Development and Characterization of Magnetoliposomes for Drug Delivery Applications

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Abstract

The objective of the present work was to synthesize and characterize magnetoliposomes, in which a stable aqueous ferrofluid with magnetite nanoparticles covered with a surfactant, is incorporated into liposomes. Two different reagents were tested as surfactants, tetramethylammonium hydroxide and phosphatidylcholine. The incorporation of these particles and synthesis of what are called magnetoliposomes was made successfully, with distribution of the magnetite associated with the lipidic membrane of the synthesized oligolamellar structures. The lipid used for the formation of the liposomes was soybean phosphatidylcholine and the concentration of ferrofluid was varied, between 1.25 g of magnetite per mole of lipid, 25 g/mol and 75 g/mol. Cholesterol was also added to the lipid, to evaluate its influence in the retention of ferrofluid and more than one method for the synthesis of liposomes was tested (Classical Film Method plus extrusion and Dried Rehydrated Vesicles). After synthesis, all the samples were characterized through Transmission Electron Microscopy, Dynamic Light Scattering, Fourier Transform Infrared Spectroscopy and SQUID magnetometry. The results were satisfactory in all techniques, with TEM and DLS defining the structure of the magnetoliposomes very clearly and with the distribution of the ferrofluid very discernible in the TEM images. All the samples showed significant magnetization evaluated by SQUID magnetometry, a result particularly important for the magnetoliposomes samples. In FTIR, some interesting conclusions were drawn, namely the increase of membrane ordering and decrease of permeability with cholesterol and with ferrofluid.

Keywords

Nanoparticles, Magnetoliposomes, TEM, DLS, FTIR, SQUID.
Resumo

O objectivo do presente trabalho consiste na síntese de magnetolipossomas, onde um ferrofluido aquoso estável de nanopartículas de magnetite cobertas com um surfactante, é incorporado em lipossomas. Foram usados dois reagentes diferentes como surfactante, hidróxido de tetrametilamônio e fosfatidicolina. A incorporação dessas partículas e síntese do que são chamados magnetolipossomas foi feita com sucesso, com distribuição da magnetite associada à membrana lipídica das estruturas oligolamelares sintetizadas. O lípido usado para formação dos lipossomas foi fosfatidicolina de soja e a concentração de ferrofluido foi variada entre 1.25 g de magnetite por mole de lípido, 25 g/mol e 75 g/mol. Colesterol também foi adicionado ao lípido, para avaliar a sua influência na retenção de ferrofluido e foi testado um segundo método para a síntese de lipossomas. Após a síntese, as amostras foram caracterizadas por microscopia electrónica de transmissão, dynamic light scattering, espectroscopia de infravermelho e magnetometria com SQUIDs. Os resultados foram satisfatórios em todas as técnicas, com a MET e o DLS a definirem a estrutura dos magnetolipossomas muito claramente e com a distribuição do ferrofluido muito visível nas imagens obtidas. Todas as amostras apresentaram magnetização significativa, avaliada por magnetometria com SQUIDs, um resultado particularmente importante nos magnetolipossomas. Em FTIR, algumas conclusões interessantes foram retiradas, nomeadamente a diminuição da desordem e da permeabilidade com o colesterol e com o ferrofluido.

Palavras Chave

Nanopartículas, Magnetolipossomas, TEM, DLS, FTIR, SQUID
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List of Abbreviations

\( \tau_0 \) : Attempt Time
\( k_B \) : Boltzmann Constant
Chol : Cholesterol
\( H_C \) : Coercive Field
DSPE : Diesteroylphosphatidylethanolamine
DOX : Doxorubicin
DRV : Dried Rehydrated Vesicles or Dried Reconstituted Vesicles
DLS : Dynamic Light Scattering
K : Effective Magnetic Anisotropy
EPC : Egg Phosphatidylcholine
EPR-effect : Enhanced Permeability and Retention effect
fcc : Face Centered Cubic
FTIR : Fourier Transform Infrared Spectroscopy
d(H) : Hydrodynamic Diameter
DPPC : L-alpha-dipalmitoyl phosphatidylcholine
LUV : Large Unilamellar Vesicles
MR : Magnetic Resonance
MRI : Magnetic Resonance Imaging
\( \chi \) : Magnetic Susceptibility
MUV : Multilamellar Vesicles
MVV : Multivesicular Vesicles
\( \tau_N \) : Néel Relaxation Time
OLV : Oligolamellar Vesicles
PC : Phosphatidylcholine
PTA : Phosphotungstic Acid
PEG : Polyethyleneglycol
PCA : Principal Component Analysis
RF : Radiofrequency
\( M_R \) : Remanent Magnetization
RES : Reticuloendothelial System
SEM : Scanning Electron Microscope
SEC : Size Exclusion Chromatography
SUV : Small Unilamellar Vesicles
SPC : Soy bean Phosphatidylcholine
SQUID : Superconducting Quantum Interference Device
SPION : Superparamagnetic Iron Oxide Nanoparticle
TEOS : Tetraethoxysilane
TMAOH : Tetramethylammonium Hydroxide
D : Translational Diffusion Coefficient
TEM : Transmission Electron Microscope
USPION : Ultrasmall Superparamagnetic Iron Oxide Nanoparticle
\( \eta \) : Viscosity

\(^1\)Note: This list is in alphabetical order
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1.1 Motivation and Objectives

As the population worldwide grows older, the concern over health issues and methods to increase life expectancy is more important everyday. Particularly, the development of new drugs and new forms of drug delivery has received increased attention during the past decades. It is a growing and fast developing field, and each year brings up new drug delivery systems, with improvement in triggering of drug release in a controlled and targeted way. Nowadays, the capability of measuring particle sizes in the nanometer range and the advent of analytical techniques has made the drug delivery systems research evolve from the micro to the nanosize scale, in order to reduce the dose and reactive nature of the molecule, thus improving its efficacy and reducing side effects.

An ideal drug delivery system is supposed to be both efficient and discreet, delivering the drug to a specific location, without being cleared off by the patient's immune system and without interfering with cells other than the target cells. A lot of the present research focuses on cancer chemotherapy, trying to find a vehicle for the strong chemicals administered to cancer patients that target only the cancer cells, thus diminishing the drug’s severe and harmful toxic effects on normal organs. Also, it is convenient that this system is biocompatible and preferably biodegradable.

Liposomes seem ideal as they have the potential of satisfying all these requisites. They consist of an aqueous core entrapped by one or more bilayers composed of natural or synthetic lipids, and natural lipids are biologically inert and weakly immunogenic, having low intrinsic toxicity. Their composition can be varied and they can encapsulate drugs with different lipophilicities, either inside the core or inside the membrane. Also, their versatility in terms of active targeting, such as coupling with antibodies and coating with ligands targeting proteins expressed on cancer cell membranes, for example, is a great asset, as is their susceptibility to biological triggers such as temperature. All this factors can be manipulated and studied and make this drug delivery system a grand focus of current research in this area.

Superparamagnetic nanoparticles, on the other hand, have also been extensively studied for numerous applications, including drug delivery. The objective of these particles is to provide a targetable drug delivery through an application of an external magnetic field or to be used as contrast enhancers in magnetic resonance imaging. Another application of these particles could be hyperthermia, local heating of a specific tissue to yield its local destruction without affecting other healthy cells.

In the present work, one aims to add the benefits of the two systems, synthesizing magnetoliposomes, that is to say, liposomes containing magnetic nanoparticles in their interior. The advantage of magnetoliposomes, either bacteria-derived magnetosomes or magnetite nanoparticles incorporated into liposomes, over the currently used dextran-coated iron oxide nanoparticles, is stressed by some authors [1]. They add the liposomes biocompatibility and versatility already
proved in vivo to the magnetic nanoparticle’s possible ability to help with a controlled drug release, by heating the tissue after the arrival of the system to the target area, and disrupting the liposome membrane thus releasing the drug or help enhancing contrast in imaging systems. Also, the high cellular uptake efficiency of magnetoliposomes, when compared to dextran-coated iron oxide nanoparticles, offers interesting potential for therapy and imaging.

The level of success of this synthesis in the present work had to be evaluated with the use of characterization methods, mainly Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS), Fourier Transform Infrared Spectroscopy (FTIR) and SQUID magnetic measurements. All these methods provided an assessment of the physical structure, size, constitution and magnetization of the synthesized vesicles, yielding valuable conclusions and clearing a pathway to follow with this technology. The applications of these particles are diverse and if a stable and specific formulation can be obtained, the possible uses for this system are too wide to enumerate. This work intends to be a useful step towards the controlled synthesis of these nanoparticles, exploring this recent and very promising area of research. It is an ideal theme to explore in a master thesis, as it provides a challenge with ambitious goals, which were met to the fullest given the time available and hopefully it can be a stepping stone for further research and development.

1.2 Fundamentals

1.2.1 Magnetic Materials

The use of magnetic nanoparticles, particularly for medical applications, began only around the mid 1970s [2]. These applications include magnetic drug delivery, magnetic fluid hyperthermia and contrast enhancement for diagnostic imaging procedures.

Materials can be classified according to their response to external magnetic solicitations. One very important material property that should be defined is the magnetic susceptibility (\( \chi \)), which is a dimensionless quantity that relates the magnetization to the externally applied magnetic field. In the case of linear and isotropic media, the magnetic susceptibility can be seen as the initial slope of the magnetic curve that presents the magnetization response (\( M \)) as a function of the applied magnetic field (\( H \)), as follows:

\[
\chi = \frac{M}{H}.
\]  

Magnetism in magnetic materials has its origin in the spin of the electrons and their orbital movement around their nucleus. Taking this into account, one can establish several types of magnetism, to be described as follows. Diamagnetism is common to all materials, resulting from the fact that, though they present no net magnetic moment, an external magnetic field disturbs the angular velocity of electrons orbiting around their nucleus, inducing a magnetic moment opposite to the applied field. This effect results in a very weak negative magnetic susceptibility, therefore
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has little practical utility [3].

Paramagnetism results from the alignment of the magnetic dipoles with the applied field and it naturally disappears when the field is removed. Paramagnetic materials present a small positive magnetic susceptibility in the presence of a magnetic field.

Ferromagnetic and ferrimagnetic properties appear when paramagnetic ions are incorporated into a crystal structure. Ferromagnetism results from the cooperative alignment of the magnetic moments from adjacent sites and antiferromagnetism results from the cancellation of magnetic moments by anti-parallel interactions between cations on adjacent sites. Unlike paramagnetism described previously, in these two effects the magnetic moment is maintained even when the external magnetic field is removed. This phenomenon is called hysteresis and is caused by the presence of magnetic domains or Weiss domains, which reflects the tendency of the magnetic dipoles moments to align inside small regions [4]. After the external field is switched off, a remanent magnetization can be observed ($M_R$) and in order to achieve once more a random domain orientation, an external magnetic field in the opposite direction must be applied, which is called the coercive field ($H_C$). An increase in temperature of ferromagnetic materials, causes a disordering in their magnetic dipoles so that from a certain temperature forwards (Curie temperature), the material becomes paramagnetic. In ferrimagnetism, different ions present magnetic moments with different values, so that when these moments align in an antiparallel fashion, there is a remaining net magnetic moment.

When ferro or ferrimagnetic materials are divided, the obtained nanoparticles can be small
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enough to show a single domain structure with a non-zero net magnetic moment [2]. This behavior is called superparamagnetism (figure 1.1). It happens because there is a critical volume below which it costs more energy to create a domain wall than to support the external magnetostatic energy of the single-domain state. Due to thermal activation or by interaction with an external magnetic field, the magnetic moment may overcome an energy barrier \( E = KV_p \), where \( K \) is the effective magnetic anisotropy constant and \( V_p \) is the volume of the particle core, and change its orientation within the particle. This phenomenon has a time constant called Néel relaxation time and for small particles this time is so small that the magnetization vector oscillates extremely fast, giving the perception that the particles exhibit no net magnetization. The Néel relaxation time is given by:

\[
\tau_N = \tau_0 \exp\left(\frac{KV_p}{k_BT}\right).
\]  

(1.2)

Where \( \tau_0 \) is a length of time, characteristic of the material, that has values between \( 10^{-9} \) s and \( 10^{-11} \) s, \( k_B \) is the Boltzmann constant and \( T \) is the temperature. When the particle magnetic moment reverses at times shorter than the experimental time scales, the system is in a superparamagnetic state. Otherwise, it is in the blocked state, and the temperature which separates these two regimes is called blocking temperature, \( T_B \), and it depends on the effective magnetic anisotropy constant, the size of the particles, the applied magnetic field and the experimental measuring time [4].

When subjected to an alternating magnetic field, particles overcome the mentioned energy barrier and dissipate the energy through Néel relaxation, generating heat. This phenomenon reaches its maximum when the field’s driving frequency and the Néel relaxation time match, although this is difficult to achieve in reality due to the broad distribution of particle sizes [7, 8]. Heating can also be due to the friction generated by the rotational Brownian motion within a carrier liquid.

1.2.2 Structure of Magnetite

Magnetite, \( Fe_3O_4 \), as part of the magnetic ferrites group, presents a spinel structure. This structure is based on a face centered cubic (fcc) close-packed oxygen sublattice, so that each unit cell presents eight fcc oxygen subcells. There are two types of interstitial sites, tetrahedral and octahedral. In a normal spinel, one-half of the octahedral sites are occupied by the trivalent cations (\( Fe^{3+} \)) and one-eight of the tetrahedral sites are occupied by the divalent cations (\( Fe^{2+} \)) [5, 6, 9]. Taking into account the fact that there are 32 octahedral sites, 64 tetrahedral sites and only 32 oxygen anions per unit cell, one can observe that only 16 octahedral sites and 8 tetrahedral sites are occupied, which leaves out a lot of space. In fact, a more common variation of this structure, the inverse spinel, is often found in nature, as the divalent cations are generally larger than the trivalent ones, they tend to occupy preferably the larger octahedral positions.
So, the occupancy in an inverse spinel, is established by one half of the trivalent cations in the one-eight-filled tetrahedral sites, and the divalent cations as well as the other half of the trivalent cations in the octahedral positions. In reality, the spinel structure is found somewhere between normal and inverse, in the sense that there is some degree of disorder by the exchange of the divalent and trivalent cations. This spinel structure can be seen in figure 1.2, namely with examples of an octahedral site and a tetrahedral site.

![Figure 1.2: Spinel structure (taken from [6]).](image)

Electron spins of trivalent cations in octahedral sites tend to align antiparallel to those in tetrahedral sites. The divalent cations, however align their spins parallel to the trivalent cations in adjacent octahedral sites. As described previously, this arrangement of spins that don’t cancel each other completely is referred to as ferrimagnetism. Obviously, as the nanoparticles used have a size below the critical diameter, they present a superparamagnetic behavior.

