UNIVERSIDAD AUTÓNOMA DE BAJA CALIFORNIA
Facultad de Ciencias Químicas e Ingeniería

TESIS PARA OBTENER EL GRADO DE MAESTRÍA EN CIENCIAS E INGENIERÍA

Microcápsulas biodegradables para la liberación de la enzima catalasa mediante estimulación de ultrasonido

PRESENTA
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Declaración de Colaboración Institucional

Esta tesis ha sido realizada en el marco de un programa de doble titulación de maestría en investigación, fruto de la colaboración académica entre la Universidad Autónoma de Baja California (UABC) y Queen Mary University of London (QMUL). Este esfuerzo colaborativo permitió desarrollar una investigación integral que forma parte tanto del programa Research Master's (ResM) de QMUL como del programa Maestría en Ciencias e Ingeniería de UABC.

El trabajo de investigación presentado en esta tesis fue llevado a cabo durante un período de dos años. En el primer año, completado en UABC, se realizó un estudio profundo de las herramientas y de la teoría fundamental necesarias para el desarrollo de este proyecto de investigación, titulado "Microcápsulas biodegradables para la liberación de la enzima catalasa mediante la estimulación de ultrasonido". Esta etapa inicial del proyecto, enfocada en el establecimiento de los fundamentos teóricos y metodológicos, estuvo bajo la supervisión del Dr. José Manuel Cornejo Bravo (UABC), el Dr. Eduardo Alberto López Maldonado, quienes brindaron orientación clave para definir el marco y los objetivos de la investigación.

Durante el segundo año, el proyecto fue acogido por QMUL, bajo la supervisión del Profesor Gleb Sukhorokov donde se profundizó en la fase experimental y se implementaron metodologías avanzadas de encapsulamiento de cargos altamente sensibles además del entrenamiento en Microscopía Confocal y Microscopía Electrónica de Barrida.

La presente tesis cumple con los requisitos académicos y científicos de ambas instituciones y permite una evaluación integral en ambas universidades. Reflejando los altos estándares de rigor que estas instituciones exigen para la obtención de la doble titulación. Esta colaboración internacional es un esfuerzo conjunto que busca fomentar la excelencia académica y proporcionar una experiencia investigativa enriquecida, aprovechando la sinergia entre los recursos y conocimientos especializados de ambas universidades.

Este documento, que constituye el esfuerzo conjunto de ambas instituciones y el fruto de la dedicación académica vertida este tiempo, quedará registrado en los archivos académicos de UABC y QMUL, reflejando un compromiso con la innovación científica y la cooperación internacional en la formación de investigadores.

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Pasante de: Maestría en Ciencias
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El cual deberá usted desarrollar, de acuerdo con el siguiente orden:

I. INTRODUCTION
II. LITERATURE REVIEW
III. MATERIALS AND METHODS
IV. RESULTS AND DISCUSSIONS
V. CONCLUSIONS

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Master by Research

SEPTEMBER 2024

Declaration

Biodegradable microcapsules for catalase enzyme release via ultrasound stimuli.

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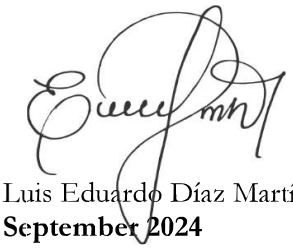

Luis Eduardo Díaz Martínez
September 2024

Abstract

Despite the promising advancements made by sophisticated drug delivery systems, several critical issues remain in the field. More challenges arise when it comes to encapsulating sensitive cargoes such as proteins and enzymes. Proteins and enzymes are often sensitive to environmental conditions such as temperature, pH, and degradation and can undergo denaturation or degradation during encapsulation, storage, and release. This research proposes two types of printed microcapsule structure, the first based on the combination of soft lithography and layer-by-layer techniques, and the second based on soft lithography encapsulation only.

The first printed microcapsule structure mentioned as Layer-by-Layer (LbL) assembly is a versatile and widely used method for fabricating microcapsules with precise control over composition, structure, and functionality. This technique involves the sequential deposition of oppositely charged polyelectrolytes, enabling the creation of multi-layered shells around core materials. Microcapsules constructed through LbL assembly offer unique advantages, including tuneable permeability, controlled release, and the ability to encapsulate sensitive cargos, such as enzymes, drugs, or nanoparticles, while preserving their structural integrity.

A key application of LbL microcapsules is the encapsulation and protection of highly sensitive biological molecules, such as the catalase enzyme. Catalase, known for its vulnerability to environmental conditions, can be stabilized within the LbL shells, ensuring its activity is retained until triggered release. This makes LbL microcapsules particularly promising for biomedical applications where controlled enzyme delivery is required.
On the other hand, the second printed microcapsule mentioned is based on soft lithography using PLGA (poly (lactic-co-glycolic acid)) microcapsules as the main and only polymer in its structure. In recent years, PLGA (poly (lactic-co-glycolic acid)) microcapsules have emerged as a highly promising biodegradable and biocompatible platform for drug and enzyme delivery. The combination of PLGA with LbL techniques allows for the creation of hybrid microcapsules that offer enhanced protection for sensitive proteins and enzymes, such as catalase while providing controlled and stimuli-responsive release. The biodegradability of PLGA makes it particularly attractive for clinical applications, where it safely degrades to lactic and glycolic acid, both naturally metabolized by the body. Integrating of PLGA with the LbL assembly enhances the microcapsules' mechanical strength and can improve their biodegradability and ability to release cargo in response to external triggers such as pH, temperature or ultrasound.

For instance, High-Intensity Focused Ultrasound (HIFU) has been demonstrated as an effective trigger for non-invasive, on-demand release of encapsulated enzymes, further expanding the potential of these systems in therapeutic applications.
Both encapsulation methods provide flexibility in the design and manufacture of the microcapsules, enhancing the protection of the enzyme and shielding it from degradation. Maintaining the stability of encapsulated enzymes over time is critical for their efficacy in drug delivery applications. Therefore, this study focuses on the preparation and characterization of printed biodegradable polymer delivery systems to overcome the challenges of enzyme cargo encapsulation and release.

Key words: Layer-by-Layer (LBL), soft lithography, sensitive cargoes, microcapsules, drug loaded, Controlled release.

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List of Symbols and Abbreviations

CLSM – confocal laser scanning microscopy
DDS's – drug delivery system
DI – Deionized water
DMSO – Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
DS – Dextran Sulphate
FDA – Food and Drug Administration
FITC – fluorescein isothiocyanate
HA – Hyaluronic Acid
HIFU – High – Intensity Focus Ultrasound
HIV – Human Immunodeficiency Virus
LbL – Layer-by-Layer
LIFU – Low – Intensity Focus Ultrasound
PAH – poly (allylamine hydrochloride)
PBS – potassium buffer saline
PCL - Polycaprolactone
PDDA – poly (diallyl dimethyl ammonium)
PDMS – polydimethylsiloxane
PEI – poly (ethylene imine)
PEG - Polyethylene glycol
pH – Measure of acidity/basicity of aqueous solution
PHA - Polyhydroxyalkanoates
PLA – Polylactic acid
PLGA – polylactic-co-glycolic acid
PSS – Polystyrene Sulfonate
SEM – Scanning Electron Microscopy

Introduction

Background

Microcapsules have become a crucial technology in Drug Delivery Systems (DDSs) due to their ability to enhance the efficacy, stability, and controlled release of therapeutic agents. These small structures encapsulate active pharmaceutical ingredients and highly sensitive cargoes within a protective shell, which can be engineered to offer controlled and sustained release. [1,2]

One of the key advantages of microcapsules is their capability to protect sensitive drugs from environmental degradation, this is one of the main challenges in DDSs. Many pharmaceutical compounds, particularly biologics and peptides, are susceptible to factors such as pH and temperature. The catalase enzyme is considered one of the most important and sensitive biological compounds. Catalase enzyme plays a critical role in breaking down hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). This reaction is crucial because hydrogen peroxide, although a byproduct of many metabolic processes, is toxic to cells. If not broken down, H_2O_2 can lead to oxidative damage, which can harm proteins, DNA, and cell membranes. [3]

The catalase enzyme, like many biological molecules, has specific environmental conditions under which it remains stable and functional. If these conditions are not met, catalase can lose its activity due to denaturation or degradation, one of the main conditions are optimal temperature close to 37°C, higher temperature (above 50 – 60°C) can cause denature, and optimal pH around 7, however it can function in a pH range between 6.8 and 7.5. [3]

The encapsulation approach offers many advantages to protect the catalase enzyme from other environments conditions and prevent it denature. Nevertheless, in the stimuli used to trigger the microcapsules release is by High – Intensity Focus Ultrasound, here another problem arises because the sonication elevates the temperature higher than 50 – 60 °C, one of the main goals is being able to protect the catalase enzyme from the sonication heat and finding the optimal point that promotes the release of the catalase from the microcapsules.

Recent advancements in microcapsule technology, including the use of novel materials and innovative fabrication techniques, have further expanded their applications in drug delivery systems, like the soft lithography and the Layer – by – Layer approach. For example, the integration of biodegradable polymers such as poly (lactic-co-glycolic acid) (PLGA) has improved the environmental sustainability and biocompatibility of microcapsules, while maintaining their functionality and effectiveness. The next challenge is to try alternative biodegradable materials that can show better performance in the protection of highly sensitive cargoes. [24,16].

Another encapsulation technique is the use of the Layer – by – Layer (LbL) approach, this methodology involves the sequential deposition of alternating layers of opposite charged polyelectrolytes. This approach is one of the most common methodologies in the protection of highly sensitive cargoes. Through this research, the polyelectrolytes used were poly (sodium styrene sulfonate) (PSS) and Poly – allylamine hydrochloride (PAH), this combination is one of the most popular combinations used in LbL.

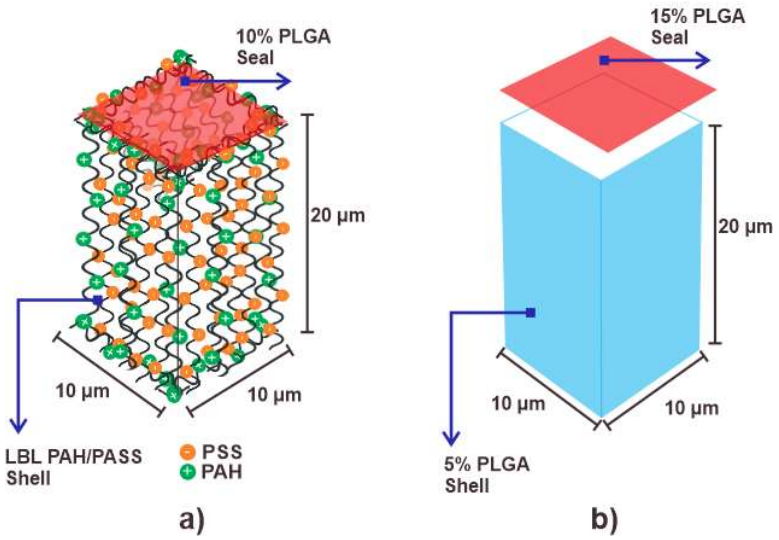

Figure 0.1. – 1 Microcapsules structures used in the protection of highly sensitive cargoes (a) Layer - by - Layer (LBL) microcapsules made from PAH/PSS polyelectrolytes and sealed with PLGA 75:25, (b) Microcapsule using the soft lithography approach made from PLGA 75:25.

Motivation and aim

The development of drug delivery systems (DDSs) faces significant challenges, particularly in the protection and controlled release of sensitive therapeutic agents. Many biologics, enzymes, and peptides are prone to degradation from environmental factors such as pH, temperature, and oxidative stress, which limits their therapeutic potential. One such biologically important molecule is the catalase enzyme, which plays a crucial role in breaking down harmful hydrogen peroxide into water and oxygen. However, catalase is highly sensitive to environmental changes, especially heat, making it essential to develop systems that protect its integrity while enabling controlled release.

Microcapsules have emerged as a promising solution for encapsulating sensitive cargoes, providing a protective shell that can release drugs in a controlled manner. Recent advancements, including the use of biodegradable polymers like poly (lactic-co-glycolic acid) (PLGA) and innovative fabrication techniques such as soft lithography and Layer-by-Layer (LbL) assembly, have expanded the applications of microcapsules in DDSs. These systems offer enhanced stability and controlled release for sensitive molecules like catalase and addressed some of the key challenges, particularly in the use of High-Intensity Focused Ultrasound (HIFU) as a trigger for drug release, because, the heat generated by HIFU can exceed the optimal temperature for catalase, leading to its degradation.

The aim of this research is to develop and evaluate microcapsule-based DDSs capable of encapsulating and protecting the catalase enzyme under challenging conditions, such as the heat produced by HIFU. By employing biodegradable polymers like PLGA and using LbL assembly, this study seeks to enhance the stability and release profile of the catalase enzyme, ensuring its functionality is maintained during the drug delivery process. Through the exploration of novel materials and optimized methodologies, this research aims to improve the overall efficacy and safety of DDSs for sensitive cargoes.

Chapter I | Literature Review

1.1 Drug Delivery Systems (DDS's)

1.1.1. Drug Delivery Systems (DDS's) Applications

Drug delivery systems (DDS's) are technologies designed to deliver therapeutic agents (drugs) or biological agents to specific sites within the body, ensuring that the cargo is released at the right time, at the right dose, and in the right location. Drug Delivery System (DDS's) is an interface between the patient and the drug. It may be a formulation of the drug to administer it for a therapeutic purpose or a device used to deliver the drug. They accelerate the transport of drugs to the precise target location in the body, optimizing therapeutic outcomes while minimizing accumulation in non-target areas. [4,5]. Drugs can be administered through a variety of routes, including but not limited to oral administration [6,7], buccal and sublingual routes [8], nasal and ophthalmic delivery [9,10], transdermal and subcutaneous methods [11,12], as well as anal, transvaginal, and intravesical routes [13,14]. The drug's components determine its physicochemical properties, which in turn influence the effects it produces within the body.

