



**Development of hybrid nanosystems
based on membrane lipids as SPIONs
nanocarriers for *Staphylococcus aureus*
biofilms disruption**

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Mestrado Integrado em Bioengenharia

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July 22, 2019

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Agradecimentos

Terminada mais uma etapa, chegou a altura de agradecer a todos os que dela fizeram parte e que, de uma forma ou de outra, tornaram a realização desta dissertação possível.

Em primeiro lugar, um grande obrigada à Dra.Claúdia Nunes e à Dra.Catarina Pereira Leite pela orientação, por todo o apoio, motivação e conhecimento transmitido ao longo deste percurso. Aos restantes Mikes Wazowskis (aka membros do grupo MB²) por toda a disponibilidade, pelos chocolatinhos nos momentos mais conturbados e pelo positivismo e alegria sempre presentes. Acima de tudo, obrigada por tão bem saberem acolher a Boo e por lhe mostrarem os cantos a casa como ninguém.

Mas como nem só da tese se fez o Curso, seguem-se os agradecimentos a todos que fizeram destes 5 anos o melhor que poderiam ter sido.

Às minhas mitocôndrias, por todo o ATP. Porque somos energia!

Aos complexos de Golgi e aos recém adicionados retículos, que como membros oficiais da Célula, a mantêm em funcionamento. (Em especial, à eterna criança pelas tentativas de motivação diárias e pelas explicações de *machine learning*.)

A todos os frequentadores assíduos da B118, desde 2014, pela companhia, pelas pausas (demasiado prolongadas) no estudo, pelas conversas sobre a casa, pela Zebra Fish, pelos gritos às 22h da noite.

Queria ainda aproveitar para expressar o meu agradecimento a algumas entidades, que mesmo sem saber, contribuíram substancialmente para a minha formação académica:

À Google, em especial ao tradutor e ao scholar.

Ao Spotify.

Ao sci-hub.

Ao Therausus.com.

A todas as marcas de chocolate, em especial à Snickers.

À Dona Beatriz.

À Super2000.

Aos SASUP.

À FAP (pela contribuição monetária, ainda inexistente, para a impressão das cópias necessárias desde documento).

Para terminar, fica um eterno agradecimento aos meus pais por todo o sacrifício e tempo investido na minha educação.

Obrigada!

Filipa Soares

Abstract

In the last decades, the inefficiency of several classes of antibacterial agents against pathogenic bacteria has increased. The ability of some of those bacteria to form biofilms, leads to an increased concern, since infections associated with biofilms are still extremely difficult to treat. Not only due to the mechanisms of resistance developed by bacteria, but also due to the presence of the dense extracellular polymeric matrix. The Gram-positive bacterium *Staphylococcus aureus* biofilms are one of the most common causes of human bacterial infections worldwide.

Among several strategies already reported in this area, nanotechnology, through the use of liposomes, namely fusogenic liposomes, and superparamagnetic iron oxide nanoparticles (SPIONs), gave promising results. Liposomes were the first delivery systems to make the transition from concept to clinical application, and they are now an established technologic platform with considerable clinical acceptance. Yet, there is still potential to improve them and the combination of liposomes with magnetic nanoparticles enables the formation of multifunctional vesicles used for magnetic targeting, for drug or gene delivery, for magnetic fluid hyperthermia controlled drug release and contrast in imaging, which allows theranostic applications. In particular, the combination of liposomes with SPIONs is promising because of their magnetic behaviour and their biocompatibility.

In this context, this work aimed to optimise SPIONs loaded fusogenic-liposome formulations using a Box–Behnken design. The synthesis of SPIONs was performed through a microwave assisted method and liposomes were prepared by the thin film hydration method followed by ultrasonication. In a quality by design (QbD) concept to assist formulation and process design, the first step is to pre-define the desired final product quality profiles. Thus, the effects of lipid concentration, SPIONs amount and sonication time on encapsulation efficiency, size and polydispersity of the formulations were assessed. Through the application of the mathematical model 2-way interactions (linear $\times$ quadratic), the optimal formulation was obtained for a lipid concentration of 15 mM, using 0.5 ml of SPIONs and 0.5 minutes of sonication time. The final formulation results are in accordance with the predictions, allowing the validation of the used model. Dynamic light scattering measurements allowed the size and polydispersity characterisation of the formulation as well as the determination of the lipid phase transition temperature, in the case of the liposomal formulation. For the magnetoliposomes, other methods should be carried out to assess this value. A stability study showed that the optimised formulation remained stable, at least, for 3 days, when stored at 4 °C. Moreover, encapsulation showed to be an advantage, reducing the toxicity of SPIONs considering the same concentrations.

Resumo

Nas últimas décadas, a ineficácia de várias classes de agentes antibióticos contra bactérias patogénicas tem aumentado drasticamente e o facto de algumas dessas bactérias serem capazes de formar biofilmes torna esta problemática ainda mais preocupante. Infecções bacterianas causadas por biofilmes continuam a ser extremamente difíceis de tratar, não só devido aos mecanismos de resistência desenvolvidos pelas próprias bactérias, mas também devido à presença de uma matriz densa de polímeros extracelulares. Biofilmes formados pela bactéria Gram-positiva *Staphylococcus aureus* são uma das maiores causas de infeções em todo o mundo.

De entre as possíveis estratégias já reportadas para erradicação de biofilmes, a nanotecnologia, através do uso de lipossomas, nomeadamente, lipossomas fusogénicos, e nanopartículas superparamagnéticas de óxidos de ferro (SPIONs), deu já provas da sua eficácia. Lipossomas são considerados os primeiros sistemas de entrega a fazerem a transição entre o conceito e a sua aplicação clínica e estão atualmente estabelecidos como uma tecnologia com considerável aprovação a nível clínico. No entanto, existe ainda potencial para melhorá-los, sendo a sua combinação com nanopartículas magnéticas, como os SPIONs, uma abordagem bastante promissora, devido à sua biocompatibilidade e propriedades magnéticas únicas. Assim, é possível produzir vesículas multifuncionais com as quais é possível fazer direcionamento magnético, permitindo também a libertação controlada de fármacos, hipertermia magnética, melhoria de contraste em exames de imagem para diagnóstico, entre outras possibilidades.

Neste contexto, este trabalho teve como objetivo otimizar formulações de magnetolipossomas fusogénicos usando um design experimental do tipo Box-Behnken. A síntese de SPIONs foi realizada através de um método de precipitação assistida por microondas e os lipossomas pelo método de hidratação do filme lipídico, seguido de ultrasonicação. Usando o conceito de *quality by design* (QbD) para o design do processo, o primeiro passo passou pela definição de objetivos para as propriedades consideradas críticas do produto final: maximização da eficiência de encapsulação de SPIONs e a obtenção de tamanhos de partículas próximos de 200 nm. Para tal, foram considerados os efeitos da concentração de lípido, da quantidade de SPIONs e do tempo de sonicação.

Através da aplicação do modelo matemático *2-way interactions* (linear $\times$ quadrático), foi feita a otimização, obtendo-se a formulação ótima para 15 mM de lípido, 0,5 ml de SPIONs e tempo de sonicação 0,5 minutos. Verificou-se que os resultados desta formulação estavam de acordo com as previsões feitas pelo modelo, o que permitiu a sua validação. Medições de espectroscopia de correlação fotónica permitiram caracterizar a formulação em termos de tamanho das partículas e polidispersão, assim como determinar a temperatura de transição de fase. Para os lipossomas vazios a temperatura encontrada foi de aproximadamente 23 °C. Para os magnetolipossomas, outros métodos devem ser considerados para esta determinação. Através do estudo de estabilidade conclui-se que a formulação de magnetolipossomas é estável, pelo menos, por 3 dias, a 4 °C. Testes de citotoxicidade mostraram que a encapsulação aumenta a viabilidade celular em relação à mesma concentração de SPIONs não encapsulados.

“How about if I sleep a little bit longer and forget all this nonsense?”

Franz Kafka, *Metamorphosis*

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Abbreviations and Symbols

AC	Alternating Current
CHEMS	Cholesteryl hemisuccinate
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle Medium
DMPG	<i>1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)</i>
DMSO	Dimethyl sulfoxide
DOE	Design of Experiments
DOPE	<i>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine</i>
DPPC	<i>1,2-dipalmitoyl-sn-glycero-3-phosphocholine</i>
DSC	Differential Scanning Calorimetry
LOD	Limit of Detection
LOQ	Limit of Quantification
LP	Liposome
LPS	Lipopolysaccharide
LUV	Large Unilamellar Vesicle
%EE	Encapsulation Efficiency
EMA	European Medicines Agency
EPS	Extracellular Polymeric Substances
FDA	Food and Drug Administration
FRET	Förster Resonance Energy Transfer
FT	Freeze-and-thaw Cycles
IONP	Iron Oxide Nanoparticle
MIC	Minimum Inhibitory Concentration
MLP	Magnetoliposome
MLV	Multilamellar Liposome Vesicle
MNP	Magnetic Nanoparticle
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MTT	<i>3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide</i>
NBD-PE	<i>N-(7-nitrobenzen-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine</i>
NP	Nanoparticle
PBS	Phosphate Buffered Saline
PdI	Polydispersity Index
PE	Phosphatidylethanolamine
PEG	Polyethyleneglycol
PG	Phosphatidylglycerol
L-PG	Lysyl-phosphatidylglycerol
QbD	Quality by design
Rho-PE	<i>N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine</i>
RSM	Response Surface Methodology
SEC	Size-exclusion Chromatography
SEM	Scanning Electron Microscopy
SPION	Superparamagnetic Iron Oxide Nanoparticle
ST	Sonation Time
SUV	Small Unilamellar Vesicles
TEM	Transmission Electron Microscopy
T_m	Transition Temperature
VAN	Vancomycin
WGA	Wheat Germ Agglutinin

Chapter 1

Introduction

1.1 Motivation

Since the 1950s, a decrease of untreatable diseases, due to the introduction of antimicrobial agents was verified (Höjgård, 2012). However, after the 1980s, morbidity and mortality due to respiratory infections, and other bacterial infections has risen again as the common pathogens started to develop increased antibiotics resistance (Ventola, 2015).

Initially, drug-resistant microorganisms occurred only in hospitals but due to the irrational and unavailing application of antimicrobial agents in human and veterinary medicine and in agriculture, they expanded to the community (Jampílek and Král'ová, 2017). Currently, bacterial resistance became an issue, representing, nowadays, a worldwide threat as well as a huge financial burden on healthcare services.

Among the most frequently resistant bacterial strains there are: methicillin-resistant *Staphylococcus aureus* (*S. aureus*), vancomycin-resistant *S. aureus*, vancomycin-resistant enterococci, penicillin- and macrolide-resistant *Streptococcus pneumoniae*, cotrimoxazol-resistant *Escherichia coli*, the third generation of cephalosporin-resistant *E. coli* and *Klebsiella pneumoniae*, and carbapenem-resistant *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Jampílek and Král'ová, 2017).

To make this problem even more concerning, some bacteria of these bacteria, such as *S. aureus*, *E. coli*, *P. aeruginosa* and others, are capable of forming the so-called biofilms, which are bacterial communities, irreversibly attached to a surface and enclosed in a matrix of primarily polysaccharide material. It is known that, in biofilms, bacteria have an increased ability to resist naturally to antimicrobials, when compared to bacteria in the sessile state (Donlan, 2002), and it is reported that the number of infectious diseases caused by biofilms, as well as device-related infections are increasing dramatically (Rukavina and Vanić, 2016).

Therefore, as a way of providing novel and effective biofilm control strategies, a better understanding of biofilm processes and structure should be done. Several therapeutic strategies have already been developed and tested against these structures. Nevertheless, more recently, alternative approaches to antibiotics, such nanotechnological approaches, have been considered promising

in overcoming the antimicrobial resistance developed by several pathogenic microorganisms (Huh and Kwon, 2011). For instance, the development of several classes of antimicrobial nanoparticles (NPs) and nanosized carriers for antibiotics delivery, has shown effective results in treating bacterial infectious diseases, both *in vitro* and in animal models (Huh and Kwon, 2011).

However, the constant increase of the resistance to antibiotics can make these strategies inefficient in a very short time. With these in mind, it is crucial to keep looking for new alternatives, possibly without using antibiotics, in order to mitigate the bacterial resistance problem, while preventing or controlling the formation and proliferation of detrimental biofilms.

1.2 Objectives

As a first goal, it is intended to provide a literature review and analyse the state-of-the-art of the major topics related to biofilm control strategies, including conventional and nanotechnological approaches.

Based on literature review, and considering magnetoliposomes (MLPs) as a potential strategy to *S. aureus* biofilms disruption, the main goal of this work is to develop an optimised method for the production of these nanoparticles. In detail, fusogenic liposomes encapsulating superparamagnetic iron oxide nanoparticles (SPIONs) will be prepared and optimised using an experimental design. This study will focus on two critical formulation qualities, namely, the SPIONs encapsulation efficiency and formulation particle size. These two product qualities are considered very important for both manufacturers and clinical application, since a higher percentage of SPIONs encapsulation can reduce the manufacturing cost and increase the triggering of the heat rise, while allowing greater flexibility in dosing. Another goal will be to achieve a particle size range near 200 nm, to enable administration and permeation through body tissues.

The optimised formulation will be characterised by FTIR and UV-Vis spectroscopy as well as by dynamic light scattering (DLS) measurements, for size, polydispersity index (PdI) and phase transition temperature (T_m) determination. Cell toxicity will also be evaluated for MLPs as well as empty liposomes (LPs) and SPIONs.

1.3 Structure

The remainder of this work is structured as follows: Chapter 2 presents an overview on the biofilms structure and their formation process, the characterisation of detrimental biofilms associated to healthcare-associated infections and some traditional and already used biofilm control strategies. Regarding nanotechnological approaches, the use of LPs (conventional and fusogenic LPs) and SPIONs are reviewed in Chapter 3. Concerning the intended production of MLPs, an overview of the current applications of such hybrid nanosystems is also presented. In Chapter 4 the methods used for the preparation of SPIONs, LPs and MLPs were described, as well as their further characterisation and application. The results and its discussion are presented in Chapter 5 and the main conclusions and future work proposals are described in Chapter 6.

Chapter 2

Biofilms

2.1 Structure and Formation

Primarily, microorganisms have been described and characterised only as planktonic cells. Until, in the 18th century, Antonie Van Leeuwenhoek observed and examined the first biofilm (so-called “animalcules”) in the dental plaque, collected from his own teeth (Donlan, 2002) (Deng and Lv., 2016).

Later, Costerton et al. (1978) postulated the general theory of the biofilm formation, based on observations of dental plaque and sessile communities formed in the natural aquatic ecosystems (Deng and Lv., 2016). In this theory, the mechanisms whereby microorganisms adhere to living and nonliving materials were explained as well as the benefits accrued by this ecologic niche (Donlan, 2002). Since that time, numerous studies have been carried out about biofilms in industrial and ecologic settings and in environments more relevant for public health (Donlan, 2002).

Nowadays, a biofilm is defined as an assemblage of microbial cells attached irreversibly to the substrate at the interface or to each other, embedded in an exopolysaccharidic (EPS) matrix produced by the cells, which show phenotypic changes (Lazar et al., 2003). Numerous factors, like surface properties, nutrients availability and the microorganism, can influence the structure and composition of biofilms (Rukavina and Vanić, 2016).

Bacteria seem to initiate biofilm formation in response to diverse nutritional and environmental conditions (Jamal et al., 2015). Firstly, bacterial adherence occurs, in consequence of transportation, through the movement of the cells in the environment toward the substrate surface. The cells initially attach, in a reversible way, with the possibility of detaching either by their own mobility or by gentle agitation. Then, they create a permanent irreversible binding with the possibility of removing the bacteria adhering only under the action of strong shaking forces (Deng and Lv., 2016). After some bacteria adhered to a surface, growth and quick multiplication takes place, resulting in the formation of microcolonies. As a result of quorum sensing (QS) signals, the mechanism of EPS production is activated, forming the mature biofilm (O’Toole et al., 2000) (Jamal et al., 2015). The development of the formed biofilm continues as long as fresh nutrients are available. When the cells are nutrient deprived, they detach from the surface and return to a planktonic mode

of growth (O'Toole et al., 2000). A schematic drawing of the process of biofilm formation is presented in Figure 2.1.

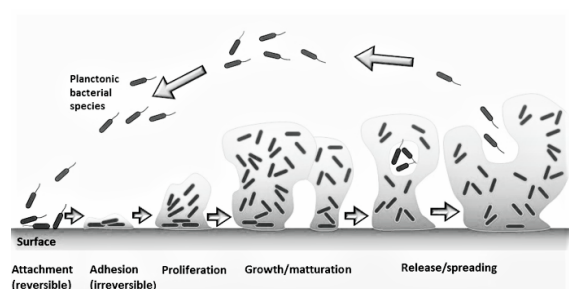


Figure 2.1: Schematic drawing of biofilm formation. Adapted from Rukavina and Vanić (2016)

A mature biofilm is formed by about 10% of microorganisms covered with an EPS matrix, which represents 50–90% of the total organic carbon atoms of the biofilm structure (Deng and Lv., 2016). The EPS amount increases with biofilm age, which influences microbial adherence to the substrate surface and protects the biofilm from physicochemical factors of the external environment, such as antimicrobial agents (Donlan, 2002). Hence, it is not surprising that chronic infections associated with biofilms are strongly resistant to antibiotics and to the clearance mechanisms of the host organism (Lazar et al., 2003).

2.2 Mechanism of Biofilms Resistance

The protective mechanisms against antibiotics, in biofilms, are distinct from those reported for conventional antibiotic resistance in planktonic cells (Singh et al., 2017). In biofilms, cells became 10 to 1000 times more resistant to the effects of antimicrobial agents (Mah and O'toole, 2001), due to the combination of intricate mechanisms, related to the biofilm structure and physiology, as well as innate induced mechanisms, developed by the cells in response to the antimicrobial agent itself (Anderson and O'toole, 2008).

Briefly, intricate mechanisms include the poor antibiotic penetration through the EPS matrix, the decreased oxygen and nutrient availability (which leads to an altered metabolic activity and slowed growth of bacteria), the formation of persister cells, among others (Anderson and O'toole, 2008) (Singh et al., 2017).

Regarding the innate induced mechanisms, genetic adaptations are observed in the cells within the biofilm, which allow the expression of a distinct phenotype with increased resistance (Mah and O'toole, 2001). Thus, the enhanced expression of multidrug efflux pumps, the alteration of the membrane-protein composition as well as the expression of enzymes for inactivation of the bactericide are some examples of innate induced mechanisms of bacteria within biofilms (Mah and O'toole, 2001) (Stewart and Costerton, 2001) (Singh et al., 2017) .

Biofilm resistance to antibiotics has several economic and health implications, as further explored in Section 2.3. Aiming towards the reduction of these implications, a lot of research has

been carried out to better understand the already reported, as well as the new-found, mechanisms of resistance expressed by bacteria in biofilms.

2.3 Detrimental Biofilms and Healthcare-associated Infections

Biofilms can be found on a wide variety of surfaces, including living tissues, medical devices, industrial places, water system pipes and commonly occur on hard surfaces submerged in or exposed to an aqueous solution (Donlan, 2002) (Jamal et al., 2015).

Among these, healthcare-associated infections are, currently, associated with increased patient morbidity and mortality, as already referred. It is estimated that more than 60% of hospital-associated infections results from biofilms formed on indwelling medical devices, which results in more than 1 million cases of infected patients annually (Rukavina and Vanić, 2016). This situation represents a huge financial burden on healthcare services worldwide as well as a concerning public health issue.

Regarding medical devices, biofilms composed by *S. aureus*, *S. epidermidis*, *E. faecalis* and *K. pneumoniae* can be mostly found in infected biomaterial implant surfaces, such as central venous catheters, artificial joints and heart valves (Donlan, 2001) (Subbiahdoss et al., 2012). Urinary catheters frequently develop *E. coli*, *S. aureus*, which can cause septicemia and possible device failure (O'Toole et al., 2000) (Donlan, 2001).

Most commonly reported biofilm-associated infections appear with the use of urinary and central venous catheters (O'Toole et al., 2000). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have shown that all indwelling catheters are colonised by microorganisms embedded in a biofilm matrix (Donlan, 2001). In the case of central venous catheters, the microorganisms came from patient's skin microflora, exogenous microflora from health-care personnel, or contaminated infusates. Bacteria migrate externally from the skin along the exterior catheter surface or internally from the catheter hub or port (Donlan, 2001) (Subbiahdoss et al., 2012). Regarding urinary catheters, adhesion to catheter materials depends on the hydrophobicity of both the organisms and the surfaces (Brisset et al., 1996). Due to the fact that catheters display both hydrophobic and hydrophilic regions, a huge variety of microorganisms is able to colonise these devices (Brisset et al., 1996). Moreover, the increase in urinary pH and ionic strength, particularly due to divalent cations (calcium and magnesium), result in an increase of bacterial attachment (Donlan, 2001).

The colonisation and formation of biofilm depend strongly on the duration of catheterisation (Donlan, 2001). The longer the catheters remain in place, the greater the tendency of bacteria to develop biofilms, resulting in possible associated infections.

Bacteria such as *S. epidermidis*, *S. aureus*, *Streptococcus spp.*, gram-negative bacilli, diphtheroids, enterococci, and *Candida spp.*, may also attach and develop biofilms on heart valves, artificial joints and surrounding tissues of surgical-sites. Implantation of the external prosthesis and other surgical processes, in general, damage living tissues, which leads to an increased tendency of microorganisms to colonise these locations.

Although biofilms develop preferentially on inert surfaces such as medical devices, or on dead tissue, they can also appear directly on living tissues, without the presence of indwelling medical device or the occurrence of any surgical procedure (Costerton et al., 1999).

As already seen, microbial biofilms may pose a public health problem regarding the fact that they can possibly develop on medical device surfaces or even directly in living tissues, causing severe infections. Their high-level resistance to antimicrobials makes the treatment of these infections very difficult. Furthermore, detachment of single or clustered cells can cause a significant risk of microbial dissemination within the host and an increased risk of a chronic infection (Percival et al., 2015) (Wu et al., 2015).

