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Magnetoliposomes: recent advances in the field of controlled drug delivery

Abstract

Magnetoliposomes have gained increasing attention as delivery systems, as they surpass many limitations associated with liposomes. The combination with magnetic nanoparticles provides a means for development of multimodal and multifunctional theranostic agents that enable on-demand drug release and real-time monitoring of therapy.

Areas covered: Recently, several magnetoliposome structures have been reported to ensure efficient transport and delivery of therapeutics, while improving magnetic properties. Besides, novel techniques have been introduced to improve on-demand release, as well as to achieve sequential release of different therapeutic agents. This review presents the major types and methods of preparation of magnetoliposomes, and discusses recent strategies in the trigger of drug release, development of theranostic formulations, and delivery of drugs and biological entities.

Expert opinion: Despite significant advances in efficient drug delivery, current literature lacks an assessment of formulations as theranostic agents and complementary techniques to optimize thermotherapy efficiency. Plasmonic magnetoliposomes are highly promising multimodal and multifunctional systems, providing the required design versatility to optimize theranostic capabilities. Further, photodynamic therapy and delivery of proteins/genes can be improved with a deeper research on the employed magnetic material and associated toxicity. A scale-up procedure is also lacking in recent research, which is limiting their translation to clinical use.

Keywords

Magnetic nanoparticles; Liposomes; Magnetoliposomes; Magnetic hyperthermia; Photothermia; Drug delivery; Theranostics; Cancer therapy

1. Introduction

Cancer remains a major public health problem worldwide, with lung, colorectal, stomach, and liver cancer being the leading causes of death [1]. Despite the improvement of survival rates, conventional chemotherapy's lack of selectivity, specificity, and required high drug concentration lead to both healthy and cancer cells being indiscriminately affected [2–4].

Drug delivery systems have provided a route to improve conventional chemotherapy by the incorporation of drugs into nanoparticles. Such enables better drug stability and solubility, reduction of systemic clearance, and enhancement of the specificity [5]. The latter can be attained either through active (e.g. surface functionalization with groups that interact with specific receptors located in tumour) or passive (e.g. via enhanced permeability and retention effect, EPR) targeting [4,5]. Particularly, lipid formulations are highly advantageous owing to the encapsulation of both hydrophilic and hydrophobic drugs, which can be functionalized with different targeting ligands, such as proteins, peptides, nucleic acids, or small molecules [6]. This potentiality led to various patents, such as Onivyde™, Marqibo®, Visudyne®, Depocyt®, and Doxil® [7]. The latter consists of doxorubicin encapsulated in polyethylene glycol-coated liposomes and was the first US Food and Drug Administration (FDA)-approved liposomal formulation [5,7]. The reader is recommended to references [6,7] for an overview of liposomes in drug delivery.

However, conventional liposomes (consisting of a lipid bilayer encapsulating an aqueous phase) usually display slow and uncontrolled drug release [8]. Further, exploring the EPR effect is not enough to ensure proper targeting, as the porosity of tumour vessels and degree of tumour vascularization is not observed in all tumours [6]. Thus, the need to optimize targeting and release at the right time and period, to reduce side effects, and enable efficient treatment as the main objectives of drug delivery [5], led to the combination of liposomes with magnetic nanoparticles into liposome-based nanostructures, the so-called magnetoliposomes.

The presence of magnetic nanoparticles provides a means for nanoscale level manipulation and control of liposomes through a magnetic field gradient, such as conduction of signals [9], and magnetic targeting if the system displays a strong magnetic moment [4,5], besides the passive and active targeting. Moreover, an alternating magnetic field (AMF) can be applied as an exogenous stimulus to trigger drug release, besides exploring endogenous stimuli (response to physiological conditions), and the use of magnetic hyperthermia as adjuvant therapy [10]. Importantly, magnetoliposomes can work as theranostic agents (i.e. provide a means for both diagnosis and therapy administration) through monitorization by magnetic resonance imaging (MRI) due to the T_2 shortening effect of the magnetic nanoparticles [11,12].

Hereby, considering the current interest of developing multimodal and multifunctional materials that fulfil complex functions, this review initially introduces the reader to the types and methods of preparation of magnetoliposomes, followed by advances in the biomedical applications, mainly hyperthermia, and delivery of therapeutic agents, besides pointing out strategies for future developments.

2. Types of magnetoliposomes

Currently, magnetoliposomes can be classified according to the distribution of nanoparticles in the liposome structure in four major classes: solid magnetoliposomes (SMLs), which either consist of a single particle coated by a lipid bilayer membrane [13,14], or a cluster of magnetic particles [15–18]; aqueous magnetoliposomes (AMLs), in which magnetic nanoparticles are dispersed in the inner aqueous lumen of liposomes [15–20]; nanoparticles embedded in the lipid bilayer membrane [21–24]; and nanoparticles bonded to the liposomes' surface [25–27]. Further, the class of plasmonic magnetoliposomes has been recently reported, which allies both the magnetic and photothermal approaches. These systems can be fabricated either through AMLs encapsulating core/shell magnetic/plasmonic nanoparticles [15], or through the incorporation of plasmonic and magnetic nanoparticles in different compartments of the liposomes (e.g. magnetic nanoparticles in the inner aqueous lumen and plasmonic nanoparticles bonded to the liposome surface) [28]. Figure 1 summarizes the main architectures of magnetoliposomes.