According to the target place in the body and the nature of the drug, the delivery system is engineered and designed. This can also be developed to release the drug into the body in a specific place and/or rate at which it must be released [15]. There is a constant evolution of methods of DDS's, which involves modifications of conventional methods and the discovery of new routes and devices. Nevertheless, there are relevant issues in the release and absorption in the body according to the route that is being used to administer the drug. In **Table No. 1.1.1 – 1**, it is addressed some of the issues and perks of the four most common Drug Delivery Systems (DDS's) related to the delivery to blood circulation, onset of action, bioavailability, dose control, and adverse effects [16].

Table No. 1.1.1 - 1 Comparison of most common routes of DDS's [16]

Issue	Oral	Intravenous	Intramuscular / Subcutaneous	Transdermal
Delivery to blood circulation.	Indirect through Gastrointestinal tract.	Direct.	Indirect absorption from tissues.	Indirect.
Onset of action.	Slow	Rapid	Moderate to rapid.	Moderate to rapid.
Bioavailability	Low to high.	High.	High.	Low.
Dose control.	Moderate	Good.	Moderate.	Poor.
Administration.	Self.	Health Professional.	Self or health professional.	Self.
Adverse effects.	High.	Low	Low.	Moderate,
Use for proteins and peptides.	No.	Yes.	Yes.	No.

Through the routes mentioned above, many DDSs has been developed, the most common and traditional are oral pills and injections. There are some other more sophisticated methods used to treat more dangerous and deadly diseases such as cancer [17], HIV [18], and neurodegenerative disorders [19]. These advanced systems are designed to improve precision, safety, and efficacy of therapeutic agents, particularly for diseases where traditional methods may fall short like it is show in **Table No. 1.1.1 – 1.**

For instance, in cancer treatment, nanoparticle-based delivery systems allow for targeted therapy [20], ensuring that chemotherapy drugs are directed specifically to tumour cells, minimizing damage to healthy tissues and reducing side effects. Liposomes, microspheres, and polymer-based nanoparticles are also employed to deliver drugs in a controlled manner, ensuring sustained release over time, which is critical for maintaining therapeutic levels of potent drugs while reducing toxicity [21].

In the case of HIV, antiretroviral drugs encapsulated in biodegradable polymers [22] or microparticles can enhance patient adherence by providing long-acting formulations that reduce the need for frequent dosing. Similarly, for neurodegenerative diseases like Parkinson's or Alzheimer's, blood-brain barrier-permeable nanoparticles are used to deliver drugs directly to the brain, overcoming one of the most significant challenges in treating such disorders [20,22].

Even though nanoparticle – based delivery systems and antiretroviral encapsulations are novel methods within the DDS's, there are many other, some of them traditional and common than other, like it is shown on **Figure No. 1.1.1 – 1** [27].

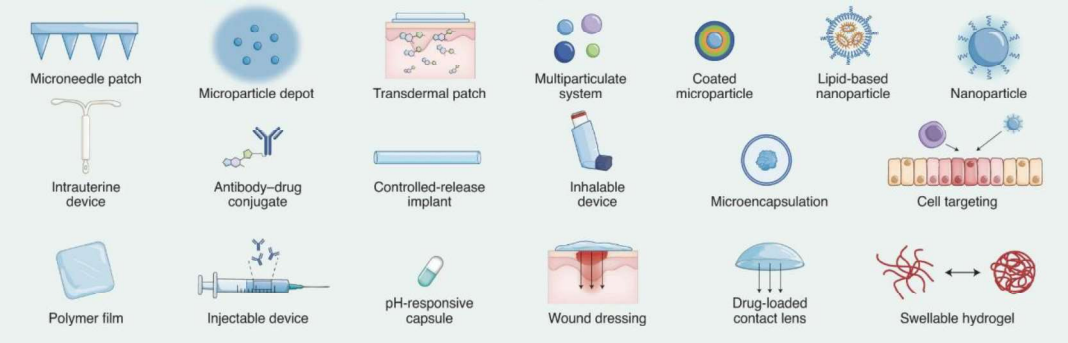

Figure 1.1.1 - 1 Different types of Drug Delivery Systems [27]

Each drug delivery system is tailored to the drug's characteristics, the target site, and the therapeutic needs, optimizing efficacy and patient outcomes.

1.1.2. Microcapsules in DDS's.

In drug delivery, microcapsules are small structures particles that consist of an active drug or therapeutic agent enclosed within a protective shell or coating [16,25]. These capsules typically range in size from a few micrometres to millimetres and are designed to provide controlled release of the encapsulated drug over time or at a specific target site in the body. The shell material, often made from biodegradable polymers, protects the active substance from degradation, ensures stability, and allows for precise release control in response to environmental factors such as pH, temperature, or external triggers like ultrasound. This feature is essential in conditions that require long-term medication, such as chronic pain, diabetes, or hypertension, as it reduces the need for frequent dosing and helps maintain a stable therapeutic concentration in the body [16, 25].
Some of the advantages of working with microcapsules in drug delivery systems are the following:

a) **Controlled release:** Microcapsules allow for the sustained or timed release of drugs, ensuring that therapeutic agents are delivered over an extended period rather than all at once. This feature is essential in conditions that require long-term medication, such

as chronic pain, diabetes, or hypertension, as it reduces the need for frequent dosing and helps maintain a stable therapeutic concentration in the body [23,25].

b) **Protection of sensitive drugs or therapeutic agents:** Many drugs, such as proteins, enzymes, and peptides, are highly sensitive to environmental factors like pH, temperature, and enzymatic degradation. Microcapsules provide a protective barrier that shields these sensitive drugs from degradation until they reach their target site. This is especially important for biologics, which are often unstable in the gastrointestinal tract or bloodstream.
c) **Targeted drug delivery:** Microcapsules can be engineered to release drugs at specific sites in the body, such as tumour cells, inflamed tissues, or the central nervous system [18,25]. This targeted delivery reduces the exposure of healthy tissues to potent drugs, minimizing side effects and enhancing therapeutic outcomes. For example, in cancer therapy, microcapsules can deliver chemotherapy drugs directly to tumour sites, reducing damage to surrounding healthy tissues.
d) **Improved patient compliance:** By enabling long-acting formulations or controlled-release profiles, microcapsules can reduce the frequency of dosing. This improves patient compliance, especially for treatments requiring regular medication, such as diabetes (insulin) or cardiovascular diseases. Patients benefit from fewer doses and more convenient treatment regimens [25]
e) **Reduction of side effects:** Microcapsules can help minimize drug-related side effects by controlling the release rate and localizing the drug's action. In traditional drug delivery, large doses may lead to systemic side effects or toxicity. Microencapsulation allows for the gradual release of drugs, reducing the likelihood of high peak concentrations in the bloodstream and minimizing side effects [24,25].
f) **Versatility and customization:** Microcapsules are highly versatile and can be customized for different drug delivery routes, including oral, injectable, transdermal, inhalation, and ocular delivery [4-12]. This adaptability allows pharmaceutical companies to develop new formulations for a variety of therapeutic needs, expanding the possibilities for personalized medicine [25].
g) **Stimuli – Responsive release:** Advanced microcapsules can be designed to respond to specific external stimuli such as pH, temperature, or ultrasound, allowing for on-demand or triggered drug release [24]. For instance, High-Intensity Focused Ultrasound (HIFU) can trigger drug release from microcapsules at a specific site in the body, providing a non-invasive and controlled therapeutic approach [26].
h) **Enhanced bioavailability:** Many drugs, particularly those that are poorly soluble or unstable in the body, suffer from low bioavailability when delivered through traditional methods. Microcapsules enhance the bioavailability of these drugs by protecting them from degradation and ensuring they reach their target site in a form that can be effectively absorbed by the body.
i) **Application in combined therapies:** Microcapsules can be used to co-deliver multiple drugs or therapeutic agents, allowing for combination therapies that target different pathways of a disease simultaneously. This is particularly useful in complex diseases like cancer or HIV [17,18], where multiple drugs need to be delivered in a controlled and synergistic manner.

Microcapsules manufacturing is a research field that has gain a lot of recognition through the year for overcoming some of the most important issues in many treatments and therapy among different diseases. Nevertheless, challenges such as manufacturing complexity, limited drug loading capacity, potential for burst release, and cost con hinder their widespread application. Therefore, optimizing and further research need to be done to enhance their performance among their current applications and more other can be explore.

1.1.3. Biodegradable Polymers in DDS's.

Biodegradable polymers play a crucial role in drug delivery systems due to their ability to degrade into non-toxic byproducts that are naturally eliminated by the body. This property makes them highly suitable for creating controlled and targeted drug delivery systems, reducing the need for surgical removal of drug carriers and minimizing long-term side effects. There is a wide range of polymers used in drug delivery applications, and according to the purpose and route of delivery they can be natural, synthetic, biodegradable and /or non – biodegradable [29]. Some of them are showed in **Table No. 1.1.3. – 1.**

Table No. 1.1.3. – 1 Representative list of polymers used in drug delivery. [29]

Classification		**Polymer**
Natural polymers	**Protein – based polymers**	Collagen, albumin, gelatine.
	Polysaccharides	Agarose, alginate, carrageenan, hyaluronic acid, dextran, chitosan, cyclodextrins
Synthetic polymers	**Biodegradable**	
	Polyesters	Poly (lactic acid), poly (glycol acid), poly (hydroxy butyrate), poly (ε – caprolactone), poly (β – malic acid), poly (dioxanones).
	Polyanhydrides	Poly (sebacic acid), poly (adipic acid), poly (terephthalic acid) and various copolymers.
	Polyamides	Poly (imino carbonates), polyamine acids.
	Phosphorous – based polymers.	Polyphosphates, poly phosphonates, polyphosphazenes.
	Others	Poly (cyano acrylates), polyurethanes, polyortho esters, Poly dihydropyrans, polyacetals.
	Non – Biodegradable	
	Cellulose derivatives	Carboxymethyl cellulose, ethyl cellulose, cellulose acetate, cellulose acetate propionate, hydroxypropyl methyl cellulose.
	Silicones	Polydimethylsiloxanes, colloidal silica.
	Acrylic polymers	Polymethacrylates, poly (methyl methacrylate), poly hydro (ethyl – methacrylate).
	Others	Polyvinyl pyrrolidone, ethyl vinyl acetate, poloxamers, poloxamines.

Deciding between polymers for drug delivery depends on several factors related to the drug, the target site, the delivery method, and the desired release profile. Some of the key considerations are show in **Table No. 1.1.3. -2.**

Table No. 1.1.3. – 2 Factors to consider when deciding between polymers for drug delivery.

Consideration	Fundamental
Biocompatibility	The polymer must not cause an adverse reaction in the human body (e.g., inflammation, toxicity, or immune response) [16, 25] For example: Poly (lactic-co-glycolic acid) (PLGA), Polylactic acid (PLA), Polyethylene glycol (PEG), and Chitosan.
Biodegradability	Biodegradable polymers are often preferred for drug delivery because they break down in to non – toxic byproducts that are naturally eliminated by the body, avoiding the need for surgical removal. For example: PLGA, PLA, PCL, Polyhydroxyalkanoates (PHA).
Drug Stability and compatibility.	• **Chemical stability:** The polymer must protect the drug from degradation (e.g., pH, enzymes, temperature) during the formulation, storage, and delivery process. • **Avoiding drug – polymer interactions:** The polymer should not react with the drug, causing degradation or loss of function. • **Drug encapsulation efficiency.**
Release profile	• **Controlled release:** ability to provide controlled or sustained release of the drug over time • **Burst release:** initial rapid release followed by slower release • **Stimuli responsive release:** respond to environmental triggers such as pH, or temperature.
Target drug delivery	Polymers can be functionalized with targeting ligands (e.g., antibodies, peptides) that bind specifically to receptors on the target cells or tissues, ensuring the drug reaches its intended site and reducing off-target effects.
Drug release environment.	• **pH Sensitivity**: Some drugs need to be protected from acidic environments (e.g., stomach) and released in more neutral pH regions like the intestines or tissues [24]. • **Temperature Sensitivity**: Polymers used in thermosensitive hydrogels can release drugs in response to changes in body temperature, useful in localized therapies [24]
Molecular weight of the polymer	• **Higher molecular weight** polymers degrade more slowly, providing sustained release over longer periods. • **Lower molecular weight** polymers degrade faster, providing shorter release times, suitable for short-term therapies. • **Molecular weight also** influences the viscosity and mechanical properties, which can be crucial for injectable or implantable systems.
Administration route	Drugs can be administered through a variety of routes, including but not limited to oral administration [6,7], buccal and sublingual routes [8], nasal and ophthalmic delivery [9,10], transdermal and subcutaneous methods [11,12], as well as anal, transvaginal, and intravesical routes [13,14].
Degradation byproducts	The byproducts of polymer degradation should be non-toxic and easily metabolized or eliminated by the body. For example, PLGA degrades into lactic acid and glycolic acid, both of which are metabolized in normal biological pathways [16,25]

Consideration	Fundamental
Manufacturing and scalability	The polymer should be amenable to scalable manufacturing processes and produce drug formulations that maintain their stability during production and storage. Factors such as cost, ease of synthesis, and ability to be processed into the desired form (e.g., nanoparticles, hydrogels, microspheres) are important for practical applications.
Regulatory approval	Polymers that have been approved by regulatory agencies (e.g., Food and Drugs Administration (FDA, US), Medicines and Healthcare products Regulatory Agency (MHRA, UK)) for use in drug delivery systems provide additional assurance of safety and efficacy.

Selecting the most suitable polymer for drug delivery systems is a complex and critical process that demands a careful balance between the polymer's chemical and physical properties and the specific requirements of the therapeutic agent, as well as the desired application.