Hence, nowadays, biofilm associated infections became a serious challenge for modern medicine and pharmacy. Effective biofilm control strategies should be investigated, and strategies such as antimicrobial coating and surface alterations of medical devices provide promising opportunities in the prevention of biofilm formation (Percival et al., 2015).

2.4 Biofilm Control Strategies

There are several reported strategies to control the formation and proliferation of detrimental biofilms.

Current prevention and eradication techniques can be divided into two main groups: physical-mechanical approaches (using water sprays and jets), aiming biofilm disruption and removal, and surface-coating or eluting substrates, which aim to prevent the adhesion of bacteria to the surfaces (Koo et al., 2017). Regarding coatings impregnation with antibiotics, Mistry et al. (2016) showed that the use of acrylic beads with absorbable antibiotic-loaded bone cement can prevent orthopaedic infection. Alternatively, it is also possible to use several antimicrobial metal or inorganic coatings, with already reported efficiency in the prevention of biofilm formation (Lemire et al., 2013). This approach allows higher localised antibiotic concentrations, for longer periods of time, with *in situ* antibiotic delivery, which is a huge advantage in comparison to the systemic administration of antibiotics.

Despite these and other advances in biofilm-specific clinical therapies, the systemic administration of antibiotics is still an important and effective measure to control the infections caused by biofilms. Due to toxicity issues, it is impossible to reach the effective antibiotic minimum bactericidal concentration (MBC), by conventional antibiotic administrations, for biofilm eradication *in vivo* (Wu et al., 2015). Thus, antibiotic treatment is considered inefficient in biofilm eradication. Current antibiotic applied strategies involve the use of a combination of antibiotics, with tested ability to penetrate into the biofilm matrix, to ensure an effective action of antibiotic at the site of biofilm infection (Wu et al., 2015).

If the infection involves a foreign body, such as central venous catheters, urinary catheters, joint prostheses, and other infected indwelling devices, removal and, if possible, periodical substitution of the material is highly recommended (Wu et al., 2015). If infected devices removal

is not possible, an attempt to reduce the biofilm burden with antibiotics followed by continued suppressive antibiotic treatment must be applied (Wu et al., 2015).

Biofilm formation is a dynamic process that involves several crucial stages, as previously explained: microbial cell migration, cell-to-cell signalling and interactions, EPS synthesis, and cell-EPS interactions (Yang and Givskov, 2015). While our understanding of these processes and mechanisms is evolving, technological advances are providing tools to develop new chemical-biologic therapeutic approaches.

One of the most interesting strategies is related to the interference in EPS formation and integrity. As already seen, EPS matrix is responsible for cell cohesion and adhesion to the surface in biofilms, providing mechanical stability and protection against host effectors and antimicrobial therapies (Simões et al., 2010) (Wu et al., 2015). Thus, targeting the EPS matrix may be an effective strategy to disaggregate bacteria and, possibly, disrupt the biofilm. In this perspective, EPS-targeting can be achieved by disrupting EPS synthesis and secretion (Sambanthamoorthy et al., 2012), binding of EPS adhesins (Mann and Wozniak, 2012) or by degrading EPS in established biofilms, using enzymes, such as glucanohydrolases (Pleszczyńska et al., 2015).

The most studied regulatory mechanism that has been found to control the production of EPS, as well as biofilm formation and differentiation is quorum sensing (QS) regulation (Vu et al., 2009). Hence, another way to promote biofilm self-disassembly could be through the targeting of QS signalling pathways.

Multiple strategies have been developed to interfere with bacterial QS including competitive binding of inhibitors to the QS receptors (Rasmussen et al., 2005), inhibiting synthesis of QS signals (Chung et al., 2011) (Rasmussen and Givskov, 2006), enzymatic degradation of QS (Dong et al., 2001), and signals posttranscriptional control of QS genes via sRNA (Pérez-Martínez and Haas, 2011).

More recently, chemical engineering had emerged as a promising approach, providing means to control surface properties, such as composition, size, shape, surface area and surface chemistry. Donelli et al. (2002) developed a new polymer-antibiotic system to inhibit bacterial biofilm formation in catheters, through an experimental model based on the adsorption of a beta-lactam antibiotic (cefamandole nafate) on functionalised urethane polymers. A polymer-antibiotic system was capable of inhibiting bacterial growth up to 7 days (Donelli et al., 2002).

In another perspective, Breuing et al. (2005) applied a new strategy of healing in chronic wounds, using low-frequency ultrasonic debridement, promoting bacterial biofilm destruction. It was performed on 17 patients over 8 months, with a minimum follow-up of 3 months (Breuing et al., 2005). As a result, it was observed that some patients completely healed and a small percentage had a reduction in wound area of 20%-30%. Moreover, none of the patients required initiation of antibiotic treatment after starting low-frequency ultrasonic debridement (Breuing et al., 2005).

Besides, all the above referred approaches, nanotechnology can also be considered as a promising strategy for biofilm control, for instance, through the production of functionalised nanostructures designed to enhance penetration, to selectively target or to release drugs locally after bacterial attachment or within biofilms.

Chapter 3

Nanotechnological Approach

Recently, nontraditional control strategies have been investigated in order to overcome the resistance that several pathogenic bacteria have been developed against most of the commonly used antibiotics. Nanotechnology is playing a crucial role in the search of new strategies for destruction and inhibition of biofilms proliferation. Especially, a variety of nanosystems with antimicrobial activity and several nanocarriers for antibiotics delivery have proven their effectiveness for treating infectious diseases (Huh and Kwon, 2011).

LPs and metal NPs, such as SPIONs, are particularly interesting nanoparticles, due to their specific properties and biocompatibility, as further explained in Sections 3.1 and 3.2.

3.1 Liposomes

Liposomes (LPs) are spherical phospholipid-based nanocontainers that are composed by one or more phospholipidic bilayers concentrically oriented around an aqueous compartment (Fielding, 1991). These vesicles can vary from very small ($0.025\ \mu\text{m}$) to large ($2.5\ \mu\text{m}$) vesicles (Akbarzadeh et al., 2013). Based on their size and number of bilayers, LPs can be classified into multilamellar vesicles (MLVs) and unilamellar vesicles (Sharma and Sharma, 1997). Unilamellar vesicles, according to their size, can also be divided into large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs) (Sharma and Sharma, 1997).

The similar structure between lipid bilayer of unilamellar vesicles and the cell membranes allows effective interaction and possible fusion between these nanoparticles and the bacterial membranes (Fielding, 1991) (Rukavina and Vanić, 2016). Moreover, such a structure facilitates encapsulation of both lipophilic drugs, into the phospholipid double layers, and hydrophilic drugs, encapsulated into the aqueous core (Forier et al., 2014).

Due to their advantages, LPs are, by far, the main considered delivery nanosystem (Huh and Kwon, 2011). In fact, LPs were the first delivery systems to make the transition from concept to clinical application, and they are now an established technological platform with considerable clinical acceptance. Currently, there are about 15 therapeutic products available on the market, and there are several other LP preparations in various stages of clinical trials (Bulbake et al., 2017).

Yet, there is still potential for the application of these nanoparticles in other areas. In this context, it has been widely reported the study of LPs as antimicrobial drug delivery systems, for the treating of biofilm infections (Drulis-Kawa and Dorotkiewicz-Jach, 2010) (Pelgrift and Friedman, 2013) (Forier et al., 2014), namely, *S. aureus* biofilms related infections (Huh and Kwon, 2011).

Although LPs have proven to be promising delivery systems, their poor stability is still a concern. In order to overcome this limitation, several studies have been investigating the possibility of surface modification in these NPs, using different stabiliser ligands. For instance, Berti et al. (2016) presented a study where the coating of LPs with calcium phosphate was used to improve the delivery capacity and the mechanical stability of LPs. Two dyes, acridine orange and 5,10,15,20-tetrakis(1-methyl-4-pyridinio)porphyrin were loaded into the aqueous core of LPs (Berti et al., 2016). The nanosystem adhesion capacity and the leaking of fluorophore molecules to *S. aureus* were then evaluated. By TEM and SEM imaging, it was observed that the calcium phosphate-coated vesicles remained adhered to the bacterial cell walls, even after intense washing treatment (Berti et al., 2016). Furthermore, epifluorescence experiments showed that calcium phosphate-coated LPs have good biofilm staining capability after two hours incubation of the biofilms with the LPs, which means that a significant controlled release of the fluorophores occurs, when in contact with *S. aureus* biofilm (Berti et al., 2016). This study proved the potential of calcium phosphate-coated LPs as selective drug carriers to *S. aureus* biofilms.

In association with antibiotics, Hou et al. (2017) recently developed a lysozyme-associated liposomal formulation encapsulating gentamicin and tested its efficiency against *P. aeruginosa* and *S. aureus* biofilms. The surface functionalisation of the negatively charged LP with the cationic lysozyme was possible through electrostatic attraction (Hou et al., 2017). It allows not only the stabilisation of the vesicle but also facilitates the interactions of the LPs with the negatively charged matrix of the bacterial biofilm (Hou et al., 2017). Thus, the lysozyme-associated LPs proved to be effective at damaging established biofilms and inhibiting biofilm formation of pathogens (Gram-positive and Gram-negative bacteria) than free gentamicin (Hou et al., 2017).

In order to study the targeting effect in bacterial biofilm, using different ligands, Vyas et al. (2007) developed metronidazole-loaded LPs covered with hydrophobic derivates of mannan, such as cholesteryl mannan and sialo-mannan. The main aim of this study was to promote receptor-mediated uptake of liposomal contents into the vicinity of the biofilms. When tested against *S. aureus* biofilms, the mannosylated LPs showed a percentage of bacterial growth inhibition of 98% for sialo-mannan-anchored, 86% for cholesteryl mannan-anchored, and 70% for plain LPs (Vyas et al., 2007). *In vivo* results, using an infection model, also showed a higher efficiency of sialo-mannan-anchored LPs when compared with other formulations. Based on that, it is possible to conclude that sialo-mannan ligand mediates the interactions between the LPs and the biofilm, leading to a local release of metronidazole (Vyas et al., 2007).

Besides the targeting of the nanocarrier to increase local drug release, it is also possible to simultaneously promote an immune response. Meng et al. (2016) evaluated the *in vitro* and *in vivo* efficacy of wheat germ agglutinin (WGA)-modified LPs encapsulating clarithromycin against methicillin-resistant *Staphylococcus aureus* (MRSA). WGA promotes the interaction of LPs with

the bacterial cell walls and subsequent drug release, as well as it contributes for enhanced antibacterial activity of the immune system by binding to the surface of macrophages (Meng et al., 2016) (Corradin et al., 1991). Results revealed that the entrapped drug in non-modified LPs could not enhance the antibacterial activity against MRSA, when compared to the free drug. Moreover, only WGA-modified LPs led to a significant decrease of biofilm, comparing to the drug-loaded non-modified LPs (Meng et al., 2016). *In vivo* assays showed a decrease of 16 to 24-fold of the viable bacteria that remained in the kidneys and the spleens of infected animals after treated with WGA-modified LPs, comparing to the control group (Meng et al., 2016). In addition, cellular uptake studies showed not only an enhanced targeted delivery but also an increased immune response in MRSA treated with WGA-modified LPs, comparing to the non-modified LPs (Meng et al., 2016).

In another perspective, besides antibiotics' encapsulation, it is already reported the use of natural compounds as promising alternatives to traditional antimicrobial agents. For instance, Cui et al. (2016) evaluated the anti-biofilm effect of LPs encapsulating cinnamon oil, also against MRSA. Cinnamon oil is a natural essential oil obtained from a plant resource with reported antibacterial properties (Mathew and Abraham, 2006). To assess its *in vitro* antimicrobial activity, the formulation was tested against MRSA biofilms formed on several surfaces (stainless steel, gauze, nylon membrane and non-woven fabrics). Obtained results showed a significant reduction of the amount of MRSA, comparing to free cinnamon oil, and a reduction of the thickness and sizes of MRSA biofilms after treatment with the liposomal formulation. Therefore, it was proved that the encapsulation of cinnamon oil into LPs improved its chemical stability, enhancing its antimicrobial activity against tested biofilms.

Altogether, these data showed that surface stabilised LPs can be considered an efficient drug delivery system, namely in the treatment of biofilm infections, through the destruction of already formed biofilms or by inhibiting their formation, depending on the active substance involved. Moreover, by overcoming the stability issue, the encapsulation of antimicrobial agents into LPs might bring several benefits, such as the protection of the antimicrobial agent from binding to matrix material and enzymatic inactivation, enhancing its antimicrobial effect.

However, to our purpose, the LP properties could be improved in order to obtain the so-called fusogenic LPs. The accurate design of lipid composition allows LPs to fuse with the bacterial outer membrane, enabling the content delivery to the cytoplasm, constituting as additional advantage in the combat of biofilms formation (Forier et al., 2014). Moreover, it may be possible to reduce the dose of antimicrobial agents by targeting the fusogenic LPs to the biofilm through surface functionalisation, maximising therapeutic benefits and reducing the possible side effects (Forier et al., 2014).

3.1.1 Fusogenic Liposomes

Fusogenic liposomes (also known as FluidosomesTM) are conventional LPs with enhanced ability to fuse with cell membranes. Their bilayers contain an optimised ratio of certain lipids, which cause disturbance in the packing of the phospholipid bilayer. Cone-shaped lipids (e.g. phosphatidylethanolamine) or lipids with shorter acyl chains and increased number of double bounds

induce the lowering of the membrane transition temperature (T_m) or disorders the lipid packing. Also, the addition of cholesterol lowers the T_m , increasing the fluidity of the bilayers and, consequently, favouring the fusion process (Forier et al., 2014). Hence, LPs containing this type of lipids can be characterised as relatively fluid bilayers, unlike the typical conventional LPs (Rukavina and Vanić, 2016).

The fusion process between vesicles and cell membranes occurs in three main steps, as represented in Figure 3.1. First, the adhesion or aggregation of the vesicle to the membrane takes place, due to different interaction forces (electrostatic interactions, attractive Van der Waals interactions and hydration forces). Then, at the site of contact, the packing of the lipid membrane is perturbed by the fluid bilayers of the LPs, leading to the initiation of the merging of the outer monolayers of the two membranes. Finally, the complete coalescence of the two vesicles occurs with the mixing of the membrane lipids and the inner content of the vesicles (Rukavina and Vanić, 2016).



Figure 3.1: Model of fusion between fluid LPs and membrane. Adapted from Rukavina and Vanić (2016).

This process depends on several factors, such as the constitution of both vesicle and cell membranes, surface charge, pH, temperature, size of the LPs and the presence of the so-called fusogens (such as calcium ions).

Rukavina and Vanić (2016) had studied the influence of these factors in the fusion process of DPPC:DMPG:cholesterol (Chol) (8:1:1) and DPPC:DMPG (9:1) LPs with bacterial membranes (*S. aureus*, *P. aeruginosa* and *E. coli*). First, it was observed similar degrees of fusion for LPs with sizes ranging from 100 to 800 nm, showing that size does not seem to have a strong influence in the fusion process. A significant increase in the degree of fusion was observed at both low pH and high pH and the highest percentage of fusion was obtained at a temperature near the T_m of the tested LPs. When cholesterol was added to LPs, at concentrations as low as 10% the degree of fusion is dramatically decreased. In addition, it was demonstrated that LP–bacteria fusion is directly proportional to the amount of calcium in the medium. Naturally, negatively charged phospholipid vesicles can not fuse or aggregate due to the long-range electrostatic repulsion. So, divalent cations, by binding to negatively charged vesicles, working as a fusogen, reduce electrostatic repulsion and induce aggregation and the subsequent fusion. The main driving force for the LP–bacterial fusion showed to be the nature of bacterial membranes. Tested LPs fused with

both Gram-positive and Gram-negative bacteria but, for the studied strains, Gram-negative bacteria showed a higher degree of fusion than Gram-positive bacteria. It is suggested that different mechanisms, for Gram-negative and Gram-positive bacteria, can be involved in LP–bacterial fusion. Considering the two main differences between Gram-negative and Gram-positive bacteria, it is suggested that the outer-leaflet layer of lipopolysaccharide (LPS) in Gram-negative bacteria membranes can act as an inducer. Another possibility is related to the high amount of phosphatidylethanolamine (PE) in the membranes of Gram-negative bacteria, which could facilitate the fusion process because of its fusogenic nature.

Also, some studies on the use of lipid and lipid-polymer hybrid nanoparticles (NPs) for the treatment of bacterial infections have been published. Nicolosi et al. (2010) studied a new strategy using fusogenic LPs to localise a glycopeptide antibiotic, vancomycin (VAN), to the periplasmic space of bacteria, thereby allowing it to exert its bactericidal activity. A DOPE/DPPC/CHEMS (4:2:4) lipid mixture was used to prepare LUVs. With high-efficiency loading, it was possible to inhibit the growth of wild and standard Gram-negative bacterial strains, with a minimum inhibitory concentration (MIC) of approximately 6 mg/mL against both *E. coli* and *Acinetobacter baumannii*. Unlike the free antibiotic and the VAN-loaded LPs (non-fusogenic), fusogenic LPs loaded with VAN were able to adhere and fuse with the external membrane of *E. coli*, enlarging the spectrum of activity of the antibiotic.

Hadinoto and Cheow (2014) proved that DPPC/DMPG LPs, with a T_m of approximately 30 °C showed a significant decrease in viability of *P. aeruginosa* biofilms compared to the free antibiotic. Also against *P. aeruginosa*, a study reported the complete eradication of a chronic pulmonary infection by fluid fusogenic LPs encapsulating tobramycin (Beaulac et al., 1996). The antimicrobial activity of the fusogenic LPs was also compared to free tobramycin in *in vitro* biofilms of *P. aeruginosa*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, *E. coli* and *S. aureus*. In comparison to the free antibiotic, fusogenic LPs encapsulating tobramycin showed an increased antimicrobial effect in all tested biofilms (Beaulac et al., 1998). That proved that the used LPs are capable of fusing with different bacteria and be effect against the considered bacteria.

This brief overview shows that fusogenic LPs may be valuable for antibiofilm therapy. As seen above, the majority of the reported studies are based on antibiotics improved delivery to bacterial cells. This approach allows the increase of antimicrobial agents activity, by protecting the antibiotic against degrading enzymes, preventing inactivation by binding to components surrounding the biofilm and increasing the diffusion of the nanoformulation into the biofilm. Moreover, through the fusion of LPs with bacterial membranes, it is allowed the direct release of antimicrobials into the cells, increasing the contact between the bacteria and the antibiotic and giving the possibility to reduce the dose (Forier et al., 2014).

However, the use of antibiotics in these approaches could make them useless, if bacterial resistance to such agents keeps improving. So, it is crucial to find effective alternatives, in this scope, and nanotechnology keeps giving possible ways. The combination of LPs with magnetic NPs, such as superparamagnetic iron oxide nanoparticles (SPIONs) enables the formation of multifunctional vesicles that seem to be promising antimicrobial agents due to their magnetic behaviour as

well as biocompatible. This approach will be explored in the following sections.

3.2 SPIONs

Magnetic nanoparticles have been of great scientific and technological interest over the past decades, due to their unusual electronic, optical and, especially, magnetic properties (Laurent et al., 2008) (Mahmoudi et al., 2011). Among them, superparamagnetic iron oxide nanoparticles (SPIONs) remain as one of the most versatile nanosystems as they represent the best compromise between good magnetic properties and very reduced toxicity (Tombácz et al., 2015).

Iron oxide nanoparticles (IONPs) consist of cores of iron oxides, such as magnetite (Fe_3O_4) or its oxidised form, maghemite ($\gamma-Fe_2O_3$), with a close-packed cubic lattice form in which the iron ions are located at interstices between the oxygen ions (Seabra et al., 2017) (Mahmoudi et al., 2011) (Akbarzadeh et al., 2012).

In sizes smaller than 30 nm, these IONPs, at room temperature, present a magnetic behaviour by applying an external magnetic field (Demirer et al., 2015) (Seabra et al., 2017). After the removal the external magnet, the NPs became nonmagnetic (Justin et al., 2017). This property of IONPs is called superparamagnetism and occurs due to the reduced size and increased surface-to-volume ratio of these NPs in comparison to the ones found in bulk state (Seabra et al., 2017), as further explored in Section 3.2.1.

Currently, SPIONs are already used in clinical application for diagnostic purposes (Gupta and Gupta, 2005). In fact, numerous studies have proven the capability of these NPs to be used for a plethora of uses. With the appropriate surface chemistry, they have the potential to be used in a several *in vivo* applications, such as magnetic resonance imaging (MRI) contrast enhancement, magnetic drug delivery systems, tissue repair, hyperthermia, early detection of inflammatory cancer and diabetes, gene therapy, cell separation, among others (Laurent et al., 2008) (Demirer et al., 2015).

However, these IONPs dispersed in a fluid or in a biological medium tend to aggregate, due to their hydrophilic surfaces, surface charge, and large surface area to volume ratio (Cruz et al., 2017). It becomes a problem when talking about their potential biomedical applications. In order to reduce agglomeration, allowing the formation of a colloidal dispersion with longer time stability, adequate molecules can be used to cover their surface (Gupta and Gupta, 2005). Besides stability, the correct coating also improves biocompatibility and provides either further functionalisation for drug delivery or enables specific therapeutic practices (Reddy et al., 2012).

In another perspective, the physical and chemical properties of SPIONs can also be very attractive for antimicrobial applications, namely in the prevention and control of biofilms formation (Seabra et al., 2017), as explained in Section 3.2.2.

3.2.1 Magnetic Properties

The magnetic materials may be divided in different classes in terms of their volumetric magnetic susceptibility, χ , as well as their feedback to AC magnetic field. Most materials present magnetism

only in the presence of an applied field (Laurent et al., 2011) (Hedayatnasab et al., 2017). So, they can be classified as paramagnets, with χ in the range of 10^{-6} - 10^{-1} , or diamagnets with a negative χ values. The materials that exhibit ordered magnetic states and present a magnetic behaviour without requiring a magnetic field are classified as ferromagnets and ferrimagnets (Laurent et al., 2011).

