

## Preparation of Magnetoliposomes with a Green, Low-Cost, Fast and Scalable Methodology and Activity Study against *S. aureus* and *C. freundii* Bacterial Strains

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A novel, fast, low-cost and scalable methodology to prepare stable magnetoliposomes (MGLs), without the use of organic solvents, is described. The concept of the work is based on the dual use of soy lecithin associated to a new liposome preparation methodology. Soy lecithin was used to coat the nanoparticles of magnetite ( $\text{Fe}_3\text{O}_4$ @lecithin) and for encapsulation of  $\text{Fe}_3\text{O}_4$ @lecithin (Lip- $\text{Fe}_3\text{O}_4$ @lecithin). Liposomes with size less than 160 nm, polydispersity index of 0.25 and zeta potential of  $-41$  mV, were prepared with the use of autoclave and sonication. The liposomal formulations containing magnetite and stigmasterol (Lip- $\text{Fe}_3\text{O}_4$ @lecithin, Lip-Stigma and Lip-Stigma- $\text{Fe}_3\text{O}_4$ @lecithin) were shown to be promising for the application as antibacterial. The liposomal formulation and magnetite were characterized by the following techniques: conventional and high-resolution transmission electron microscopy (TEM/HRTEM), energy-filtered transmission electron microscopy (EFTEM), proton nuclear magnetic resonance ( $^1\text{H}$  NMR), Fourier transform infrared spectroscopy (FTIR), X-ray powder diffraction (XRPD), dynamic light scattering (DLS) and zeta potential. The Lip- $\text{Fe}_3\text{O}_4$ @lecithin had a minimum inhibitory concentration (MIC) of  $8.4 \mu\text{g mL}^{-1}$  in the presence of 200 Oe magnetic field against *S. aureus*.

**Keywords:** soy lecithin, magnetite, liposomes, *S. aureus*, *C. freundii*

### Introduction

The disordered use of antibiotics has increased the inefficiency of several classes of antibacterial agents in combating microorganisms called multiresistant. Such microorganisms are present in hospitals of large metropolises or even diverse environments of small communities.<sup>1-5</sup> The Gram-positive bacterium *Staphylococcus aureus* is the leading cause of human bacterial infections worldwide, being endemic and readily resistant to antimicrobials. *S. aureus* is listed in the World Health Organization (WHO)<sup>6</sup> as one of the six pathogens

with priority II (high) in the development of new antibiotics. Regarding Gram-negative bacteria, the WHO warns of resistance to multiple antibiotics and the ability to transmit genetic material to other bacteria. An example of Gram-negative bacteria, *Citrobacter freundii* is a fecal coliform that resides in water, soils, and intestines of humans.<sup>7</sup> Inorganic pharmaceuticals containing metal oxides may utilize different action mechanisms and can, therefore, be considered a viable alternative to traditional antibiotics.<sup>8</sup> One option is the use of iron oxide magnetic nanoparticles (IOMNPs). IOMNPs have been extensively explored in several areas, such as nanocatalysis,<sup>9</sup> hyperthermia,<sup>10</sup> magnetic resonance imaging<sup>11</sup> and cancer treatment.<sup>12</sup> The properties of IOMNPs are suitable for a wide diversity of

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applications, such as high surface area and heat generation capacity by the application of an external magnetic field.<sup>9-13</sup> Several studies on the antibacterial activity of IOMNPs, in particular the magnetite nanoparticles (NPs) are described in the literature.<sup>14-17</sup> Limitations due to agglomeration and interactions with biological media have been overcome by coating the NPs with several classes of stabilizing agents.<sup>18-21</sup> Another strategy to increase the interaction of NPs with biological media is encapsulation in liposomes, preparing so-called magnetoliposomes (MGLs). The encapsulation in liposomal vesicles permits reduced contact between drugs and healthy cells; as well as increased efficiency in traversing the diseased cell membrane to reach the target.<sup>18,22</sup>

In this paper, soy lecithin, a combination of phospholipids from renewable sources, was used to coat NPs ( $\text{Fe}_3\text{O}_4$ @lecithin) and then as liposome for encapsulating the  $\text{Fe}_3\text{O}_4$ @lecithin. Two previous studies<sup>23,24</sup> using soy lecithin as the coat of NPs have been described. However, none of the studies used lecithin as a liposome. The addition of the second step for encapsulation of  $\text{Fe}_3\text{O}_4$ @lecithin was determinant to obtain more stable MGLs than  $\text{Fe}_3\text{O}_4$ @lecithin in the aqueous medium. Another factor that distinguishes our paper from the previous publications is the order of addition of reagents. In our work, soy lecithin was added after the co-precipitation, reducing the possibility of encapsulation residuals of initial reagents in the soy lecithin clusters. Therefore, dual use of the soy lecithin biomimetic agent<sup>25</sup> increased the interaction between  $\text{Fe}_3\text{O}_4$  and liposome vesicles in the Lip- $\text{Fe}_3\text{O}_4$ @lecithin, creating new possibilities for the preparation of MGLs. Another enhancement was enabling the MGLs to be highly water-dispersive using a novel methodology that is fast, low-cost and scalable without the use of organic solvents, which were not previously described in the literature. Traditional methodologies such as film hydration<sup>26</sup> and sonication<sup>27</sup> require a lot of preparing time and the use of organic solvents to achieve small quantities of product.<sup>28</sup> Subsequently, they are still subjected to a sterilization step.<sup>29</sup> Therefore, a new methodology was proposed to prepare sterile liposomes. This methodology involves hydrating the soy lecithin in an autoclave at 120 °C for 15 min, cooling to 4 °C, liposome size reduction, encapsulating stigmaterol and/or  $\text{Fe}_3\text{O}_4$ @lecithin and using ultrasound tip. The use of an initial autoclave stage allows preparation of large amounts of the liposomes. The production is only limited by to the capacity of the autoclave equipment that can reach industrial scales. In addition, the influence of use of stigmaterol (Stigma), a molecule similar to cholesterol, was also investigated.<sup>28,29</sup> The liposomes prepared were tested against *S. aureus* and *C. freundii* microorganisms in

the presence of a magnetic field (MF41) of 200 Oe, 315 kHz and also in its absence at room temperature (WMF25) and at temperature of 41 °C (WMF41).