1.1.4. Highly Sensitive Cargoes in DDS's

In drug delivery systems, highly sensitive cargoes refer to therapeutic agents that are particularly vulnerable to degradation or inactivation due to environmental conditions like temperature, pH, enzymatic activity, or oxidative stress. The encapsulation and protection of these cargoes are critical for ensuring their efficacy. It exists many sensitive cargoes that are widely used in drug delivery that aim to treat complex and dangerous diseases; the most common types of sensitive cargoes used are listed in **Table No. 1.1.4. -1**, which includes some of the main challenges that scientists face when it comes to work with these cargoes.

Table No. 1.1.4. – 1 Types of highly sensitive cargoes commonly used in DDS's

Sensitive cargo	Challenges	Common Delivery System used	Examples
Proteins and peptides	Proteins and peptides are prone to denaturation and enzymatic degradation. They require protection from harsh environments in the body, such as the stomach's acidic pH or proteolytic enzymes in the bloodstream.	• Nanoencapsulation [30, 31] • Microparticles [32] • Liposomes [35,36] • Hydrogels [37]	Insulin, growth factors [33]).
Enzymes	Enzymes are highly susceptible to degradation and loss of activity under physiological conditions, such as high temperatures, pH, or oxidative stress.	• Layer-by-layer (LbL) encapsulation • PLGA-based microparticles • Hydrogels [37]	Catalase [34] superoxide dismutase, lipase.
Nucleic Acids	Nucleic acids are highly susceptible to degradation by nucleases in the body and require protection for successful gene therapy.	• Nanoparticles [39] • lipid-based systems (liposomes and lipid nanoparticles) [40, 41]	DNA, RNA, mRNA [41].

Sensitive cargo	Challenges	Common Delivery System used	Examples
Vaccines	Vaccine components, especially mRNA, are highly sensitive to degradation by enzymes and need to remain stable to elicit a proper immune response.	• Lipid nanoparticles (LNPs) [40, 41] • Biodegradable microsphere	mRNA vaccines [41] (e.g., COVID-19 vaccines).
Hormones	Hormones, especially peptide-based ones, degrade quickly due to enzymatic activity and changes in temperature or pH.	• Polymer-based Microspheres [45] • Hydrogels [42] • Transdermal patches [44]	Insulin [42], human growth hormone (HGH) [43]
Antibodies	Antibodies are large, complex proteins that can be degraded by proteases or denatured in suboptimal environments, limiting their therapeutic action.	• Nanocarriers, • hydrogels • Encapsulation within liposomes or biodegradable polymers [46]	Monoclonal antibodies antibody-drug conjugates (ADCs).
Live Cells and tissues	Live cells are highly sensitive to environmental conditions, such as temperature, oxygen levels, and the immune system's response. They require advanced encapsulation for survival and effective delivery.	• Cell membrane – coated nanovesicles (CMNVs) [47] • Nanoparticles [47]	Stem cells, genetically modified cells (e.g., CAR-T cells), cell-based therapies. [47]
Small molecule drugs (prone to degradation)	Small molecule drugs that are prone to oxidation, hydrolysis, or photodegradation require protection during delivery to maintain their efficacy.	• Liposomes • Nanocarriers [48] • Hydrogel [48] • Nano capsules	Doxorubicin (an anticancer drug) [48]

The development of **advanced drug delivery systems** like biodegradable polymers, nanocarriers, and microcapsules is essential for overcoming these challenges and ensuring that sensitive cargoes can be delivered safely and effectively to their target sites in the body and offering better treatments and therapies for patients with serious diseases.

1.1.4.1. Catalase Enzyme

Catalase enzyme has gained significant interest in the field of drug delivery, particularly due to its sensitivity to environmental factors like pH, temperature, and oxidative stress. Encapsulation of catalase in drug delivery systems (such as microcapsules) is being explored to protect the enzyme from degradation and enhance its therapeutic potential. These systems aim to deliver catalase to specific sites in the body where oxidative stress is high, such as in inflammation, cancer, or neurodegenerative diseases, allowing the enzyme to neutralize harmful reactive oxygen species (ROS) and protect tissues from damage [34,49].

Catalase is also studied for its potential use in enzyme therapies, where its ability to rapidly detoxify hydrogen peroxide may offer therapeutic benefits in conditions where oxidative stress plays a pathogenic role.

Is an important enzyme found in nearly all living organisms exposed to oxygen, including plants, animals, and most bacteria. Its primary function is to catalyse the decomposition of hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) [50], a process that is vital for protecting cells from oxidative damage caused by reactive oxygen species (ROS). Hydrogen peroxide is a byproduct of many metabolic reactions, and if allowed to accumulate, it can lead to cellular damage or death. [34,49].

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase is a large, tetrameric enzyme, meaning it consists of four subunits. Each subunit contains a heme group, an iron-containing structure responsible for binding hydrogen peroxide. The enzyme works by first binding a molecule of H_2O_2 to the iron in the heme group, splitting it into water and oxygen. This reaction occurs extremely rapidly, allowing catalase to process millions of hydrogen peroxide molecules per second, making it one of the most efficient enzymes known. Some of the most significant biological functions that the catalase does are the following [34,49]:

a) **Protection from Oxidative Stress:** By breaking down hydrogen peroxide, catalase plays a critical role in the detoxification of ROS, which can cause damage to DNA, proteins, and lipids. This function is particularly important in organs such as the liver, where metabolic activity produces significant amounts of hydrogen peroxide.

b) **Immune Response:** Catalase also has an important role in immune defence. Immune cells like neutrophils generate ROS to kill pathogens, and catalase helps regulate this process by preventing excessive hydrogen peroxide buildup, thereby protecting healthy cells.

c) **Antioxidant Defence:** As a key part of the cellular antioxidant defence system, catalase works alongside other enzymes like superoxide dismutase (SOD) and glutathione peroxidase to neutralize ROS and maintain cellular health.

1.2. Photolithography and Soft Lithography

1.2.1. Photolithography Methodology

Photolithography is a widely utilized and established technique in the semiconductor industry, but it can also be employed for the fabrication of metal nanoparticles. The process involves projecting a pattern, defined by a lithographic mask, onto a light-sensitive resist layer that has been applied to a substrate.

According to Hubenthal, F. et. al (2011) [51], here are two approaches to this method: using either a positive resist or a negative resist. The standard process using a positive resist is schematically depicted in **Figure No. 1.2.1. – 1.**

At first, a metal film is deposited on the substrate **(Figure 1.2.1. – 1a).** Afterwards, a photoresist is spin coated on the sample and a soft bake process (up to 30 min at temperatures between 60 and 100 °C) of the resist is accomplished **(Figure 1.2.1.-1b).** The sample is then illuminated through a mask with suitable light **(Figure 1.2.1.-1c).** Thus, the structure of the mask is imaged on the resist and causes photochemical changes therein.

Afterwards, the resist is developed and hard baked for 20–30 min at temperatures between 120 and 180 °C **(Figure 1.2.1.-1d).** Finally, the open metal areas are etched away **(Figure 1.2.1.-1e),** and the photoresist is removed, leaving the metal nanoparticles on the substrate **(Figure 1.2.1.-1f)** [51].

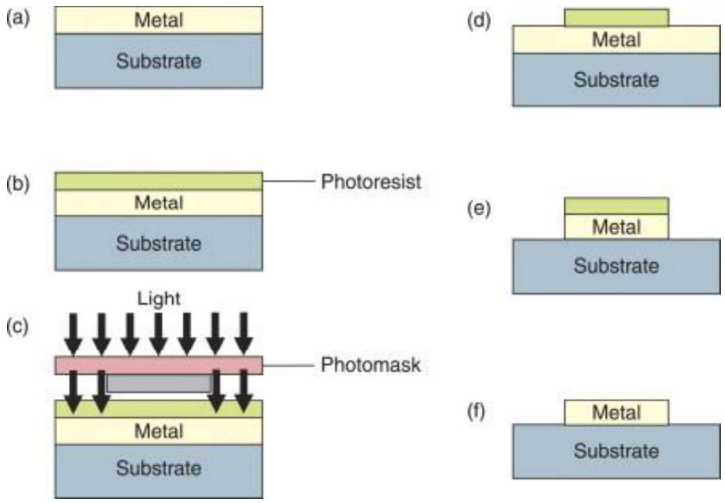

Figure 1.2.1 - 1 Schematic of photolithography with a positive resist [51]

The process for the negative resist is very similar to what is presented in **Figure 1.2.1 - 1**. For a nanoparticle generation with a negative resist, the photoresist is directly deposited on the substrate and soft baked. Afterwards, the resist is exposed via the mask with light, developed, and hard baked. Void areas are created **(Figure 1.2.1 - 2a),** which are filled due to the deposition of a metal film onto the sample **(Figure 1.2.1. – 2b).** Finally, the residual resist with the metal on top is chemically removed, leaving behind the metal nanostructures **(Figure 1.2.1. – 2c).**

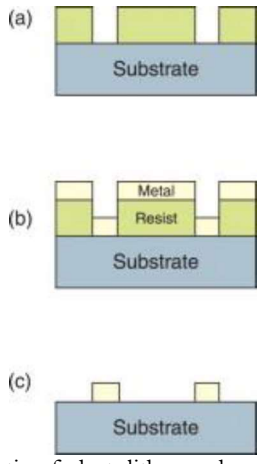

Figure 1.2.1 - 2 Schematic of photolithography with a negative resist [51]

Photolithography, traditionally used in the semiconductor industry for microfabrication, has found innovative applications in the field of drug delivery systems. Its ability to create highly precise and controlled micro- and nanostructures makes it an ideal technique for designing advanced drug delivery platforms. Photolithography enables the fabrication of structures that can encapsulate, protect, and release drugs in a controlled and targeted manner, offering significant advantages in terms of precision and efficiency. Even though it offers a lot of advantages, this technique it still has some challenges, some of them are listed in **Table 1.2.1. – 1** in the drug delivery research field.

Table No. 1.2.1. – 1 Advantages and limitations of Photolithography in Drug Delivery.

Advantages		**Limitations**	
Precision	Photolithography allows for the creation of drug delivery systems with highly controlled geometry, which is essential for achieving targeted and sustained drug release [51,52,53]	**Cost**	The equipment and materials required for photolithography are expensive, which may limit its widespread use in drug delivery, especially for low-cost applications [51,53]
Scalability	The process is compatible with mass production, making it suitable for manufacturing large quantities of drug delivery devices, such as microneedles or nanoparticles [51,52]	**Complexity**	The process can be technically challenging and requires specialized knowledge, limiting its accessibility compared to other drug delivery fabrication techniques [51,52].
Versatility	Photolithography can be used to pattern a wide range of materials, including polymers, metals, and hydrogels, allowing for the design of multifunctional drug delivery systems [51,52]		

Despite it costs and complexity, photolithography offers a novel approach for the fabrication of microcapsules and nanoparticles due to their ability to make precise shapes and sizes, which can be tailored to enhance drug encapsulation and release profile. Some other application of Photolithography in drug delivery is the creation of microneedles arrays, lab – on – a – chip devices and hydrogels and polymer networks, which states that this technique offers a significant potential in the development of sophisticated delivery systems, providing precise control over the structure and function of delivery platforms.

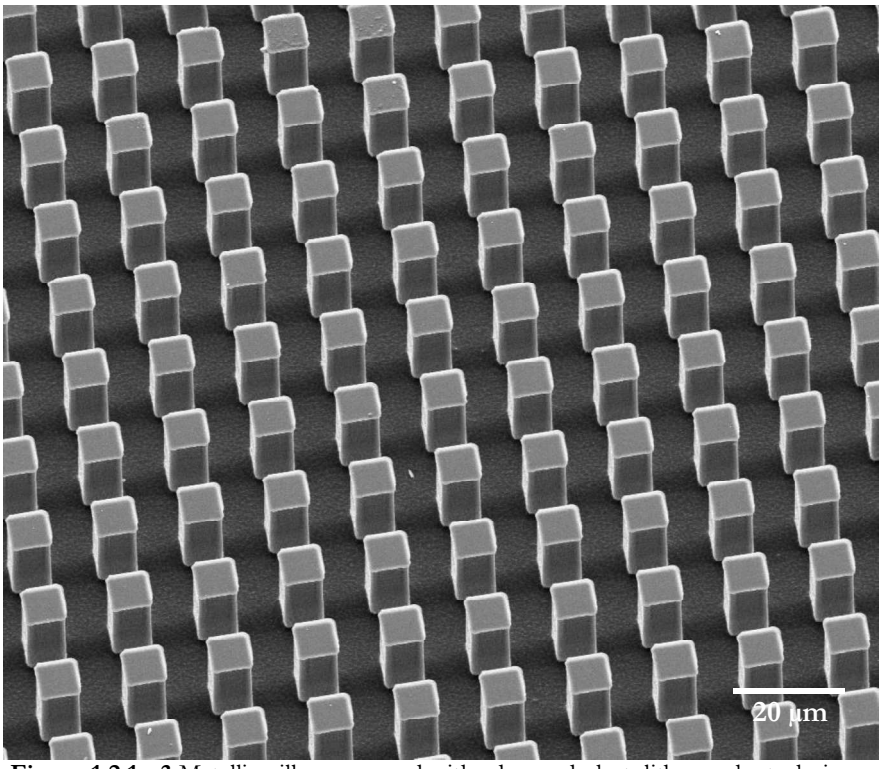

Figure 1.2.1 - 3 Metallic pillars prepared with advanced photolithography technique.

1.2.2. Soft Lithography Methodology

Soft lithography is a set of microfabrication techniques used to pattern surfaces or create micro- and nanoscale structures, typically using elastomeric (flexible) materials. Unlike traditional photolithography, which relies on rigid masks and high-energy light sources, soft lithography uses a flexible mould, often made of polydimethylsiloxane (PDMS), to transfer patterns onto a substrate. These methods are widely used in areas like microfluidics, tissue engineering, and drug delivery systems due to their flexibility, cost-effectiveness, and ability to produce intricate structures with high resolution [52].

There exist many types of techniques in soft lithography and are widely using in different applications, some of the most common techniques are shown in **Table No. 1.2.2. – 1.**

Table No. 1.2.2. -1 Techniques in soft lithography.

