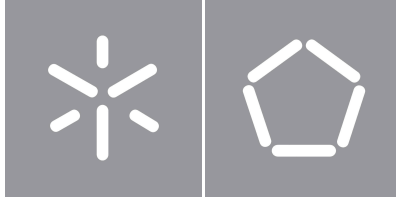


University of Minho
School of Engineering

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Magnetoliposomes as New Approach for Bone Cancer Therapies

january 2024



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Magnetoliposomes as New Approach for Bone Cancer Therapies

Masters Dissertation
Masters in Physics Engineering

Dissertation supervised by
Dr. Vanessa Fernandes Cardoso
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Abstract

Cancer, notably osteosarcoma, poses detection and treatment challenges, with its relentless progression and resistance to conventional therapies. Innovative approaches are imperative to overcome limitations like toxicity and adverse effects on healthy cells. Nanotechnology, particularly magnetoliposomes, offers promise in this pursuit. Osteosarcoma's intricate nature demands precision and enhanced effectiveness in treatment strategies. Conventional therapies, such as radiotherapy, often fall short of delivering optimal results due to their limitations, such as the resistance of bone cancer to radiation requiring higher doses, prompting the exploration of alternative methods. Notably, liposomes have proven to be a successful vehicle for drug delivery, significantly improving the efficacy of chemotherapeutic agents like doxorubicin. The integration of superparamagnetic nanoparticles further enhances the potential of these systems by enabling localized drug delivery. This innovative approach not only addresses the challenges associated with conventional treatments but also marks a significant stride towards improving the precision and overall effectiveness of combating osteosarcoma. This project involves the processing of magnetoliposomes containing encapsulated doxorubicin, magnesium, and calcium ferrites (with a diameter of $8.75 \pm 0.35nm$, saturation magnetization of $4.39emu/g$, and $E_g = 1.35eV$), along with a comprehensive study of their respective physical characteristics. The results reveal magnetoliposomes with a diameter of $542.54 \pm 71.78nm$ and a polydispersity index of $27.48 \pm 3.84\%$. Notably, the systems exhibit nearly complete encapsulation of doxorubicin and a magnetic nanoparticle encapsulation efficiency of $49.00 \pm 5.17\%$. The investigation into drug release kinetics under static and dynamic conditions unveils significant variations in release rates. Particularly, when subjected to an alternating magnetic field with different frequencies and on-cycles (time the magnetic stimuli are applied), the drug release rates are nearly 30% higher under magnetic stimulation over $5h$ compared to static conditions. This emphasizes how external stimuli affect controlled substance release, showcasing their potential in targeted drug delivery.

Keywords: Magnetoliposomes, Osteosarcoma, Cancer, Doxorubicin, Magnetic Nanoparticles, Magnetic Bioreactor.

Resumo

O cancro, incluindo o osteossarcoma, apresenta desafios na detecção precoce e tratamento eficaz. Abordagens inovadoras são necessárias devido à resistência às terapias convencionais. A nanomedicina, como os magnetolipossomas, surge como uma alternativa promissora, superando limitações dos tratamentos tradicionais. O tratamento do osteossarcoma requer precisão e eficácia aprimoradas, pois terapias convencionais, como a radioterapia, são pouco eficazes. Isso impulsiona a exploração de métodos alternativos, como os magnetolipossomas na nanotecnologia. Lipossomas têm se mostrado eficazes na entrega de fármacos, melhorando a eficácia de agentes quimioterapêuticos como a doxorrubicina. A integração de nanopartículas superparamagnéticas eleva ainda mais o potencial desses sistemas ao permitir a entrega localizada de fármacos. Esta abordagem inovadora não só enfrenta os desafios associados aos tratamentos convencionais, mas também representa um avanço significativo para melhorar a precisão e a eficácia global no combate ao osteossarcoma. Este projeto envolve a síntese de magnetolipossomas contendo doxorrubicina encapsulada e nanopartículas de ferrites de cálcio e magnésio (com um diâmetro de $8.75 \pm 0.35nm$, magnetização de saturação de $4.39emu/g$, e $E_g = 1.35eV$), juntamente com um estudo das suas características físicas. Os resultados revelam magnetolipossomas com um diâmetro de $542.54 \pm 71.78nm$ e um índice de polidispersividade de $27.48 \pm 3.84\%$. Notavelmente, os sistemas exibem uma encapsulação quase completa da doxorrubicina e uma eficiência de encapsulação de nanopartículas magnéticas de $49.00 \pm 5.17\%$. A investigação das cinéticas de liberação de fármacos sob condições estáticas e dinâmicas revela variações significativas nas taxas de liberação. Em particular, quando sujeitos a um campo magnético alternado com diferentes frequências e ciclos-ON (tempo em que os estímulos magnéticos são aplicados), as taxas de liberação de fármacos no caso com estimulação magnética é $\approx 30\%$ superior relativamente ao estático ao fim de 5h. Isso realça o impacto de estímulos externos na liberação controlada de substâncias encapsuladas, destacando as potenciais aplicações desses sistemas na entrega direcionada de medicamentos.

Palavras Chave: Magnetolipossomas, Osteossarcoma, Cancro, Doxorrubicina, Nanopartículas Magnéticas, Biorreator Magnético.

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List of Abbreviations

AML Aqueous Magnetoliposome	FTIR Fourier Transform Infrared Spectroscopy
AMF Alternating Magnetic Field	HBP Hydroxybenzophenone
CHEMS Cholesteryl Hemisuccinate	IR Infrared
DLS Dynamic Light Scattering	MLP Magnetoliposome
DNA Deoxyribonucleic Acid	MPI Magnetic Particle Imaging
DOX Doxorubicin	MRI Magnetic Resonance Imaging
DPPC Dipalmitoylphosphatidylcholine	NP Nanoparticle
DSPE-PEG2000 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]	OS Osteosarcoma
DSMA Dimercaptosuccinic Acid	PDI Polydispersity Index
DMSO Dimethyl Sulfoxide	RNA Ribonucleic Acid
DSC Differential Scanning Calorimetry	SML Solid Magnetoliposome
DSPC Distearoylphosphatidylcholine	SPION Superparamagnetic Iron Oxide Nanoparticle
EE Encapsulation Efficiency	TEM Transmission Electron Microscopy
EFS Event Free Survival	UV-Vis Ultraviolet to Visible
EPR Enhanced Permeability and Retention	VSM Vibrating Sample Magnetometer
FDA Food and Drug Administration	XRD X-ray Diffraction

Chapter 1

Introduction

1.1 Contextualization

In 2019, cancer emerged as the primary cause of death in 57 countries and the second most prevalent cause of mortality among individuals under 70 years old in 55 countries (Figure 1.1). Globally, it was estimated that there were approximately 19.3 million cases and 10 million deaths attributable to cancer in 2020 [1].

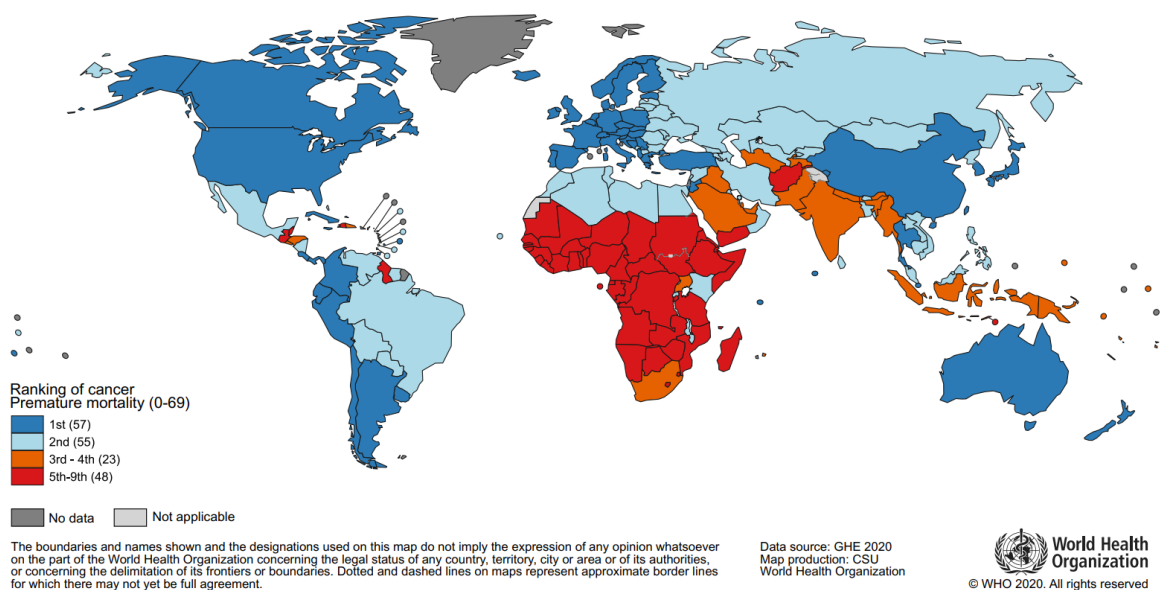


Figure 1.1: Global ranking of cancer as a cause of death at ages <70 years in 2019 [1].

Among all the cases of cancer in both children and adults, bone tumours make up a small percentage, approximately 3-5% in children and less than 1% in adults. Osteosarcoma (OS) is the most common type of malignant bone tumour that is diagnosed. [2].

The treatment methodology has remained largely unchanged for several decades. It yields a 5-year

event-free survival (EFS) rate of approximately 70% for localized tumours, while patients with metastatic or recurrent disease experience less than 20% [3].

The current treatment approach for OS involves performing a surgical resection to eliminate all cancerous tissue, in combination with systemic chemotherapy and occasionally radiotherapy to address any micrometastatic disease (cases where cancer cells spread to distant areas of the body unrelated to the primary tumours) [1].

Despite its effectiveness in eliminating significant cancer cell masses, surgical intervention is an invasive procedure that carries a heightened risk of infections and the potential for incomplete tumour removal, which may result in cancer reappearing [4]. Furthermore, chemotherapy poses an additional challenge for OS therapy. The drugs typically exhibit systemic effects, affecting the entire body rather than exclusively targeting cancer cells, leading to significant side effects [2].

Magnetoliposomes (MLPs) have emerged as a highly promising and versatile tool in the field of oncology, offering a large potential for the treatment of various types of cancer. MLPs possess unique characteristics that enable them to transport anti-cancer drugs, facilitate hyperthermia treatment [5], and aid in disease detection through advanced imaging techniques [6]. They demonstrate exceptional biocompatibility, ensuring their compatibility with biological systems. The use of biodegradable and non-toxic lipid components in MLP formulations significantly reduces the risk of adverse reactions and immune responses. This biocompatibility profile enhances the safety of these systems, making them suitable for clinical applications [7].

MLPs offer a valuable contribution to disease detection through their incorporation of superparamagnetic nanoparticles (NPs). These NPs enable the visualization of MLPs using imaging techniques such as magnetic resonance imaging (MRI) or magnetic particle imaging (MPI) [8]. This allows for accurate and non-invasive assessment of tumour size, location, and response to therapy. MLPs serve as effective imaging agents, providing critical information for monitoring disease progression and evaluating treatment effectiveness. Integrating MLPs with imaging techniques represents a significant advancement in diagnostic capabilities, facilitating personalized and precise imaging [9].

One of the most significant advantages of MLPs lies in their ability to act as carriers for anti-cancer drugs. They can also offer a controlled and targeted drug delivery system by encapsulating therapeutic agents within their lipid bilayer structures. This approach allows for the precise administration of high drug concentrations directly to the tumour site while minimizing systemic toxicity and reducing adverse side effects. MLPs effectively enhance drug efficacy and bio-availability, thus improving therapeutic outcomes [10]. MLPs can be classified based on the molecules added to their structure. Active targeting MLPs

can be modified with targeting compounds that have a higher affinity for specific types of cells, such as cancerous cells. On the other hand, they can be locally administered, and possess a passive targeting [11].

MLPs and similar systems tend to accumulate more in tumorous tissues compared to normal tissues. This phenomenon is known as the Enhanced Permeability and Retention (EPR) effect. It is caused by abnormal molecular and fluid transport dynamics in tumour tissues [12]. The magnetic targeting of MLPs can enhance the therapeutic agents of these systems in tumourous regions using an external magnetic field. This helps guide the MLPs to the targeting site, thereby synergizing the EPR effect [13]. In addition to their drug delivery capabilities, MLPs have shown great promise in hyperthermia treatment, a therapeutic approach that utilizes heat to selectively target and destroy cancer cells. By taking advantage of their magnetic properties, MLPs can be guided and localized within the tumour region using external magnetic fields. Once accumulated at the desired site, MLPs can be subjected to an alternating magnetic field, leading to the generation of heat and the induction of hyperthermia. This localized hyperthermia treatment approach holds immense potential as a non-invasive and targeted strategy for eliminating cancer cells while minimizing damage to surrounding healthy tissues [14].

1.2 Motivation and Objective

The motivation behind this project lies in the necessity to develop new methods for more efficient cancer therapy, particularly for targeting OS. Drug delivery systems have become increasingly important, particularly in the field of nanomedicine. Their primary purpose is to improve the pharmacokinetic and pharmacodynamic properties of drugs by ensuring targeted drug delivery, controlled drug release, and improved drug stability. Overall, these systems have been instrumental in enhancing therapeutic efficacy [15]. The chosen approach for study involves the use of MLPs due to their numerous advantages, as outlined in the contextualization and further discussed in upcoming chapters. The objective of the project is to develop these systems and characterize them physically and chemically, while also analysing their ability to release drugs passively and in a controlled manner through magnetic stimulation using a magnetic bioreactor.

The study on MLPs is a promising approach for improving cancer treatment. By studying their physicochemical properties and drug-release capabilities, the research of these systems hopes to develop more effective methods for combating OS.

1.3 Dissertation Structure

The dissertation will consist of the following chapters, which can be summarized as follows:

Chapter 1 - Introduction

This chapter will provide context, motivation and objective for the project, as well as present the structure of the dissertation.

Chapter 2 - Theoretical Framework

This chapter will present the theoretical concepts of the project, including nanomedicine, OS, liposomes, NPs, doxorubicin (DOX), MLPs and the magnetic bioreactor.

Chapter 3 - Experimental Procedures, Methods and Characterization Techniques

In this part it will be presented the various laboratory techniques and materials used for processing superparamagnetic NPs and MLPs. Also, this chapter explores theoretical concepts related to physically characterizing systems and equipment used in the project.

Chapter 4 - Results and Discussion

This chapter will present and discuss the results and their analysis.

Chapter 5 - Conclusion and Future Perspectives

In the final chapter, the project's conclusion and future work will be presented.

Chapter 2

Theoretical Framework

2.1 Nanomedicine

Nanotechnology is a rapidly advancing interdisciplinary field that consists in the manipulation and creation of systems and devices at the nanoscale [16]. It holds the potential to revolutionize various domains such as medicine [17], communication [18], electronics [19], robotics [20] and many more. It possesses evident advantages such as cost-effectiveness, due to less material used, and accelerated functionality in mechanical, chemical, and biological components [21].

Furthermore, nanotechnology offers a group of advantages when materials are approached to the nanoscale. However, as materials shrink to this scale, their properties change. These changes encompass alterations in structural, magnetic, thermal, optical, electronic, mechanical, and even biological aspects [22]. These modifications are intricately tied to the interactions of a limited number of atoms at surfaces and interfaces [22].

At this scale, the dominance of electromagnetic energy quantization plays a more pronounced role, molecular motion follows a more unpredictable pattern, and the concept of wave-particle duality becomes increasingly apparent. This realm showcases a pivotal shift in the ratio of surface area to volume, significantly influencing reactivity and the point at which materials melt. The phenomenon of quantum confinement emerges, imposing restrictions on the movement of electrons. Simultaneously, fluctuations in ionization energy occur as a function of the size of these types of materials. The ionization energy can either increase or decrease depending on the specific material and its size at the nanoscale [23, 24].

Nanotechnology has revolutionized various fields, including nanomedicine, by introducing innovative techniques and tools. This branch of medicine uses nanotechnology in order to prevent diseases [25] and it is applied to other fields such as imaging [26], diagnosis [27], monitoring [28], treatment [29], repair, and regeneration of biological systems [30]. Research so far has focused on the development of biosensors to aid in diagnostics and vehicles to administer vaccines, medications [15], and gene therapy,

including the development of nanosystems to assist in cancer treatment [31, 32].

It is crucial to the evolution of disease treatment, especially in the case of oncologic diseases such as OS, that is understood and utilized the concept of nanomedicine. Traditional methods have shown limited success in treating this condition, highlighting the need for new and improved ways to combat this disease.

2.2 Osteosarcoma

2.2.1 Cancer Overview

Cancer is a term used to describe a group of diseases that can affect any part of the body. It is also known as malignant tumours or neoplasms [33]. Typically, human cells undergo growth and multiplication, achieved through the process of cell division, to generate new cells in accordance with the body's needs. In cases where cells age or become damaged, they enter a state called apoptosis (cell death) followed by the introduction of fresh cells to take their place. However, there are instances when this well-regulated process malfunctions, leading to the abnormal proliferation of flawed or impaired cells in situations where such growth should not occur [34]. A fundamental indicator of cancer is the accelerated proliferation of irregular cells that surpass their typical confines and have the capacity to infiltrate neighbouring body regions. This infiltration can result in metastasis, a phenomenon where cancer spreads to distant organs. Generally, metastases are the leading cause of death from these diseases. This process is described by multiple stages: from 0 to 4 describing the evolution of the disease in the body [35]:

- **Stage 0** signifies a localized state of cancer referred to as "*in situ*", indicating that cancer remains confined to its original location. At this point, cancer has not extended to neighbouring tissues and is frequently treatable. Surgical intervention typically results in the complete removal of the tumour.
- **Stage I** characterizes cancer that has not significantly penetrated surrounding tissues and has not metastasized to lymph nodes or distant areas within the body. This phase is commonly identified as early-stage cancer.
- **Stage II and Stage III** denote cancers that have penetrated surrounding tissues to a greater extent. In some cases, these stages also involve the spread to nearby lymph nodes but not to other body regions.
- **Stage IV** designates a critical point where cancer has disseminated to other organs or body parts. This phase is also referred to as advanced or metastatic cancer, indicating an advanced spread

beyond the original site.

The development of cancer involves a multi-stage process, starting from a pre-cancerous lesion and progressing to a malignant tumour. The change in a person's genetic configuration is influenced by external factors that fall into three categories: physical carcinogens (like UV and ionizing radiation), chemical carcinogens (such as asbestos, tobacco smoke, alcohol, aflatoxin, and arsenic), and biological carcinogens (including infections caused by viruses, bacteria, or parasites) [36].

2.2.2 Bone Cancer

Primary bone cancers are very uncommon tumours, making up less than 0.2% of all cancers. However, it is hard to determine their true incidence due to their rarity. These cancers have a diverse range of clinical presentations, but with appropriate treatment, they are usually curable. The most frequently occurring types of bone cancer are OS (35%), chondrosarcoma (30%), and Ewing sarcoma (16%) [37].

Tumours of this type often cause a range of symptoms, including pain, hypercalcaemia (abnormally high levels of calcium in the blood), bone fractures, and more. These symptoms have a significant impact on the quality of life of patients [38].

Diagnosing these specific tumours is frequently challenging due to their initial symptoms resembling those of other muscle and skeletal conditions. X-rays are commonly relied upon for diagnosis, given their widespread utilization in such cases [39].

2.2.3 Osteosarcoma

The most widespread form of bone cancer among children and adolescents is OS, a type of primary malignant bone tumour. This disease causes abnormal bone cell growth, resulting in the creation of malignant bone tissues in the affected area, as seen in Figure 2.1 (provided as an example). OS primarily targets the bones surrounding the knees, tibias, femurs, legs, or arms, but it can also appear in rare instances in the spinal cord, pelvis and other regions.

One of the most common signs of OS is the presence of a mass, which is almost always firm and tender to the touch [41].

Patients with OS may also experience functional impairments, such as limping or difficulty moving the affected limb. These limitations in mobility can arise from the tumour's location and size, impacting the surrounding tissues and bone structure [42]. Consequently, loss of function and a decreased range of motion are often observed in affected individuals.



Figure 2.1: Representative X-ray of OS in the femur. The arrows represent an abnormality in bone growth [40].

While these symptoms are frequently observed in cases of OS, they can also appear in other muscle or skeletal conditions, as mentioned previously, adding complexity to the already demanding task of precisely diagnosing this aggressive form of cancer. One important factor in enhancing effective treatment and more favourable patient outcomes lies in early detection and fast intervention.[43, 44]. Approximately 10% to 20% of diagnosed patients already exhibit metastasis, predominantly affecting the lungs (around 85%). The remaining 80% to 90% are believed to carry micro-metastases, as current diagnostic tests are unable to detect them [45].

This type of cancer exhibits a particularly malignant nature, with a high potential for metastasis, especially to the lungs. Studies have shown that within 18 months to 2 years after diagnosis, patients experience a distressingly low EFS rate of less than 20% over 5 years. This alarming statistic highlights the aggressive and challenging nature of the disease, underscoring the urgent need for effective treatments and interventions to improve patient outcomes [44].

OS is categorized into high grade, intermediate grade, or low grade based on their behaviour. The tumor's grade serves as an indicator of the cancer's potential growth and spread in the body. High-grade OS are the most aggressive form. Under a microscope, they exhibit distinct differences from normal bone tissue, often revealing numerous actively dividing cancer cells. While various types of high-grade OS exist, the most common are osteoblastic, chondroblastic and fibroblastic. These three types are defined by the type of molecules they secrete for structural or biochemical support, in this case, bone, cartilage and collagen respectively [46]. Intermediate-grade OS are relatively uncommon. They are typically managed similarly to low-grade ones. In the case of low-grade OS is the slowest-growing form, these tumours closely

resemble normal bone tissue. When examined, they reveal a lower proportion of dividing cells [47].

The microenvironment of this type of tumour plays a critical role in its development. It consists of various types of cells, including tumour cells, immune cells (such as white blood cells like lymphocytes, macrophages, and neutrophils, which protect the body from infection and disease), fibroblasts, and endothelial cells (which maintain the integrity of blood vessels and regulate blood flow). In addition, the microenvironment includes the extracellular matrix, which is produced by the fibroblasts and surrounds and supports the cells. The combination of these cell types greatly influences the progression of the disease, affecting its growth, survival, and the spread of tumour cells [48].

2.2.4 Current Osteosarcoma Therapies

The predominant approach to treating OS mainly revolves around a synergistic combination of surgical intervention, chemotherapy, and, in some cases, radiation therapy.

Surgery

This treatment strategy aims to completely remove tumorous tissue from the affected region using two main approaches: amputation and limb salvage surgery [49].

