptly portrayed by Kumar and colleagues (28), who demonstrated that magnetic NPs – injected in the tail of mice – were successfully directed to the heart and kidneys via an external magnetic field.

Stimuli-responsive nanoparticles

Stimuli-responsive NPs are becoming more and more prominent in the medical sciences and increasingly encouraging in the development of next-generation disease therapies (29, 30). To name some auspicious examples, applications may include diagnostic imaging, targeted drug release, hyperthermia treatment or a combination of them. In general, multifunctional materials,

which aim at providing both treatment options and diagnostic potential are particularly sought after to complement the emerging field of theranostics.

In regard to targeted drug delivery, stimuli-responsive NPs are visionary concepts to deliver and release a drug exactly where it is needed. However, the release needs to be modulated, as passive diffusion out of the carrier alone is usually slow. Drug release by an external stimulus (e.g., a magnetic field, infrared light, pH etc.) is an ideal approach, as it enables a spatial and temporal control over the drug release. As triggers, SPIONs are again ideal candidates due to their size- and material-dependent physicochemical properties, which in turn bestow them with superlative conditions to confer any nanocarrier the ability to fulfill additional tasks.

One of the most intriguing stimuli-responsive NP-based drug carriers is arguably the magnetoliposome, i.e., a combination of a liposomal drug carrier and magnetic NPs. First described by De Cuyper and Joniau in 1988 (31), magnetoliposomes have become remarkable hybrids due to the multivalent properties of both the carriers and the triggers. Liposomes may be designed to be thermosensitive, i.e., to undergo a phase transition from an impermeable gel state to a permeable liquid-crystalline state when a defined temperature barrier is reached (32). As mentioned before, magnetic NPs exhibit remarkable heating effects when exposed to an AMF (9). In regard to magnetoliposomes, this inherent property is pivotal: If generated close to the main release barrier (i.e., the phospholipid membrane), the resulting thermal energy may be used to alter the membrane and render it permeable to an encapsulated drug.

Combining these two independent systems yields a versatile nanoplatform, which may provide combined drug delivery and hyperthermia treatment at a specific target site under co-instantaneous tracking via MRI (Figure 1).

In short, this covers practically the entire scope of application, which is desirable for third-generation nanocarriers. Although still far away from direct clinical application, there has been significant progress in the development and understanding over the past decade, ranging from general biophysical investigations to triggered release demonstrations. Nevertheless, a basic understanding of all materials involved still remains the prerequisite stage to fulfill before moving to the next step.

This review aims at presenting the most recent developments in the field, the most common materials used and the hybrids in general. Moreover, recent biophysical findings by the authors will be commented on to provide a general overview on what is possible, what has been done and – last but not least – what is still possible in the future.

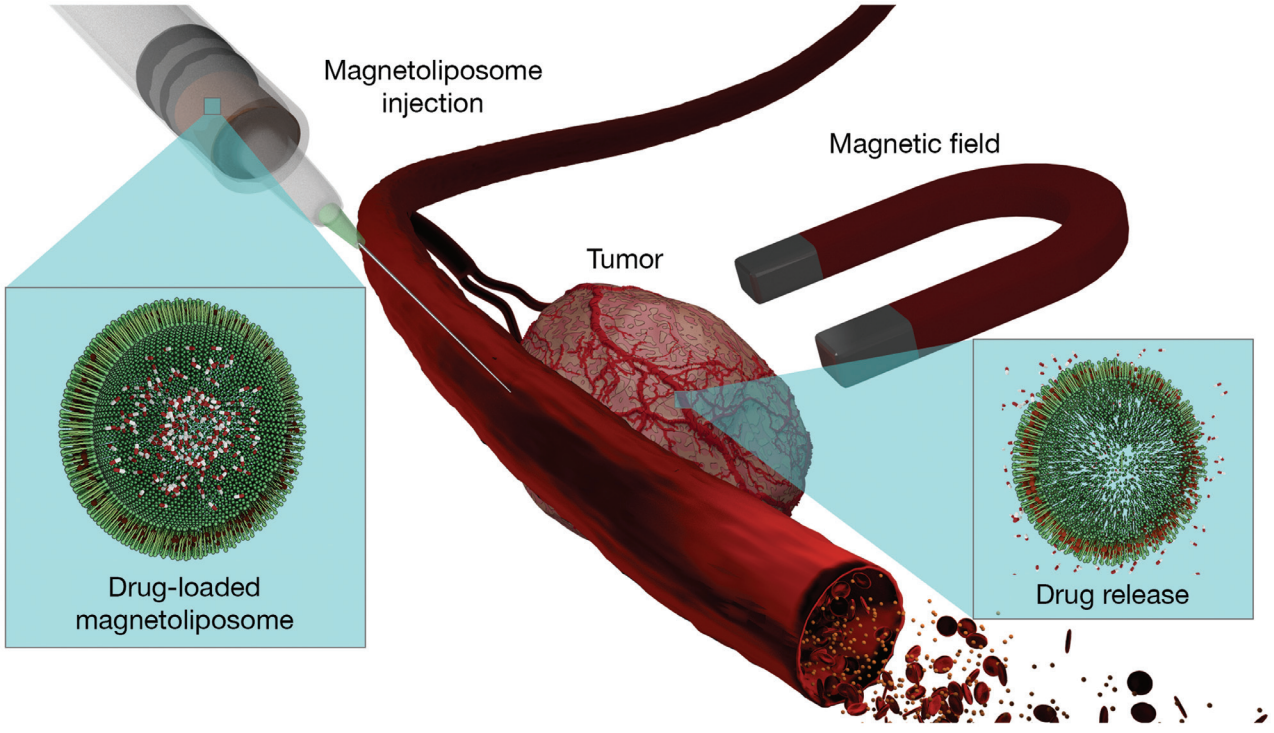


Figure 1 Schematic representation of a SPION-liposome hybrid drug delivery system specifically designed for the triggered release of an encapsulated hydrophilic drug.