## Experimental

### Materials

Stigmaterol (Sigma-Aldrich Chemical Company, USA), soy lecithin (CRQ, Brazil), iron sulfate II ammoniacal hexahydrate (Vetec, Brazil), iron chloride III hexahydrate (Vetec, Brazil),  $\text{CDCl}_3$  (Sigma-Aldrich Chemical Company, USA), ammonium hydroxide PA (Synth, Brazil), sodium hydroxide PA (Synth, Brazil), citric acid (Synth, Brazil), brain heart infusion (BHI) agar (Kasvi, Brazil), BHI broth (Kasvi, Brazil) were used without any purification. The Gram-positive and Gram-negative bacteria used were *Staphylococcus aureus* (ATCC 29212), and *Citrobacter freundii* (ATCC 8090), respectively. The ultra-purified water was obtained by Milli-Q system (Millipore, Brazil).

### Preparation of the bare liposome (Lip)

Bare liposomes were prepared by hydration of soy lecithin (100 mg  $\text{mL}^{-1}$ ) in ultra-purified water. The autoclavable flask containing the components were autoclaved (CS Prismatec Autoclave, Brazil) for 15 min at 120 °C. Bare liposomes were maintained at 4 °C for 24 h. Then, the bare liposomes were sonicated on tip ultrasound (Disruptor Unique Model DES 500, Brazil) for reducing sizes.<sup>26,30</sup> Standards of 4 cycles  $\times$  4 min were performed with intervals of 1 min for each sequence, at 95% power (500 W). The temperature of sample was maintained below 75 °C. The sample was noted Lip.

### Synthesis of $\text{Fe}_3\text{O}_4$ @lecithin

Magnetite coated with soy lecithin ( $\text{Fe}_3\text{O}_4$ @lecithin) was prepared in a one-pot reaction using the modified co-precipitation methodology of the literature.<sup>31,32</sup> Briefly, in a two-neck round bottom flask, under nitrogen flow, 500 mL of ultra-purified and degassed water were mixed to  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  (0.0459 mol), and  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (0.08879 mol). The solution was stirred for 5 min at room temperature. After that, 200 mL of  $\text{NH}_4\text{OH}$  was added under stirring for synthesis of the nanoparticles magnetite ( $\text{Fe}_3\text{O}_4$ ) and stirred for additional 20 min. Sequentially, 100 mL of Lip (100 mg  $\text{mL}^{-1}$ ) was added under constant stirring for 10 min in the bottom flask. The  $\text{Fe}_3\text{O}_4$  coated by soy lecithin was washed with ultra-purified degassed

water.<sup>31,33</sup> The sample was denominated  $\text{Fe}_3\text{O}_4@$ lecithin and was conditioned in Falcon tubes, lyophilized and stored at 0 °C. The yield of this operation was 92%.

#### Preparation of liposome formulations

Aliquots of 10 mL of the Lip were separated for stigmaterol and/or magnetite components incorporation. The liposomal preparations were named according to their composition: stigmaterol (Lip-Stigma), stigmaterol and coated magnetite (Lip-Stigma- $\text{Fe}_3\text{O}_4@$ lecithin), and the coated magnetite (Lip- $\text{Fe}_3\text{O}_4@$ lecithin). A bare liposomal formulation containing the phospholipid (Lip) was also prepared as standard sample. Lip, Lip-Stigma, Lip-Stigma- $\text{Fe}_3\text{O}_4@$ lecithin, Lip- $\text{Fe}_3\text{O}_4@$ lecithin were submitted to the same ultrasound cycle defined previously. Then, the formulations were ultracentrifuged<sup>34</sup> for 20 min at 10000 × g in Eppendorf model 5810R centrifuge (Germany). The supernatant was separated and the formulation resuspended in ultra-purified water. The procedure was performed until all unencapsulated components of Lip, Lip-Stigma, Lip-Stigma- $\text{Fe}_3\text{O}_4@$ lecithin and Lip- $\text{Fe}_3\text{O}_4@$ lecithin were removed from the formulations. The formulations were suspended in phosphate buffered saline (PBS buffer) at pH 7.3 ± 0.1, leading to the final concentrations (Table 1) determined by atomic absorption spectroscopy (AAS) and proton nuclear magnetic resonance (<sup>1</sup>H NMR).

**Table 1.** Content of soy lecithin, stigmaterol and magnetite

Sample	Lecithin / (mg mL <sup>-1</sup> )	Stigmaterol / (mg mL <sup>-1</sup> )	$\text{Fe}_3\text{O}_4$ / (mg mL <sup>-1</sup> )
Lip	92	–	–
Lip- $\text{Fe}_3\text{O}_4@$ lecithin	92	–	7.0
Lip-Stigma	92	39	–
Lip-Stigma- $\text{Fe}_3\text{O}_4@$ lecithin	92	39	9.7