### 1.2.3 Ferrofluids

Ferrofluids are kinetically stable colloidal dispersions of nano-sized ferromagnetic particles in a carrier liquid [7][10]. These particles need to be of approximately 10 nm in diameter, and they possess a single magnetic domain, so that a long range magnetostatic attraction exists between them, leading to their agglomeration and subsequent sedimentation. In order to prevent this, an impeditive mechanism such as the addition of a surfactant needs to be developed. The synthesis of a ferrofluid is then divided into two parts, making the magnetic nanoparticles and their dispersion into a carrier liquid, creating a kinetically stable colloidal suspension. The surfactant adheres to the nano-sized particles and stabilizes them through a chemical steric effect. Aqueous, oil and liquid metal based ferrofluids have been developed with the appropriate choice of surfactant [11].
1.2.4 Principles of Colloid Science

Any particle that has at least one linear dimension between $10^{-9}$ m (nano) and $10^{-6}$ m (micro) is considered a colloid [12]. Colloidal systems can be defined as an intermediate class of materials lying between bulk and molecularly dispersed systems, where although one component is finely dispersed in a continuous external phase, the degree of subdivision does not approach that in simple molecular mixtures [13]. Colloidal materials are composed of at least two phases, one dispersed and one continuous phase, and present special properties that will be addressed very briefly in this section. The nature of these phases determines some of the properties of the colloid and determines their different names, such as aerosols (dispersion of fine liquid droplets or solid particles in a gas), emulsions (dispersion of liquid droplets in a liquid or a solid), foams (dispersion of gas bubbles in a liquid or a solid) and sols or colloidal dispersions (dispersion of solid particles in a liquid or solid) [14]. The present work focuses only on colloidal dispersions, in which the nano-sized particles (dispersed phase) are dispersed in an aqueous medium (continuous external phase).

The nano dimensions of the colloidal structures translate into a large surface-to-volume ratio, meaning that a significant number of molecules of the nanoparticles lie at the surface, in the interface between the nano-sized structures and the medium, thus having properties different from those in the bulk. This fact explains clearly the importance of surface chemistry in the study of these phenomena. One important concept is the kinetic stability of a colloidal dispersion. The system is considered stable if it remains well dispersed for long periods of time (this definition of long is subjective and related to the intended application). Kinetically unstable colloidal dispersions will tend to aggregate. If the attractive forces holding the aggregate together are very large compared with the typical forces used in stirring, mixing, milling or ultrasonic probes, the system is regarded as permanently aggregated. This irreversible aggregation is called coagulation [14]. Flocculation refers to a weaker and more reversible aggregation.

A colloidal dispersion represents a state of higher free energy than the bulk material [13]. The colloidal dispersion will tend to lower its free energy unless there's some substantial energy barrier to prevent this thus keeping the system in a metastable state, in which it will remain for a certain time. If this energy barrier disappears or becomes negligibly small, the colloidal particles start to sediment. In the case of colloidal dispersions the system will be carried over the energy barrier by the Brownian motion of the colloids, which results from the random bombardment of the surface of the colloids by molecules in the medium. However, the energy of the colloids undergoing Brownian motion equals $(3/2)k_B T$ per colloid, which isn’t much [13]. If one considers the small chances that a colloid has of undergoing collisions involving energies on the order of $10k_B T$, one can see that the probability of overcoming the barrier is quite small, and the dispersion will remain in a metastable state. This barrier doesn’t have a permanent value, it is a sensitive function of several factors including the composition of the medium, temperature and pressure. So, instability of a
colloidal dispersion is normally caused by the reduction of height of the barrier (figure 1.3), mainly by influence of media parameters, rather than a rise in the energy of the particles undergoing Brownian motion.

Figure 1.3: Two possible forms of total interaction free energy (iii) in function of the distance between two particles, H, resulting from a combination of attractive (i) and repulsive (ii) contributions. In a), P represents the primary maximum, a high energy barrier that results from strong repulsive interaction. In b), the reduction of these repulsive interactions or their range results in a decrease of the energy barrier and the system passes into the primary minimum, $M_1$ (adapted from [13]).

1.2.4.A Stability of a Colloidal Dispersion

To control the kinetic stability of a colloidal dispersion, one must know the factors that contribute to the overall free energy, depicted in figure 1.3, which are the attractive van der Waals interactions (a) i)) and the electrostatic repulsions (a) iii)).

Concerning the first ones, it is well known that attractive forces exist between non-polar molecules, called London forces, due to the motion of the electrons in all atoms, causing rapidly fluctuating dipoles. As large assembles of atoms, colloidal particles will suffer attraction due to the sum of the interactions of one atom in a colloid with each atom of a neighboring colloid, assuming pairwise additivity. This results in interactions within a range comparable to the colloidal particles radii. This attractive potential energy is directly proportional to a particle radius, a material constant called the Hamaker constant, and is inversely proportional to the distance of separation of two colloidal particles [14]. This constant is a function of the density of the material and the electronic polarisability and presents values in the range of $10^{-20}$. 
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To take a look at the electrostatic repulsion, one must first address the concept of the electrical double layer. Colloids in most colloidal dispersions in aqueous media carry a surface electric charge, and this charge plays a role in stabilizing colloidal dispersions. The surface charge of colloids can be induced through various mechanisms, including bond cleavage at the nanocrystal (or amorphous) surface, ionization of surface groups and isomorphous substitution (replacement of one atom by another of similar size in a crystal lattice). In an electrolyte, the solvated ions surround the colloids, shield their surface charge and polar molecules orientate in the electric field. This is called the electrical double layer, illustrated in figure 1.4.

![Figure 1.4: Schematic representation of the electrical double layer structure at the solid/electrolyte interface. Adapted from [14].](image)

The distribution of ions in this double layer is dependent upon the concentration of the electrolyte, the formal charge of the electrolyte ions, the solvent and the potential at the boundary between the compact inner layer of ions and the diffuse outer layer of ions. The extent to which this ionic atmosphere characteristic of the electrical double layer extends is commonly referred to as the double layer thickness, and can be assessed by measuring the zeta potential, which is the potential at the external double layer boundary, also called shear plane. The charge on the colloid is balanced by the charge of the double layer, with an excess of counter-ions. When two colloids approach each other, their layers may overlap and the resultant repulsive force may stabilize the colloidal dispersion, by outweighing the van der Waals attraction forces. One can
then conclude that, by adding the attractive potential due to van der Waals interactions and the repulsive electrostatic potential, a typical curve for charge-stabilized colloidal particles is obtained.

1.2.5 Liposomes

Liposomes were first described by Bangham in 1965 while studying cell membranes. He found that when phospholipids were dispersed in water in definite ranges formed spontaneously vesicular structures of hydrated bilayers, afterwards known as liposomes. They can be as small as 20 nm or as large as 10 μm. The phospholipids, as the main components of naturally occurring bilayers are amphiphilic, that is they have defined polar and non-polar regions, so that, when in aqueous medium, the non-polar regions orientate towards the interior while the polar regions are in direct contact with the aqueous phase. This results in a double-walled hollow sphere, illustrated in figure 1.5.

Figure 1.5: Structure of the liposome - a double walled, hollow sphere, constituted mainly of phospholipid molecules with polar hydrophilic heads and non-polar hydrophobic tails.

Liposomes can be unilamellar (with only one bilayer surrounding the aqueous core) or multilamellar (with several bilayers oriented concentrically around an aqueous core) [15]. According to these structural properties, they can have different classifications, as shown on table 1.1.

<table>
<thead>
<tr>
<th>Vesicle Types</th>
<th>Abbreviation</th>
<th>Diameter Size</th>
<th>Number of Lipid Bilayers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small Unilamellar Vesicles</td>
<td>SUV</td>
<td>20 - 100 nm</td>
<td>One</td>
</tr>
<tr>
<td>Large Unilamellar Vesicles</td>
<td>LUV</td>
<td>&gt; 100 nm</td>
<td>One</td>
</tr>
<tr>
<td>Multilamellar Vesicles</td>
<td>MLV</td>
<td>&gt; 0.5 μm</td>
<td>Five to Twenty</td>
</tr>
<tr>
<td>Oligolamellar Vesicles</td>
<td>OLV</td>
<td>0.1 - 1 μm</td>
<td>Approximately five</td>
</tr>
<tr>
<td>Multivesicular Vesicles</td>
<td>MVV</td>
<td>&gt; 1 μm</td>
<td>Multicompartmental Structure</td>
</tr>
</tbody>
</table>
Also, the choice of phospholipids determines the rigidity and the charge of the bilayer. Saturated phospholipids with long acyl chains form a rigid, rather impermeable bilayer structure, while unsaturated phosphatidylcholine (PC) species from natural sources (egg or soy bean phosphatidylcholine) give much more permeable and less stable bilayers.

Another important concept is the phase transition temperature, which is affected by the hydrocarbon length, unsaturation, charge and headgroup species. The phase transition temperature is defined as the temperature required to induce a change in the lipid physical state, from the ordered gel phase, where the hydrocarbon chains are fully extended and closely packed, to the disordered liquid crystalline phase, where the hydrocarbon chains are randomly oriented and fluid. van der Waals interactions increase with hydrocarbon length, making the disruption of the ordered packing more difficult, thus increasing the phase transition temperature. In unsaturated lipids, double bonds can produce a kink in the alkane chain, disrupting the regular periodic structure, which allows additional flexibility in the adjacent chains, leading to lower transition temperatures. The membrane permeability decreases with the addition of cholesterol, making the membrane more rigid, as it intercalates the phospholipid molecules, causing a decrease in the flexibility of surrounding lipid chains. Stability of the bilayer components is also affected by the degree of unsaturation. The more unsaturated a compound, the easier the product is oxidized and thus, the less stable it is. This explains why lipids from biological sources which typically contain significant levels of polyunsaturated fatty acids are less stable than their synthetic counterparts.

1.3 State of the Art

1.3.1 Liposomes as Drug Delivery Systems

Liposomes were discovered by Bangham and coworkers in 1965 [16] and suggested as drug carriers in cancer chemotherapy by Gregoriadis et al. [17] in 1974. Since then, liposomes have been extensively used as carriers for pharmaceutical, diagnostic and cosmetic agents, with already a few commercially available products [18 [19]. Nevertheless, they still have not attained their full potential as drug and gene carriers. There are improvements to be made namely in the design and functionality of liposomes to allow them to bypass the multiple anatomic and cellular barriers and reach the target site, to synthesize ligands that are selective for particular cell types and to devise mechanisms for the liposomes to transfer the encapsulated drug into the target [20]. The liposomes must remain stable until they reach the target site and the drug is released at a high enough dosage. The use of these drug delivery nanosystems results primarily in lower systemic toxicity, which is a big issue in cancer therapy, through selective delivery of active drug in disease sites. This selective delivery can be achieved by either passive or active targeting [18 [21]. Passive targeting uses the physical properties of the liposomes together with the microanatomy of the target tissue to obtain selective localization, and active targeting requires some kind of ligand
to bind the liposome surface to the pathological cells. There are already drugs that demonstrate selective tumor localization in animal models and humans, such as DOXIL [22, 23], which is a good example of passive targeting.

Initially, liposome drug delivery nanosystems suffered from the very fast blood clearance by the reticuloendothelial system (RES). The possibility of coating the liposome with the synthetic polymer polyethyleneglycol (PEG) increased significantly the liposome’s half-life in the blood [24], establishing these vesicles as successful drug delivery vehicles. The pegylated liposomes are long circulating in blood due to a highly hydrated and protected liposome surface, constituted by the hydrophilic polymers that inhibit protein adsorption and opsonization of the liposomes. PEG provides the liposomes with up to 72 h half-life in blood. These long circulating liposomes accumulate significantly in tumors due to their leaky vasculature and the lack of an effective lymphatic drainage system, called the enhanced permeability and retention effect (EPR -effect) [24]. One way of increasing drug bioavailability at the tumor target site is the use of site-specific triggers that can release drugs specifically in the diseased tissue. The combination of this active triggering with the active targeting can lead to an enhanced specific drug release. Strategies for active liposome targeting include liposomes coupled to specific antibodies [25, 26], as well as liposomes coated with ligands targeting proteins expressed on cancer cell membranes or endothelial cells lining the newly generated blood vessels in the tumor. However, site-specific targeting has not yet been sufficient to obtain a significantly increased efficacy in the treatment of cancer, when compared to passively accumulating liposomes [18, 19]. This can be justified by the destructive uptake of the liposomes possibly due to lysosomal degradation. Some examples of site-specific biological triggers include pH, temperature and redox microenvironment [27, 28]. The extracellular and intracellular pH is greatly affected by diseases, so that in solid tumors the extracellular pH tends to be significantly more acidic (6.5) than the pH of the blood at 37 °C, which is (7.5) [27]. Also the pH of the endosomal and lysosomal vesicles inside the cells are also significantly lower than the cytosolic pH [27]. It is possible to engineer vehicles for drugs with the right material composition so that the delivery occurs in specific extracellular or intracellular sites. Temperature is also exploited as a trigger, using a delivery system that releases the payload at temperatures above 37 °C. Applying an hyperthermic stimuli would cause local drug release [27, 29]. Physical targeting of drugs and local hyperthermia is also achievable using external stimuli such as a magnetic field. The typical in vivo dose of 100–120 kHz alternating magnetig field is applied to experimental tumor models for about 30 min to achieve temperatures between 40 and 45 °C, as tumor cells seem to be more sensitive to heat-induced damage than normal cells [27]. Recent studies have used superparamagnetic iron oxide nanoparticles encapsulated by liposomes or nanoparticles in order to test the efficiency of hyperthermia, and assuring only the intended target is heated. Radiofrequency (RF) ablation in combination with heat-sensitive liposomal doxorubicin (DOX) has been examined as an approach to promote the DOX accumulation in highly refractory tumors [30]. This combination
1.3 State of the Art

proved highly effective, with a significant increase in tumor necrosis of the liposome encapsulated drug combined with RF ablation when compared with RF ablation alone.

1.3.2 Iron Oxide Nanoparticles in Magnetic Resonance Imaging and Drug Delivery

The synthesis of superparamagnetic nanoparticles has been extensively developed in the past decade with numerous applications such as magnetic storage media, biosensing applications and medical applications, including targeted drug delivery and contrast agents in Magnetic Resonance Imaging (MRI) [31]. Focusing on medical applications, these nanoparticles need to have appropriate surface chemistry and/or adequate coating (nontoxic and biocompatible), allowing a targetable delivery with particle localization in a specific area. They can bind to drugs, proteins, antibodies, and be directed to an organ, tissue or tumor, using an external magnetic field [31]. These targeted particles constitute a powerful ally in molecular imaging, testing for example the specific binding of a ligand to a tumor.