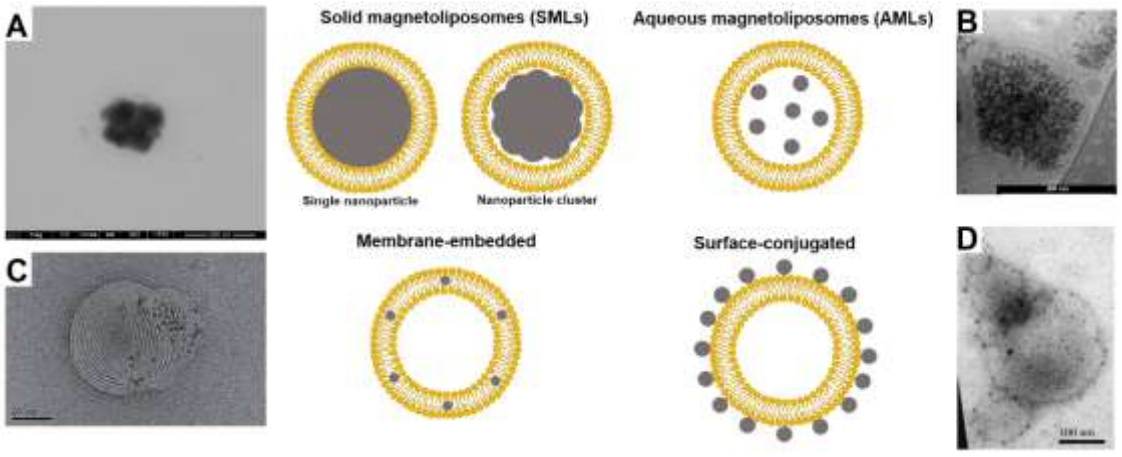

Figure 1. Schematic representation (not scaled) of the main architectures of magnetoliposomes and respective electron microscopy images, as examples: (A) solid magnetoliposomes (SMLs); (B) aqueous magnetoliposomes (AMLs) (adapted from reference [20] with permission from American Chemical Society, 2021); (C) magnetoliposomes based on membrane-embedded nanoparticles (adapted from reference [22] with permission from Royal Society of Chemistry, 2021); and (D) magnetoliposomes based on surface-conjugated nanoparticles (adapted from reference [25] with permission from Royal Society of Chemistry, 2021).

Other lesser common structures include the plasmonic magnetoliposomes and vesicle assemblies.

The abovementioned architectures need to be selected according to the applications, as each presents advantages and disadvantages. For instance, the high iron-to-lipid ratio of SMLs enables high cellular iron uptake [11], and similar magnetic properties to the neat nanoparticles [29]. However, the system only allows encapsulation of hydrophobic compounds as it does not contain an inner aqueous cavity, while the AMLs can transport both hydrophobic and hydrophilic drugs. The major drawbacks of AMLs are the reduced magnetic properties due to the extra water mass, which can also affect the thermal transition, as it requires the heating of all system, besides that the preparation requires properly stabilized nanoparticles as they can react with the membrane and cause undesired leakage [30]. The embedding of nanoparticles in the membrane surpasses AMLs limitations, as it enables the direct heating of the membrane, though the nanoparticle encapsulation efficiency is limited by the nanoparticle's size [22,30]. Although less explored, the nanoparticle-decorated magnetoliposomes make the entire inner cavity of liposomes available for drug loading and enables higher drug release efficiency/trigger [28], besides the potential use of sequential and complementary strategies (magnetic hyperthermia and photothermia). For example, Salvatore *et al*. [27] developed magnetoliposomes comprised of iron oxide nanoparticles embedded in the membrane and bonded to the membrane surface through double-stranded DNA conjugated with a cholesteryl unit. The application of a low-frequency AMF (LF-AMF) enabled the release of the content in the aqueous cavity and the DNA strand, which could be sequentially released by varying the frequency and the application time of the applied field.

3. Methods of magnetoliposomes preparation

Fabrication of magnetoliposomes includes the synthesis of the magnetic nanoparticles, which can be *in-situ* or *ex-situ*. The *in-situ* method consists on the use of phospholipid vesicles as nanoreactors (constraining the size of the particle to the liposome size), which can afford single-particle SMLs [14]. On contrary, the *ex-situ* method requires the initial synthesis of the nanoparticles and posterior combination with phospholipids/liposomes into magnetoliposomes.

The iron oxide-based nanoparticles are a class of major biomedical interest owing to the good magnetic properties, stability, biocompatibility, and flexibility of chemical modification [5,30]. The synthesis can be carried out through top-down or bottom-up approaches, though the bottom-up methods are preferentially used, which include chemical strategies such as

coprecipitation, thermal decomposition, sol-gel, hydrothermal and solvothermal, and microemulsion synthesis [5,30,31]. Particularly, coprecipitation provides an easy and affordable way to produce nanoparticles with different chemical compositions. For instance, different bare ferrite nanoparticles were synthesized through coprecipitation, including ferrites doped with manganese [16], calcium [32], magnesium [17], and nickel [15], and posteriorly formulated into magnetoliposomes. However, considering the inherent toxicity of transition metals and that ferrites are prone to the generation of reactive oxygen species (ROS), calcium and magnesium have been recently proposed as substitutes of the commonly used doping transition metals to improve biocompatibility [31,32].

Concerning the development of magnetoliposomes, common techniques include the ethanolic injection (EI) [15–18], thin film hydration (TFH) [19], reverse-phase evaporation (RPE) [10], and the double emulsion method (DE) [33]. The EI method has been demonstrated to be a simple, low cost and reproducible method for the fabrication of homogeneous AMLs, which consists in the injection of a lipid solution in ethanol to an aqueous solution of nanoparticles, above the melting temperature of the lipids and under vigorous agitation [34]. The TFH method consists on the formation of a thin lipid film by drying an organic solution of lipids, hydrating with an aqueous solution of nanoparticles, reducing its size through sonication and extrusion, to produce uniformly-sized magnetoliposomes [2,10]. Similarly, in the RPE method, an organic solution of lipids is initially mixed with an aqueous solution of nanoparticles and sonicated to form a water-in-oil emulsion. Posteriorly, the organic solvent is evaporated to form a gel phase and hydrated with a suitable buffer [10]. Nonetheless, it has been recently demonstrated, using the TFH method, that different parameters affect the loading efficiency of hydrophilic nanoparticles, such as the buffer type, concentration, pH, the liposome's composition, and components' stoichiometry [19]. For instance, formulations displayed in Table 1 demonstrate that the encapsulation efficiency is clearly influenced by the lipid composition of the liposome. The TFH method is more efficient when using Egg-PC rather than DPPC lipids. Besides ethanolic injection has lower encapsulation efficiency than TFH, it is chosen sometimes for its easier process. In another work, the payload was also demonstrated to potentially affect the encapsulation of nanoparticles [35].