Amputation is a crucial option for early OS cases, especially when the tumour has not spread to other parts of the body and when other therapies like chemotherapy have proven ineffective. As a consequence of using this treatment strategy, there is often a need to use internal prostheses to effectively address the resulting changes in bone structure. These internal prostheses serve an important purpose by providing support and strength to the affected area, compensating for the removed tumorous tissue. Additionally, they may act as substitutes for the abnormal bone tissue that has been excised during the surgical procedure. [43, 2]

Limb salvage procedures are more recent and have demonstrated the ability to minimize the removal of healthy bone tissue. To ensure the effectiveness of this approach, precise determination of the tumour region is crucial, and this is achieved through the utilization of X-rays, computed tomography scans, and MRIs. By employing these imaging techniques, surgeons can accurately assess the full dimensions of the tumour and perform surgical removal while preserving healthy bone tissue [50].

Compared to amputation, limb salvage procedures result in a functional and durable limb for patients. This technique allows for the salvage of the affected area, contributing to better functional outcomes and improved quality of life for the patients [51].

Chemotherapy

This therapy involves the use of medication to eliminate any cancer cells that may have been left behind after surgery. It also works to target the entire system and prevent any new metastasis while halting the growth of potential new tumours [52].

Preoperative chemotherapy is often used as a treatment method for cancer patients. This involves the administration of chemotherapy drugs throughout the entire body for a period of 6 to 18 weeks before surgery. The goal of this treatment is to shrink the tumour, making it easier to remove during surgery. While it is a common practice, it is not without its risks and side effects [53]. During the treatment process, various drugs are administered, including widely recognized and effective medications like methotrexate, cisplatin, and DOX, as documented in the source [54]. However, the extended use of these drugs carries side effects throughout the body, including neurotoxicity, gastrointestinal reactions, bone marrow complications, liver and kidney damage and cardiomyopathy [55, 56].

Radiotherapy

In cases where chemotherapy proves to be less effective, local radiotherapy can be employed to exert an impact on the tumour and control its growth [57]. Radiotherapy employs ionizing radiation to create minute breaks in the deoxyribonucleic acid (DNA), thereby rendering normal cellular functions, such as cellular division, impossible [58].

However, a challenge arises when dealing with OS, as this type of tumour is not very sensitive to radiation. Consequently, higher doses of radiation must be administered to the tumour site, which inevitably increases the risk of side effects for the patient [49].

2.2.5 Challenges in Cancer Therapy

Cancer therapy has witnessed remarkable progress in recent years, with advances in understanding the intricate biology of the disease and the development of innovative treatment strategies [59]. Despite these significant advancements, the field still has many challenges that inhibit more effective and targeted cancer treatments including:

- **Lack of specificity** when it comes to targeting cancer cells while preserving healthy ones. Conventional treatment methods such as chemotherapy and radiation do not have the precision required to discriminate between cancerous and non-cancerous cells. The development of therapies that possess selectively is crucial to destroy cancer cells while preserving as many normal cells as possible

[60].

- **Cytotoxicity** presents another significant challenge. While these treatments effectively induce tumorous cell death, they also inflict damage on healthy tissues, resulting in many side effects such as hair loss, nausea, and immune suppression. Finding the right balance between making treatments effective and reducing harm to healthy cells is a tough challenge in developing therapies [61].
- **Multi-drug resistance** is another obstacle in cancer therapy. Cancer cells can adapt and develop resistance to multiple drugs, rendering previously successful treatments ineffective. This ability creates the need for innovative approaches that can circumvent or counteract drug resistance mechanisms [62].
- **Stem-like cancerous cells** represent a particularly challenging target. These cells possess the ability to self-renew and differentiate into various cell types within the tumour. These cells are often resistant to conventional therapies, demanding novel strategies to effectively target and eliminate them [63].

2.2.6 New Approaches for Osteosarcoma Therapy

Gene Therapy

Gene therapy involves introducing DNA sequences into foreign cells to modify their behaviour. While some examples of this therapy have been utilized, only a limited portion of it has been specifically used on OS. Research in this domain has explored genes adapted for this type of cancer, including suppressor genes that hinder specific cellular functions, suicide genes that trigger cell apoptosis, and immune genes [64]. Research in this field includes the investigation of liposome-delivered *thymidine kinase* and *ganciclovir*, suicide genes, in the context of OS cells [65], as well as the utilization of *TP53*, a tumour suppressor gene responsible for regulating cell division and inhibiting further cancer development [66].

Immunotherapy

Immunotherapy presents another promising approach for OS treatment. This strategy seeks to regulate the immune system's ability to distinguish between cancerous and healthy cells, eradicating or restraining cancer growth. This approach has been employed through various methods, including tumour vaccines, immune modulators, modified T cells (a type of white blood cells central to the immune system),

and modified cytokines (proteins secreted by immune cells to trigger an immune response) [67]. Some examples of these types of therapy are presented in Table 2.1

Table 2.1: Studies of immunotherapy adaptations for OS

Approach	Description	Article
<i>Cytokines</i>	Use of interleukin-18, a type of cytokine, for mouse OS	[68]
<i>Cytokines</i>	Use of interleukin-34, a type of cytokine, for mouse OS	[69]
<i>Tumour Vaccine</i>	Vaccine efficacy against primary and metastatic cancer with in vitro-generated in OS cells in mice	[70]
<i>Modified T Cells</i>	Anti-CD166/4-1BB chimeric antigen receptor T cell therapy for the treatment of OS	[71]

Drug Delivery Strategies

In recent times, there has been a growing interest in the field of new cancer therapies towards enhancing drug delivery systems for targeting cancer cells, while concurrently minimizing the toxicity and adverse effects associated with therapies. Cleavable hydroxybisphosphonate (HBP) linked DOX presents a promising approach for treating OS. This innovative bone-specific medication is created by chemically bonding DOX to hydroxy bisphosphonates, which naturally exhibit a strong affinity for bone tissue. The primary goals of this treatment are to enhance its effectiveness while minimizing its impact on the rest of the body. Notably, this formulation demonstrates rapid and consistent targeting of both bone tissue and tumour-related abnormal bone growth. This precise targeting enables the delivery of a higher concentration of DOX directly to the OS region. The release of DOX from this nanosystem is triggered by the acidic pH levels characteristic of OS tumorous regions [72].

Liposomes have demonstrated a significant impact on the study of OS cases. Researchers are intensively investigating liposome-based delivery systems that encapsulate substances such as DOX and cisplatin. These studies aim to push advancements in localized and targeted chemotherapy for patients with OS [73]. This approach holds promise for enhancing the efficacy and precision of chemotherapy while minimizing systemic side effects.

Another innovative approach is the use of hydrogels. Self-assembling silk hydrogels has emerged as a promising breakthrough in the targeted treatment of OS. With the help of X-ray, these hydrogels can be accurately placed in the affected tissue, offering advantages including excellent biosafety, minimal systemic toxicity, and a significantly non-invasive nature. This novel approach holds substantial potential

in enhancing the management of OS at the local level [74].

While the application of MLPs in the context of OS remains unknown, considering the established efficacy of liposome-based systems and the proven effectiveness of DOX in OS treatment, the clear potential of superparamagnetic iron oxide nanoparticles (SPIONs) presents an innovative avenue for addressing this cancer type.

2.3 Liposomes

In 1965, while testing a new electron microscope, Alec Douglas Brangham of the Institute of Animal Physiology in Cambridge accidentally discovered that phospholipids could create spherical vesicles when suspended in water [75]. These systems consist of one or more concentric bilayers of phospholipids, similar in composition to cell membranes, which provide excellent biocompatibility and allow them to easily fuse with cell membranes [76].

This marked the first description of liposomes, which have become highly useful systems with multiple applications in drug delivery [77], gene therapy [78], cosmetic formulations [79], and vaccine carriers [79].

2.3.1 Phospholipids Composition

Phospholipids are molecules with both hydrophilic and hydrophobic properties. The hydrophilic polar group, also called "head", consists of a phosphate group linked to a glycerol molecule, while the hydrophobic tail consists of two fatty acid chains, illustrated in Figure 2.2 [80].

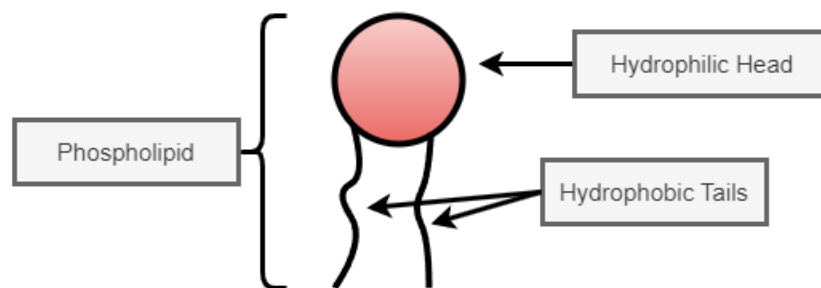


Figure 2.2: Illustrative representation of a phospholipid.

Phospholipids are classified based on their origin, either natural or synthetic. They also can be characterized by their transition temperature (T_m), that is the temperature required to induce a change in the

lipid's physical state from the ordered gel phase, where the hydrocarbon chains are fully extended and closely packed, to the disordered liquid crystalline phase, where the hydrocarbon chains are randomly oriented and fluid [81].

Dipalmitoylphosphatidylcholine (DPPC) (Figure 2.3) is the most commonly used phospholipid due to its drug encapsulation capacity, thermosensitivity ($T_m = 41^\circ\text{C}$), and suitability for biomedical applications and controlled release [82].

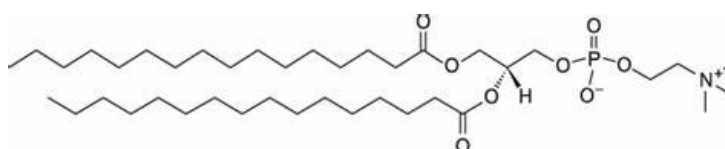


Figure 2.3: DPPC molecular structure.

Also, Distearoylphosphatidylcholine (DSPC) (Figure 2.4) has a higher transition temperature ($T_m = 55^\circ\text{C}$). When combined with DPPC in a binary formulation, it results in more rigid vesicles, which in turn enhances the amount of drug release [83]. It has also been found that adjusting the ratio between DPPC and DSPC can regulate the number of nanoscale gaps induced in liposome membranes, leading to improved release kinetics. These findings suggest that the careful selection and manipulation of lipid components can significantly impact drug delivery efficacy [84].

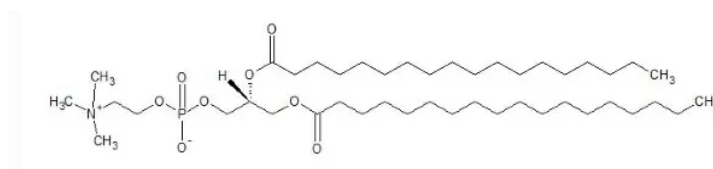


Figure 2.4: DSPC molecular structure.

Additionally, when 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000) (Figure 2.5) is added to the lipid formulation it prevents the aggregation and enhances stability and half-life comparatively to conventional liposomes [85].

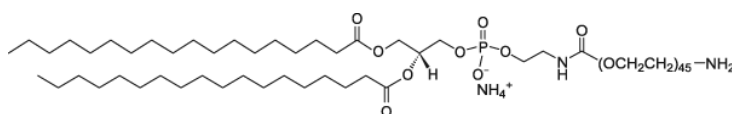


Figure 2.5: DSPE-PEG2000 molecular structure.

Furthermore, an acidic cholesterol ester known as cholesteryl hemisuccinate (CHEMS) can also be used, whose molecular structure is presented in Figure 2.6. This will allow for the acquisition of a lamellar structure of liposomes upon hydration in aqueous solutions with a neutral or alkaline pH [86].

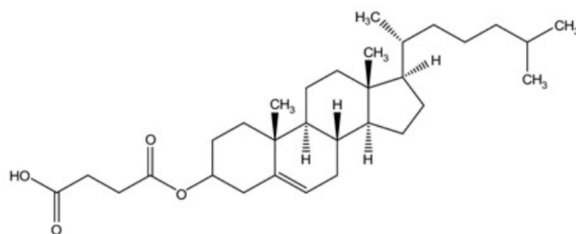


Figure 2.6: CHEMS molecular structure.

Thus, within the scope of this dissertation and considering the aforementioned, the previously mentioned combination was utilized due to the benefits presented by the combination of these lipids.

2.3.2 Liposome Organization

Phospholipids, when in contact with an aqueous environment, arrange themselves spontaneously. This organization is a consequence of the hydrophilic interaction among polar groups and the *Van der Waals* forces that come into play between hydrocarbon chains. This intricate process can be explained from a thermodynamic perspective: it is primarily driven by the natural tendency of hydrophobic lipid chains to avoid contact with water, causing them to repel each other [87].

The result of this interaction is the formation of thin, flexible lipid bilayers, as illustrated in Figure 2.7. These bilayers establish a unique structure that acts as a semi-permeable barrier, selectively allowing certain substances to pass through while restricting the movement of ions and other compounds such as polar molecules.

These bilayers are immersed in an aqueous medium on both their outer and inner sides, effectively creating a water-filled environment on both surfaces. This arrangement has significant implications in various biological and chemical contexts [87].

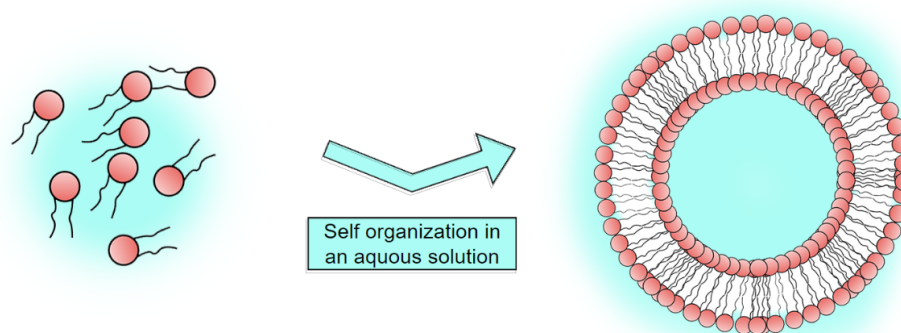


Figure 2.7: Illustration of phospholipid self-arrangement in an aqueous solution.

Furthermore, during their formation, liposomes can encapsulate hydrophilic materials such as certain drugs in the aqueous solution, or hydrophobic compounds within the layers of their lipid bilayer, as illustrated in Figure 2.8 [88].

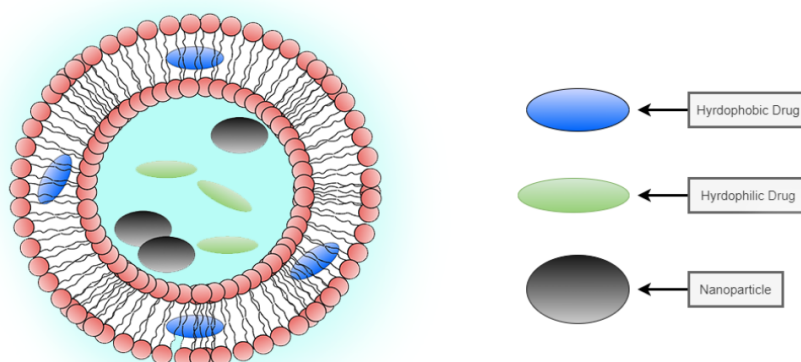


Figure 2.8: Carrier liposome with hydrophilic drug and NPs loaded in the core region of liposome and hydrophobic drug loaded.

The biomedical potential of various drugs and compounds can be significantly improved by enclosing these systems, which can help to protect them from degradation and enhance their therapeutic efficacy.

2.3.3 Liposomes as Nanocarriers

Liposomes are versatile and promising nanocarriers for drug delivery and other biomedical applications. As research in this area continues, liposomes play an increasingly important role in nanomedicine due to numerous advantages presented next [89]:

- *Biocompatibility*: Liposomes are composed of the same material as cell membranes, making them highly biocompatible.
- *Drug Protection*: It is possible to protect drugs from degradation caused by exterior factors by encapsulating them inside the bilayer or its core, extending the half-life of the drug.
- *Controlled Release*: By exposing liposomes to external factors, such as heat, their contents can be released in a controlled manner. Additionally, they can offer a sustained release of medication passively or via stimulation.
- *Site-targeting*: These systems can transport their contents to the affected area through either passive or active targeting methods. This can help to reduce side effects throughout the body and increase the maximum dose that can be tolerated, enhancing the therapeutic benefits.

Although liposomes have been successful in therapeutic applications, they still present some disadvantages, such as [90, 91]:

- *Low Lifespan*: Despite extensive research, liposomes still face issues such as short shelf life and poor circulation in the blood. This can cause drugs to leak out too early or liposomes to fuse together.
- *High Production Cost*: Phospholipids' high cost makes mass production of these systems difficult.
- *Unwanted Reactions*: At times, phospholipids can experience oxidation and reactions similar to hydrolysis with certain compounds.
- *Low solubility*: Some liposomes may have low solubility, which can affect their ability to deliver drugs effectively.

In medicine, liposomes are used as drug carriers to enhance drug effectiveness and minimize side effects. Liposomes have several uses, including chemotherapy, as presented in Table 2.2:

Table 2.2: Liposome-encapsulated drugs and their uses.

Drug	Application	Article
<i>Doxil</i>	Ovarian, Kaposi's sarcoma and breast cancer	[92]
<i>Myocet</i>	Metastatic breast cancer	[93]
<i>Marqibo</i>	Acute lymphoblastic Leukaemia	[94]
<i>Onivyde</i>	Metastatic adenocarcinoma of the pancreas	[95]
<i>Lipo-dox</i>	Ovarian and breast cancer	[96]

Although liposomes are extensively used in chemotherapy, they are also widely used in delivering drugs for infections. For example, *Mosquirix* is used in the treatment of malaria via vaccination [97], and *AmBisome* is used for fungal infections [98].

The research on new liposome formulations is ongoing, as their potential for cancer therapy continues to be proven. *Prometil* and *Lipocurc* are two such formulations being studied for the treatment of solid tumours [99, 100], which are abnormal masses of tissue without cysts or liquid areas. Other liposome-based in development include *PTX-LDE* for ovarian carcinoma [101].

2.4 Magnetic Nanoparticles

Superparamagnetic NPs are extensively researched systems with large potential in the field of biomedical applications. Their capabilities encompass the detection of bacteria, viruses, and proteins, as well as their utility in advancing MRI technology among other areas [102].

2.4.1 Types of Magnetism

In order to understand superparamagnetism it is important to understand the different types of magnetism, and their differences in aspects of electron spin, their orbital movement and how an external magnetic field (H) affects it.

Magnetization (M), in Ampere per meter (A/m), can be defined by the following Equation 2.1:

$$M = H\chi_m \quad (2.1)$$

Where χ_m is the magnetic susceptibility of a certain material that indicates a measure of how susceptible the material is to becoming magnetized [103].

There are materials with five different types of magnetic properties: ferromagnetism, antiferromagnetic, ferrimagnetism, diamagnetism and paramagnetism.

Materials that exhibit ferromagnetism have positive magnetic susceptibilities and high values, in the order of 10^2 and 10^6 , so they are strongly attracted to external magnetic fields and have the ability to retain magnetic properties after the removal of an external field like it is observed in Figure 2.9. The remnant magnetic flux density, B_r , is the magnetic flux density that remains in a ferromagnetic material after an external magnetic field has been removed. The coercive field strength, H_c , is the value of the external magnetic field that must be applied in the opposite direction to reduce the magnetic flux density in the material to zero.

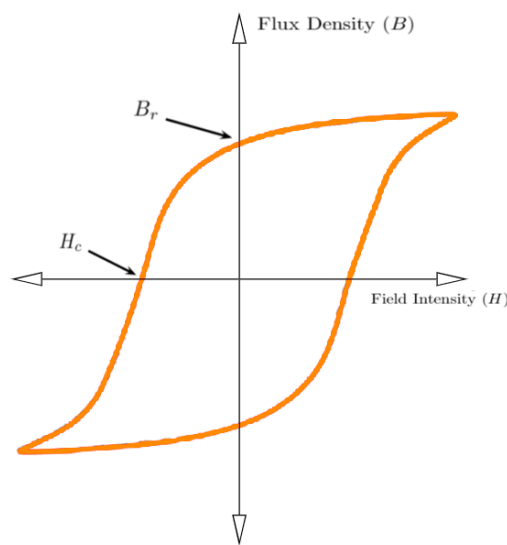


Figure 2.9: Schematic representation of the $B - H$ curve of a ferromagnetic material. The magnetic flux density that remains in a ferromagnetic material after removing an external magnetic field is represented by B_r , while H_c represents the coercive field strength, adapted from [104].

This happens because these materials, such as iron or nickel, have some unpaired electrons in their constitution and, as a result, their atoms have a permanent atomic magnetic dipole. Since the magnetic dipole moments of the atoms exert strong forces on the moments of neighbouring atoms, there is a small region of space where the moments are aligned with each other, even without an external field, as shown in Figure 2.10, the phenomenon occurs.

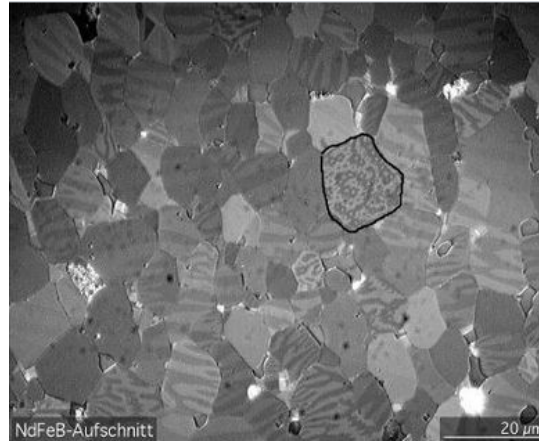


Figure 2.10: Microcrystalline grains within a piece of $Nd_2Fe_{14}B$ (the alloy used in neodymium magnets) with magnetic domains made visible with a Kerr microscope. The domains are the light and dark stripes visible within each grain [105].

Another important aspect of ferromagnetic materials is a specific temperature known as the Curie point, which varies for each substance. At this temperature, ferromagnetic materials undergo a transformation, losing their distinctive magnetic properties and no longer exhibiting magnetism. Nevertheless, upon cooling, they once again acquire their ferromagnetic attributes [106].

For the case of antiferromagnetic materials, such as manganese oxide (MnO). Neighbouring ions act as tiny magnets and spontaneously arrange in opposing, or anti-parallel, orientations at lower temperatures within the material as demonstrated in Figure 2.11. As a result, the material does not exhibit significant external magnetism. Under specific conditions, the material demonstrates antiferromagnetic characteristics, where the magnetism from aligned magnetic atoms or ions in one direction is balanced by those in the opposite direction [107].

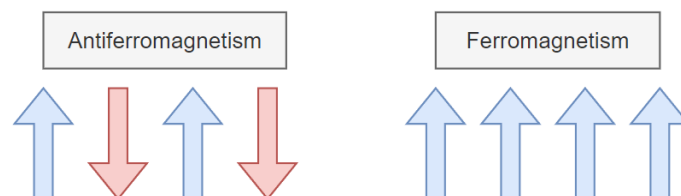


Figure 2.11: Schematic of alignment of the material's polarization for antiferromagnetic and ferromagnetic materials.

