Solid and aqueous magnetoliposomes as nanocarriers for a new potential drug active against breast cancer

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Abstract

Iron oxide nanoparticles, with diameters around 12 nm, were synthesized by coprecipitation method. The magnetic properties indicate a superparamagnetic behavior with a coercive field of 9.7 Oe and a blocking temperature of 118 K. Both aqueous and solid magnetoliposomes containing magnetite nanoparticles have sizes below 150 nm, suitable for biomedical applications. Interaction between both types of magnetoliposomes and models of biological membranes was proven. A new antitumor compound, a diarylurea derivative of thienopyridine, active against breast cancer, was incorporated in both aqueous and solid magnetoliposomes, being mainly located in the lipid membrane. A promising application of these magnetoliposomes in oncology is anticipated, allowing a combined therapeutic approach, using both chemotherapy and magnetic hyperthermia.

1. Introduction

The potential of magnetic nanoparticles for biomedical applications has been recognized, as they offer major advantages due to their unique size and physicochemical properties [1]. Nanoparticles with superparamagnetic behavior are preferred for these purposes, as they exhibit a strong magnetization only when an external magnetic field is applied [2-4]. Liposomes entrapping magnetic nanoparticles (magnetoliposomes) can be guided and localized in the therapeutic site by external magnetic field gradients and used in cancer therapy by hyperthermia [5-8]. Moreover, liposomes revealed advantageous in overcoming issues of solubility, pharmacokinetics, toxicity and *in vivo* stability and toxicity of other delivery systems [9-11]. Magnetoliposomes have been used as a chemotherapy alternative through magnetically-controlled drug delivery and thermotherapy [12-15]. Iron oxide nanoparticles (Fe_3O_4 , $\gamma\text{-Fe}_2\text{O}_3$) have been widely used due to their proven biocompatibility and low toxicity [16-20]. Magnetoliposomes containing magnetite nanoparticles have been used in MRI [21], environmental applications [22], thermo/chemotherapy [23] and treatment of other diseases [24]. These nanosystems take advantage of the very large saturation magnetization reported for magnetite nanoparticles [16,17,22], higher than the one observed for nickel ferrite [25] and manganese ferrite nanoparticles [26].

Thienopyridines including their diarylurea derivatives have shown different biological activities, namely as antitumoral agents [27] and receptor tyrosine kinase inhibitors [28]. Recently, a strong VEGFR-2 inhibition activity, with very low IC₅₀ values (on the order of 10 nM) was reported for a series of new arylurea derivatives of thienopyridines [29]. Also, a potent antiproliferative activity on two human breast cancer cell lines of distinct types, a hormone-dependent MCF-7 and hormone independent MDA-MB-231 cell lines, was described for this type of compounds [30].

In this work, both aqueous and dry magnetoliposomes based on magnetite nanoparticles were prepared by soft templating methods and characterized. The interaction between the prepared magnetoliposomes and models of cell membranes (giant unilamellar vesicles, GUVs) was evaluated using FRET (Förster Resonance Energy Transfer).

A new diarylurea derivative of thienopyridine **1** (Figure 1), with potent activity against breast cancer cell lines [30], was incorporated in both types of magnetoliposomes. Compound **1** exhibits a strong antitumor activity in MCF-7 and MDA-MB-231 breast tumor cell lines (IC₅₀ of 1.2 μM and 5.0 μM, respectively [30]), the latter being the most aggressive and difficult to treat. Specifically, the therapy against the highly metastatic MDA-MB-231 cancer cells could strongly benefit from the synergistic effect of chemotherapy and magnetic hyperthermia. Therefore, these studies are of main importance for the development of a combined thermo/chemotherapy for breast cancer.

Figure 1. Structure of the arylurea derivative of thienopyridine.

2. Experimental

All the solutions were prepared using spectroscopic grade solvents and ultrapure water (Milli-Q grade).

2.1. Magnetite nanoparticles preparation

2.1.1. Coprecipitation method

Magnetite nanoparticles (NPs) were prepared by a coprecipitation method in aqueous solution, using fresh Fe²⁺ and Fe³⁺ solutions in a 1:2 stoichiometry [31]. A 0.15 M iron

(II) sulfate solution ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) and a 0.3 M iron (III) sulfate solution ($\text{Fe}_2(\text{SO}_4)_3 \cdot 9\text{H}_2\text{O}$) were used. 27 μL of each solution were added to 2 mL of water and 200 μL of sodium hydroxide (2.3 M) and the mixture was kept at 80 °C for 2 hours, in absence of oxygen. The magnetite nanoparticles formed through the reaction:

After the synthesis, the precipitated nanoparticles were washed several times with ultrapure water, recovered by magnetic decantation and redispersed in the initial volume of water.