There are several methods available for the synthesis of magnetic nanoparticles, namely microemulsions, sonochemical reactions, hydrothermal reactions, hydrolysis and thermolysis of precursors, flow injection syntheses and electrospray syntheses. The most advantageous method for producing magnetite nanoparticles is, however, chemical coprecipitation of iron salts [31], used in the present work. This technique is probably the simplest and most efficient chemical pathway to obtain magnetic nanoparticles.

Iron oxides are usually prepared by an aging stoichiometric mixture of ferrous and ferric salts in aqueous medium. The chemical synthesis of $Fe_3O_4$ may be written as:

$$Fe^{2+}_{(aq.)} + 2Fe^{3+}_{(aq.)} + 8OH^-_{(l)} \rightarrow Fe_3O_4_{(s)} + 4H_2O_{(l)}$$ (1.3)

Note that magnetite is very sensitive to oxidation, in which case it transforms into maghemite ($\gamma Fe_2O_3$). This oxidation happens to every surface $Fe^{2+}$ within magnetite nanoparticles.

The main advantage of the coprecipitation process is that a large amount of nanoparticles can be synthesized in open air [31]. There are two stages in this process, first a short burst of nucleation occurs when the concentration of the species reaches critical supersaturation and secondly, there is a slow growth of the nuclei by diffusion of the solutes to the surface of the crystal. The size and shape of the nanoparticles can be tailored with relative success by adjusting pH, ionic strength, temperature, nature of the salts or the $Fe^{II}/Fe^{III}$ ratio, giving particles with size ranging from 2 to 17 nm [31].

Another method for synthesis of magnetic nanoparticles, with a more rigorous control over size and shape has been developed, called thermal decomposition [4]. Monodisperse magnetic nanocrystals with smaller size can essentially be synthesized through thermal decomposition of organometallic compounds in high-boiling organic solvents containing stabilizing surfactants. This
organometallic compounds include metal acetylacetonates, metal cupferronates or carbonyls. Fatty acids, oleic acid and hexadecylamine are often used as surfactants. The ratios of the starting reagents are the decisive parameters for the control of the size and morphology of magnetic nanoparticles. The reaction temperature, reaction time, as well as aging period may also be crucial for the precise control of size and morphology [4].

Concerning microemulsions, it is well established that large amounts of two immiscible liquids can be brought into a single phase (macroscopically homogeneous but microscopically heterogeneous) by addition of an appropriate surfactant or surfactant mixture [4, 32]. By mixing two identical water-in-oil microemulsions containing the desired reactants, the microdroplets will continuously collide, coalesce and break again, and finally a precipitate forms in the micelles. This precipitate can be extracted by filtering or centrifuging the mixture, so that a microemulsion can be used as a nanoreactor for the formation of nanoparticles. These nanoparticles can be prepared as spheroids or as tubes, but although many types of magnetic nanoparticles have been synthesized in a controlled manner using this method, the nanoparticle size and shapes usually vary over a relatively wide range. The yield is generally low compared to the other two methods described above and large amounts of solvent are necessary to synthesize a good amount of material.

Overall, co-precipitation is the preferred method due to its simplicity and satisfactory results, although thermal decomposition seems to be the best method developed to date in terms of size and morphology control of the nanoparticles. Alternatively, microemulsions can also be used to synthesize monodispersed nanoparticles with various morphologies, however, the low yield and large quantities of solvent needed are discouraging. The nanoparticles synthesized by any of these methods need to be stabilized or protected in some way, so that agglomeration is prevented [4]. Pure metals or alloys are also very unstable towards oxidation in air, and this susceptibility towards oxidation becomes higher as the particles become smaller. All the protection strategies can be divided into two major groups, coating with organic shells, including surfactant, polymers and liposomes, or coating with inorganic components, including silica, carbon, precious metals or oxides [4].

A very simple method to protect the magnetic nanoparticles is to induce a controlled oxidation of the surface of the pure metal core, a technique known as passivation. This oxidation can be achieved in several ways, for example using a plasma-gas-condensation-type cluster deposition apparatus to oxidize the surface of the nanoparticles, exposure of cobalt nanoparticles to an oxygen plasma or use of synthetic air to smoothly oxidize the surface of the synthesized cobalt nanoparticles to form a stable CoO outer layer. ([33, 34]).

Surfactants or polymers can also be employed to passivate the surface of the nanoparticles during or after the synthesis to avoid agglomeration [4]. Electrostatic repulsion or steric hindrance can be used to disperse nanoparticles and keep them in a kinetically stable colloidal dispersion.
1.3 State of the Art

Such systems are known as ferrofluids, developed by Papell in 1965 [35] and is the approach used in the present work. Considering ferrofluids in aqueous media, the surface properties of the magnetic nanoparticles are the main factors determining the kinetic stability of the colloidal dispersion. In the case of co-precipitation of \( \text{Fe}^{2+} \) and \( \text{Fe}^{3+} \) in ammonia, the magnetite nanoparticles synthesized have a very large and negative surface area so that they will tend to agglomerate. Stabilization is accomplished with aqueous tetramethylammonium hydroxide [4]. In general, surfactants and polymers can be chemically anchored or physically adsorbed on magnetic nanoparticles to form a single or double layer, which creates steric hindrance between them to balance the van der Waals attractive forces. Surface modified magnetic nanoparticles with biocompatible polymers are studied intensively for magnetic field targeting of drugs or as contrast agents for MRI [36, 37]. Besides providing a steric barrier, polymeric coatings can upon intravenous injection help the particle’s uptake evasion by the RES and maintain a long plasma half-life (as was mentioned with PEG in the previous section).

Inorganic core-shell structures, utilizing biocompatible silica or gold to encapsulate the magnetic nanoparticles have become an attractive option for application of these particles as drug delivery agents or MRI contrast agents [38], namely for molecular imaging or image-guided therapy. Silica shells are attractive due to their ease of synthesis and stability under aqueous conditions. Sol-gel processes using tetraethoxysilane (TEOS) are generally utilized to produce coatings of controlled thickness [38]. Surface reactive groups can be easily added to these core-shell structures and the ability to encapsulate functional molecules, such as therapeutic agents or imaging agents such as fluorophores and contrast agents is an interesting option [39].

Gold has other advantages, namely its low chemical reactivity. However, this chemical inertness may also lead to difficulty in forming gold shells over magnetic nanoparticles. These magnetic nanoparticles are synthesized through a variety of methods, namely reversed microemulsion, combined wet chemical and laser irradiation [4].

Liposomes and micelles were already mentioned in the previous section as outstanding vehicles for drug delivery. Encapsulation of magnetic nanoparticles inside liposomes or micelles has the advantage that their \textit{in vivo} behavior is well established with processes such as PEGylation resulting in long blood circulation times. They are able to encapsulate large numbers of magnetic nanoparticles and deliver them together, avoiding dilution, to the target site. Magnetic-fluid-loaded liposomes or magnetoliposomes were developed by encapsulating maghemite nanocrystals within unilamellar vesicles of egg phosphatidylcholine and \( \text{DSPE – PEG}_{2000} \) (distearoylphosphatidylethanolamine - polyethylene glycol), by Martina et al. in [40]. Their hydrodynamic size was around 195 nm and the vesicles were formed by film hydration coupled with sequential extrusion. However, they demonstrate an encapsulation efficiency of only 1.8% and the inexistence of specific interactions of maghemite with the unilamellar liposome membrane. The term magnetoliposomes was first mentioned by De Cuyper and Joniau [41] in 1988, but their group focuses
on a different coating approach, as they synthesize small liposomes consisting of nanoparticles stabilized by a phospholipidic bilayer, without an internal aqueous compartment.

### 1.3.3 Applications

As mentioned before, the main biomedical applications of magnetic nanoparticles, liposomes and magnetoliposomes focused in the previous two sections are as contrast agents for MRI (particularly for magnetic nanoparticles) and/or drug delivery systems. MRI is one of the most powerful non-invasive imaging modalities utilized in clinical medicine today and magnetoliposomes with the benefits of the magnetic nanoparticles could have applications in molecular imaging by connecting them with different antibodies or peptides directed to several types of receptors, to evaluate specific binding to tumors for example. MRI is based on the property that hydrogen protons will align and process around an externally applied magnetic field, $B_0$ and upon application of a transverse radiofrequency pulse, the protons will be perturbed from $B_0$. The process of relaxation, which is the return of the protons to their original state is monitored to generate an MR image. Image contrast is given by local variations of relaxation, that arise from proton density and chemical and physical nature of the tissues within the specimen [36]. Magnetic nanoparticles enhance contrast by shortening the relaxation times (both longitudinal ($T_1$) and transverse relaxation ($T_2$)). The effect of the particles in $T_2$ shortening results from the large susceptibility difference between the particles and the surrounding medium, resulting in microscopic magnetic field gradients. These gradients lead to dephasing of the proton magnetic moments and thus decrease the transverse relaxation time of protons. Therefore, iron oxides (mainly magnetite and maghemite) have been used as contrast agents for 20 years [42], namely due to shortening of $T_2$ relaxation times in the liver, spleen and bone marrow, through selective uptake by the Kupffer cells. Particularly, Superparamagnetic Iron Oxide Nanoparticles (SPIONs), with a diameter of a few hundred nanometers have as usual clinical targets liver diseases, as Kupffer cells in this organ are destroyed when a hepatic disease takes place, thus a strong contrast between normal and abnormal tissue is produced. Ultrasmall Superparamagnetic Iron Oxide Nanoparticles (USPIONs), on the other hand can be used for example in lymph node imaging. Due to their reduced size (less than 50 nm), they can extravazate from the blood vessels into interstitial spaces and reach lymph nodes. Nodes with malignant cells cannot undergo phagocytosis and so, nanoparticles are uptaken only by the normal nodes [42]. These particles have already been proved to successfully detect lymph-node metastases in patients with prostate cancer, as shown by Harisinghani et al. [43]. They conducted a study with eighty patients with prostate cancer and examined them by MRI before and 24h after administration of superparamagnetic nanoparticles and stated that the sensitivity of MRI with the nanoparticles increased significantly. Liver tumors and metastases as small as 2-3 mm have been distinguished, as well as lymph node metastases with a diameter of 5-10 mm, with the use of magnetic nanoparticles [44, 45]. Improvement of the delineation of
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brain tumor boundaries and quantification of tumor volumes is also being evaluated. Active targeting magnetic nanoparticles are also being used to improve tumor detection and localization by exploring the molecular signatures of these diseases [46]. In cardiovascular imaging, magnetic nanoparticles have been used in myocardial injury, atherosclerosis and other vascular diseases. Nahrendorf et al. [47] used functionalized monocristalline iron oxide nanoparticles with vascular cell adhesion molecule-1 targeting peptides, which accumulated in cells that overexpressed this molecule, as confirmed through MR imaging in mice. Smith et al. [47] have decorated SPIONs with a protein to target apoptosis in atherosclerotic plaques, which also manifested through a signal hypointensity in the lesions in a rabbit model. These atherosclerotic plaques imaging can therefore be useful in evaluating the risk of acute ischaemic events [48].

As mentioned in the section 1.3.1, the primary disadvantage of chemotherapy in cancer treatment is the non-specificity of the chemotherapeutic agents and the resulting side effects. Magnetic drug targeting might be a solution to this problem, using an external magnetic field to increase site-specific delivery of drugs. The drug is attached to a magnetic nanoparticle carrier, a colloidal suspension of these magnetic targeted carriers is injected intravenously, an external magnetic field gradient is applied to guide the carriers and the drug is released on the desired location. Of course there are several parameters influencing this process, physicochemical properties of the drug-loaded nanoparticle, field strength and geometry, depth of the target tissue, rate of blood flow and vascular supply [49]. These targeted magnetic nanoparticles still present several problems, including the embolization of the blood vessels, difficulty in scaling up from animal models due to limited field penetration of commercial magnets, control of drug diffusion after release from the particles and toxic responses. Grief and Richardson studied several of these parameters (hydrodynamics within blood vessels, particle volumes, magnetic field strength and effects of cells within the plasma) and concluded that magnetic drug targeting could only be used effectively for targets close to the surface of the body [50]. However, recent articles explore the use of hyperthermia to modify local tumor environment and increase tumor microvascular permeability [51]. Park et al. [52] proposed a system with two components, gold nanorods to act as photothermal antennas to increase local tumor hyperthermia and targeted nanoparticles consisting of either magnetic nanoworms or doxorubicin-loaded liposomes, the recruitment of which is increased by the tumor heating. They concluded that mice with tumors treated with this combined technique displayed significant tumor volume reduction. They also show that targeted liposomes display greater accumulation in the tumors and deliver more payload relative to untargeted liposomes, and that the heating is also related with higher levels of liposome accumulation. In a recent paper, Thomas et al. [53], successfully described killing of bacteria through heating of superparamagnetic nanoparticles synthesized in the presence of oxamic acid, succinic acid or tiopronin by applying an AC magnetic field of around 12 kA/m at a frequency of 1.05 MHz. This illustrates a possibly different application of magnetic nanoparticles in the treatment of a range of infectious diseases.
2.1 Ferrofluid Synthesis

2.1.1 Stabilization of magnetite nanoparticles with tetramethylammonium hydroxide

Ferrofluid synthesis is composed by two steps, the first is to make the magnetite nanoparticles and the second is to disperse these particles into a carrier liquid by utilizing a surfactant to create a colloidal suspension. The procedure chosen to synthesize the magnetite nanoparticles was coprecipitation of iron chlorides, as it is simple and gives satisfactory results with a good yield. The synthesis is based on the one made by Berger et al [7], with some changes that will be addressed shortly. All the reagents and their properties for the ferrofluid and magnetoliposomes syntheses are described on table 2.1.