Table 1. Encapsulation efficiencies of magnetoliposomes prepared by different methods and variable composition.

Lipid composition	Preparation method	Encapsulation efficiency (%)	Reference
Egg phosphatidylcholine	Ethanolic injection	47%	[34]
ASO (25% soy phosphatidylcholine)	Reverse phase evaporation (RPE)	58% ([MNP]=0.116 mg/mL)	[35]
Hydrogenated soybean phosphatidylcholine	Reverse phase evaporation (RPE)	36 % (10.1 mg Fe^{2+}/mol lipid)	[36]
Egg-PC/cholesterol 2:1	Thin film hydration	70%	[33]
	Double emulsion	40%	
DPPC/cholesterol/PEG	Thin film hydration	39%	[19]Lipid

Further, considering that the liposome's internal volume is just a small fraction of the total sample volume, the fabricated magnetoliposomes need to be purified, which can be carried out through magnetic separation [32], size exclusion chromatography, centrifugation, and salt-induced aggregation [37]. However, it is worth to point out that magnetic separation can be unable to properly separate non-encapsulated from encapsulated nanoparticles. If hydrophobic nanoparticles are used, the self-assembly with the lipid bilayer membranes can drive to the fabrication of magnetoliposomes with membrane-embedded nanoparticles. For instance, Shaghasemi *et al*. [23] developed PEGylated magnetoliposomes of variable lipid composition embedded with palmityl-nitrodopamine-coated iron oxide nanoparticles, through a scalable solvent inversion-sonication method. The inclusion of nanoparticles was demonstrated by Chen *et al*. [21] to improve the bilayer stability and thus, reduce the spontaneous leakage of the payload from the aqueous compartment. Yet, the main hurdle in the development of this nanosystem architecture is the insertion of nanoparticles being constrained by the thickness of the membrane, which limits the inclusion of larger particles (> 3.4 nm) and can lead to the formation of micelles [22,30]. Concerning this limitation, Choi *et al*. [22] recently developed a solvent-guided approach that enabled the insertion of 6 nm or 15 nm nanoparticles, and further demonstrated the potential of the system in the magnetic field-guided separation of cancer cells.

Although less explored, some advances have been reported in the fabrication of surface conjugated magnetoliposomes. For instance, Floris et al. [25] explored electrostatic interactions between cationic nanoparticles and anionic phospholipids to self-assemble into liposomes bearing magnetic nanoparticles onto the surface. An electrostatic self-assembly strategy was also reported by Haša et al. [26] consisting of anionic liposomes and iron oxide

magnetic nanoparticles and a positively charged polyelectrolyte (poly-L-lysine) that assembled in sub-micrometre aggregates. An alternative to the use of the electrostatic interactions was reported in the work of Salvatore et al. [27]. The strategy consisted in using cholesteryl units conjugated with double-stranded DNA linked to the magnetic nanoparticles, which enabled the spontaneous anchorage of nanoparticles in the outer leaflet.

4. Advances in antitumour drug delivery

4.1. Thermotherapeutic strategies using magnetoliposomes

The presence of magnetic nanoparticles enables the use of magnetic hyperthermia, which main objective is the exposure of tissues to high temperatures (42 – 45 °C), so to induce cancer cell death by apoptosis or to enhance the susceptibility to radiation and antitumour drugs [38]. Such heating effect is associated with the Néel (spin reversal) and Brownian (particle rotation) relaxation of the nanoparticles magnetic moments upon application of an AMF, as a consequence of the delay of the magnetic nanoparticles relaxation time compared to the oscillating AMF. Further, the former is strongly size-dependent, occurring at sizes that only require some energy to induce rotation of the magnetic moment, while the latter is affected by the medium viscosity, as it consists on the physical rotation of the nanoparticle.

Nonetheless, when dealing with biological applications, the maximum field amplitude and frequency are limited so to avert the occurrence of eddy currents. Hereby, different extrinsic and intrinsic properties need to be considered to increase the heating efficiency of the nanoparticles and thus reduce the required dose and prevent undesirable cytotoxic effects [31,39]. In fact, optimizing the magnetic properties of nanoparticles is crucial for magnetoliposomes application, as both the diamagnetic mass contribution and clustering of nanoparticles in the core lead to a reduced saturation magnetization compared to neat magnetic nanoparticles [16,29].

Besides the potential use to prompt cancer cell death, magnetic hyperthermia can trigger drug release in materials that display a phase transition. Regarding the magnetoliposomes, the combination of magnetic hyperthermia with temperature-sensitive liposomes (thermosensitive liposomes, TSLs) provides a means for controlled drug release through the increase of temperature above the phase transition temperature (T_m) of lipids. As examples, some commonly used TSLs composition includes dipalmitoyl phosphatidylcholine (DPPC, $T_m \approx 41$ °C) and distearoyl phosphatidylcholine (DSPC, $T_m \approx 54$ °C) [17]. Above T_m, thermosensitive lipids undergo a transition from the gel (low permeability to hydrophilic

molecules) to a liquid-crystalline state (high permeability to hydrophilic molecules), facilitating the release of the liposome content. This control enables a synergy between chemotherapy and magnetic hyperthermia, which together with the magnetic targeting, enhances the efficacy of cancer therapy [2]. For instance, Ferreira *et al*. [40] developed magnetoliposomes (DPPC:cholesterol 12:3) loaded with the chemotherapeutic drug gemcitabine, and citric acid-functionalized iron oxide nanoparticles, which drug release reached 70% after 5 min of exposure to an AMF, while only 17% was released after 72 h at 37 °C. Recently, Ribeiro *et al*. [41] were able to co-encapsulate both paclitaxel and gemcitabine and improved the controlled release. The system (DPPC:cholesterol 10:1) displayed less than 10% drug release after 72 h of incubation, while a 30 min application of AMF induced a release enhancement up to 94% and 42% of gemcitabine and paclitaxel, respectively. The authors also demonstrated *in vitro* that the combined therapy was more effective against breast cancer cells (MGSO-3) than the separate use of chemotherapy and hyperthermia. The synergy between chemotherapy and hyperthermia has been assessed in other systems comprising different payloads, including doxorubicin [42], curcumin [43], methotrexate [44], and cisplatin [36].