#### Characterization of $\text{Fe}_3\text{O}_4@$ lecithin and liposomes

Zeta potential ( $\zeta$ ) was performed for  $\text{Fe}_3\text{O}_4@$ lecithin, Lip, Lip-Stigma, Lip- $\text{Fe}_3\text{O}_4@$ lecithin and Lip-Stigma- $\text{Fe}_3\text{O}_4@$ lecithin (Zetasizer Nano ZS equipment, United Kingdom) in triplicates at 25.0 °C for 14 days. The same apparatus was used for analyses of liposome size using dynamic light scattering (DLS). The samples were lyophilized in a Terroni Lyophilizer model LS 3000 (Brazil) for <sup>1</sup>H NMR, infrared spectroscopy technique (FTIR), X-ray powder diffraction (XRPD) and for magnetics measurements. The initial and final mass of all lyophilized samples were measured on analytical balance (Shimadzu, model AUW220D, Brazil). Crystallographic

phases of  $\text{Fe}_3\text{O}_4@$ lecithin, Lip- $\text{Fe}_3\text{O}_4@$ lecithin, Lip-Stigma- $\text{Fe}_3\text{O}_4@$ lecithin were characterized by XRPD, using Rigaku-Geigerflex equipment (Japan) and Siemens-D5000 diffractometer (Germany), with  $\text{CuK}\alpha$  radiation in the range of 5 to 80° and 1° min<sup>-1</sup>. The iron oxide phase was determined by fitting (311) peak by Voigt function.<sup>35,36</sup> FTIR (PerkinElmer FT-IR GX model, USA) were performed to evaluate the coating of  $\text{Fe}_3\text{O}_4@$ lecithin, iron oxide phase and to verify the absence of hydrolysis of the esters or oxidation in Lip, Lip-Stigma, Lip- $\text{Fe}_3\text{O}_4@$ lecithin and Lip-Stigma- $\text{Fe}_3\text{O}_4@$ lecithin. The solids were homogenized in KBr (1% m/m) and disc-pressed. The FTIR spectra were recorded in transmittance mode in the 4000 to 400 cm<sup>-1</sup> range. Morphologies, particle size distribution, and phases by micrographs were recorded on transmission electron microscope Tecnai G2-20 SuperTwin FEI, 200 kV (USA). The samples were diluted in water and one drop was deposited on holey carbon-Cu, 300 mesh, 50 micron screens.<sup>37</sup> The micrographs were obtained in the conventional and high-resolution transmission electron microscopy (TEM/HRTEM) and energy-filtered transmission electron microscopy (EFTEM) modes. The mean diameter of  $\text{Fe}_3\text{O}_4$  in the  $\text{Fe}_3\text{O}_4@$ lecithin was calculated by the ImageJ software package. The HRTEM images were processed in the Digital Micrograph program in the fast Fourier transform mode (FFT) for determination of the interplanar spacing.<sup>38</sup> Spectroscopic analyses of <sup>1</sup>H NMR were recorded on DPR 400 Bruker Avance II DRX 400 equipment (USA) operating at 400 MHz. This technique was used to determine phospholipids/steroids molar ratio of soy lecithin, stigmaterol in the Lip and Lip-Stigma and to verify the absence of phospholipid hydrolysis. The samples Lip and Lip-Stigma were lyophilized and solubilized in  $\text{CDCl}_3$ . The hysteresis curves of  $\text{Fe}_3\text{O}_4@$ lecithin and Lip- $\text{Fe}_3\text{O}_4@$ lecithin at 310 K were obtained on a SQUID (superconducting quantum interference device) Magnetometer Cryogenic S700X (Argentina) with magnetic field capacity up to 7 T. FAAS was used to determine iron content in  $\text{Fe}_3\text{O}_4@$ lecithin, Lip- $\text{Fe}_3\text{O}_4@$ lecithin and Lip-Stigma- $\text{Fe}_3\text{O}_4@$ lecithin on a Hitachi-Z8200 spectrometer (Japan) coupled to a graphite furnace.

Biological activity without magnetic field at room temperature (WMF25), without magnetic field at 41 ± 1 °C (WMF41) and with magnetic field at 41 ± 1 °C (MF41)

Antibacterial activity of the liposomes was assayed by the agar dilution method.<sup>39</sup> Bacteria *S. aureus* (ATCC 29213) and *C. freundii* (ATCC 8090) were individually inoculated in vials containing BHI broth and incubated in an oven at 37 °C

for 24 h. Inoculum containing approximately  $1 \times 10^6$  colony forming units (CFU) of each microorganism *per* mL of sterile water were prepared in sterile vials (InocSol). Liposomes samples described in Table 1 and InocSol were added to Eppendorfs containing BHI broth. The final concentrations of liposomes were presented in the Table 2 with total volume of the 1 mL *per* Eppendorf.

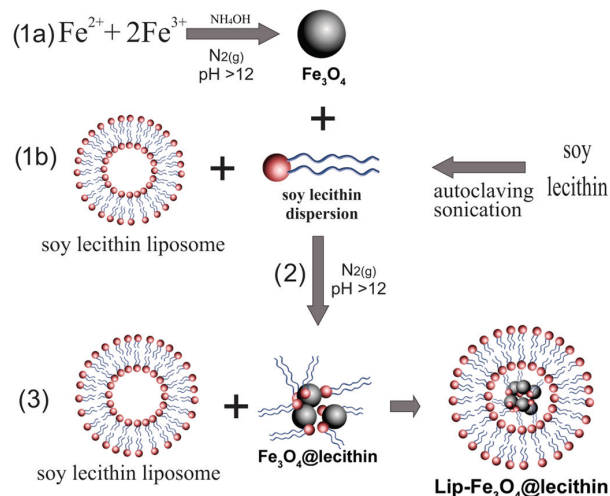
**Table 2.** Content of lecithin, stigmaterol and magnetite

Sample	Lecithin / (mg mL <sup>-1</sup> )	Stigmaterol / (mg mL <sup>-1</sup> )	Fe <sub>3</sub> O <sub>4</sub> / (mg mL <sup>-1</sup> )
Lip-Fe <sub>3</sub> O <sub>4</sub> @lecithin1	0.80	–	0.059
Lip-Fe <sub>3</sub> O <sub>4</sub> @lecithin2	0.11	–	0.0084
Lip-Stigma1	0.80	0.32	–
Lip-Stigma2	0.11	0.045	–
Lip-Stigma-Fe <sub>3</sub> O <sub>4</sub> @lecithin1	0.80	0.32	0.081
Lip-Stigma-Fe <sub>3</sub> O <sub>4</sub> @lecithin2	0.11	0.045	0.012