This anti-parallel alignment of atomic magnets can be disrupted by heating and disappears completely beyond a distinct temperature known as the Néel temperature (T_N). This temperature, which depends on the antiferromagnetic material, was named after the pioneering work of French physicist Louis Néel in 1936, who played a key role in understanding antiferromagnetism. While some antiferromagnetic materials have Néel temperatures that go well beyond room temperature, more commonly, these temperatures are lower [108].

When antiferromagnetic solids are exposed to an external magnetic field, their behaviour varies depending on the temperature. At extremely low temperatures, the material remains unresponsive to the external field due to the unwavering anti-parallel alignment of the atomic magnets. As the temperatures increase, certain atoms break free from this organized arrangement and align themselves with the external field. The atoms line up and create some magnetism in the solid, strongest at the Néel temperature. But when the temperature increases past that point, the excessive movement of atoms prevents them from aligning with the magnetic field, causing the small amount of magnetism present in the solid to gradually decrease.

Ferrimagnetism is a specific type of permanent magnetism observed in solids. In this type of material, individual atomic magnetic fields arrange themselves spontaneously, with some aligning parallel or in the same direction (similar to ferromagnetism), while others adopt an anti-parallel configuration, pairing off in opposite directions (similar to antiferromagnetism). This behaviour is particularly evident in single crystals of ferrimagnetic substances, where the dominant influence comes from the parallel alignment described in Figure 2.12. With this, the presence of atoms in the anti-parallel arrangement limits the overall magnetic strength of these materials compared to purely ferromagnetic materials. This spontaneous alignment responsible for ferrimagnetism is disrupted entirely when the material surpasses a specific Curie temperature, just like ferromagnetic materials. Once the material's temperature drops below the Curie point, the phenomenon of ferrimagnetism becomes active again [109].

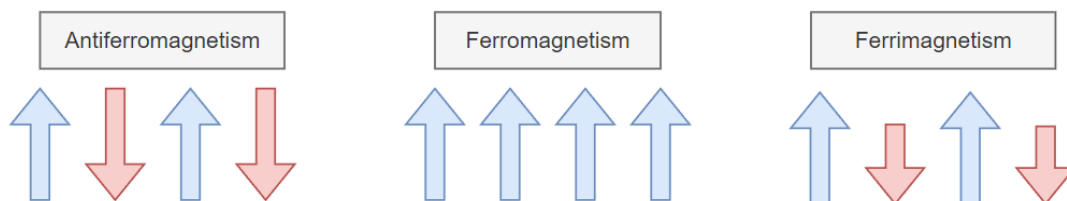


Figure 2.12: Schematic of the alignment of the material's polarization for antiferromagnetic, ferromagnetic materials and ferrimagnetic material.

In the case of diamagnetic materials, they exhibit negative susceptibility, with magnetic susceptibility values ranging from -10^{-6} to -10^{-3} [110]. They are slightly repelled in the presence of external magnetic fields. The susceptibility of diamagnetic materials arises from the realignment of electron orbitals under the influence of a magnetic field. This results in a weak magnetization that opposes the applied external magnetic field as observed in Figure 2.13. As all their electrons are paired, they lack a permanent magnetic moment. Furthermore, they do not retain their magnetic properties when the external magnetic field is removed [111, 103].

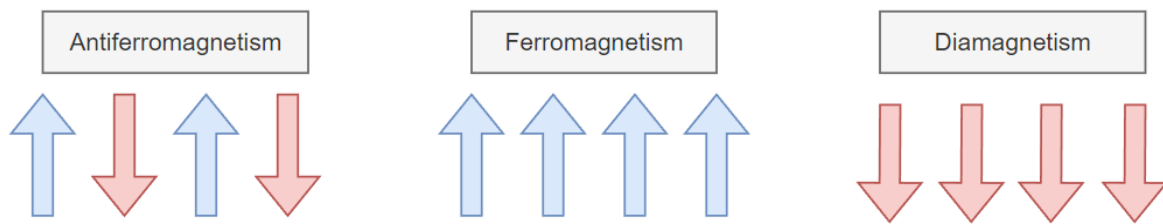


Figure 2.13: Schematic of the alignment of the material's polarization for antiferromagnetic, ferromagnetic materials and diamagnetic materials.

Finally paramagnetic materials exhibit a positive magnetic susceptibility with reduced values ($10^{-5} < \chi_m < 10^{-3}$), causing them to be slightly attracted to external magnetic fields [112]. Their magnetic properties arise due to the presence of some unpaired electrons and the realignment of electrons caused by the external magnetic field. In the absence of these fields, the magnetic moments tend to align with the field, but this tendency is counteracted by the random orientations of the moments due to thermal motion described in Figure 2.14. The fraction of moments that become aligned with the field depends on the field's intensity and temperature. After the field is removed, they do not retain their magnetic properties [113, 103].

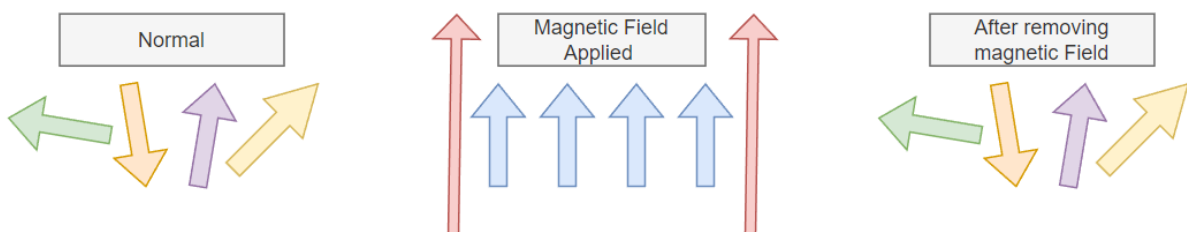


Figure 2.14: Schematic of the alignment of the material's polarization before, during and after applying an external magnetic field represented as the two red arrows.

With a better understanding of the complexities of paramagnetism, it is now possible to comprehend materials with superparamagnetism. These phenomena occur at the nanometric level and result from the small size of particles, which give rise to this effect. .

2.4.2 Superparamagnetism

Ferromagnetic materials consist of discrete regions known as domains, each comprising a large number of atomic dipoles, typically around 10^{12} to 10^{18} [114], with inherent magnetic moments. In the absence of an external magnetic field, the orientations of these moments within a domain are randomly oriented.

However, when an external magnetic field is applied, the domains begin to align their magnetic moments in parallel with the field direction. This alignment process continues until all the domains are aligned, resulting in saturation magnetization. At this point, the material's overall magnetic properties cannot be significantly enhanced by increasing the external magnetic field strength (saturation magnetization) [115].

Between these aligned domains, there exist interfaces referred to as Bloch walls. These Bloch walls represent regions of magnetic transition between neighbour domains. They are essential for accommodating the gradual change in magnetization orientation from one domain to the next [116]. This transition can be visualized in Figure 2.15.

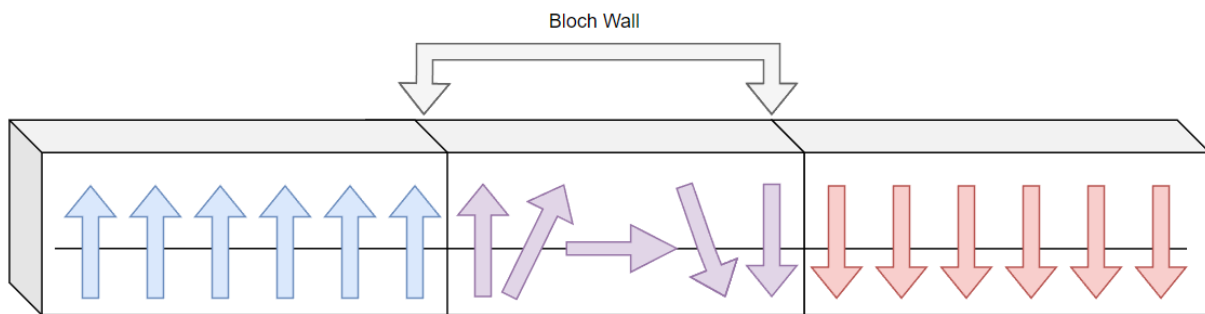


Figure 2.15: Illustration representing a Bloch Wall featuring the magnetization transition between two distinct domains.

The emergence of domains is a result of a delicate equilibrium between magnetostatic energy, which originates from an applied external magnetic field (and increases proportionally with the material's volume), and the energy associated with Bloch walls (which escalates with the surface area between the domains) [117]. This interplay is responsible for the formation process and since the creation of domains is defined by the particle size, there is a specific size at which the magnetostatic energy becomes equal to the energy

between domains. This critical size is known as the critical diameter (D_c). Below this critical threshold, the energy required for the formation of Bloch walls surpasses the energy necessary to maintain the magnetostatic energy of the single-domain state [118].

Furthermore, when particle dimensions are smaller than the critical diameter, these ferromagnetic particles are composed of only one domain and a superparamagnetic diameter can be achieved, which varies depending on the material. Below this specific point, ferromagnetic particles exhibit superparamagnetic behaviour [119].

In Figure 2.16, the magnetization curves of ferromagnetic, paramagnetic, and superparamagnetic materials are compared. Ferromagnetic materials exhibit a hysteresis loop in their magnetization curve. As the external magnetic field increases, the material's magnetization increases rapidly and reaches saturation. When the external field is removed, the material retains some residual magnetization. In contrast, the magnetization of paramagnetic materials increases with the external magnetic field without a hysteresis loop. The material does not retain magnetization once the field is removed. The magnetization curve for superparamagnetic materials is similar to that of paramagnetic materials without hysteresis. However, it responds faster to changing magnetic fields and has a saturation magnetization similar to a ferromagnetic material [120].

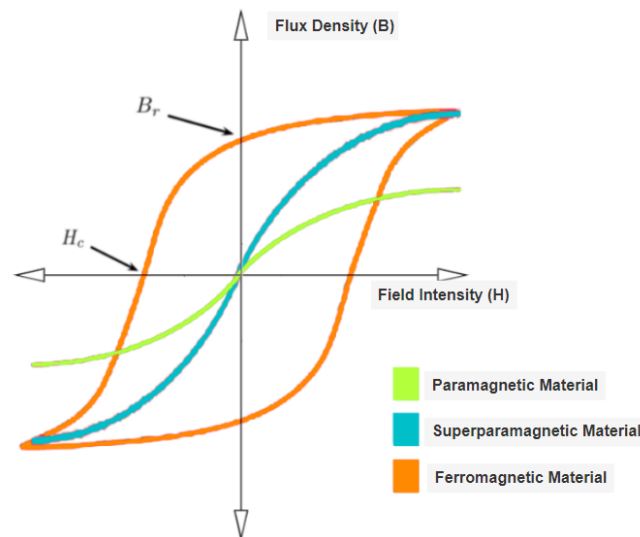


Figure 2.16: This figure schematically represents the B-H curve of three different materials: paramagnetic, superparamagnetic, and ferromagnetic. The magnetic flux density that remains in a ferromagnetic material after removing an external magnetic field is represented by B_r , while H_c represents the coercive field strength, adapted from [104].

The phenomenon discussed above has a wide range of practical applications, and its ability to create superparamagnetic nanoparticles has opened up new possibilities in various fields.

2.4.3 Superparamagnetic Iron Oxide Nanoparticles

SPIONs are widely recognized as the predominant superparamagnetic materials. These magnetic NPs have gained significance due to their remarkable stability at physiological pH levels and affordable and straightforward creation process[121].

These SPIONs have found extensive application in the biomedical domain, particularly as contrast agents for X-rays [122] and MRI in the diagnosis of tumours and other medical conditions [123]. Notable examples of SPIONs approved for such purposes include Feraheme®, Feridex I.V.®, and Gastromark® [124]. Furthermore, the versatility of SPIONs allows for functionalization with various drugs or other systems, including cytotoxic agents for chemotherapy and also in the pursuit of enhancing treatment efficacy, these NPs can be used to induce magnetic hyperthermia under alternating magnetic fields [125] among other applications referenced in Figure 2.17 [126].

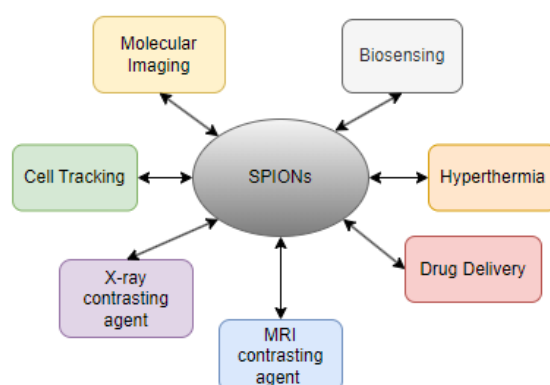


Figure 2.17: Schematic representation of the multiple uses of SPIONs.

Although the extensive use of SPIONs is important, it is crucial to consider certain downsides associated with their utilization. Notably, these NPs have been associated with various potential mechanisms of toxicity, including cytotoxicity, alterations in metabolic activity, induction of oxidative stress on cells, inflammation, and even DNA damage [127, 128].

To mitigate the cytotoxic effects of SPIONs, multiple modifications have been explored. These include strategies such as encapsulation of the NPs or the application of a surface coating [129].

2.4.4 Magnetic Hyperthermia

In the process of magnetization in SPIONs, there are primarily two mechanisms for returning to the original state once the magnetic field is withdrawn called Néel and Brownian relaxations. In Néel relaxation, the magnetization of an individual NP can swiftly change direction, and this is quantified by the Néel relaxation time (τ_N). This time interval is influenced by factors like particle size, magnetic anisotropy, and the surrounding temperature. This relationship is mathematically expressed in Equation 2.2.

$$\tau_N = \tau_0 \exp \left(\frac{KV}{k_B T} \right) \quad (2.2)$$

Here, τ_0 represents a material-specific characteristic termed the "attempt time", K stands for the anisotropy constant, while V denotes the NPs's volume. Additionally, k_B refers to the Boltzmann constant, and T indicates the temperature of the medium.

Brownian relaxation involves the NPs and its magnetic moment rotating, leading to interactions with the surrounding liquid in which the particles are suspended. The duration of Brownian relaxation is denoted as the Brownian relaxation time (τ_B), which can be expressed using Equation 2.3.

$$\tau_B = \frac{3\eta V_H}{k_B T} \quad (2.3)$$

Here, η signifies the viscosity of the surrounding medium, while V_H represents the hydrodynamic volume of the NPs. In this relaxation process, the viscosity of the medium is of paramount importance. Notably, Brownian relaxation becomes markedly less significant for small particles immersed in high-viscosity environments, wherein Néel relaxation overwhelms. The prevalence of either mechanism predominantly hinges on factors such as particle size, magnetic anisotropy, and the viscosity of the liquid medium.

When subjected to a high-frequency alternating magnetic field (AMF), wherein the magnetic field direction changes more rapidly than the relaxation time of the NPs, the reorientation of magnetic moments is postponed. This delay results in energy losses and subsequent dissipation of heat [130].

2.5 Magnetoliposomes

MLPs represent highly promising nanosystems with significant potential across diverse biomedical applications. These types of liposomes grouped superparamagnetic NPs, enhancing their functionality. The several types of MLPs depend on the position of these NPs in relation to the liposome. Their versatile capabilities encompass crucial roles in bio-imaging and efficient drug delivery.

MLPs exhibit dual benefits, functioning as contrasting agents for advanced MRI scans and as efficient carriers for chemotherapeutic agents. Their magnetic properties enable precise magnetic field-guided targeted drug delivery, ensuring accurate drug localization [131]. Moreover, MLPs offer the advantage of controlled and on-demand drug release, facilitating personalized therapeutic approaches.

The potential applications of MLPs span various domains such as hyperthermia therapy for targeted tissue heating. Furthermore, these systems contribute to the advancement of cell tracking and sorting techniques, enhancing our understanding of cellular behaviour [132, 133].

As previously highlighted MLPs represent lipid systems formulated for the agroupation of superparamagnetic NPs and other compounds such as pharmaceutical drugs. These specialized systems can be categorized according to the specific configuration of the NPs they encompass as it is depicted in Figure 2.18. Aqueous magnetoliposomes (AMLs) are composed of magnetic NPs dispersed in the aqueous core of the liposome and solid magnetoliposomes (SMLs): where a cluster of magnetic NPs is surrounded by the lipid bilayer. NPs can attach to liposomes by either adhering to the exterior or being encapsulated within the lipid bilayer.

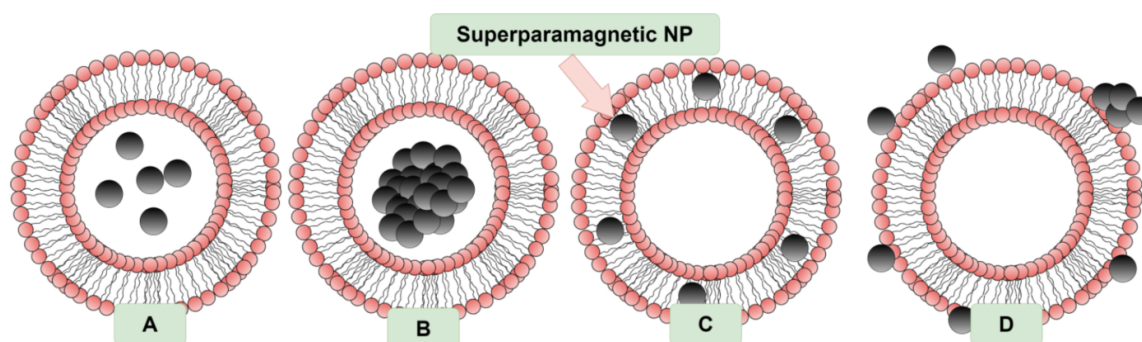


Figure 2.18: Illustration depicting various types of MLPs: AML (A), SML (B), magnetic NPs embedded in the phospholipid bilayer (C), and surface-conjugated NPs (D).

Different types of molecules can be co-encapsulated with NPs, including DOX, which is of particular relevance to this project. These systems have the ability to infiltrate cells due to their similar composition. The mechanisms of entry include fusion, endocytosis, pinocytosis, and others, each involving different mechanisms. As a result, they can release their contents within a targeted set of cells, particularly cancerous ones. Figure 2.19 illustrates and describes the process through pinocytosis, where the cell membrane encapsulates the MLP. This process involves: 1. Initial approximation of a cell by SML loaded with DOX. 2. Penetration process as SML gains entry into the cell. 3. Internal residence of SML within the cell. 4. Controlled release of SML's payload triggered by external stimulation [134].

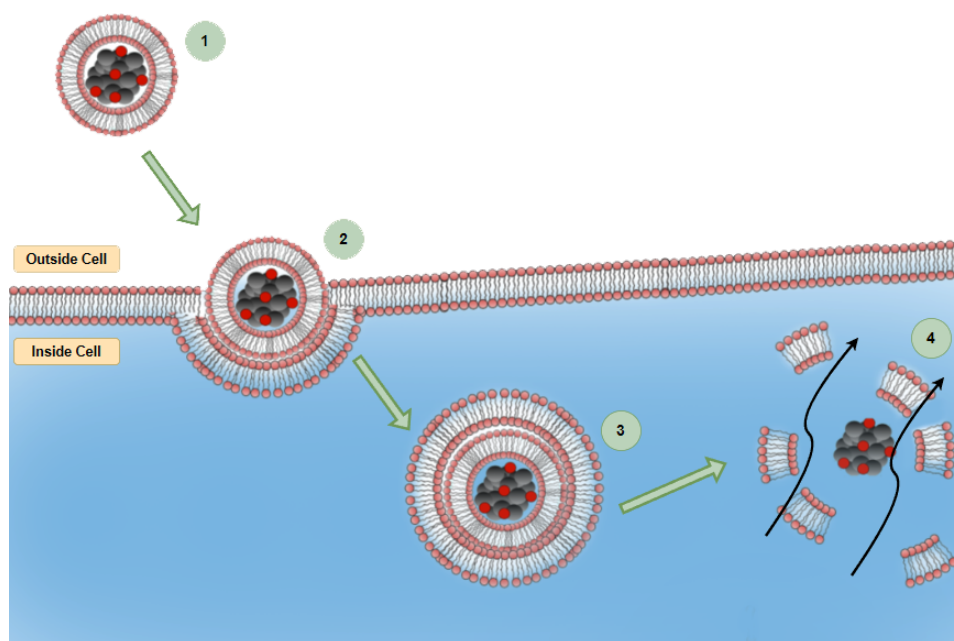


Figure 2.19: Illustration depicting the sequential stages of encapsulated DOX infiltration within a cancerous cell through an SML.

2.5.1 Mechanisms of Action of Magnetoliposomes

As mentioned earlier, MLPs have extraordinary potential in the field of oncologic therapy. One therapeutic approach involves the utilization of hyperthermia. This method takes advantage of the inherent sensitivity of cancer cells to temperature variations compared to healthy cells. In the temperature range of 42°C to 45°C, cancerous cells undergo a state of cellular death, making hyperthermia a viable strategy for treatment.

This process can be applied in the field of MLPs where it is possible to sustain a localised heat source and use the thermal sensitivity of cancerous cells to eliminate them. This application of MLPs is highly studied for multiple cases of cancer. Notable research includes studies involving MLPs with encapsulated DOX for combined chemotherapy and hyperthermia targeting rat C6 glioma cells [135], as well as investigations into hyperthermia utilizing MLPs loaded with gemcitabine and paclitaxel to target human primary breast cancer cells (MGSO-3 line) [12]. These studies underscore the promising potential of utilizing MLPs for hyperthermia therapy.

MLPs offer promising applications in medical imaging, particularly in the context of MRI scans. They have the potential to aid in the delivery of specific contrasting agents such as SPIONs into targeted cells, as highlighted by Faria *et al.* [136]. Notably, studies have demonstrated the improved contrast of MRI

images through the utilization of MLPs, as shown by Carvalho *et al.* [137]. Furthermore, the modulation of this contrast could potentially be achieved by external stimuli, such as changes in temperature or the application of focused ultrasound, as explored by Lorenzato *et al.* [138].

MLPs have also a huge potential in the field of drug delivery system. Using liposomes as carriers to encapsulate active agents allows their targeted delivery and controlled release. This approach ensures that the payload is dispensed only under precise conditions. In domains like oncology, particularly in chemotherapy, existing methods of drug delivery frequently result in the non-localised impact of the drugs, resulting in numerous side effects. The exploration of MLPs offers a more focused and concentrated dose within the affected area. This not only amplifies the therapeutic effect of the drug used but also minimizes side effects related to systemic toxicity, contributing to a more refined and effective treatment strategy [139]. Research, exemplified by Babincová *et al.* uses AMF to regulate the release of DOX, showcasing the potential for site-specific release mechanisms with promising implications for chemotherapy applications [140]. Additionally, a laser-triggered drug was released due to the temperature increase on the MLPs [141]. Furthermore, techniques used in drug delivery can also be extended to transport other agents, such as proteins and genes. This expansion serves to mitigate adverse reactions arising from physiological conditions, while concurrently facilitating cellular entry [139].

2.6 Doxorubicin

DOX is an antibiotic with an important role in chemotherapy, recognized for its effectiveness in cancer treatment. Often integrated into a comprehensive combination of chemotherapy drugs, DOX contributes significantly to the creation of a powerful and efficacious anti-tumour treatment.