SPION-liposome hybrids – obtaining the materials

The centerpiece of the magnetoliposome is unmistakably the type of NP used. SPIONs are the most evident candidate. To obtain them, there are numerous established wet-chemical methods including microemulsions or hydrothermal syntheses (2) in addition to gas phase methods such as thermal decomposition in hot-wall reactors or flame synthesis (33). While the wet-chemical bottom up approaches typically better control particle characteristics such as size or shape, flame syntheses allow for continuous and therefore large-scale production of magnetic NPs. The most dominant and widely used technique in the biomedical field is the co-precipitation method of aqueous Fe^{2+}/Fe^{3+} salt solutions by the addition of a base under inert atmosphere (34). This approach yields magnetite NPs, which are easily oxidized to maghemite (Figure 2A). Adjusting particle size and size distribution is extremely challenging with this process, and the control of pH, ionic strength and seed concentration is crucial. Since the blocking temperature depends on the size (distribution) of the NPs, large polydispersity (i.e., a broad particle size distribution) results in a wide range of blocking temperatures and consequently suboptimal magnetic behavior for many applications (34). Nonetheless, this method is arguably the most popular source of SPIONs for magnetoliposomes, as large quantities can be synthesized at once.

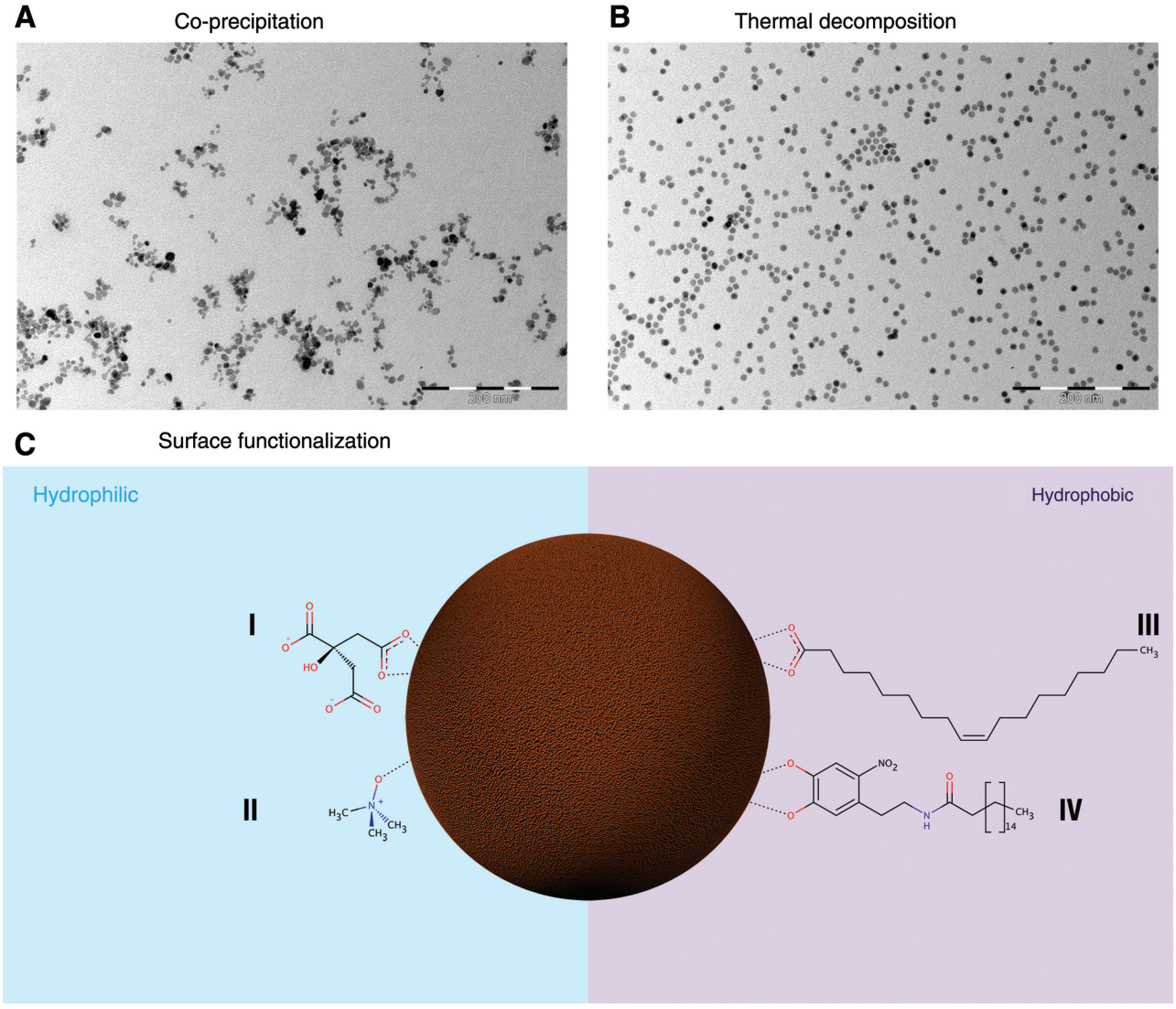


Figure 2 Transmission electron micrographs of SPIONs obtained by co-precipitation (A) or by thermal decomposition (B). Figure 2C illustrates possible SPION surface functionalizations to render them hydrophilic, using e.g. carboxylates (I) or tetramethyl-ammonium hydroxide (II) or hydrophobic using fatty acids (III) or dopamine derivatives (IV).

To obtain much more monodisperse SPIONs, synthesis by thermal decomposition (35) has become the leading approach. In short, an organometallic precursor (e.g., iron oleate (36), iron acetylacetonate, iron carbonyls) is thermally decomposed in a high boiling point solvent (e.g., octyl ether, hexadecene, eicosane). This approach yields highly crystalline NPs with narrow size distributions (Figure 2B) (35). Furthermore, the size can be tuned by the choice of solvent, the reaction time and the reactant ratios. The produced SPIONS are stabilized by a surface-attached oleate molecule and dispersed in an organic solvent. Consequently, additional steps might be required to transfer the SPIONs to an aqueous environment. This phase transfer relies on NP surface derivatization strategies replacing the originally grafted hydrophobic molecule with hydrophilic compounds, or direct functionalization of the surface-grafted hydrophobic molecules themselves (37). Surface chemistry not only determines the colloidal stability of the NPs, but also their association to the liposome, i.e., whether they will be embedded in the hydrophobic bilayer or within the hydrophilic lumen. An arsenal of molecules and surface chemistry strategies are available, and several candidates were used to date. A selection is highlighted in Figure 2C.