Tests for *S. aureus* and *C. freundii* inhibition were done against the liposomes samples prepared under three different conditions: (i) 25 °C for 30 min (WMF25); (ii) oven heating at  $41 \pm 1$  °C for 30 min (WMF41); and (iii) magnetic field for 30 min at  $41 \pm 1$  °C (MF41). The sample with number 2 was tested against *S. aureus* and *C. freundii* in the condition: (iii) magnetic field for 30 min at  $41 \pm 1$  °C (MF41). The liposome samples were diluted in BHI agar plates and maintained at 37 °C for 24 h. Then the CFUs were quantified. The procedure was performed in triplicate. Quantification of inhibition of bacterial growth was done according to the literature.<sup>39,40</sup> Samples submitted to MF41 treatment were accompanied by a reference sample (no inoculum) of the same concentration and volume for temperature control. Samples (1 mL) were placed in the center of the three-turn coil with internal diameter of 3.2 cm and a magnetic field of 200 Oe, 300 A and frequency of 315 kHz (Easyheat Ambrell, models 0224, USA) was applied for 30 min. When the temperature reached about 41.8 °C, the equipment was switched off and restarted only when the temperature was below 40.5 °C. The total time of 30 min in the magnetic field did not consider the time required for the sample to be cooled so that the temperature did not reach values higher than 42 °C. The samples were kept inside the coil throughout the treatment. The temperature variation in the reference sample was measured through a fiber optic thermometer (New Star 5kW RF Power Supply, Ameritherm, Inc., USA) located in the center of the Eppendorf (reference sample). The heat rate measurements were recorded by a Photon Control software TPMeter 10FTC-DIN-GT-HT.

## Results and Discussion

The first step in preparing the MGLs involved the synthesis of magnetite through a modified co-precipitation method previously described<sup>31,32</sup> and then the modification of its surface in a one-pot reaction is shown in Figure 1 (steps 1a, 1b and 2) to prepare Fe<sub>3</sub>O<sub>4</sub>@lecithin.



**Figure 1.** Preparation of magnetoliposomes.

### Characterization of Fe<sub>3</sub>O<sub>4</sub>@lecithin

The TEM image (Figure S1a, Supplementary Information (SI) section) of the Fe<sub>3</sub>O<sub>4</sub>@lecithin shows the nanoclusters formation.<sup>9,23</sup> NPs of the Fe<sub>3</sub>O<sub>4</sub> in the Fe<sub>3</sub>O<sub>4</sub>@lecithin (Figure S1b, SI section) have a mean size of 13 nm. The magnetite phase of Fe<sub>3</sub>O<sub>4</sub>@lecithin (Figure 2) was determined by X-ray powder diffraction (XRPD) according to JCPDS card 19-629. The mean diameter of 15 nm of the crystallite was determined by the Debye-Scherrer equation.<sup>41,42</sup> The structure determined was of cubic face centered (fcc) Fd-3m without deformation. The calculated net parameter was  $a = 8.41$  Å and the density was  $5.18 \text{ g cm}^{-3}$ .<sup>9,10</sup>

The FTIR spectra for Fe<sub>3</sub>O<sub>4</sub>@lecithin (Figure 3) show the vibration frequency related to the Fe–O bond ( $561 \text{ cm}^{-1}$ ) of magnetite and the spectra confirm the surface functionalization by soy lecithin of Fe<sub>3</sub>O<sub>4</sub>@lecithin. Magnetite ( $88 \pm 1\%$  m/m) contents in Fe<sub>3</sub>O<sub>4</sub>@lecithin were determined by AAS.

The saturation magnetization ( $M_s$ ) of the Fe<sub>3</sub>O<sub>4</sub>@lecithin was  $62 \text{ emu g}^{-1}$  (Figure 4), magnetic moment of  $\mu = 1.86 \times 10^{-19} \text{ J T}^{-1}$  and magnetic susceptibility at high fields was  $0.96 \times 10^{-4} \text{ emu g}^{-1}$  at 310 K. The hysteresis curves were adjusted using the Fortran 90 program based on the Langevin model in the super paramagnetic region (equation S1, SI section).<sup>43</sup> The average magnetic core

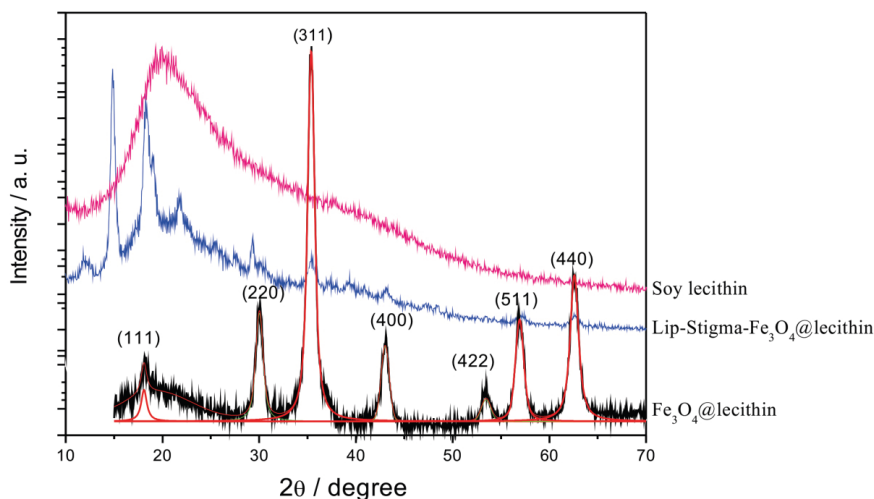


Figure 2. XRPD of soy lecithin,  $\text{Fe}_3\text{O}_4$ @lecithin and Lip-Stigma- $\text{Fe}_3\text{O}_4$ @lecithin.