Having received the stamp of approval from the Food and Drug Administration (FDA), DOX serves as a crucial therapeutic option for multiple types of solid tumours. These include OS, breast cancer, small lung tumours, ovarian cancer, and bladder cancer. The versatile application of DOX underscores its importance as a cornerstone in the arsenal of treatments available to oncologists, offering hope and improved outcomes for patients battling various forms of cancer [142, 143]. Figure 2.20 illustrates the molecular structure of DOX.

This molecule has a complex structure with distinct components that contribute to its unique properties. It consists of an anthracycline ring which is a flat, planar arrangement of four fused rings. These rings adopt a quinone structure and give the molecule a distinct red colour by having a benzene ring with two oxygen atoms bound to it. Attached to the anthracycline ring is a sugar residue, specifically the

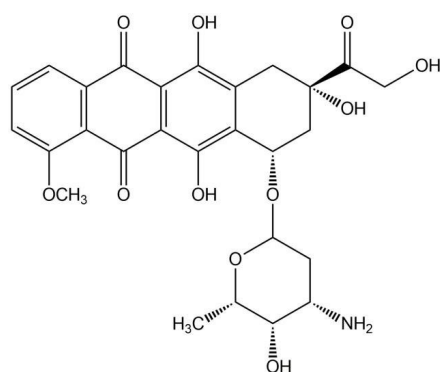


Figure 2.20: Schematic representation of the molecular structure from DOX [144].

daunosamine molecule, composed of carbon, hydrogen, oxygen, and nitrogen atoms. The daunosamine and the anthracycline ring are connected through a glycosidic bond, which is a specific type of chemical linkage connecting a sugar molecule to another molecule. Additionally, the molecule includes a carboxylic acid group located at the end of the anthracycline ring [145]. This functional group consists of a carbon atom, two oxygen atoms, and a hydrogen atom. It plays an important role in rendering DOX soluble in water, which helps it to dissolve and disperse effectively in aqueous solutions [146].

2.6.1 Mechanism of Action of Doxorubicin

DOX mechanism of action is complex and not fully understood, but it is known to interact with DNA in several ways. This drug can insert itself between the base pairs of the DNA double helix. This can cause breaks in the DNA and inhibit the replication of both DNA and ribonucleic acid (RNA). This will inhibit normal cellular processes leading to cellular death (apoptosis) [147].

Furthermore, DOX also inhibits the function of topoisomerase II, an enzyme that helps to relax the double helix formation in DNA during transcription, as illustrated in Figure 2.21. By inhibiting this enzyme, DOX can prevent the DNA from being properly transcribed, which can lead to cell death [147].

DOX can also generate free radicals and cause oxidative stress, which can damage multiple cell components. [143].

The administration is commonly given intravenously at 21-day intervals, typically, in liposomal formulations. This approach serves the purpose of mitigating the numerous side effects associated with its use. They include a large number of conditions such as fatigue, vomiting, nausea, oral sores, bone marrow suppression, hair loss, and the potential emergence of new tumours. Additionally, in the case of some patients, cardiac toxicity is a side effect that limits its extended usage [143].

In unfortunate instances of extravasation, where the intravenously administered drug leaks into sur-

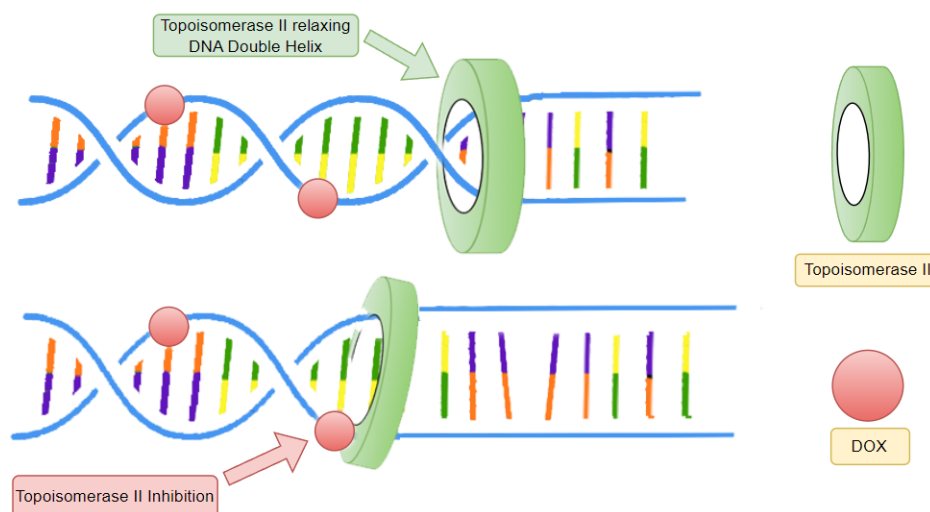


Figure 2.21: Schematic representation inhibition of Topoisomerase II by its combination with DOX.

rounding tissues, leads to severe tissue ulceration and, in the most severe cases, necrosis, underscoring the importance of careful administration procedures [148].

DOX is an effective chemotherapy drug, but its systemic action can cause severe side effects, as previously mentioned. Such side effects can restrict the use of the drug and lead to discontinuation of treatment. Therefore, enclosing DOX in liposomes, such is the case of DOXIL, is a better approach. This encapsulation technique has already been proven to safeguard patients from side effects and ensure the completion of treatments [92].

2.7 Magnetic Bioreactor

Magnetic stimulation is a possible way to stimulate drug release from MLPs. When a low-frequency AMF is applied to these systems, it can induce motion of the magnetic NPs embedded in the liposome membrane. This movement can cause changes in the structure and permeability of the membrane, resulting in the release of the encapsulated drug. The motion of the magnetic NPs generates mechanical stress, which can cause the formation of local pores or defects in the liposome membrane. The alignment of the magnetic NPs in the direction of the applied magnetic field increases the permeability of the membrane.

According to [149], the rate of drug release from MLPs can be controlled by varying the frequency and intensity of the applied AMF. The authors suggested that the slow release of the drug observed at low frequencies is due to the formation of local pores or defects in the liposome membrane. These changes

increase the permeability of the membrane, allowing the drug to diffuse out more easily.

Magnetic stimulation of MLPs has the potential to be a valuable tool for controlled drug delivery, as it is non-invasive and contactless. It can also be used to deliver drugs to specific tissues or organs in the body. Additionally, the rate of drug release can be controlled by varying the parameters of the AMF, such as the frequency and intensity.

Bioreactor systems have become increasingly important in tissue engineering applications, as they can accurately replicate the native cell/tissue microenvironment. By exposing the tissue culture to various chemical or physical stimuli, bioreactors can create a synergistic environment that stimulates cell response. The effectiveness of this approach depends on the bioreactor technology used and the response of scaffold structures to the stimuli.

Bioreactors have an important application in bone tissue engineering. Bone material is piezoelectric, which means that it can create an electric signal when it undergoes mechanical stress and vice-versa. This effect is essential for bone growth and development. Bioreactors that can electromechanically stimulate bone cells can mimic this important element of the bone microenvironment, and encourage tissue growth and differentiation. In [150] it is described a magnetic bioreactor that can be used for bone tissue engineering. In their study, they used a sinusoidal magnetic field with an amplitude of 0.22T and a frequency of 0.3Hz. This device applies magnetic, mechanical, and electrical stimuli to the cells in culture. It works by utilizing a magnetic field to a magnetically responsive scaffold that contains magnetostrictive nano/microparticles embedded in a specific matrix. The matrix can be electrically responsive or not, depending on the desired application frequency until certain limits, in order to get the required alternated magnetic field at the culture plate.

To create an AMF a magnet is moved at a specific frequency for a set amount of time. To ensure mechanical protection, limit switches and precision sensors were implemented. These components permitted electronic control of the table's position through a linear sensor and magnetic encoder for speed. These components are illustrated in Figure 2.22.

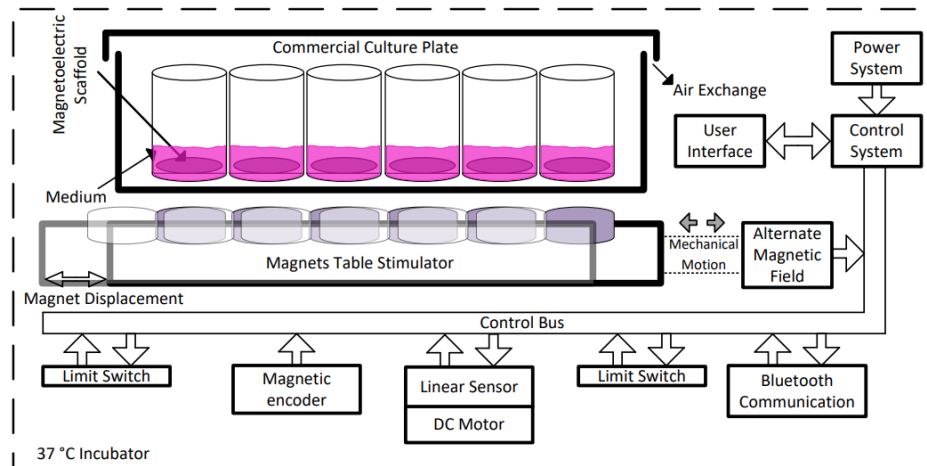


Figure 2.22: Magnetolectric bioreactor system operating principle through the use of electrical and mechanical controls to produce an AMF [150].

Bioreactors such as this one offer a number of advantages for tissue engineering applications. First, they allow us to recreate the complex physical and chemical environment of the native tissue, which is essential for promoting cell growth and differentiation. Second, they allow the application of controlled stimuli to the cells, which can be used to direct their behaviour in a desired way. Third, they allow us to scale up tissue production, which is important for clinical applications.

Chapter 3

Experimental Procedures, Methods and Characterization Techniques

3.1 Materials and Methods

3.1.1 Magnesium and Calcium Ferrite Magnetic Nanoparticles Synthesis

In order to prepare magnesium and calcium ferrite ($\text{Ca}_{0.25}\text{Mg}_{0.75}\text{Fe}_2\text{O}_4$) magnetic NPs with shape anisotropy, a number of steps, were taken.

First it was measured the following reagents from Sigma-Aldrich in Table 3.1.

Table 3.1: Reagents used with the respective molar mass, concentration used and mass.

Reagent	Molar Mass (g/mol)	Molar Ratio (mM)	Mass (mg)
Magnesium acetate tetrahydrate	214.45	0.75	161
Calcium acetate hydrate	158.16	0.25	39
Iron(III) citrate tribasic monohydrate	544.9	2	526
Oleic acid	282.46	3.1	875

Next, $15mL$ of octadecene was poured into a double-neck flask, which was then heated to $120^\circ C$ while being stirred continuously with a magnetic stirrer.

The pre-measured reagents were then added to the preheated octadecene, and the temperature was maintained at $120^\circ C$ for an hour. Following this, the reflux condenser was attached, and the temperature was gradually increased to $200^\circ C$ at a rate of $5^\circ C/min$. It was kept at $200^\circ C$ for $10min$ before the temperature was raised to $290^\circ C$ at a rate of $1^\circ C/min$ and refluxed for $60min$. The NPs obtained were then washed with dimethyl sulfoxide (DMSO) and ethanol in order to remove oleic acid residues. Oleic acid was then replaced with dimercaptosuccinic acid (DMSA) to make the NPs hydrophilic. This was

done by preparing a solution of 60mg DMSA in 5mL of DMSO and combining it with 50mL of toluene. The mixture was then subjected to sonication for 5min and mechanical stirring for 24h . Toluene was introduced, and the mixture was centrifuged. The supernatant containing oleic acid-coated particles was discarded, and multiple washing cycles with ethanol and acetone were performed to remove any remaining oleic acid molecules. Finally, the particles were dried and immersed in ultrapure water. By following these steps carefully, the desired magnetic $\text{Ca}_{0.25}\text{Mg}_{0.75}\text{Fe}_2\text{O}_4$ NPs with shape anisotropy were obtained.

3.1.2 Magnetoliposome Synthesis

The Sigma-Aldrich lipids were separately dissolved in chloroform to create a 20mM solution. The respective lipids used are present in Table 3.2.

Table 3.2: Components used in the procedure.

Lipid	Molar Mass (g/mol)	Lipid Mass (mg)	Volume of Chloroform (μL)
DPPC	734.53	0.147	10
DSPC	746.94	0.158	10
DSPE-PEG2000	3532.41	0.2	5
CHEMS	1112.79	0.0974	10

A lipid composition was formulated with each lipid present in Table 3.3 in the quantities specified.

Table 3.3: Quantities and volumes of each lipid from chloroform solution to achieve the desired percentage.

Lipid Name	Lipid Ratio (%)	Volume (mL)
DPPC	60	90
DSPC	20	30
DSPE-PEG2000	5	7.5
CHEMS	15	22.5

The chloroform solvent was completely removed from the lipid solution using a nitrogen stream.

Next, it was introduced $150\mu\text{L}$ of ethanol to the lipid mixture and in a separate procedure, it was combined 2mg of magnetic NPs with 3mL of ultrapure water.

Then, the suspension of water and magnetic NPs was heated to 50°C and added DOX in water to achieve a concentration of $50\mu\text{M}$.

Gradually, the lipid solution was added gradually into the NP solution, using constant vortexing, at $\approx 1500rpm$, to ensure a uniform and controlled incorporation of the lipids into the magnetic NPs.

By slowly adding the lipid solution to the water solution, the formation of MLPs is observed around the DOX and magnetic NPs as shown in Figure 3.1.

Finally, the resulting formulation was sonicated for 30min, ensuring the proper MLP size.

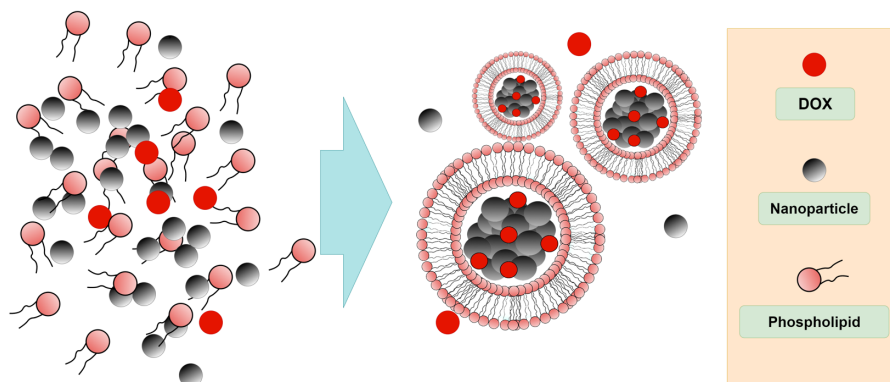


Figure 3.1: Process of formation of lipids into liposomes around the magnetic NPs and DOX and the remaining non-encapsulated components.

It is possible to repeat this procedure using varying quantities of lipids, magnetic NPs, and DOX.

3.2 Characterization Methods

3.2.1 Dynamic Light Scattering

Dynamic Light Scattering (DLS) is a powerful technique used to accurately determine the size of particles in solutions and emulsions at the nanometric scale. The principle of this technique lies in the Brownian motion, also called "random walk", exhibited by particles, where smaller particles exhibit faster, and more erratic movements compared to larger ones [151].

Exploiting the phenomenon of Brownian motion, DLS utilizes a laser beam that interacts with the particles in the sample. As the laser light encounters the particles, it undergoes scattering, resulting in a pattern of scattered light that contains valuable information about the particles' size distribution [151, 152].

By analysing the intensity fluctuations of the scattered light, the DLS instrument can derive the particles' diffusion coefficient, which is directly related to their size as depicted in Figure 3.2. The technique provides a detailed size distribution profile of the particles present, offering valuable insights into their characteristics [152].

One of these parameters is the polydispersity Index (PDI). It is a metric used to measure the heterogeneity of particle sizes within a sample. It helps to determine the diversity in particle dimensions among a population of molecules, particles, or other entities [153]. On the other hand, it is also possible to obtain the hydrodynamic radius that represents the average radius of the vesicles.

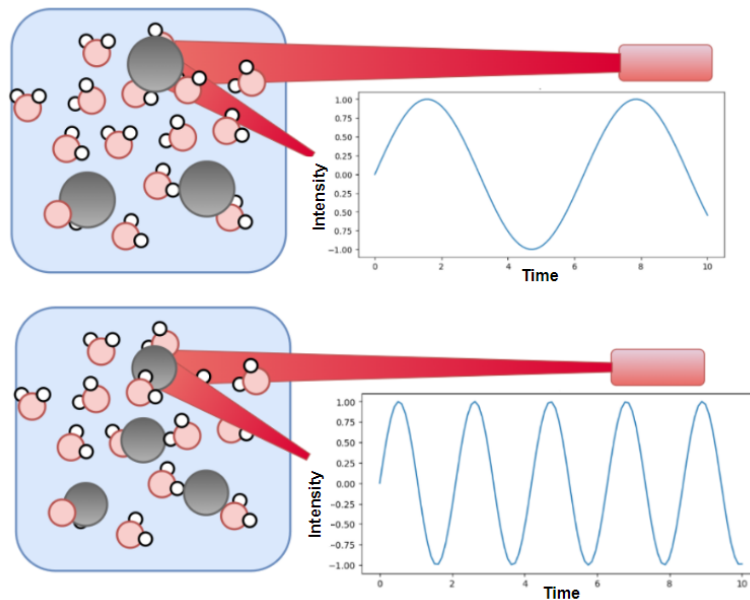


Figure 3.2: A basic representation of the frequency dependence on the particle size in a DLS system.

The connection between the velocity of particles and their size can be determined using the *Stokes-Einstein* equation (Equation 3.1). The velocity of particles is indicated by the diffusion coefficient, D . This equation takes into account the viscosity of the dispersant (η) and the temperature (T), since these factors directly impact particle motion. The *Stokes-Einstein* equation must be valid that particle movement is solely governed by Brownian motion:

$$D = \frac{k_B T}{6\pi\eta R_H} \quad (3.1)$$

Where R_H is the hydrodynamic radius.

Figure 3.3 illustrates the configuration of a DLS device. In this setup, a laser emitting light at a specific frequency is directed towards the sample contained within a cuvette. If the sample contains particles, the incoming laser light is scattered in various directions. The scattered light is captured and detected at a specific angle over some time. This collected signal is then utilized to calculate the diffusion coefficient and particle size using the *Stokes-Einstein* equation [154].

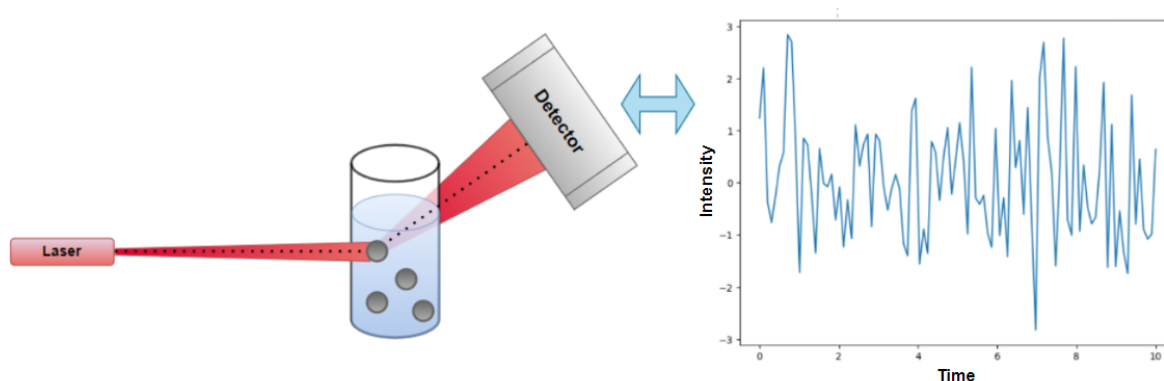


Figure 3.3: Basic setup of a DLS measurement system where the sample is contained in a cuvette. The scattered light of the incident laser can be detected at different angles.

Aside from its dimensions, this DLS allows for the determination of the Zeta potential. This particular factor is linked to the surface charge of particles when they are suspended in a fluid. It offers valuable information about the stability of the dispersion and its units are in Volt (V). The extent of electrostatic repulsion and, therefore, the quality of particle dispersion improves as the absolute value of the Zeta potential increases and when this value approaches figures closer to zero, the probability of aggregate formation becomes more pronounced [155].

Magnetoliposome Average Size Procedure

As previously mentioned, DLS is a powerful technique that can be used to determine the average size of particles, such as MLPs. In this study, it was used a DLS Litesizer 500 to estimate the size of MLPs. To ensure the accuracy and reliability of the obtained measurements, multiple tests were conducted on a single sample with a concentration of $50\mu M$ of DOX and $2mg$ of NPs.

Stability Over Time Procedure

As part of the research to evaluate the shelf life of these systems, the zeta potential of the sample was studied over a month-long period, including storage in a refrigerator and periodic measurements at room temperature. Understanding the temporal behaviour of zeta potential is key to assessing the stability and performance of these systems over time.

During the observation period, zeta potential measurements were conducted at specified intervals in the DLS Litesizer 500 to comprehensively track the evolution of the sample's surface charge after synthesizing the MLP sample with $50\mu M$ of DOX and $2mg$ of NPs.

Storing the sample in a refrigerator was a significant choice, as it simulates a controlled storage environment that is relevant for various applications, such as pharmaceuticals, cosmetics, or colloidal suspensions. Moreover, bringing the sample back to room temperature before each measurement mimics real-world scenarios where products are stored under different temperature conditions and are then subjected to environmental changes during use.

Influence pH in Magnetoliposomes Procedure

The aim of this study was to investigate the impact of pH on different factors in the systems, ranging from pH 7.3 to simulate physiological conditions [156]. In this procedure, two 200mL aqueous solutions were prepared, namely "Solution A" and "Solution B", using the components listed in Table 3.4.

Table 3.4: Reagent for each solution with respective molar mass, concentration and mass used in the 200mL solution.

	Reagent Name	Molar Mass (g/mol)	Concentration (M)	Mass (g)
Solution A	Sodium Phosphate	177.9	0.1	3.56
Solution B	Boric Acid	61.83	0.2	2.47
	Citric Acid	210.14	0.05	2.10

Using the volume ratios of each solution presented in Table 3.5, multiple buffer solutions were produced with varying pH values.

Table 3.5: Resulting pH after combining the respective volumetric ratio of each solution.

pH	3	4	5	5.5	6	6.5	7	7.4	8	9
Solution A	12	22.5	33	37	41	45.75	50.5	53.4	57.5	65.5
Solution B	88	77.5	67	63	59	54.25	49.5	46.6	42.5	34.5

600μL of the respective pH buffer solution and 300μL of the MLP sample were applied into its respective *Eppendorf* tube. The DLS Litesizer 500 was used to analyze the zeta potential of each sample three times to ensure more accurate results.

3.2.2 Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) is an important technique used to study structures at a nanometer scale by using an electron beam. It provides insights into the morphology, size, and distribution

of magnetic particles in MLPs. TEM is crucial in providing detailed information about the lipid bilayer thickness, the presence of aggregates, the formation of multilayer structures, and other characteristics of MLPs. It is widely recognized for its role in characterizing materials for biomedical applications and serves as a comprehensive tool for directly assessing the structural attributes and distributions within these nanostructures [157].