These magnetic materials are organised in the so-called magnetic domains, which are portions of the material where the magnetic moments are all aligned in the same direction (Akbarzadeh et al., 2012). It is this domain structure that determines the size dependence of the magnetic behaviour of ferro and ferrimagnetic materials (Hedayatnasab et al., 2017). When exposed to an external magnetic field, ferro and ferrimagnetic materials' behaviour follows a hysteresis loop (Figure 3.2), characterised by two main parameters: remanence and coercivity (Akbarzadeh et al., 2012).

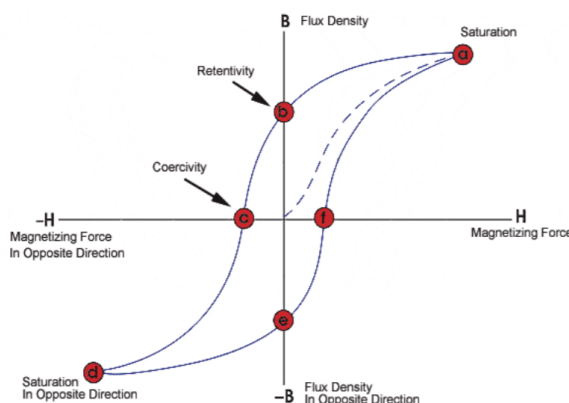


Figure 3.2: Hysteresis loop. Figure from Wright et al. (2008)).

Dealing with NPs, the coercivity becomes the most relevant property, because it is size-dependent. When the size of ferromagnetic materials is reduced, the coercivity progressively increases to a maximum and then decreases (Akbarzadeh et al., 2012). For sizes below a critical diameter, the single-domain particles present a superparamagnetic behaviour, where coercivity reaches zero and no hysteresis loop is verified, when exposed to an external magnetic field (Tombácz et al., 2015). In the case of magnetite or maghemite, i.e. the main materials that compose the core of SPIONs, the superparamagnetic behaviour is reached for diameters below 30 nm (Akbarzadeh et al., 2012).

Under an external magnetic field, the domains of a ferromagnetic NP as well as the magnetic moment of single domain superparamagnetic NPs align with the applied field. In the absence of an external field, ferromagnetic NPs will maintain a net magnetisation, whereas superparamagnetic NPs will exhibit no net magnetisation due to rapid reversal of the magnetic moment. In another words, for superparamagnetic NPs, the magnetic moment changes its orientation rapidly, averaging to zero if no magnetic field is applied.

This way, it is possible to control the magnetic behaviour of these superparamagnetic NPs,

as well as their location in living systems, which becomes a huge advantage for the application of SPIONs in biological environments. Besides magnetic targeting and control of these NPs, the magnetic properties of SPIONs can be also used for the generation of localised heat, through a phenomenon called hyperthermia. A detailed explanation of this application is carried out in Section 3.2.1.1. Furthermore, it is known that both magnetite and maghmite meet the magnetic moment and biocompatibility requirements for usage in nanomedicine.

3.2.1.1 Hyperthermia

Hyperthermia is commonly defined as the elevation to abnormal body temperature artificially induced by external medical devices (Hedayatnasab et al., 2017). It is typically used to relieve cysts, inflammation and arthritis pain, due to the increase of the blood flow, providing nutrition to the affected tissues and reducing muscle spasms (Hedayatnasab et al., 2017). More recently, hyperthermia was also applied as a cancer treatment, using a local injection of magnetic fluid or tumour specific antibody targeting, to locally heat the affected area (Nikitin et al., 2019). Upon excitation with an AC field, the magnetic NPs transform electromagnetic energy to heat. The heat supplied in this transformation is a function of the NPs' size, shape, and composition and of their interactions, being also dependent on the applied AC magnetic field (Cruz et al., 2017). The energy generated in this process can then be utilised to destroy cancer cells (Abenojar et al., 2016).

As the magnetic field periodically reverses its direction, it induces change in orientation of the NP magnetic moment. The dissipative reorientation of the NPs magnetic moment under an alternate magnetic field is the heating source in magnetic hyperthermia (Cruz et al., 2017). Heat dissipation can occur by four mechanisms: (1) eddy current heating due to the effects of induction from the application of an alternating pulse magnetic field (for NPs with size $> 1 \mu\text{m}$); (2) hysteresis losses (in multi-domain magnetic particles); (3) relaxation losses (in single-domain magnetic NPs); and (4) frictional losses induced by the interaction between the NPs and the surrounding medium, in viscous suspensions (Abenojar et al., 2016) (Hedayatnasab et al., 2017).

In single-domain particles, such as SPIONs, relaxation heating loss mechanisms presents more relevance. The atomic magnetic moments are aligned inside the particle, changing in orientation collectively. Applying a magnetic field to the NPs will align their magnetic moments, either by rotating the magnetisation within the particle (Néel relaxation) or rotating the particle itself (Brown relaxation), as shown in Figure 3.3. When in the presence of an AC field, magnetic moments are continuously relaxing, leading to heat production (Cruz et al., 2017).

Concerning the clinical application of magnetic hyperthermia, important requirements should be take in account in order to make it both harmless and well tolerated by patients and also effective in treatment (Cruz et al., 2017). For that, high frequency (F) and amplitude (H) of the alternate magnetic field (of the order of tens to hundreds of kHz and a few kA/m, respectively) are required. However, to avoid the damage of the biological tissues, these parameters must be kept below critical values (Cruz et al., 2017).

Many efforts have been devoted in the last 20 years to improve hyperthermia techniques for clinical applications. Advances in the area of nanotechnology have contributed to the development

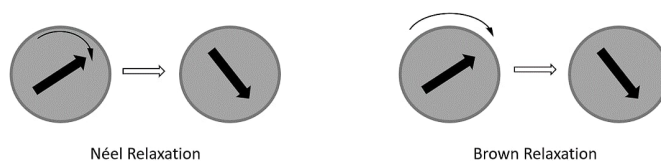


Figure 3.3: Representation of the two mechanisms involved in the heat release of single-domain magnetic NPs.

of magnetic fluid hyperthermia and its expansion to other possible applications, such as biofilm eradication (Laurent et al., 2011). In fact, the use of SPIONs as hyperthermia agents as well as “shield breakers” represents an effective strategy for the disruption of biofilms, combining the penetration ability of these NPs with their magnetic and antimicrobial properties (Li et al., 2019). Throughout the next section, a summary of the literature review on the antimicrobial potentialities of SPIONs is presented.

3.2.2 Antimicrobial activity

As already seen, it is urgent to find alternatives to existing antimicrobial drugs in the control and eradication of biofilms, in order to prevent and treat some severe bacterial infections. Application of nanotechnology seems to represent an excellent alternative through the use of metal and metal oxide NPs, such as SPIONs, that can be considered as antimicrobial agents as well. Particle size is the essential parameter determining the antimicrobial effectiveness of these NPs (Jampílek and Král'ová, 2017).

Bacteria, in biofilms, are protected from environmental stresses, such as desiccation, attack by the immune system, protozoa ingestion, and antimicrobials, which allows them to significantly reduce the efficacy of therapeutic agents (Solano et al., 2014). One of the factors that allow that resistance is the inability of the drugs to efficiently penetrate the EPS barrier, decreasing the amount of antimicrobial reaching the targeted microorganism. It has been already proven that small NPs have a faster rate of diffusion through mucus in comparison with larger particles (Armijo et al., 2014) (Sanders et al., 2000). With this in mind, the use of SPIONs as antimicrobial agents or its combination with antibiotics can be considered.

Metal oxide NPs have proven effectiveness against resistant strains of microbial pathogens and have considerable heat resistance (Dizaj et al., 2014). SPIONs, in particular, have the additional advantage of, through the application of an external magnetic field, allowing a site-specific nanoparticle penetration into the bacteria biofilm (Jampílek and Král'ová, 2017).

Furthermore, it was already reported the ability of SPIONs to inhibit bacterial growth and, consequently, biofilm formation. Armijo et al. (2014), using the disk diffusion method, showed

how this NPs were capable to create a 17 mm radius inhibition (MIC = 4 $\mu\text{g/mL}$) against *P. aeruginosa* biofilm. Also, Chen et al. (2012) synthesised SPIONs functionalised with carboxymethyl chitosan, and tested them against biofilms of *S. aureus* and *E. coli* under a magnetic field. The obtained reduction of cells viability, after 48 h of exposure to 2.0 mg/mL of the formulation, was 84% for *S. aureus* and 95% for *E. coli*.

In a different approach, SPIONs can also be used as an antibiofilm agent through the generation of local hyperthermia, by the application of an alternating current (AC) magnetic field. This effect leads to a local increase of temperature in the biofilm (between 20 °C to 60 °C), depending on the NPs concentration (Seabra et al., 2017). This property was tested against *P. aeruginosa* biofilms, by Park et al. (2011), using a concentration of SPIONs of 60 mg/mL. This strategy resulted in a higher than 4 log biofilm inactivation in the first 8 min.

Recently, Fang et al. (2017) studied the hyperthermia effect induced by magnetic Fe_3O_4 NPs with the aim of destroying *in vivo* *S. aureus* biofilms, for the treatment of osteomyelitis. For that purpose, a metallic 18G needle was implanted into the bone marrow cavity of distal femur of sixty 12-weeks-old male Wistar rats, after the injection of Methicillin-sensitive *S. aureus*. The inserted implants were heated to 75 °C, by automatically switching an on/off device of the AC, promoting a better distribution of the particles within the biofilm structure. As a result, it was observed an eradication of the biofilm and an enhanced efficiency of the plain vancomycin, which was co-administrated with the magnetic NPs (Fang et al., 2017). This study highlights not only that *S. aureus* biofilms can be destroyed by magnetic nanoparticles-induced hyperthermia but also that the therapeutic effect of systemic antibiotics could be enhanced, when used in combination with MNPs (Fang et al., 2017).

Besides the combination with antibiotics, SPIONs can also be combined with Ag, as an antimicrobial agent for the treatment of *S. aureus* biofilms. Durmus and Webster (2013), in their study, combined the unique magnetic and antibacterial properties of SPIONs and Ag, through the design of Ag-conjugated SPIONs. A decrease of 1.57% was observed in planktonic MRSA growth when exposed to 1 mg/mL of Ag-conjugated SPIONs. Furthermore, it was also observed a significant biofilm biomass decrease to less than 47% in the presence of an external magnetic field (Durmus and Webster, 2013). This study demonstrated that combining the properties of SPIONs and Ag, through the creation of the referred nanosystem, can be considered as an alternative to antibiotics for the eradication of MRSA biofilms (Durmus and Webster, 2013).

The state of the art analysis carried out in this section showed that SPIONs have emerged as promising therapeutics, with proved efficacy as antibiofilm agents against both Gram-positive and Gram-negative bacteria. Moreover, these nanoparticles can be guided to the specific site of infection by applying an external magnetic field. Furthermore, SPIONs surface can be functionalised with other agents, like polymers (e.g. chitosan), enhancing the interaction between the nanoparticles and cell membranes.

3.2.3 Potential cytotoxicity

SPIONs' unique magnetic properties along with their reported biocompatibility confer many advantages in a vast variety of biomedical applications. However, understanding the potential negative effects of the exposure to SPIONs remains a concern, since new applications of these NPs keeps emerging. So, in this section the effects of the exposure to SPIONs (*in vitro* and *in vivo*) are reviewed.

Once the SPIONs are administered intravenously, they enter liver and spleen (Raynal et al., 2004). At cellular level, after being internalised, SPIONs are degraded by enzymes present in lysosomes, by breaking the NPs assembly, producing ions. The generated Fe^{2+} lead to the production of reactive oxygen species (ROS) by altering mitochondrial and other organelle functions, which can lead to different cellular damage effects (Patil et al., 2018). Reported toxicities in experimental studies include reduced mitochondrial activity, cellular stress mediated generation of ROS, inflammation and chromosome condensation and other DNA damage (Singh et al., 2010) (Figure 3.4).

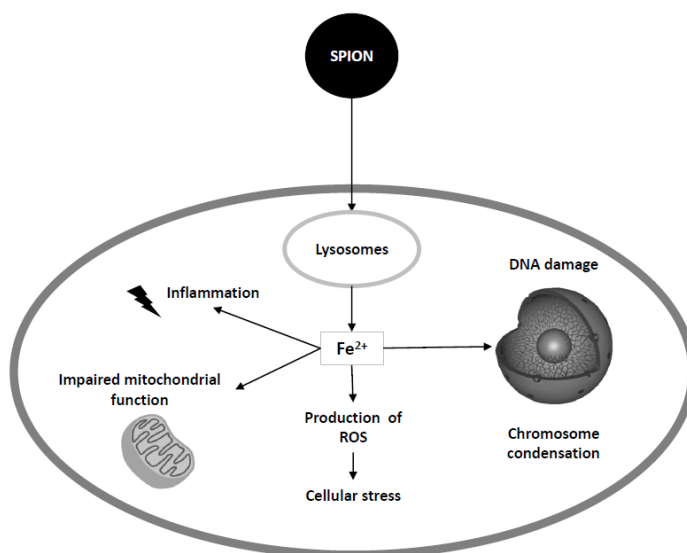


Figure 3.4: Schematic representation of possible mechanisms of SPIONs cytotoxicity at cellular level.

Karlsson et al. (2008) showed that several metal oxide NPs, SPIONs included, were safe and non-cytotoxic below 100 $\mu\text{g/ml}$. Another study tested Fe_3O_4 NPs coated with a bipolar surfactant, on normal, glia and breast cancer cells. It revealed that the toxicity of these NPs is concentration-dependent with the particles being non-toxic in the concentration range of 0.1-10 $\mu\text{g/ml}$ and showing toxicity at 100 $\mu\text{g/ml}$ (Ankamwar et al., 2010).

In the human body, other systemic problems can be found. SPIONs are very small in size, comparable with the biomolecules. It can lead to a sequestration of these NPs into various body

systems, interfering with their normal functioning (Patil et al., 2018). For instance, SPIONs can cross the blood brain barrier and damaged neural functions, or they can cross nuclear membrane and cause mutations. Also, bare SPIONs, presenting not sufficient small size have low solubility, which leads to agglomeration and precipitation, causing possible obstruction of blood vessels (Singh et al., 2010) (Patil et al., 2018). Thus, it seems that SPIONs on their own are not sufficiently biocompatible (Janko et al., 2019). One strategy to overcome this issue is to coat the SPIONs with biocompatible molecules such as silicon, dextran, citrate and PEGylated starch (Singh et al., 2010).

In fact, some formulations of surface coated magnetic-based NPs have already gained approval for use in humans. Resovist (ferucarbotran) and Feraheme (ferumoxytol) are two examples of clinically approved dextran-coated SPIONs formulations (Jin et al., 2019) (Janko et al., 2019). Resovist was approved for the imaging of small focal liver lesions in the European, Japanese, Australian and Chinese market (Jin et al., 2019). Feraheme was approved by FDA (U.S. Food and Drug Administration), in 2009, and EMA (European Medicines Agency) for treatment of iron deficiency anemia, in 2012 (Jin et al., 2019).

Although the potential toxicity of SPIONs is proved, these NPs can be considered biocompatible, concerning *in vitro* and *in vivo* applications. However, there is evidence that some of the used coatings, like dextran, do not bound strongly to the iron oxide core, possibly leading to detachment and consequent aggregation of the NPs (McCarthy and Weissleder, 2008).

Therefore, another possible way to get around the biocompatibility issue could be the encapsulation of SPIONs in LPs, by increasing the overall size and also functioning as a coating, allowing the production of nanosystems known as magnetoliposomes (MLPs). These nanosystems and their advantages are explored in the following Section 3.3. Few works have been published reporting methods for the production of this hybrid nanosystem, but there is still a need for optimisation of the procedure as well as evaluation of their toxicity *in vitro* and *in vivo*, before their application.

3.3 Magnetoliposomes

Nanotechnology is advancing at a fast pace and holds promise to overcome many of current therapeutic problems, such as antibiotics resistance and control of biofilm formation. Through the combination of both LPs and SPIONs, it is possible to create multifunctional nanosystems with high biocompatibility and high therapeutic potential, the so-called magnetoliposomes (Bonnaud et al., 2013) (Gonzales and Krishnan, 2005). As mentioned before, LPs are lipid vesicles mostly used as drug carriers, hampering degradation problems of the drug and improving diffusion rate into the biofilms EPS matrix. SPIONs can be manipulated by an external magnetic field gradient, providing external guidance of the therapy and acting as antimicrobials. The combination of all these advantages enables the creation of a new hybrid nanosystem with great potential in this field.

SPIONs surface properties determine where the particles will spatially be located within the liposome (Figure 3.5) (Monnier et al., 2014). The most common strategies are either to encapsulate the magnetic NPs directly within the liposome lumen (Beaune et al., 2008) (Cintra et al., 2008) or

to embed them in between the lipid bilayer (Chen et al., 2010b) (Bonnaud et al., 2014). It is also possible to directly conjugate SPIONs with the liposome surface (Floris et al., 2011).

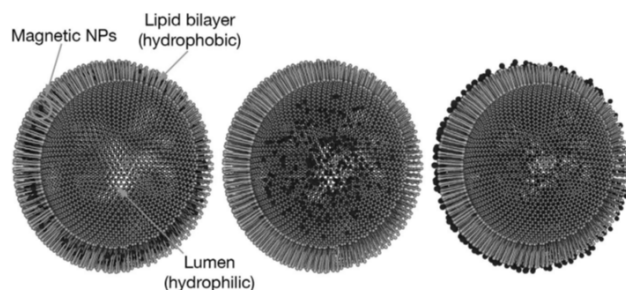


Figure 3.5: Different possible location of SPIONs in LPs. Adapted from Monnier et al. (2014)).

So, the first step to develop this type of nanosystem is choosing the nature of surface coating of SPIONs, depending on their final application. Due to the hydrophilic nature of their surface, uncoated SPIONs tend to be located into the liposome vesicle (Sakulkhu et al., 2015). To increase the hydrophilicity and their stability, SPIONs can be covered with molecules such as citrate (Beaune et al., 2008) and oleate (Gonzales and Krishnan, 2005). For embedding them in between the lipid bilayer, the stabilisation of SPIONs must be done with hydrophobic molecules, such as oleic acid (Chen et al., 2010b) or almityl-nitroDOPA (Amstad et al., 2011).

Although there are different possibilities, embedding SPIONs directly in between the bilayer allows a more efficient destabilisation of the membrane and an optimised magnetically triggered release, by relaxation processes of the magnetic nanoparticles that results in heat production (Bonnaud et al., 2013). Moreover, SPIONs in the liposome lumen might impair or affect any other encapsulated drug even before the membrane actually becomes permeable (Monnier et al., 2014). However, considering the final aim of this work, the encapsulation in the lumen of the fusogenic LPs is intended. The presence of SPIONs between the bilayer could interfere in the fusion process of the fusogenic LPs, compromising the release of SPIONs into the cytoplasm of bacteria. Besides, the encapsulation into the aqueous compartment allows the capture of a higher amount of uncoated SPIONs.

Also, regarding the final objective of the present work, the choice of the lipid constitution of the LPs determines their T_m and their ability to fuse with cell membranes. If the final application was magnetic resonance imaging (MRI) or magnetically guided drug delivery, T_m would be set around 42 °C, a few degrees above normal body temperature. (Monnier et al., 2014). Since the nanosystem was design to prevent and control biofilm formation, it is useful to choose the right lipids, in an optimised ratio, in order to obtain MLPs with the intended fusogenic properties. Hereby, in addition to the above-referred properties of common MLPs, it is also possible to improve the diffusion rate of the nanosystems into biofilms structure as well as improve the interaction between bacteria and antimicrobial agents. Whereas the reported antimicrobial activity of SPIONs by themselves, the development of a hybrid nanosystem based on lipid membranes and SPIONs could

be seen as a new nanotechnological approach to biofilm therapy.

So far, there is not a lot of work carried out concerning the application of MLPs as antimicrobial agents against *S. aureus*. Very recently, Silva et al. (2018) tested a formulation composed by LP encapsulating stigmasterol and magnetite NPs, coated with lecithin, against *S. aureus* and *Citrobacter freundii*. These tests were performed for different formulations, in the presence of a magnetic field of 200 Oe, 315 kHz and also in its absence at room temperature and at temperature of 41 °C (Silva et al., 2018). The obtained results showed that stigmasterol not only increased the encapsulation capacity of NPs of the LPs but also allowed a better response the magnetic NPs to the magnetic field. Although the formulation composed by stigmasterol-encapsulated LPs lead to a inhibition of 92% of *S. aureus* colonies, it were the LPs encapsulating only magnetic NPs that showed the higher inhibiting growth. With this formulation, in the presence of the magnetic field at 41 °C, a 98% of inhibition of the *S. aureus* colonies was achieved, corresponding to a minimum inhibitory concentration (MIC) of 8.4 µg/mL.

Concerning the use of MLPs for *S. aureus* biofilm eradication, to the extent of our knowledge, no experimental work has been reported. Therefore, the proposed work represents an innovative approach on two levels: by exploring MLPs, as well as their fusogenic abilities, as a potential strategy for biofilms disruption, alternatively to antibiotics.

Chapter 4

Materials and Methods

In this chapter, the used methods for the synthesis, characterisation and optimisation of the SPIONs and MLPs formulations are explained and described.

First, in Section 4.1, the microwave assisted method for the synthesis of SPIONs is described. Then, in Sections 4.2 and 4.3, the preparation of fusogenic LPs and MLPs, by the thin lipid film hydration method, is explained. The characterisation methods, such as DLS, FTIR and the developed iron quantification method, are also explained in Section 4.4. The performed preliminary assays and the subsequent optimisation of the MLPs formulation are described in Sections 4.5 and 4.6, respectively. Considering the optimised formulation, the MTT assay carried out for the evaluation of the citotoxicity is also explained in Section 4.7.

In Table A.4, a list of the used reagents, along with their purity and brand, is presented.

4.1 Synthesis of SPIONs

SPIONs were prepared using a microwave-assisted synthesis. Briefly, it is based on the traditional co-precipitation technique associated with a microwave heating. The co-precipitation technique is a simple method in which iron oxides can be synthesised through the co-precipitation of Fe^{2+} and Fe^{3+} aqueous salt solutions by addition of a base. Conventionally, magnetite (Fe_3O_4) is prepared by adding a base to an aqueous mixture of Fe^{2+} and Fe^{3+} chloride at a 1:2 molar ratio, according to the following equation (Laurent et al., 2008).