2.1.2. Reverse coprecipitation method

In the reverse coprecipitation method, only one iron solution is used [32,33]. 121 μL of NaOH solution (50%, 18.9 M) were added to 10 mL of ultrapure water to adjust pH to ~ 13 . A fresh 0.2 M solution of iron (II) sulfate was prepared. 5 mL of the latter solution were added, drop by drop, to the NaOH solution, under magnetic stirring. On an alternative procedure, the 5 mL of Fe^{2+} solution were added, also drop by drop, in five portions of 1 mL each, each addition separated by 10 minutes. In both cases, the total concentration of iron was 66 mM. The reaction was kept for one hour at room temperature.

The precipitated nanoparticles were then washed several times with ultrapure water and recovered by magnetic decantation.

2.2. Preparation of magnetoliposomes

2.2.1. Aqueous magnetoliposomes

Aqueous magnetoliposomes (AMLs) are formed when the magnetic nanoparticles are encapsulated in liposomes. Egg yolk phosphatidylcholine (Egg-PC), from Sigma-Aldrich, was used for lipid vesicle formation. A 10 mM Egg-PC solution in ethanol was injected, under vigorous vortexing, to an aqueous solution of nanoparticles (ethanolic injection method [34,35]). After encapsulation, the ferrofluid was washed with water and purified by magnetic decantation and centrifugation to remove all the non-encapsulated NPs.

2.2.2. Solid magnetoliposomes

DPPC (1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine) and DOPG (1,2-Dioleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) sodium salt) from Avanti Polar Lipids, was used to produce a double lipid layer around the magnetite nanoparticles, following the procedure previously developed by us for nickel ferrite nanoparticles [25]. First, 760 μ L of the synthesized magnetite NPs (in 2.1.1) were centrifuged. Then, the deposited particles were redispersed in 10 μ L water in an ultrasonicator for one minute at 189 W, and 3 mL of chloroform were added to the aqueous dispersion of NPs. After vigorous agitation, 165 μ L of a 20 mM solution of DPPC (or DOPG) were added under vortexing, to form the first lipid layer of solid magnetoliposomes (SMLs). The particles sedimented using a small magnet and the supernatant was discarded. The particles were then washed twice by magnetic decantation with pure water, in order to remove the lipid that was not attached to the NPs. The second lipid layer was then formed by the injection of 165 μ L of lipid (20 mM), under vortexing, in a 3 mL aqueous dispersion of the particles with the first lipid layer. The resulting SMLs were then washed twice, purified with pure water by centrifugation and redispersed in 3 mL.

Compound **1** was incorporated into aqueous magnetoliposomes by the co-injection method, as already described [26]. In solid magnetoliposomes, compound **1** was incorporated by injection of an ethanolic solution together with the formation of the second lipid layer [26].

2.3. Preparation of Giant Unilamellar Vesicles (GUVs)

Soybean lecithin (*L*- α -Phosphatidylcholine), from Sigma-Aldrich, was used for GUVs preparation, using a procedure previously described [36,37]. A film of soybean lecithin was obtained by evaporation under an ultrapure nitrogen stream of a 1 mM lipid solution. This film was incubated with 20 μ L of water at 45 °C for 45 minutes. Then, 3 mL of 0.1 M glucose solution was added, and the resulting mixture was again incubated at 37 °C.

2.4. Spectroscopic measurements

2.4.1. General methods

Absorption spectra were recorded in a Shimadzu UV-3101PC UV-vis-NIR spectrophotometer. Fluorescence measurements were performed using a Fluorolog 3 spectrofluorimeter, equipped with double monochromators in both excitation and

emission and a temperature controlled cuvette holder. Fluorescence spectra were corrected for the instrumental response of the system.