<table>
<thead>
<tr>
<th>Product</th>
<th>Abbreviation</th>
<th>Purity</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron (II) Chloride Tetrahydrate (FeCl₂₄H₂O)</td>
<td>-</td>
<td>99%</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Iron (III) Chloride Hexahydrate (FeCl₃₆H₂O)</td>
<td>-</td>
<td>97%</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ammonia (NH₃)</td>
<td>-</td>
<td>25% (diluted)</td>
<td>Pronalab</td>
</tr>
<tr>
<td>Hydrochloric Acid (HCl)</td>
<td>-</td>
<td>37% (diluted)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Tetramethylammonium Hydroxide ((CH₃)₄NOH)</td>
<td>TMAOH</td>
<td>25% (diluted)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>L - α phosphatidylcholine (egg)</td>
<td>EPC</td>
<td>-</td>
<td>Avanti Polar Lipids</td>
</tr>
<tr>
<td>Soybean Phosphatidylcholine</td>
<td>SPC</td>
<td>-</td>
<td>Lipoid</td>
</tr>
<tr>
<td>Phosphotungstic Acid Hydrate (H₃PO₄₁₂WO₃)</td>
<td>PTA</td>
<td>-</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

The first step in the procedure consists in preparing the solutions of iron (II) chloride and iron (III) chloride in hydrochloric acid (HCl) 2 M. With that in mind, and in order to obtain solutions of 2 M, one needs to dissolve 0.3975 g of iron (II) chloride tetrahydrate (FeCl₂₄H₂O) in 1 mL HCl and 0.2702 g of iron (III) chloride hexahydrate (FeCl₃₆H₂O) in 4 mL HCl. The next step is to combine the two solutions, place a magnetic stirring bar in the flask and begin stirring vigorously. A solution of aqueous ammonia (NH₄OH) 0.7 M was also prepared previously by dilution of 4.8 mL of concentrated ammonia to 100 mL of water. 50 mL of this solution were added dropwise with a pipette to the previous mixture, with a very slow rhythm, approximately 1 mL every 10 seconds, still under continuous vigorous magnetic stirring. A black precipitate, magnetite, started to form almost immediately. When all the ammonia is added, the stirring is ceased and the magnetic stirring bar removed. The precipitate is allowed to settle, with the help of a strong magnet underneath the flask, then one decants and disposes of most of the liquid. The remaining solution is then centrifuged during one minute at 1000 rpm and the supernatant is decanted afterwards.
2.1 Ferrofluid Synthesis

In order to get rid of any remaining ammonia, the cited article used vacuum. In the present work, that step was replaced by a washing and centrifugation step, in order to save time and simplify the procedure. The washing was made with bi-distilled water, and the centrifugation was made during two minutes at 1000 rpm. Figure 2.1 contains a diagram of the magnetite synthesis used in the present work.

![Diagram of magnetite synthesis process]

Figure 2.1: Diagram representing the procedure used for synthesizing magnetite.

Initially, this magnetite was incorporated directly into lipid vesicles. However, there was a clear problem due to the agglomeration of the magnetite nanoparticles, when they were dispersed in water to incorporate into the liposomes, and when the extrusion was performed as part of the liposome synthesis, the polycarbonate membranes retained practically all magnetite. So the lack of stability of the magnetite nanoparticles in solution clearly made it impossible to synthesize magnetoliposomes as their agglomeration caused the colmatation of the membranes. This fact
imposed the necessity of adding a surfactant to the prepared magnetite, synthesizing a stable aqueous ferrofluid that could be successfully incorporated. So, after taking a small portion of magnetite for further characterization, the synthesis of an aqueous ferrofluid took place. As can be seen in figure 2.2, 8 mL of the surfactant, 25% tetramethylammonium hydroxide \((\text{CH}_3)_4\text{NOH}\) solution (TMAOH), were divided between the two centrifuge tubes used, with vigorous stirring with a glass rod, until the solid was completely suspended in the liquid. The contents of the tubes were poured into a flask and, with the help of the strong magnet that attracted the ferrofluid to the bottom, the excess liquid was poured off. The volume of added surfactant was varied between 1 mL and 8 mL (vide table 2.2), the ferrofluid with 8 mL of surfactant was the one that yielded better results, and thus was chosen to be incorporated into liposomes.

![Diagram representing the procedure used for synthesizing a ferrofluid.](image)

TMAOH surrounds magnetite nanoparticles with hydroxide anions and tetramethylammonium cations creating an electrostatic interparticle repulsion in an aqueous environment and thus stabilizing the colloid \[11\]. This can be seen in figure 2.3.

### 2.1.2 Stabilization of magnetite nanoparticles with phosphatidylcholine

Towards the end of this thesis, another interesting alternative to using TMAOH as surfactant (due to the toxicity of this compound) was implemented, based on the procedure by Giri et al. \[54\]. In this procedure, magnetite is also synthesized through the coprecipitation method, but it is coated with a phospholipid, in this case phosphatidylcholine (PC), that is added during the synthesis of the magnetite and, according to the authors, contributes to stop the nanoparticles from growing too large. The first step is preparing a 0.2 M solution of \(\text{FeCl}_2\) and \(\text{FeCl}_3\) with a molar ratio 2:1, in 5 mL of water. To this solution, one adds a solution of PC in methanol \((\text{CH}_3\text{OH})\), obtained by dissolving 0.046 g of PC in 1 mL of methanol. Afterwards, 50 mL of a 0.93 M solution of \(\text{NH}_3\text{OH}\) were added, dropwise to the previous solution with vigorous stirring. Two different procedures were developed on the next step, in one case, the stirring of this mixture continued with
Figure 2.3: Representation of the ideal interaction between tetramethylammonium hydroxide and magnetite in order to obtain a stable ferrofluid. Surfactant adheres to magnetite nanoparticles through coating with the hydroxide anions, which attract tetramethylammonium cations, creating a diffuse shell and promoting repulsion between particles and consequently stabilization of the colloid. Taken from [11].

A temperature of $60 ^\circ C$ for 15 minutes before being decanted and washed, and on the other, one proceeded immediately with decanting, as this yielded a good result on the first magnetite synthesis, described previously. This protocol was experimented with both egg phosphatidylcholine (EPC) and soybean phosphatidylcholine (SPC). The supernatant was discarded and the remaining product was washed four times with water, one with acetone and one with methanol [54]. A diagram of this procedure is shown in figure 2.4.

A summary of the ferrofluid synthesis is presented in table 2.2. The preparations written in bold were the ones optimized to be incorporated into liposomes. In the case of the ferrofluid with TMAOH, the size was the optimization factor and in case of the ferrofluid with PC, the behavior of the nanoparticles in solution was the chosen criteria.

2.2 Magnetoliposome Synthesis

2.2.1 Traditional film method with sequential extrusion

Generally speaking, liposomes are formed when thin films of lipids are hydrated and stacks of liquid crystalline bilayers become fluid and swell. These hydrated lipid sheets detach during agitation and self-close to form large multilamellar vesicles, that prevent interaction of water with the hydrophobic chains at the edges [55]. After these particles are formed, reducing their size
2.2 Magnetoliposome Synthesis

Figure 2.4: Diagram representing the procedure used for stabilizing magnetite nanoparticles with phosphatidylcholine. The procedure was followed both with and without the stirring and heating described in the box surrounded with the red line.

requires energy, in the form of sonic energy (sonication) or mechanical energy (extrusion). The lipid or lipids are first mixed and dissolved in an organic solvent (in the present work chloroform)
2.2 Magnetoliposome Synthesis

Table 2.2: Summary of the Ferrofluid Synthesis.

<table>
<thead>
<tr>
<th>Sample Designation</th>
<th>TMAOH : Fe₃O₄ (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:0.3</td>
</tr>
<tr>
<td></td>
<td>2:0.3</td>
</tr>
<tr>
<td></td>
<td>4:0.3</td>
</tr>
<tr>
<td></td>
<td>6:0.3</td>
</tr>
<tr>
<td></td>
<td>7:0.3</td>
</tr>
<tr>
<td></td>
<td>8:0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SPC : Fe₃O₄ (mg)</th>
<th>0.046:0.077</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC : Fe₃O₄</td>
<td>Heat Treated</td>
</tr>
<tr>
<td>EPC : Fe₃O₄</td>
<td>Heat Treated</td>
</tr>
</tbody>
</table>

and this solvent is removed afterwards to yield a lipid film in a rotary evaporator. It is important that the lipid film is thoroughly dried to remove all organic solvent. After the lipid film is obtained, its hydration takes place, simply by adding an aqueous medium to the container of dry lipid and agitating. The hydrating medium should have a temperature higher than the phase transition temperature of the lipid in order for the hydration to take place in the fluid phase of the lipid, and this temperature should be maintained during the complete hydration period. The chosen hydration medium depends on the application of the lipid vesicles, and includes mainly distilled water or buffer solutions. At the end of hydration, one has large multilamellar vesicles, with several lipid bilayers separated by water layers, which can be downsized by several methods, including extrusion and sonication. Sonication, as the name implies, uses sonic energy to disrupt the large multilamellar vesicles into small unilamellar vesicles with diameters in the range of 15 - 50 nm. This is either accomplished by bath sonication or probe tip sonication. Probe tip sonicators deliver high energy input to the lipid suspension but cause overheating of the suspension leading to degradation and also release of titanium particles into the suspension, that have to be removed afterwards by centrifugation. This leaves bath sonication, the conditions of which are nearly impossible to reproduce, as factors such as composition and concentration, temperature, sonication time and power, volume and sonicator tuning, are all an influence on the final state of the lipid suspension [55]. For these reasons, extrusion was the method adopted on the present work, although 10 minutes of sonication were applied prior to extrusion.

Extrusion is a procedure in which the lipid suspension is forced through a polycarbonate membrane with a defined pore size to yield particles with a diameter near this pore size. Extrusion should also be done above the phase transition temperature of the lipid to improve its fluidity and thus facilitate its passage through the membranes.

Particularly in the present work, magnetoliposomes of soy bean phosphatidylcholine were
2.2 Magnetoliposome Synthesis

Prepared, based on the protocol in [56]. Initially, PC was dissolved in 4 mL of chloroform in a round-bottom flask and dried in a rotary evaporator (ROTA VAPOR R - 144 of BÜCHI) under reduced pressure at 40°C to form a thin film. The hydration medium was distilled water with dissolved ferrofluid, in order to encapsulate the magnetic nanoparticles in the liposomes. The best results were found when the ferrofluid was recently made (preferably on the previous day) and left in solution for 24 h, which led to deposition of some of the larger particles, that were discarded, so that only the supernatant was used to hydrate the lipid film. 5 mL of this solution were used to hydrate the film, composed by 0.076 g of SPC, in order to obtain a lipid concentration of 20 mmol/L. The film was hydrated for 24 h and afterwards 10 minutes of bath sonication were applied prior to extrusion. Extrusion was made progressively with membranes of 800 nm, 600 nm, 400 nm and 200 nm, twice for each membrane, except for the 200 nm, which was used four times, with membrane exchange between the second and the third. After this process, sample size and other characterization methods were assessed. This protocol is presented in figure 2.5.

![Diagram](image_url)

**Figure 2.5:** Diagram representing the first magnetoliposome synthesis, using only phosphatidylcholine and with 1.25 g of ferrofluid per mole of lipid.

The main problem was clearly found when extruding the sample. Most ferrofluid seemed to get stuck on the polycarbonate membranes when passing from 400 nm to 200 nm. This is probably due to an inefficient action of the surfactant and consequent agglomeration of the magnetic nanoparticles, as this problem had already occurred before the addition of the TMAOH. Differ-
ent concentrations of ferrofluid were tested, and although obviously in the higher concentration this problem got worse, in the lower concentrations it still occurred. The timing of the ferrofluid synthesis and its incorporation in the liposomes also appeared to have some influence on this phenomena, as the ferrofluid seemed to lose stability as time went by, so that after a week it started to precipitate more rapidly when dissolved in water. Having stated this, ideally the ferrofluid should be synthetized as closely as possible to incorporation, to minimize agglomeration of the magnetic nanoparticles. Also, another way to fight this was to leave the ferrofluid in water overnight and to use only the supernatant to incorporate into the liposomes. This appeared to have a positive effect when the extrusion was performed, and the final solution after the 200 nm membranes still retained some colour, which corroborated the fact that there was still some ferrofluid present. The liposomes were then subjected to size-exclusion chromatography (SEC), with a chromatography column (10 mL) of Econo Pac 10 DG from Bio-Rad, to exclude the ferrofluid nanoparticles that weren’t encapsulated, so that the characterization methods would reveal only the presence of the ferrofluid incorporated into vesicles. The eluent used was bidistilled water.

2.2.2 Dried Rehydrated Vesicles

Another method for preparing liposomes is through Dried Reconstituted Vesicles or Dried Rehydrated Vesicles (DRV). The liposomes prepared through this method have the capability to entrap larger quantities of hydrophilic solutes, when compared to other types of liposomes [57]. These vesicles were developed in 1984 by Kirby and Gregoriadis, a technique that involves vesicle formation in conditions that do not cause decomposition or loss of activity of active substances and thus is usually used to prepare liposomes with active substances as enzymes, proteins or peptides. In the case of the present work, the advantage that was aimed for was the high entrapment efficiency of the ferrofluid. This efficiency is useful, not only in economy of reagents but also as less lipid is required to achieve a good local concentration of magnetite. Generally speaking this technique involves freeze-drying of the empty vesicles obtained by the regular procedure of film formation and hydration described on the beginning of this section, with their consequent reorganization after rehydration. The vesicles suffer two hydrations, the first one with around 20 % of the final hydration volume with a concentrated solution of the substance to entrap and the second one, performed after a certain amount of time with the remaining hydration volume of distilled water. This results in theory in a higher encapsulation efficiency precisely due to the controlled rehydration step with a concentrated solution of the substance to be encapsulated when the preformed empty vesicles are disrupted. A more detailed version of the applied procedure is represented in figure 2.6. In the diagram is represented the first synthesis, with soybean phosphatidylcholine and a break of 24 h between the first and second hydrations. Another synthesis was performed with a break of 2 h between hydrations. Two more synthesis with the same differences in the hydration period were made with phosphatidylcholine and cholesterol (m= 0.0511 g
2.3 Characterization Methods

Four methods were used to characterize the obtained nanoparticles, Dynamic Light Scattering (DLS), Transmission Electron Microscopy (TEM), Fourier Transform Infrared Spectroscopy (FTIR) and SQUID magnetometry. TEM was the one explored more thoroughly, as it gives more realistic perception of what our sample looks like, and allows one to measure its size and address roughly the status of one’s experiment, thus providing a standing point from which to evolve. The other three techniques were very useful to evaluate the size of the nanoparticles and prove the effective existence of vesicles, to assess through FTIR whether there was some detectable difference and $m=0.0126$ g respectively). The final lipid concentration was $20 \text{ mmol} L^{-1}$ in all cases.

A summary of all the magnetoliposomes syntheses is presented in Table 2.3, with the different variations in ferrofluid constitution and concentration, as well as the inclusion of cholesterol and differences in the hydration period of the DRVs.