The main advantage of the introduction of microwaves into the reaction system is the extremely rapid kinetics of crystallisation. Hence, microwave-assisted synthesis is faster and very energy efficient, compared to the traditional co-precipitation method (Sreeja and Joy, 2007).

In this work, 250 mg of ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and 138 mg of ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) were dissolved in 20 mL of double distilled water, in a glass vessel, under vigorous magnetic stirring. In a closed hotte, 3mL of 25% ammonium hydroxide (NH_4OH) were added, constantly and drop-wise, to the ferrous solution, creating a black solution.

The glass vessel was then sealed with a Teflon cap and removed from the stirring, for the precipitation to occur. The resultant suspension was placed in the microwave system, setting the following parameters: Temperature: 100°C; Stirring: maximum; Time: 10 minutes; Maximum wattage: 300 W; Maximum pressure: 200 psi.

After this time in the microwave system, back in a closed hotte, the solution was placed in a 50 mL Falcon® and it was added water as needed. In this phase, by approaching a strong permanent magnet, it was possible to quickly dispose all the supernatant, remaining just the black particles aggregates near the magnet. These black particles were then resuspended in 25 mL of double distilled water. The disposal and resuspension were performed two more times.

The final SPIONs formulation was stored at 4 °C.

4.2 Preparation of Fusogenic Liposomes

Since the aim was to produce LPs with enhanced fusogenic ability, based on the literature review, the formulation DOPE/DPPC/CHEMS (4:2:4) was chosen.

The used method for the preparation of LPs was the thin film hydration method. This method comprises three main steps, as showed in Figure 4.1: (1) the preparation of the lipid film, (2) the hydration of the film and (3) a size reduction step.

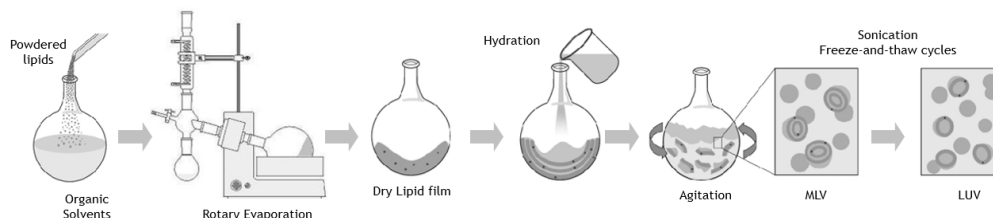


Figure 4.1: Scheme of the preparation of LPs by the thin film hydration method.

First, the adequate amount of lipids was dissolved in a solution of methanol/chloroform (2:3) to assure a homogeneous mixture. The solvents were then removed through rotary evaporation under nitrogen stream, followed by 1h of vacuum environment, to ensure the complete removal of the organic solvents. The produced thin lipid film could be stored at -20 °C until hydration.

The hydration was then made with phosphate buffered saline (PBS) under vigorous agitation, leading to the spontaneous formation of MLVs. In order to create a LUVs suspension, freeze-and-thaw (FT) cycles (freeze with dry ice and thaw in hot water at 55°C, ten times) or ultrasonication (sonicator Vibra Cell™, 70% of amplitude) or both were performed. The resulting formulation was characterised using the techniques explained in Section 4.4.

4.3 Preparation of the Magnetoliposomes

For the preparation of the MLPs, the thin film hydration method was also applied, using the same lipid composition (DOPE/DPPC/CHEMS (4:2:4)).

The first step of the procedure was also the preparation of the lipid film. Two different methods were tested, resulting in films with different compositions: (1) films only with DOPE/DPPC/CHEMS (4:2:4); (2) films with DOPE/DPPC/CHEMS (4:2:4) incorporating SPIONs.

Films (1) were produced using the same methodology described for empty LPs (Section 4.2), and SPIONs were added to the formulation in the hydration step, along with the PBS buffer.

For films (2), a defined volume of SPIONs suspension was dried using a nitrogen stream and then resuspended in methanol/chloroform (2:3). The resuspended SPIONs were added to the mixture of lipids, also in methanol/chloroform (2:3), and the organic solvents were removed through rotary evaporation under nitrogen stream, followed by 1h of vacuum environment. In this case, the subsequent hydration step was performed with PBS buffer only.

After the formation of the MLVs, in both cases, the size reduction step were also carried out. Depending on the formulation intended to be prepared, FT cycles (freeze with dry ice and thaw in hot water at 55°C, ten times) and ultrasonication, or only ultrasonication, were performed.

Beyond the previous referred three steps, for MLPs, an additional purification step was required, in order to separate the SPIONs-encapsulated LPs from free SPIONs. It was performed through a size-exclusion chromatography (SEC), in a Sephadex column, in which a fraction of 1 ml of the formulation was added. The volume containing the SPIONs-encapsulated LPs was collected to further characterisation.

4.4 Characterisation of Formulations

4.4.1 Dynamic Light Scattering

DLS, also known as Photon Correlation Spectroscopy, is one of the most popular methods used to determine the size of particles in a suspension. It measures the fluctuations in the intensity of the light scattered by particles in a colloidal solution, in a microsecond time range, which are directly related to the Brownian motion of the solute (Berne and Pecora, 2000).

4.4.1.1 Particle size and Polydispersity index

Diffusion of larger particles will be slower than smaller particles and, measuring the time dependence of the scattered light, the DLS equipment generates a correlation function that can be mathematically linked to the particle size (NanoComposix, 2012). DLS can also determine the polydispersity index (PdI), which is a measure of the width of the particle size distribution (NanoComposix, 2012).

PdI less than 0.2 are typically referred to monodisperse suspensions, in which the particles have approximately the same size. Otherwise, PdI higher than 0.2 are referred to polydisperse colloidal solutions (Nobmann, 2016) (NanoComposix, 2012).

The result package of a DLS measurement typically includes sizing distributions by intensity, volume and number. Since the intensity distribution is what is actually measured, this is the most accurate and reliable distribution. The other distributions are also very useful but to generate that data, there are more assumptions involved, such as optical properties of the material, which are not often known (Instruments, 2012). Thus, the intensity distribution simply describes how much light is scattered by the particles in different size bins.

In this work, a Nanobrook ZetaPALS (Zeta Potential Analyser Instruments) was used for the characterisation of the size and size distribution of the samples, using 6 runs of 2 minutes, for each sample, at room temperature.

4.4.1.2 Phase Transition Temperature

The phase transition temperature (T_m) of the empty LPs and the MLPs were also obtained by DLS measurements, through the analysis of the variation of the count rate with temperature. For these assays, two different formulations were prepared: one composed by MLPs with the considered optimal lipid concentration (15 μM), sonication time (0.5 min) and SPIONs volume (0.5 ml); and the equivalent formulation composed only by empty LPs.

Nanobrook ZetaPALS (Zeta Potential Analyser Instruments) was also used to perform these measurements. The suspensions were diluted to a lipid concentration of 0.25 μM . The cuvettes were filled with approximately 3 ml of the formulations and placed in the DLS equipment. In an automatic mode, the temperature was allowed to increase between 7 $^{\circ}\text{C}$ and 50 $^{\circ}\text{C}$, with temperature steps of 1 $^{\circ}\text{C}$, and 3 minutes of temperature stabilisation. For each formulation, the 'normalised mean count rate vs. temperature' plot was obtained.

These plots typically fit sigmoid curves from which it is possible to extract a value of T_m from the inflection point, as well as the cooperativity (B) parameter from the slope of the curve. With this in mind, the obtained data was fitted to sigmoid curves, using the following equation (Grancelli et al., 2002):

$$r_{SS} = r_{S1} + p_1 T + \frac{r_{S2} - r_{S1} + p_2 T - p_1 T}{1 + 10^{B(1/T - 1/T_m)}} \quad (4.1)$$

where T corresponds to the absolute temperature, T_m is the midpoint of phase transition, B is the cooperativity parameter, p_1 and p_2 represent the slopes of straight lines at the beginning and at the end of the plot, and r_{S1} and r_{S2} represent the intercepting values at the y axis.

4.4.2 Determination of Encapsulation Efficiency

In order to determine the SPIONs encapsulation efficiency (%*EE*), a colorimetric assay for quantification of iron in the nanoformulations, by using 1,10-phenanthroline, was carried out (Braunschweig et al., 2012) (Hedayati et al., 2018) (Costo et al., 2019).

Phenanthroline is a bidentate ligand capable of coordination to metal centres through the nitrogen atoms (Lever, 2004). This chromogenic compound, in the presence of Fe(II) reacts to produce a complex ion (Figure 4.2), which is orange in colour and absorb very strongly in the UV-Vis range, with a maximum absorbance value near 511 nm (Larionova and Bulygina, 2015) (Vakh et al., 2015).

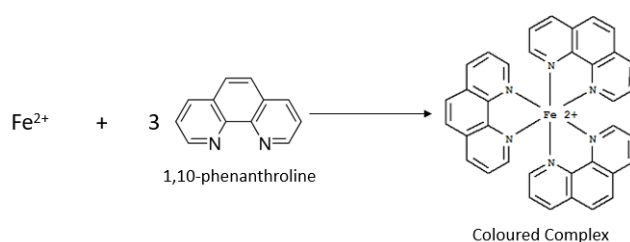


Figure 4.2: Reaction of Fe(II) with 1,10-phenanthroline used for the colorimetric quantification of iron in the formulations. Adapted from Royal Society of Chemistry (2019).

On this basis and considering the properties of the formulations, a quantification protocol was designed. In the first place, a calibration curve was obtained, from which the unknown iron content of the samples was determined. The procedure is explained in more detail below (4.4.2.1 and 4.4.2.2).

4.4.2.1 Samples Quantification

Samples of 0.5 ml of the formulations were digested with 0.5 ml of 1M HCl, for 2h, at 60 °C. The digested samples were then allowed to cool to room temperature.

Since SPIONs are mostly composed by Fe(III), these ions must be quantitatively reduced to Fe(II) by a redundant agent, such as hydroxylamine hydrochloride ($\text{NH}_2\text{OH}\cdot\text{HCl}$) (Royal Society of Chemistry, 2019).

Thus, 0.25 ml of a 10% hydroxylamine hydrochloride solution were added to the samples. After approximately 15 minutes, 0.25 ml of a 0.25% 1,10-phenanthroline solution, was also added.

After 42h at room temperature, 0.7 ml of Milli-Q water were added and the samples were centrifuged for 20 minutes, at 14 000 rpm, leading to the formation of a lipid pellet. The absorption spectrum (between 380 nm and 600 nm) of the supernatant was obtained. The iron concentration values were then calculated using the maximum absorbance value (at 511 nm) by means of a calibration curve obtained from iron standard solutions.

Considering the different volumes of SPIONs used in each formulation, this protocol was applied to the nanoformulations, obtaining the concentration of Fe(II) in the MLPs formulations ($[Fe(II)]_{MLPs}$) as well as the SPIONs' suspension, with the same dilution. From this, it was possible to determine the concentration of Fe(II) in the initial SPIONs suspension ($[Fe(II)]_{SPIONs}$). The %EE was then calculated through the ratio between $[Fe(II)]_{MLPs}$ and $[Fe(II)]_{SPIONs}$.

4.4.2.2 Calibration Curve

50 ml a solution of 100 ppm of Fe(III), prepared from $FeCl_3$, were digested with 2 ml of 1M HCl, during 2h, at 60 °C, with constant agitation. From 100 ml of this digested solution, already at room temperature, 0, 0.5, 1, 2, 4, 6 and 8 ppm of Fe(III) standards were prepared, in 10 ml volumetric flasks. In each volumetric flask, it was also added 0.5 ml of a 10% hydroxylamine hydrochloride solution and 0.5 ml of a 0.25% 1,10-phenanthroline solution.

The Fe(III) ions, that were reduced to Fe(II) by the hydroxylamine hydrochloride, react immediately with phenanthroline to produce a complex ion, leading to the appearance of orange colour. The absorption spectra (between 380 nm and 600 nm) of the standards were obtained and maximum absorbance at 511 nm was considered to build the calibration curve, based on the Lambert-Beer Law of spectroscopy.

This procedure was carried out in triplicate in order to perform the analyses of calibration curve, linearity, range, limit of detection (LOD) and limit of quantification (LOQ) of the method.

4.5 Preliminary Assays

As a first step for the further optimisation of the method, three sets of preliminary assays were carried out. Regarding their possible influence in the size, PDI and encapsulation efficiency (%EE) of the final formulation, the following variables were considered: film preparation method (1 or 2); SPIONs volume (ml); use (C) or not use (S) of FT cycles, lipid concentration (mM); sonication time (ST) (minutes). In Tables A.1, A.2 and A.3, there are specified the different variables for the different formulations prepared in the performed set of assays.

4.6 Box–Behnken experimental design for MLPs optimisation

The method of production of MLPs was optimised by using a design of experiments (DOE) methodology, applying a 3-factor, 3-level Box–Behnken statistical design.

The Box–Behnken design is a response surface experimental methodology (RSM), frequently applied to the optimisation of analytical procedures as a multivariate optimisation strategy. Using this design it is possible, by applying a polynomial mathematical model, to predict the levels of the considered independent variables, also called factors, to achieve the desired responses. It allows not only for testing a large number of factors simultaneously but also avoids the use of a huge number of independent runs required in step-by-step optimisation approaches (Motwani et al., 2008).

Viewing this model as a cube (Figure 4.3), the set of experimental points can be seen as middle points of the edges and central points replicates. In fact, the "missing corners" help to avoid extreme experimental conditions, for which unsatisfactory results might occur (Ferreira et al., 2007) (Motwani et al., 2008).

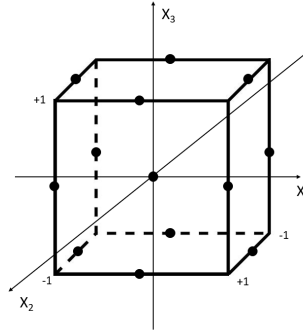


Figure 4.3: Geometric view of a three-factor Box-Behnken design. Adapted from Peixoto et al. (2018).

On the basis of the preliminary assays, the selected factors were the lipid concentration (X_1), the used volume of SPIONs (X_2) and the sonication time (X_3). It was seen that these factors can be considered critical parameters affecting the mean size of the nanoparticles (Y_1), the polydispersity index (Y_2) and the encapsulation efficiency (Y_3). Thus, these variables were selected as dependent variables (or responses) for the application of the experimental design. The factors and their low (-1), medium (0) and high levels (+1) were defined, in accordance with the values performed in the preliminary assays (Table 4.1).

The experimental runs required under Box-Behnken design (N) can be calculated using the Equation 4.2 (Jain et al., 2011),

$$N = k^2 + k + C_p \quad (4.2)$$

where k is the factor number and C_p is the replicate number of central point. Thus, for a $k = 3$, a minimum of 15 experiments, including 12 factorial points at the midpoints of the edges and 3 replicates at the centre point are required (Ferreira et al., 2007) (Elmizadeh et al., 2013). Based on the code ranges of levels of the factors applied in a 3-level, 3-factorial Box–Behnken experimental design, the 15 experimental runs were generated. The replicates are used for estimation of pure error sum of squares (Elmizadeh et al., 2013).

Best-fitting experimental model, among the tested quadratic no interaction, 2-way interaction (linear $\times$ linear), and 2-way interaction (linear $\times$ quadratic) models, were statically evaluated according to the correlation coefficient (R^2) and the adjusted correlation coefficient (adjusted R^2) computed by the StatSoft[®] STATISTICA 12 software. Also the statistical significance of the coefficients of the polynomial equation was evaluated from the p -value.

Table 4.1: Considered factors and responses for the appliance of the Box-Behnken experimental design.

Factors	Level of Variables		
	Low (-1)	Medium (0)	High (+1)
X_1 = Lipid Concentration (mM)	10	20	30
X_2 = SPIONs Volume (ml)	0.5	1.0	1.5
X_3 = Sonication Time (min)	0.5	1.5	2.5
Responses	Goals		
Y_1 = Mean Size (nm)	~100		
Y_2 = PdI	Minimise		
Y_3 = %EE	Maximise		

The obtained results were treated with the StatSoft® STATISTICA 12 software. Response surface plots were drawn and the statistical validity of the model and the polynomials were established. For the optimisation of the nanoformulation, the desirability function (D) approach was applied (Derringer and Suich, 1980). In this approach, each response is changed into individual desirability function (d_i) that can vary between 0 (undesirable response) and 1 (completely desirable response) (Rahman and Nasir, 2018). The desirability values for each response were established aiming a formulation with mean size of the NPs near to 100 nm, PdI as lower as possible as well as maximising the encapsulation efficiency (Table 4.1). D was computed combining the individual desirability values by applying the geometric mean (Equation 4.3) (Ferreira et al., 2007) (Rahman and Nasir, 2018):

$$D = (d_1 \times d_2 \times d_3 \times \dots \times d_m)^{1/m} \quad (4.3)$$

where m is the number of responses. The set of factor values that maximise the D function are obtained, along with the predicted correspondent responses, with an associate confidence range of 95%.

The considered optimal formulations were again prepared, in triplicate, and the obtained responses evaluated in comparison to the predicted responses given by the model. The selected optimal formulation of MLPs along with the corresponding formulation composed by empty LPs were then characterised by DLS measurements, for size distribution, polydispersity, T_m , and UV-vis spectrum determination. Stability studies, through size and PdI measurements, were also performed for both formulations, stored at 4 °C, during 3 days after the preparation.

4.7 Toxicity and Cell Viability Assay

To conclude about the toxicity of the nanoformulations, a MTT assay was performed.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) is a yellow water-soluble tetrazolium dye that is reduced by live cells to a purple formazan product, through mitochondrial activity (Plumb, 2004), as shown in Figure 4.4. For most cell populations, the total

mitochondrial activity can be related to the number of viable cells. So, this assay is frequently used to assess the *in vitro* cytotoxic effects of different substances on cell lines (Plumb, 2004) (Van Meerloo et al., 2011).

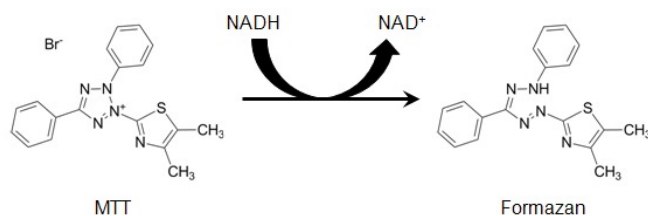


Figure 4.4: Structures of MTT and coloured formazan product. Adapted from Riss et al. (2016).

For the chemosensitivity test, exponentially growing L929 cells (fibroblasts) were harvested into a 50 ml Falcon[®]. The suspension was centrifuged (300g, 5 min) and pellet cells were resuspended in growth medium (DMEM), for counting. 100 μ l of the diluted suspension (5×10^4 cells/well) were placed into each well of a 96-well plate and incubated in a humidified atmosphere, at 37 °C. After 24h of incubation, the culture medium was replaced for four replicates of five dilutions of three different freshly prepared formulations (SPIONs, MLPs and LPs) as well as a negative control using Triton X-100 solution 1% (v/v).

The treated cells were then incubated during 24h, in a humidified atmosphere, at 37 °C. After that, the formulation was replaced by 100 μ l of a MTT solution 10% (v/v) in each well and the cells were allowed to contact with the MTT solution during 2h, at 37 °C. After this period, 100 μ l of dimethyl sulfoxide (DMSO) were added to each well, in order to solubilise the formazan produced by the living cells, for 15 minutes at 37 °C in a cell culture incubator. Finally, the absorbance at 570 nm, corresponding to the maximum absorbance value of the coloured compound, and the reference absorbance at 630 nm, relative to the solvent, were measured, using a plate reader.

The cell viability percentage was calculated according to the Equation 4.4

$$\text{Cell viability (\%)} = \frac{(Abs_{570\text{ nm sample}} - Abs_{630\text{ nm sample}})}{(Abs_{570\text{ nm untreated cells}} - Abs_{630\text{ nm untreated cells}})} \times 100\% \quad (4.4)$$

Chapter 5

Results and Discussion

In this chapter, the obtained results are presented as well as their analysis and discussion. First, for the determination of the %EE, an iron quantification method was developed. The obtained standard spectra along with the calibration curve and its validation are shown in Section 5.1. Considering the properties of the suspensions, an optimisation of the method was carried out by evaluating the absorbance of samples with known iron content over time. Before the preliminary results, a simple characterisation of synthesised SPIONs is made in Section 5.2. With the conclusions drawn from the preliminary assays, an experimental design was applied, and in Section 5.4 the chosen model and predictions are validated for the optimisation of the properties of the final formulation of MLPs. In Section 5.5, a characterisation of the optimal formulation is carried out, namely in terms of size, polydispersity and T_m . After the characterisation, the cytotoxicity results are presented in Section 5.6.

5.1 Iron Quantification Method

5.1.1 Calibration Curve

A phenanthroline-based colorimetric assay was developed to quantify the total iron content of the formulations, as a mean of obtaining %EE values. For this to be possible, a calibration curve was obtained using $FeCl_3$ standards ranging from 0.5-8 ppm of Fe , prepared in triplicate. This range of concentrations was selected covering low, medium and high concentrations in order to include the samples concentration, from the SPIONs suspensions (high iron concentration) to final MLPs formulations (lower iron concentration). From the obtained UV-Vis spectra, it is possible to observe that, as reported in literature, the maximum value for the absorbance was found at 511 nm for all the standards. Besides that, it is clear that the increase of the iron concentration of the standards lead to an increase in the absorbance at the referred wavelength.

To confirm the linearity of this relation between the peak absorbance and the iron concentration, a calibration curve was built, by plotting the absorbance values at 511 nm (y) *versus* the iron concentration (x), in ppm. Then, a linear regression was applied to the experimental data. The obtained calibration curve is represented in Figure 5.1.