For NPs encapsulated in the lumen or grafted to the surface, citrate (38, 39) and oleate (overcoated by a hydrophilic ligand, (40) e.g., a second lipid layer) stabilized SPIONs are the most frequently used candidates. For NPs embedded in the lipid bilayer, SPIONs coated with oleic acid (41–43) are the favored choice. Another alternative was presented by Amstad and colleagues (44) by introducing SPIONs stabilized with palmityl-nitroDOPA (Figure 2C, IV) into the lipid membrane, arguing that such particles were less prone to aggregation than standard oleic acid-coated SPIONs and that they embed themselves more willingly in between the bilayer.

In all, choosing the synthetic approach and surface coating of the NP is the first step to develop SPION-liposome hybrids, and should not be taken lightly. Other factors such as overall NP geometry are of equal importance and might contribute in yielding more basic information on lipid-nanoparticle interactions in general.

On the other hand, the choice of lipids determines the phase transition temperature, which is typically set only a few degrees above body temperature (e.g., around 42°C). Changing the composition of the liposome bilayer, e.g., by including cholesterol, is known to reduce the leakage of drug molecules from the liposomes by "tightening" the bilayer (45). As a long blood circulation time is generally desirable for any vesicle intended for medical usage, adding a small percentage of Polyethylene glycol (PEG)-derivatized lipids in the membrane is an option to obtain this property. PEG chains reduce the overall uptake efficiency by macrophages, and liposomes with this attribute are termed "stealth" (45). In all, the available selection – counting both natural and synthetic phospholipids – is immense and way beyond the scope of this review.

Magnetoliposomes and the state of the art

The NP surface properties determine where the particles will spatially be located within the liposome. Over the past years, numerous variations of magnetoliposomes have been presented, and a selection is highlighted in Table 1. Principally, research is concentrated on controlling the release of an encapsulated drug. However, their utility as MRI contrast agents has been presented on several occasions (39, 52, 55). Other applications, such as cell sorting and gene delivery, have also been addressed (64).

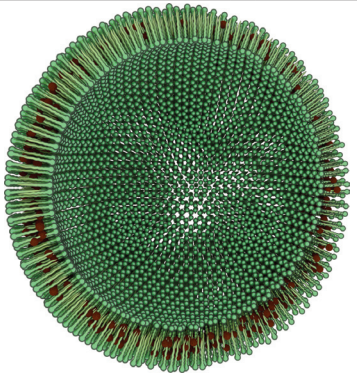
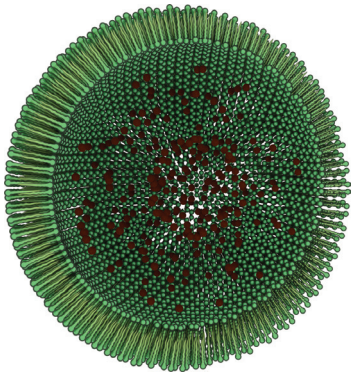
Three different approaches are possible to associate the SPIONs to the liposomes (Figure 3). The two strategies which are increasingly becoming seminal are either to encapsulate the magnetic NPs directly within the liposome lumen (38, 48, 49), the other to embed them in between the lipid bilayer (41, 43, 44). Although pursued with other inorganic NPs [e.g., gold (65)], directly conjugating SPIONs to the liposome surface has only marginally been done (42).