This type of imaging is composed of three fundamental parts: an electron gun, an anode and a condenser system, to generate an electron beam and precisely focus it onto the target specimen, an image-forming setup that will focus electrons that traverse the sample, and an image-capturing mechanism that transforms the electron rendered image into a more usable data. A simple TEM system can be depicted in Figure 3.4 adapted from [158].

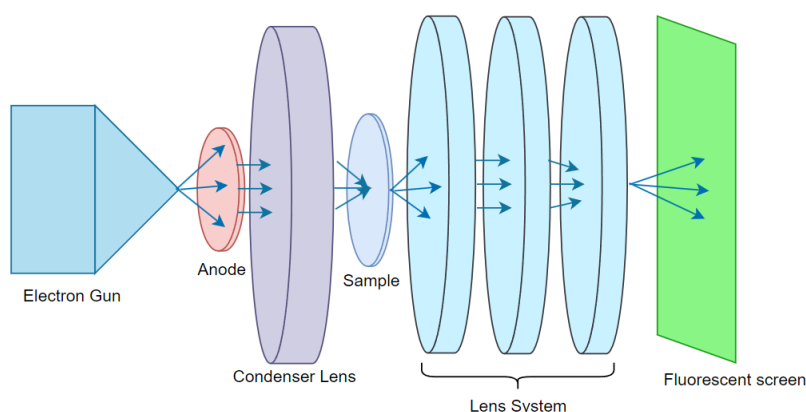


Figure 3.4: Simplified TEM setup with an electron gun, anode, condenser lens, sample, lens system, and fluorescent screen. The electron path is shown by blue arrows.

In order to observe the structure of the MLPs and the NPs right after synthesis, a fresh batch of MLPs with DOX was produced for the TEM sample, alongside a sample of $2mg$ of NPs in $3mL$ of ultrapure water. The TEM images were obtained using a JEOL JEM 1400 Plus electron microscope, operating at $100kV$.

3.2.3 X-Ray Diffraction

The X-ray diffraction technique (XRD) is used to gather information about the crystalline structure of materials. This allows for the characterization and identification of materials based on the presented diffraction pattern. This is a result of constructive interference between monochromatic X-rays and the

crystalline sample. When Bragg's Law ($n\lambda = 2d\sin\theta$) is satisfied, the interaction between the incident rays and the sample produces constructive interference (Figure 3.5). This law relates the wavelength of electromagnetic radiation with the diffraction angle and the lattice spacing in the crystalline sample. The direction of possible diffractions depends on the size and shape of the material's unit cell. In contrast, their intensity depends on the type of arrangement between atoms in the crystalline structure [159].

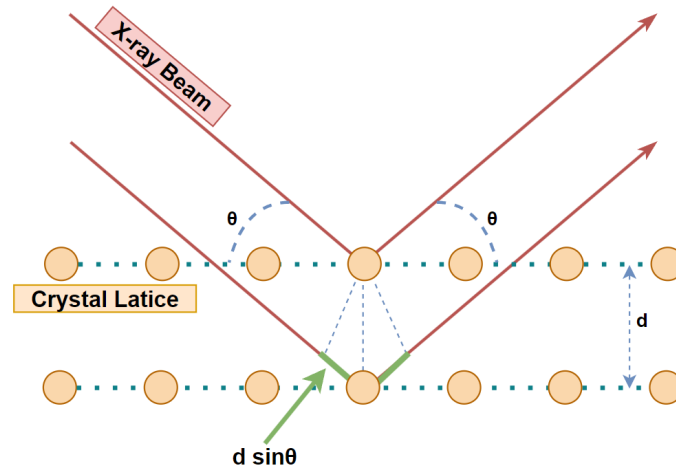


Figure 3.5: Bragg's Law representation with an X-ray beam hits a crystal lattice.

X-ray diffractometers consist of five basic components: an X-ray tube, a sample holder, an analyzing crystal, a goniometer, and an X-ray detector.

X-rays are generated within a cathode ray tube by heating a filament to produce electrons. These electrons are then accelerated towards a target material by applying a voltage and bombarding the target with electrons [160]. This process leads to the emission of characteristic X-ray spectra, with common components being κ_α and κ_β . The specific wavelengths of these X-rays are dependent on the target material (e.g., Cu, Fe, Mo, Cr). To obtain monochromatic X-rays necessary for diffraction, filtering by foils or crystal monochrometers is required [161].

Copper is the most commonly used target material in single-crystal diffraction, with Cu κ_α radiation having a wavelength of $1.5418\text{\AA}$ [162]. These collimated X-rays are directed onto the sample, which is rotated and its position is controlled by a goniometer, ensuring that the sample is properly aligned with the incident X-ray beam.

As both the sample and the detector rotate, the intensity of the reflected X-rays is recorded. When the geometry of the incident X-rays impinging on the sample satisfies Bragg's law, constructive interference occurs, resulting in an intensity peak. The detector records and processes this X-ray signal, converting it into a count rate. The count rate can then be displayed [161].

The geometry of an X-ray diffractometer involves the sample rotating in the path of the collimated X-ray beam at an angle θ , while the X-ray detector is mounted on an arm and collects the diffracted X-rays at an angle of 2θ . The range of angles at which data is collected is typically present in the X-ray scan, with the standard range being from 5° to 70° for powder patterns [161]. The full system can be observed in Figure 3.6

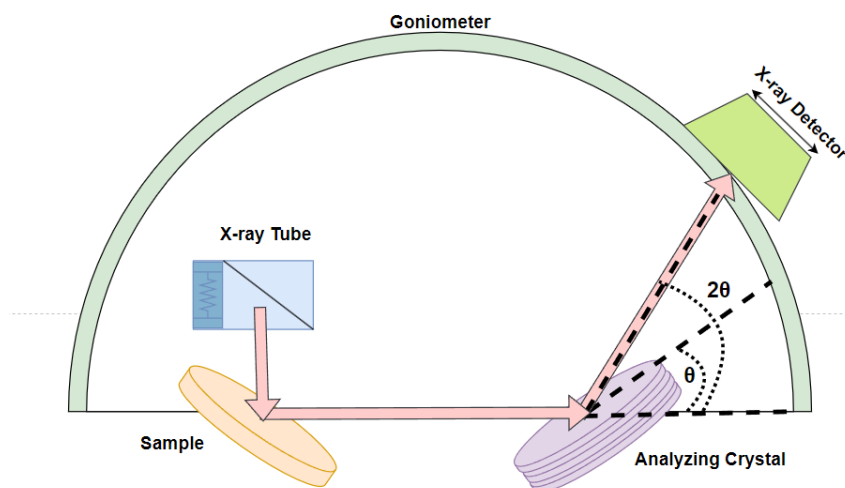


Figure 3.6: Representation of the XRD system.

X-ray powder diffraction is widely used for the identification of unknown crystalline materials and determining their structure, such as minerals and inorganic compounds [163]. This technique plays a crucial role in various fields, including geology [163], environmental science [164], material science [165] and biology [166].

To examine the crystalline structure of the magnetic NPs, an XRD analysis was performed using a dried sample weighing 2mg . It was used a Philips PW 1710 diffractometer to process the samples. The diffractometer operated in a Bragg–Brentano configuration and used $\text{CuK}\alpha$ radiation from Royal Philips. Data was processed using Profex software.

3.2.4 Absorption spectroscopy

Absorption spectroscopy is an important scientific technique that measures the intensity of light before and after it interacts with a sample. Specifically, UV-Vis absorption spectroscopy uses electromagnetic radiation with wavelengths ranging from 190nm to 750nm as the light source [167]. When this radiation interacts with molecules, it triggers electronic transitions that lead to the absorption of quantized energy. UV-Vis absorption spectroscopy is a tool for investigating electronic transitions, providing data

on the intensity of absorption bands, which directly correlate with the probability of specific transitions. Higher intensity bands indicate allowed transitions, while weaker bands indicate forbidden transitions in the spectrum [168]. In order to analyze the absorption or transmission of light by samples, a spectrophotometer system requires several components that work together. These components include a light source, monochromator, beam splitter, rotating disc, detector, controller, sample, and reference.

The light source is responsible for providing radiation across different wavelengths. The monochromator selects a single wavelength of light. The beam splitter splits the light into two beams, one directed at the sample and the other at the reference. Between the sample and the detector, there is a rotating disc that directs and alternates the light coming from the reference and the sample. The detector measures light intensity after it passes through both the sample and reference. The controller processes the collected data [169]. Figure 3.7 shows a simplified configuration of this system.

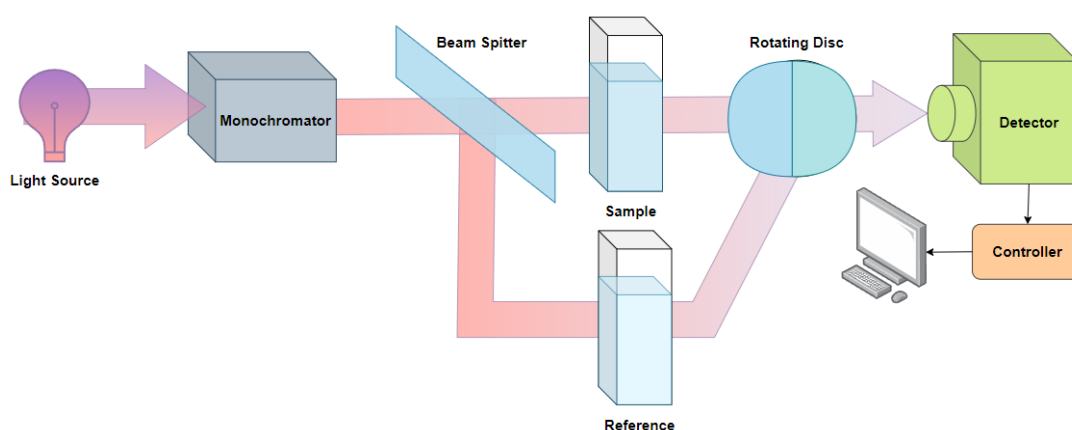


Figure 3.7: A basic absorption spectroscopy system consists of a light source, monochromator, beam splitter, sample and reference, rotating disc, detector, and a computer controller.

Another piece of equipment utilized for absorbance spectroscopy is the microplate reader, in this case, the Infinite M Nano+ by Tecan. This is a dual-mode microplate reader that uses monochromator-based optics for both absorbance and fluorescence applications. Its working principle is similar to the one mentioned above, but it does not require a reference sample [170]. The intensity of light passing through a material is absorbed at certain frequencies, which explains the mechanism behind this equipment. These frequencies are unique to each material and the type of radiation that is passed through it. Absorption occurs due to inelastic collisions (without conservation of kinetic energy) between photons and the constituent electrons of the material. The energy absorbed from photons is fixed by atoms, which consequently undergo excitation, moving from the fundamental electronic state to higher energy electronic states. This process occurs when the energy of photons is equal to the difference in energy between the excited (E_{ES})

and fundamental states (E_{FS}), as described in Equation 3.2:

$$h\nu = E_{ES} - E_{FS} = \Delta E \quad (3.2)$$

Where h is the Plank's constant, ν is the frequency of light and ΔE the variation in energy.

Molecules possess quantified rotational and vibrational energy, in addition to electronic energy. Rotational energy is associated with the molecule's rotation around its centre of gravity, while vibrational energy is related to atoms' vibration in molecules. Electronic energy, on the other hand, is related to the distribution of electrons around atomic nuclei. Each of these energy types contributes to the molecule's total energy, and therefore, for each electronic state, there exists a set of rotational and vibrational levels [171, 168]. This can be visualised in Figure 3.8. In addition to the substance itself, the medium in which it is suspended, and its concentration also influence the absorption of parallel light beams, as described by Lambert-Beer's law. The term transmittance (T) is the ratio of the radiant power exiting the sample under study (P_λ) to the incoming radiant power (P_λ^o) (Equation 3.3).

$$T = \frac{P_\lambda}{P_\lambda^o} \quad (3.3)$$

And absorbance (A) is the negative decimal logarithm of the transmittance (Equation 3.4).

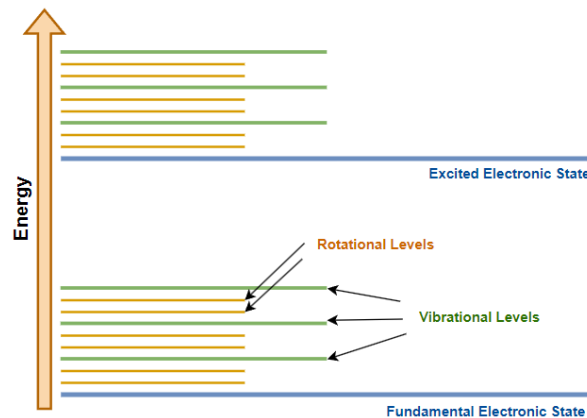


Figure 3.8: The energy levels of rotational and vibrational states are represented, along with the positions of fundamental and excited electronic states.

$$A = -\log(T) \quad (3.4)$$

The equation below describes *Lambert-Beer's law* where the relationship between absorbance, molar concentration (c) in $\text{mol}\cdot\text{m}^{-3}$, path length (l) in meters, and molar absorption coefficient (ϵ) in $\text{m}^2\cdot\text{mol}^{-1}$ in SI units) for a species in solution (Equation 3.5) [172]. This effect can be visualized in Figure 3.9.

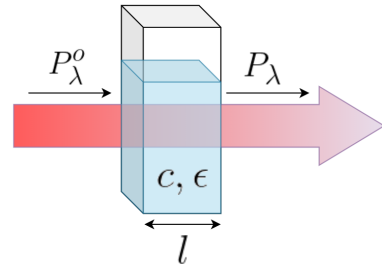
$$A = cl\epsilon \quad (3.5)$$


Figure 3.9: Diagram illustrating the reduction in radiant power of monochromatic radiation after passing through a cuvette of width l , containing a solution with concentration c of the component under study and a characteristic molar absorption coefficient ϵ .

UV-Visible Absorption Spectrum of Nanoparticles Procedure

To determine the bandgap of the magnetic NPs, absorption spectroscopy was employed as the chosen technique. The UV-Vis absorption spectroscopy was conducted using an ultrapure water solution containing a concentration of $0.2\text{mg}/\text{mL}$ of magnetic NPs. Subsequently, the sample underwent analysis utilizing a Shimadzu UV-3101PC spectrophotometer, covering a wavelength range from 300nm to 700nm .

Encapsulation Efficiency of Nanoparticles in Magnetoliposomes Procedure

The encapsulation efficiency (EE) of magnetic NPs on the MLPs was also measured using absorption spectroscopy. In this experimental procedure, the necessary volumes of an NP solution were calculated and transferred to Falcon tubes to obtain various quantities of NPs ranging from 3mg to 0.1mg .

To each tube, was added 1.2mL of a 37% hydrochloric acid (HCl) solution from Sigma-Aldrich, subjected the samples to 10min of sonication, and allowed the NPs to digest overnight. After digestion, dilute the acid to a 1M solution by adding 10.8mL of ultrapure water.

The process relies on the creation of iron(III) chloride complexes within the samples. This is achieved by adding concentrated HCl to the magnetic NPs, which promotes the formation of iron(III) chloride complexes with a strong signal in the UV-Vis range [173].

Each solution was placed into four wells of a microplate as it is visualized in Figure 3.10, and analyzed using a microplate reader @Infinite M Nano+. According to [173], Iron(III) chloride complexes have their

highest absorbance peak at $344nm$. Using this wavelength, values were obtained and a calibration slope was processed with a Python script.

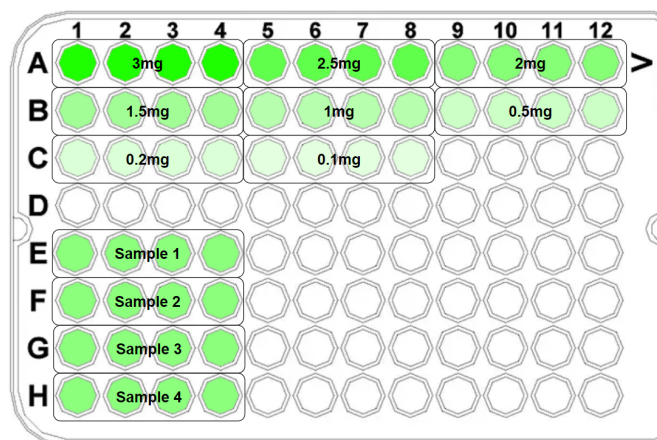


Figure 3.10: Layout used in the microplate for processing the calibration slope.

After it was synthesized MLPs under standard conditions, using $2mg$ of magnetic NPs, and repeating this process in quadruplicate. Subsequently, it was magnetically decanted the non-encapsulated NPs from the MLPs (depicted in Figure 3.11), and pipet the remaining encapsulated magnetic NPs and the non-encapsulated ones while evaporating any remaining water.

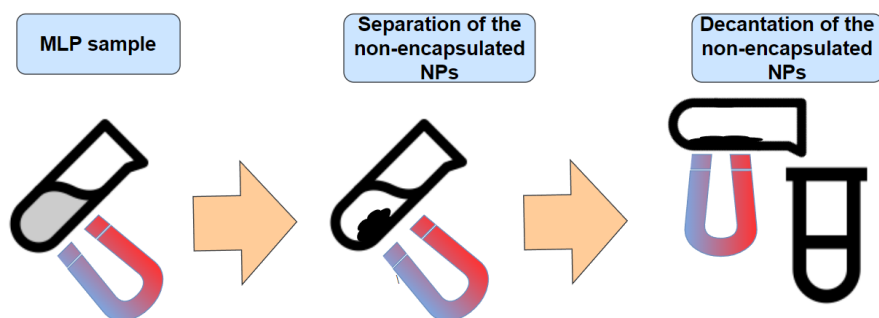


Figure 3.11: Process of magnetic decantation that involves using a magnet to separate non-encapsulated NPs at a faster rate than encapsulated ones.

Next, it was introduced $1.2mL$ of a 37% HCl solution to the dried SMLs and non-encapsulated NPs. The solution was sonicated for $10min$ and allowed the NPs to digest overnight. Then, dilute the acid to a $4M$ concentration by adding $1.8mL$ of ultrapure water to each sample. The NPs were analysed using the same equipment and conditions, and the non-encapsulated mass was determined with a Python script.

Drug Release Over Time Under Static and Dynamic Conditions

As previously highlighted, a primary objective of this project is to investigate the effects of magnetic stimuli on drug release from the MLPs, utilizing the magnetic bioreactor detailed in Section 2.7. This study aims to compare these effects with those observed under static conditions. To achieve this, absorption spectroscopy was utilized as the analytical technique.

For the procedure it was prepared several Eppendorf tubes with predetermined concentrations of DOX, including $100\mu M$, $50\mu M$, $25\mu M$, $12.5\mu M$, $6.25\mu M$, $3.125\mu M$, $1.5625\mu M$, and $0M$. Subsequently, each of these solutions was meticulously placed into five wells of a microplate, as depicted in Figure 3.12, and subjected to analysis in a microplate reader ®Infinite M Nano+. The mean values for each concentration were calculated, and the calibration slope was determined using the data obtained.

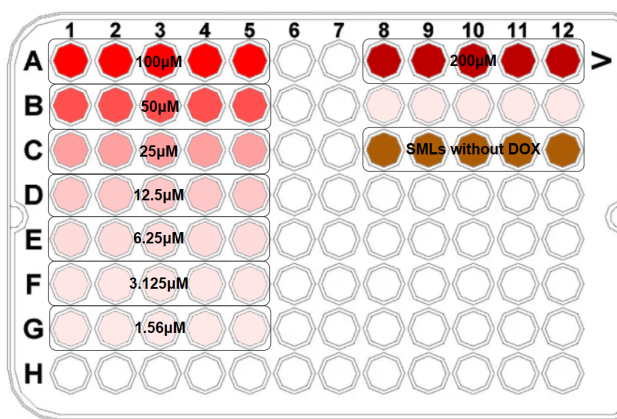


Figure 3.12: Layout used in the microplate for processing the calibration slope.

Subsequent to this, drug release under static conditions involved the preparation of a 6 mL solution of MLPs with the standard concentration of NPs, lipids, and DOX. Utilizing a larger microplate, $550\mu L$ of the solution was dispensed into each well. The microplate was then incubated at $37^{\circ}C$ to mimic physiological conditions. After $30min$, the contents of the first three wells were filtered using a $0.45\mu m$ syringe filter, determined to be the best means of filtration in the Appendix A, and transferred to an Eppendorf tube. This procedure was repeated 2 more times at $3h$ intervals and $5h$ intervals. For dynamic conditions, the same protocol was implemented on 3 separate days. On each day, the microplate was positioned above a homemade magnetic bioreactor (similar to Figure 3.13) with distinct settings.

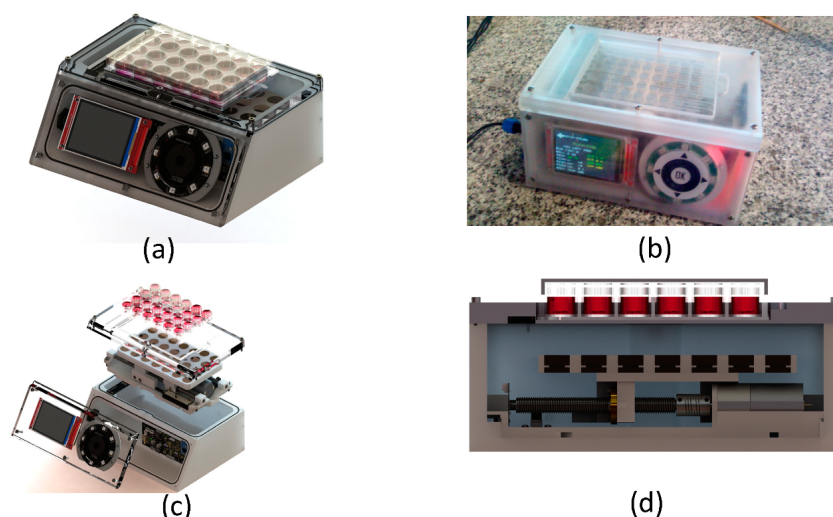


Figure 3.13: (a) Illustration of the bioreactor assembled with a microplate; (b) detailed mechanism of the bioreactor; (c) Schematic display of the disassembled primary electric and mechanical components; and (d) Cross-sectional view of the mechanical component [174].

On the first day, a magnetic field was applied at a frequency of $0.3Hz$, with a $20min$ "on" period followed by a $40min$ "off" period. On the second day, the same frequency was maintained, but the "on" period extended to $40min$, followed by a $20min$ "off" period. On the last day, the frequency was increased to $0.6Hz$, with a $40min$ "on" period and a $20min$ "off" period. Following the collection of filtered samples, each specimen was distributed into 4 wells of a microplate for subsequent analysis using the microplate reader.