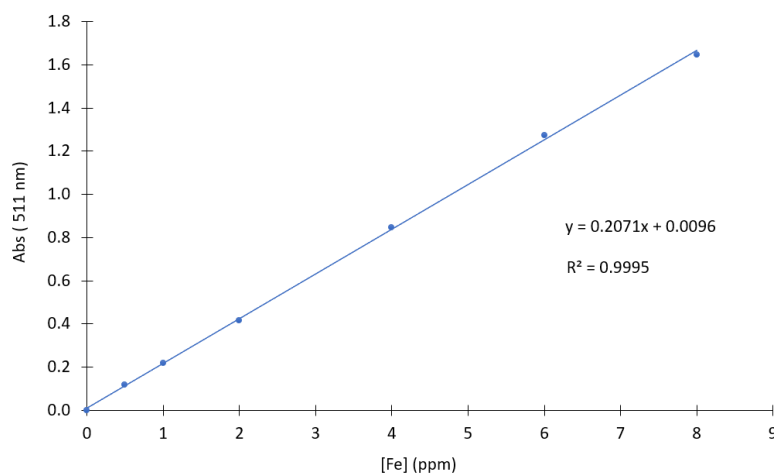


Figure 5.1: Calibration curve (0.5-8 ppm *Fe*) measured at the peak absorbance for the coloured compound (511 nm).

The linearity of the calibration curve was assessed through the regression equation ($y = (a \pm ts_a)x + (b \pm ts_b)$) and the corresponding correlation coefficient (R^2), calculated by the least squares method. Considering the standard deviations of a and b and a confidence level of 90% for 4 ($n - 2$) freedom degrees, through the t -Student value, the calculated confidence intervals of a (s_a) and b (s_b) were 0.0024 and 0.0109, respectively. Thus, the obtained linear regression was $y = (0.2071 \pm 0.0052)x + (0.0096 \pm 0.0233)$ with $R^2 = 0.9995$.

Since the correlation coefficient is greater than 0.999, it means that there is a good correlation of linearity through all the concentrations used. The limits of quantification (LOQ) and detection (LOD) were also mathematically calculated by the relationship between the standard deviation (s_d) of the calibration curve and its slope (S), represented in Equations B.1 and B.2.

The obtained LOQ was 0.53 ppm which means that, with this method, it is possible to quantify iron concentrations as low as this value. On the other hand, the calculated LOD of 0.16 ppm indicates the minimum concentration from which it is possible to assess the presence of iron in the samples.

In conclusion, we can affirm that a linear equation was fitted to the experimental data, according to the Lambert-Beer law. Moreover, the obtained linear correlation was considered valid for the quantification of the total content of iron on the formulations, since LOQ is higher than the minimal concentration found in any sample.

5.2 Characterisation of SPIONs

In the present work, SPIONs were synthesised by a microwave-assisted method. The procedure was very simple and fast, due to the extremely rapid kinetics of crystallisation achieved through

the use of microwave heating. Another advantage of this process is the production of easily reproducible results due to the well-defined and previously optimised parameters introduced in the microwave system.

During the implementation of the protocol, after the addition of the NH_4OH to the ferrous solution, it was possible to detect an evident colour change to black, in consequence of the formation of SPIONs. Then a strong permanent magnet was put near the final formulation. It was observed a movement of the black particles towards the magnet (Figure B.7). After the removal of the magnet and, with some agitation, it was possible to disperse the aggregate, obtaining the suspension as it was before.

The characterisation of the size of the obtained SPIONs was not possible to perform using DLS because, at very small sizes (< 20 nm), the nanoparticles are mostly absorbing and scatter very little light. Although it has not been possible to measure SPIONs size, it is estimated that the size of the produced SPIONs varies in the range of less than 10 nm to greater than 40 nm, based on previous TEM analyses reported on literature for similar experimental conditions (Iida et al., 2007) (Hui et al., 2008).

Another fundamental aspect associated with SPIONs produced by co-precipitation methods is the possible aggregation and coagulation of obtained NPs. As the procedure does not include surfactants and leads to the production of extremely fine nanocrystals in a high ionic strength aqueous medium, the SPIONs tend to aggregate during the synthesis, due to their high surface energy (Yoffe et al., 2013). Consequently, a reduction of magnetisation capacity and degradation of the NPs can occur. In the case of the SPIONs produced in this work, aggregation or coagulation were not visible, in this phase.

However, as a future approach, the colloidal stability of these NPs should be taken into account. Thus, as a mean of increasing the shelf-life of SPIONs suspensions, surface coating could be a possibility. The appropriate coating would allow not only to reduce oxidation reactions of these NPs, preserving the molecular structure of magnetite, but also to decrease the aggregation phenomenon, as well as improve the biocompatibility.

Nonetheless, considering that, in this work, SPIONs will be encapsulated in the aqueous vesicles of liposomes, the protective lipid bilayer is expected to increase the biocompatibility as well as minimise iron oxidation reactions. Hence, the use of uncoated SPIONs for this application was considered accurate. However, in this scenario, aggregation may still be a concern.

5.3 Preliminary Results

Using a freshly prepared SPIONs suspension for the production of MLPs, three sets of preliminary assays were carried out. As a first step for the optimisation of the preparation method, several parameters and some steps of the process were changed, in order to evaluate their influence in the final formulation properties. As a mean of quantitatively evaluating the properties of these formulations, the mean size and size distributions as well as the PdI and the %EE values were obtained and analysed. From these results some conclusions were drawn regarding the effect of

the sonication time, the lipid concentration, the SPIONs volume, the use of FT cycles and the film preparation method on the process.

5.3.1 Assay 1

In the first assay, three different sonication times were tested, for both film compositions, using 2 ml of formulation with a lipid concentration of 3 mM, a SPIONs volume of 0.5 ml and using FT cycles. At this stage, only mean size and PdI were measured, immediately after the ultrasonication step, in order to conclude about the achieved size reduction for each formulation. The DLS results for the mean size and PdI of each formulation are presented in Table 5.1.

Table 5.1: Mean size and PdI of formulations prepared in preliminary assay 1, measured after the ultrasonication step.

Formulation	ST (min)	Film Method **	Mean Size $\pm$ SD (nm) *	PdI $\pm$ SD *
L1	0.5	1	189.6 $\pm$ 4.6	0.194 $\pm$ 0.006
L2	1.0		174.9 $\pm$ 2.5	0.175 $\pm$ 0.011
L3	1.5		233.5 $\pm$ 3.0	0.254 $\pm$ 0.060
S1	0.5	2	178.1 $\pm$ 1.2	0.198 $\pm$ 0.040
S2	1.0		174.3 $\pm$ 1.8	0.183 $\pm$ 0.003
S3	1.5		197.2 $\pm$ 1.1	0.238 $\pm$ 0.006

* Measured after the ultrasonication step

** Film Method 1 - lipid film; Film Method 2 - lipid film incorporating SPIONs

Constant parameters: Lipid concentration = 3 mM; SPIONs volume = 0.5 ml; Using FT cycles

After MLVs encapsulating SPIONs are formed, by the hydration of the lipid films, they may be reduced in size by ultrasonication. This method uses acoustic energy to induce larger LPs fragmentation in the so-called bilayer phospholipid fragments (BPF) (Korgel et al., 1998). These fragments then fuse to fold up into thermodynamically stable LPs with a smaller size that may be either unilamellar or multilamellar in composition (Woodbury et al., 2006) (Lapinski et al., 2007). The time over which lipid solutions are sonicated determines the size of the vesicles. The result of such random, uniform fragmentation can normally be fitted using the Weibull distribution, which describes a narrow unimodal size distribution of particles, similar to the lognormal distribution (Tenchov and Yanev, 1986).

From the obtained results, it is possible to observe that, for both film preparation methods, lower values of mean size and PdI were obtained for the intermediate sonication time (1 min). It seems that the reduction in size that takes place using 0.5 minutes or 1.5 minutes is not so efficient. In fact, for longer sonication times, it was expected more effective size reductions as well as a decrease in polydispersity of the suspension. Thus, to better understand the size reduction phenomenon, a deeper analysis to the size distribution plots was carried out.

The corresponding size distributions obtained by DLS for all formulations are presented in Figure B.3.

In all distributions, it is possible to identify two different and well-defined populations. These bimodal distributions can not be adjusted to the referred Weibull distribution, which means that the dynamics observed during the ultrasonication cannot be described by the previous explained

mechanism. Besides that, it seems that the increase in the mean size observed for the higher sonication time is caused by the increase in the mean size of both populations.

In fact, the presence of the magnetic NPs or even the lipid composition can be responsible for this phenomenon. On one hand, the ultrasonic treatment not only induce fragmentation of the phospholipids but also the dispersion of the SPIONs, inside and outside the LPs, interfering in the rearrangement process of the LPs (Kharisov et al., 2014). Also, the presence of possible aggregates in the SPIONs suspension can lead to the formation of larger LPs around them.

On the other hand, the used lipid mixture can lead to the formation of structures other than LPs. DOPE is a cone-shaped lipid with a small phosphorylethanolamine (PE) head group and two unsaturated (18:1) hydrocarbon chains. Considering the molecular structure of DOPE, it is known that this lipid alone cannot form a LPs, because its inverted cone-shape leads to a tendency to adopts inverted hexagonal phase (Liu, 2011). So, in order to form stable LPs with this lipid, materials containing titratable acidic groups must be added. In the case of the used formulation, cholesteryl hemisuccinate (CHEMS) and DPPC, in a defined proportion, were chosen to stabilise DOPE in the produced bilayers (Li et al., 2015). Nonetheless, if the right proportion of lipids is not achieved, the nature of the used lipid may induce the formation of other structures.

Moreover, it can be also seen from the size distributions that, for all formulations, populations corresponding to the highest intensity presented higher mean diameters (varying between 220.50 and 434.18 nm), while populations relative to the smaller intensities had smaller mean sizes (varying between 63.35 and 129.27 nm). Considering the possible final application of the MLPs in biofilms eradication, it was desired that a great amount of the NPs in the suspension remain with size close to 100 nm, allowing a easier penetration through the EPS matrix. Since the presented size distributions are in function of the intensity, it means that, for all formulations, the population with smaller sizes represents the majority of the NPs in the suspensions, as desired.

However, the reported rapid clearance of circulating LPs from the bloodstream, along with their high uptake by liver and spleen, can be considered an obstacle to the targeting to biofilms in the human body (Gabizon and Papahadjopoulos, 1988). Thus, to overcome this problem, techniques based on surface modification have been developed in order to increase the LPs circulation time. For instance, polyethyleneglycol (PEG) has often been used not only to avoid opsonisation but also to lower the toxicity, by improving the stability and pharmacokinetics characteristics of the LPs (Nunes and Barros, 2015). Nonetheless, the addition of molecules to the surface of LPs increases their overall size, complicating their intravenous administration and also the penetration in the EPS matrix of the biofilms. So, considering the functionalisation of the MLPs a possibility as a future approach, non-functionalised LPs produced, in this stage, should present a size close to 100 nm or lower (Bozzuto and Molinari, 2015). Since the obtained size distribution always presented a population of larger MLPs, a mean size of 200 nm was considered accurate.

From this assay, it was possible to verify that, for the selected lipid concentration and SPIONs volume, also using FT cycles, 1 minute of ultrasonication was enough to achieve suitable size and polydispersity results. This result were observed for both film preparation methods. Therefore, formulations L2 and S2 were selected to continue the procedure.

The formulations L2 and S2 were subject to SEC and new measurements of mean size and PDI were carried out. The obtained results for both formulations are shown in Table 5.2 and the size distributions presented in Figure B.6.

Table 5.2: Mean size and PDI of the final formulations L2 and S2, prepared in preliminary assay 1, after SEC.

Formulation	Mean Size $\pm$ SD (nm)	PDI $\pm$ SD
L2	311.1 $\pm$ 3.3	0.172 $\pm$ 0.015
S2	246.0 $\pm$ 3.8	0.204 $\pm$ 0.012

The size distribution remained bimodal with narrow populations of different mean sizes. It means that the collected volume from the SEC, corresponding to the darker fraction visible during the separation (Figure B.5), allows the recovery of both populations of size populations already present before SEC. However, this separation step led to an increase in the mean size by an increase in both mean sizes of these two populations.

This may be explained by the retention of the smaller LPs inside the pores of the resin. In fact, during the elution, the column becomes darker, as showed in Figure B.4. This may suggest the retention of SPIONs and other smaller structures associated with SPIONs such as larger SPIONs aggregates or smaller MLPs. This phenomena and the rearrangement of the liposomal structures promoted by the chromatographic process can explain the variation in the mean size of the NPs caused by SEC.

At the end of the procedure, the final formulations presented PDI values smaller than 0.3 and mean sizes, for the numerous population, of about 100 nm, as desired.

5.3.2 Assay 2

In the experiments performed in assay 2, four different formulations were prepared with the aim of evaluating the effect of the FT cycles, while comparing the two film preparation methods. 10 FT cycles were performed in formulations A2 and B2 (C), while for formulations A1 and B1, FT cycles were not used (S). The lipid concentration was increased to 15 mM and 1 ml of the SPIONs suspension was used. Each formulation was prepared in duplicate to evaluate the reproducibility of the obtained results in terms of mean size, PDI and %EE. The obtained results are shown in Table 5.3.

Hydration of lipid films results in polydisperse MLVs suspensions (Colletier et al., 2002). FT cycling is a technique frequently used not only to increase encapsulation efficiency, but also to reduce lamellarity of the formed MLVs and to reduce the polydispersity of the nanosystem, by promoting the disruption and subsequent fusion of the liposomal bilayer (Costa et al., 2014). The performed procedure was freezing the LPs suspension with dry ice and thawing at 55 °C, a temperature above the phase transition temperature of the lipids.

However, comparing mean size values between formulations with FT cycles and without this step, it is possible to observe that no effective improvement in size, PDI or %EE values were achieved in the formulation in which the 10 FT cycles were performed. In fact, the highest %EE

Table 5.3: Mean size, PDI and encapsulation efficiency (%) of the final formulations, prepared in preliminary assay 2.

Formulation	FT Cycles	Film Method *	Mean Size $\pm$ SD (nm)	PdI $\pm$ SD	%EE
A1	S	1	393.4 $\pm$ 3.5	0.314 $\pm$ 0.010	24.4
	S		527.9 $\pm$ 23.7	0.269 $\pm$ 0.014	10.1
A2	C		225.7 $\pm$ 5.7	0.329 $\pm$ 0.006	14.1
	C		544.3 $\pm$ 5.5	0.270 $\pm$ 0.006	13.3
B1	S	2	364.7 $\pm$ 2.3	0.228 $\pm$ 0.008	17.4
	S		235.1 $\pm$ 2.2	0.208 $\pm$ 0.010	19.6
B2	C		304.2 $\pm$ 4.7	0.246 $\pm$ 0.019	13.9
	C		183.3 $\pm$ 2.3	0.221 $\pm$ 0.007	18.6

* Film Method 1 - lipid film; Film Method 2 - Lipid film incorporating SPIONs

Constant parameters: Lipid concentration = 15 mM; SPIONs Volume = 1 ml; ST = 1 min

values (24.4% and 19.6%) were obtained, respectively, for formulations of type A1 and B1, in which FT cycles were not performed.

It is known that the number of FT cycles required to increase encapsulation depends on the system and varies greatly in the literature. However, it is reported that a significant effect is achieved using up to 10 cycles (Hwang et al., 2012). Maybe, the number of cycles or the duration of each cycle were not enough for a significant improvement in the tested formulations. Thus, since the effect of the cycles was not noticed, this step was excluded from the procedure to be optimised, in an attempt of simplifying the method.

The film preparation method was also evaluated, comparing the results of the formulations of type A1 and A2 with the type B1 and B2 (Table 5.3). Both mean size and PDI presented lower values when SPIONs are included in the lipid film (method 2). Moreover, it seems that encapsulation is also favoured by the incorporation of SPIONs in the lipid film. %EE values for method 2 were globally higher and presented less variability between the replicates.

From these results, it was possible to make a first choice related to the exclusion of FT cycles and then verify that, with method 2, more suitable and consistent results could be obtained.

5.3.3 Assay 3

To verify the reproducibility of the previous results, a new assay was carried out, testing the two film preparation methods, using the same lipid concentration (15 mM), SPIONs volume (1 ml), sonication time (1 min) and without FT cycles. The results are presented in Table 5.4.

Table 5.4: Mean size, PDI and encapsulation efficiency (%) of the final formulations, prepared in preliminary assay 3.

Formulation	Film Method *	Mean Size $\pm$ SD (nm)	PdI $\pm$ SD	%EE
F1	1	175.3 $\pm$ 0.9	0.254 $\pm$ 0.007	9.6
F2	2	267.3 $\pm$ 0.6	0.267 $\pm$ 0.002	23.1

* Film Method 1 - lipid film; Film Method 2 - lipid film incorporating SPIONs

Constant Parameters: Lipid concentration = 15 mM; SPIONs Volume = 1 ml; ST = 1 min; Without FT Cycles

For both methods, the range of the obtained mean size and PDI values were accurate and similar to the previous equivalent formulation types (A1 and B1). As desired, PDI values were lower than

0.3. Again the %EE was higher for the second film composition. Therefore, for the optimisation of the method, the second film preparation method was selected.

As a final observation, it is possible to notice that the highest obtained %EE was 24.4%, in all sets of preliminary assays. In fact, there is a limit in the number of encapsulated SPIONs inside the aqueous volume of the LPs. Moreover, since SPIONs have a hydrophilic surface and they are not coated, their size is increased by the presence of the attached water molecules leading to a larger average hydrodynamic diameter which decrease the number of NPs that could be encapsulated.

5.4 Box–Behnken Experimental Design

With the aim of optimising the MLPs formulations and study the effects of the chosen factors on the final parameters of the nanoformulations, a 3-factor, 3-level Box–Behnken experimental design was applied. On the basis of the preliminary results, the critical effects that seemed to affect the main parameters of evaluation (mean size, Pdl and %EE) were the lipid concentration, the volume of SPIONs and the sonication time. As already said, for the optimisation, the procedure did not included FT cycles and the selected film preparation method was incorporating SPIONs in the lipid film (method 2).

Considering the three selected factors and the defined value of their levels, the 15 experimental runs suggested by the StatSoft® STATISTICA 12 software, applying the referred experimental design, were carried out. The responses for each formulation is presented in the Table 5.5.

Table 5.5: Box-Behnken design matrix and experimental responses, for each generated formulation.

Formulation	Factors			Responses		
	X_1 (mM)	X_2 (ml)	X_3 (min)	Y_1 (nm)	Y_2	Y_3 (%)
R1	10	0.5	1.5	317.2	0.301	18.36
R2	30	0.5	1.5	182.4	0.302	21.62
R3	10	1.5	1.5	338.7	0.300	2.96
R4	30	1.5	1.5	361.6	0.304	8.21
R5	10	1.0	0.5	273.1	0.303	17.78
R6	30	1.0	0.5	184.5	0.295	25.80
R7	10	1.0	2.5	178.6	0.333	12.99
R8	30	1.0	2.5	130.4	0.295	15.69
R9	20	0.5	0.5	195.0	0.259	14.24
R10	20	1.5	0.5	284.5	0.311	14.78
R11	20	0.5	2.5	145.8	0.318	18.75
R12	20	1.5	2.5	275.0	0.352	12.49
R13	20	1.0	1.5	210.5	0.344	12.10
R14	20	1.0	1.5	246.6	0.333	15.53
R15	20	1.0	1.5	206.3	0.342	13.32

X_1 - Lipid concentration (mM); X_2 - SPIONs Volume (ml); X_3 - Sonication Time (min)

Y_1 - Mean Size (nm); Y_2 - Pdl; Y_3 - Encapsulation Efficiency (%)

As shown, the values of the measured responses varied between 130.4 nm and 361.6 nm, for the mean size (Y_1), from 0.259 to 0.352 for Pdl (Y_2), and between 2.96 and 25.80% for the %EE (Y_3). Considering the obtained ranges for all the responses and the desired values for the final

optimal formulation, the selected levels for the three factors could be considered accurate for the application of the experimental design.

5.4.1 Fitting of Data to the Model

The experimental data obtained from Box–Behnken design experiments were first fitted to three different models: no interaction, 2-way interactions (linear $\times$ linear) and 2-way interactions (linear $\times$ quadratic) models. The suitability of models to describe each response was evaluated by comparing model R^2 and adjusted R^2 (Table 5.6).

Table 5.6: Summary of results of regression analysis for the responses Y_1 , Y_2 and Y_3 .

Response	Models	R^2	Adjusted R^2
Y_1	No interactions	0.8689	0.771
	2-way interactions (linear $\times$ linear)	0.970	0.915
	2-way interactions (linear $\times$ quadratic)	0.986	0.901
Y_2	No interactions	0.734	0.534
	2-way interactions (linear $\times$ linear)	0.771	0.358
	2-way interactions (linear $\times$ quadratic)	0.992	0.942
Y_3	No interactions	0.682	0.444
	2-way interactions (linear $\times$ linear)	0.730	0.245
	2-way interactions (linear $\times$ quadratic)	0.985	0.896

Y_1 - Mean Size (nm); Y_2 - PdI; Y_3 - Encapsulation Efficiency (%)

It is known that high R^2 values indicate a satisfactory representation of the process by the model and a good correlation between experimental and theoretical values provided by the model equation. However, a high R^2 does not necessarily imply that the model is capable of yield good predictions of new observations. Therefore, it is also beneficial to calculate adjusted R^2 in order to conclude about the goodness of the prediction, along with the goodness of the fit given by the regular R^2 . So, a good prediction model should have R^2 values, at least, of 0.80 and adjusted R^2 values as close as possible to the corresponding R^2 (Sahu and Andhare, 2018).

Based on these criteria, the best-fitted model was the 2-way interactions (linear $\times$ quadratic) model, showing R^2 values > 0.98 for all the regression equations generated and higher adjusted R^2 values, comparing with the other models. Moreover, adjusted R^2 were considerably close to the respective R^2 values. It means that the interactions between the considered factors and their effects in the responses presented such complexity that a cubic model must be used for the fit and for achieve a valid prediction.

After the selection of the model, the polynomial equations involving the main effects and interaction factors for mean size, PdI and %EE were obtained. The regression coefficients are presented in Table 5.7, along with their p -values for a 95% confidence level.