Figure 2.6: Diagram representing the synthesis of magnetoliposomes using Dried Rehydrated Vesicles method.
2.3 Characterization Methods

Table 2.3: Summary of the Magnetoliposomes Synthesis.

<table>
<thead>
<tr>
<th>Classical Film Method Plus Extrusion</th>
<th>Ferrofluid (Dark Sludge)</th>
<th>Ferrofluid Suspension (g ferrofluid/mole lipid)</th>
<th>Liposome Composition**</th>
<th>Sample Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMAOH : Fe₃O₄ (8 mL:0.3 g)</td>
<td>1.25 ; 25 ; 75 25</td>
<td>SPC</td>
<td>SPC : Chol</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPC : Fe₃O₄ [0.046 g:0.077 g]</td>
<td>25</td>
<td>SPC : Chol</td>
<td>SPC : SPC : Chol</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMAOH : Fe₃O₄ (8 mL:0.3 g)</td>
<td>75</td>
<td>SPC : Chol</td>
<td>SPC : Chol</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Hydration Period.
** Lipid Concentration equals 20 mmol² in all cases, with 20 % Cholesterol in the SPC : Chol compositions.
*** The hydration period for all samples synthesized through the Classical Film Method Plus Extrusion was 24 h.

between empty vesicles and vesicles loaded with ferrofluid and to measure the magnetization curves of the samples with a SQUID magnetometer.

2.3.1 Dynamic Light Scattering

Dynamic Light Scattering (DLS), also referred as Quasi-Elastic Light Scattering or Photo Correlation Spectroscopy is a technique suitable for measuring the size of particles in the sub micron region [58]. It basically measures Brownian motion of particles, usually suspended within a liquid, and relates it to particle size, so that the larger the particle, the slower the Brownian motion. The temperature also needs to be accurately measured and stable, otherwise convection currents will cause non-random movements and compromise the measurement. The velocity of the Brownian motion is defined by a property known as the translational diffusion coefficient ($D$). The size of a particle is then obtained using this coefficient and the Stokes-Einstein equation, as follows:

$$d(H) = \frac{k_BT}{3\pi\eta D} \quad (2.1)$$

Where $d(H)$ is the hydrodynamic diameter, $k_B$ is the Boltzmann’s constant, $T$ is the absolute temperature and $\eta$ is the viscosity. The diameter obtained through this technique is the hydrodynamic diameter, which is the diameter of a sphere that has the same translational diffusion coefficient as the particle. This coefficient depends on the size of the particle, on any surface structure and on the concentration and type of ions in the medium. In DLS, the speed at which
the particles are diffusing due to Brownian motion is measured, by measuring the fluctuations of the scattered light. The smaller particles cause the intensity of light to fluctuate more rapidly than the larger ones. To measure the spectrum of frequencies contained in the intensity fluctuations caused by the Brownian motion, one uses a digital auto correlator that will compare the intensity of the signal at a time \( t \) with that at a time \( t + \delta t \), \( t + 2\delta t \), etc., returning a correlogram, with the correlation coefficient in function of time. If the particles are large, their Brownian motion is slower and the signal will change slowly, allowing the correlation to persist for a long time. The reverse is verified by the small particles. Therefore, the time at which the correlation starts to decay is an indication of the mean size of the sample. Also, the steeper the line, the more monodisperse the sample is [58]. A size distribution is then obtained, which can be plotted as the relative intensity of light scattered by particles in various size classes. In the present work, measurements were taken by a Zetasizer nano S series equipment by Malvern.

2.3.2 Transmission Electron Microscopy

Electron Microscopes have a higher resolution when compared with light microscopes due to the wavelengths of the electrons used for microscope illumination, about five orders of magnitude smaller than that of visible light [59]. The resolution of electron microscopes can reach the order of 0.1 nm. There are two main types of electron microscopes, transmission electron microscopes (TEM) and scanning electron microscopes (SEM). The mechanism of a transmission electron microscope is similar to that of a transmission light microscope, and has the following components: light source, condenser lens, specimen stage, objective lens and projector lens (figure 2.7).

The visible light is replaced by an electron ray, glass lenses for visible light are replaced by electromagnetic lenses - because glass does not deflect or focus an electron beam - and a vacuum environment is required to eliminate influence from collisions between high energy electrons and air molecules. Going through the several parts of the TEM system, first an electron gun generates a high energy electron beam by accelerating electrons emitted from a cathode. This acceleration voltage, usually greater than 100 kV, determines the microscope's resolution. After the accelerator, there are condenser lenses to control beam diameter and convergence angles of the incident beam on a specimen. The TEM has three lenses to ensure good magnification capability, and the intermediate lens is used to switch the TEM between an image mode and a diffraction mode. TEM specimens must be thin foils (about 100 nm) because they should be able to transmit electrons, and this thin specimen is mounted in a specimen holder with a 3 mm diameter disc. The specimen preparation depends on the type of sample and needs to be made carefully, so it is a time consuming procedure.

Concerning the present work, the procedure for sample preparation and also the chemical contrast optimization was a very challenging task. The microscope used was the model H-8100 from Hitachi and the beam’s energy went only as low as 100 keV. To image biological structures as
sensitive as liposomes, the ideal energy would be on the order of 80 keV, if one doesn’t possess the best alternative that is cryo-TEM. Aside from that first problem, the chemical contrast also presented its issues. The contrast agent chosen was Phosphotungstic Acid (PTA), as it images the liposomes membranes quite accurately and it partially preserves their structure from being damaged by the electron beam. However, this contrast doesn’t react very well with the magnetite and ferrofluid, as it tends to accumulate around them, yielding a very dark image of inferior quality. Some images turned out to be valid and to provide interesting conclusions, but the optimization of these procedures was rather harsh. The main protocol used consisted in placing one drop of the sample on top of the carbon grid, letting it dry completely, then placing one drop of contrast on top of it for 45 s and finally wiping off the excess contrast with some filter paper. Afterwards, one tried reducing by half the exposure of the sample to the contrast, trying to attain a compromise
2.3 Characterization Methods

between enough contrast to discern the liposomes membranes, and not too much contrast, so as not to obtain an image too dark.

2.3.3 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) is a vibrational spectroscopy technique used very often for materials characterization [59]. Vibrational spectroscopy uses wavelengths in the order of $10^{-7} \text{m}$, typically infrared light, but the electromagnetic waves in this technique are normally characterized in terms of wavenumber, which is simply the reciprocal of wavelength and takes the unit of $\text{cm}^{-1}$. Infrared spectroscopy is based on the phenomenon of infrared absorption by molecular vibrations [59]. When irradiating a molecule with electromagnetic waves within infrared frequency range, there will be one particular frequency that by matching the vibrational frequency of the molecule, will cause excitation of the molecular vibration. This will cause the electromagnetic radiations of that specific frequency to be absorbed, as the photon energy is transferred to excite molecular vibrations, resulting in deep valleys on the infrared spectra of that same molecule. By analyzing these valleys and their frequency values, one can discover to which molecule they correspond.

In case of FTIR, the Fourier transform method is used to obtain an infrared spectrum in a whole range of wavenumbers simultaneously. The key component of this system is the Michelson Interferometer, composed of one beam-splitter and two mirrors. The beam-splitter transmits half of the infrared beam from the source and reflects the other half. The two split beams strike a fixed mirror and a moving mirror and after their reflection, they combine again at the beam-splitter in order to irradiate the sample. The moving mirror intends to change the optical path lengths of the beams in order to generate light interference between them. As the optical path difference changes, the beams will show constructive and destructive interference periodically. A plot of light interference intensity as a function of optical path difference is called an interferogram. The radiation from the FTIR source is composed of numerous wavelengths and the detector receives the interferogram signals which are transmitted to a sample. Through the fast Fourier transform algorithm, this interferogram is converted into an infrared spectrum, which is a plot of light intensity versus wavenumber.

In a FTIR analysis with the pellet method, such as the ones used in the present work, a background is collected first, to which contribute the detector, beam splitter, mirror, IR source, atmospheric conditions and the pellet itself, and then subtracted to the spectra of the actual sample. The pellets are usually made from KBr, and the sample is ground together with this salt and the powder mixture is pressed to form a pellet. In the present work, the equipment used was a FTIR spectrophotometer Nicolet 5700 and the samples were prepared by the described pellet method. TMAOH with magnetite was analyzed in the liquid form and, as such, a drop was placed between two KBr pellets and measured.
Initially the pure spectra were normalized and the offsets removed in order to compensate the inhomogeneity of the samples and allow a better comparison between them. After the analysis of these spectra, Principal Component Analysis (PCA) was performed, inspired by what is done by several authors to differentiate and automatically classify yeast strains [60]. They are able to take spectra with a high degree of similarity and, through PCA, differentiate several bacterial isolates based upon the small differences between the spectra that this method enhances. This method is commonly employed in the interpretation of the infrared spectral data variance and was thus applied on the samples analyzed by FTIR on the present work to check whether it allowed differentiation of the several magnetoliposomes among them or of the liposomes with and without ferrofluid. If successful, it would allow for a fast classification of these vesicles, with a method that, as far as the author’s knowledge, wasn’t yet applied to this area of research.

PCA is a statistical analysis technique that basically reduces a multidimensional data set to its most dominant features, removes noise and retains the principal components of the data, which are basically the original spectra expressed in a rotated coordinate system. It shows whether there are similarities in the data, grouping the multivariate data sets into natural clusters. In the present work, based on [60], PCA was applied to the second derivative of the spectra obtained and the process was developed in MatLab, using an internal function of this software. The data was first written as a matrix S with n spectra, in which each column represented one spectral vector of m intensity data points [61]. An intensity correlation matrix C is then computed from this spectral matrix, $\mathbf{C} = \mathbf{S} \mathbf{S}^T$, and the diagonalization of this matrix yields the eigenvalues, expressing the variance contained in each of the principal components and the eigenvectors from which these principal components can be obtained. The original spectra are then expressed as a linear combination of the principal components, with the scores as weighting coefficients. The largest fraction of the spectral variance is usually contained in the first loading vectors, that is to say the first principal component accounts for as much of the variability in the data as possible. The scores determine how much each principal component contributes to each spectrum, similar spectra exhibit similar scores, and they are used to group the spectra. The score values are plotted against each other and each datapoint represents one spectrum and usually an ordered grouping is observed, which leads to useful conclusions about spectra similarities and automatic and preliminary classification of samples. In the present work, the objective was to apply the same principle to liposomes, and see whether some sort of classification and differentiation of spectra was obtained, based on any of the characteristics of these vesicles, either presence of ferrofluid, concentration of ferrofluid or inclusion or lack thereof of cholesterol.

### 2.3.4 SQUID Magnetometer

A Superconducting Quantum Interference Device (SQUID) magnetometer is frequently used to quantify the magnetization of materials due to its very high sensitivity. It is based on supercon-
ducting loops containing Josephson junctions. The d.c. Josephson effect occurs when two superconducting materials are placed next to each other separated only by a thin insulating layer, and a current flows in the absence of any applied voltage. As the barrier is very thin, electron pairs cross the junction from one superconductor to the other without dissociating [62]. The a.c. Josephson effect predicts that the application of a small d.c. electric potential to a junction such as the one described would produce a small alternating current [62]. The Josephson junction will oscillate with a characteristic frequency which is proportional to the voltage across the junction. There are two basic types of SQUIDs, a two-junction dc SQUID and a single-junction rf SQUID [63]. The two junction squid, as the name implies consists of two Josephson junctions connected in parallel. Two bulk superconductors, brought together by the two Josephson junctions form a ring, as in figure 2.8.

![Figure 2.8: The two-junction dc SQUID, taken from [63]. Josephson junctions a and b are connected in parallel and a magnetic coil in the interior of the ring generates a magnetic flux $\phi_e$.](image)

The flux through the loop of the SQUID is generated by a magnetic coil placed in the interior of the ring. In the absence of any external magnetic field, the input current splits into the two branches equally, without developing a voltage. When an external flux is applied, electrical screening currents flowing on the surface of the superconductors will generate a magnetic field to cancel it. If the current in one of the branches exceeds a critical current for the Josephson junction, the superconducting ring becomes resistive and a voltage appears across the junction. This voltage will be a function of the applied magnetic field.

The single-junction rf SQUID is presented in figure 2.9. The rf SQUID is based on the ac Josephson effect and is less sensitive but cheaper than dc SQUID. It is coupled to the inductor of an LC-tank circuit, and the rf voltage across this tank circuit oscillates as a function of the magnetic flux in the loop.

The model used in the present work was the Magnetic Property Measurement System by Quantum Design, with a sensitivity up to $10^{-8}$ emu. The sample was placed in a nonmagnetic
2.3 Characterization Methods

Figure 2.9: The single-junction rf SQUID, taken from [63]. A single Josephson junction J closes a superconducting ring.

gelatin capsule and compressed with paper to prevent movement and breakage during vacuum. The magnetic field was ramped from 0 to 60000 Oe at 300 K.
3 Results and Discussion

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To initiate the results section, a few considerations need to be addressed. Concerning the ferrofluid synthesis, it is composed of two steps, as mentioned in the methods section: synthesis of the magnetite nanoparticles and covering of those particles by a chosen surfactant, in the first case tetramethylammonium hydroxide and in the second case the lipid phosphatidylcholine.

Throughout the synthesis of magnetite nanoparticles the change in colors was clearly visible. Initially, the iron chloride solutions were green and brown (for iron II and III respectively) and as the addition of ammonia proceeded, a black phase started to precipitate until the solution turned completely black, thus confirming the presence of magnetite (vide figure 2.1). The need of a surfactant was initially detected by the malfunction of the extrusion when incorporating magnetite into liposomes, as the membranes retained the agglomerated magnetite. Ferrofluid was intended to be a stable colloidal dispersion of magnetite nanoparticles in a carrier liquid, with a nanoparticle with surfactant size around 50 nm.