2.4.2. FRET measurements

The interaction of magnetoliposomes with models of biological membranes (GUVs) was evaluated by Förster Resonance Energy Transfer (FRET). FRET efficiency, Φ_{RET} , defined as the proportion of donor molecules that have transferred their excess energy to acceptor molecules, was calculated through donor emission quenching, by taking the ratio of the donor integrated fluorescence intensities in the presence of acceptor and in the absence of acceptor [38]. The distance between donor and acceptor molecules was determined through the FRET efficiency (equation 1),

$$r = R_0 \cdot \left[\frac{1 - \Phi_{\text{RET}}}{\Phi_{\text{RET}}} \right]^{1/6} \quad (1)$$

where R_0 is the Förster radius (critical distance), that can be obtained by the spectral overlap, $J(\lambda)$, between the donor emission and the acceptor absorption, according to equations (2) and (3) (with R_0 in Å, λ in nm, $\epsilon_A(\lambda)$ in $\text{M}^{-1} \text{cm}^{-1}$), [38]

$$R_0 = 0.2108 [k^2 \Phi_D^0 n^{-4} J(\lambda)]^{1/6} \quad (2)$$

$$J(\lambda) = \int_0^\infty I_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda \quad (3)$$

where $k^2 = 2/3$ is the orientational factor assuming random orientation of the dyes, Φ_D^0 is the fluorescence quantum yield of the donor in the absence of energy transfer, n is the refraction index of the medium, $I_D(\lambda)$ is the fluorescence spectrum of the donor normalized so that $\int_0^\infty I_D(\lambda) d\lambda = 1$, and $\epsilon_A(\lambda)$ is the molar absorption coefficient of the acceptor.

FRET assays were employed to confirm the formation of the second lipid bilayer in the solid magnetoliposomes (SMLs). For that purpose, the rhodamine B labeled lipid Rhodamine-DHPE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (from Avanti Polar Lipids, structure in Figure S1 of the Supplementary Material) was included the first lipid layer, while the nitrobenzoxazole labeled lipid NBD-C₆-HPC (1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine) (from Avanti Polar Lipids, structure also in Figure S1) was included in the second lipid layer.

For the study of the interaction of magnetoliposomes with GUVs, the former were labeled with both NBD-C₆-HPC and the hydrophobic probe Nile Red (from Fluka, structure in Figure S1).

The fluorescence quantum yield, Φ_s , of the energy donor (in both cases, the dye NBD) in magnetoliposomes was determined by the standard method (equation 4) [39,40],

$$\Phi_s = \frac{A_r F_s n_s^2}{A_s F_r n_r^2} \Phi_r \quad (4)$$

where A is the absorbance at the excitation wavelength, F the integrated emission area and n the refraction index of the solvents used. Subscripts refer to the reference (r) or sample (s). The absorbance at the excitation wavelength was always lower than 0.1 to avoid the inner filter effects. The NBD-C₆-HPC molecule intercalated in lipid membranes was used as reference, $\Phi_r=0.32$ at 25 °C, as reported by Invitrogen [41].

2.4.3. Fluorescence anisotropy measurements

The steady-state fluorescence anisotropy, r , is calculated by

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (5)$$

where I_{VV} and I_{VH} are the intensities of the emission spectra obtained with vertical and horizontal polarization, respectively (for vertically polarized excitation light), and $G = I_{HV}/I_{HH}$ is the instrument correction factor, where I_{HV} and I_{HH} are the emission intensities obtained with vertical and horizontal polarization (for horizontally polarized excitation light).

2.6. Structural characterization

Scanning electron microscopy (SEM) images of magnetite nanoparticles and solid magnetoliposomes were recorded using a Scanning Electron Microscope FEI - Nova 200 NanoSEM. In the case of SMLs, a negative staining was employed. For that, a 2% aqueous solution of ammonium molybdate tetrahydrate was prepared. Then, 20 μ L of sample and 20 μ L of staining solution were mixed and a drop of this mixture was placed onto a Formvar grid, held by tweezers. After 20 seconds, almost all the solution was removed with filter paper and left dry.

The processing of SEM images was performed using ImageJ software. It consisted in enhancing local contrast followed by automatic local thresholding and particle analysis. The area of each particle allowed an estimation of its size. The resulting histograms were fitted to Gaussian distributions.

Liposomes mean diameter and size distribution (polydispersity index) were measured using a Dynamic Light Scattering (DLS) equipment (NANO ZS Malvern Zetasizer) at 25 °C, using a He-Ne laser of $\lambda=632.8$ nm and a detector angle of 173°. Five independent measurements were performed for each sample.

X-Ray Diffraction (XRD) analyses were performed using a conventional Philips PW 1710 diffractometer, operating with Cu K $_{\alpha}$ radiation, in a Bragg-Brentano configuration.