Table 5.7: Regression coefficients for the mean size, PdI and %EE, and the corresponding p -value (95% confidence level), applying the 2-way interaction (linear $\times$ quadratic) model.

Factor	Mean size (nm)		PdI		%EE	
	Coefficient	p -value	Coefficient	p -value	Coefficient	p -value
Intercept	738.956	0.0665663	0.242469	0.044157	83.5766	0.033286
X_1	-36.498	0.171813	-0.014292	0.090505	-7.4665	0.032085
X_2	-463.592	0.255997	0.225167	0.1001669	-39.3200	0.230888
X_3	11.950	0.884118	0.016375	0.482698	-20.5438	0.068749
X_1X_2	6.505	0.750615	0.006950	0.279070	5.1595	0.066530
X_1X_3	10.000	0.256677	0.006250	0.065471	1.5790	0.087149
X_2X_3	19.850	0.464816	-0.009000	0.264335	-3.4000	0.189690
$X_1X_2^2$	2.490	0.729495	0.005100	0.091360	-0.2210	0.697148
$X_1^2X_2$	-0.090	0.800946	-0.000425	0.035977	-0.1154	0.042489
$X_1^2X_3$	-0.225	0.287834	-0.000175	0.051742	-0.0428	0.073526
X_1^2	0.654	0.250232	0.000480	0.046984	0.1903	0.027185
X_2^2	174.733	0.320871	-0.170833	0.040185	-3.3050	0.782352
X_3^2	-52.192	0.045496	-0.012458	0.055013	3.3463	0.065963

The effect of the factors in the dependent variables can be inferred from the magnitude and the sign of the regression coefficients. Effects were considered statistically significant if their p -value were ≤ 0.05 , for the considered confidence interval. Additionally, a positive sign indicates that the value of the response increases with an increase in the level of the factor (synergistic effect). For the opposite situation, a negative sign (antagonistic effect) means that an increase in the level of a factor leads to a decrease in the value of the response.

Thus, analysing the Table 5.7 values, it is possible to deduce that the lipid concentration (X_1) has a significant antagonistic effect on %EE (Y_3). It means that the encapsulation is not favoured by a linear increase of the lipid concentration, considering the same sonication time and the same SPIONs volume.

Looking at the interactive coefficient between the quadratic term for lipid concentration and the SPIONs volume ($X_1^2X_2$), another conclusion can be drawn. It seems that this term presents an antagonist effect on %EE, being more sensitive to lipid concentration than to SPIONs volume. However, an increase of any of these factors lead to a decrease in %EE. However, if we exclude the term corresponding to SPIONs volume, the quadratic term of lipid concentration (X_1^2) starts to favour the encapsulation. So, it is possible to conclude that depending on the magnitude of the increase, lipid concentration can decrease or increase the encapsulation efficiency. From this, we can say that, in general, an increase in the lipid concentration does not lead to better encapsulation efficiencies, even conjugating this increase with a higher SPIONs volume.

Additionally, it is also possible to observe that the PdI values increase with a quadratic increase of the lipid concentration (X_1^2). Therefore, high lipid concentrations do not seem to present advantage in the properties of the final formulation.

The SPIONs volume (X_2) also seems to present a nonlinear relationship with the PdI values, since the corresponding regression coefficient present a p -value lower than 0.05. Thus, it is possible to infer that an increase in the SPIONs volume will lead to a decrease in the PdI of the formulations. The linear term of this factor conjugated with the quadratic term of the lipid concentration ($X_1^2X_2$)

has also statistical significance, and presents a negative sign for PdI values. This means that, increasing the overall cubic term, by increasing the lipid concentration or the SPIONs volume, it is possible to obtain lower PdI values, being the lipid concentration more relevant in this effect.

The effect of the sonication time (X_3) is only statistical significant for the mean size of the NPs, specifically for the quadratic regression coefficient. It presents a high magnitude as well as negative sign, which means that while sonication time increases, the mean size of the NPs strongly decrease, as expected.

From this analysis, it was possible to understand that the individual effects and the interactive effects of the selected factors on the responses are complex and it can not be inferred only from the significant coefficients of the regression equations. It allows an interesting analysis of the significance of the factors on the final prediction. However, opposite effects of the same variable can be observed, so it is difficult to draw valid conclusions from that. Therefore, another type of analysis of the interaction of the factors was carried out, by plotting response surface plots, relating the factors, in pairs, with a determined response.

5.4.2 Response-Surface Analysis

Three-dimensional response surface plots were drawn for the graphical analysis of the interactive effect of two factors on a responses, at one time, when the third factor is kept at a constant level (Figures 5.2, 5.3 and 5.4).

It is important to highlight that, regardless the considered level of the constant factor, the behaviour of the interactive effect of the two other factors remained equivalent. As it can be inferred by the observation of the plotted surfaces, all the relationships among the three variables were non-linear.

From Figure 5.2, it can be observed that for higher lipid concentrations and lower SPIONs volumes, lower mean sizes can be reached. Decreasing the lipid concentration or increasing the volume of SPIONs, mean size increases. For all lipid concentrations covered by the model, an increase in the sonication time allows a decrease in the mean size, being the values lower for higher lipid concentrations.

A more complex relationship between the SPIONs volume and the sonication time with the mean size was found. It was observed that, regardless the SPIONs volume, lower mean sizes are obtained when very low or high sonication times are used. This effect is more evident for higher amounts of SPIONS.

Considering the analysis carried out for mean size, the optimal formulation should have SPIONs volume below 1.0 ml, high lipid concentrations (close to 30 mM) and sonication times above 2 minutes.

Looking at the effect of lipid concentration and sonication time on the PdI (Figure 5.3a), another conclusion can be draw. It seems that higher lipid concentrations and lower sonication times do not favour the minimisation of the PdI. Moreover, relating SPIONs volume and sonication time with PdI (Figure 5.3b), once more, lower SPIONs volumes and lower sonication times show better results, regarding PdI values. Thus, based on that, lower PdI values are reached using lipid

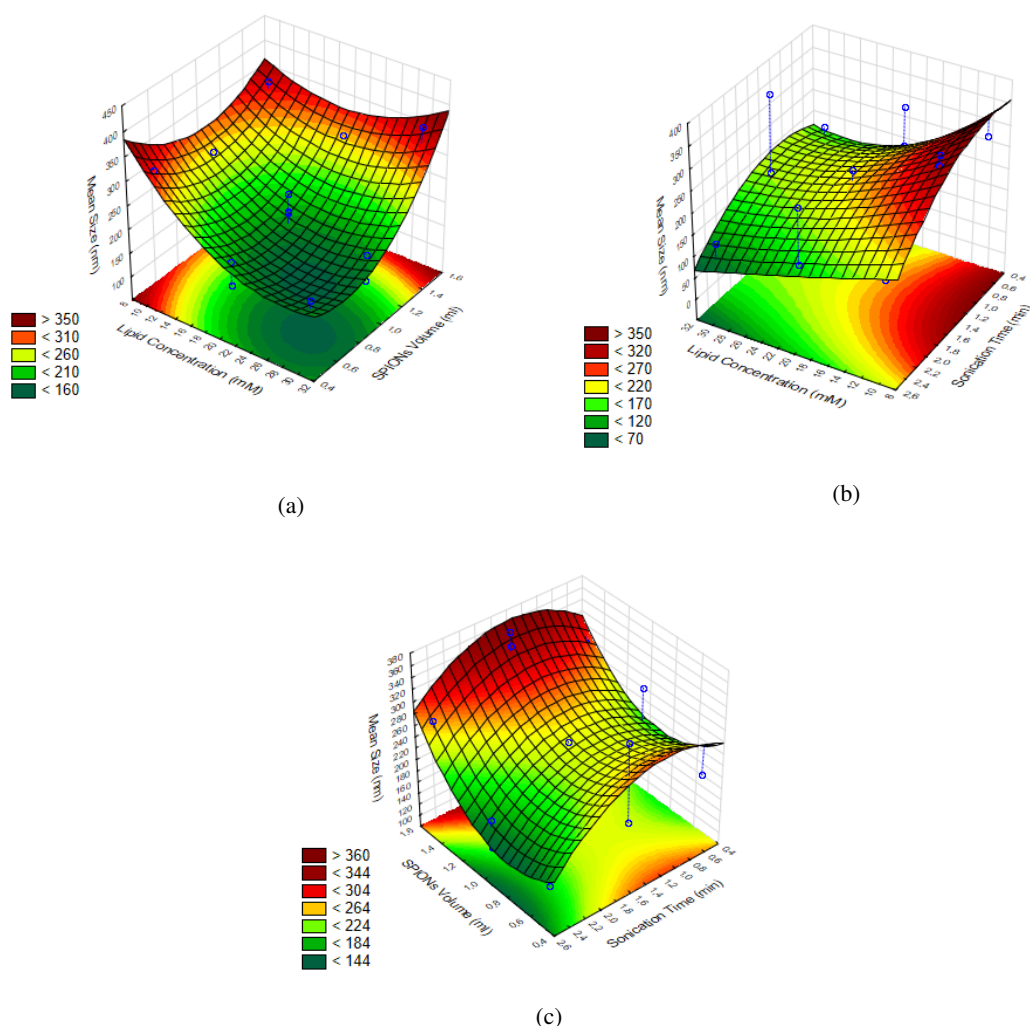


Figure 5.2: Response surface plots showing effect of (a) lipid concentration (mM) and SPIONs volume (ml); (b) lipid concentration (mM) and sonication time (min); (c) SPIONs volume (ml) and sonication time (min) on mean size (nm).

concentrations below 30 mM, sonication times below 0.8 minutes and SPIONs volumes below 0.8 ml.

The quadratic influence of the lipid concentration on the %EE is visible on both Figure 5.4a and Figure 5.4b, for low SPIONs volumes and low sonication times, respectively. It was observed that maximum %EE is obtained for high lipid concentrations and low sonication times, considering SPIONs volumes below 0.6 ml.

From this analysis, it is possible to verify that the optimal formulation must be chosen, not only based on the response surface relationships, since some factors, such as mean size and PdI, presented opposite effects depending on the analysed response. It must be taken into account all the possible interactions that the model predicts. So, in order to obtain the responses as closer as possible to the desired, a balance of all possible effects, considering the 2-way interactions (linear $\times$ quadratic) model, was carried out.

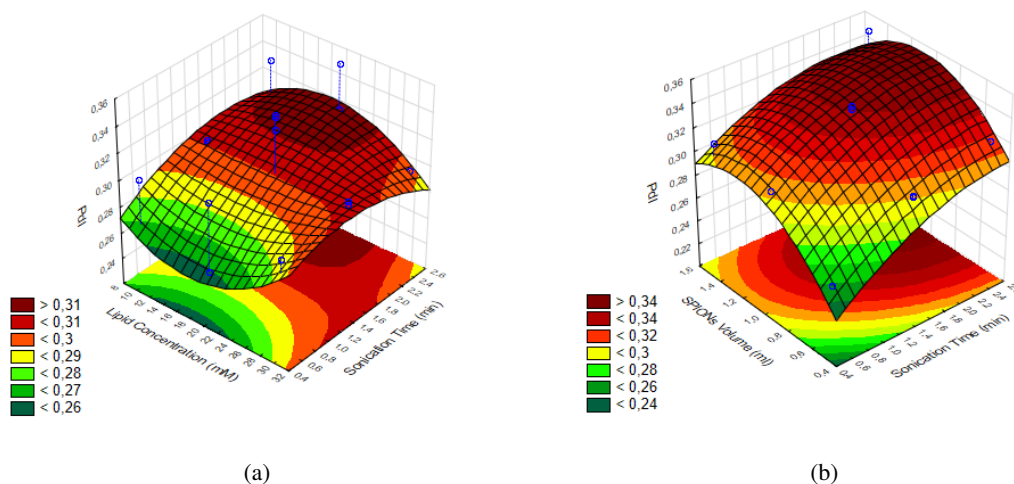


Figure 5.3: Response surface plots showing the (a) effect of lipid concentration (mM) and sonication time (min); and (b) effect of SPIONs volume (ml) and sonication time (min) on PdI.

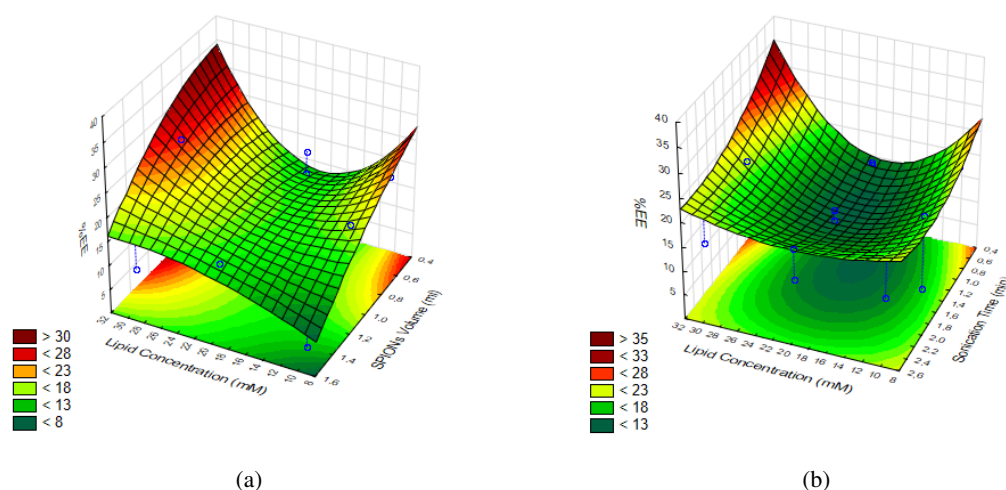


Figure 5.4: Response surface plots showing the (a) effect of lipid concentration (mM) and SPIONs volume (ml); and (b) effect of lipid concentration (mM) and sonication time (min) on %EE.

5.4.3 Optimisation and Validation of the RSM

The optimum formulation was selected based on the criteria defined in Section 4.6 (maximum %EE, minimising the PdI and a mean size close to 100 nm), applying the desirability function.

Following these considerations, desirability values were attributed to the three levels of all factors. The computed formulation (15 mM of lipid concentration, 0.5 ml of SPIONs and 0.5 minutes of ultrasonication), with the desirability levels presented in Figure B.9, was considered a suitable checkpoint formulation. Thus, in order to validate the experimental model and polynomial equations, the considered formulation was prepared in triplicate and the mean experimental results compared with the predicted responses given by the model. The experimental and predicted values for the responses of these formulations, along with the 95% of confidence intervals and the

corresponding prediction error are presented in Table 5.8.

Table 5.8: Composition of the checkpoint formulation, the experimental and predicted values, with the corresponding confidence interval and percentage prediction error.

Optimised Formulation Composition ($X_1 : X_2 : X_3$)	Response Variable	Experimental Value	Predicted Value	-95% CI	+95% CI	Prediction Error
15:0.5:0.5	Mean Size (nm)	241.2 $\pm$ 26.1	246.2	153.0	339.3	2%
	PdI	0.315 $\pm$ 0.007	0.260	0.235	0.284	21%
	%EE	22.56 $\pm$ 0.05	15.54	8.23	22.85	45%

CI - confidence interval

The mean particle size and %EE values were found to be within the defined limits, while the experimental PdI was out the predicted range. However, the reasonably similarity between the experimental values and the predicted ones allows us to judge this model as suitable for this optimisation. It was possible to obtain MLPs with mean size, PdI and %EE values as desired.

5.5 Characterisation of the Magnetoliposomes

The considered optimal formulation of MLPs, with 15 mM of lipid concentration, 0.5 ml of SPI-ONs and 0.5 minutes of ultrasonication, was characterised using several techniques. For comparison purposed, in some cases, the corresponding formulation containing empty LPs was also characterised, in some cases.

5.5.1 Size Distribution and Polydispersity

After the optimisation of the preparation method, the resulting formulation was characterised also in terms of size distribution and polydispersity. It was possible to verify that all prepared replicates of the considered optimal formulation (15:0.5:0.5) presented a similar bimodal size distribution, with slightly variations in the mean size of the two population. In the Section A.1, an analysis of the size distribution and polydispersity of the formulations prepared in preliminary assay 1 was already carried out. From this analysis, two possible hypothesis for the existence of the referred two well-defined populations: the interference of the magnetic NPs on the size reduction step or the lipid composition of the vesicles. However, to better understand the reason for this type of distribution, considering the optimal formulation, DLS measurements were performed for both MLPs and empty LPs suspension, with the same composition and lipid concentration. In Figure 5.5 are presented representative size distributions of these formulations.

Comparing the size distributions of empty LPs suspension (Figure 5.5a) and the equivalent formulation containing SPIONs (Figure 5.5b), it is possible to conclude that the bimodal distribution is not caused by the presence of SPIONs. Both formulations presented a distribution with two well-defined populations of NPs. Considering this, the hypothesis of lipid composition is still a possibility. The association effect of the used proportion of the lipids and the ultrasonic energy interaction with those lipids lead to the formation of two stable populations of LPs.

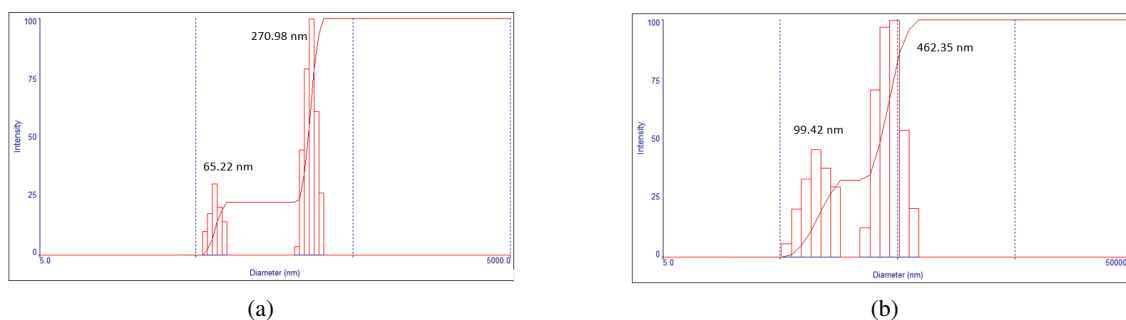


Figure 5.5: Intensity size distributions of (a) empty LPs (DOPE/DPPC/CHEMS (4:2:4)) and (b) MLPs (15:0.5:0.5) suspensions obtained by DLS.

Nonetheless, since smaller LPs, with sizes near 100 nm, represent the numerous population, the size reduction step, as it is performed, can be considered efficient and the resultant formulation suitable for the proposed biological application.

5.5.2 Colloidal Stability

LPs are lipid vesicles with known short half-life time, when no surface modification is used (Akbarzadeh et al., 2013). Since the MLPs formulations prepared in this work do not have coated surface, it is important to evaluate the structural stability of the produced nanosystem, in order to define a viability range of time in which they can be considered stable MLPs. With this in mind, a short stability study was carried out, through the measurement of the mean size and PDI of a MLPs formulation (15:0.5:0.5), over 3 days after the preparation of the suspension. Reported TEM studies showed that maximum stability is achieved when LPs are stored at refrigeration temperatures (about 4 °C) (Bhalerao and Raje Harshal, 2003). Therefore, after the preparation of the formulations, they were stored at 4 °C, in an attempt of maximising the viability time. Also, as previously mentioned, the suspensions were prepared in PBS, at pH 7.4 as a way of mimic the physiological environment. Figure B.12 shows the obtained evolution of these parameters with time, for the MLPs formulation.

To conclude about the influence of the presence of SPIONs, inside the aqueous core of the LPs, on the stability of these NPs, the same study was performed for the equivalent formulation, containing empty LPs. The results are shown in Figure B.13.

Observing the obtained plots of the mean size and PDI in function of time, it is possible to verify that, for the considered 3 days after the preparation, no significant variation were noticed. The formulation of MLPs, after the preparation, had a mean size of 218.0 nm and PDI of 0.300. After 3 days, the values for these parameters remained almost unaltered. However, a slight sedimentation was observed, after 2 days, which can be related with some SPIONs leakage.

In the case of the empty LPs, after the preparation, the suspension presented a mean size of 313.8 nm. Over time, a slight decrease in mean size (to 286.1 nm) was verified as well as a slight variation on PDI values, between 0.319, in day 0, and 0.222, in day 3. From these results, it can be

inferred that some rearrangement is possibly taking place, but the structural stability of the LPs is not compromised.

This analysis allows us to say that fusogenic empty LPs, composed of DOPE/DPPC/CHEMS (4:2:4), with a lipid concentration of 15 mM, remained stable for, at least, 3 days in PBS, at pH 7.4. The equivalent formulation containing SPIONs encapsulated LPs also seem to preserve its stability for the same period of time.

As future approach to improve both LPs and MLPs stability over time, several techniques could be applied, such as lyophilisation, freezing, spray-drying and supercritical fluid technology (Chen et al., 2010a). Among these, lyophilisation is considered the more suitable technique for the produced formulations, since it extends the shelf-life of the LPs with minor damage for the encapsulated substances (Franzé et al., 2018).

5.5.3 Phase Transition Temperature

During the phase transition, the bilayer of LPs undergoes conformational changes. It is known that below their main T_m , the phospholipids are closely packed, and form a rigid ordered gel phase. When the temperature gets close to the T_m , the tightly packed phospholipids start to loosen up, resulting in more empty space between them. The structure of the membrane becomes looser until a disordered fluid crystalline phase is established at the end (Chen et al., 2018).

Understanding the effect of the phase transition on the structure of the liposomal membranes as well as knowing the corresponding value of T_m are important steps to take before the application of LPs formulations. More recently, DLS has been adapted to perform this measurement. It has been found that the shape alterations, induced by the phase transition can be correlated with a variation on the mean count rate (number of photons detected per second), measured by the DLS equipment (Michel et al., 2006). Therefore, the detection of a discontinuity in these values, as the temperature increases, correspond to a change in the optical properties of a suspension, characteristic of an emerging macroscopic phenomenon of phase transition (Michel et al., 2006).