Soft Lithography technique	Fundament
Microcontact printing (μCP)	Microcontact printing (μCP) is a simple and versatile tool for transferring and patterning extremely small quantity of molecules on a substrate. However, the quantity of molecules transferred by typical μCP process is estimated to be less than about 10 ng mm−2. [54]
Micromolding in capillaries (MIMIC)	MIMIC involves filling microchannels within a PDMS mould with a liquid polymer that solidifies into a desired structure. This method is particularly useful for fabricating microfluidic devices and microreactors [55].
Nanoimprint Lithography (NIL)	NIL involves pressing a patterned mould into a thin polymer film and then curing or hardening the film to create nanostructures. While considered part of soft lithography, NIL typically uses harder moulds than other techniques [56].

Like photolithography, this methodology has both advantages and limitations, among their advantages stands out their flexibility, due to the use of elastomeric moulds (such as PDMS) that allows soft lithography to be applied to non-flat surfaces, expanding its potential applications. Also, their resolution is one of this distinction as well as their low cost if we compare it with the traditional photolithography, as requires simpler equipment and materials.

On the other hand, some of their limitations are their durability, the flexible nature of elastomeric moulds, such as PDMS, can limit their durability especially for high – resolution or repetitive applications. Also, not all materials can be patterned using soft lithography, as some may not adhere well to the mould or substrate, limiting the choice of materials in certain applications.

Nevertheless, soft lithography is a versatile and accessible technique that enables the creation of complex micro- and nanostructures across a wide range of disciplines such as microfluidics [57], tissue engineering [58], drug delivery systems [54,55,56], and biosensors.

1.2.2.1. Microcapsules with Soft Lithography Methodology.

Microcapsules created using soft lithography are a novel approach in drug delivery and material science, providing precise control over capsule size, shape, and surface characteristics. Soft lithography, due to its flexibility and cost-effectiveness, is ideal for fabricating microcapsules with highly defined structures, which can encapsulate sensitive substances like drugs, enzymes, or other therapeutic agents.

There have been several approaches in using the soft lithography methodology to encapsulate different cargoes. On the research of Kudryavtseva et. al. (2022) it is mentioned the use of this methodology in the fabrication of printed asymmetric microcapsules enhancing loading capacity and evaluating different non – uniform size and shape of carriers made from a biodegradable polymer like PLGA. To demonstrate the applicability of asymmetric microcapsules made from soft lithography, Kudryavtseva et. al. (2022) encapsulated dexamethasone which has low solubility in water (100 mg/ 100 mL), and it demonstrated efficient encapsulation and controlled released of the drug [24].

In another study performed by Kudryavtseva et. al. (2021) describes the preparation and characterization of this type of microcapsules shapes made form soft lithography. Both types of capsules showed monodisperse size and shape distribution and were found to provide sufficient stability to encapsulate small water-soluble molecules and to retain them for several days and ability for intracellular delivery [59].

Despite all their advantages and applications, there is still some challenges and limitations of soft lithography in the microcapsule's applications such as:

- **Durability of Molds:** The flexible PDMS moulds used in soft lithography may wear out after multiple uses, potentially affecting the consistency of microcapsule production [52,59].
- **Encapsulation of Hydrophilic Cargo:** It can be more challenging to encapsulate highly hydrophilic drugs or proteins using soft lithography, as they may interact unfavourably with certain polymers [52].

Nevertheless, soft lithography offers a flexible, precise, and cost-effective method for fabricating microcapsules in drug delivery applications. Its ability to tailor capsule size, structure, and release profiles makes it a valuable tool for developing advanced drug delivery systems, particularly for sensitive and biodegradable cargoes [24,59].

1.3. Layer – by – Layer (LbL) Methodology

Layer-by-Layer (LbL) methodology is a versatile technique used to fabricate thin films or microcapsules by sequentially depositing alternating layers of oppositely charged materials, typically polyelectrolytes **(Figure No. 1.3. – 1)** [60, 61].

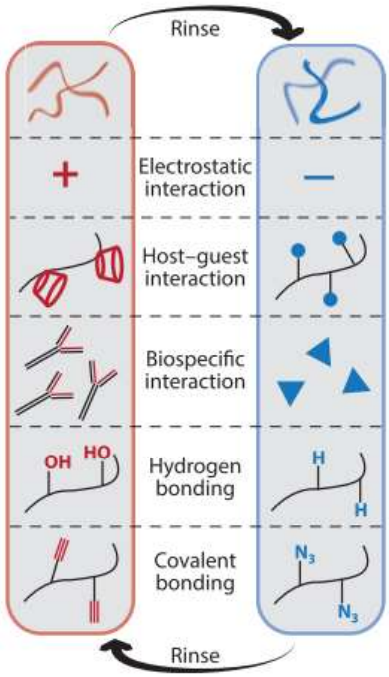

Figure 1.3. – 1 Sequence of alternate deposit of layers of oppositely charged material in layer - by layer methodology. [61]

As shown in **Figure 1.3. – 1,** there are some common interactions that occurred during a Layer-by-layer methodology.

The first mentioned called **"Electrostatic interaction"**, is where the alternating layers of positively and negatively charged materials (polyelectrolytes) are deposited on a substrate. Electrostatic interactions drive the assembly process [61 - 64].

The second mentioned called **"Hydrogen- Bonding"**, in this stage the layers are assembled based on hydrogen bonding between materials [61 - 64].

And finally, the third mentioned called **"Covalent bonding"**, layers are connected through covalent bonds, leading to stronger and more stable structures. After the previous stages described, rinse should be made [61 - 64].

This process results in highly structured and customizable multilayer assemblies that can encapsulate a variety of cargoes such as drugs, proteins, or enzymes, making it widely used in drug delivery, tissue engineering, and biosensing.

There is many advantages around layer – by – layer, but there is also some challenges the stand out, this challenges are still under study and there have been some progress but still exist limitations, some of them are mentioned in the **Table No. 1.3. – 1.**

Table No. 1.3. – 1 Advantages and limitations of layer – by – layer methodology.

Advantages		Limitations	
Precision and control.	LbL allows precise control over the thickness and composition of the films or capsules, enabling fine-tuning of drug release profiles or other functionalities.	**Time consuming.**	Building LbL films is a repetitive, layer-by-layer process, which can be time-consuming for large-scale applications.
Versatility	A wide range of materials, including polymers, nanoparticles, and biomolecules, can be used, allowing the method to be adapted to various applications.	**Material compatibility.**	The technique relies on electrostatic interactions, so it may not be suitable for materials that lack sufficient charge or those that are incompatible with the solvents used in the process.
Stimuli – responsiveness	The multilayer structures can be engineered to respond to environmental stimuli (such as pH, temperature, or light), enabling controlled and targeted release of encapsulated agents.		
Biocompatibility	LbL can incorporate biocompatible and biodegradable materials, making it suitable for medical and drug delivery applications.		

Another limitation that is worth mentioning is the permeability, these features can be a key challenge in certain applications in drug delivery systems in which control release in critical. While LBL provides precise control overt thickness and composition of layers, several limitations in permeability can arise.

a) **Limited control over fine – tuning permeability.**
   - **Layer thickness variability:** Achieving precise control over the thickness of individual layers is critical for adjusting permeability. Small changes in deposition conditions (e.g., concentration, pH, ionic strength) can lead to non-uniform layer formation, making fine-tuning difficult.
   - **Difficulties in predicting permeability:** The permeability of LbL films depends on a combination of factors such as layer thickness, the charge of materials, and environmental stimuli. Predicting how these variables interact to control permeability can be complex, leading to challenges in tailoring release profiles accurately.
   - **Layer number (excessive layering):** Adding too many layers to increase encapsulation stability or modify permeability can lead to thick, rigid films that are less permeable than needed. Balancing the number of layers to achieve optimal permeability without compromising the release profile can be challenging.

b) Molecules encapsulated in LbL

- **Size selectivity:** The multilayer structure of LbL films often acts as a molecular sieve, allowing small molecules to pass through but hindering the release of larger molecules, proteins, or nanoparticles. This can be a limitation when encapsulating and releasing large biomolecules in drug delivery systems.
- **Long – term retention of cargo:** In some cases, the LbL system may provide overly slow or delayed release of encapsulated cargo, particularly for hydrophilic or charged molecules. This may limit the system's ability to provide therapeutic concentrations of a drug in a timely manner.

1.3.1. Methodologies and applications of LbL

The **Layer-by-Layer (LbL)** assembly process can be carried out using several different fabrication techniques, each with its own advantages and specific applications. The three most common methods are dip coating, spray coating, and spin coating.

a) Dip coating.
This method involves immersing a substrate in a solution containing one type of polyelectrolyte or material, then rinsing, and dipping it into a second solution containing the oppositely charged material. This process is repeated to build up alternating layers.

b) Spray coating.
In this method, the substrate is sprayed with alternating solutions of polyelectrolytes, rinsed between layers. It's faster than dip coating and can be applied to large areas.

c) Spin coating
In this process, the substrate is placed on a rotating platform, and polyelectrolyte solutions are dropped onto the spinning surface. Centrifugal force spreads the solution evenly across the surface.

All the above mentioned are novel and widely used methodologies in many drug delivery applications and offer plenty of advantages, nevertheless, they show some limitations and a analyse according to the needs of the application needs to be done [61,62,63]. Some of these advantages and limitations are shown in **Table No. 1.3.1. – 1.**

Table No. 1.3.1. – 1 Advantages and limitations in LbL coating methodologies

Method	Advantages	Limitations
Dip coating	1. Allows the uniform on substrates with complex geometries. 2. Provides good control over layer thickness.	• Time – consuming due to the multiple dipping and rising steps.
Spray coating	1. Faster than dip coating. 2. Suitable for large, flat substrates or irregularly shaped surfaces. 3. Can control thickness and uniformity of the layers effectively.	• Less precise control over the amount of material deposited compared to dip coating.
Method	**Advantages**	**Limitations**

Spin coating	1. Very fast and suitable for thin and uniform coatings. 2. Provides excellent control over films thickness, especially for thin layers.	• Not suitable for coatings in non-flat or irregular surfaces. • Limited to small substrates.

Deciding which of the above-described coating methodology depends on the application of interest. These applications can be for the formation of multilayered filmed to protective sensitive cargoes, for microneedles, implants, cells, liposomes and nanoparticles [61] (**Figure No, 1.3.1. – 1**)

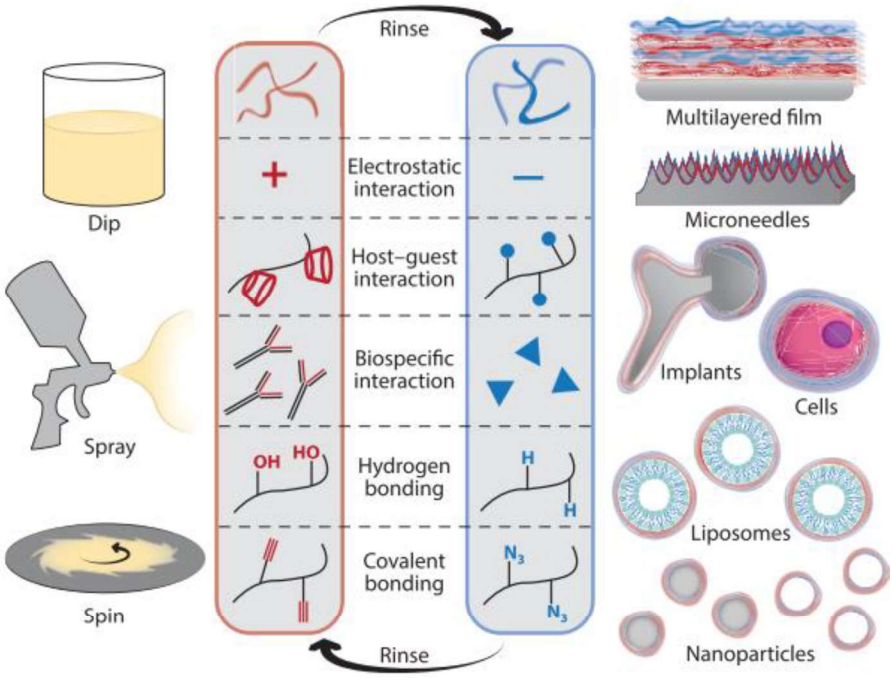

Figure 1.3.1. -1 (Left) Schematic of common LbL approaches, (middle) common interactions exploited in LbL self-assembly, and (right) examples of substrates coated for biomedical applications. [61]

1.3.2. Polyelectrolytes used in LbL Methodology.

LbL assembly is usually driven by electrostatic interactions between oppositely charged compounds. Hydrophobic, van der Waals, hydrogen bonding, covalent bonding, host–guest, and bio specific interactions can all contribute to film growth and may even serve as the primary driver of film assembly [64].

In Layer-by-Layer (LbL) assembly, polyelectrolytes are essential because they interact through electrostatic forces, enabling the sequential layering of materials. Here are some of the most used **polyelectrolytes** in LbL assembly (**Table No. 1.3.2. – 1**).

Table No. 1.3.2. – 1 Common polyelectrolyte used in LbL.