Concerning the potential harmful effects to surrounding tissues due to hyperthermia and eddy currents, the use of low-field AMF (LF-AMF) has been suggested as a safer approach for drug delivery applications. For example, Spera *et al*. [45] developed magnetic liposomes with a high melting transition temperature (hydrogenated soybean phosphatidylcholine:cholesterol 8:2), which content was reproducibly released at each cycle of exposure to a 20 kHz and 60 A/m AMF. This strategy has also been assessed in nanosystems comprising large unilamellar liposomes [46], or hydrophobic iron oxide nanoparticles embedded in a lipid bilayer [47]. In the latter, the presence of the nanoparticles increases the stiffness and morphological inhomogeneity, which facilitates the rupture due to the mechanical vibration of the nanoparticles upon application of the LF-AMF. Recently, Vlasova *et al*. [48] assessed the mechanism of drug release of liposomes embedded with nanoparticles (*N*-palmitoyl-6-nitro-dopamine-coated iron oxide) using different liposome compositions and nanoparticle sizes. The authors found that the addition of cholesterol to saturated lipid-based liposomes or replacement of saturated lipids by unsaturated ones decreased the dye release upon application of AMF, besides being dependent on the field strength, but not on the frequency (figure 2). Such was associated with the rupture of the gel phase membranes of saturated lipids, while the defects and deformations induced in the unsaturated ones are likely to heal. Other methods have also been explored to trigger the fast release of the liposomes' payload, such as the use of short magnetic pulses [28] or pulsed electromagnetic fields [49].

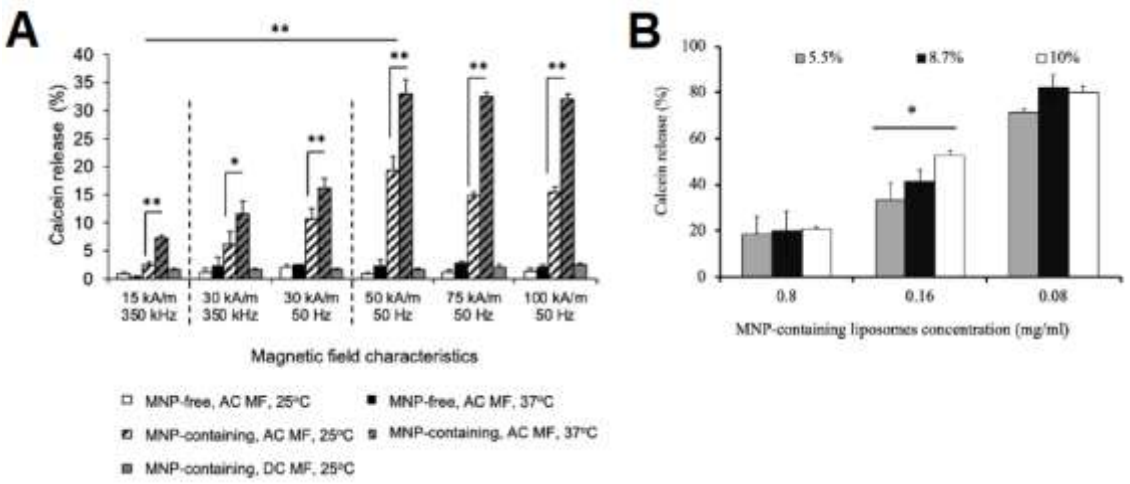

Figure 2. (A) Effect of AMF strength and frequency, and temperature on calcein release from magnetoliposomes of DSPC/DSPE-PEG2000 (95:5) without (MNP-free) and with (MNP-containing) 5 nm magnetic nanoparticles. (B) Effect of sample concentration and nanoparticle content on calcein release from magnetic liposomes under an AMF (50 kA/m, 50 Hz) at 37 °C for 30 min. Adapted from [48] with permission from Elsevier, 2021.

A strategy that has been recently explored consists of the photothermal effect upon laser irradiation of the nanoparticles with near-infrared (NIR, 700 – 1000 nm) light [8,50]. Photothermia explores the phenomenon of localized surface plasmon resonance and the most commonly used material is gold nanoparticles [51,52]. The photothermia phenomenon consists in the enhancement of the local electromagnetic field upon application of an external oscillating electric field, from which the heating effect is associated with the fast phase loss of the coherently excited electrons *via* electron-electron collisions. Interestingly, besides the commonly used plasmonic gold nanoparticles, magnetite nanoparticles have also displayed good photothermal conversion efficiency [53,54], which can be used as an alternative or in combination with an AMF. In ferrites, the photothermal effect has been associated with the photoexcited electrons temporarily transit from the valence band to the conduction band, followed by electron-hole recombination (bridged by intrinsic mid-band gap states or trap-gaps due to internal defects) that can occur through non-radiative relaxation [55]. Hereby, photothermia can be used as an alternative or in combination with an AMF. For example, Shen *et al*. [8] developed magnetic liposomes (DPPC:cholesterol 4:1) loaded with iron oxide nanoparticles and doxorubicin, which after intravenous injection in mice, was slowly enriched in the tumour and allowed both the photothermal effects through irradiation with a continuous 808 nm laser light (2 $W.cm^{-2}$) and contrast in magnetic resonance imaging (MRI).