Depending on the final application, the spatial location of the SPIONs within the hybrid is a determining factor: for MRI tracking, NPs encapsulated in the lumen are preferred. However, when using such hybrids as drug carriers, embedding the SPIONs directly in between the lipid bilayer seems more beneficial, as SPIONs in the liposome lumen might impair or affect any co-encapsulated drug even before the membrane actually becomes permeable. Moreover, the energy, which is required to permeate the membrane, should be delivered directly where it is needed.

Characterizing the vesicles – options and caveats

Visualizing and characterizing magnetoliposomes is arguably the most important step in developing such hybrids and is indispensable in detecting the exact NP locations or whether the condition applies to all specimens in the

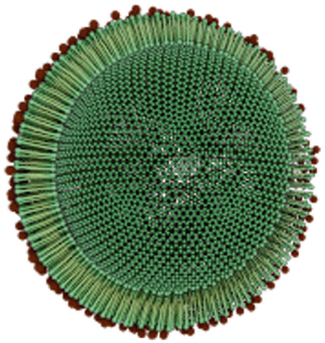
Table 1 Magnetoliposomes: SPION synthesis, surface specifications, lipid composition, functionality and analytical methods used to characterize the hybrid.

Reference	SPIONs specifications	SPION synthesis	Lipid composition	Hybrid functionality	Analytics
Embedded in the lipid bilayer					
Amstad et al. (44)	Palmityl-nitroDOPA or oleic acid coated	Microwave-assisted non-aqueous sol-gel route	DSPC-PEG2000 PE	Magnetic trigger calcein release	Cryo TEM, TEM, STEM, SANS, DLS, TGA, EDX, fluorescence spectroscopy
Bonnaud et al. (43)	Oleic acid coated (clusters)	Thermal decomposition	DPPC DPPG DMPE DPPE-PEG2000 Cholesterol	Magnetic trigger Model for membrane energetics	DLS, SAXS, Cryo TEM, Cryo ET
Chen et al. (41)	Oleic acid coated	Industrial provenance	DPPC	Magnetic trigger carboxyfluorescein release	Cryo TEM, DSC, fluoresence spectroscopy
Floris et al. (42)	Oleic acid coated, acetylacetonate	Thermal decomposition, co-precipitation	Soy PC	Magnetic trigger	TEM, XRD, DLS, Zeta potential
Qiu et al. (46)	AOT coated	Microemulsion	Lecithin	Magnetic trigger	TEM, AFM, DSC, steady-state fluorescence spectroscopy
Qiu and An (47)	AOT coated	Microemulsion	Lecithin	Magnetic trigger calcein release	TEM, fluorescence spectroscopy
Encapsulated in the lumen					

(**Table 1** Continued)

Reference	SPIONs specifications	SPION synthesis	Lipid composition	Hybrid functionality	Analytics
Beaune et al. (38)	Citrate coated	Co-precipitation	DOPC	Magnetic trigger	Magnetophoresis, photobleaching, CLSM, elasticity measurements
Bothun and Preiss (48)	Ferrotec GmBH	Industrial provenance	DPPC Cholesterol	Radio frequency- induced drug release	Cryo TEM, SAR measurements
Cintra et al. (49)	Carboxyl-dextran coated	Co-precipitation	PC Cholesterol	Magnetic trigger	TEM, powder diffraction, DLS, static magnetic birefringence, electron magnetic resonance
Conde et al. (50)	Silica sulfonate coated	Industrial provenance	POPC DDAB DSPE-PEG2000	Magnetic trigger	Zeta potential, TEM, DLS
Faria et al. (51)	Tetramethyl-ammonium hydroxide coated	Industrial provenance	SPC Cholesterol	Magnetic trigger	TEM, DLS, SQUID magnetometry, FTIR, MRI
Fortin-Ripoche et al. (52)	Citrate coated	Co-precipitation	PC DSPE-PEG2000	Magnetic trigger MRI contrast agent	Cryo TEM, DLS, magnetophoresis, in vivo MRI
Prassl et al. (53)	Polar surfactant coated (EMG 1500, FerroTec)	Industrial provenance	POPC DSPE-PEG2000	Magnetic trigger MRI contrast agent	TEM, DLS, fluoresence polarization, Zeta potential, absorbance, in vivo MRI
Garcia-Jimeno et al. (54)	Anionic coated (EMG 707, FerroTec)	Industrial provenance	Soy PC	Magnetic trigger in vivo injection and biodistribution analysis	TEM, DLS, SQUID magnetometry, Zeta potential
Garnier et al. (55)	Citrate coated (clusters)	Industrial provenance	DOPC Cholesterol	Magnetic trigger MRI contrast agent	Cryo TEM, DLS, MRI
Giri et al. (56)	PC coated	Co-precipitation	PC Cholesterol	Magnetic trigger	TEM, SQUID magnetometry, XRD, FTIR
Gonzales and Krishnan (40)	Oleic acid, trimethyl-amine *N*-oxide coated	Thermal decomposition	DPPC	Magnetic trigger	TEM, SQUID magnetometry, XRD
Linemann et al. (57)	Chitosan-lipid coated	Industrial provenance	Soy PC DDAB DSPE-PEG2000 -MAL	Magnetic trigger in vitro magneto-transfection	Zeta-potential, DLS
Martina et al. (39)	Citrate coated	Co-precipitation	EPC DSPE-PEG2000	Magnetic trigger MRI contrast agent (in vivo)	Cryo TEM, CLSM, QELS, magnetization measurements, relaxometry,magnetophoresis
Meledan-dri et al. (58)	DOPG coated	Co-precipitation	DOPG DOPC	Magnetic trigger	Cryo SEM, TEM, ATR IR, PCS, NMR, AAS
Nappini et al. (59)	$CoFe_2O_4$, TMAOH coated	Co-precipitation	PC	Magnetic trigger	TGA, DLS, SAXS, steady-state fluorescence spectroscopy
Pradhan et al. (60)	FluidMag-HS, chemicell GmbH	Industrial provenance	DPPC Cholesterol DSPE-PEG2000 DSPE-PEG2000 -folate	Magnetic trigger	Cryo TEM, TEM, fluorescence microscopy, DSC, XRD, magnetometry