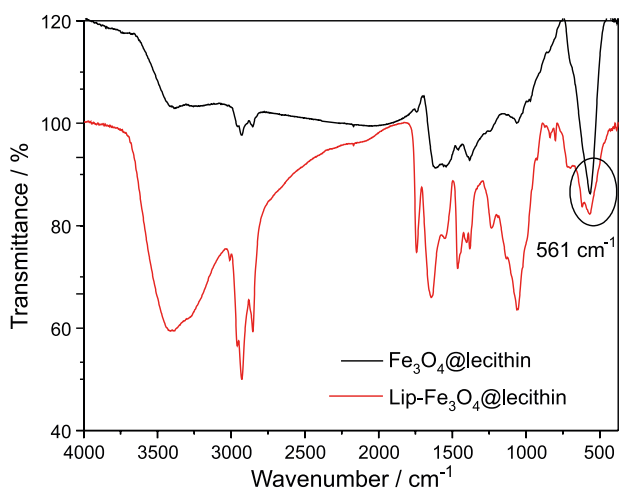


Figure 3. FTIR spectra of  $\text{Fe}_3\text{O}_4$ @lecithin and Lip- $\text{Fe}_3\text{O}_4$ @lecithin.

diameter calculated using the Fortran 90 software program was  $12.7 \pm 0.2$  nm confirming the values determined by TEM and XRPD. This value is consistent with nanoparticle super paramagnetic behavior.<sup>40</sup> The zeta potential ( $\zeta$ ) values found for  $\text{Fe}_3\text{O}_4$ @lecithin were  $-19 \pm 1$  mV. The zeta potential ( $\zeta$ ) confirms the stability of NPs with high water-dispersion in an aqueous medium.

The second step in MGLs preparation involves the encapsulation of the stigmaterol (Lip-Stigma), of  $\text{Fe}_3\text{O}_4$ @lecithin (Lip- $\text{Fe}_3\text{O}_4$ @lecithin) and encapsulation of the stigmaterol and  $\text{Fe}_3\text{O}_4$ @lecithin (Lip-Stigma- $\text{Fe}_3\text{O}_4$ @lecithin) as shown in Figure 1 (step 3). The samples were compared to the bare liposome (Lip).

#### Characterization of liposomes

The liposome size, polydispersity index (PI), zeta potential ( $\zeta$ ), pH and the FTIR of the liposomes (Table 1)

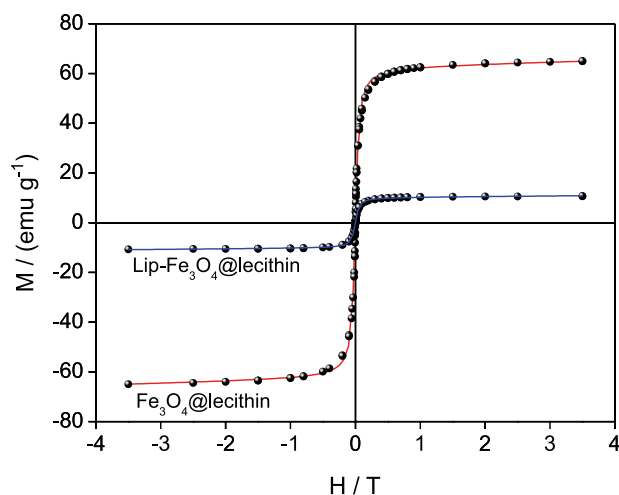


Figure 4. Hysteresis curve of  $\text{Fe}_3\text{O}_4$ @lecithin and Lip- $\text{Fe}_3\text{O}_4$ @lecithin at 310 K.

were evaluated for 14 days (Table 3). Liposomes exhibited temporal stability in aqueous medium and PI compatible with pharmaceutical applications. The zeta potential of the liposomes shows high values without the tendency toward agglomeration corroborating with the observed stability.<sup>44,45</sup> The pH of the liposome samples remained stable at  $\text{pH } 7.3 \pm 0.1$ .

Figures 5a and 5b show the overlap of lecithin spectra with Lip-Stigma (Figure 5a) and lecithin with stigmaterol (Figure 5b). The overlap of the FTIR spectrum of lecithin and Lip-Stigma (Figure 5a) shows characteristic bands of soy lecithin components:  $1741 \text{ cm}^{-1}$  (ester carbonyl);  $1261$  and  $1044 \text{ cm}^{-1}$  (P-O-R bonds);  $1630 \text{ cm}^{-1}$  ( $-\text{C}=\text{C}-$ , linoleic acid). It was possible to observe that there were no changes in the spectra pattern due to autoclaving and ultrasound of the sample in the Lip-Stigma. The absence of new bands in the  $1600\text{-}1800 \text{ cm}^{-1}$  in relation to soy lecithin corroborates