Nonetheless, despite the advances in the combination of plasmonic nanoparticles and liposomes [56], current literature lacks the combination of both magnetic and plasmonic

modalities. The potential use of plasmonic magnetoliposomes (liposomes entrapping nanoparticles with both plasmonic and magnetic components) for phototherapy was demonstrated by Rodrigues *et al*. [57] and Rio *et al*. [15] for both AMLs and SMLs. These types of magnetoliposomes combine both photothermal (generation of heat) and magnetic properties (magnetic hyperthermia, magnetic drug delivery), creating a synergistic effect. Particularly, the magnetic-plasmonic combination is of interest for theranostics (the combination of diagnostics and therapy) as it surpasses limits associated with imaging modalities and hyperthermia [58,59]. For instance, Tomitaka and co-workers [58] developed plasmonic magnetoliposomes bearing core/shell magnetic/plasmonic nanoparticles, and loaded with tenofovir disoproxil fumarate (TDF), that not only provided a strong negative and positive contrast in MRI and magnetic particle imaging (MPI), respectively, but also enabled a bright positive contrast in X-ray computed tomography (CT). The plasmonic magnetoliposomes are expected to be more advantageous than neat magnetoliposomes considering the properties of the magnetic-plasmonic nanoparticles. Concerning thermotherapy, Das et al. [60] demonstrated that the combined magnetic hyperthermia and photothermia could enhance the heating efficiency (expressed as specific loss power, SLP, or specific absorption rate, SAR) in at least one order of magnitude using lower intensity requirements compared to the separate use of the employed techniques. Furthermore, Jin *et al*. [61] suggested a new imaging technique using magnetic-plasmonic nanoparticles, the magnetomotive photoacoustic imaging, which consists on the use of photoacoustic imaging technique and the application of a pulsed magnetic field. Through the detection of the magnetic nanoparticles induced motion in response to a time-varying magnetic field, contrast can be improved by identifying sources associated with the nanoparticles and rejecting the background signals (static background and incoherent absorbers), whether from diffuse or localized sources.

4.2. Theranostic applications

Magnetoliposomes provide a versatile nanosystem for theranostics by providing a means for on-demand drug release, chemotherapy-hyperthermia synergy, and contrast for imaging techniques, including the new modalities discussed in previous sections. Particularly, the presence of the magnetic nanoparticles provides a means to improve both T_1 and T_2 magnetic resonance imaging [31]. The combination of magnetic nanoparticles with the lipid membranes enables the tuneability of different factors so to optimize the efficiency as contrast agents [12,62], such as the permeability of the lipid bilayer, the water exchange rate and cholesterol content, and the presence of a fixed aqueous layer thickness, which can be induced by the

presence of PEG. In fact, PEGylation has been demonstrated to provide various advantages in the development of magnetoliposomes as theranostic agents, such as not significantly affect the magnetophoretic mobility of magnetoliposomes compared to free nanoparticles [63], improve hemocompatibility and reduce toxicity [64]. However, it can also be a hurdle in the targeting of the system and consequent accumulation, as reported by Estelrich *et al*. [65]. The authors verified *in vitro* that the targeting capability of PEGylated magnetoliposomes functionalized with arginine-glycine-aspartic acid (RGD) to HeLa cells expressing RGD receptors was comparable to PEGylated and bare magnetoliposomes. Hereby, a strategy was suggested for optimization of cell interaction and internalization of magnetoliposomes consisting in the addition of short PEG chains and other longer chains carrying the ligand. In the absence of PEG chains, a higher uptake of RGD-functionalized magnetoliposomes was reported by Ribeiro *et al*. [66] for different tumour cell types *in vitro*, including glioma and ovarian cancer.

Nevertheless, other polymer coatings can be used in the development of magnetoliposomes to improve stability and drug delivery properties. For instance, Guo and co-workers [67] developed carboxymethyl dextran-coated magnetoliposomes loaded with doxorubicin that displayed an enhanced release upon application of a LF-AMF, besides providing a means for efficient T_2-weighted contrast with an r_2/r_1 ratio of 57. Methotrexate-modified magnetoliposomes loaded with doxorubicin and bearing nanoparticles embedded in the lipid bilayer were developed by Guo *et al*. [44], which afforded a transverse relaxivity of 60.06 $mM^{-1}s^{-1}$ and rapidly reached in 5 min a 4.2-fold enhancement of the heating effect upon the combination of both AMF and 808 nm laser irradiation. The system displayed a significant release of doxorubicin, which ensured a decrease of doxorubicin associated toxicity and improved *in vivo* therapeutic effectiveness. Other strategies in theranostics include the use of pro-drugs or anti-inflammatory agents. For instance, Calle *et al*. [68] developed magnetoliposomes loaded with the anti-inflammatory agent omega-3 polyunsaturated fatty acid ethyl ester, which besides an enhanced MRI contrast *in vivo*, significantly improved the effect against inflammation associated with glioma growth, where the liposomal formulation slowed down proliferation and induced remission. Recently, in a work by Thébault *et al*. [69], magnetoliposomes were loaded with Combretastatin A4 phosphate (CA4P), a water-soluble pro-drug of the vascular disrupting agent Combretastatin A4, which can lead to the starvation of the tumour and modify its microenvironment. The system was monitored through MRI and the payload release was triggered by local heating High-Intensity Focused Ultrasound (HIFU) (figure 3). The authors reported a 150-fold improvement using the combined therapy (compared with the chemotherapy alone) 24 h post-therapy after one single treatment.

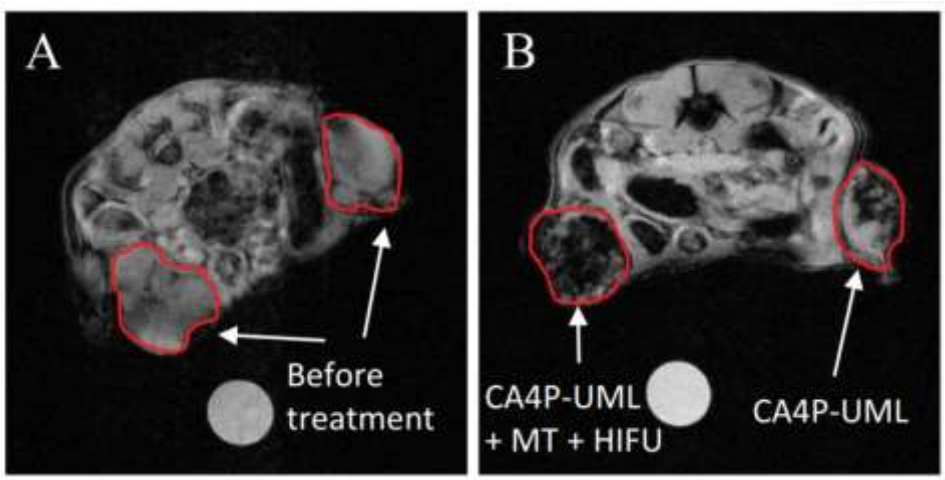

Figure 3. T_2*-weighted MRI images comparing tumours (A) before and (B) after treatment with ultramagnetic liposomes (UML) loaded with Combretastatin A4 phosphate (CA4P) through combined magnetic targeting (MT) and High-Intensity Focused Ultrasound (HIFU) on the left tumour (CA4P-UML+MT+HIFU), and solely administration of the magnetic liposomes on the right tumour (CA4P-UML). Adapted from [69] with permission from Elsevier, 2021.