(**Table 1** Continued)

Reference	SPIONs specifications	SPION synthesis	Lipid composition	Hybrid functionality	Analytics
Sabate et al. (61)	TMAOH coated	Co-precipitation	PC	Magnetic trigger	TEM, XRD, DLS, Zeta potential
Skouras et al. (62)	Hydrophilic-coated-USPIO-P00904	Industrial provenance	PC DSPC PG DSPE-PEG2000 DSPE-Biotin cholesterol	Magnetic trigger	TEM, Zeta potential, relaxometry
Tai et al. (63)	Dextran-coated	Industrial provenance	DPPC DSPC Cholesterol	Magnetic trigger in vivo carboxyfluorescein release	TEM, fluoresence spectroscopy
Surface attached					
Floris et al. (42)	Oleic acid coated, acetyl-acetonate	Thermal decomposition, co-precipitation	Soy PC	Magnetic trigger	TEM, XRD, DLS, Zeta potential

Abbreviation of chemicals: DDAB, dimethyldioctadecylammonium bromide; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-(phospho-*rac*-(3-lysyl(1-glycerol))) chloride, DPH diphenylhexatriene; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-Distearoyl-sn-glycero-3-phosphocholine; DSPE, 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine; DSPE-PEG-2000, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(amino(polyethylene glycol)-2000); DSPE-Biotin, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(biotinyl(polyethylene glycol)-2000) ammonium; DSPE-PEG-2000-Mal, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(maleimide (polyethylene glycol)-2000); DSPE-PEG-2000-Folate, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(folate(polyethylene glycol)-2000) ammonium; EPC, 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine chloride; PC, phosphatidylcholine; PEG 2000 PE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-((metoxy polyethylene glycol) 2000 Da); PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; Soy PC, L-α-phosphatidylcholine; SPC, sphingosyl-phosphorylcholine; TMAOH, tetramethylamonium hydroxide.

Abbreviation of Methods: AFM, atomic force microscopy; Cryo TEM, cryo transmission electron microscopy; Cryo ET, cryo electron tomography; Cryo SEM, cryo scanning electron microscopy; DLS, dynamic light scattering; DSC, synamic scanning calorimetry; EDX-FS, energy dispersive X-ray fluorescence spectroscopy; FTIR, Fourier transform infrared spectroscopy; QELS, quasi elastic light scattering; MRI, magnetic resonance imaging; SANS, small angle neutron scattering; SAXS, small angle X-ray scattering; SQUID, superconducting quantum interference device; STEM, scanning transmission electron microscopy; TEM, transmission electron microscopy; TGA, thermo gravimetric analysis; XRD, X-ray diffraction; SAR, specific absorption rate.

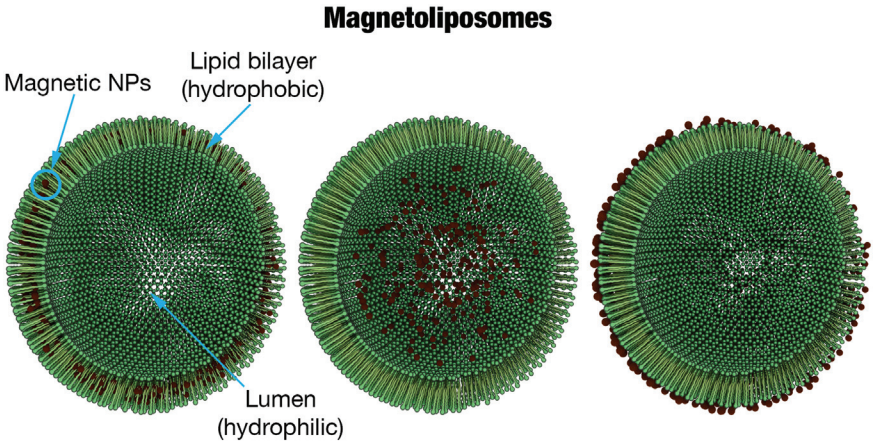


Figure 3 SPION-liposome hybrids. SPIONs acting as triggers to release a cargo (e.g., drug molecules) can be located in the lipid bilayer, the lumen, or can be grafted to the surface of the liposome (from left to right).

solution. Structural and architectural details, such as the SPION distribution or arrangement within the hybrids, are also relevant in studying the interactions of NPs and membranes in general. However, the challenge lies in providing convincing data, which is both qualitative and quantitative, while assuring that the hybrids are in their native state.