3.2.5 Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) is a thermal analysis technique frequently used in the pharmaceutical field. It can also be used for studying the influence of temperature on systems (such as MLPs). It is essential to observe their behaviour in various conditions, including room temperature and physiological environments. It works via a dual chamber configuration: in one chamber, there is a sample to be analyzed while the other serves as a reference. This technique effectively distinguishes between endothermic (heat-absorbing) and exothermic (heat-releasing) transformations. This makes it easier to determine critical parameters such as transformation temperatures and enthalpy changes in both solids and liquids, as they relate to changes in temperature [175]. The system shown in Figure 3.14 is a simplified DSC system. The oven heats the sample and reference crucibles to a desired temperature. The sample crucible holds the material being analyzed, while the reference crucible holds a baseline reference material. The

thermometer, denoted as PT100, measures the temperature of both crucibles. The heating and cooling system controls the temperature of the oven, allowing it to be heated or cooled as needed. The purge gas system removes any unwanted gases or vapours from the oven that may interfere with the analysis. The temperature difference measurement system measures the difference in temperature between the sample and reference crucibles, which is used to calculate the heat flow into or out of the sample.

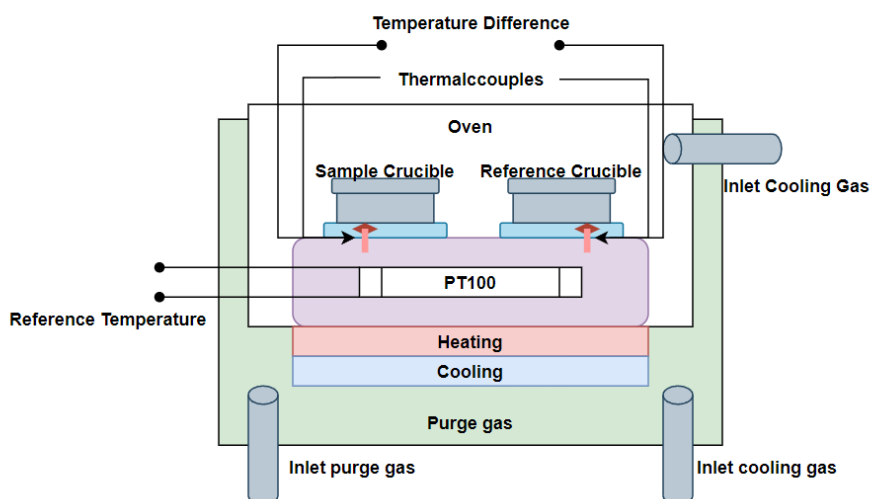


Figure 3.14: Simplified DSC setup. The setup consists of an oven, a sample crucible, a reference crucible, a PT100, a heating and cooling system, a purge gas system, and a temperature difference measurement system, adapted from [176].

In order to observe the influence of temperature on MLPs, the DSC samples included one MLP sample with DOX and another without any drug. These samples underwent drying until approximately $500\mu L$ of water evaporated. The samples were analysed in a DSC822e from Mettler Toledo DSC822e using N_2 as a purging gas at a temperature range of $30 - 60^\circ C$.

3.2.6 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy is a technique with a wide range of applications including analysis of small molecules and complexes, cells and tissues, and mapping of cellular components like carbohydrates, lipids, and proteins. This even helps in the detection of abnormal cells, such as cancerous ones. Moreover, FTIR spectroscopy has progressively expanded to encompass protein investigations, delving into their conformation, folding dynamics, and intricate molecular attributes.

When a sample is traversed by infrared (IR) radiation, a portion of the radiation is absorbed, while the

remainder is transmitted. The ensuing signal captured by the detector manifests as a spectrum, akin to a molecular "fingerprint" unique to the sample. By employing the Fourier Transform, the output from the detector is translated into a comprehensible spectrum [177]. A simple and possible FTIR can be depicted in Figure 3.15.

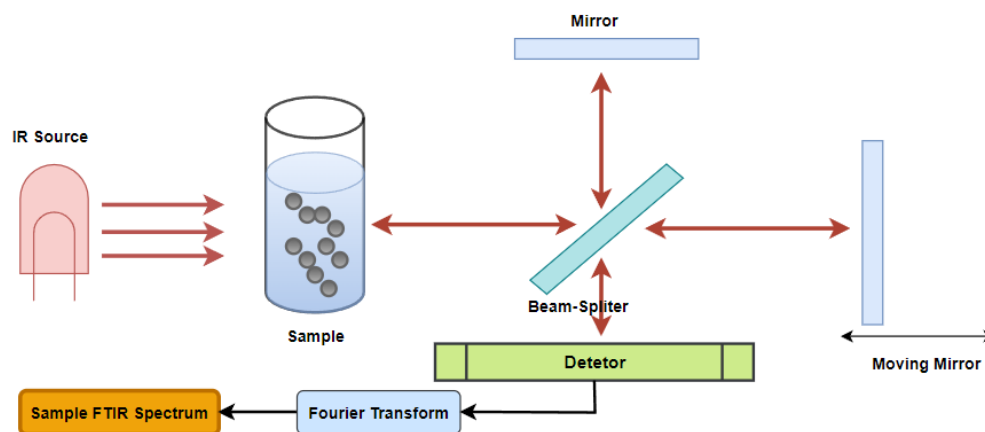


Figure 3.15: Basic FTIR system.

This test aims to detect the presence of Iron oxide (from the $\text{Ca}_{0.25}\text{Mg}_{0.75}\text{Fe}_2\text{O}_4$ NPs), phospholipids, and DOX.

In order to detect the presence of the multiple compounds from these systems (DOX, phospholipids and iron oxide from the $\text{Ca}_{0.25}\text{Mg}_{0.75}\text{Fe}_2\text{O}_4$), the FTIR sample involved a sample of 2mg of magnetic NPs, two samples of MLPs with DOX, and two samples without DOX. These samples were dried in an oven overnight at a temperature of 90°C . FTIR-ATR spectrometer model Jasco FT/IR-4100. The spectrum was recorded in the $4000\text{--}600\text{cm}^{-1}$ wavenumber range for 64 scans with a resolution of 4cm^{-1} .

3.2.7 Vibrating Sample Magnetometry

The Vibrating Sample Magnetometry (VSM) technique is used to measure the magnetic properties of materials, including MLPs. It allows the determination of magnetic characteristics of these systems, such as magnetization, coercivity, and magnetic susceptibility [178].

When performing VSM, the sample is placed on a support capable of vibrating in a specific direction. Subsequently, a magnetic field is applied to the sample, and its magnetic response is measured while the field is varied. This allows for the determination of the sample's magnetization in relation to the applied magnetic field [178, 179]. In Figure 3.16, a simple VSM system is depicted.

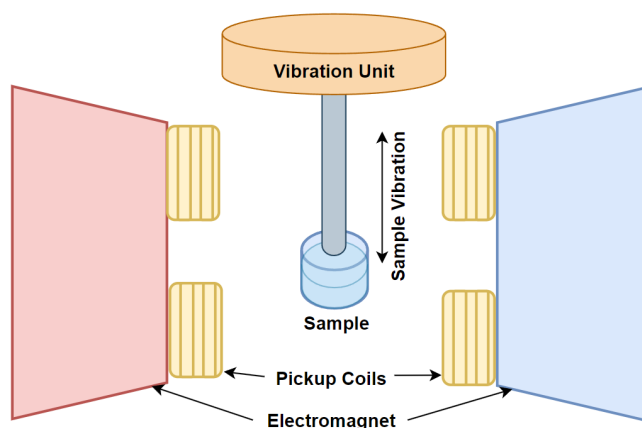


Figure 3.16: Basic VSM system. Composed of a vibration unit, an electromagnet, and pickup coils, that pick up current created by the varying magnetic created by the sample.

VSM offers crucial insights into the magnetic properties of materials and other systems such as MLPs, including the presence and strength of magnetization. This information can be utilized to assess the effectiveness of the magnetic particles incorporated into the liposomes, as well as to investigate the influence of parameters like particle size and concentration on the magnetization of the MLPs.

For the VSM samples, a 2mg sample of magnetic NPs was combined with three MLP samples containing different amounts of NPs (1mg , 2mg , and 4mg). The samples were dried in an oven at 90°C overnight. The samples were analysed using a MicroSense EZ7 vibrating sample magnetometer.

3.2.8 Fluorescence Spectroscopy

A spectrofluorometer uses fluorescence or phosphorescence properties in certain materials for measuring. Fluorescent molecules contain specific electronic structures called fluorophores, which are responsible for their fluorescence. These fluorophores are usually found in their lowest energy state, known as the ground state, at room temperature. Within this ground state, there are several vibrational energy levels, with molecules naturally residing in the lowest one. When exposed to UV or visible light, the fluorophore absorbs photons. The absorption elevates the molecule's electrons to higher molecular and vibrational states, often referred to as S1 and S2, the first and second excited singlet states, respectively. With this extra energy, the molecule can dissipate some of it as kinetic energy. As a result, these energized molecules collide with other molecules within the sample, resulting in the loss of their vibrational energy. These energy losses are called non-radiative transitions. Once a certain threshold of energy loss is reached, the molecule returns to the lowest vibrational level within the excited state. At this point, no further energy

can be lost kinetically, and the molecule must undergo relaxation through the process of fluorescence to return to the ground state. This process lifetime is relatively slow, taking about $10ns$.

Phosphorescence, similar to fluorescence, involves specific molecules called phosphors that emit light when exposed to energy sources like UV or visible light. These phosphors start in their lowest energy state, the ground state, and absorb photons to reach excited triplet states (T_1). However, phosphorescence is distinct due to "spin-forbidden" transitions, making the return to the ground state slower. This delay results in longer-lasting excitation and the emission of lower-energy light, often in the visible or IR range, making phosphorescence valuable in various scientific and practical applications. Compared to fluorescence, this process takes longer and can take seconds or even milliseconds to complete [180, 181]. The types of emissions can be better understood by looking at the visual representation in Figure 3.17.

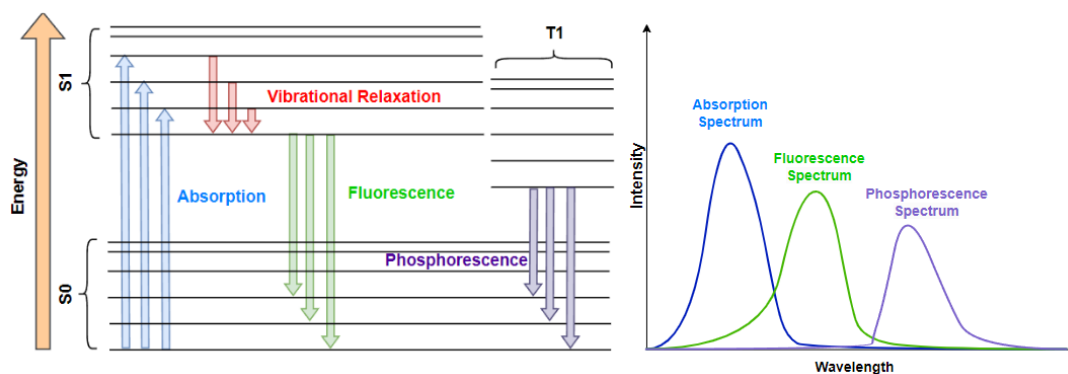


Figure 3.17: Fluorescence emission and phosphorescent emission in the triplet state (T_1). Also the wavelength pattern of fluorescence and phosphorescence emission

A spectrofluorometer is a piece of equipment that includes a light source, usually a xenon arc lamp, although other options such as a laser port, NanoLED solid-state pulsed sources, and a triple-illuminator option are also available. The equipment also consists of an excitation monochromator that filters the source light and allows a single wavelength of light to reach the sample. Additionally, there is an emission monochromator that filters the light coming from the sample and transmits the filtered signal to a detector, which then transmits the signal to a controller. Lastly, the controller sends the signal to a computer to turn it into more usable data [182]. The system's fundamental diagram is illustrated in Figure 3.18.

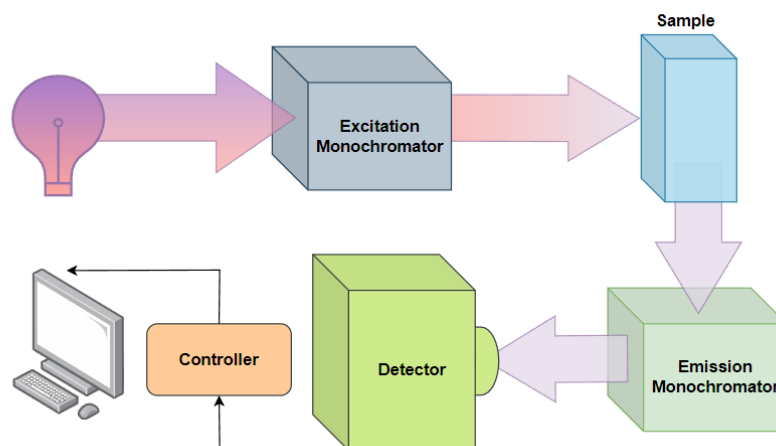


Figure 3.18: Basic spectrofluorometer system. Composed of a light source, an excitation monochromator, an emission monochromator, a detector and a controller.

In a spectrofluorometer, a light source is filtered by an excitation monochromator, allowing only a single wavelength of light to reach the sample. If the sample is fluorescent or phosphorescent, it responds to the incoming radiation by emitting light after being excited with a certain wavelength. The resulting radiation from the sample is filtered by an emission monochromator. Finally, a spectrum is produced by recording the variation in intensity according to wavelength [182]. The system's fundamental diagram is illustrated in Figure 3.18.

Encapsulation Efficiency of Doxorubicin in Magnetoliposomes

To determine the EE of DOX in MLPs fluorescence spectroscopy was used. In this experimental procedure, a series of aqueous DOX solutions with specific concentrations were meticulously prepared to establish a calibration curve. These concentrations ranged from $50\mu M$ down to significantly low concentrations, including $8.33\mu M$, $1.38\mu M$, $0.23\mu M$, $0.038\mu M$, $0.0064\mu M$, $0.0011\mu M$, $0.00017\mu M$, $2.97 \times 10^{-5}\mu M$, $4.96 \times 10^{-6}\mu M$, and $8.27 \times 10^{-7}\mu M$. Each distinct DOX concentration was carefully divided into separate Eppendorf tubes.

To determine the calibration slope, a highly sensitive fluorescence spectrometer was employed. Each Eppendorf tube, containing its respective DOX concentration, was placed within a quartz cell, and a fluorescence measurement was acquired. A fluorescence of DOX is structured, with peaks centred at 555 and $590nm$ and a slight increase at $\approx 630nm$ when excited at $480nm$ [183]. This fluorescence measurement was repeated three times for each concentration to ensure accuracy and reliability.

In a separate experiment, three distinct solutions of MLPs were prepared, each loaded with varying concentrations of DOX. These concentrations included $50\mu M$, $500\mu M$, and $5mM$. To isolate the DOX-loaded MLPs and remove any unincorporated drug, the solutions were processed through a $100nm$ Amicon filter. This filtration step was conducted using a centrifuge at $3000rpm$ for a duration of $8min$, ensuring efficient separation of the components. This process can be visualized in Figure 3.19

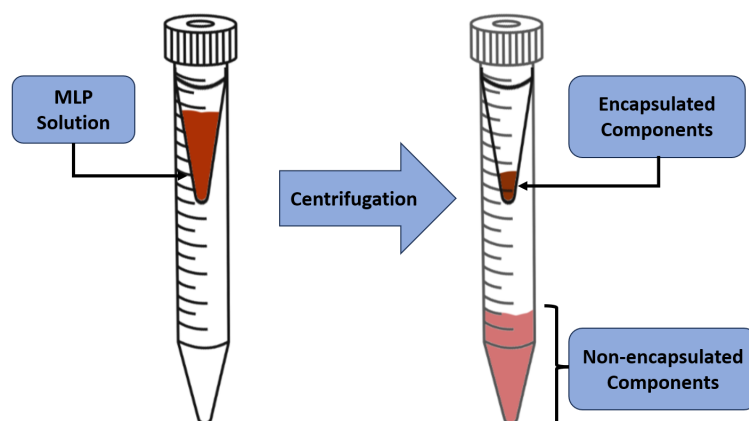


Figure 3.19: Solution of MLPs was subjected to centrifugation both before and after passing through an Amicon.

Subsequently, the non-isolated DOX solutions were subjected to fluorescence analysis in the same fluorescence spectrometer mentioned earlier. The resulting fluorescence data were collected and processed using a Python script, allowing for the quantification and comparison of DOX concentrations within the various MLP solutions. This comprehensive experimental approach ensures the accuracy and precision of the obtained results.

Chapter 4

Results and Discussion

4.1 Magnetoliposome Average Size

This analysis provides a crucial role in understanding the physical characteristics and behaviour of MLPs, contributing to a broader comprehension of their properties and potential applications. The obtained results, of several syntheses carried out on different days, are presented in Table 4.1.

Table 4.1: Combined table with size and PDI.

Hydrodynamic Radius (nm)	PDI (%)
467.9 ± 95.93	31.2 ± 2.26
523.3 ± 105.26	29.2 ± 2.05
469.2 ± 129.28	31.0 ± 2.59
643.1 ± 114.3	24.1 ± 2.94
469.6 ± 92.34	31.3 ± 2.68
577.7 ± 90.82	27.9 ± 2.97
630.6 ± 106.3	22.7 ± 3.13
560.5 ± 149.0	22.4 ± 4.37

Based on the analysis of the experimental results the hydrodynamic radius and PDI of the MLPs can be determined. The MLPs have a hydrodynamic radius of $542.74 \pm 110.40nm$ with a PDI of $27.48 \pm 2.87\%$.

Upon comparison, it is possible to observe that the systems obtained are larger in size, and similar PDI compared to those presented in other research papers, as shown in Table 4.2.

Table 4.2: Combined table with size and PDI.

Research Paper	Hydrodynamic Radius (nm)	PDI (%)
[10]	163 ± 23	20.3 ± 2
[82]	153 ± 8	21 ± 3
[133]	187 ± 2.1	24.8 ± 24.8
[184]	197 ± 9	21.8 ± 3

4.2 Stability Over Time

During the study, the zeta potential of MLPs was monitored for 43 days, with the test being executed three times each day to ensure an accurate result. The data was represented graphically with error bars, as shown in Figure 4.1. A high zeta potential absolute value indicates that the particles are less likely to aggregate due to increased electrostatic repulsion.

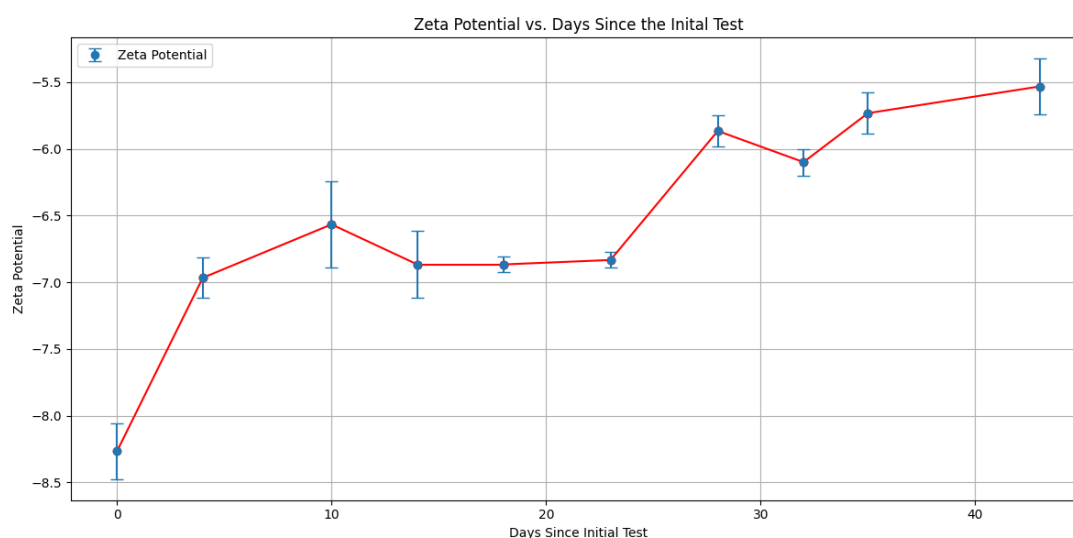


Figure 4.1: Zeta potential of MLPs over 43 days.

The plotted data indicates a clear trend in the zeta potential of the MLPs over the 43-day observation period. On day 1, the zeta potential was $-8.3mV$, which gradually its absolute value decreased to $-5.5mV$ by day 43. This decline in zeta potential is a significant finding, indicating that the stability of the MLP suspension has changed over time.

The reduction in zeta potential increases the likelihood of particle aggregation. With decreased electrostatic repulsion, the particles are no longer as effective in preventing close contact, making aggregation

a more probable outcome [155].

In the context of this study, a related investigation found similar results [14]. In the latter, a distinct pattern unfolded over a two-week span, revealing a noteworthy decline in the absolute value of the zeta potential, plummeting from -8.5mV to -1mV . When drawing a comparative analysis between the two scenarios, a conspicuous disparity in the rate of zeta potential reduction surfaces. Notably, the developed system exhibits a more gradual decrease, suggesting a heightened level of stability in comparison to the observed case. This discrepancy unveils valuable insights into the dynamic behaviour of these systems and underscores the nuanced nature of their stability profiles.

4.3 Influence of pH in Magnetoliposomes

As it was mentioned before, an Anton Paar Litesizer 500 was used to measure the zeta potential of MLPs under different pH levels ranging from 3 to 9. A batch of MLPs was distributed among various Eppendorf in a buffer solution with respective pH levels. 3 examinations were performed for each pH level to obtain accurate results. This information could be used to study MLPs with DOX that have optimal stability and cellular uptake in different environments. For example, MLPs with DOX could be designed to be more stable in the bloodstream and have better cellular uptake by cancer cells. As shown in Figure 4.2, the zeta potential of MLPs decreased with an increase in pH level, whereas the opposite was observed in the systems without DOX. However, the zeta potential of MLPs with DOX consistently showed lower absolute values compared to those without DOX at all pH levels. This indicates that DOX interacts with the lipid bilayer, thereby reducing the surface charge of the MLPs.

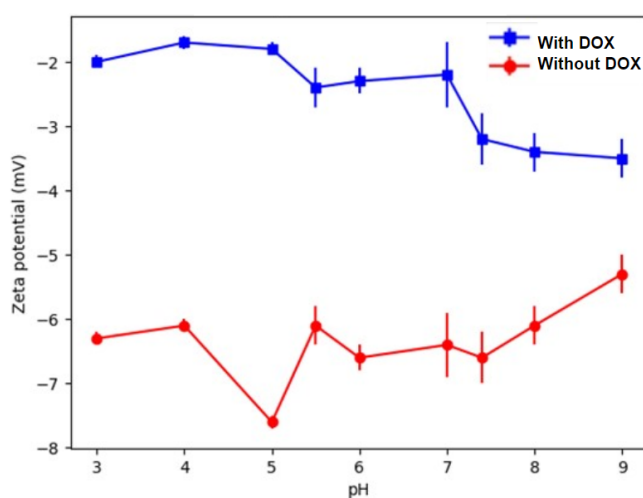


Figure 4.2: Zeta potential of MLPs with (Blue) and without DOX (Red) at different pH levels

In this plot, it is observed that MLPs exhibit a consistent negative surface charge across a range of pH values. This characteristic feature can be attributed to the prevalence of negatively charged phospholipids within the MLP formulation [185].