**Table 3.** Physico-chemical parameters of the liposomal formulations for 14 days, stored at 4 °C

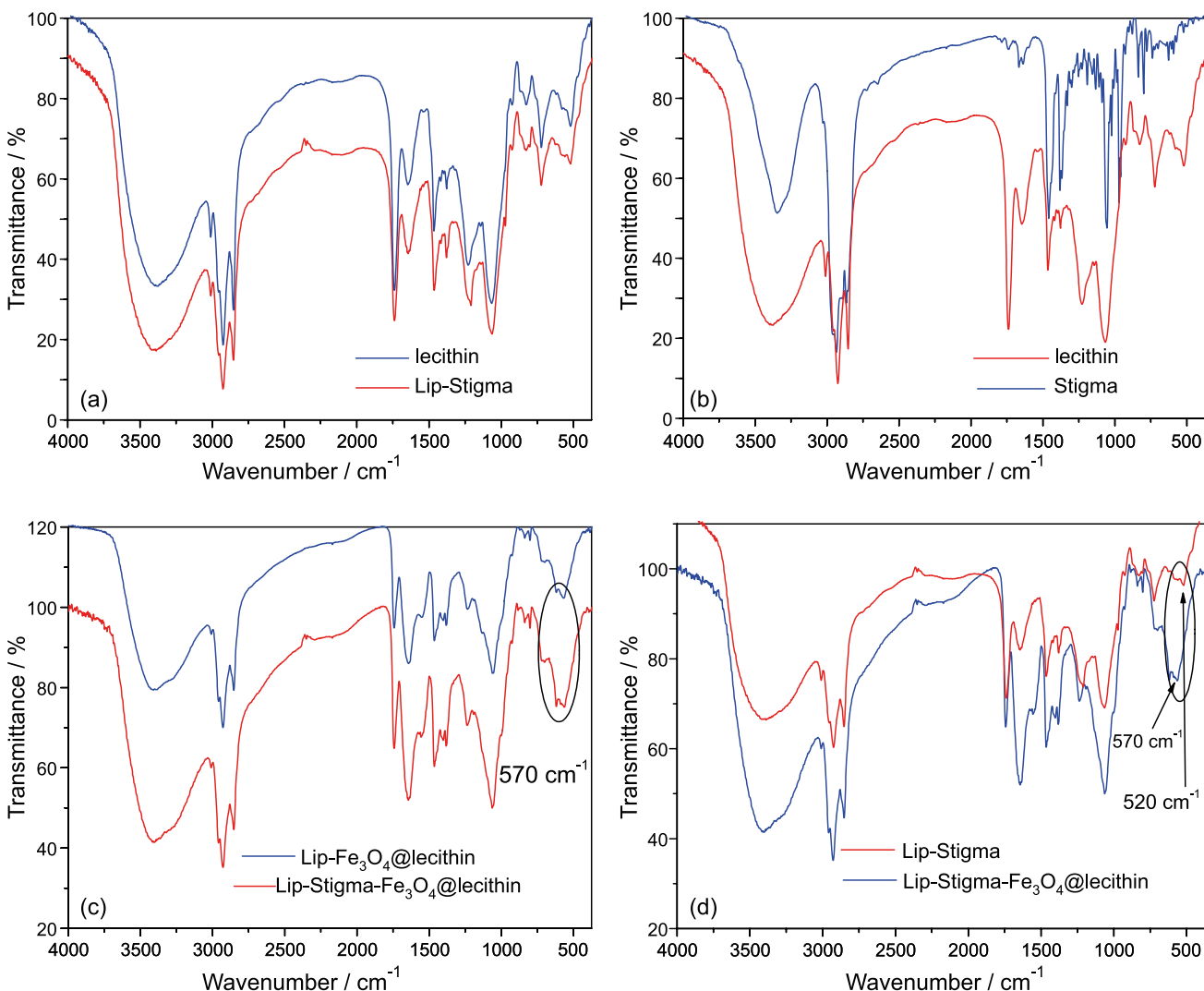
Liposomal formulation	Size / nm	PI <sup>a</sup>	$\zeta^b$ / mV	Weight loss <sup>c</sup> / %
Lip	189 ± 4	0.325	-41 ± 1	-
Lip-Stigma	160 ± 4	0.250	-49 ± 1	-
Lip-Fe <sub>3</sub> O <sub>4</sub> @lecithin	142 ± 4	0.198	-53 ± 1	5
Lip-Stigma-Fe <sub>3</sub> O <sub>4</sub> @lecithin	146 ± 4	0.230	-69 ± 3	5

<sup>a</sup>Polydispersity index deviations less than 3% in the period; <sup>b</sup>zeta potential; <sup>c</sup>loss of mass corresponding to Fe<sub>3</sub>O<sub>4</sub> for 14 days.

with the observation of absence of free fatty acids, due to the process of the autoclaving and sonication in the Lip and Lip-Stigma.<sup>46-48</sup> Comparison of the soy lecithin sample with the stigmaterol pattern (Figure 5b) shows an overlap of the stigmaterol bands with the soy lecithin bands. This

can be explained by the presence of steroids in the initial lecithin sample hindering the detection of stigmaterol. The band at 570 cm<sup>-1</sup> (Figures 5c and 5d) confirms the presence of the Fe–O bond of the magnetite phase showing that the NPs coating coverage was efficient in maintaining the magnetite as the only phase.<sup>49-53</sup> The comparison of Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin and Fe<sub>3</sub>O<sub>4</sub>@lecithin spectra (Figure 3) reveals the intensification of the bands pertaining to lecithin due to the encapsulation of Fe<sub>3</sub>O<sub>4</sub>@lecithin.

The TEM image and EFTEM maps<sup>54</sup> of C coated (of soy lecithin) (Figures 6a and 6b) of Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin show a dense population of NPs surrounded by phospholipids. The carbon of phospholipids on the surface of NPs were differentiated by energies lines. HRTEM images of Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin (Figure 7) confirm a population of NPs surrounded by phospholipids. The surface coating of Fe<sub>3</sub>O<sub>4</sub> NPs with lecithin in the Fe<sub>3</sub>O<sub>4</sub>@lecithin sample favored

**Figure 5.** FTIR spectra of (a) Lip-Stigma and soy lecithin; (b) stigmaterol (Stigma) and soy lecithin; (c) Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin, Lip-Stigma-Fe<sub>3</sub>O<sub>4</sub>@lecithin; and (d) Lip-Stigma and Lip-Stigma-Fe<sub>3</sub>O<sub>4</sub>@lecithin after 14 days at 4 °C.

the increase of the interactions of NPs with liposomes. It was observed that the coating facilitated the entry of NPs into the vesicles and reduced the ultrasound time duration. The reduced ultrasound time duration minimized the risks of oxidation of NPs and hydrolysis of phospholipids esters.