Polyelectrolyte	Type	Properties	Applications
Poly (allylamine hydrochloride) (PAH)	Cationic	PAH is a widely used cationic polyelectrolyte due to its strong positive charge and good solubility in water.	Drug delivery systems [24, 26], and biosensors [67,73]
Poly (styrene sulfonate) (PSS)	Anionic	PSS is a highly charged, water-soluble polyanion that pairs well with cationic polymers like PAH [24, 26].	Drug delivery systems [24, 26], and biosensors [67]
Poly (diallyl dimethyl ammonium chloride) (PDADMAC)	Cationic	PDADMAC is a cationic polyelectrolyte with high water solubility and a strong positive charge.	Water treatment [67] and drug delivery [69].
Poly (acrylic acid) (PAA)	Anionic	PAA is a weak polyanion that forms layers under specific pH conditions, making it useful for creating pH-responsive LbL films.	Drug delivery systems [70]
Poly(ethyleneimine) (PEI)	Cationic	PEI is highly positively charged and often used as the first layer in LbL films due to its strong adhesion to surfaces.	Drug Delivery [24,26], and surface modification [71].
Chitosan	Cationic	A natural, biodegradable, and biocompatible polymer derived from chitin; chitosan is widely used in biomedical applications.	Drug delivery systems, wound healing, and tissue engineering [72].
Hyaluronic Acid (HA)	Anionic	HA is a naturally occurring polyanion with excellent biocompatibility and is often used in biomedical applications.	Frequently used in drug delivery, tissue engineering, and hydrogels for medical applications [72]
Poly(l-lysine) (PLL)	Cationic	PLL is a positively charged polypeptide that is biocompatible and biodegradable, making it suitable for biomedical applications.	Gene delivery, LbL films for medical devices, and drug encapsulation [70].
Dextran Sulphate	Anionic	A highly charged polyanion used for building multilayers with biocompatibility and biodegradability.	Mainly used in drug delivery systems and biomedical coating [72,70].
Poly (methacrylic acid) (PMA)	Anionic	PMA is a weak polyanion, sensitive to pH changes, making it useful in stimuli-responsive LbL films.	Used in pH-sensitive drug delivery systems [73]

There are some important considerations when choosing a pair of polyelectrolytes to work with. In Lbl assembly, a cationic and anionic polyelectrolyte need to be paired to create alternating layers. Some polyelectrolytes (e.g., PAA, PMA) have pH – dependent charges, which can leverage to create pH sensitive systems. And finally, biodegradability and biocompatibility, like chitosan and hyaluronic acid, this are often favoured in biomedical applications

1.4. Ultrasound in Drug Delivery Systems (DDS's)

Ultrasound is a unique and exciting theragnostic modality that can be used to track drug carriers, tigger drug release and improve drug deposition with high spatial precision [65]. is an advanced approach in drug delivery systems that utilizes ultrasound waves to control the release of therapeutic agents from carriers, such as microcapsules, liposomes, or nanoparticles. This method provides a non-invasive, targeted, and controlled drug release mechanism, offering significant advantages in treating various medical conditions, especially in localized therapies.

There is a series of factors that arise when using ultrasound for Drug Delivery Systems (DDS's), these are called as "Mechanism of Ultrasound" and make possible the stimuli that helps release the cargoes on specific sites.

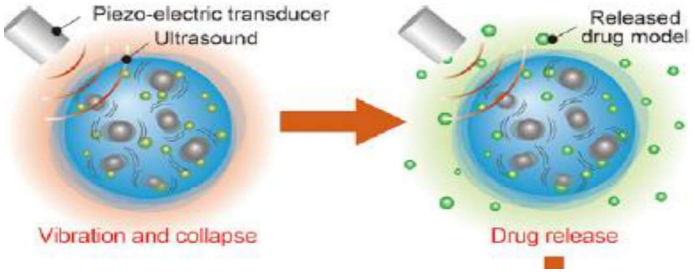

Figure 1.4. – 1 Example of ultrasound applied to a DDS's and the mechanism of cargo release [66].

The key mechanism that occurs when ultrasound is applied to drug delivery systems are the following:

Cavitation

Cavitation refers to the formation, growth, and collapse of bubbles in a liquid medium due to the pressure changes induced by ultrasound waves. It plays a critical role in drug delivery, especially in targeted therapies where localized effects are needed. There are two types of cavitation that are relevant in ultrasound applications:

- **Stable Cavitation**: Low-intensity ultrasound causes the formation and oscillation of small bubbles, which can enhance permeability of cell membranes and improve drug uptake.
- **Inertial Cavitation**: High-intensity ultrasound generates larger bubbles that collapse, creating micro-jets and shockwaves. This can temporarily disrupt tissues, allowing drugs to penetrate deeper or cross biological barriers like the blood-brain barrier.

Acoustic Radiation Force: Ultrasound waves exert mechanical forces that can push drug-loaded particles, such as liposomes or microcapsules, toward target tissues. This can improve the accumulation of drugs at specific sites, such as tumours.

Thermal Effects: High-Intensity Focused Ultrasound (HIFU) can locally heat tissues, triggering the release of temperature-sensitive drugs or altering the permeability of tissues to facilitate drug diffusion. This mechanism is particularly useful for **triggered release** in thermo-sensitive carriers like liposomes.

Enhanced Permeability and Retention (EPR): Ultrasound can enhance the EPR effect by increasing the permeability of blood vessels at tumours sites, allowing nanoparticle drug carriers or macromolecules to accumulate more efficiently in tumours tissues.

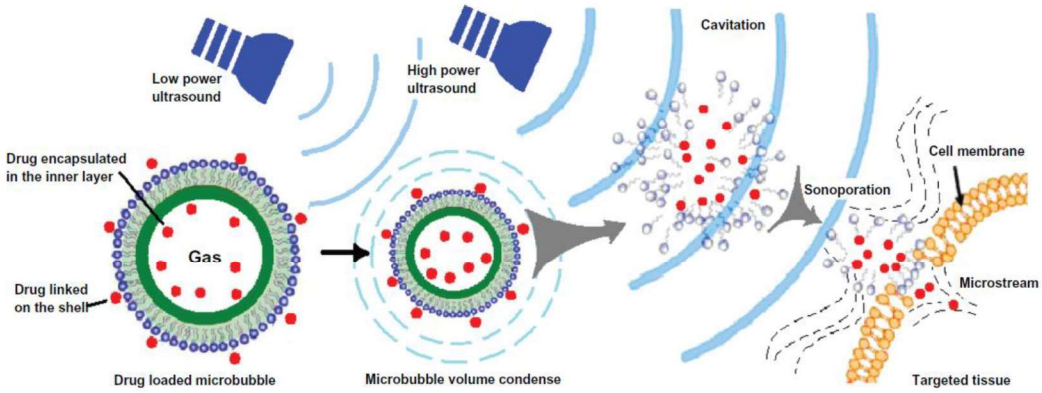

Figure 1.4. – 2 Sonication of HIFU and LIFU to a encapsulated capsule for targeted release.

1.4.1. Ultrasound Frequency Intensity

Ultrasound waves are presented typically in the form of High Intensity Focus Ultrasound and Low Intensity Focus Ultrasound.

1.4.1.1. Low – Intensity Focus Ultrasound

Low-Intensity Focused Ultrasound (LIFU) is a non-invasive therapeutic technique that uses ultrasound waves at low energy levels to stimulate specific tissues or organs without causing significant thermal or mechanical damage. LIFU is primarily known for its applications in **neuromodulation**, **drug delivery**, and **non-invasive surgery**, offering a gentler alternative to High-Intensity Focused Ultrasound (HIFU).
Low-Intensity Focused Ultrasound (LIFU) typically operates in the frequency range of **200 kHz to 1.5 MHz**, depending on the specific application and the target tissue. The choice of frequency affects the penetration depth, and the type of mechanical effects produced by the ultrasound waves.

Lower frequencies (around 200 kHz to 500 kHz) offer **deeper penetration** into tissues, making them suitable for targeting deeper organs or regions of the brain.

1.4.1.2. High – Intensity Focus Ultrasound

High-Intensity Focused Ultrasound (HIFU) is a non-invasive therapeutic technique that uses focused ultrasound waves at high energy levels to precisely target and destroy tissue through heat and mechanical effects. It is primarily used in medical fields like oncology for tumours ablation, but it also has applications in cosmetic treatments and non-invasive surgeries.

HIFU typically operates in the **0.8 MHz to 3.3 MHz frequency range**, with higher frequencies used for superficial targets and lower frequencies for deeper tissues. The frequency and intensity are adjusted based on the application:

- **0.8 MHz to 1.6 MHz**: Commonly used for targeting deep tissues, such as liver or kidney tumours.
- **1.6 MHz to 3.3 MHz**: Suitable for more superficial applications like skin tightening or tumours ablation closer to the surface.

1.4.2. Ultrasound-Triggered Drug Delivery Systems

Ultrasound-triggered drug delivery systems offer a promising approach to achieving precise, targeted, and controlled release of therapeutic agents. By utilizing sound waves to activate drug carriers at specific locations within the body, these systems minimize off-target effects and enhance the efficiency of drug therapies. Various types of carriers, including microbubbles, microcapsules, nanoparticles, and liposomes, have been developed to respond to ultrasound stimuli. These carriers can be engineered to release drugs when exposed to ultrasound, making them particularly useful for treating diseases such as cancer, where localized drug delivery is crucial. This innovative method enables non-invasive, on-demand release, improving the overall therapeutic outcomes.

- Microbubbles: Microbubbles are gas-filled, ultrasound-responsive particles often used in conjunction with ultrasound imaging and drug delivery. Upon exposure to ultrasound, microbubbles oscillate and can collapse (cavitation), releasing the drug at the target site. Microbubbles are widely used in cancer therapies for tumours targeting and vascular drug delivery.

- Microcapsules and Nanoparticles: Ultrasound can trigger the release of drugs encapsulated in biodegradable microcapsules or nanoparticles. These carriers can be designed to release their cargo only when exposed to specific ultrasound frequencies, which allows for spatially controlled drug release. For example, High-Intensity Focused Ultrasound (HIFU) is used to trigger the release of drugs from temperature-sensitive liposomes or PLGA-based microcapsules.

- Liposomes: Ultrasound-sensitive liposomes can be engineered to release their drug payloads in response to focused ultrasound. The thermal or mechanical effects of ultrasound disrupt the liposome membranes, allowing for the release of the encapsulated drug at the site of interest.

Chapter II. | Materials and Methods

2.1. Materials

Silicon wafer used for the microcapsule's fabrication, was made using a lift-off lithographic chromium mask, via cryogenic reactive ion etching.
Polymer stamps were created using a silicon mould of Poly (dimethyl siloxane) (PDMS) kit (Sylgard 184, Dow-Corning, USA) according to the soft lithography method [24]. The mould has a grid of rectangular pillars measuring 20 × 10 × 10 meters spaced 20 microns apart.
Poly (D, L-lactide-co-glycolide) (PLGA, lactide: glycolide (75:25), mol wt. 66,000-107,000), gelatine bovine, sodium chloride, poly (fluorescein isothiocyanate allylamine hydrochloride), poly (sodium 4-styrene sulfonate) (PSS Mw~70,000), poly (ethylenimine) (PEI, Mw~750,000), and poly (allylamine hydrochloride) (PAH, Mw 50,000) were all purchased from Sigma Aldrich.
Amplex ® Red Reagent (5 mg) and Dimethyl Sulfoxide (DMSO), Anhydrous. (500 µL) were purchased from Invitrogen ™. Horseradish Peroxide, Trizma ® Hydrochloride Solution (100 mL Hydrogen Peroxide Solution (500 mL), and Catalase from bovine liver (powder, 2,000-5,000 units/mg protein) was purchased from Sigma Aldrich.

2.2. Methods

2.2.1. PDMS Polymer Stamp Manufacturing.

The general procedure for the PDMS polymer stamps manufacturing is shown in **Figure 2.2.1. – 1.** To produce the PDMS Stamp the elastomer and the crosslinker from Sylgard 184 Silicone were mixed in a 1:10 ratio, cast into master wafer (pillar template), leave in vacuum incubator for one hour and then degassed and cure at 70°C for around 1-2 hours.

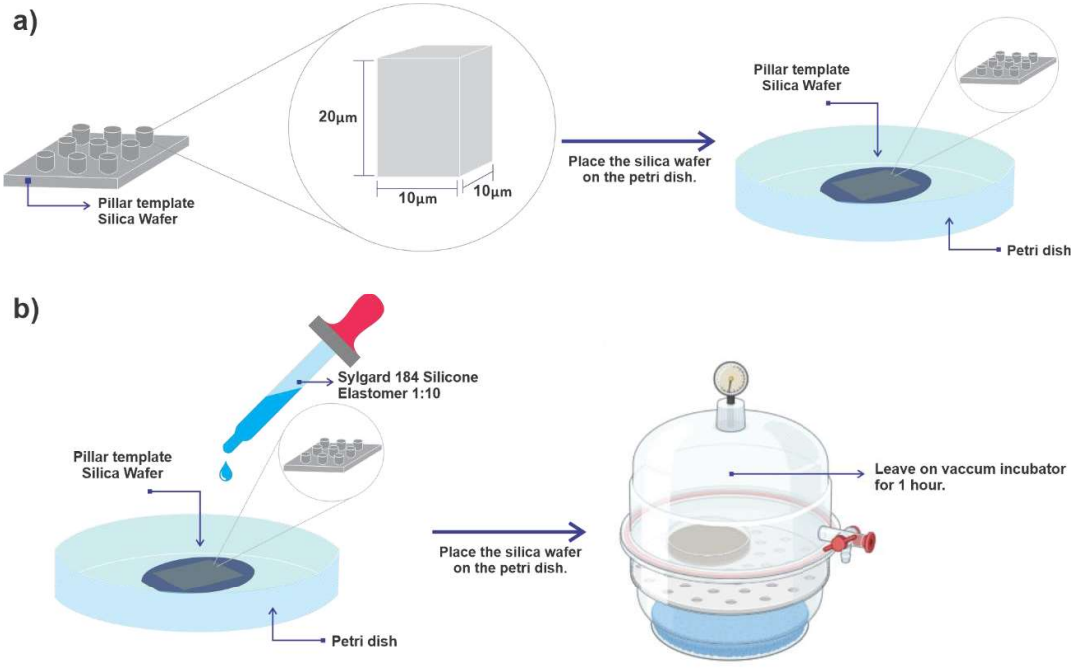

Figure 2.2.1. - 1 PDMS Polymer stamp manufacturing process. a) Polymer casting b) Degassed and cured of the polymer.