The zeta potential of MLPs without DOX is more negatively charged compared to the case with DOX. A possibility of this factor is caused by alterations in the membrane layer.

When the pH is increased, it introduces more negatively charged ions into the system, which leads to a gradual decrease in the zeta potential of the MLPs with DOX. In the case without DOX, the slight increase in the zeta potential could be due to the reduction in system stability caused by the pH increase, which increases the likelihood of aggregation in these systems.

4.4 Transmission Electron Microscopy

In this procedure, fresh samples of magnetic NPs in water were tested, along with samples of MLPs containing 50M of DOX and others without the drug. The TEM results show that the magnetic $\text{Ca}_{0.25}\text{Mg}_{0.75}\text{Fe}_2\text{O}_4$ NPs are nearly square-shaped and range from a few to dozens of nm that form small clusters (Figure 4.3).

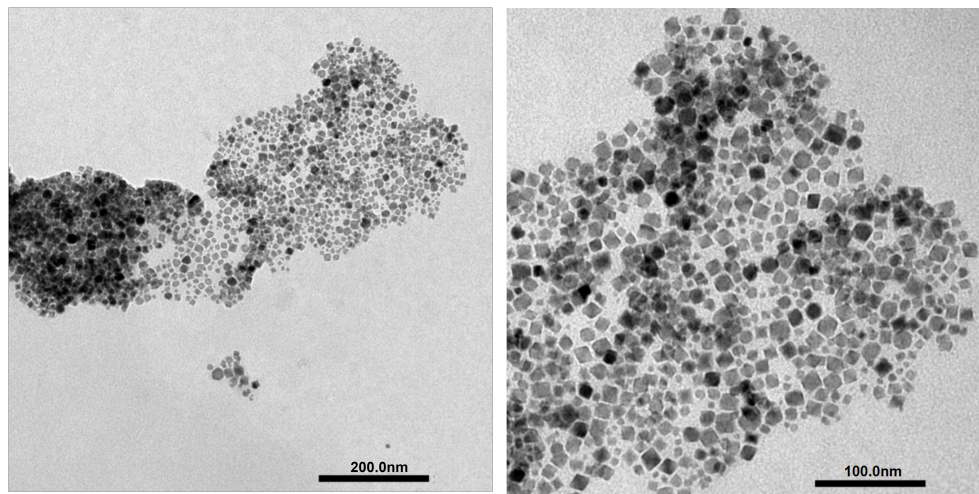


Figure 4.3: This is a cluster of square-shaped magnetic $\text{Ca}_{0.25}\text{Mg}_{0.75}\text{Fe}_2\text{O}_4$ NPs, and the second image is a close-up view of the same cluster.

It was applied a bandpass filter to the first image and adjusted its contrast using *ImageJ* software. Afterwards, it was conducted particle size analysis using the same software and determined the NPs' average length. After a Python script processed the results and generated a histogram with its corresponding Gaussian approximation, as shown in Figure 4.4. The analysis indicates that the average size of the NPs is $8.75 \pm 0.35\text{nm}$.

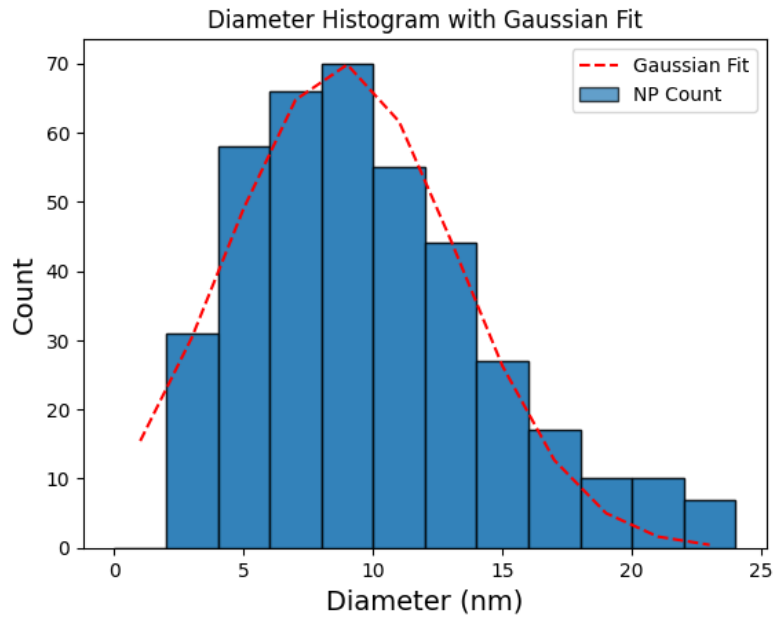


Figure 4.4: Histogram representation of the average size of the NPs with its respective Gaussian approximation

The findings support those in [186]. The TEM analysis revealed that the magnetic NPs with a similar metal composition had sizes below 10nm . The study resulted in two groups of particles with sizes of $7.0 \pm 0.7\text{nm}$ and $9.9 \pm 2.5\text{nm}$. The larger group is probably made up of NP aggregates appearing in larger sizes in the images.

When observing the TEM figures of the MLPs, two different scenarios are noticed: liposomes with clusters of $\text{Ca}_{0.25}\text{Mg}_{0.75}\text{Fe}_2\text{O}_4$ NPs surrounding them and liposomes encapsulating the NPs (Figure 4.5).

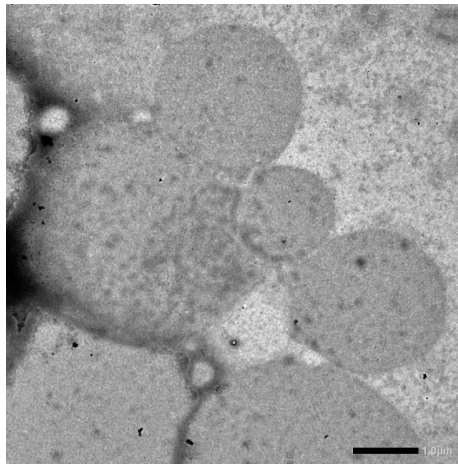


Figure 4.5: MLPs with encapsulated $\text{Ca}_{0.25}\text{Mg}_{0.75}\text{Fe}_2\text{O}_4$ NPs.

It is possible to observe that the $\text{Ca}_{0.25}\text{Mg}_{0.75}\text{Fe}_2\text{O}_4$ NPs with the intended shape and encapsulated them inside liposomes were successfully formed.

4.5 X-Ray Diffraction

A test was conducted to analyze the crystallinity and purity of a sample of magnetic $\text{Ca}_{0.25}\text{Mg}_{0.75}\text{Fe}_2\text{O}_4$ NPs that were produced for this purpose. The XRD analysis results are shown in Figure 4.6.

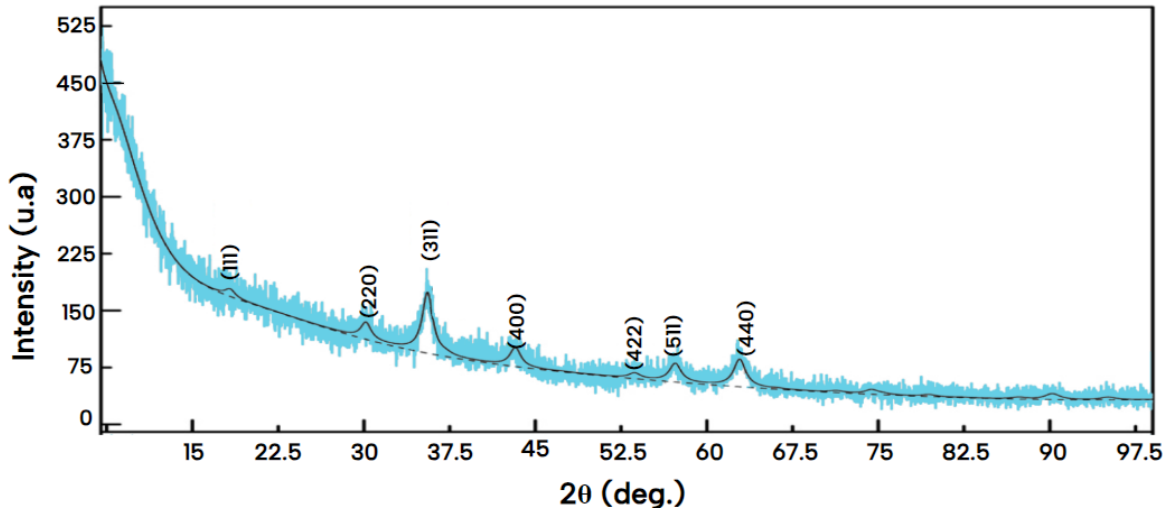


Figure 4.6: XRD results with a blue outline around each point indicating the associated error.

XRD analysis of magnesium and calcium ferrites revealed distinct peaks that matched the findings reported by Heidari *et al.* in 2021 [187]. The observed XRD patterns had peaks corresponding to the (111), (220), (311), (400), (422), (511), and (440) crystallographic planes. It is worth noting that the 2θ values obtained closely matched those reported in the reference mentioned above, confirming the structural properties of the magnesium and calcium ferrite samples being studied.

4.6 UV-Visible Absorption Spectrum of Nanoparticles

Based on Equation 4.1, a Tauc plot was used to determine the energy band gap.

$$(\alpha h\nu)^n \propto (h\nu - E_g)^n \quad (4.1)$$

Where α is the absorption coefficient, which is proportional to the absorbance, n is an exponent that depends on the nature of the transition ($n = 2$ for a direct semiconductor and $n = 1/2$ for an indirect

semiconductor) and E_g is the energy band gap.

It was found that a linear relationship could only be obtained for $n = 2$, as observed in Figure 4.7, which indicates that the magnetic NPs behave as direct semiconductors. The energy band gap was estimated from the intersection of the Tauc plot, and the value obtained was 1.35eV .

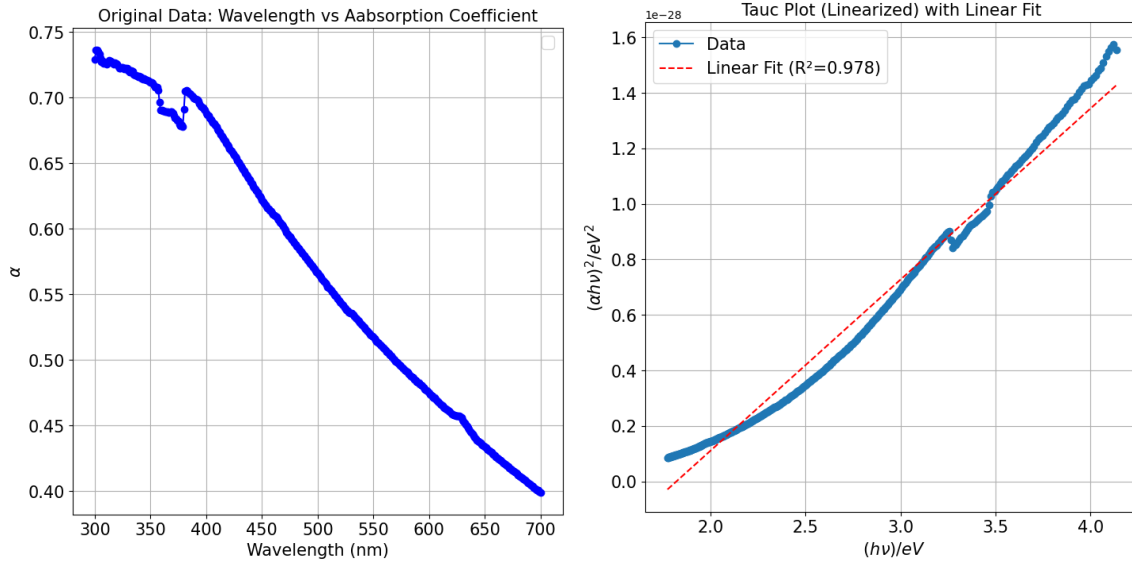


Figure 4.7: UV-Visible Absorption Spectroscopy results: Absorption vs. wavelength on the left, Tauc plot with linear regression on the right.

This test is crucial in understanding the optical properties of magnetic NPs. A bandgap of 1.35eV corresponds to a wavelength of approximately 918nm , by using Equation 4.2, which is within the near-IR region of the electromagnetic spectrum.

$$\lambda(\text{nm}) = \frac{hc}{E(\text{eV})} \quad (4.2)$$

Where λ is the wavelength, h is the Planck constant, c is the speed of light, and E is the energy (E_g in this case).

This means that the magnetic NPs would mainly interact with light in the near-infrared region. Materials with a bandgap in this range can absorb and emit light in the near-IR region, which makes these NPs suitable for other types of applications such as near-IR imaging or sensing.

This value exceeds the value reported in [82] (1.29eV), but is slightly higher than their findings. However, it is lower than values found in other studies such as [188] (1.42eV), [189] (2.00eV), and [186] (1.51eV).

4.7 Thermal Characteristics

In this analysis, using a DSC model DSC822e, it was observed intriguing results that provide valuable insights into the behaviour of lipids at different temperatures, the results are presented in Figure 4.8.

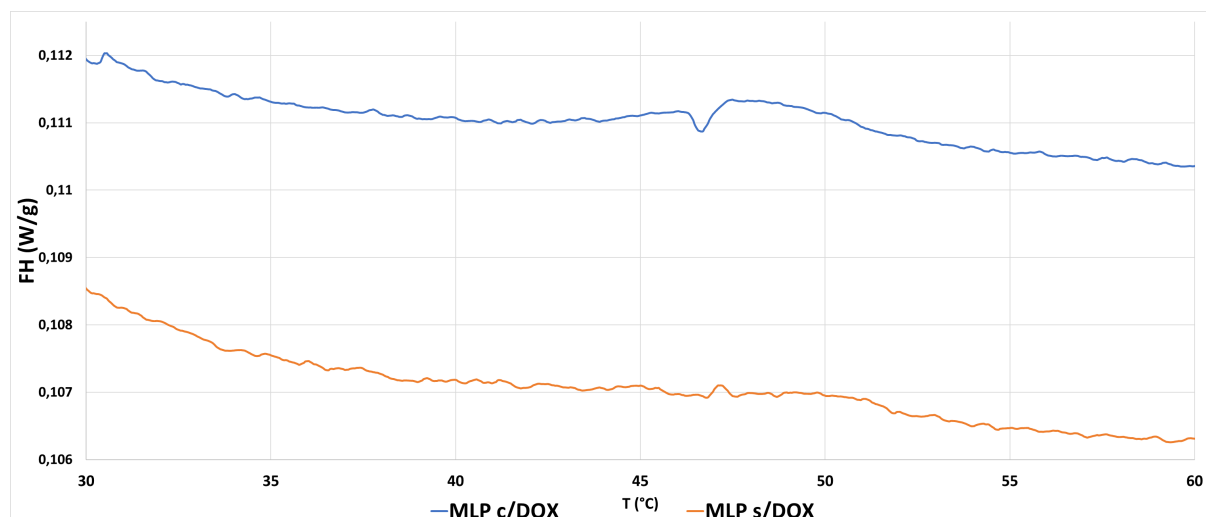


Figure 4.8: Results of the DSC analysis of the MLPs without DOX (orange) and MLPs with DOX (blue).

In these results, the presence of two distinct peaks in the heat flow (measured in W/g) for both cases is observed, and it is particularly interesting to note that these peaks occur at around $47^{\circ}C$. This temperature is significant because it closely aligns with the transition temperature of the lipids being studied.

In the study [190], it was observed that DPPC vesicles exhibit a pronounced peak in heat flow at $41^{\circ}C$, which aligns well with the known transition temperature of DPPC lipids, as described in Section 2.3.1.

The systems under investigation contain other types of lipids which affect the transition behaviour of the vesicles. The presence of these additional lipids or molecules could cause the vesicles to transition at higher temperatures [190], indicating that they may be influencing the phase transition of the lipid bilayers within the vesicles.

Moreover, it is worth noting that the presence of lipids with higher transition temperatures, such as DSPC, could be a key contributing factor to this phenomenon [191]. DSPC, being known for its higher transition temperature compared to DPPC, could be driving the overall transition of the lipid system to occur at higher temperatures. This finding underscores the intricate interplay between different lipid components within vesicles and their impact on the thermal behaviour of the system. Additionally, a higher overall heat flow in the MLPs containing DOX is observed. This suggests the influence of this molecule on the system.

4.8 Fourier Transform Infrared Spectroscopy

This test aims to detect the presence of iron oxide (from the $\text{Ca}_{0.25}\text{Mg}_{0.75}\text{Fe}_2\text{O}_4$ NPs), phospholipids, and DOX. A Python script was used to detect the most pronounced peaks of the obtained plot in Figure 4.9.

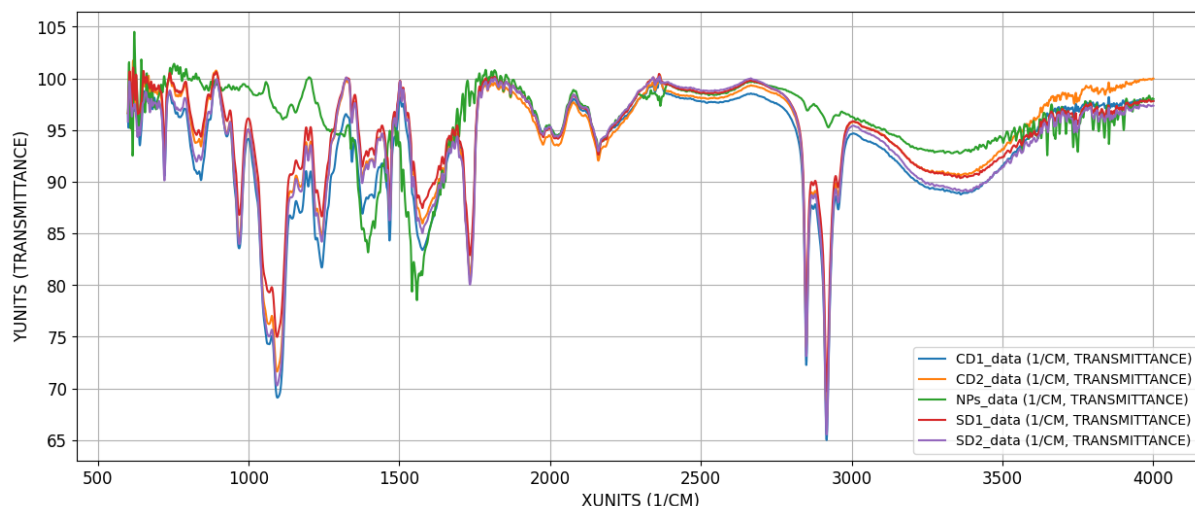


Figure 4.9: Results of the FTIR analysis: CD1_data and CD2_data represent two batches of MLPs with DOX, SD1_data and SD2_data represent two batches of MLPs without DOX and NPs_data represent NPs.

One of the significant findings was the presence of magnetic NPs, as evidenced by a strong band around 602cm^{-1} . This band is a characteristic of vibrations between iron (Fe) and oxygen (O) atoms, indicating the existence of Fe-O bonds, which is a telltale sign of NPs in the sample [192].

Another observation is the absorption band at 3354cm^{-1} , which is attributed to the moisture content. [192].

The FTIR spectrum also displayed distinct peaks in the range of 1095cm^{-1} , 1242cm^{-1} , and 1222cm^{-1} , indicating the presence of lipids. These peaks correspond to the stretching vibrations of the P=O group in the phosphate group of lipids, suggesting that lipids are a significant component of the sample.

Moreover, The FTIR peaks at 2916cm^{-1} and 2849cm^{-1} are due to the C-H stretching of alkane groups. These peaks are usually medium to strong in intensity and indicate the presence of carbon atoms bonded to hydrogen atoms. This further supports the presence of lipids due to their composition of long hydrocarbon chains, which are essentially alkanes [193, 194].

In [195], a graph is presented that compares the peaks of MLPs at analogous wavelengths. The article notes that the peaks at 575cm^{-1} and 572cm^{-1} are indicative of the Fe-O bonds in the NPs.

Additionally, a peak at 1092cm^{-1} is observed, representing the P=O bonds in lipids. Another band in the $3200 - 3600\text{cm}^{-1}$ range, as well as a peak at 1239cm^{-1} , represents other hydroxyl groups, including moisture. The peaks at 2848cm^{-1} and 2921cm^{-1} indicate the presence of C-H bonds, consistent with the presented results.

4.9 Magnetic Properties

The test results showed that all samples exhibited superparamagnetic behaviour. This behaviour is due to the absence of hysteresis in the plot presented in Figure 4.10.

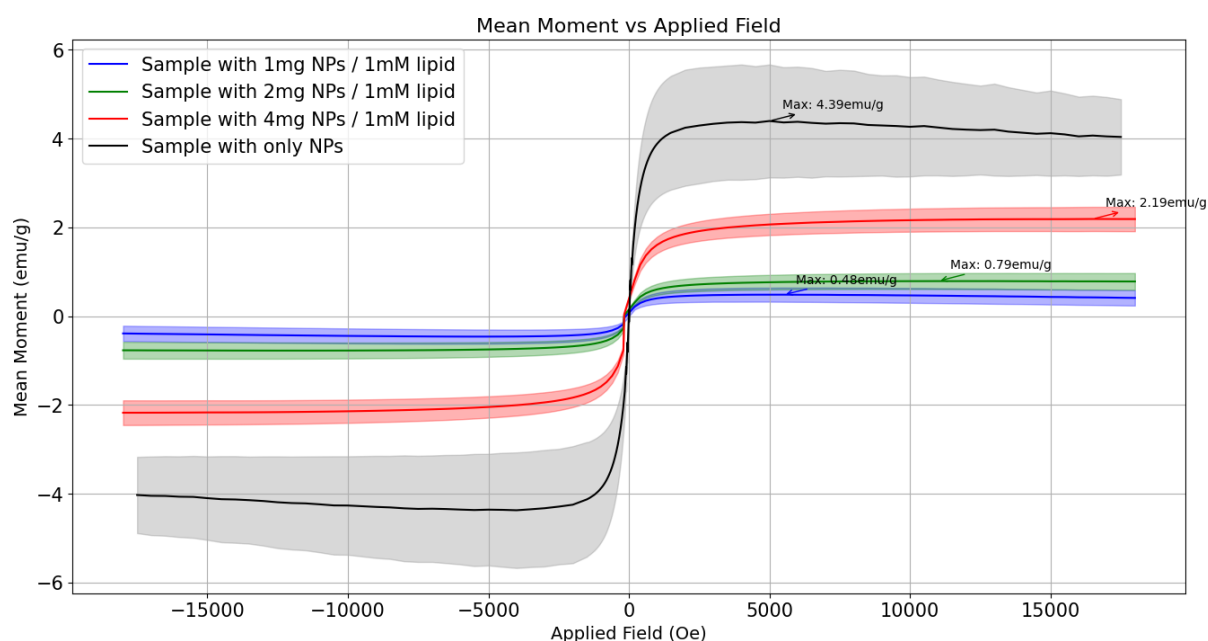


Figure 4.10: Results of the VSM analysis: The samples are presented in blue (1mg of NPs), green (2mg of NPs), red (3mg of NPs) and black (only NPs) and it's max value of magnetisation. The shaded region in the plots represents the associated error the mean moment.