2.7. Magnetic measurements

Magnetic measurements were performed at room temperature in a Superconducting Quantum Interference Device (SQUID) magnetometer (Quantum Design MPMS5XL), using applied magnetic fields up to 5.5 T.

The temperature dependence of the magnetization was measured in the temperature range from 5 K to 380 K. The curves were obtained by initially cooling the sample under an applied magnetic field of $H = 100$ Oe (field cooled, FC) and then measuring its magnetization with increasing temperature (applied field of $H = 50$ Oe). Subsequently, after reaching 380 K, the sample was re-cooled, this time with no applied magnetic field (zero-field-cooled, ZFC) and the magnetization measurements were again performed with increasing temperature, under the same magnetic field of $H = 50$ Oe. From the behavior of the FC and ZFC curves, the blocking temperature (T_B) of the superparamagnetic nanoparticles can be obtained [42]. The magnetization hysteresis loop measurements were made by fixing the temperature and measuring the magnetization at a series of different applied magnetic fields. This type of study gives information about the maximum magnetization and the degree at which the sample remains magnetized when the applied field is removed, and how easily the sample magnetization can be reversed, the so-called coercive field.

2.8. Nanoparticles encapsulation efficiency in AMLs

The nanoparticles encapsulation efficiency in aqueous magnetoliposomes (AMLs) was estimated from the spectrophotometric determination of iron (III) content, through the

formation of a phenylfluorone complex sensitized with Triton X-100 [43]. To obtain iron (III) from the magnetoliposomes, the latter were digested by heating at 500° C overnight, to remove all the biological components. Then, 1 mL of concentrated nitric acid (from Sigma-Aldrich) was added and the sample was heated to 80° C for 2 hours. The temperature was then raised to 150° C for 48 h, and the pH was increased until 5.5 by successive cycles of addition and evaporation of ultrapure water (Milli-Q grade). Finally, 1 mL of water was added to the digested sample and, after ultrasonication for one hour, the iron (III) content of magnetoliposomes was released.

For the spectrophotometric measurements, the standard addition method was employed. 100 µL of the digested sample and 2×10^{-5} M, 3×10^{-5} M, 4×10^{-5} M, 5×10^{-5} M or 6×10^{-5} M of iron (III) stock solution were added to 1.6×10^{-4} M phenylfluorone and 4×10^{-3} M Triton X-100 solutions. The pH was then adjusted to 9 using 0.05 M borax buffer [43]. A calibration curve for the determination of iron (III) concentration was previously obtained. Three independent measurements were performed and standard deviations (SD) were calculated. The encapsulation efficiency, $EE(\%)$, of iron oxide nanoparticles (NPs) in AMLs was determined by equation:

$$EE(\%) = \frac{(total\ amount\ of\ NPs - amount\ of\ non-encapsulated\ NPs)}{total\ amount\ of\ NPs} \times 100 \quad (6)$$

3. Results and discussion

3.1. Characterization of magnetite nanoparticles

3.1.1. Scanning electron microscopy (SEM)

SEM images of the nanoparticles prepared by coprecipitation in aqueous solution (preparation method described in 2.1.1) revealed generally spherical nanoparticles uniform in size, with a size distribution of 11.6 ± 1.6 nm (Figure 2).

Figure 2. SEM image of magnetite nanoparticles prepared by coprecipitation method in aqueous solution. Inset: Particles size histogram and fitting to a Gaussian distribution.

Magnetite nanoparticles obtained by reverse coprecipitation method in aqueous solution (protocol 2.1.2.) exhibit larger sizes and a higher polydispersity (Figure 3), with some “fibrous” structures in the case of adding iron (II) in five times, with 10 minutes intervals (Figure 3B). For nanoparticles prepared adding all Fe^{2+} at once, a size distribution of 21.7 ± 3.8 nm was obtained, while a diameter of 20.9 ± 2.7 nm was determined for the nanoparticles obtained with addition of Fe^{2+} in five portions.

Figure 3. SEM images of magnetite nanoparticles prepared by reverse coprecipitation method in aqueous solution. **A:** Addition of all Fe^{2+} at once; **B:** Addition of Fe^{2+} in five portions, with 10 minutes intervals. Insets: Particles size histograms and fitting to Gaussian distributions.

According to these results, magnetite nanoparticles prepared by coprecipitation method in aqueous solution (Figure 2, corresponding to protocol 2.1.1) were chosen for further

characterization and for the preparation of aqueous and solid magnetoliposomes, considering their small size, low polydispersity and low degree of aggregation.