2.2.2. Soft Lithography Microcapsules Manufacturing.

For the fabrication of printed microcapsules, the PDMS stamps prepared in **Section 2.2.1. PDMS Polymer Stamp Manufacturing** were used. The PDMS stamp with the printed pattern was dipped into a polymer solution of 5% PLGA 75:25 in acetone like it is shown on **Figure 2.2.2. – 1a,** by doing this a thin polymer film is formed and it has taken the shape of the Patterned PDMS. After this, cargo which was previously milled, was loaded into the microwells of the PDMS. The cargo used was catalase from bovine skin 2,000 – 5,000 U/mg like it is shown on **Figure 2.2.2. – 1b.** Then, a flat PDMS (no pattern) was dipped in a polymer solution of 15% of PLGA 75:25 in acetone, then placed above the Patterned and loaded PDMS **(Figure 2.2.2. – 1c).** Finally, after a few seconds of letting the solution dry, the flat PDMS was removed and the excess of polymer solution left over the Patterned PDMS was removed with a 1.5% solution of PLGA 75:25 in acetone. Then, the now sealed patterned PDMS was placed up-side down in a petri dish with a 10% gelatine from bovine skin solution and was stored at 5°C for 10 – 15 minutes. Then the gelatine was dissolved with deionized water at 50°C **(Figure 2.2.2. – 1d).**

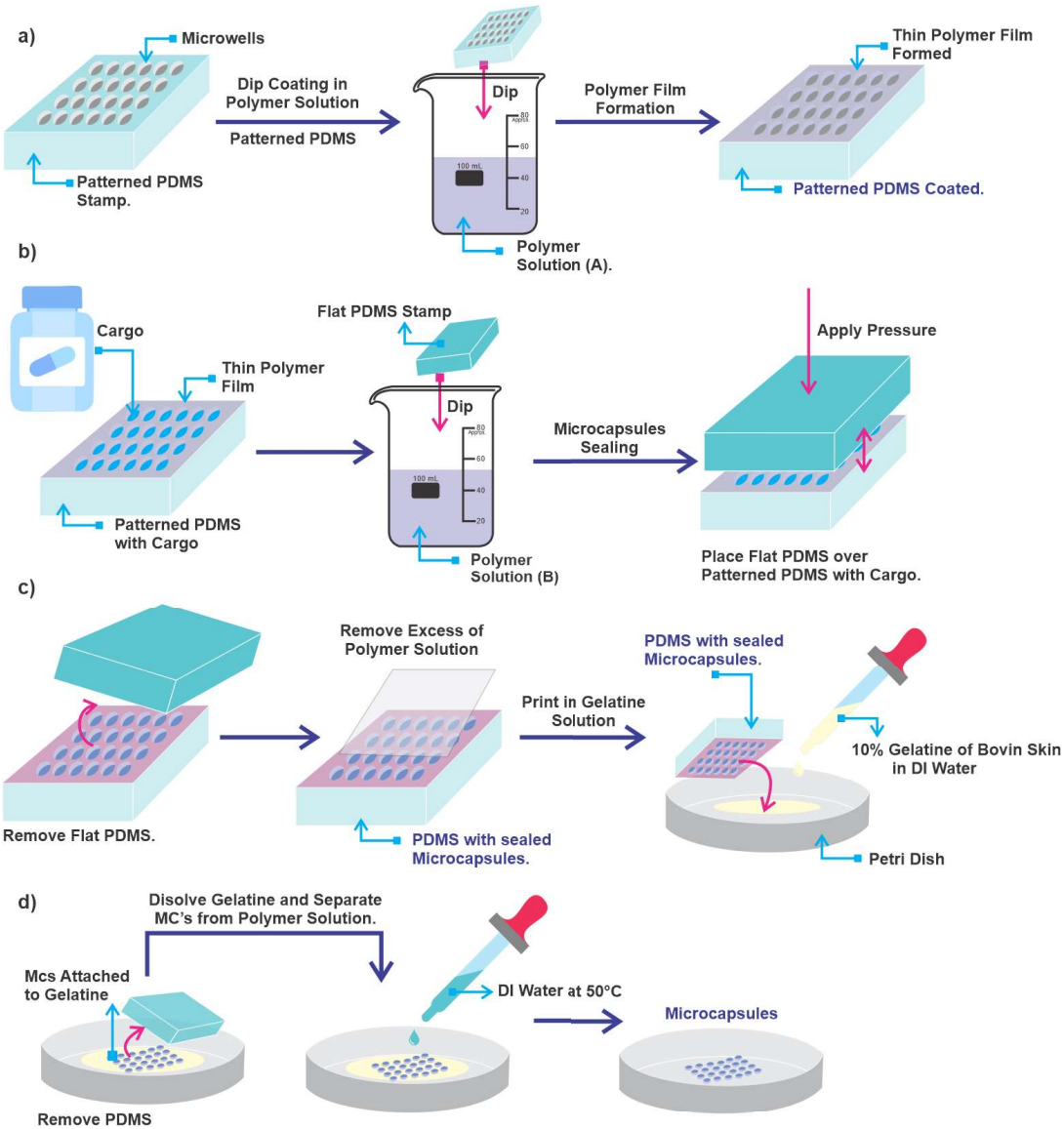

Figure 2.2.2. – 1 Manufacturing process of PLGA - PLGA Microcapsules based on soft lithography methodology.

2.2.3. Layer – by – Layer Microcapsules Manufacturing

For the fabrication of printed microcapsules, the PDMS stamps prepared in **Section 2.2.1. PDMS Polymer Stamp Manufacturing** were used. The PDMS Stamps were dipped in a pre – treatment solution of poly (ethylenimine) (PEI) (2mg/mL) in NaCl 2.0 M for 10-15 minutes. Then, they were placed in a Layer – by – layer coating machine that consists of a dipping robot and a turning table. The LbL coating is set up with eight stations, 6 of them are Deionized water used for rinse stages and the other two were set up with poly (allylamine hydrochloride) (PAH) 2mg /mL in NaCl 0.5 M and poly (sodium 4-styrene sulfonate) (PSS) 2mg /mL in NaCl 0.5 M with the distribution that is shown on **Figure 2.2.3. – 1a.**

The coating process consists of 4 stages, **Table No. 2.2.3. – 1,** mentions the stages and dipping times used.

Table No. 2.2.3. – 1 Coating stages and parameters for LBL Coating

Position	Stage	Solution	Dipping Time (s)	Drying Time (s)
1	Anionic Coating	PSS solution	600	30
3	Rinse for anionic coating	Deionized Water	60	10
4		Deionized Water	60	10
5		Deionized Water	60	10
7	Cationic Coating	PAH Solution	600	30
9	Rinse for cationic coating	Deionized Water	60	10
10		Deionized Water	60	10
11		Deionized Water	60	10

The average time taken for each cycle of bilayers (PSS/PAH) coating was 30 minutes and 40 bilayers was made for the microcapsules.

After this, cargo was loaded into the microwells of the PDMS. The cargo used was catalase from bovine skin 2,000 – 5,000 U/mg like it is shown on **Figure 2.2.3. – 2a.** Then, a flat PDMS (no pattern) was dipped in a polymer solution of 10% of PLGA 75:25 / acetone, then placed above the Patterned and loaded PDMS **(Figure 2.2.3. – 2b).** Finally, after a few seconds of letting the solution dry, the flat PDMS was removed and the excess of polymer solution left over the Patterned PDMS was removed with a 1.5% solution of PLGA 75:25 in acetone. Then, the now sealed patterned PDMS was placed up-side down in a petri dish with a 10% gelatine from bovine skin solution and was stored at 5°C for 10 – 15 minutes. Then the gelatine was dissolved with deionized water at 50°C **(Figure 2.2.3. – 2c).**

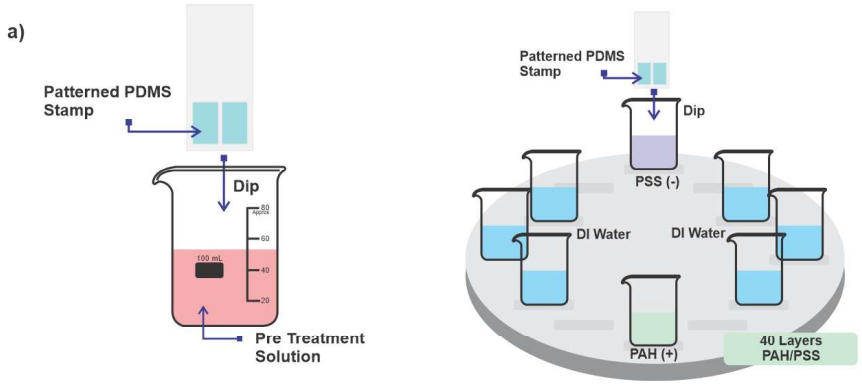

Figure 2.2.3. – 1 Pre - treatment for PDMS before LbL coating (left) and coating solution set up for layer - by -layer (right).

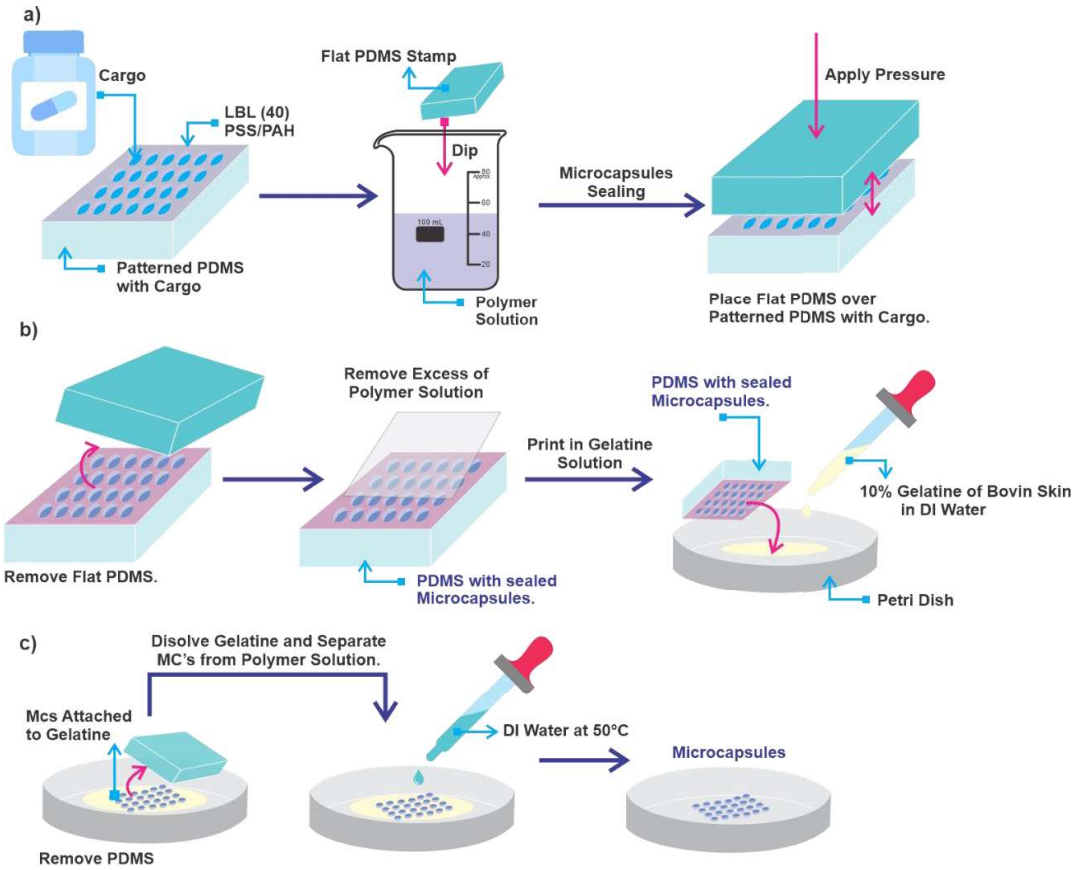

Figure 2.2.3. – 2 (a) Catalase encapsulation in LbL coated MCs' and sealing with PLGA solution, (b) Polymer excess removal and (c) MC's printing.

2.2.4. Microcapsules Count with Haemocytometer

The microcapsules were made following the protocols mentioned in **Section 2.2.2 Soft Lithography Microcapsules Manufacturing**, and **Section 2.2.3. Layer – by – layer Microcapsules Manufacturing.** The microcapsules made where wash and centrifuge to remove impurities and separate supernatant. Once the microcapsules were washed, a Neubauer ® Haemocytometer 0.100 mm Thickness was used to count the number of microcapsules produced. A small drop of microcapsules solution (around 10 µL) was placed in the counting chambers and with a Microscope at 10x magnification the microcapsules were counted **(Figure No. 2.2.4. – 1).**

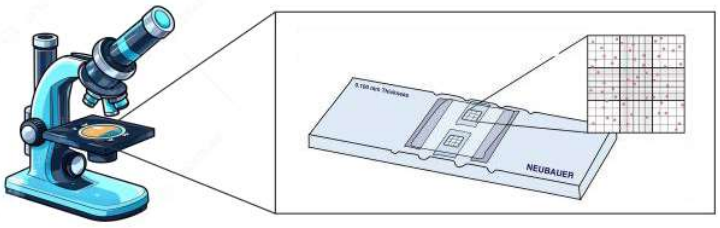

Figure 2.2.4. – 1 Haemocytometer for MC's count.

Is important to mention that microcapsule solution must be homogenous, so the sample has an even distributed number of microcapsules and provide a more accurate count. By looking through the microscope and counting the number of microcapsules in the gridlines, the total concentration can be calculated using the following formula:

$$Microcapsules\left(\frac{MC's}{mL}\right) = \frac{Total\ MC's\ per\ square.}{Total\ of\ squares\ counted.}$$

If the sample was diluted, then the amount of MC's obtained needs to be multiplied by the dilution factor. If not, express the obtained value in x 10^4 Microcapsules /mL

2.2.5. Amplex Red ® Catalase Assay

To determine the Catalase Activity before and after the ultrasound sonication, an Amplex Red ® Catalase Assay was performed. The concentrations of the solutions needed were produced by diluting concentrations from the stock solutions.