To estimate the T_m of the LPs with the selected lipid composition and to evaluate if encapsulated SPIONs interfere with the phase transition phenomenon, two different formulations, with the optimal composition, were subjected to DLS measurements: the considered optimal MLPs formulation; and the equivalent formulation composed by empty LPs. The state of molecular packing in phospholipid bilayers can be affected not only by the temperature and by the lipid components but also by the ionic composition of the aqueous environment (Jacobson and Papahadjopoulos, 1975). So, for both experiments, the biological environment was mimicked by the preparation of the formulations in PBS at pH 7.4. The curves representing the variation of the normalised count rate with temperature increase (between 7 and 50 °C) for both formulations are presented in Figure 5.6.

For the formulation composed by empty LPs, it can be observed that between 21 °C and 35 °C, the variation of the count rate is more pronounced than the variation observed for lower or higher temperatures. So, this curve fits a sigmoid curve typically obtained for phase transition phenomena (Michel et al., 2006). It means that, fitting the data to the previously referred Equation

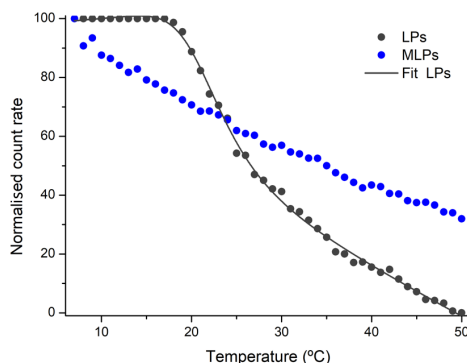


Figure 5.6: Evolution of the normalised count rate as temperature function for the tested LPs and MLPs formulations. Black line shows the sigmoid fit to the LPs data.

4.1 (Section 4.4.1.2), it is possible to assess the value of T_m for the tested lipid composition. In Table 5.9, the values of each parameter of the fitted sigmoid model are presented, along with the respective standard deviation error (SD) and the achieved adjusted R^2 .

Table 5.9: Parameters of the fitted sigmoid model equation (4.1), for the tested LPs formulation, the respective SD and obtained adjusted R^2 .

	Value	SD
p_1	0.26	0.17
p_1	-1.62	0.10
r_{S1}	97.46	2.02
r_{S2}	79.25	4.93
B	88.6	7.25
T_m	22.54	0.27
Adj. $R^2 = 0.998$		

Considering the obtained adjusted R^2 (close to 1) and from the visual observation, it is valid to affirm that the model fitted well to the experimental data. Hence, from this analysis, it is possible to conclude that the produced LPs, composed by DOPE/DPPC/CHEMS (4:2:4), presented a T_m of approximately 22.5 °C. Below this temperature, the lipids in the bilayer are more tightly packed and the acyl chains are ordered, forming the called gel phase. With temperature increase, the vesicles gradually increase their fluidity, by the promoted movement of the phospholipid tails, until the a fluid phase is reached.

Since the T_m of DPPC and DOPE are 41 °C and -16 °C, respectively, it seems that, in fact, the presence of DOPE in the vesicle membranes largely contributes to the decrease of the T_m , in comparison to the one that would be obtained for DPPC vesicles. The presence of double bounds in the hydrocarbon chains of DOPE molecules promotes a disturbance in the packing of the phospholipid bilayer, lowering the T_m of the vesicles with this lipid in their composition.

From the observation of the same variation curve for the MLPs, a different scenario can be found. For the same range of temperatures, in the case of the MLPs, the variation of the count rate with the temperature does not present a sigmoidal profile and the total decrease of count rate is

lower than the verified for the empty LPs. Thus, for this data, the sigmoid fitting was not possible. The first conclusion that we could draw from these results is that the presence of SPIONs in the formulations interferes with the phase transition phenomenon.

From the analysis of Figure 5.6, it is possible to observe that in the MLPs formulation the two well-defined levels, characteristic of gel and fluid phases, do not exist. From the profile, it seems that there is a major decrease in cooperativity of the phase transition process, and that the transition occurs within a vast range of temperatures.

In the case of the empty LPs, the strong packing of the lipid molecules hampers a gradual phase transition, and thus, this phenomenon occurs as a cooperative process, despite the different lipids present, in which the lipid molecules increase their movements with the temperature increase. When SPIONs are present in the formulation the slow variation of the count rates can be indicative of a decrease in the cooperativity of the phase transition, turning the lipid melting into a more gradual and smooth process. Since SPIONs present an hydrophilic surface, they tend to interact with the head region of the phospholipids. The possible retention of some of these NPs near the hydrophilic head of the lipid molecules may decrease the Van der Waals interactions between the phospholipid and, consequently, increase the lateral mobility of the molecules on the bilayers.

In order to confirm the obtained results for empty LPs and to assess a value of T_m for the MLPs formulation, other methods, such as DSC, should be performed.

5.6 Toxicity and Cell Viability Results

As already seen, SPIONs are potential candidates in biomedical applications, namely in biofilms eradication, due to their reported antimicrobial activity. However, these magnetic NPs can be considered potentially toxic above certain concentrations. This toxicity is essentially determined by the physical and chemical characteristics of SPIONs (Thorat et al., 2016). Hence, despite of the already reported cytotoxicity tests using SPIONs, it remains important to study the overall toxicity associated with them and determine, if possible, a minimum non-toxic concentration for the produced NPs, since they may present different characteristics from the ones reported in literature. Besides that, it is crucial to also evaluate the toxicity of these NPs when encapsulated in LPs. So, a MTT assay was carried out, using a SPIONs suspension, a MLPs formulation with the considered optimal parameters (15:0.5:0.5) and the corresponding empty LPs suspension.

Figure B.14 shows the 96-well plate after the performed assay. From the observation of the plate, it was possible to infer that all cells were able to metabolise the MTT into formazan, creating the purple colour, except the ones treated with Triton (right column), as expected. In Tables 5.10, 5.11 and 5.12 are shown the calculated cell viability percentages and the respective standard deviation, considering the four replicates of each concentration.

Considering the marked similarity of the LPs vesicles and the cell membranes, it was expected that the tested formulation does not induce toxicity. Indeed, the cell viability values for all the tested concentrations, were all close to 100%, being slightly lower for higher lipid concentration.

Table 5.10: Mean value and respective standard deviation of cell viability percentages for each lipid concentration, considering the tested formulation.

Lipid concentration	3 mg/ml	1.5 mg/ml	0.75 mg/ml	0.375 mg/ml	0.1875 mg/ml
Cell Viability LPs	(94±15)%	(87±17)%	(90±13)%	(100±5)%	(100±14)%

Table 5.11: Mean value and respective standard deviation of cell viability percentages for each lipid concentration and the corresponding Fe concentration, considering the tested formulation of MLPs.

Lipid concentration	0.7 mg/ml	0.35 mg/ml	0.175 mg/ml	0.0875 mg/ml	0.0438 mg/ml
Fe concentration	0.053 mg/ml	0.027 mg/ml	0.013 mg/ml	0.007 mg/ml	0.003 mg/ml
Cell Viability MLPs	(100±8)%	(99±23)%	(98±10)%	(98±11)%	(98±9)%

Table 5.12: Mean value and respective standard deviation of cell viability percentages for each lipid concentration, considering the tested SPIONs.

Fe concentration	0.744 mg/ml	0.372 mg/ml	0.186 mg/ml	0.093 mg/ml	0.047 mg/ml
Cell Viability SPIONs	(64 ± 13)%	(62 ± 10)%	(75 ± 9)%	(63 ± 6)%	(69 ± 4)%

For the MLPs formulation, lower lipid concentrations were tested. It was verified that, regardless both lipid and iron concentrations, cell viability values were almost 100% once again. It means that the iron concentration due to the encapsulated SPIONs do not affect the cell metabolism, for an incubation time of 24h.

The tested iron concentration for SPIONs formulation led to significant decrease in cell viability, to values close to 65%. In fact, the iron concentration for SPIONs are significantly higher than the ones selected for MLPs as well as close to the referred as the maximum non-toxic concentration (0.1 mg/ml) in literature, which explains the reduced cells viability obtained for SPIONs (Ankamwar et al., 2010). However, comparing the higher concentration tested for MLPs (0.053 mg/ml) and the lower iron concentration tested for SPIONs (0.047 mg/ml), it is possible to conclude that the encapsulation reduce the toxicity of SPIONs, being an advantage for the application of these NPs in living systems, such as for biofilm eradication.

Chapter 6

Conclusions and Future Work

As seen through the literature review carried out, the emergence of resistance among several bacterial pathogens and the possibility of some pathogens forming biofilms are recognised as a major public health threat worldwide.

Throughout the years, several strategies to target biofilms have been reported and more recently, nanotechnology has also provided good alternatives. The production of MLPs, through the combination of LPs and SPIONs, has already been made for a variety of applications. However, concerning the use of these hybrid nanosystem as antimicrobial agents for biofilms eradication, without the use of any antibiotics, no experimental work has been reported.

Thus, the aim of this work was to develop a hybrid nanosystem to target *S. aureus* biofilms, combining the potentialities of fusogenic LPs, as nanocarriers, and the magnetic and antimicrobial properties of SPIONs. With this in mind, a sequential division of work was made and the obtained results explored throughout this document.

From the three sets of performed preliminary assays it was possible to develop a new method for the MLPs preparation, incorporating the SPIONs on the lipid film, as well as simplifying the procedure, by the exclusion of FT cycles. Based on the obtained results, the lipid concentration, the used volume of SPIONs and the sonication time were selected as factors for the further performed experimental design, affecting the formulation in terms of mean size, PdI and %EE.

For the calculation of %EE, a good correlation of linearity between iron concentration and absorbance was found and the obtained calibration curve was validated for quantification of the total content of iron on the formulations.

The method of synthesis of the MLPs was then optimised using a 3-factor, 3-level Box–Behnken experimental design. Considering the levels defined for each factor and the obtained results, applying the desirability function, the formulation with 15 mM of lipid concentration, 0.5 ml of SPIONs and 0.5 minutes of sonication was computed as the optimal formulation. Reasonably predictor errors was observed in the experimental responses for this formulation. Therefore, the model was considered accurate, with a good predictive ability, except for PdI values that showed almost random variations that could not be well fitted to the considered model.

From the physico-chemical characterisation of the optimal formulation, it was seen that the referred nanosystem presented stability, at least, for the 3 measured days and SPIONs seemed not to interfere in the stability of the LPs. The obtained T_m for the LPs formulation was around 23 °C while for MLPs it was not possible to determine a value of T_m . For the used range of temperatures, DLS measurements were not enough to determine what happened to the MLPs with the increase of temperature. In addition, from the cytotoxicity assay, it was possible to conclude that the encapsulation of SPIONs into the LPs showed advantages in terms of an increased cell viability, for an incubation time of 24h.

Considering the proposed objective, testing the efficiency of the formulation in the biofilm disruption of *S. aureus* is still missing. However, additional work that was not in the initial plan was performed, like the cytotoxicity studies, which are essential to establish the suitable concentrations to be used in the biofilms assays.

Moreover, in addition to the work already done, some additional characterisation should be performed. For example, it would be important to perform magnetisation measurements, in order to confirm the existence of superparamagnetism and obtain values of saturation magnetisation of the produced NPs. The same measurements should be performed for the MLPs formulation, as a mean of understanding if the encapsulation affects the magnetic properties of SPIONs and, consequently, their antimicrobial activity.

Also, a characterisation of the formulations should be carried out by TEM, to confirm the size distribution of SPIONs and MLPs as well as to confirm the encapsulation of SPIONs inside the LPs. If an increase in SPIONs stability was desired, a suitable coating should also be used. Considering that an encapsulation inside the aqueous vacuole is aimed, an hydrophilic molecule should be chosen, which would also work as a way of increase the %EE.

For the T_m determination, the DLS measurements should again be carried out using a larger range of temperatures, which could possibly give more information about the phase transition phenomenon and how SPIONs are influencing it. To ensure reproducibility for the results obtained for LPs formulation, the performed DLS measurements should also be performed in triplicate. In alternative, as previously mentioned, other methods for T_m determination (DSC, p.e.) should be used, in order to confirm the calculated T_m for LPs formulation as well as to allow a more extent understanding of the MLPs phase transition.

To confirm if the produced LPs and MLPs presented the desired fusogenic enhanced abilities, fusion assays should be carried out. A strong possibility to consider would be the use of a lipid mixing assay based on the principles of Förster resonance energy transfer (FRET) to monitor membrane fusion of the prepared LPs and MLPs with other vesicles.

Concerning the cytotoxicity assays, longer incubation times should be tested as well as lower concentrations for SPIONs and higher concentrations of MLPs, in order to determine the maximum limit for dosage of the formulation. Additionally, more replicates and other viability assays should be performed to confirm the obtained results in the presented MTT assay as well as understand the mechanism that led to such low cell viability values, for the tested SPIONs concentrations.

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Appendices

Appendix A

Materials and Methods

A.1 Methods

A.1.1 Preliminary Assays

The effect of the film preparation method (1 or 2), SPIONs volume (ml), use (C) or not use (S) of FT cycles, lipid concentration (mM), sonication time (ST) (minutes) on the properties of the formulations were evaluated during the three sets of preliminary assays.

Table A.1: Specifications of the variables for the formulations prepared in preliminary assay 1.

	Film Method	ST (min)	SPIONs Volume (ml)	FT Cycles	Lipid concentration (mM)
L1	1	0.5	0.5	C	3
L2	1	1.0			
L3	1	1.5			
S1	2	0.5			
S2	2	1.0			
S3	2	1.5			

Table A.2: Specifications of the variables for the different formulations prepared in preliminary assay 2.

	Film Method	ST (min)	SPIONs Volume (ml)	FT Cycles	Lipid concentration (mM)
A1	1	1.0	1.0	C	15
A2	1			S	
B1	2			C	
B2	2			S	

Table A.3: Specifications of the variables for the different formulations prepared in preliminary assay 3.

	Film Method	ST (min)	SPIONs Volume (ml)	FT Cycles	Lipid concentration (mM)
F1	1	1.0	1.0	S	15
F2	2				

A.2 Materials

In Table A.4, the reagents used in the described protocols are listed with the corresponding purity and brand.

Table A.4: List of the used reagents and their corresponding purity and brand

Reagent	Purity	Brand
DOPE	$\geq 97\%$	SIGMA [®]
DPPC	-	Avanti [®] Polar Lipids, Inc.
CHEMS	-	SIGMA [®]
PBS buffer (10× concentrate)	-	Sigma-Aldrich [®]
Chloroform	stabilised with 0.6% ethanol	VWR Chemicals
Methanol	$\geq 99.9\%$	Fisher Chemical
Sephadex [®] G-25	-	Sigma-Aldrich [®]
Iron(II) chloride tetra-hydrate	$\geq 99.0\%$	Sigma-Aldrich [®]
Iron(III) chloride hexa-hydrate	97%	Sigma-Aldrich [®]
Ammonium hydroxide	-	Fisher Chemical
1,10-Phenanthroline hydrate	$\geq 99\%$	Fisher Chemical
Hydrochloric acid	-	Fisher Chemical
Hydroxylamine hydrochloride	99%	Sigma-Aldrich [®]
Dulbecco's Modified Eagle Medium (DMEM)	-	Gibco [®]
Dimethyl sulfoxide	$\geq 99.9\%$	Sigma-Aldrich [®]
MTT	98%	Sigma-Aldrich [®]
Triton [™] X-100	-	Sigma-Aldrich [®]

Appendix B

Results and Discussion

B.1 Iron Quantification Method

B.1.1 Optimisation Step

By visual observation of the colour change, it is possible to notice that the formation of iron-phenanthroline complex is fast for $FeCl_3$ standards and it is known that, once formed, this complex is stable at least 1 week (Hedayati et al., 2018). However, for the samples containing SPIONs or SPIONs-encapsulated LPs, the formation of the coloured complex is much slower. This phenomenon can be due to the fact that the iron in this suspensions is present as iron oxides in a bulky structure (as NPs) which, even using the acid digestion step, take more time to completely react with phenanthroline. Thus, for these formulations a monitorisation of the complexation reaction was required, in order to improve their sensitivity and accuracy.

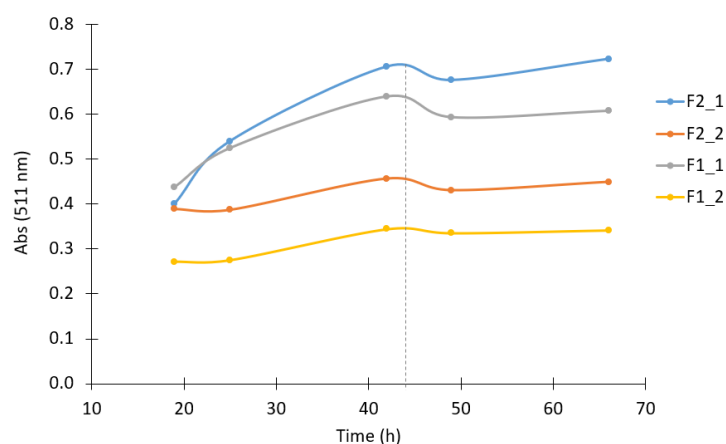


Figure B.1: Evolution of the absorbance (at 511 nm) of samples with different iron concentration over time.

Two formulations with different lipid concentrations (F1 with 10 mM and F2 with 30 mM) and the same volume of SPIONs (0.5 ml) were prepared. From each formulation, two samples

collected after SEC, presenting different iron content, were subject to the quantification procedure and their absorbance measured, at 511 nm. Five time points were tested: 19, 25, 42, 49 and 66 hours, after the addition of the phenanthroline solution. The results are presented in Figure B.1.

It is possible to observe that a maximum value of absorbance is obtained, for all cases, near 42 hours. This means that, regardless the lipid concentration or the amount of SPIONs in the samples, the reaction of Fe(II) with phenanthroline takes approximately 42 hours to occur. After 42 hours, the absorbance starts to decrease, possibly due to the degradation of the coloured complex in solution. Therefore, for the quantification of the total iron content, the absorbance of the samples were measured after 42 hours of the addition of phenanthroline.

B.2 Calibration Curve

Figure B.2 shows the obtained UV-Vis spectra of phenanthroline complex for the prepared standards.

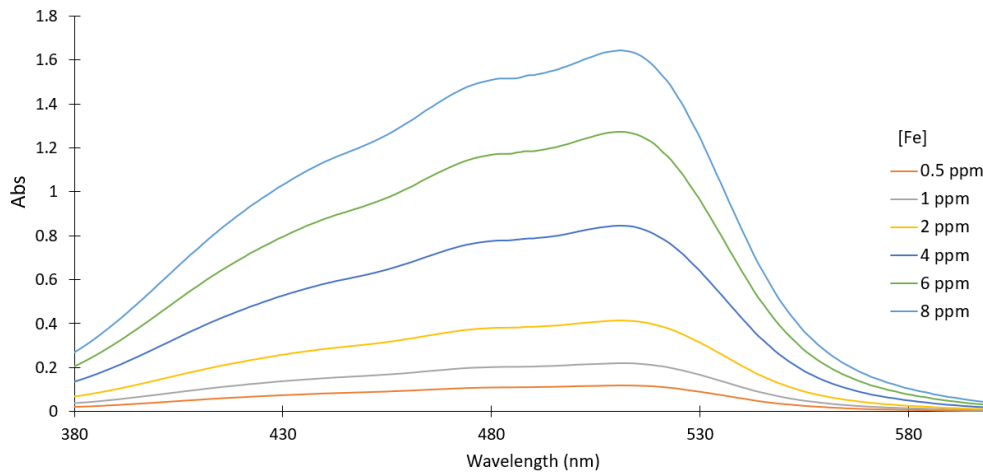


Figure B.2: UV-Vis spectra of phenanthroline complex with Fe(II) (0.5-8 ppm *Fe*).

The limits of quantification (LOQ) and detection (LOD) of the method were obtained by the following equations:

$$LOQ = 3 \times \frac{s_d}{S} \quad (B.1)$$

$$LOD = 10 \times \frac{s_d}{S} \quad (B.2)$$

where s_d corresponds to the standard deviation of the calibration curve and S to its slope.

B.3 Preliminary Assays

B.3.1 Assay 1

The obtained size distributions for all the prepared formulations, before SEC, are presented in Figure B.3.

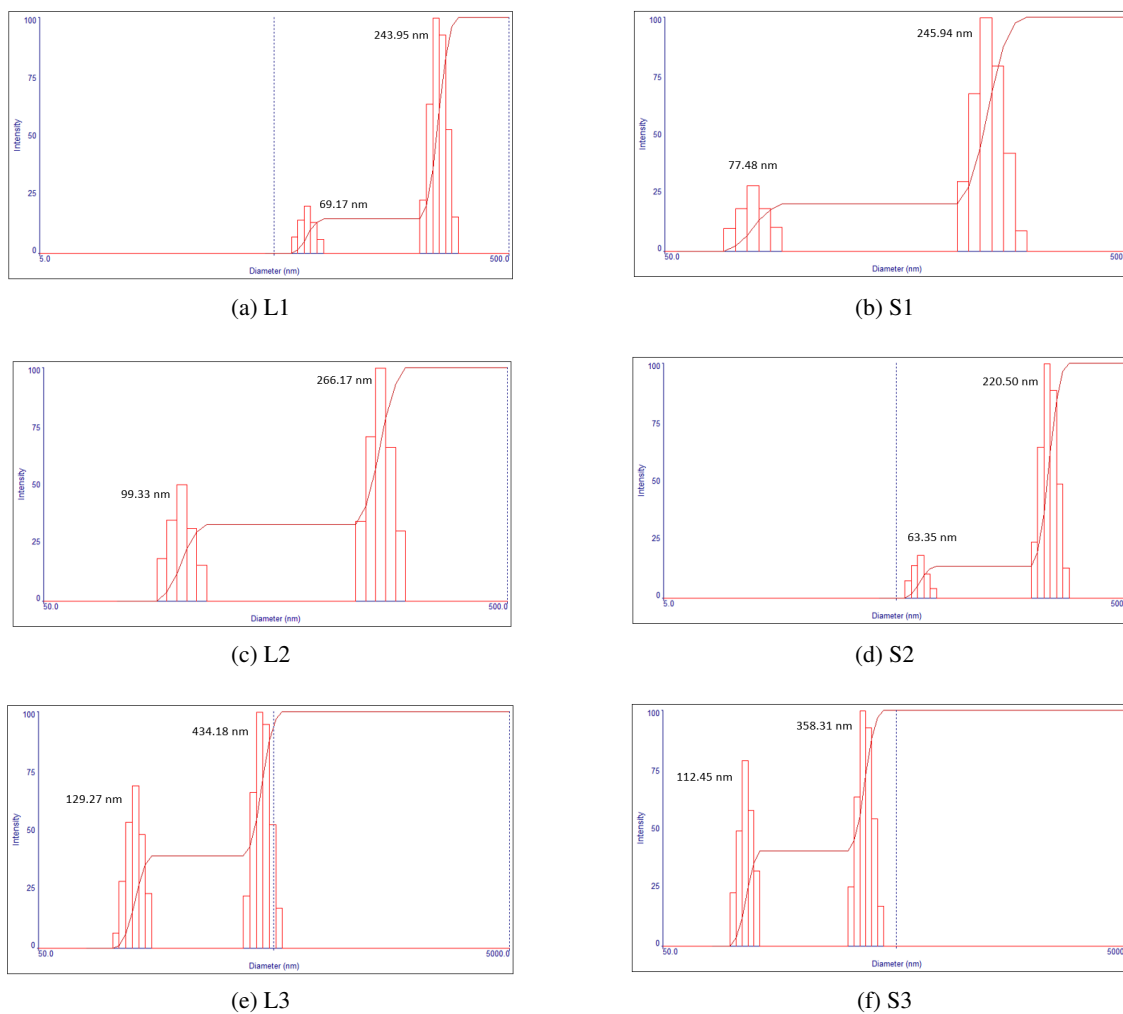


Figure B.3: Intensity size distributions of formulations (a) L1, (b) S1, (c) L2, (d) S2, (e) L3 and (f) S3 obtained by DLS, after the ultrasonication step.