The liposome synthesis, as mentioned in the methods section, was performed mainly and successfully through the method introduced by Bangham, in which multilamellar vesicles are formed through hydration of a lipid film. This method was chosen mainly due to its simplicity, with the aim of improving the existing synthesis of magnetoliposomes, by retaining the magnetite nanoparticles into the liposome structure and studying the effects of the surface properties of these nanoparticles in a possible interaction with the lipidic membrane. Also, the effect of the phospholipid composition for the synthesis of these structures and their process of preparation was explored, based on the results of other authors. The work by Martina et al. [40], already mentioned on the state of the art section, showed the synthesis of magnetoliposomes with maghemite nanoparticles incorporated into liposomes with an egg phosphatidylycholine and $\text{DSPE} - \text{PEG}_{2000}$ lipid formulation. However, they demonstrated an inexistent interaction of the nanoparticles with their unilamellar lipid membranes and had a very low encapsulation efficiency of 1.8%. In the present work, we decided to simplify the phospholipid formulation to soybean phosphatidylcholine (SPC) alone, due to the low transition temperature of this phospholipid and advance afterwards to the inclusion of cholesterol to try to enhance the retention of ferrofluid. Also, the use of an aqueous ferrofluid was preferred to be easily incorporated in the liposomes. Other authors, such as Chen et al. [64], use magnetite covered with oleic acid. They incorporate these particles directly into the lipidic membrane, yielding more rigid and less permeable membranes, using a lipid formulation of DPPC alone, with the drawbacks that the synthesis of ferrofluid with oleic acid is much more elaborate and yields a non aqueous ferrofluid that, in principle, would effectively have more affinity with the liposome membrane. De Cuyper and Soenen [65] have a different approach, aiming to synthesize magnetoliposomes constituted by magnetite nanoparticles individually wrapped with a phospholipidic membrane. They synthesize magnetite nanoparticles surrounded by lauric acid, and claim that this surfactant is replaced by the liposome, during a dialysis procedure, with spontaneous adhesion of phospholipids to the surface of magnetite [66]. They all describe different
interactions of the nanoparticles with the phospholipids and use different lipid formulations and methods. In the present work, the lipid formulation and synthesis are simplified in order to clarify several issues, based on a different work by R. Sabaté et al. [56]. They studied the encapsulation efficiency of ferrofluid loaded liposomes by a method similar to ours and concluded that for 1.25 g of $Fe_3O_4$ per mol of SPC, translated into 96.6% of encapsulation, although given the small amount of ferrofluid (in the case of this work 0.000125 g) there was quite a small chance of visualizing it in the small area analyzed through TEM. Nevertheless, we decided to start with that concentration and test increasing concentrations of ferrofluid. This phenomenon reaches a threshold, around 25 g of magnetite per mol of SPC, where the final concentration is 11.49 g of magnetite per mol of SPC, over which the increase of initial concentration doesn’t manifest in a great increase in the final concentration. In the present work, the concentrations analyzed were of 1.25, 25 and 75 g of ferrofluid per mol of SPC. In the last two, the ferrofluid was left in solution overnight to allow deposition of aggregates, that were discarded, so that only the supernatant was used. This increased greatly the quality of the images obtained by TEM and permitted the observation of interesting conclusions.

After these few considerations, TEM, DLS, FTIR and magnetization measurements of the synthesized samples are shown, analyzed and discussed on the next sections.

### 3.1 TEM and DLS Analysis

#### 3.1.1 Ferrofluid with TMAOH

The characterization of the magnetite nanoparticles, before being covered with any surfactant started with a TEM analysis, as illustrated in figure 3.1.

The magnetite nanoparticles were relatively monodisperse and presented a size around 10-15 nm. Their size and monodispersity are not very easy to control through synthesis by coprecipitation as the removal of the supernatant with a pasteur pipette might not be fully efficient, and the centrifugation is the decisive step to stop the particles growing further.

When covering the magnetite nanoparticles with the surfactant, the main goal was, as previously mentioned, to obtain a kinetically stable colloidal suspension of the synthesized nanoparticles. The size measurements were addressed using DLS. Focusing on the first ferrofluid synthesis, using TMAOH as surfactant, several TMAOH volumes were tested, from 1 mL to 8 mL (referred to reagent volumes in protocol of figure 2.2), taking into account the values found in literature. From 4 mL to 8 mL of surfactant volume, a scale factor of 10 was used in all reagents, in order to reduce lab costs. The macroscopic behavior of the prepared ferrofluids was identical in all the volumes. When measuring the size of each preparation through DLS, the values obtained were far higher than the ones intended for the incorporation into liposomes. A clear correlation between the ferrofluid size and the volume of surfactant added wasn’t obtained. However, the
ferrofluid containing 8 mL of surfactant was the one that yielded better results, presenting a peak of intensity around 50 nm, although a higher, unexpected, valued peak (around 400 nm) was also present (vide figure 3.2). A graphic display of the size distribution of the particles by intensity of scattered light of the best result obtained, which was that of 8 mL, is presented in figure 3.2. As there is more than one peak present, it is useful to look at the size distribution by volume, a conversion made automatically by the Malvern software. Around 50 % of the sample volume is occupied by particles with 55.69 nm. A further conversion into a size distribution by number of particles was also made through the Malvern software, and around 99 % of the number of particles presented the desirable size (results not shown). The remaining graphs corresponding to different volumes of TMAOH are presented in the appendix section (appendix I). The bigger nanoparticles have a much higher intensity of scattered light, as intensity of scattering is proportional to $d^6$, $d$ being the diameter of the particle, from Rayleighs approximation. In terms of volume this difference is not as noticeable, as the volume of a sphere is proportional to $d^3$, and it is logical that even though we have around 99 % of the particles in number of the smaller size, the bigger particles in case of a volume distribution will become more noticeable and in case of an intensity distribution will scatter much more light than the smaller particles. However, the surfactant’s aim was to avoid the magnetite nanoparticles aggregation, so that we had all the nanoparticles on the smaller size.
3.1 TEM and DLS Analysis

Figure 3.2: DLS analysis of ferrofluid TMAOH: $Fe_3O_4$ (8mL:0.3 g). On the top is presented a size distribution by intensity of scattered light and on the bottom a size distribution by sample volume. In size distribution by intensity, the peaks present diameters of 371.9 nm with 84.8% intensity and 61.68 nm with 13.5% intensity. The polydispersion index is 0.559 and the Z-average equals 280 nm. In size distribution by volume, the peaks have diameters of 401.2 nm with 46.8% sample volume and 55.69 nm with 49.7% sample volume.

The ferrofluid sample seems relatively monodisperse with a size around 50-60 nm, although in some regions there appears to be some agglomeration and particles of bigger size. It was also observed that the kinetic stability of the ferrofluid suspension wasn’t as good as expected as it started to settle when left to rest overnight. Optimization of the ferrofluid colloidal properties and/or attempts to synthesize different ferrofluids should be done in future work. In the present work, the ferrofluid was left in suspension overnight to settle and the supernatant was incorporated into the liposomes. This procedure raised a number of issues, the main one being the impossibility of having an accurate measurement of ferrofluid concentration introduced in the liposomes, however, the liposome suspensions retained more colour (and thus more ferrofluid, one assumes) at the end of extrusion in the liposome synthesis when this procedure was carried. To illustrate
3.1 TEM and DLS Analysis

Figure 3.3: TEM imaging of the ferrofluid with TMAOH:Fe$_3$O$_4$ (8mL:0.3 g) synthesized using the procedure shown in figure 2.2.

Concerning TEM imaging of the ferrofluid TMAOH:Fe$_3$O$_4$ (8 mL:0.3 g), the chemical contrast

the ferrofluid settling overnight, two ferrofluid TMAOH:Fe$_3$O$_4$ (8mL:0.3 g) solutions are presented in figure 3.4 with 0.0025 g of ferrofluid in 5 mL of distilled water (on the left) and 0.0075 g of ferrofluid (on the right), also in 5 mL of distilled water. The solutions were photographed immediately after synthesis of the ferrofluid, left to settle overnight, and photographed again on the following day. The deposition of ferrofluid on the bottom of the flask is clearly visible. After the supernatant

was removed to incorporate into liposomes, the flask with the deposited ferrofluid was weighted. It was extremely hard to make this weighing accurate, as some water couldn’t be removed and so contributed to the weight measured, and this is an aspect that needs to be improved in future work.

Concerning TEM imaging of the ferrofluid TMAOH:Fe$_3$O$_4$ (8 mL:0.3 g), the chemical contrast
agent had a tendency to accumulate on top of the magnetite and ferrofluid, as was previously referred in the characterization methods section. This conclusion was first reached with imaging of the magnetoliposomes, as the ones that had a presumably larger amount of ferrofluid (and were effectively darker macroscopically), yielded the poorer images in terms of quality and clarity, the sample was too dark to be perceptible. An image of the ferrofluid TMAOH:Fe$_3$O$_4$ (8 mL:0.3 g) was taken to assess whether the contrast adsorption was effectively with this component of the sample and it turns out that even fairly diluted, once viewed with contrast, the ferrofluid was hardly visible, and the only images that could be obtained are presented in figure 3.5. This made it very challenging later on to obtain clear images of the liposomes, once loaded with ferrofluid.

Figure 3.5: TEM image of the ferrofluid TMAOH:Fe$_3$O$_4$ (8 mL:0.3 g) with PTA contrast. Although the ferrofluid was diluted, the sample with contrast was extremely dark. The accumulation of the contrast around the ferrofluid particles is clear.

### 3.1.2 Ferrofluid with PC

The main objective of this work was the synthesis of a ferrofluid to be introduced into liposomes. In a second attempt, the magnetite nanoparticles were stabilized by another amphiphilic molecule, phosphatidylcholine (PC). The magnetite particles stabilized with PC were imaged by TEM and afterwards incorporated into liposomes. They appeared to be spheric and fairly monodisperse, with size distribution form 5 nm to 15 nm, similar to what was found with the ferrofluid obtained with the TMAOH. This observation was common to all the samples synthesized through the protocol described in figure 2.4. The TEM images are presented in figure 3.6.

The TEM images presented are rather heterogeneous concerning PC distribution, and the phospholipid seems to lead to agglomeration of the magnetic nanoparticles, instead of stabilization. This was common to the four procedures, and differences amongst them weren’t found, at least microscopically. Macroscopically, when suspended in chloroform or water, the magnetic nanoparticles stabilized with SPC without heat treatment yielded the apparently most stable sus-
3.1 TEM and DLS Analysis

Figure 3.6: TEM image of the magnetite nanoparticles stabilized with phosphatidylcholine. In subfigures (a) and (b), ferrofluids SPC:Fe₃O₄ as prepared and heat treated are presented. In subfigures (c) and (d), ferrofluids EPC:Fe₃O₄ as prepared and heat treated are shown. There was a clear heterogeneity in the PC distribution, which wasn’t ideal for the purpose of the present work, although they seem to present the right size (from 5 nm to 15 nm) and to be spherical in shape.

...
3.1 TEM and DLS Analysis

this application. However, TEM images from the samples were collected without contrast, to assess whether some magnetite was still present, and to evaluate the particle distribution around liposomes. Between the samples analyzed with different hydration periods and different liposome formulations (SPC or SPC:Chol), the differences in color, particle size and particle distribution (from TEM or DLS analysis) weren’t very discernible. All these samples passed through a chromatography column in order to eliminate the ferrofluid that wasn’t incorporated into the liposomes. TEM images of the magnetoliposomes SPC:Chol(24 h)∥TMAOH:\(Fe_3O_4\)(75g/mol) are presented in figure [3.7].

![TEM images of magnetoliposomes](image)

(a) 1000 nm (b) 500 nm

Figure 3.7: TEM image of the magnetoliposomes SPC:Chol(24 h)∥TMAOH:\(Fe_3O_4\)(75g/mol) synthesized through the DRV method without contrast.

The presence of magnetite nanoparticles is very clear in these samples, although in a low concentration. The magnetite nanoparticles are present in agglomerates and their distribution doesn’t appear to be ordered or have any specific interaction with the liposome membrane. This is a very curious fact, as if the samples were passed through the chromatography column, one can assume that the ferrofluid is in fact inside the liposomes. With the addition of chemical contrast, the images obtained weren’t very clear, the film became very dark possibly due to the presence of ferrofluid, making it almost impossible to draw conclusions from the images, although phospholipidic membranes were discerned in some of them. The presence of vesicles in magnetoliposomes SPC:Chol(24 h)∥TMAOH:\(Fe_3O_4\)(75g/mol) was confirmed by DLS measurements, an example of which is presented in figure [3.8] with structures around 180.7 nm present.

The DLS measurements for the other syntheses with the DRV method (samples SPC:Chol(2 h)∥TMAOH:\(Fe_3O_4\)(75g/mol), SPC(24 h)∥TMAOH:\(Fe_3O_4\)(75g/mol) and SPCl(2 h)∥TMAOH:\(Fe_3O_4\)(75g/mol)) can be found in appendix I. DRV method can be studied for magnetoliposomes synthesis in a further step of this work, as, although not totally successful, there were still some magnetite nanoparticles present and with an optimization of the ferrofluid procedure better results may be achieved.
3.1 TEM and DLS Analysis

Figure 3.8: DLS measurements magnetoliposomes SPC:Chol(24 h)∥TMAOH:Fe\(_3\)O\(_4\)(75g/mol) synthesized through the DRV method. The diameter obtained was 180.7 nm and the z-average was 149.1 nm.

3.1.3.B Classical Film Method Followed by Extrusion

Recurring to the classical film method followed by extrusion, magnetoliposomes samples SPC∥TMAOH:Fe\(_3\)O\(_4\) (1.25g/mol), SPC∥TMAOH:Fe\(_3\)O\(_4\) (75g/mol), SPC:Chol∥TMAOH:Fe\(_3\)O\(_4\) (25g/mol) and SPC:Chol∥SPC:Fe\(_3\)O\(_4\) (25g/mol) were synthesized and observed through TEM. A TEM image of the magnetoliposome sample SPC∥TMAOH:Fe\(_3\)O\(_4\) (1.25g/mol) is shown in figure 3.9. This image was obtained with PTA contrast. The non incorporated ferrofluid was separated through SEC, so the magnetite present is necessarily incorporated into liposomes.

The liposome membranes are clearly visible and allow their classification as oligolamellar structures. There are some structures, pointed out with the red arrows, that could be ferrofluid, due to their size and distribution, presenting a possible interaction with membrane of the vesicles. This distribution of the ferrofluid associated with the phospholipidic membrane, other than inside the aqueous core was also observed with higher concentrations of magnetite. DLS measurements for magnetoliposomes SPC∥TMAOH:Fe\(_3\)O\(_4\) (1.25g/mol) are shown in figure 3.10 with a diameter of around 182 nm.

TEM images for the magnetoliposomes sample SPC∥TMAOH:Fe\(_3\)O\(_4\) (75g/mol) are shown in figure 3.11. The images stress the distribution of ferrofluid on the vesicles and although its distribution is rather irregular, it is mostly found associated with the lipidic membrane. Once again the samples were submitted to SEC in order to exclude the non incorporated ferrofluid.