HRTEM images of the Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin was used for the determination of the interplanar spacing and mean size of Fe<sub>3</sub>O<sub>4</sub>. The values of interplanar spacing found were consistent with magnetite (JCPDS 19-0629) described in Table 4. The values found are in agreement with the XRPD technique (Figure 2). However, in the XRPD spectra of Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin and Lip-Stigma-Fe<sub>3</sub>O<sub>4</sub>@lecithin, one can observe lower intensity of magnetite peaks due to the presence of soybean lecithin (amorphous) and it is also possible to observe peaks attributed to stigmasterol (JCPDS 10-638). The HRTEM technique enabled nanoscale analysis and the reduction of interferences observed in the XRPD spectrum due to the increase in the amount of soy lecithin and stigmasterol. Therefore, the results found by the

two techniques enabled the confirmation of the maintenance of the magnetite phase in the liposomal samples.

Hysteresis curve of the Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin (Figure 4) shows super paramagnetic behavior with values of saturation magnetization of  $M_s = 10.3 \text{ emu g}^{-1}$ , magnetic moment of  $\mu = 1.87 \times 10^{-19} \text{ JT}^{-1}$  and linear susceptibility of  $\chi = 0.2 \times 10^{-4} \text{ emu g}^{-1}$  at 310 K. The coercivity and remanent magnetization values were the same as for Fe<sub>3</sub>O<sub>4</sub>@lecithin with values close to zero, corroborating the literature.<sup>53</sup> The liposome size, zeta potential ( $\zeta$ ), pH and characterization by FTIR, HRTEM, EFTEM/electron energy loss spectroscopy (EELS) and magnetics measurements show promising stability for suitable pharmaceutical.<sup>55</sup>

The use of soy lecithin as the encapsulating agent offers advantages of commercial availability, low-cost, high encapsulation capacity, high zeta potential and physicochemical stability. The usual components described in the literature are phospholipids (PL) and steroids (S).<sup>56,57</sup> The <sup>1</sup>H NMR was used for the determination of molar ratio

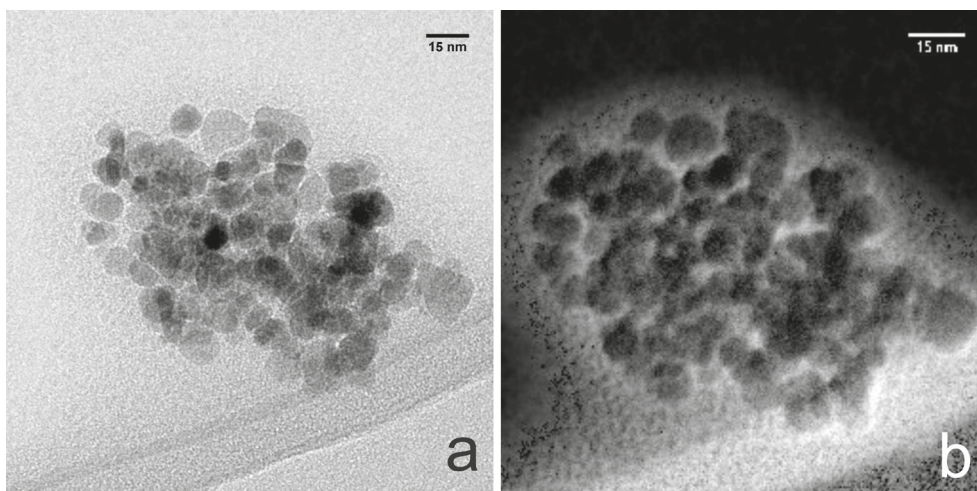


Figure 6. TEM image (a) and EFTEM maps (b) of Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin.

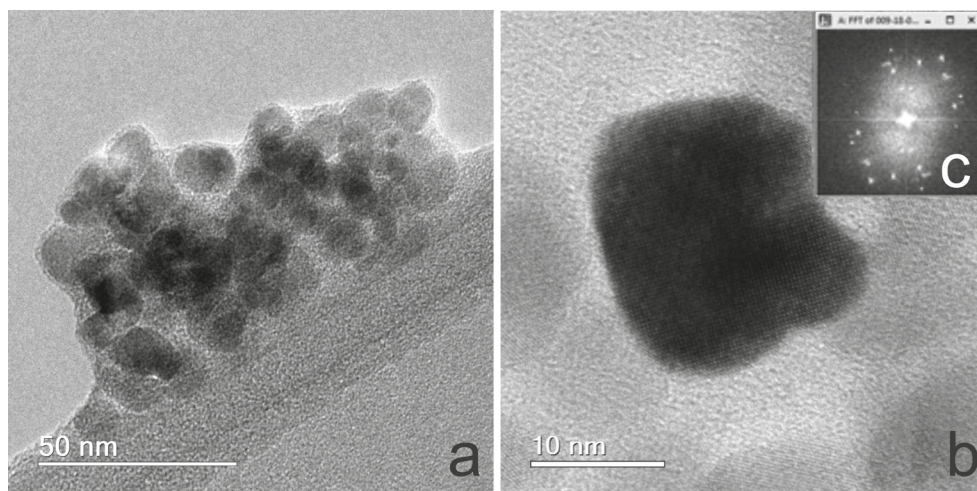


Figure 7. HRTEM images (a, b) and calculated FFT (c) of Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin.

**Table 4.** Interplanar spacing of Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin by fast Fourier transform (FFT)

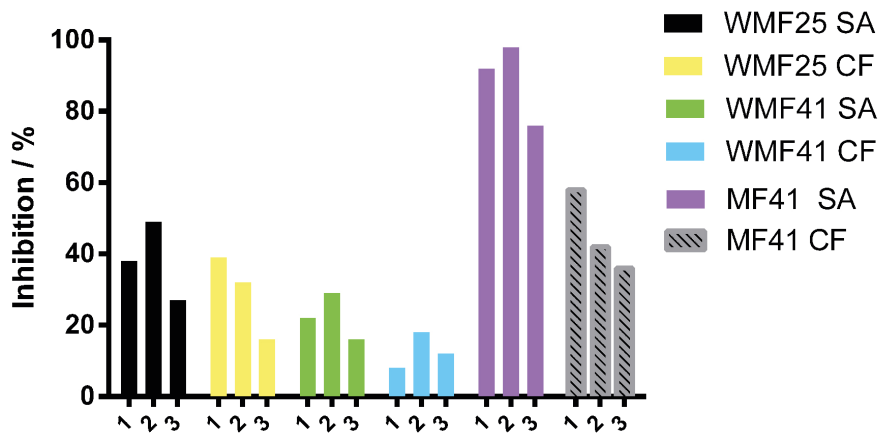
Plane	d / nm <sup>-1</sup>	1/d / nm	Theoretical / nm	Deviation / %
533	7.841	0.1275	0.1281	0.4
220	4.164	0.2402	0.2420	0.7
622	7.944	0.1259	0.1266	0.6
440	6.787	0.1473	0.1485	0.8
440	6.729	0.1486	0.1485	0.1
800	9.528	0.1050	0.1050	0

d: interplanar spacing.