3.1.2. XRD measurements

The synthesis of magnetite NPs consisting of a pure crystalline phase was confirmed by XRD analysis (Figure 4), since all their characteristic peaks (CIF 9000926), marked by their indices, are observed. The absence of high temperature thermal treatment results in a significant amorphous background in the XRD pattern. Rietveld analysis using FullProf software [44] was performed by modeling the background as a linear interpolation between a set of points at constant scattering angles, but with fitted intensities. A good fit was possible by using preferred orientation correction at (1 1 0) plane, as it can be observed in the residuals (Figure 4B). This correction is based on the March function [45], equation 7,

$$\left(r^2 \cos^2 \alpha + \frac{\sin^2 \alpha}{r}\right)^{-3/2} \quad (7)$$

where, for a platy habit, α is the acute angle between the scattering vector and the normal to the crystallites. The obtained fitted value indicates a degree of preferred orientation of $(1 - r) = 0.41$. Preferred orientation of crystallites within a powder sample was also reported for the case of Cd doped ZnO [46] and Mn doped ZnO [47]. The obtained reduced χ^2 was 1.20, with an R_F value of 7.49%, and the estimated size of the Fe_3O_4 crystallites based on the Scherrer formula was 5.6 nm. The value of R_F improves to 5.67% if the overall isothermal factor, B_{over} , is optimized, but an unreasonable value of -2.95 is obtained. A summary of the results of Rietveld analysis is shown in Table S1 of the Supplementary Material.

Figure 4. Rietveld analysis of XRD patterns of the sample, with (A) and without (B) preferred orientation correction at (1 1 0) plane.

3.1.3. Magnetic Properties

The magnetic properties of the nanoparticles synthesized by coprecipitation method (Figure 2) were characterized by measuring their corresponding magnetic hysteresis loop, which shows the relationship between the induced magnetic moment and the applied magnetic field (H). Figure 5 shows the hysteresis cycle measured on the prepared magnetite nanoparticles. A magnetic squareness value (ratio between the residual magnetization, M_r , and the saturation magnetization, M_s) of 0.023 was obtained, indicating that the synthesized nanoparticles are superparamagnetic at room temperature [42,48]. Typical magnetic properties were observed, with a very slight hysteresis, a saturation magnetization of 62 emu/g (Figure 5A) and a coercive field of 9.7 Oe (Figure 5B). Reported values for saturation magnetization of Fe_3O_4 nanoparticles typically vary from 50 to 80 emu/g, depending on their size [49-56].

Figure 5. A: Magnetization hysteresis cycle of magnetite nanoparticles at $T=300$ K. B: Enlargement of the hysteresis loop of Figure 5A, in the low field region.

Previous reports found negligible coercivity values for magnetite NPs of 8 nm size, while for nanoparticles of 12 nm a coercivity of 18 Oe was observed [49,50]. It was also shown that Fe_3O_4 nanoparticles with 16 nm and a coercive field of 27 Oe were able to produce heat with high efficiency in hyperthermia applications [49]. The low coercive field and the very reasonable saturation magnetization of the synthesized Fe_3O_4 nanoparticles point to a promising utility for hyperthermia applications. In fact, the inductive heating capability of magnetic nanoparticles, under AC magnetic field, is directly proportional to the area of the hysteresis cycle [55] and thus decreases as the coercive field is reduced, but increases as the saturation magnetization gets higher. A low coercive field in superparamagnetic particles is important to avoid extensive permanent agglomeration. Its negative effect on inductive heating capability can however be compensated by the occurrence of small clusters of magnetic nanoparticles, that originate an additional and significant heating capacity, enough to reach therapeutic temperatures [57].

The superparamagnetic particles are characterized by distinct behaviors of the temperature dependence of zero-field-cooling and field-cooling magnetization curves. Starting from the low temperatures on the ZFC curve, as the temperature increases the blocked magnetic moments align with the applied measuring magnetic field, leading to an initial increase of the sample magnetization. However, as soon as thermal fluctuations are able to allow the moments to overcome the magnetic anisotropy energy barrier, the thermal randomization of the intraparticles magnetic moments produces a subsequent decrease of the magnetization curve, with increasing temperature. The zero-field-cooling curve peak corresponds to the blocking temperature, T_B [42], and here a value of $T_B=118$ K was obtained for Fe_3O_4 NPs (Figure S2 in Supplementary