These TEM images were taken employing a different sample preparations concerning the chemical contrast agent. The time allowed for the agent to act on the sample was reduced by half, due to the fact mentioned previously that with samples that have higher ferrofluid concentration, the contrast agent made the image dark and difficult to discern. With this low exposure time, the vesicles are clearly discerned, as is the ferrofluid with the sizes corresponding to the expected values. The magnetoliposomes sample SPC∥TMAOH:Fe\(_3\)O\(_4\) (75g/mol) size, measured through DLS is presented in figure 3.12 and has the value of 211.2 nm.

This reduction of the exposure time of the sample to the chemical contrast was successful in this case, allowing a clear observation of the oligolamellar structures with ferrofluid aggregates.
3.1 TEM and DLS Analysis

Figure 3.9: TEM image of contrasted magnetoliposomes sample SPC∥TMAOH:Fe₃O₄(1.25g/mol). Oligolamellar structures are clearly observed. Red arrows identify structures that due to their localization and size could be identified as ferrofluid.

Figure 3.10: DLS measurements of magnetoliposomes sample SPC∥TMAOH:Fe₃O₄(1.25g/mol). The diameter is 182 nm and the z-average is 165.4 nm.

interacting with the membrane. It is an interesting and innovative result, specially when compared with the state of the art in this area. The already mentioned work by Chen et al. [64] where magnetite covered with oleic acid was introduced inside lipidic membranes, doesn’t show TEM images with this visible strong interaction between the magnetite nanoparticles and the liposome membranes, also because they present unilamellar structures instead of oligolamellar. Their interaction is justified mainly by studies of membrane fluorescence leakage, that decreases with incorporation of increasing ferrofluid concentrations and by the fact that their ferrofluid particles
3.1 TEM and DLS Analysis

Figure 3.11: TEM images of contrasted magnetoliposomes sample SPC∥TMAOH:Fe₃O₄(75g/mol). The distribution of the ferrofluid is rather irregular, but it is mostly found associated with the lipidic membrane.

Figure 3.12: DLS analysis of magnetoliposomes sample SPC∥TMAOH:Fe₃O₄ (75g/mol). The diameter is 211.2 nm and the z-average is 175.7 nm.

are hydrophobic. On the work by Sabaté et al. [56], where oligolamellar vesicles were synthesized, the membranes aren’t as clearly discernible as ours and they state only that their ferrofluid distribution is rather irregular, although with prevalence inside the aqueous core. The reasons behind our ferrofluid distribution are still unclear, and it could possibly be attributed to a specific interaction of the lipid headgroups and magnetite, already mentioned in several works [65, 66], where the chemisorption of the polar headgroups to the iron oxide surface was described. Also, on [67], a similar interaction with the lipidic membrane was found with iron ions, and they also document the affinity between the phosphate groups of SPC and the iron ions, with attractive Coulombic interactions playing the main role. The not complete stability of our ferrofluid and the possibly incomplete coverage of the magnetite nanoparticles, might have also lead to this curious distribution, as it allowed magnetite to contact directly with the phospholipids and thus promote the referred interaction.

Cholesterol was also included in the membrane to check whether this enhanced the retention of the ferrofluid, as it is a component known to diminish the fluidity of the membranes and thus help to make liposomes less permeable. TEM images for the magnetoliposome sample
3.1 TEM and DLS Analysis

SPC:Chol∥TMAOH:Fe₃O₄(25g/mol) are presented in figure 3.13. TEM images for this sample were acquired with and without contrast agent, as the one obtained with contrast was very dark in some areas, presumably the ones that contained more ferrofluid.

(a)

Figure 3.13: TEM images of the magnetoliposomes sample SPC:Chol∥TMAOH:Fe₃O₄(25g/mol). No contrast agent was used and the ferrofluid interaction with the membranes is observed clearly.

(b)

Without contrast agent, the membranes of the liposomes are completely indistinguishable from the grid. However, the ferrofluid's disposition in an organized manner around structures with approximately 200 nm allows us to visualize and predict the presence of lipidic vesicles. The ferrofluid interaction with the liposome membranes is clear. The decision not to add contrast intended to confirm the presence of magnetite in the SPC:Chol samples, as the contrast systematically evidenced dark and thick zones in which liposomes were discerned but the magnetite presence was masked. Comparing figures 3.13 and 3.11, although the sample SPC:Chol∥TMAOH:Fe₃O₄ (25g/mol) was obtained with a lower ferrofluid concentration, the ordered distribution of the fer-
rofluid is still present. In fact, upon the analysis through TEM, the image needed to be focused continuously, which corroborates the magnetization of the sample, through constant shifting of the beam. With contrast, the TEM image obtained for sample SPC:Chol∥TMAOH:Fe$_3$O$_4$ (25g/mol) is presented in figure 3.14.

![TEM image](image)

Figure 3.14: TEM image of contrasted magnetoliposomes sample SPC:Chol∥TMAOH:Fe$_3$O$_4$ (25g/mol).

With chemical contrast added, one can guess that the only regions where the liposomes are clearly discerned is where the ferrofluid is present in small amounts. Nevertheless, between the compromise of reducing the exposure time of the sample to the contrast, or removing the contrast in all, very satisfactory results were produced. Concerning the structure of the vesicles, the most characteristic images are obtained with contrast, in which their oligolamellar nature is clearer. However, the absence of chemical contrast or the reduction of the sample’s exposure to the contrast allowed a better observation of the ferrofluid interaction with the lipidic bilayer.

DLS analysis was also made on sample SPC:Chol∥TMAOH:Fe$_3$O$_4$ (25g/mol) and the liposome size was taken to be around 242.9 nm, as can be seen in figure 3.15.

![DLS measurement](image)

Figure 3.15: DLS measurements of magnetoliposomes sample SPC:Chol∥TMAOH:Fe$_3$O$_4$ (25g/mol). The diameter is 242.9 nm and the z-average is 204.1 nm.
3.2 FTIR Measurements

The incorporation into liposomes of the second ferrofluid, SPC:$Fe_3O_4$, revealed little success when the sample was observed through TEM. The magnetite nanoparticles seemed to remain aggregated and not disposed around the liposomes as seen on the other samples with the magnetite stabilized with TMAOH. TEM images for the magnetoliposomes SPC:Chol∥SPC:$Fe_3O_4$(25g/mol) are presented with and without exposure to chemical contrast in figure 3.16.

![TEM images of magnetoliposomes sample SPC:Chol∥SPC:$Fe_3O_4$(25g/mol).](image)

Figure 3.16: TEM images of the magnetoliposomes sample SPC:Chol∥SPC:$Fe_3O_4$(25g/mol). The left side image was taken with exposure to chemical contrast and the right side image was obtained with no contrast added.

The same problem was observed with chemical contrast, images were generally dark but vesicles were found as seen on figure 3.16. When seen without chemical contrast, however, magnetite appeared in small clusters with SPC around, with images similar to the ones obtained through TEM for the non incorporated ferrofluid (vide figure 3.6) although in a much lower concentration. The sample seems quite thick, which corroborates the fact that there is lipid around the particles. However, SPC doesn’t seem to be doing enough to keep the nanoparticles stabilized and individualized as much as possible, and it is also not clear whether these clusters are inside or outside of liposomes. In the image without contrast, greater agglomerates of magnetite nanoparticles were found, that darkened the film completely, which makes this experiment a little inconclusive. It is a method that should be explored further as the biocompatibility of the surfactant used (SPC) is a very positive asset for applications of this technology in vivo.

DLS results for the magnetoliposomes sample SPC:Chol∥SPC:$Fe_3O_4$(25g/mol) are presented in figure 3.17 and the size obtained was around 216.3 nm.

3.2 FTIR Measurements

The FTIR measurements were performed on seven samples: magnetite, ferrofluid TMAOH:$Fe_3O_4$ (8 ml:0.3 g), liposomes and magnetoliposomes, SPC∥TMAOH:$Fe_3O_4$ (1.25g/mol), SPC∥TMAOH:$Fe_3O_4$(25g/mol), SPC∥TMAOH:$Fe_3O_4$(75g/mol) (two samples were
3.2 FTIR Measurements

Figure 3.17: DLS measurements of magnetoliposomes sample SPC:Chol||SPC:Fe₃O₄ (25g/mol). Diameter was measured as 216.3 nm with a z-average of 176.3 nm.

analyzed from the last concentration and SPC:Chol||SPC:Fe₃O₄ (25g/mol). The first image shown comprises the spectra of magnetite and ferrofluid and is depicted in figure 3.18.

Figure 3.18: FTIR spectra of magnetite and ferrofluid TMAOH:Fe₃O₄ (8 ml:0.3 g).

All the spectra were normalized and their offsets removed in order to allow a better comparison among them and try to compensate the possible inhomogeneity of the samples. Particularly in the magnetoliposomes, this may compensate for possible differences in concentrations and allow a more truthful comparison among them [60]. These operations were performed in MatLab. Analyzing first the spectrum of magnetite, a peak is seen around 2364 cm⁻¹ that is attributed to the presence of some carbon dioxide [68] that wasn’t subtracted on the background due to possible changes in the atmosphere inside the equipment when the samples were switched. The clear bands around 570 cm⁻¹ and 400 cm⁻¹ (in this spectra we only see the beginning of this peak, because the limit of our analysis was precisely 400 cm⁻¹), are attributed to the Fe-O stretching mode [69] and are therefore the most characteristic region of the magnetite spectra.
3.2 FTIR Measurements

Concerning the ferrofluid TMAOH:Fe₃O₄ (8 ml:0.3 g) spectrum, one sees a very broad band towards the end, with the highest point around 530 cm⁻¹, that is attributed to magnetite. Another very prominent peak is the one around 3450 cm⁻¹, attributed to the O-H stretch of water and to the hydroxyl groups of TMAOH [60]. The last peak around 1650 cm⁻¹ is attributed to the stretching vibration of the C-N groups [69]. On our spectra, a hint of these bands is present, particularly in the ferrofluid, which corroborates the presence of maghemite. The summary of all attributed peaks is presented in Table 3.1.

Table 3.1: FTIR peak assignment for the magnetite and ferrofluid spectra.

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>530</td>
<td>Fe-O vibration of magnetite</td>
</tr>
<tr>
<td>3450</td>
<td>O-H stretching vibration</td>
</tr>
<tr>
<td>1650</td>
<td>C-N stretching vibration</td>
</tr>
<tr>
<td>700, 630, 660, 620</td>
<td>Fe-O vibration of maghemite</td>
</tr>
</tbody>
</table>

The FTIR spectra presented next belong to magnetoliposomes samples empty liposomes and magnetoliposomes SPC∥TMAOH:Fe₃O₄ (1.25 g/mol), (SPC∥TMAOH:Fe₃O₄ (25 g/mol)), (SPC∥TMAOH:Fe₃O₄ (75 g/mol)) (two samples) and one with PC:Chol (SPC:Chol∥TMAOH:Fe₃O₄ (25 g/mol). The spectra are considered an important contribution of the present thesis, as no magnetoliposomes FTIR study was published before and the results were very satisfactory. The discussion of the results is based mainly on studies of the effect of cholesterol in the liposome membrane (as our ferrofluid location was mainly in association with the liposome membrane) and the shifts in wavenumber of the antisymmetric and symmetric CH₂ stretching regions, around 2920 cm⁻¹ and 2850 cm⁻¹ respectively. The samples’ spectra is presented in figure 3.19.

Some differences in the various spectra can be observed, although some of the more evident are presented in the empty liposomes (pink curve on the graph), when compared with the magnetoliposomes, which are comprised by all the other lines, namely on the intensity of some of the peaks. The band around 3450 cm⁻¹, common to all samples could be present due to some remaining water, despite the lyophilization. The bands around 2925 cm⁻¹ and 2853 cm⁻¹ are attributed to antisymmetric and symmetric methylene vibrations as mentioned previously [54, 59]. The peak around 2361 cm⁻¹ is assumed to be of some remaining carbon dioxide inside the equipment with the change of atmosphere since the background was captured, as mentioned previously.
3.2 FTIR Measurements

![FTIR spectra of magnetoliposomes](image)

Figure 3.19: FTIR spectra of the magnetoliposomes SPC∥TMAOH:Fe$_3$O$_4$ (1.25 g/mol), SPC∥TMAOH:Fe$_3$O$_4$ (25 g/mol), SPC∥TMAOH:Fe$_3$O$_4$ (75 g/mol) (two samples), SPC:Chol∥TMAOH:Fe$_3$O$_4$ (25 g/mol) and empty liposomes. The legend in the graph corresponds to the concentrations of ferrofluid.

with the magnetite. At 1736 cm$^{-1}$ we find the peak corresponding to the C=O bond of ester functional groups of fatty acids [60]. The band around 1465 cm$^{-1}$ is due to the bending vibration of the CH$_2$ group and bands around 1233 cm$^{-1}$, 1090 cm$^{-1}$, 970 cm$^{-1}$ and 820 cm$^{-1}$ are attributed to the presence of PO$_4^{3-}$ group vibration mode [54, 60]. Having stated and labeled all peaks, there is also one at 580 cm$^{-1}$ that could be due to the presence of magnetite on the magnetoliposomes, as it is relatively prominent on the samples that present magnetite and almost inexistent on the empty liposomes. The summmary of all attributed peaks is presented in table 3.2.

Table 3.2: FTIR peak assignment for the magnetoliposomes spectra.

<table>
<thead>
<tr>
<th>Wavenumber (cm$^{-1}$)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3450</td>
<td>O-H stretching vibration</td>
</tr>
<tr>
<td>2925</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>2853</td>
<td>C=O vibration</td>
</tr>
<tr>
<td>2361</td>
<td>CH$_2$ bending vibration</td>
</tr>
<tr>
<td>1736</td>
<td>PO$_4^{3-}$ vibration</td>
</tr>
<tr>
<td>1465</td>
<td>Fe-O vibration of magnetite</td>
</tr>
<tr>
<td>1233, 1090, 970, 820</td>
<td></td>
</tr>
</tbody>
</table>

However, the most amazing differences became apparent when analyzing more particularly the
3.2 FTIR Measurements

methylene vibration bands. Several studies [70-72] have related the information of these bands, particularly the symmetric stretching band around $2850 \text{ cm}^{-1}$, to the ordering of membrane lipids, correlating broadening and small shifts in wavenumber to membrane fluidity with noticeable differences with addition of vitamins/drugs and cholesterol. K. Rebolj et al. [71] state that the shift of this band towards higher wavenumbers and its broadening points to a higher membrane fluidity, and consequent disorder of the membrane lipids due to addition of Gauche conformers. This is corroborated in [72], which also states that a shift to higher wavenumber indicates an increase in chain disorder. They also state that the symmetric mode has more sensitivity to changes in mobility and in conformational disorder of the hydrocarbon chains, so that was the band analyzed primarily in this work. A zoom of this band of the spectra of figure 3.19 is presented in figure 3.20.