For giant magnetoliposomes, light and fluorescence microscopy offer the most straightforward options to directly observe and characterized the hybrids (38). Nappini and colleagues (66, 67) highlighted the utility of these methodologies by presenting giant unilamellar vesicles visualized by confocal laser scanning microscopy, in which both vesicles and NPs were fluorescently labeled. With this approach, NP presence and distribution as well as dye release was elegantly shown. Another useful approach was presented by Beaune and colleagues (38): The elastic properties of the magnetoliposomes were investigated by studying the deformation of the vesicles under the effect of an applied magnetic field.

When working with much smaller hybrids, the physical constraints of light come into play, and alternative methods are needed. There are several techniques available ranging beyond microscopy for investigating at the nanoscale and particularly small vesicles (i.e., <200 nm). As an example, scattering techniques – such as dynamic light scattering (DLS) or small angle X-ray and neutron scattering (SAXS and SANS) – can be used to elaborate the size of the vesicles, which in turn provides critical information on sample homogeneity. As an example, Amstad and colleagues (44) have successfully employed SANS to characterize both the sample homogeneity and the change in membrane thickness when loaded with SPIONs. Nonetheless, complementary visualization by microscopy to investigate morphology or appearance of the sample is unavoidable.

Transmission electron microscopy (TEM) is still the method of choice and has been widely used in this context (42, 46). However, conventional TEM techniques require a high vacuum environment, which is – particularly in the case of liposomes – highly destructive for any water-rich sample. Although samples can be preserved, e.g., by chemical fixation, there are still countless artifacts which are created by either the fixation procedure itself and/or sample drying. Moreover, this step inevitably leads to a randomized location of the unassociated NPs over the TEM grid. Consequently, correct and objective interpretation and discrimination between liposome associated and non-associated NPs is very challenging (Figure 4).

Although straightforward, this method is not ideal to reliably characterize such specimens. On the other hand, samples can be visualized in their native state by cryo TEM. Unlike conventional TEM, the vesicles are preserved in a layer of vitreous ice, keeping them safe from drying effects or the vacuum during visualization. Cryo TEM has been used to characterize liposomes for quite some time and has been applied on several occasions in the context of NP-liposome hybrids (41, 44). Unfortunately, the resolution was often not high enough to distinctly resolve the bilayer, a task rendered even more challenging by the variety of optical effects which may occur (68). Chen and colleagues (41) proved the presence of NPs by subsequent energy-dispersive X-ray spectroscopy (EDX) Electron microscopy in general needs to be interpreted extremely carefully: Merely a two-dimensional projection of the sample is provided by this methodology and leaves the three-dimensional aspects – such as the spatial location of the NPs in regard to the lipid bilayer – subject to speculation. Deducing architectural features by relying solely on single projections is therefore not feasible. This query can be countered by cryo-electron tomography, which finally

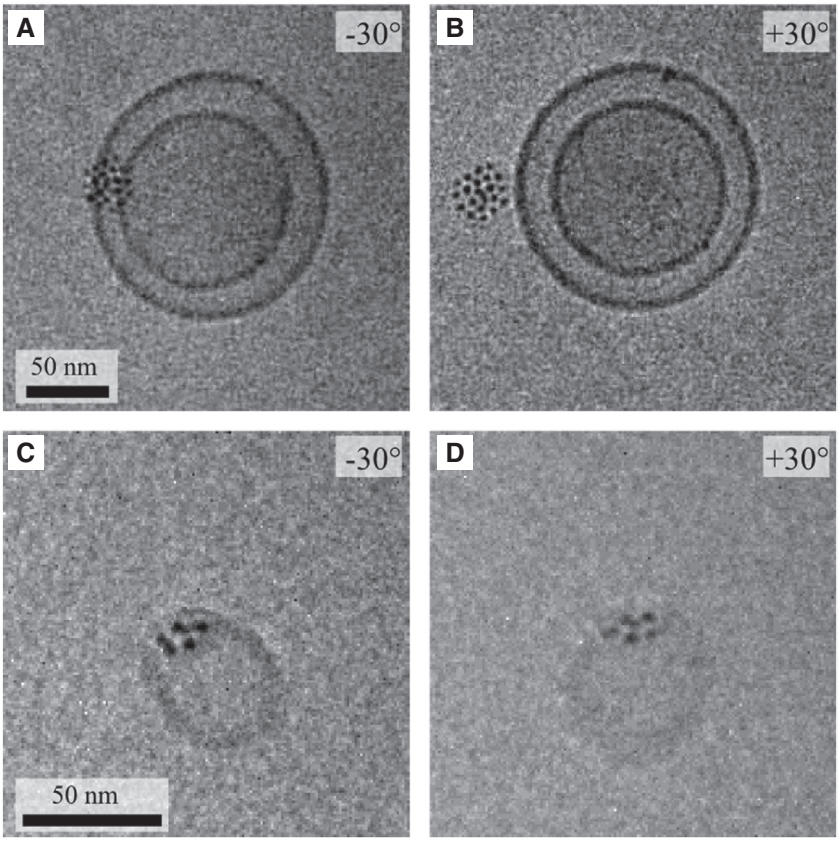


Figure 4 Cryo TEM images of SPIONs and liposomes at tilt angles of –30° (A, C) and +30° (B, D). A: Although particles at –30° seem to be associated with the liposome membrane, the tilt image at 30° (B) challenges this interpretation: it is the loss of the third dimension during the projection which leads to this misinterpretation. (C) Again, a cluster of particles seemingly interacts with the liposome membrane in the –30° tilt angle image. This interpretation is maintained, independent of the tilt angle (D). © 2013 IEEE. Reprinted, with permission, from IEEE Transactions on Magnetics, Vol. 49, No. 1, January 2013.