Material). On the FC curve, the magnetic moments were initially forced to be aligned with the magnetic field imposed during cooling. This then gives a significant overall magnetization at low temperatures, as seen on the FC curve (Figure S2), which monotonically decreases with increasing temperature. Above the blocking temperature, NPs present superparamagnetic behavior and the ZFC and FC curves show the same temperature dependence of the magnetization, which can be approached by Curie law. At lower temperature, the ZFC and FC curves separate each other indicating that some nanoparticles are thermally blocked and are not able to completely align with the applied field. Here, the nanoparticles show ferromagnetic behavior, as thermal fluctuations are not enough to randomize the intraparticles magnetic moments. For an ensemble of superparamagnetic particles above the blocking temperature, the magnetization can be described by the Langevin function [42]

$$M(H, T) = N\mu L\left(\frac{\mu H}{k_B T}\right) = N\mu\left(\coth\left(\frac{\mu H}{k_B T}\right) - \frac{k_B T}{\mu H}\right) \quad (8)$$

where μ is the particle magnetic moment, k_B is the Boltzmann constant, T is the absolute temperature, N is the number of particles per volume and H is the applied magnetic field.

The magnetization hysteresis cycle was fitted to equation (8), assuming a spherical shape and using the density of Fe_3O_4 NPs, 5.18 g/cm^3 [56]. Diameters around 5.4 nm were estimated, the high R^2 value ($R^2=0.9934$) indicating a good fit to the experimental data. This particle diameter is in excellent agreement with the one obtained from XRD analysis (section 3.1.2., Table S1).

3.2. Characterization of magnetoliposomes and interaction with model membranes

Two types of magnetoliposomes were prepared, aqueous magnetoliposomes (AMLs) and solid magnetoliposomes (SMLs) and the interaction with GUVs (giant unilamellar vesicles), used as models of cell membranes, was investigated.

3.2.1. Aqueous magnetoliposomes

Aqueous magnetoliposomes were obtained by ethanolic injection of the lipids in the aqueous ferrofluid. This method is very advantageous for hydrophobic drugs (with very limited solubility in water, as is the case of compound **1**), which can be loaded in

(magneto)liposomes by coinjection. The NPs encapsulation efficiency in these liposomes was calculated from the spectrophotometric determination of iron (III), following the procedure described in [43]. The encapsulation efficiency, obtained from three independent assays, was $EE(\%) \pm SD(\%) = 46.(9) \pm 15$. This result compares well with the ones reported for the extrusion method by Sabaté *et al.* ($EE=19\%$ in the worst case) [58] and Ferreira *et al.* ($EE=66\%$ in their best formulation) [59] and proves the suitability of the ethanolic injection method when hydrophobic drugs are considered to be loaded in AMLs. It is also probable that the encapsulation efficiency is dependent on the initial amount of ferrofluid used, as reported [58,59].

The size distribution of aqueous magnetoliposomes (AMLs) of egg phosphatidylcholine containing magnetite nanoparticles was measured by DLS. A hydrodynamic diameter of 112 ± 19 nm was determined (Figure S3 in Supplementary Material). This size is slightly larger than those obtained for AMLs of the same lipid containing nickel ferrite [25] or manganese ferrite [26] nanoparticles.

The possibility of membrane fusion between aqueous magnetoliposomes (AMLs) and giant unilamellar vesicles (GUVs), used as models of cell membranes, was evaluated by FRET. The labeled lipid NBD- C_6 -HPC and the lipid probe Nile Red were both included in AMLs, NBD acting as the energy donor and Nile Red as the energy acceptor [60]. The solvatochromic and hydrophobic dye Nile Red [61-65] exhibits a red shift in emission maximum in polar solvents. Moreover, owing to its capability to establish H-bonds with protic solvents, Nile Red fluorescence in water is very weak, exhibiting a maximum wavelength around 660 nm [65].

When both fluorophores are incorporated in magnetoliposomes, an efficient energy transfer process is observed, exciting only the donor NBD (Figure S4 in Supplementary Material). Two fluorescence bands are observed, the first ($\lambda_{\max}=535$ nm) corresponding to NBD emission and the second to Nile Red fluorescence ($\lambda_{\max}=630$ nm), arising from the energy transfer of excited NBD molecules to Nile Red. If the magnetoliposomes interact with GUVs, a larger membrane will be formed by fusion. Consequently, an increase in the donor-acceptor distance will occur with a corresponding decrease in the energy transfer efficiency from NBD to Nile Red. This is observed in Figure S4, through the increase in donor emission and the decrease of acceptor fluorescence, proving membrane fusion between both systems. The assay was performed for AMLs of Egg-PC (Figure S4-A), which is in the liquid-crystalline phase at room temperature and AMLs of DPPC (Figure S4-B), which is in the gel phase at 25 °C. Aqueous

magnetoliposomes of DPPC/cholesterol were also recently obtained by Ferreira *et al.* [59]. Our results in Figure S4 confirm that fusion with GUVs occurs for both AMLs of Egg-PC and DPPC. This result is similar to those previously observed with aqueous magnetoliposomes of Egg-PC containing different types of magnetic nanoparticles [25,26,60].