Figure 3.20: FTIR spectra of magnetoliposomes, from figure 3.19, in the symmetric methylene stretching band.

Looking more closely at this region of the spectra, there are two clear distinctions between liposomes with PC and PC:Chol and between the magnetoliposomes and PC. Analyzing first the inclusion of cholesterol, one sees that there is a small deviation to the right end of the spectra towards the lower wavenumbers, which, based on what was mentioned would indicate a decrease in chain disorder of the lipidic membrane and a higher membrane rigidity, or putting it from a different perspective, the absence of cholesterol increases chain disorder and membrane mobility. This is exactly the role that the cholesterol is supposed to be playing and is consistent with the results reported in [71, 72]. Concerning the empty liposomes when compared to the magnetoliposomes, there doesn’t appear to be a discernible shift, at least not one that we can pinpoint with certainty but there is a broadening of the empty liposomes when compared to the magnetoliposomes, either with PC or with PC:Chol. According to [72], this translates an increase of the mobility and disorder.
3.2 FTIR Measurements

of the acyl chains in empty liposomes when compared to magnetoliposomes. Other studies need to be made in order to confirm this hypothesis, namely studies with temperature variation to check whether and how this broadening and these shifts are affected by temperature, to take into account the behavior of these membranes and the exact role of the ferrofluid in terms of its stability or lack thereof. Chen et al. [64], synthesized magnetoliposomes with maghemite nanoparticles capped with oleic acid inside the lipid bilayers. They studied the fluorescence leakage with and without heating of these particles and concluded that spontaneous leaking decreased substantially with increasing number of magnetic nanoparticles. This contributes to the conclusion of the increased stability of the membrane of these magnetoliposomes and its lower permeability and possibly more ordered structure. Our FTIR result, though preliminary, points towards this increase of stability of the lipidic membrane, which could be useful in preventing spontaneous leakage from the liposomes.

As mentioned in the methods section, PCA was applied to the liposomes spectra in order to investigate similarities and differences between them and assess whether this technique would be useful in classifying liposomes subjectively in terms of detection of ferrofluid, relative concentration of ferrofluid or distinction between different lipid compositions (on the present work simply represented by PC or PC:Chol formulations). This technique was applied to the second derivative of the spectra, obtained through the finite differences method, based on what was done in [60], where they state that the second derivative enhances by itself the differences between the various spectra. This analysis was performed through MatLab and the result is presented in figure 3.21.

97.2% of the variance is accounted for by the first two principal components, so one can con-
sider the scores presented in figure 3.21 as very representative of our data. First of all, this result is to be regarded carefully, as the few samples analyzed aren’t enough to make it statistically significant, so it should only be regarded as a pointer for future attempts. Looking at this preliminary results, the outlook seems positive, as there is a clear distinction between magnetoliposomes and the empty liposomes. Even the very small concentration of 1.25 g of magnetite per mole of PC was placed in the same cluster as the other magnetoliposomes, which is a very good indication of the possible success of this method in identifying the presence or absence of any amount of ferrofluid on synthesized samples. Secondly, a rather curious observation concerning the ferrofluid concentration is observed, as the different concentrations seem to be placed on the score graph in ascendant (or descendant depending on perspective) order. This could point towards a successful classification of the vesicles through ferrofluid concentration. And finally PC:Chol, although in approximately the same concentration as the sample of PC with 25 g of magnetite per mole of lipid, remained on a lower position when compared to the other lipids, which could point towards its differentiation with respect to lipid formulation. As mentioned, this result however satisfactory and curious, isn’t to be taken as a guaranty of the success of the PCA method for the classification of liposomes, mainly due to the small number of samples analyzed, but it is a first step in the hopefully right direction.

3.3 Magnetization Measurements

Magnetization measurements were performed on the samples at 300 K, envisioning the possible application of this drug delivery system in vivo. The magnetic field was ramped from 0 to 60000 Oe. Due to the limited availability of the SQUID magnetometer, only four samples were analyzed, magnetite, ferrofluid TMAOH:Fe₃O₄ (8 ml:0.3 g) and magnetoliposomes SPC∥TMAOH:Fe₃O₄(75g/mol) and SPC:Chol∥SPC:Fe₃O₄(25g/mol). The first magnetization curve corresponds to magnetite nanoparticles and is presented in figure 3.22.

The absence of hysteresis corroborates the hypothesis of the superparamagnetism of the synthesized nanoparticles, making them ideal for biomedical applications, where a remanent magnetization isn’t desirable, and a rapid relaxation of the magnetic moments to random directions when the field is removed is important. However, more complete measurements with temperature are needed in order to conclude with certainty that the particles are in fact in the single domain state, namely the detection of the blocking temperature. A saturation magnetization of 76 emu/g was obtained. However, as the quantity of sample used in this case was smaller than 1 mg to prevent saturation of the SQUID, which corresponds to a very small volume, one has to consider an error on the magnetization values of this sample up to 25 %. Nevertheless, taking into account that the bulk magnetite saturation magnetization is 82 emu/g [73, 74]. 76 emu/g is still about 92 % of the bulk saturation magnetization. And even considering the maximum error of 25 % to the lower
3.3 Magnetization Measurements

Figure 3.22: Magnetization curve obtained for the synthesized magnetite nanoparticles. A saturation magnetization of 76 emu/g was obtained. The absence of hysteresis corroborates the superparamagnetism of these particles.

value, the saturation magnetization of the synthesized nanoparticles would still comprise about 70 % of the bulk saturation magnetization. This reduction is due to the size of the nanoparticles, as an increase on the surface area leads to an increase on the number of surface atoms with respect to bulk atoms. The magnetic moments of these surface atoms are less aligned than those of the atoms in the bulk and this leads to a reduction in magnetization. The reason for this lack of alignment is still a topic of discussion [73]. Also, the increase of the contribution from impurities and oxides at the surface layer helps decrease the saturation magnetization.

Concerning the ferrofluid, the magnetization curve obtained for sample TMAOH:\text{Fe}_3\text{O}_4 (8 ml:0.3 g) is depicted in figure 3.23.

The shape of the ferrofluid magnetization curve is, as expected, similar to the magnetite curve. The difference is mainly the saturation magnetization that reaches the value of 100 emu/g. As mentioned previously for the magnetite sample, this sample was also analyzed with a quantity smaller than 1 mg to prevent saturation of the SQUID, which once more yields an error in this saturation magnetization value up to 25 %. Nevertheless, one can compare the two analysis and discern clearly that the ferrofluid saturation magnetization is in principle higher than that of the magnetite. Guardia et al. [74] covered magnetite nanoparticles with a different surfactant (oleic acid) and obtained, similarly to the present work, a higher saturation magnetization of the ferrofluid with respect to the magnetite nanoparticles with no surfactant. They claim that the oleic acid stabilizes the surface of the nanoparticles through the new $O^{-2}$ surface ligands of the surfactant, and helps reduce the surface spin disorder, thus increasing the magnetization and
3.3 Magnetization Measurements

Figure 3.23: Magnetization curve obtained for the synthesized ferrofluid TMAOH:Fe₃O₄ (8 ml:0.3 g). A saturation magnetization of 100 emu/g was obtained.

approximating it from that of the bulk magnetite. The same explanation could be applied in the present work. Also, the increase in size of the ferrofluid particles, as agglomerates of five and six particles are formed and stabilized together, could lead to a reduction of the surface effects mentioned previously and thus increase spin alignment at the surface of the nanoparticles.

Two samples of magnetoliposomes were analyzed next, to assess only whether they presented some magnetization. The magnetization measurements for the sample magnetoliposomes SPC∥TMAOH:Fe₃O₄ (75g/mol) are shown in figure 3.24.

Taking into account that the amount of sample analyzed also revolved around a few mg and the fact that in that sample, a very small amount of ferrofluid was present as some losses are bound to occur during the incorporation process, the fact that a magnetization signal was detected is a remarkable achievement. As expected, in such a small quantity, no saturation is achieved, and larger field intensities are needed to increase small steps on the magnetization.

The magnetization measurements for the sample magnetoliposomes SPC:Chol∥SPC:Fe₃O₄ (25g/mol) are presented in figure 3.25.

The slightly smaller magnetization obtained for the magnetoliposomes with cholesterol could be due to the lower concentration of ferrofluid on this sample. In the work by S. Garcia-Jimeno et al. [75], where they try to encapsulate ferrofluid in liposomes using a reverse-phase method, a magnetization curve for magnetoliposomes is depicted. They ramped the field until 5000 Oe and found a magnetization of approximately 0.02 emu/g at that applied field. Taking that into account for comparison with the present work, the magnetization obtained by our magnetoliposomes was about 10 times higher at the same applied field in figure 3.24 and about 5 times higher in fig-
3.3 Magnetization Measurements

Figure 3.24: Magnetization curve obtained for the synthesized magnetoliposomes SPC∥TMAOH:Fe$_3$O$_4$(75g/mol).

Figure 3.25: Magnetization curve obtained for the synthesized magnetoliposomes SPC:Chol∥SPC:Fe$_3$O$_4$(25g/mol).

For some field values, more than one measurement was taken. Further work needs to be developed on behalf of the magnetic properties of these magnetoliposomes, but this is a very positive and promising result.
Conclusions and Further Work
In the present work, magnetite nanoparticles were stabilized with a surfactant, creating a ferrofluid to be incorporated into magnetoliposomes. This ferrofluid was made using two approaches, a first one with the surfactant being tetramethylammonium hydroxide, and a second one with the phospholipid phosphatidylcholine. The synthesis of a partially stable ferrofluid with TMAOH was successful, with the adequate size and shape as seen in TEM imaging. This ferrofluid presented a high magnetization, measured with a SQUID magnetometer and a spectrum in FTIR that evidenced the presence of magnetite, and the OH groups. However, its colloidal stability, when in aqueous solution, wasn’t ideal, as, when left to settle overnight, some aggregates deposited on the bottom of the flask. These aggregates were discarded and the supernatant was afterwards used successfully for the magnetoliposomes synthesis, which prevented a thoroughly accurate measurement of the ferrofluid quantity that was effectively incorporated. Future work on this area should be started by improving this ferrofluid and assuring its complete stability in solution so that not only the incorporation would be easier and more efficient, but the ferrofluid quantity could be assessed before and after incorporation in order to calculate encapsulation efficiencies and have some kind of yield for the whole magnetoliposome synthesis.

Concerning the magnetoliposome synthesis, success was attained and some curious observations were made, which could be useful in future developments of this technology. Magnetite nanoparticles were successfully incorporated into lipidic vesicles with phosphatidylcholine alone or coupled with cholesterol as the lipidic formulation. The lipidic structures were oligolamellar as proved by TEM imaging and the magnetite nanoparticles were mainly distributed associated with the membranes. This was explained due to high affinity of the phospholipid headgroups to the magnetite surface, already mentioned in several studies. The magnetoliposomes were further characterized through FTIR and magnetization measurements. In FTIR, several conclusions were drawn through the analysis of the methylene group symmetric stretching band, related to the ordering of the chains of membrane lipids and consequently to the membrane permeability. We concluded that cholesterol, as expected, induced a higher membrane rigidity with a more ordering of the lipidic chains. Ferrofluid, interestingly, also increased the stability of the lipidic membrane, thus possibly reducing spontaneous leakage of liposome content, in the possibility of a real application for this technology. Principal Component Analysis was also applied to the FTIR spectra of these vesicles in analogy to what is done with classification of yeast strains. At least two clear clusters were found of magnetoliposomes and empty liposomes, which shows that this technique might be used to verify the ferrofluid presence on these structures. A possibility of grouping the samples according to concentration of ferrofluid was also verified, so that some kind of automatic classification could perhaps be established with more of these samples. This obviously needs to be measured for a considerable number of samples to have some solid statistical meaning and that would be an interesting next step in this work in order to validate these results and possibly give them some real utility. Finally, magnetization measurements confirmed unequivocally the
presence of iron in sufficient amount to account for a measurable magnetization of these vesicles, which was a remarkable result. Compared to the only work where magnetization measurements of magnetoliposomes were found [75], the values obtained are very satisfactory. However, magnetization measurements more precise with, for instance, temperature dependence need to be made in order to assure the superparamagnetism of these nanoparticles.

Once these aspects are well established and studied, one can move on to higher objectives, such as the use of pegylated lipids to assure long blood circulation time, and the use of ligands linked to the surface of these magnetoliposomes to target these magnetic carriers to specific sites. Also, hyperthermia, either to trigger the release of the incorporated substances or to increase the local tissue temperature to help in the treatment of local pathologies as cancer, can be investigated, first in vitro and then possibly in vivo.

Taking everything into account, one concludes that the objectives of this thesis were successfully met, contributing to a better understanding of the magnetoliposomes structure and behavior and opening up new possibilities in a current and exciting area of research.
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4.1 Appendix I - Additional DLS Measurements

Figure 4.1: Size distribution by intensity of scattered light of the ferrofluid TMAOH: Fe₃O₄ (1 ml:0.3 g).

Figure 4.2: Size distribution by intensity of scattered light of the ferrofluid TMAOH: Fe₃O₄ (2 ml:0.3 g).

Figure 4.3: Size distribution by intensity of scattered light of the ferrofluid TMAOH: Fe₃O₄ (4 ml:0.3 g).

Figure 4.4: Size distribution by intensity of scattered light of the ferrofluid TMAOH: Fe₃O₄ (5 ml:0.3 g).

Figure 4.5: Size distribution by intensity of scattered light of the ferrofluid TMAOH: Fe₃O₄ (6 ml:0.3 g).

Figure 4.6: Size distribution by intensity of scattered light of the ferrofluid TMAOH: Fe₃O₄ (7 ml:0.3 g).

Figure 4.7: Size distribution by intensity of scattered light of the magnetoliposomes sample SPC(2h)||TMAOH: Fe₃O₄ (75g/mol), obtained through DLS. The diameter equals 195.5 nm and Z-average equals 163.9 nm.

Figure 4.8: Size distribution by intensity of scattered light of the magnetoliposomes sample SPC:Chol(24h)||TMAOH: Fe₃O₄ (75g/mol), obtained through DLS. The diameter equals 193.8 nm and Z-average equals 161.6 nm.
Figure 4.9: Size distribution by intensity of scattered light of magnetoliposomes sample SPC:Chol(24h) | TMAOH:Fe_3O_4 (75g/mol), obtained through DLS. The diameter equals 214.7 nm and Z-average equals 192 nm.