The plot displays a linear relationship between the addition of more magnetic NPs and its saturation magnetization. This linear relationship is clearly shown in Figure 4.11. The R-squared value of 0.97 indicates a strong linear approximation. This is due to a higher concentration of superparamagnetic NPs or, consequently, a lower concentration of lipids.

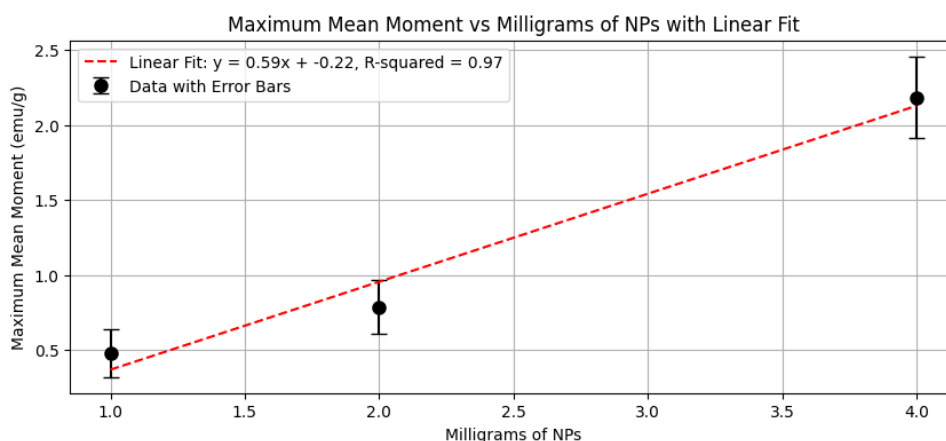


Figure 4.11: Linear regression applied to the higher mean moment value of each sample.

The saturation magnetization values obtained in this study are higher than those reported in a previous study [196], where the saturation magnetization was estimated to be 0.23 emu/g . Another study [197] found the saturation magnetization for the investigated systems to be 0.094 emu/g and 0.089 emu/g , respectively. However, the saturation magnetization observed in [198] is significantly higher, measuring 52.7 emu/g . In the case of the NPs, similar results were presented in a previous study [199], where a value of 5.35 emu/g was reported compared to the obtained 4.39 emu/g .

4.10 Encapsulation Efficiency of Doxorubicin

To study the potential of encapsulation of these systems, 3 batches of MLPs were processed with different concentrations of DOX (5 mM , $50 \mu\text{M}$ and $500 \mu\text{M}$).

In this process, a spectrofluorometer Fluorolog 3 was used to obtain the calibration slope shown in Figure 4.15.

The R-squared value obtained for this plot is 0.974, indicating a strong approximation as the value is nearly 1.

In the same plot, it shows the results obtained from three different samples with varying concentrations. After filtration using a $100 \mu\text{m}$ amicon filter and centrifugation at 3500 rpm for 8 min .

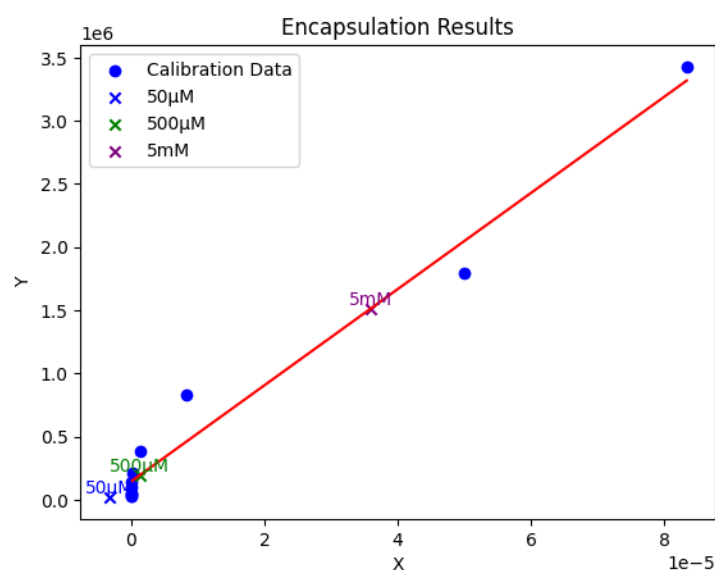


Figure 4.12: Each initial concentration measurement is denoted by a cross, with the calibration slope for DOX indicated by blue dots. The x-axis represents concentration in Molarity (M), while the y-axis depicts the average equipment reading.

The results obtained from the process are shown in Table 4.3. In the first case, the value for the case of $50\mu M$ is negative due, possibly, to the resolution of the equipment, resulting in 100% encapsulation. For the remaining cases, it is concluded that these systems are capable of encapsulating large amounts of DOX, which makes them highly efficient in this regard.

Table 4.3: Obtained results from the $EE(\%)$ of DOX.

Concentration (μM)	Non-encapsulated Amount (μM)	EE(%)
50	0	100
500	1.33	99.73
5000	$35.99\mu M$	99.28

In the research paper [200], it was found that the result achieved a very high efficiency of $96\% \pm 2\%$ encapsulation in MLPs that do not contain DPSE-PEG200 and $98.0\% \pm 0.6\%$ in MLPs at a concentration of $10\mu M$. However, in this project case, it was observed that the MLPs that contain DSPE-PEG2000 exhibited a higher encapsulation efficiency despite the higher concentration of DOX in the solution. The same for the case of [201], where an $EE(\%)$ ranging from 88.2% to 92.4% for a DOX concentration of $2mg/mL$ was obtained.

4.11 Encapsulation Efficiency of Nanoparticles

Following the defined procedure, it was possible to calculate a calibration slope that resulted in an R-squared value of ≈ 0.98 . This indicates a high degree of accuracy in estimating the results, as it is visualised in Figure 4.13, despite minor variations detected at some points. It is worth noting that these differences may indicate a reduced sensitivity for values over $2mg$ of magnetic NPs.

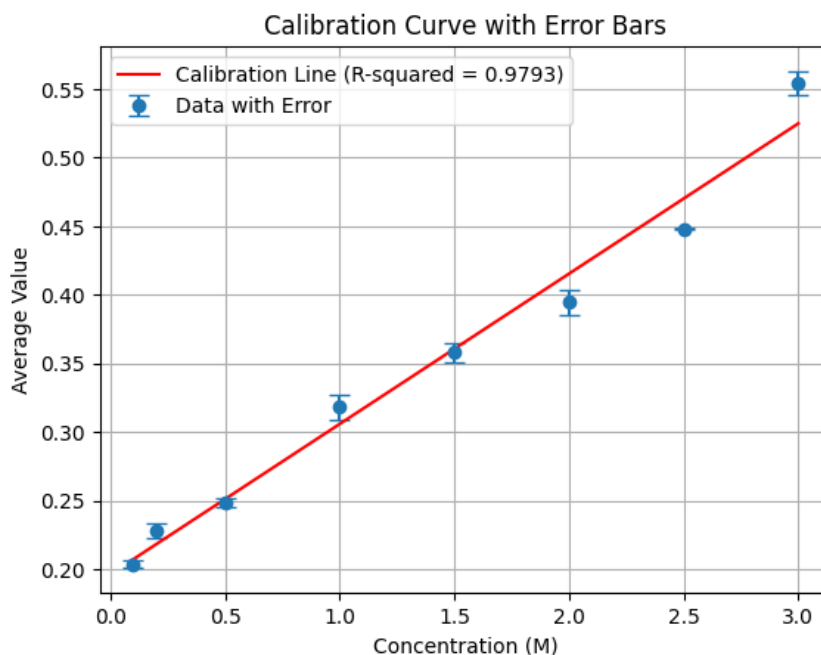


Figure 4.13: Calibration slope for the encapsulation efficiency of NPs. The x-axis represents the concentration in M and the y-axis represents the average value given by the equipment.

The non-encapsulated NPs were measured on the same reader, and their values are presented in Table 4.4. The table displays the non-encapsulated content from the total $2mg$ added to each sample.

Table 4.4: Obtained results from the absorption spectroscopy measurements for the 4 different samples.

Sample	Mean (mg)	STD (mg)	EE(%)	STD(EE(%))
Sample 1	1.14955	0.0143682	42.73	0.72
Sample 2	1.03103	0.0102042	48.49	0.5
Sample 3	0.976827	0.0124202	51.61	0.62
Sample 4	0.917926	0.00472674	54.01	0.235

The process yielded $(1.02 \pm 0.09)mg$ of non-encapsulated NPs from a total of $(2 \pm 0.1)mg$. Using

Equation 4.3 we conclude that $EE(\%) = (49.46 \pm 0.503)\%$.

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{Total Mass} - \text{Mass of Non-Encapsulated NPs}}{\text{Total Mass}} \times 100 \quad (4.3)$$

In contrast to other research findings, our results reveal a marginally elevated $EE(\%)$ compared to the system presented in [202], which reported a value of $46.(9) \pm 14\%$, and [14], which achieved a 36% efficiency for a sample with 10.1mg/mol of Fe^+ . The synthesised systems also showed a lower $EE(\%)$ compared to the 58% efficiency reported by [203]. However, the latter study utilized a lower concentration of NPs (0.116mg/mL).

4.12 Drug Release Over Time Under Static and Dynamic Conditions

To precisely measure the drug release rate, a calibration slope was created. This slope played a critical role in calculating the amount of drug released during the experiments. The calibration slope produced the following outcomes, illustrating the connection between drug concentration and measured values (Figure 4.14).

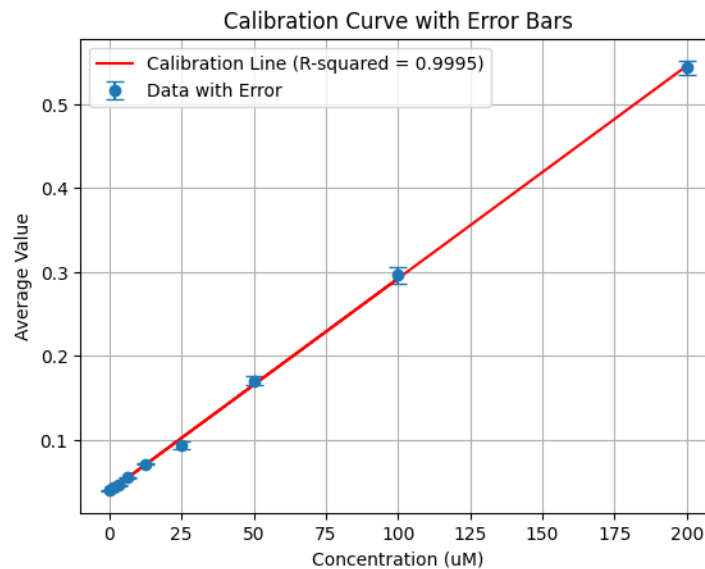


Figure 4.14: The DOX calibration slope is represented by blue dots, and the resulting points.

It shows a good linear approximation slope with a high R-squared value (0.9995). The slope was used as a reference for subsequent drug release experiments conducted under static and dynamic conditions.

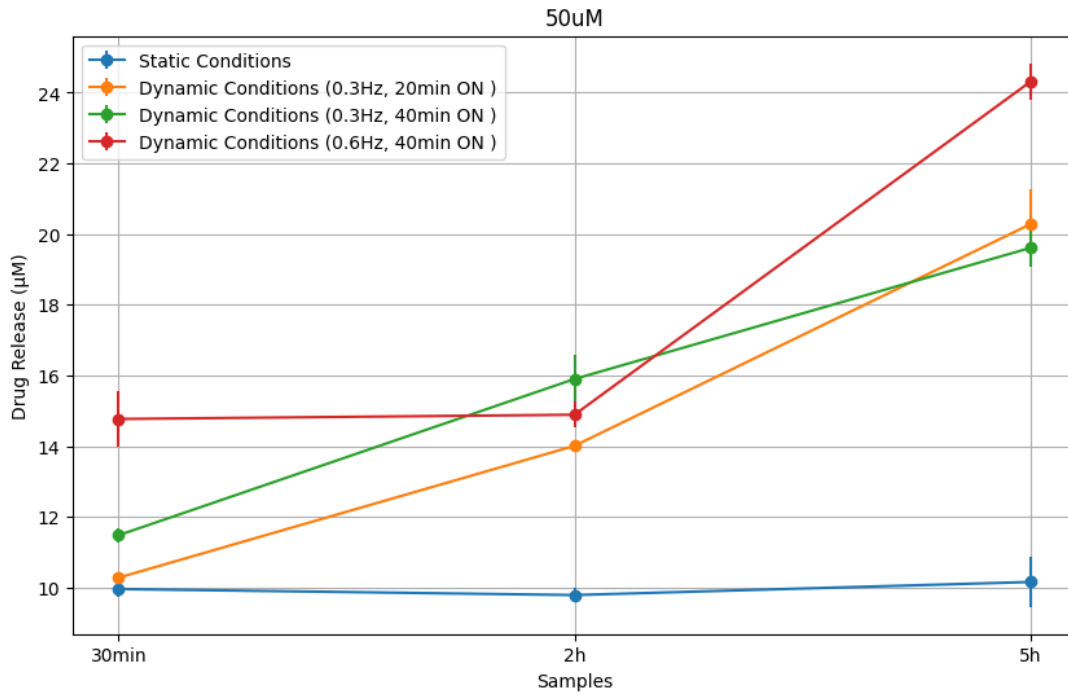


Figure 4.15: The graph shows the concentration of drug release (μM) over time. The blue line represents the static condition, while the orange line shows the drug release concentration when a frequency of $0.3Hz$ was used with a $20min$ ON cycle. The green line represents the drug release concentration when a frequency of $0.3Hz$ was used with a $40min$ ON cycle. Finally, the red line represents the drug release concentration when a frequency of $0.6Hz$ was used with a $40min$ ON cycle.

Under static conditions, the drug release rate stagnated at $10\mu M$ out of $50\mu M$, which accounts for $\approx 20\%$ over a $5h$ period.

When a magnetic field was applied with an ON frequency of $0.3Hz$, and with ON periods of $20min$, it was noticed a slightly higher release at the $30min$ mark, possibly due to the magnetic stimuli over that period. It was also observed an almost linear drug release over $5h$. It reached a final release of approximately $20\mu M$, which is nearly 40% .

In the case of the same ON frequency but with ON periods of $40min$, an even higher initial release, just under $12\mu M$, is observed, and there was a slightly higher release at the $2h$ mark. However, it achieved the same final concentration as the previous scenario.

Finally, when the same ON cycle is used, but increasing the frequency of the applied magnetic field to $0.6Hz$, at the initial $30min$ mark, it was observed a much higher initial release compared to the other scenarios. This was possibly due to the higher frequency of the magnetic stimuli. Despite that, it was observed a similar release at the $2h$ mark of the other dynamic conditions. However, the final release

exceeded $24\mu M$ (or $\approx 48\%$), which was much higher than all scenarios.

It is reasonable to assume that these systems release a very small amount of DOX in the static condition. However, significant drug release can be observed by applying a magnetic stimulation. By increasing the ON cycle time, an increase in drug release over time was noticed. Increasing the frequency resulted in a higher initial release and an overall increase in the maximum drug release. Comparing the static and dynamic conditions it is observed an increase in drug release of $\approx 28\%$.

Chapter 5

Conclusion and Future Work

5.1 Conclusion

The primary objective of this research project, centred on the development and characterization of MLPs for enhanced cancer therapy targeting OS, has been successfully achieved. Through rigorous experimentation and analysis, the study effectively established the physical and chemical properties of these innovative drug delivery systems. The developed systems are lipidic vesicles encapsulate both magnetic NPs and DOX, enhancing targeted delivery and therapeutic efficacy for this disease.

The present study on superparamagnetic $\text{Ca}_{0.25}\text{Mg}_{0.75}\text{Fe}_2\text{O}_4$ NPs provides a comprehensive characterization of their unique properties and potential applications. The TEM results showed a consistent size distribution of the magnetic NPs, averaging at around $8.75 \pm 0.35\text{nm}$. This uniformity indicates a precise synthesis method and offers promising opportunities for controlled applications. The XRD analyses further confirmed the crystalline nature of these NPs, as peaks were present, indicating their structural integrity and potential stability under various environmental conditions. Moreover, magnetic characterization through VSM showcased a saturation magnetization of 4.39emu/g . Notably, the absence of hysteresis in the VSM curves emphasizes the superparamagnetic behaviour of the magnetic NPs, which is pivotal for applications like, in this case, drug delivery. Furthermore, the bandgap of these NPs, as determined through UV-visible absorption spectroscopy, was 1.35eV , indicating a strong absorption in the near-IR region. During the study of MLPs, the DLS results showed a large hydrodynamic radius ($542.74 \pm 110.40\text{nm}$) and PDI ($27.48 \pm 2.87\%$), indicating a heterogeneous nature that could affect interactions and stability in biological systems. During a 43 day period, the zeta potential was observed to undergo a temporal evolution. It started at -8.5mV and gradually shifted to -5.5mV , indicating the sensitivity of MLPs and their degradation over time. The zeta potential was found to be affected by variations in pH, which suggests that MLPs could be used in responsive drug delivery systems under specific pH conditions. The TEM results illustrated the incorporation of NPs on the liposome surfaces and inside the bilayer, indicating

potential enhancements in therapeutic functionalities. Thermal analyses through DSC highlighted specific temperature transitions within the MLPs, suggesting potential mechanisms for controlled drug release. However, a nuanced magnetic behaviour observed in VSM results emphasized the influence of lipid components within the system that greatly affects the saturation magnetization of the MLPs. It was obtained a saturation magnetization of 4.39emu/g for the magnetic NP sample, and a linear increase of saturation magnetization from 0.48emu/g , for the MLPs with 1mg of NPs, to 2.19emu/g for the sample with 4mg . A component essential for their therapeutic potential was the encapsulation efficiency. The MLPs demonstrated notable encapsulation efficiencies for both NPs and DOX, hinting at improved therapeutic efficacy and potentially reduced side effects. During this study, the release of the drug from the MLPs was limited to only about $\approx 20\%$ over a $5h$ period under static conditions. However, it was observed interesting results when a magnetic field was introduced. By altering the frequency and period, significant changes were noticed. At a lower frequency and shorter periods, there was a moderate increase in the drug release. By changing those parameters, especially by increasing the frequency, the drug release shot up substantially, reaching close to 48% in these tests. Essentially, while static conditions did not offer much, these magnetic stimuli methods provided a promising avenue for enhanced drug release from the MLPs. MLPs possess attributes that make them promising contenders for advancing targeted therapeutic interventions. Their structural intricacies and functional capabilities enable high encapsulation efficiency and controlled drug release, demonstrating their potential for optimized drug delivery.

5.2 Future Work

This master's dissertation has successfully achieved its initial objective, which was to enhance the MLP-type nanosystems. However, there is still room for improvement in this area. As a result, the following tasks have been proposed for future work:

- Application of these systems to cells and observe their behaviour.
- Further study the drug release under static and dynamic conditions (application of other factors such as more frequencies, pH values, ON-cycle variations).
- Adaptations of these systems for possible hyperthermia applications.
- Possible encapsulation of other compounds and drugs for cancer therapies.
- Functionalization of the MLPs via interaction of over-expressive receptors in cancer cells.

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Appendix A

Optimizing Drug Release Test Filtration: Determining the Most Effective Method

To assess the efficacy of distinct filtration methods in minimizing the absorption of non-encapsulated drug, a series of experiments were conducted utilizing Amicon filtration (depicted in green) and $0.45\mu m$ syringe filters (represented in blue). The findings are illustrated in Figure A.1. The experiments encompassed solutions containing MLP devoid of any drug (MLP) and solutions with DOX at concentrations of $12.5M$, $25\mu M$, and $50\mu M$.

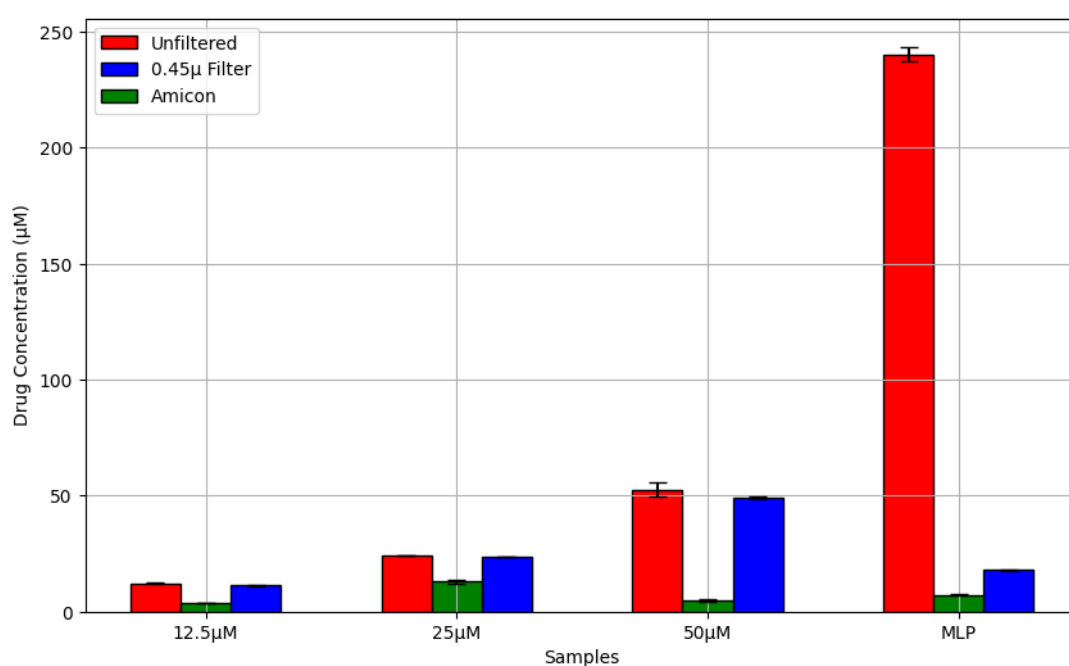


Figure A.1: Efficiency of various filtration techniques in reducing encapsulated DOX absorption.

Although the MLP solution does not contain any DOX, it shows a high absorption value because of the presence of lipids and NPs. To get accurate results, the filtration method used should allow maximum passage of non-encapsulated DOX while minimizing the passage of unwanted NPs and lipids that might affect the results.

The graph shows that Amicon filtration is effective in removing MLPs components, but it also filters a considerable amount of non-encapsulated DOX from samples with concentrations of $12.5\mu M$, $25\mu M$, and $50\mu M$. In contrast, the syringe filter slightly filters unencapsulated DOX, but it filters the MLP sample as pretended. Based on these results, The drug release procedure has been carried out using the latter technique that was selected.