3.2.2. Solid magnetoliposomes

The SMLs were synthesized by the procedure previously developed by us using nickel ferrite and manganese ferrite nanoparticles [25,26]. SMLs containing iron oxide nanoparticles and with lipid bilayers of unsaturated lipids were previously obtained by Meledandri *et al.* [21], using the lipids DOPG and DOPC (18:1 PC), and by Zhang *et al.* [22], using soybean phosphatidylcholine. In this work, clusters of magnetic nanoparticles were covered with the phospholipid dipalmitoyl phosphatidylcholine (DPPC or 16:0 PC), which possesses two saturated hydrocarbon chains and a melting transition temperature of 41 °C [66]. For comparison, SMLs containing a lipid bilayer of the unsaturated lipid dioleoyl phosphatidylglycerol (DOPG or 18:1 PG) were also prepared.

The formation of a DPPC bilayer around the nanoparticles was confirmed by FRET between NBD (donor) and Rhodamine (acceptor), using a methodology reported in previous works [25,26]. Briefly, the labeled lipid Rhodamine-DHPE was included in the first (inner) lipid layer and the labeled lipid NBD-C₆-HPC was included in the second (outer) lipid layer. A Φ_D^0 value of 0.11 for NBD-C₆-HPC in magnetite SMLs was determined. An energy transfer efficiency of 75% was obtained, corresponding to a donor-acceptor distance of 3.43 nm (equations 1-3). This result evidences the formation of the double lipid layer in DPPC SMLs, considering the usual thickness of a biological membrane (7 to 9 nm [67]).

Dynamic Light Scattering measurements revealed that the DOPG and DPPC SMLs are approximately monodisperse (Figures S5 and S6 in Supplementary Material). Hydrodynamic diameters of 136±32 nm and of 127±28 nm were obtained, respectively, for DOPG and DPPC SMLs, revealing that these nanosystems are suitable for drug delivery applications, due to their size below 150 nm and low polydispersity.

The structure of solid magnetoliposomes does not present an inner water pool and, therefore, solid magnetoliposomes can be observed by SEM microscopy. SEM image

(Figure 6) of DPPC SMLs containing Fe_3O_4 nanoparticles shows spherical structures with *ca.* 123 nm diameter, in accordance with DLS results. These SMLs are structurally similar to the ones first reported by De Cuyper and Joniau [68], but the preparation method is much simpler and expedite, as there is neither the need for an initial step of coating of the magnetic nanoparticles with lauric acid, nor the dialysis procedure, that can take up to two days.

Figure 6. SEM image of solid magnetoliposomes of the lipid DPPC containing magnetite nanoparticles.

3.3. Incorporation of the antitumor compound **1** in magnetoliposomes

The thienopyridine derivative **1** (Figure 1) presents very low growth inhibitory concentration values in MCF-7 breast adenocarcinoma cell line and in the aggressive MDA-MB-231 breast tumor cell line [30]. For this reason, compound **1** was incorporated in both AMLs and SMLs containing magnetite nanoparticles and the incorporation was monitored by fluorescence emission, taking advantage of the intrinsic fluorescence of this potential drug. Figure 7 shows the emission spectra of **1** in AMLs, SMLs and liposomes (without magnetic nanoparticles and with the same concentration of compound). It is possible to observe a quenching effect of the fluorescence emission by the magnetic nanoparticles, much more pronounced in SMLs, indicating the incorporation of the thienopyridine derivative in both AMLs and SMLs, as previously observed for another thienopyridine derivative in magnetoliposomes containing manganese ferrite NPs [26].

Fluorescence anisotropy (steady-state) values for compound **1** are presented on Table 1. For comparison, the anisotropy of the compound was measured in the highly viscous solvent glycerol ($\eta=0.9934$ Pa.s at room temperature [69]), with much larger viscosity than the one usually reported for lipid membranes (between 0.1 and 0.2 Pa.s [70,71]).