In the following figures, it is presented the separation process (SEC), used for the MLPs preparation, in its several stages of use: before, during and after some elutions.

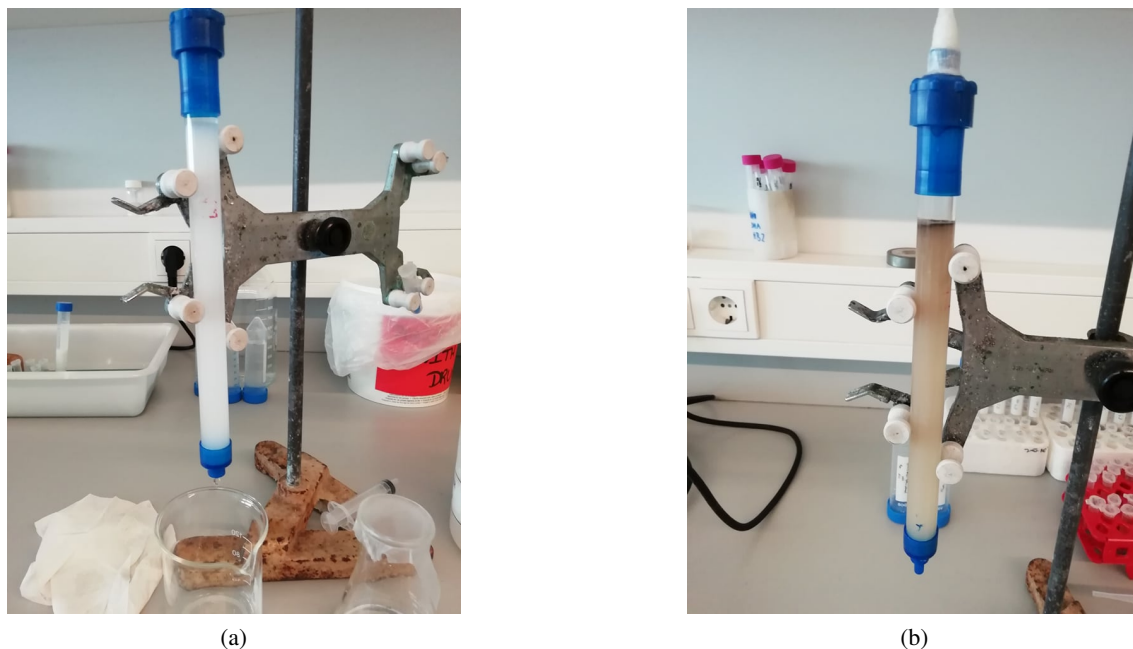


Figure B.4: SEC column (a) before being used and (b) after the elution of five formulations.

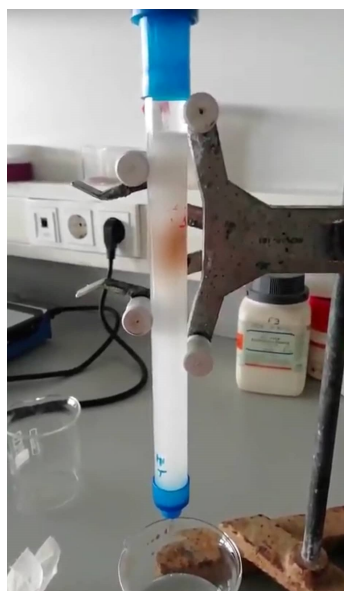


Figure B.5: SEC column during the elution.

For the previous formulations L2 and S2, already subject to SEC, new measurements were carried out and the results are present in Figure B.6.

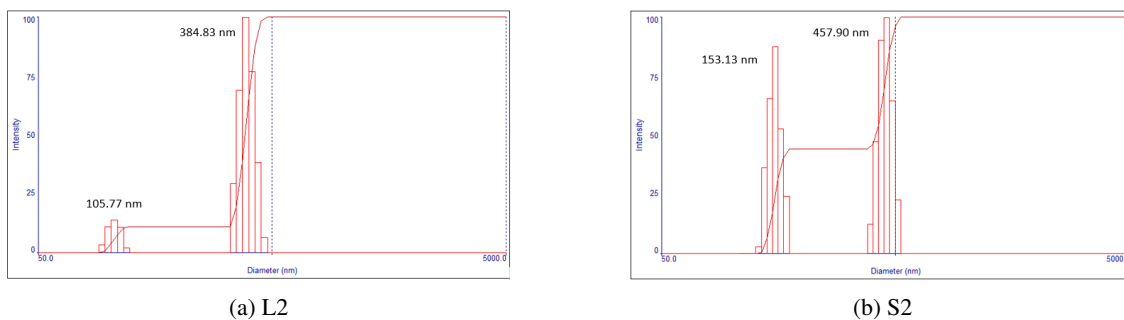


Figure B.6: Intensity size distributions of final formulations (a) L2 and (b) S2, after SEC.

B.4 Complementary Characterisation of SPIONs

In Figure B.7, it is possible to observe the influence of a strong external magnet on SPIONs.

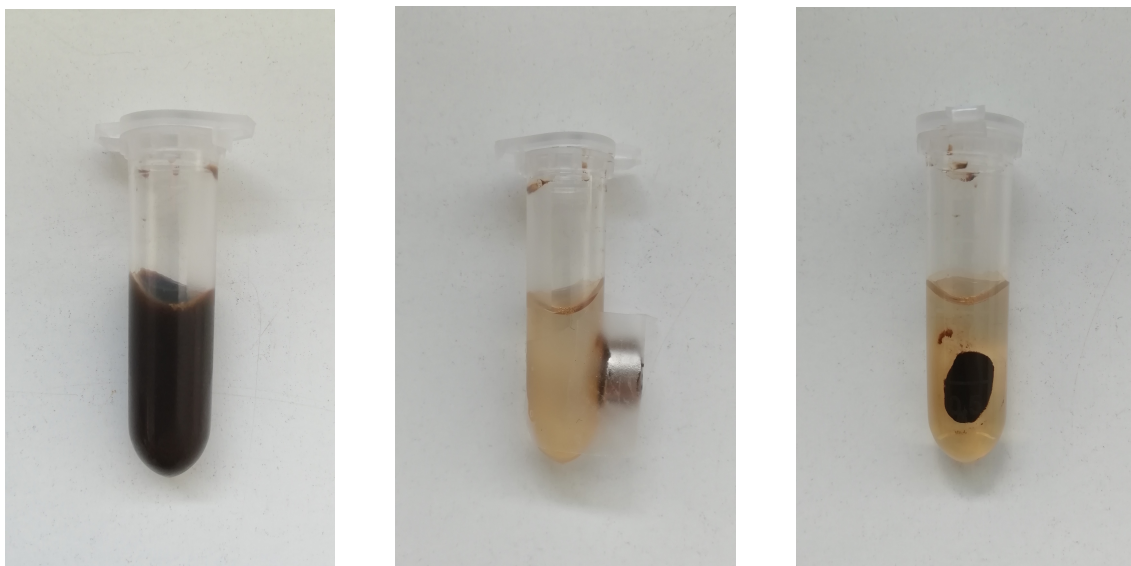


Figure B.7: Magnetic response of SPIONs, promoted by an external magnet.

B.4.1 FTIR Analysis

FTIR spectra of the Fe_3O_4 produced NPs (Figure B.8) showed characteristic three characteristic main peaks.

The peak at 1638 cm^{-1} was attributed to the bending vibration of water adsorbed on the surface of the NPs. The peak at 3356 cm^{-1} appears due to the presence of -OH groups on the surface of SPIONs (Panwar et al., 2015). In the absorption band of Fe-O, a peak at 1038 cm^{-1} near the wavelength corresponding to the Fe-O stretch vibrations (1384 cm^{-1}), can be detected (Panwar et al., 2015). Although the lower value of the transmittance was measured at 600 cm^{-1} , a significant decrease in this value can be attributed to the peak corresponding to Fe-O bending vibrations (580 cm^{-1}) (Asgari et al., 2014).

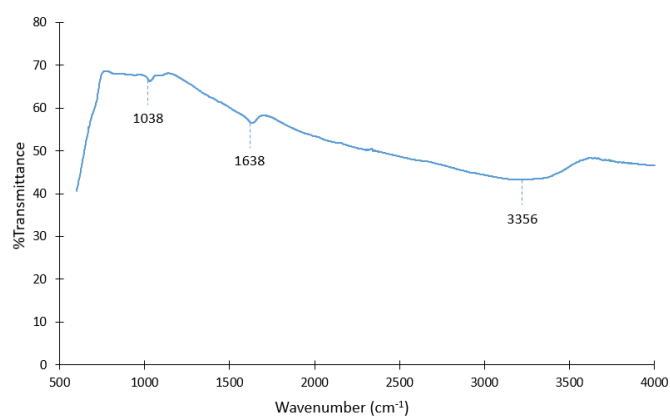


Figure B.8: FTIR spectrum of SPIONs.

B.5 Box-Behnken Experimental Design

B.5.1 Optimisation and Validation of the RSM

The graphical representation of the application of the desirability function is presented in Figure B.9.

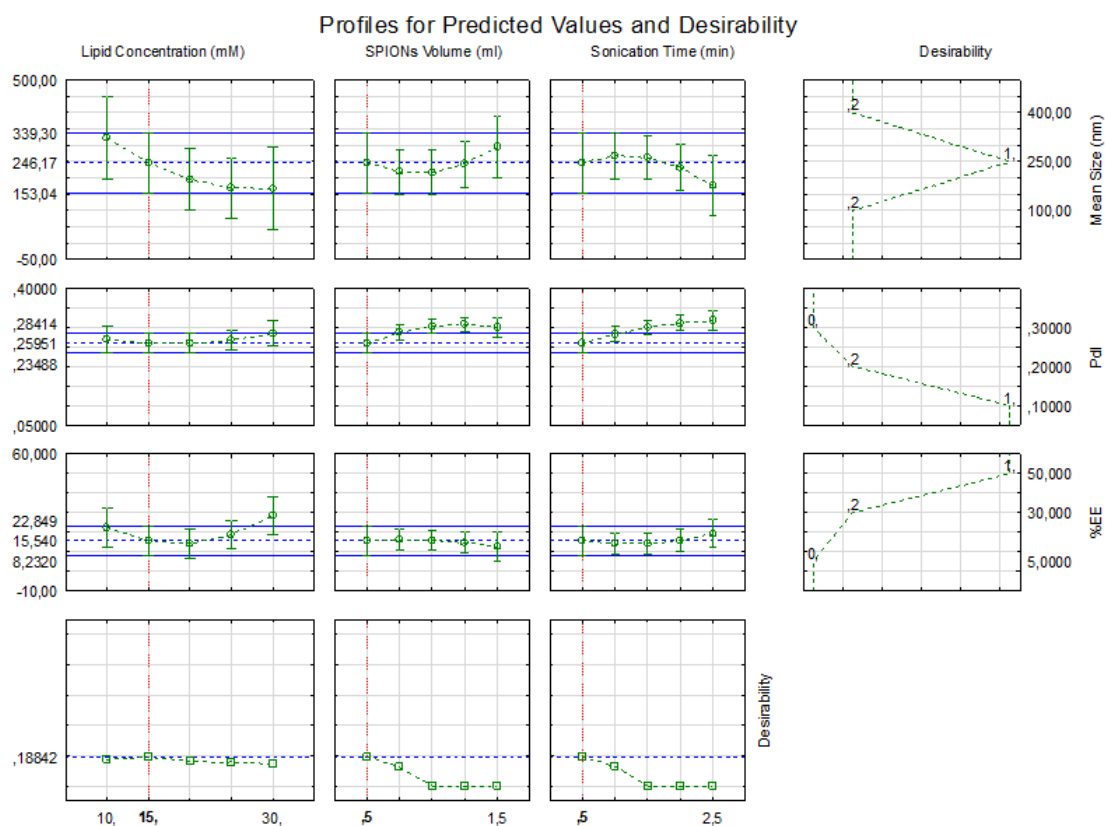


Figure B.9: Profiles for predicted values and desirability for the formulation 15:0.5:0.5.

B.6 Complementary Characterisation of the Magnetoliposomes

B.6.1 FTIR Analysis

In Figure B.10, FTIR spectrum corresponding to the MLPs formulation is presented.

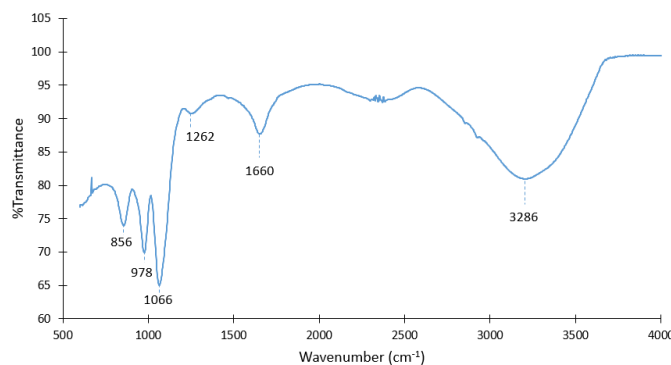


Figure B.10: FTIR Spectrum of MLPs formulation.

MLPs exhibited main infrared peaks as follows: a curved band at 3286 cm^{-1} corresponding to -OH intermolecularly and intramolecularly H bonds (Alexander et al., 2016); a peak at 1660 cm^{-1} attributed to strong bands due to C=O stretching; a small peak at 1262 cm^{-1} assigned to $[PO_2^-]$ bonds in this region (Shafaa, 2018); and below 1066 cm^{-1} , the found peaks are related to strong C-H bending.

The presence of SPIONs can not be inferred by this analysis since the absorption band of Fe-O is detected at wavenumbers below 600 cm^{-1} .

B.6.2 UV-Vis Spectra

UV-vis spectroscopy was carried out, for both empty LPs and MLPs, with the optimal formulation (15:0.5:0.5), in the wavelength range from 200 to 600 nm (Figure B.11).

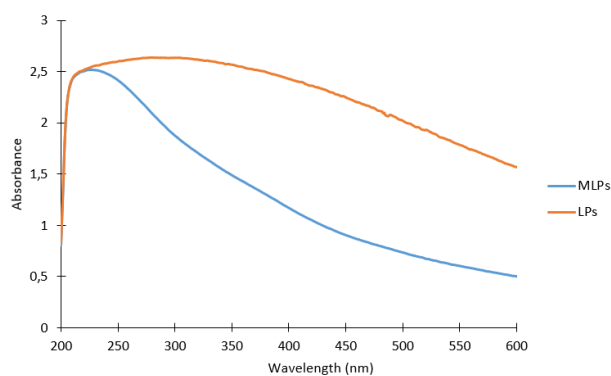


Figure B.11: UV-Vis spectra of empty LPs and MLPs composed by DOPE/DPPC/CHEMS (4:2:4).

Typically, maximum absorbance of LPs vary between 190 to 210 nm, depending on the size of the NPs (Monnard et al., 1997) (Paasonen et al., 2007) (Amendola and Meneghetti, 2009). From the obtained spectra, it can be seen that empty LUVs, composed by DOPE/DPPC/CHEMS (4:2:4), showed maximum absorption at about 280 nm while the MLPs with the same lipid composition presented an absorption peak near 230 nm. These values are in good agreement with the literature values previously referred. This identified peak shift can be related with the nanoscale of the corresponding NPs in the corresponding suspension, since LPs presented a mean size of 286.1 nm and MLPs showed a mean size of 208.0 nm.

B.6.3 Colloidal Stability

Figures B.12 and B.13 show the evolution of mean size and PdI of MLPs and LPs, respectively, over 3 days, after the preparation of the formulations.

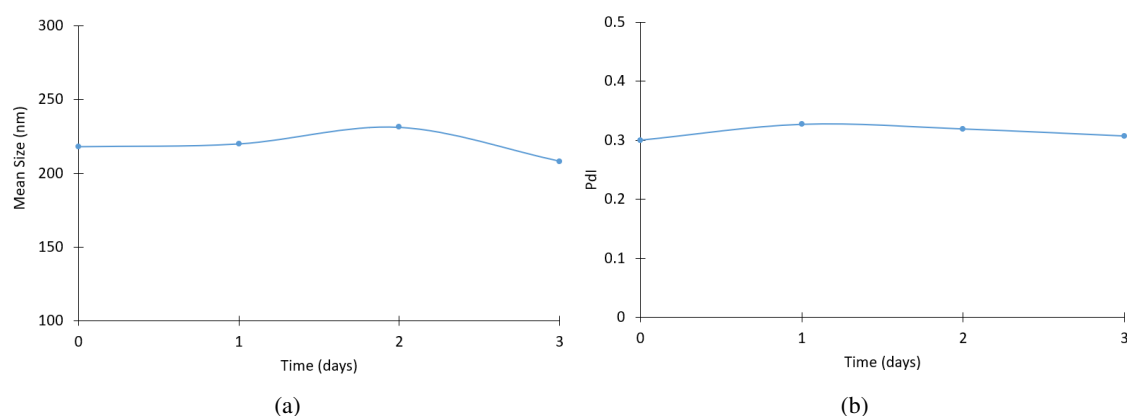


Figure B.12: Stability assay performed by the evaluation of the (a) mean size and (b) PdI values measured over time for the optimal MLPs formulation (15:0.5:0.5)

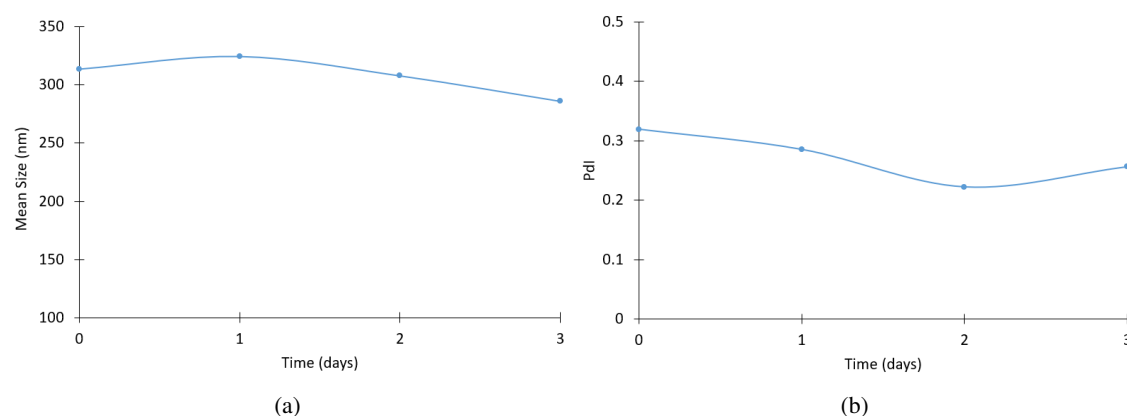


Figure B.13: Stability assay performed by the evaluation of the (a) mean size and (b) PdI values measured over time for an empty LPs (DOPE/DPPC/CHEMS 4:2:4) suspension.

B.7 Toxicity and Cell Viability Results

The following Figure shows the 96-well plate after the MTT assay. Left column corresponds to untreated cells, right column have the negative control cells, the first quadrant present the cells incubated with SPIONs, the second quadrant present the cells incubated with MLPs and the fourth quadrant present the cells incubated with LPs.

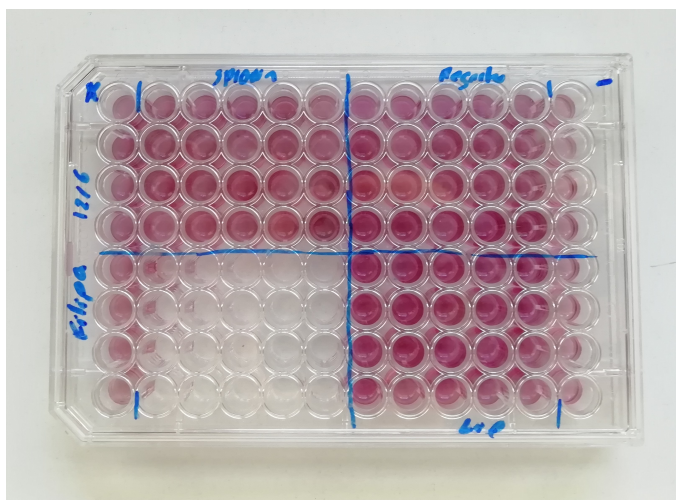


Figure B.14: 96 well plate after the performed MTT assay.