yields information on the structural and architectural aspects of the vesicles. Briefly, images of the sample are successively taken at various stage tilt angles. The collected data may then be used to reconstruct and render the three-dimensional appearance of the sample. Such data has been recently presented by the authors, with resolutions high enough to visualize the bilayer splitting around the inclusive SPIONs, along with three-dimensional renderings highlighting the NP locations and arrangements (43). Nonetheless, the investigation of small sample volumes is not sufficient for statistical relevance, which presents – in addition to the complexity of these techniques-the main limitations of cryo TEM and cryo-electron tomography.

Given the fact of the pros and cons of the aforementioned methodologies, a well-balanced combination of various techniques – i.e., scattering and spatial visualization by microscopy – is necessary to provide the information needed on both a statistical and qualitative level. In turn, these assessments are vital for any subsequent upscaling and industrial perspective.

Membrane energetics – inclusion limits between the bilayer

The incorporation of NPs into the lipid bilayer, and in particular the question of the maximum size of the NPs that can be embedded, has puzzled scientists for quite some time. No fully rigorous model is available in the literature to effectively quantify the energy needed to deform a lipid bilayer and accommodate a NP. In turn, biophysical aspects and properties of lipid bilayers also come into play.

To date, only a simplified approach has been proposed by Wi and colleagues (69). Although the variational problem-based on the Helfrich model (70) used to determine how the lipid membrane needs to deform to accommodate a NP and minimize the deformation energy – was not solved (71), they instead made some clever assumptions on the geometrical configurations of the membrane. This step drastically reduces the complexity of the problem. Their