The measured steady-state anisotropies clearly indicate that this antitumor compound is mainly located in the lipid membrane of liposomes. These values are significantly lower than the one measured in glycerol, mainly due to the distinct viscosities of both types of media (despite possible differences in excited-state lifetimes).

Table 1. Steady-state fluorescence anisotropy (r) values for antitumor compound **1** in liposomes, aqueous magnetoliposomes (AMLs) and solid magnetoliposomes (SMLs).

	Lipid	Temperature	r
Liposomes	Egg-PC	25 °C	0.182
	DPPC	25 °C	0.251
		55 °C	0.157
	DOPG	25 °C	0.061
AMLs	Egg-PC	25 °C	0.185
	DPPC	25 °C	0.258
		55 °C	0.147
	DOPG	25 °C	0.056
SMLs	DPPC	25 °C	0.169
		55 °C	0.112
	DOPG	25 °C	0.043
Glycerol	---	25 °C	0.308

Figure 7. Fluorescence spectra ($\lambda_{exc}=290$ nm) of compound **1** (3×10^{-6} M) in liposomes and magnetoliposomes (AMLs and SMLs) of DPPC containing magnetite nanoparticles, and in SMLs after interaction with GUVs.

Comparing the anisotropy values at 25 °C and 55 °C in DPPC-based systems, it must be noted that an increase of the steady-state anisotropy is predicted from a diminution of the excited-state lifetime, according to Perrin equation [38]. Upon a rise of temperature,

the excited-state lifetime is expected to decrease, due to the increment of the non-radiative deactivation pathways, mainly the rate constant for internal conversion $S_1 \rightarrow S_0$. As the anisotropy values decrease strongly at higher temperature (Table 1), this behavior can be attributed to a decrease of the rotational correlation time of the fluorescent compound. This is a result of the diminution of membrane microviscosity upon changing from the gel to the liquid-crystalline phase of the lipid DPPC ($T_m = 41\text{ }^\circ\text{C}$ [66]). The lipid DOPG is highly fluid at room temperature [72], justifying very low anisotropy values of the compound in liposomes and magnetoliposomes of this lipid. Egg lecithin is a natural mixture of phosphatidylcholines (mainly 16:0 PC, 18:0 PC and 18:1 PC [73]), this composition justifying lower anisotropy values than those of DPPC at room temperature, due to the presence of the unsaturated lipid 18:1 PC.

Comparing liposomes and magnetoliposomes, it can be observed that anisotropy values for this antitumor compound in AMLs and SMLs are, in general, similar to those determined in liposomes of the same lipids. In DPPC SMLs, the anisotropy values are lower than in DPPC liposomes or AMLs. This result points to a deeper location of the compound in SMLs membrane, as it was reported that microviscosity decreases from the interface to the interior of the membrane [74,75]. This justifies the strong quenching of compound fluorescence in SMLs. Together, these results confirm that this potential antitumor drug is fully incorporated in both types of magnetoliposomes, located mainly in the lipid bilayer and experiencing changes in fluidity between the rigid gel phase ($25\text{ }^\circ\text{C}$) and the liquid-crystalline phase ($55\text{ }^\circ\text{C}$) in the case of DPPC.

Interaction of SMLs entrapping compound **1** with model membranes was also investigated. Comparing compound emission before and after interaction with GUVs (Figure 7), an unquenching effect is detected upon interaction, confirming membrane fusion of SMLs with GUVs. Therefore, the magnetoliposomes here prepared are promising as carriers for this antitumor compound active against breast cancer. Based on the experimental results, we anticipate that both aqueous and solid drug-loaded magnetoliposomes have potential as therapeutic agents in future applications of chemo/thermotherapy of cancer.

4. Conclusions

In this work, magnetite nanoparticles with size around 12 nm were synthesized by coprecipitation method. Fe_3O_4 NPs exhibit superparamagnetic properties, with

maximum magnetization of 62 emu/g at 5 T applied field, a coercivity of 9.7 Oe and a blocking temperature of 118 K. Both aqueous and solid magnetoliposomes containing magnetite nanoparticles, with sizes below 150 nm and low polydispersity, were prepared and their interaction with model membranes by fusion was demonstrated.

A new antitumor compound active against breast cancer was successfully incorporated in both AMLs and SMLs, which is a promising result for the application of these nanocarriers in dual oncological therapy for breast cancer, using both hyperthermia and chemotherapy.

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