of the phospholipids and steroids in the Lip and Lip-Stigma samples (Figure S2, SI section). The signals observed in the soy lecithin sample prior to autoclaving/sonication and Lip coincide with the composition expected in the literature.<sup>57</sup> The molar ratio of phospholipids and steroids found in the Lip and Lip-Stigma samples were 15:1 and 13:10 (PL:S), respectively. The technique also shows absence of new signals or significant difference was not observed between the soy lecithin and Lip spectra signals after 14 days. The absence of new signals indicates the absence of free fatty acids or oxidation due to the liposome formation. <sup>1</sup>H NMR and FTIR corroborate to show the efficiency of the methodologies used to prepare and to store the liposomes.

The inhibition of microbial growth at 24 h was shown in Figure 8. The strains *S. aureus* and *C. freundii* were sensitive to the liposomal formulations studied under all conditions. The WMF41 condition was established to evaluate the effect of temperature without the presence of a magnetic field in the antibacterial activity. Comparing the three conditions: WMF25, WMF41 and MF41, it can be seen that the elevation of temperature without the presence of the magnetic field increased bacterial growth. This occurrence was also observed in the study of *Pseudomonas* biofilms by

Park *et al.*<sup>58</sup> Kim *et al.*<sup>59</sup> also observed increased antibacterial activity due to the presence of the magnetic field in the study with *S. aureus*. The most efficient condition for inhibiting growth of the microorganisms was MF41, achieving inhibitions of up to 98% of the *S. aureus* colonies for the Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin1. The specific absorption rate (SAR) calculated for samples Lip-Stigma-Fe<sub>3</sub>O<sub>4</sub>@lecithin1 and Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin1 were 1056 and 978 W g<sup>-1</sup> of iron, respectively.<sup>60</sup> The presence of stigmasterol increased the encapsulation capacity of NPs of the liposomes and the response of the NPs to the magnetic field (Figure S3, SI section). All samples presented excellent temperature responses in the presence of magnetic field (Figure S3, SI section).<sup>53,59,61</sup> The limitation of temperature to 41 ± 1 °C was used based upon literature references to avoid damage to healthy human cells.<sup>62</sup> The evaluation of Lip-Stigma1 in the MF41 condition shows that the stigma was active as antibacterial with inhibition of 92% of colonies. Traditional liposomes are accompanied by cholesterol as a steroid responsible for the control of drug release.<sup>28,29</sup> We decided to use stigmasterol because it is an abundant vegetable steroid which are proven to have biological activities.<sup>63,64</sup> These excellent responses in the presence of magnetic field prompted us to try dilution of the liposomes for determination of minimum inhibitory concentration (MIC) in the MF41 condition. The results were: Lip-Stigma2 demonstrated inhibition of 55% of *C. freundii* and 50% of *S. aureus* representing the most promising response for inhibition of *C. freundii*. The new dilutions resulted in the MIC of Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin2 against *S. aureus* (8.4 µg mL<sup>-1</sup> of magnetite) and inhibition of 49% of *C. freundii* colonies in the MF41 condition. This work was the first to evaluate the activity of NPs against *C. freundii*. The evaluation of the literature on Fe<sub>3</sub>O<sub>4</sub> activity in relation to *S. aureus* showed a variety of methodologies



**Figure 8.** Antibacterial activity of liposomes: (1) Lip-Stigma; (2) Lip-Fe<sub>3</sub>O<sub>4</sub>@lecithin; (3) Lip-Stigma-Fe<sub>3</sub>O<sub>4</sub>@lecithin under conditions: without magnetic field at room temperature (WMF25); without magnetic field and 30 min at 41 ± 1 °C (WMF 41) and with magnetic field at 41 ± 1 °C (MF41) against *S. aureus* (SA) and *C. freundii* (CF).



and results.<sup>14,16,19,20,65,66</sup> In our work, the broth micro-dilution method using UV<sup>67</sup> was initially tested, however, the results were not reproducible. Alternatively, the use of the agar dilution method<sup>39</sup> proved to be more reliable and reproducible. A prior evaluation of the literature data shows that the nature of the coating was determinant in the activity's result. In the literature consulted, the best result described without a magnetic field was 47 µg mL<sup>-1</sup>.<sup>16</sup> When compared to our work, we observed that the encapsulating of Fe<sub>3</sub>O<sub>4</sub>@lecithin into liposomes and the application of a magnetic field reduced the dosage required for MIC by approximately 6-fold.

## Conclusions

In summary, this work resulted in an enhanced methodology for preparation of magnetoliposomes in terms of time, scalability and cost. Contrary to the liposomes conventional preparation, which uses toxic organic solvents, the methodology applied in this work does not use organic solvents. Additionally, the new process led to low risk of microbial contamination due to sterilization effect resulting from the autoclave step, inherent to the methodology. Liposomes prepared with soy lecithin show stability of physicochemical properties (liposome size, temporal stability, polydispersity index, zeta potential) and excellent magnetic response *in vitro* (SAR of 1056 W g<sup>-1</sup>) allowing a wide range of biomedical applications for treatments of multi-resistant microorganisms in the presence of magnetic field.

## Supplementary Information

Supplementary data are available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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