Table No. 2.2.5. – 1 Stock presentations of solutions for Amplex Red ® Catalase Assay.

	Materials	Stock Presentation	Specifications
A	Amplex ® Red Reagent (5 mg)		Store in freezer (-5°C to -30°C) and protect form light.
B	Dimethyl Sulfoxide (DMSO), Anhydrous. (500 µL)	≥99.5% (GC)	Store in freezer (-5°C to -30°C) and protect form light.
C	Horseradish Peroxide	≈150 Units/mg Lot Result: 164 U/mg	Store in fridge (2°C to 8°C)
D	Trizma ® Hydrochloride Solution (100 mL)	1M, pH 7.4 Bio – Performance Certified	Store at room temperature.
E	Catalase from Bovine Liver (1g)	2,000-5,000 U/mg Lot Result: 3114 U/mg	Store in freezer (-5°C to -30°C)
F	Hydrogen Peroxide Solution (500 mL)	30% w/w in H_2O	Store in fridge (2°C to 8°C)

Solution A: Amplex ® Red Reagent Solution 10 mM

1) Prepare a 10mM stock solution of Amplex ® Red Reagent. Allow in one vial of Amplex ® Red Reagent (A) and DMSO (B) to warm to room temperature.
2) Dissolve the contents of the vial of Amplex ® Red Reagent (0.26 mg) in 100 µL of DMSO (A in B).
3) Each vial of Amplex ® Red Reagent in sufficient for approximately 200 assays. This stock solution should be stored and frozen at -20°C, protected from light.

Solution B: Reaction Buffer Trizma-HCl

1) Prepare a working solution of Reaction Buffer by adding 10 mL of Tris-HCl 1M pH 7.4 to 90 mL of Deionized Water (dH_2O).
2) The result will be a Reaction Buffer Tris-HCl 0.1M pH 7.5. This 100 mL volume of Reaction Buffer is sufficient for approximately 100 assays.

Solution C: H_2O_2 40 µM

1) The Stock bottle of H_2O_2 is 30%, therefore it needs to be done a serial dilution. Prepare a H_2O_2 3% by adding 10 µL of H_2O_2 30% to 90 µL of Deionized Water (dH2O).
2) Make another dilution by adding 23 µL of H_2O_2 3% to 977 µL of Deionized Water (dH_2O). This will make a 20 mM H_2O_2 Solution. Please note that although the H_2O_2 3% stock solution has been stabilized to slow degradation, the 20 mM H_2O2 working solution will be less stable and should be used promptly.
3) The final dilution will be by adding 10 µL of H_2O_2 20 µM to 4990 µL of Reaction Buffer.

Solution D: Horseradish Peroxide (HRP) 100 U/mL

1) Prepare a 100 U/mL solution of Horseradish Peroxide (HRP) by dissolving the contents of the HRP vial in 200 µL of Reaction Buffer. (The stock presentation is over 100 units needed, therefore and adjustment need to be made).
2) After use, the remaining solution should be divided into aliquots and stored frozen at -20°C.

Solution E: Catalase 10 U/mL

1) Prepare a 1,000 Units/mL solution of Catalase from Bovin Skin (E), by adding 0.0161 g of Catalase (F) in 50 mL of Deionized Water (dH2O). The amount of Catalase dissolved is determined by calculating the proportion of Units in the stock presentation (Lot result) needed to have 1,000 Units/mL.
2) Dilute an appropriate amount of the 1,000 U/mL Catalase Solution to prepare a 100 U/mL Catalase Solution. For this add 1 mL of 1,000 Units/mL Catalase Solution in 9 mL of Reaction Buffer (Solution B).

After preparing all the stock solutions, from solution E, serial dilutions need to be done to create the standard curve to analyse the sensibility of the catalase activity **(Figure No. 2.2.5. -1.)** For this, 500 µL of the catalase solution were combined with 500 µL with DI water and it was divided in half the concentration 7 times like it shown in **Table No. 2.2.5. – 1.**

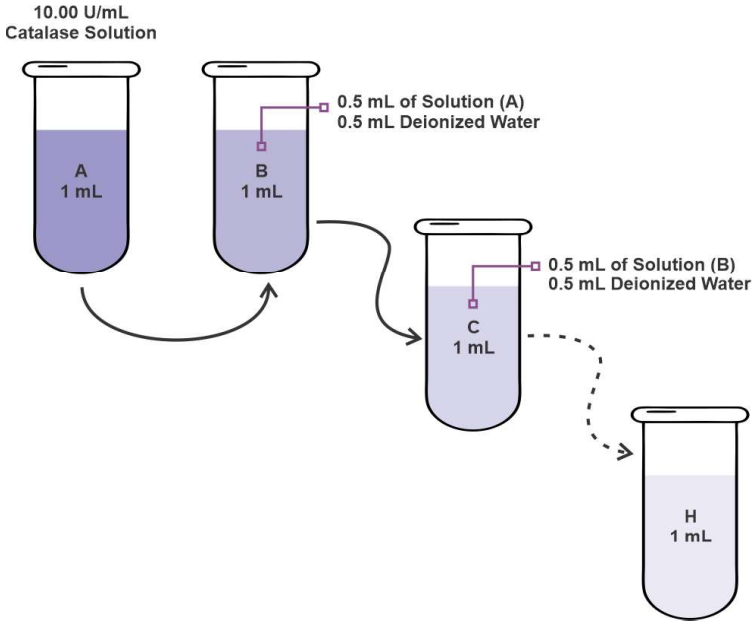

Figure 2.2.5. – 1 Serial dilutions for catalase activity standard curve.

Table No. 2.2.5. – 2 Catalase Activity concentrations after serial dilutions.

Dilution Label	Catalase Activity (U/mL)	Real Catalase Activity (mU/mL)
A	10,000	2500
B	5,000	1250
C	2,5000	625
D	1,250	312.50
E	625	156.25
F	312.50	78.13
G	156.25	39.06
H	0	0

After the dilutions been made, they were placed in a well plate CoStar 96 by triplicate and 25 µL Tris – HCl (Solution B) and 25 µL of H_2O_2 40 µM (Solution C) were added to each microwell. It was incubated at room temperature for 30 m in for the reaction between the Catalase solution and Hydrogen Peroxide solution take place. After 30 minutes, 50 µL of Amplex Red Reagent 10 mM (Solution A) were added and it was incubated at 37°C for 30 minutes **(Figure No. 2.2.5. – 2).**

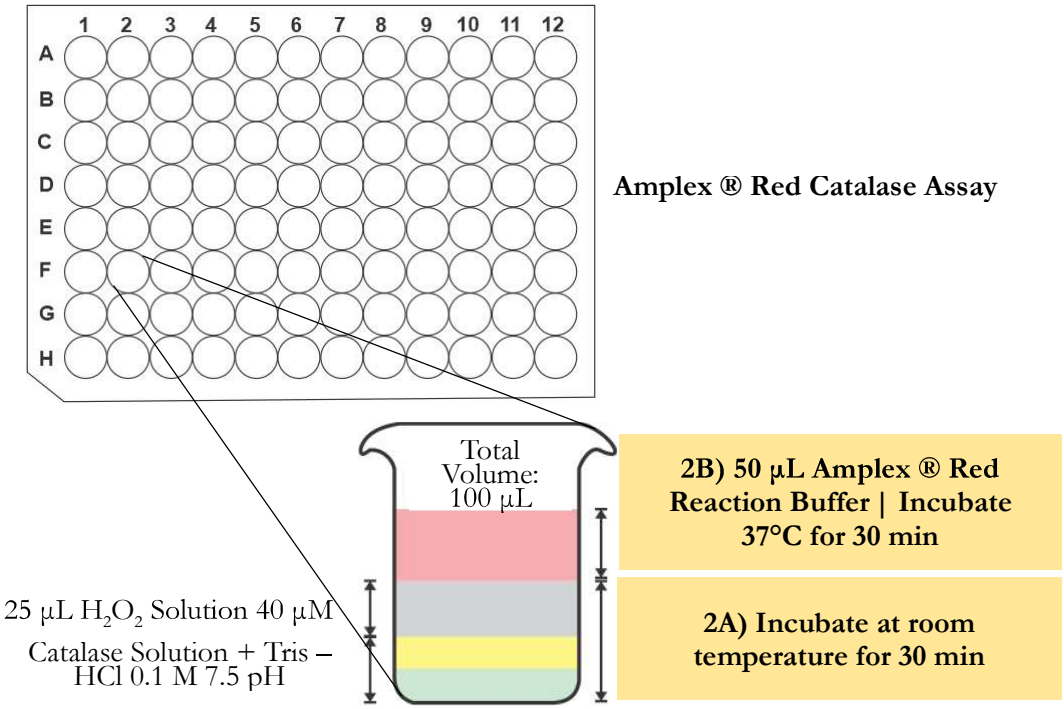

Figure 2.2.5. -2 Amplex Red Reagent reaction chain.

After both reactions were finish, Fluorescence Intensity was measured using FluoStar Optima BMG Labtech at wavelength 544/590 nm

2.2.6. Ultrasound Release.

For ultrasound stimuli release Fisher Brand ® FB120 Sonicator was used at a frequency of 20kHz, amplitude 40% Pulse ratio 3 seconds in and 3 seconds out and for 1.5 minutes **(Figure No. 2.2.6. -1).**

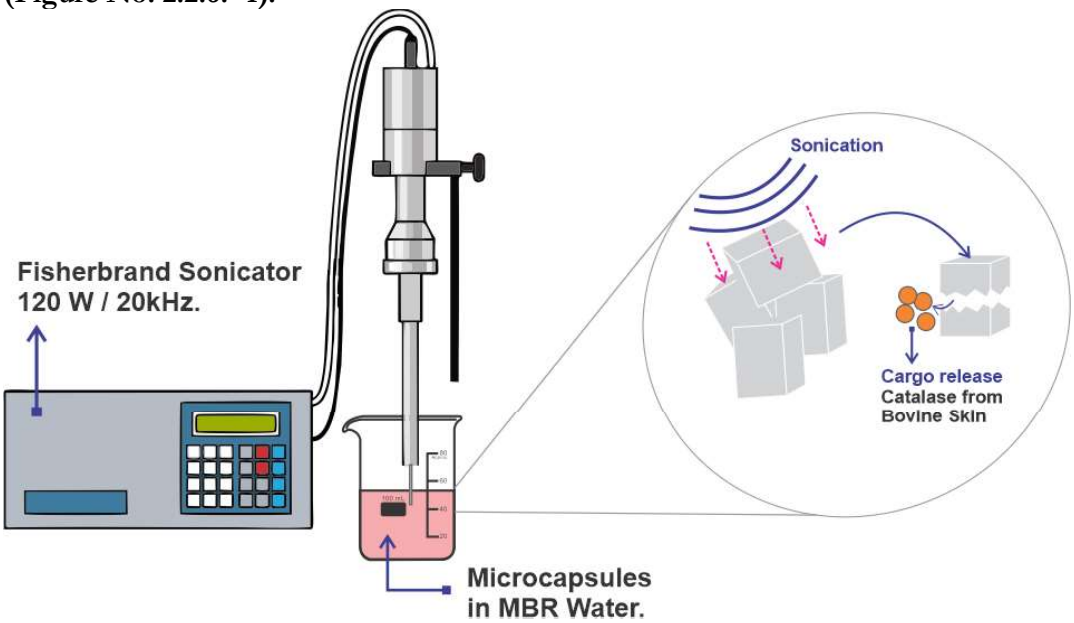

Figure 2.2.6. -1 Ultrasound stimuli for microcapsules release.

2.2.7. Kinetics Release

Following the protocols from **Section 2.2.2. Soft Lithography Microcapsules Manufacturing,** microcapsules made from PLGA – PLGA were manufactured and following the protocols from **Section 2.2.4. Microcapsules Count with Haemocytometer,** the number of the microcapsules manufactured were estimated. Two batches of microcapsules were manufactured, one with catalase cargo and another was left empty.

Both Microcapsules were placed in triplicate on the wells of a 24 Thermo Scientific ™ Nunc ™ well plate with filtered vessels (6 samples in total, 3 for each type of microcapsules batch). After a specific time, the supernatant of all vessels was taken and stored in Eppendorf tubes, after this the wells were filled back again with Deionized water **(Figure No. 2.2.7. – 1).** The specific times for supernatant removal are the following:

Table No. 2.2.7. – 1 Released period of kinetics release from PLGA – PLGA Microcapsules with catalase cargo.

Time (hrs)	
0	72
2	96
24	144
48	

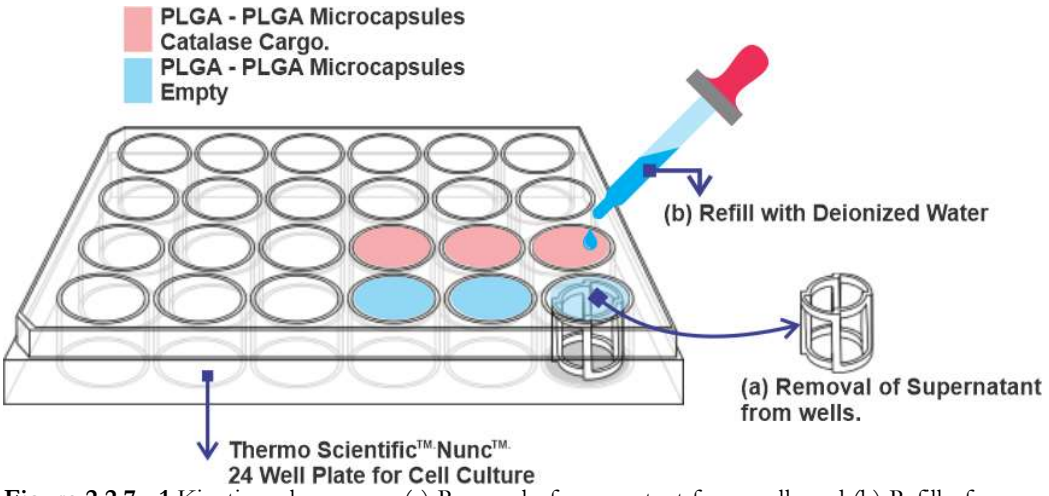

Figure 2.2.7. -1 Kinetics release assay (a) Removal of supernatant from wells and (b) Refill of removed volume with deionized water.