4.3. Photodynamic therapy

Photodynamic therapy (PDT) is a clinical treatment, recognized by US Food and Drug Administration, based on the excitation of a photosensitizer (PS) localized at the disease site with appropriated light irradiation, usually visible or NIR light. Upon irradiation, the photosensitizer molecules are excited to a singlet state, which in turn can undergo intersystem crossing to form a more stable excited triplet state. At this stage, the PS reacts with nearby molecular oxygen (O_2), producing singlet oxygen and reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and superoxide anion radical ($O_2^{\cdot -}$), that might induce cell death through apoptosis, necrosis and cell membrane disruption [70,71]. Particularly, in cancer treatment, PDT can destruct tumour cells by three main processes: (1) direct kill of cancer cells by ROS through apoptosis and/or necrosis; (2) damage of tumour vasculature, which causes cell death *via* deprivation of oxygen and nutrients; (3) induced acute inflammatory response, leading to the influx of macrophages and leukocytes, that can promote tumour destruction and recognition by the immune system [70,72]. The main advantages of PDT over conventional cancer treatments are the possibility of reducing tumour mass with minimized side effects, cost-effectiveness, safety and non-invasiveness, localized treatment, and induction of immunity [71,72]. Several photosensitizers have been clinically approved for the treatment of different cancer types, including Photofrin® (basal cell skin cancer), Foscan® (cervical, gastric and esophageal cancer), and Laserphyrin® (lung cancer, head and neck cancer) [71]. However, the clinical use of this early generation of PSs suffers from

some disadvantages like low penetration depth, low extinction coefficients (which requires high dosages of PS to achieve effective photodynamic activity), long half-life, and consequent skin phototoxicity, as well as low solubility and low cell/tissue specificity [73]. The next generation of PSs must be able to overcome these limitations and efforts are pointed out to the combination of PSs with nanocarriers as a means to potentiate photodynamic effects. This association allows increasing the solubility of PS molecules, the cell-specificity by surface functionalization of the nanoparticles, and the uptake by cancer cells through the EPR effect [74]. Several nanomaterials have been reported for a targeted and effective photodynamic treatment such as gold, silver and silica nanoparticles, quantum dots, carbon-based nanomaterials, graphene-based nanomaterials, polymeric nanoparticles, and liposomes [73,74]. Liposomes exhibit a high versatility, being able to carry both lipophilic and hydrophilic PS molecules, together with anticancer drugs, and can also be decorated with targeting ligands [75], leading to more effective PDT treatments. The incorporation of superparamagnetic nanoparticles into PS-loaded liposomes allows to selectively guide and accumulate PS agents in tumour sites using an external magnetic field [75,76]. Actually, protoporphyrin IX-loaded magnetoliposomes promoted the *in vitro* death of MCF-7 human breast cancer cells within 24 h when applying white light for 5 min [77]. A dual therapy can also be achieved with magnetoliposomes by combining PDT with magnetic hyperthermia. For instance, magnetoliposomes loaded with zinc phthalocyanine (ZnPc) complexed with cucurbituril were obtained, and the photodynamic and hyperthermia effects were tested *in vitro* in the melanoma cell line B16-F10 for 3 hours [78]. The combination of magnetic hyperthermia with the application of an alternating magnetic field (operating at 1 MHz) and PDT, with a light dose of 2.0 J/cm^2, led to a cancer cell viability of only 13% *in vitro*, about half than the observed when PDT was applied alone (30%), evidencing the synergistic effect of this dual therapy strategy [79]. Other formulations incorporating ZnPc were developed, such as DOX-loaded nanoarchitectures with a core-interlayer-shell structure, Fe_3O_4@mSiO_2@lipid-PEG-methotrexate, for combined chemo-photodynamic therapy, with improved cellular uptake and anticancer activity [80]. Based on a nanosystem with these features, the treatment strategies are vast. Anilkumar *et al.* [50] engineered photosensitive magnetoliposomes for combined photothermal and photodynamic cancer therapy. Cationic liposomes were used to encapsulate citric acid-coated magnetic nanoparticles and the photosensitizer indocyanine green (ICG) for dual-mode therapy. Hyaluronic acid-polyethylene glycol (HA-PEG) was coupled at the surface of liposomes to target U87MG glioblastoma cell line, binding the over-expressed CD44 receptors at the cell surface. *In vivo* antitumor studies showed efficient photothermal effect

(figure 4A) and tumour growth prevention (figure 4B) upon exposure to an 808 nm laser at 2 W/m^2.