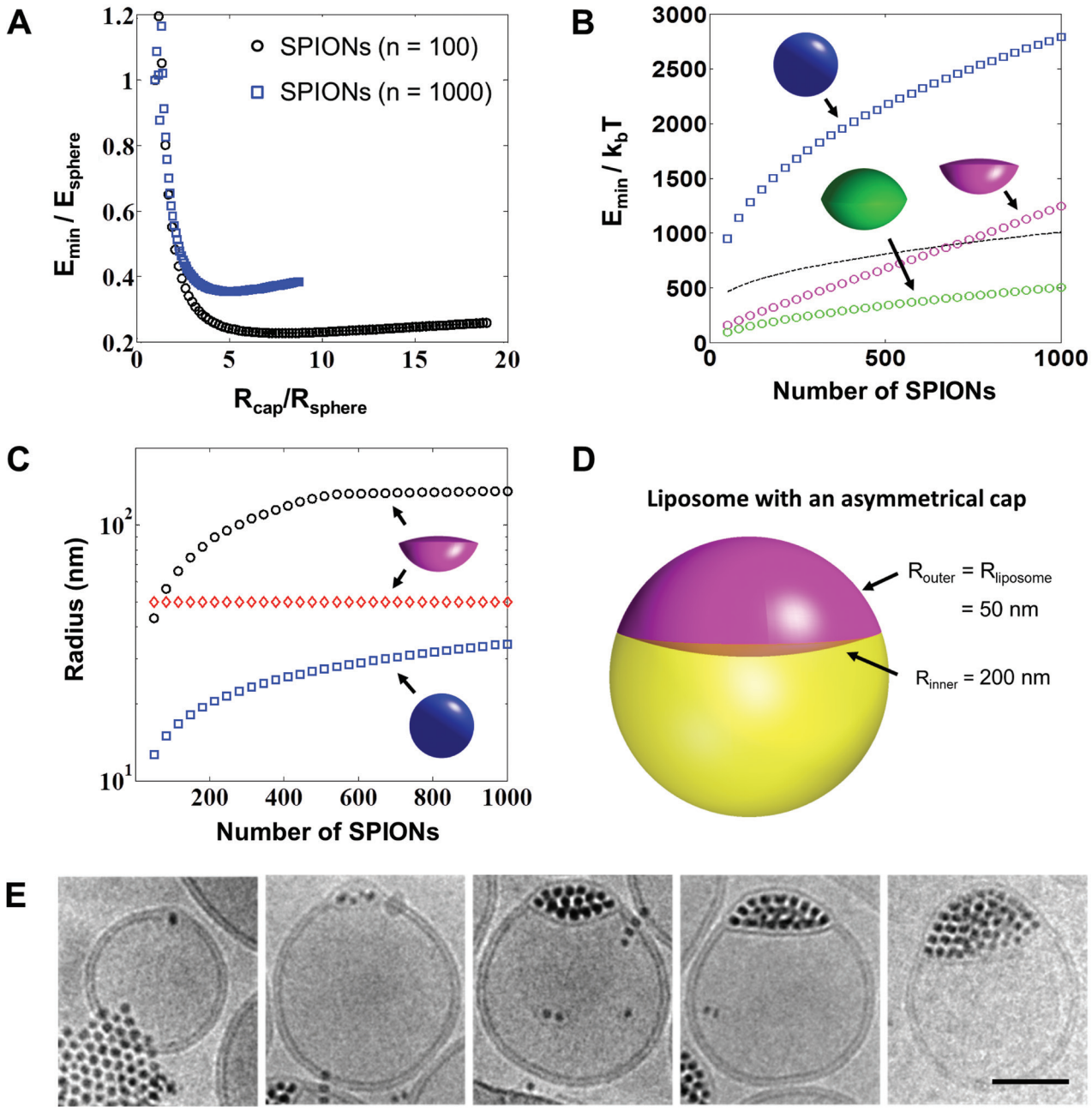


Figure 5 The energetics behind cluster-sized inclusions in between a phospholipid membrane. (A) The inclusion energy of an inclusion with a double spherical cap geometry, as a function of the spherical cap radius for both 100 and 1000 nanoparticles. Both radius and energy are normalized by the corresponding values for a spherical inclusion. (B) Energy of an inclusion with an asymmetric spherical cap geometry, with one radius equal to the liposome radius (taken equal to 50 nm) as a function of the number of nanoparticles in the inclusion. The energy of a corresponding spherical inclusion is additionally shown for comparison (blue), along with that of a spherical cluster covered by a lipid monolayer (black dashed line). (C) Radii of the asymmetric spherical cap inclusion as a function of the number of particles in the inclusion, as compared to the spherical inclusion radius. (D) Shape of a typical asymmetric inclusion with minimal energy. (E) Cryo TEM images showing the membrane deformation with increasing number of embedded SPIONs, scale bar=50 nm. Reprinted with permission from ACS Nano, Vol. 8, No. 4, 2014, Pages 3451–3460. Copyright 2014 American Chemical Society.

work has important consequences, as it allowed them to conclude that only spherical inclusions with a radius smaller than 3.5–4 nanometers can be incorporated into a lipid membrane. Larger spherical NPs are preferentially expelled by the lipid membrane and stabilized by a lipid monolayer. While these findings confirm some experimental observations made with quantum dots and SPIONs, they cannot explain the results recently obtained by the authors

of this review (43). In fact, inclusions with a diamond-like shape made of hundreds of SPIONs could be incorporated into the lipid membrane (Figure 5E). In order to explain these findings, the aforementioned theory was extended to inclusions with a non-spherical shape. In order to keep the simplicity of the original model, the model was restricted to spherical caps. This modified theory adds an additional degree of freedom, i.e., the radius of the spherical cap. For a given inclusion volume, the calculations show that an increase in the spherical cap radius – compared to a spherical inclusion – leads to a lower deformation by decreasing the bending energy of the membrane. However, a minimum is reached for a sufficiently large cap radius, as any further increase is penalized by the inclusion area becoming too large. These results, shown in Figure 5, demonstrate that NP clusters which can be organized into non-spherical assemblies are viable options to incorporate large quantities of SPIONs into a liposome membrane.

The importance of these results for hyperthermia applications is significant. In fact, the dependence of the heating rate of SPIONs exposed to an AMF is strongly dependent on the particle size, and seems to have an optimum for NPs with a diameter of about 20 nm (9). This approach offers the possibility to incorporate sufficiently large NPs to obtain optimal heating rate.

On the other hand, this also leads to new and currently unresolved problems. For example, one open question is whether a larger cluster of NPs remains superparamagnetic. Losing superparamagnetism is detrimental for the colloidal stability of the liposomes, as it would cause them to exhibit dipolar interactions. Furthermore, the heating power generated by NP clusters has not been systematically investigated to date. While there are studies showing the beneficial effect of clustering on the usage of SPIONs in MRI (72), the impact on the heating rate is a virtually unexplored area. However, this query is only a single example: A long list of questions needs to be addressed in the future, starting by which composition, particles size, size distribution is required to optimize hyperthermia performance of the magnetoliposomes. Finding answers to these intriguing questions will require a fundamental change in magnetoliposome design, and further combining the previously mentioned experimental characterization to modeling techniques might be highly beneficial for future developments.

Conclusions and perspectives

Today, liposomes are clinically established, yet there is still potential to improve them. Although significant effort has been invested in the development of magnetoliposomes, we still are only scratching on the tip of the iceberg – especially on a materials level – and the translation into the clinics is still being awaited. The efficacy of smart drug delivery systems, however, implies that the hybrids are thoroughly characterized by emerging and complementary techniques, which is – in our opinion – arguably one of the principal drawbacks in developing them. The complexity of these systems is highlighted by the multidisciplinary expertise needed, which includes organic and inorganic chemistry, bio- and magnetophysics, pharmacology and biology. Nonetheless, the last decade has yielded interesting new concepts. Some of them have been tested in laboratory settings, and further advancement will hopefully bring these hybrids a step closer to direct clinical application in the future.

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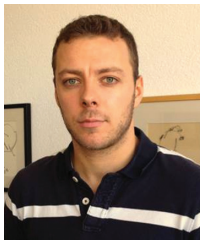

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