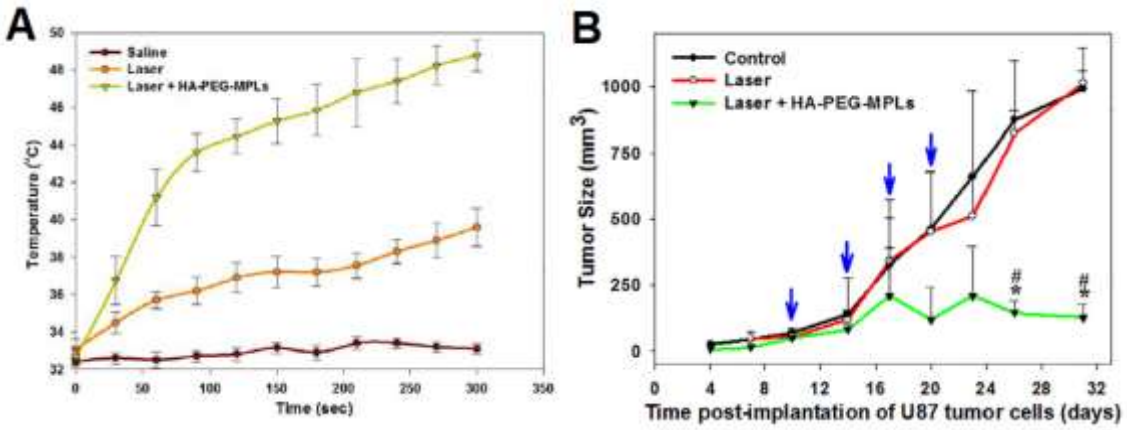

Figure 4. *In vivo* photothermal profile (A) and tumour size growth (B) of tumour-bearing mice after injection with saline solution, with saline solution followed by NIR laser irradiation and injection with HA-PEG magnetoliposomes followed by NIR laser irradiation. Adapted from [50] with permission from Elsevier, 2021.

4.4. Protein and gene delivery

Protein administration is, in general, highly susceptible to hydrolysis and enzymatic degradation [81], while nucleic acids like plasmid DNA, mRNA, and siRNA have a highly negative and polar nature that hampers their entry across the cell membrane, besides exhibiting low stability and easy degradation by endonucleases in the bloodstream [82,83]. The incorporation of these biological entities into nanodelivery systems allows a safer and more efficient delivery, characterized by low cytotoxicity and immunogenic response, minimized off-target effects, and cell-specific delivery by surface functionalization. Several nanoformulations have already been used to carry proteins and genes, including polymeric nanoparticles [84,85], liposomes [86,87] and other lipid-based nanoformulations [88-90], inorganic nanoparticles [91], micelles [92], or dendrimers [93]. Once again, magnetoliposomes join some advantageous features comparing to the mentioned nanocarriers. A guided delivery of therapeutic proteins and genes is possible through magnetic targeting due to the presence of magnetic nanoparticles. A controlled and triggered release is also achieved by the combination of magnetic hyperthermia and thermosensitive liposomes, the loading content of magnetoliposomes only being released in the target tissues. Thomsen and co-workers [94] have developed magnetic immunoliposomes aiming to cross the blood brain barrier (BBB). Magnetite nanoparticles were covered with PEGylated liposomes composed of L-α-phosphatidylcholine and the cationic surfactant dimethyldioctadecylammonium bromide (18:0 DDAB). The resulting magnetic liposomes were conjugated to antibodies against the rat

transferrin receptor (OX26), allowing a targeted delivery to the brain capillary endothelial cells (BCECs). The researchers performed *in situ* brain perfusion with external magnetic force and demonstrated a preferential accumulation of magnetic immunoliposomes in BCECs and the transport across BBB of magnetic nanoparticles subjected to a magnetic force. The entire magnetic immunoliposomes were not detectable in the brain parenchyma, leading to the hypothesis that OX26-liposomes are retained in the BCECs and magnetic nanoparticles are dragged out of the liposomes. Although the cytotoxic activity of 18:0 DDAB in mammalian cells is not clearly defined, the cell viability assays in this study showed that cell treatment with magnetic liposomes or magnetic OX26-liposomes did not affect significantly viability of rat brain endothelial cells.

Magnetic cationic liposomes can also work as non-viral nanocarriers in gene therapy [95]. Small interfering RNA (siRNA), associated with cell-penetrating peptides (CPPs), was encapsulated in thermosensitive magnetoliposomes to achieve an efficient delivery of siRNA to tumour cells, by application of an AC magnetic field. *In vitro* and *in vivo* studies showed efficient selective delivery of siRNA to MCF-7 cells, gene silencing activity, as well as enhanced tumour accumulation (figure 5), antitumor efficacy, and decreased non-specific accumulation of the siRNA-CPPs-loaded magnetoliposomes in healthy organs [96].

Besides hyperthermia and targeting functionalities, an image-guided delivery can also be achieved with magnetoliposomes, using the magnetic nanoparticles as contrast agents for T_2-weighted MRI [97], providing theranostic capabilities to magnetic liposomes [62,98]. Focusing on this, Do *et al.* [99] recently reported an optimized preparation of cationic magnetoliposomes for magnetic transfection of a pFAR4-luciferase plasmid to CT26 cells. Magnetoliposomes prepared by the cosolvent sonication method promoted both higher r_2 relaxivity and *in vitro* transfection efficiency than the ones prepared by the reverse phase evaporation method. The highest magnetic transfection enhancement was achieved at 30 min or 3 h of magnetic induction time. These results demonstrate the promising applicability of magnetoliposomes in magnetically-guided gene delivery.

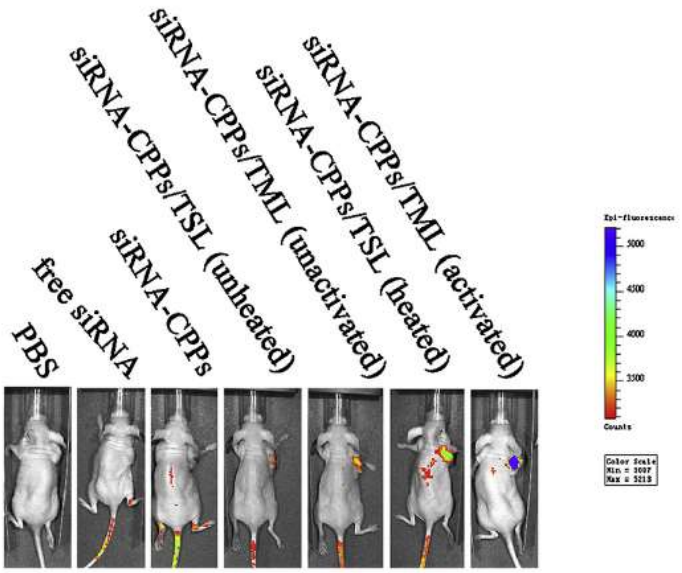

Figure 5. *In vivo* imaging of biodistribution of tumour-bearing mice treated with PBS, free siRNA, siRNA conjugated with CPPs (siRNA-CPPs), siRNA-CPPs-loaded thermosensitive liposomes (siRNA-CPPs/TSL unheated or heated) and siRNA-CPPs-loaded thermal and magnetic dual-responsive liposomes (siRNA-CPPs/TML activated or unactivated). Reproduced from [96] with permission from Elsevier, 2021.

5. Conclusion

Magnetoliposomes provide a suitable platform for the development of multimodal and multifunctional theranostic agents that enable on-demand and sequential release of therapeutic agents. Several types of architectures have been developed to allow the encapsulation of a wide array of drugs, improve the magnetic responsiveness and triggered drug release, as well as the use of complementary techniques. For this purpose, different preparation techniques have been used to optimize nanoparticle encapsulation efficiency, systems' homogeneity, and fabrication of complex structures. In addition, several chemical synthesis strategies have been reported to prepare affordable magnetic nanoparticles with improved properties. In this regard, the magnetic properties of magnetoliposomes have been explored not only to improve the release responsiveness of the system through hyperthermia, but also on the use of safer approaches, mainly the use of low frequency alternating magnetic fields or the combined magnetic hyperthermia-photothermia, reducing the magnetic field and laser intensity required for an efficient chemotherapy-hyperthermia synergy. Particularly, magnetic nanoparticles enable the use of magnetoliposomes as contrast agents and real-time monitorization of the therapy. Moreover, the tuneability of the components has stimulated the optimization of theranostic properties, besides the development of novel imaging techniques enabled by the combined use of magnetic and plasmonic materials. The multifunctionality of these nanocarriers extends to new cancer nanotherapies including

photodynamic therapy and gene therapy, being possible to combine different therapeutic modalities in a single nanocarrier. Photothermal/photodynamic therapy, chemo-photodynamic therapy, and magnetic guided delivery of proteins and genes are some of the possibilities that can be added to magnetic hyperthermia and targeted drug delivery. In fact, *in vitro* and *in vivo* assays have been more frequent and have shown reduced cytotoxicity, higher bioaccumulation in the target site (which avoids undesirable side-effects in healthy tissues), and the capacity to prevent tumour growth. These features open a new hopeful way for the rational development and clinical evaluation of magnetoliposomes for cancer therapy.

6. Expert Opinion

New materials in the field of drug delivery are emerging through the combination of existing systems, which adds complexity and enables multifunctional approaches in therapy. The potential of liposomes in nanomedicine is reflected in a large number of patents. An example is ThermoDox®, a recent patent based on doxorubicin-loaded thermosensitive liposomes, which is currently in phase III clinical trials.

Magnetoliposomes pave the way to novel opportunities in drug delivery through the combination of magnetic nanoparticle properties with the versatility of liposomes. A wide array of magnetoliposomes structures has been developed to improve their properties, enabling the selection and optimization of the system according to the application. For instance, AMLs and SMLs have been the most explored structures, mainly due to the lower complexity and not being as limited to particle sizes as the class of membrane-embedded magnetoliposomes. In addition, the simplicity of preparation of these systems enabled the efficient encapsulation and delivery of a wide range of drugs [16-18,34,56]. Consequently, despite the different methods developed for the fabrication of magnetoliposomes, ethanolic injection and thin-film hydration remain the most used strategies. More complex structures are required to enable the sequential release of therapeutic agents, like the ones developed by Salvatore *et al*. [27], although the compartmentalization of drugs in magnetoliposomes with different transition temperatures could also be explored as a means to achieve the same effect.

Recently, advances were undertaken in the optimization of membrane-embedded magnetoliposomes, surpassing previous limits of nanoparticle size that could be embedded. Further, the improved membrane stability and faster responsiveness of this system make it highly promising for future developments of on-demand drug release. Particularly, this design

can be of high relevance in drug delivery to sensitive tissues, such as the brain, considering that a low-frequency magnetic field can be used as an alternative to hyperthermia to efficiently trigger drug release, despite that post-therapy fate of the nanoparticles needs to be assessed in future works. Alternatively, the combined use of photothermia and magnetic hyperthermia through the production of AMLs and/or SMLs encapsulating core/shell magnetic/plasmonic nanoparticles might afford a suitable strategy for less harmful approaches, while attaining a higher therapeutic efficiency. In this regard, and considering the broadening of imaging modalities that can be used to follow in real-time the therapy, including MRI, computed tomography and magnetic particle imaging, as well as the potential use of novel imaging techniques, the plasmonic magnetoliposomes are envisioned as promising systems in future developments.

A great improvement was achieved through the combination of photosensitizers with magnetic nanocarriers for photodynamic therapy, enabling their targeted delivery and higher accumulation in tumour cells. Nevertheless, an efficient photodynamic effect is still a challenge. The use of near-infrared light promotes a higher penetration depth, but fails in providing high energy to excite PS agents. The use of upconversion nanoparticles, which can convert low energy NIR light into higher energy UV/visible light, shows improved ROS production efficiency and was already reported for tumour treatment [100]. The association of these nanoparticles with photosensitizers into nanocomplexes could be considered in upcoming magnetic nanocarriers, without neglecting the possible adverse effects in the physiological environment.

Gene therapy is now one of the most important therapeutic approaches for several diseases, so the development of efficient carriers for genetic material is of major concern. Non-viral vectors are safer regarding cytotoxicity and immunogenicity issues. As discussed above, magnetic liposomes show promising features as non-viral carriers for cancer gene therapy, as they can guide the genetic material to the tumour site and promote its controlled release through magnetic hyperthermia. Most studies using magnetic liposomes for this aim use magnetite as the magnetic component, which is limited in terms of the diversity of magnetic nanocarriers that can be produced. Other ferrites like manganese ferrite, calcium ferrite, or even anisotropic-shape iron-based magnetic nanoparticles, can provide a more efficient magnetic response which translates into an improved therapeutic effect. Overall, several advances have been undertaken towards the optimization of magnetoliposomes, providing unique and promising multimodal features to safe and efficient cancer therapies that can be combined in a single nanocarrier.

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