2.2.8. Ultrasound Imaging Methodology

Following the protocols from **Section 2.2.2. Soft Lithography Microcapsules Manufacturing,** microcapsules with PLGA and PLA were manufactured and used to perform the Ultrasound Imaging Assay. For ultrasound visualization, the capsules were placed in selected wells of a 6-well plate. The wells were filled with water and sealed using an Easy Seal plate sealer (Greiner bio-one). The capsules were visualized in a Vevo F2 Ultrasound Instrument (FUJIFILM Visual Sonics) using a 29 MHz probe. The probe was placed on top of the plate sealer to avoid absorption of the ultrasound waves by the plasmid. Parameters were adapted to the sample for optimal visualization. **(Figure No. 2.2.8. – 1).**

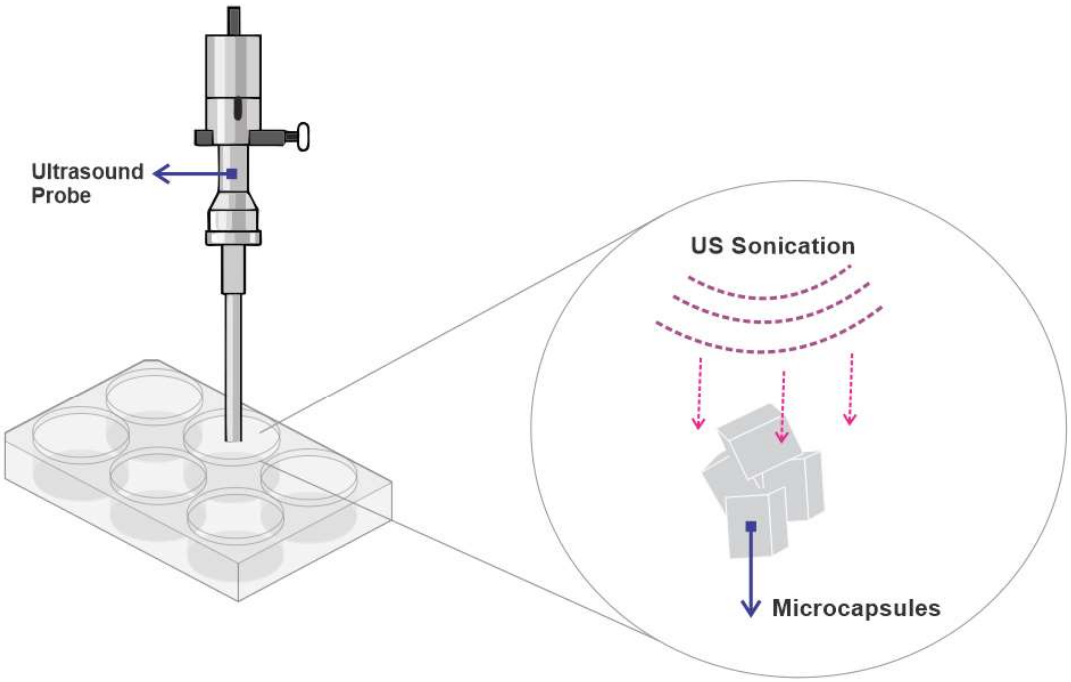

Figure 2.2.8. -1 Set up for ultrasound imaging using a 6 well plate.

2.2.9. Microcapsules Characterization

2.2.9.1. Scanning Electron Microscope (SEM)

Scanning electron microscopy (SEM, ESEM Quanta 400 FEG, FEI, USA) was used to investigate the morphology of obtained samples with imaging conditions of 10 kV accelerating voltage and 10 mm working distance. Samples were placed on a mounting stage and coated with a thin 10 nm gold layer (Agar Auto Sputter Coater, Agar Scientific, UK).

The Scanning Electron Microscope (SEM) uses a focused beam of high-energy electrons directed at a specimen. As these electrons interact with the material, they generate secondary and backscattered electrons. Detectors capture these emitted electrons and convert the signals into detailed images of the specimen's surface topography, with the process occurring in a high-vacuum environment to ensure a continuous electron flow [74].

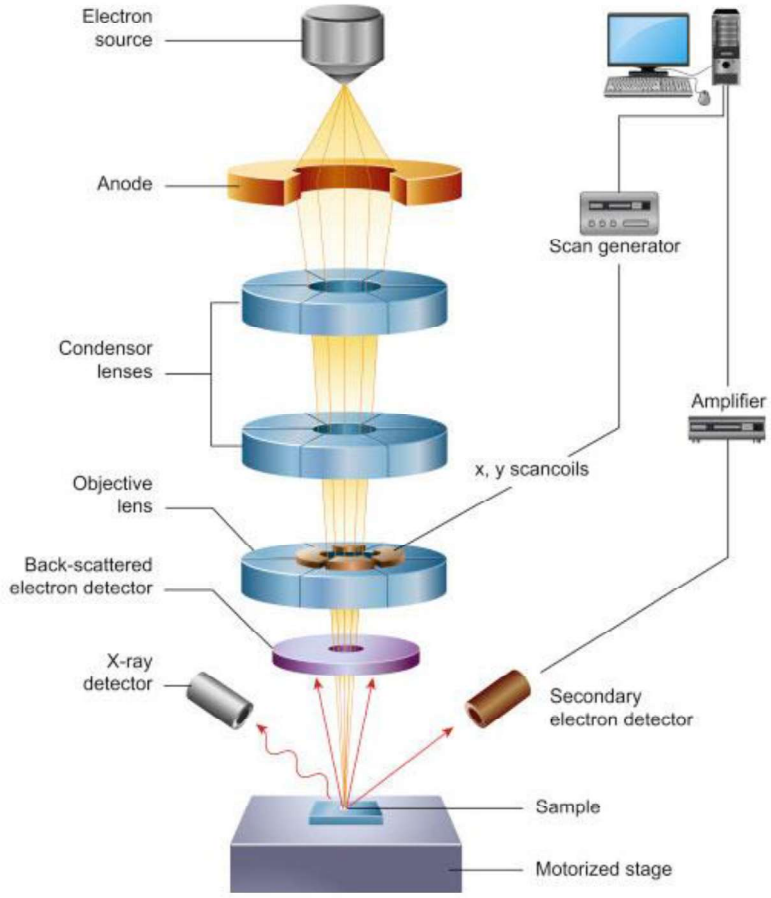

Figure 2.2.9.1 – 1 Scanning Electron Microscope properties [74].

2.2.9.2. Confocal Fluorescence Laser Scanning Microscope (CLSM)

The confocal laser scanning microscopy (CLSM) ZEISS LSM710 ELYRA PS.1 confocal laser scanning microscope was used for microcapsules and microchambers characterization and cells imaging, including live-cell imaging.

This microscope is a combination of an inverted laser scanning confocal microscopy 710 (LSM 710) with super-resolution imaging microscopy ELYRA PS.1: photo activated localization microscopy (PALM)/stochastic optical resolution microscopy (STORM) and structured illumination microscopy (SIM) is equipped with 10×, 20×, 63× oil and 100× oil

immersion objectives. For microcapsule samples, the suspensions were deposited on thin glass slide and covered with cover slip

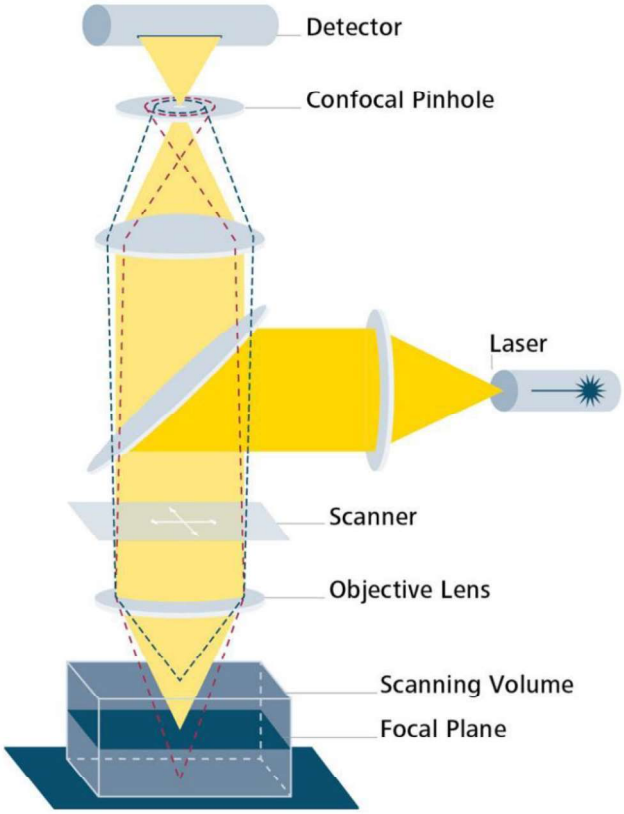

Figure 2.2.9.2 - 1 Confocal Fluorescence Laser Scanning Microscope properties.

Chapter III. | Results and Discussions

3.1. Microcapsules Characterization

Following the protocols from **Section 2.2.2. Soft Lithography Microcapsules Manufacturing** and **Section 2.2.3. Layer – by – Later Microcapsules Manufacturing,** symmetric microcapsules were produced. Even though soft lithography and layer – by – layer methodologies are well studied methods, applying these techniques created some challenges that were overcome.

3.1.1. PLGA – PLGA Microcapsules

3.1.1.1. SEM Characterization

Some of the challenges were the low microcapsules production. At the beginning many of the microcapsules broke before the gelatine printing process. This was due to excess scrubbing over the PDMS and/or on some occasion, for the creation of films. By optimizing the manufacturing process significant results were obtained **(Figure No. 3.1.1.1. – 1).**

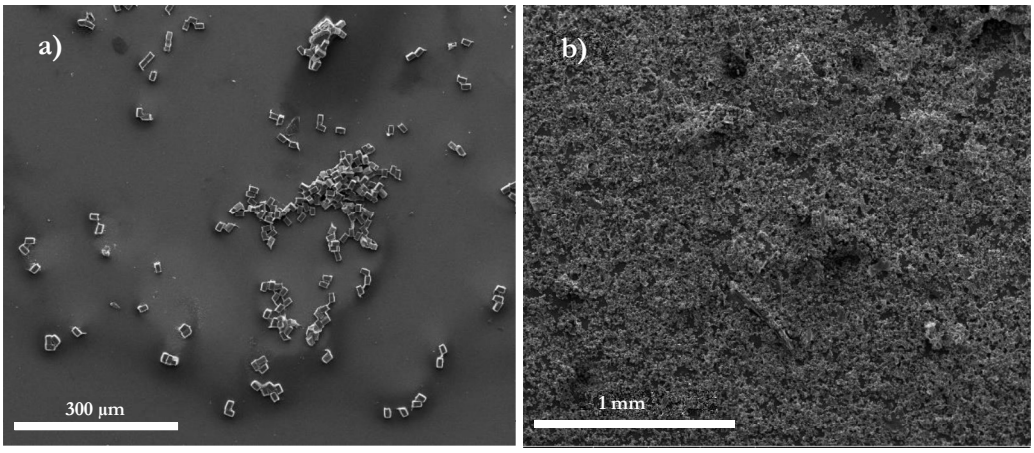

Figure 3.1.1.1. – 1 SEM micrographs from untreated PLGA - PLGA microcapsules (a) Low production of Microcapsules (<8 x 10^4 MC's/mL) and (b) High production of microcapsules (>100 x 10^4 MC's/mL).

In the development of this research, the microcapsules produced were treated with ultrasound sonication to stimuli the cargo release of catalase enzyme. Using the SEM the morphology of the microcapsules was able to be seen, and the morphology of the microcapsules can be observed before and after the ultrasound sonication treatment **(Figure No. 3.1.1.1. – 2).**

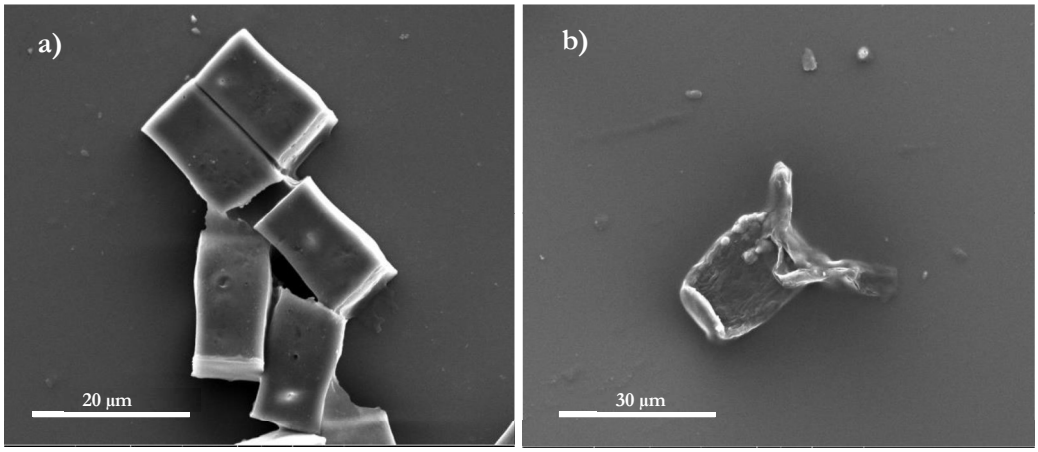

Figure 3.1.1.1. - 2 SEM Micrograph of (a) Untreated PLGA - PLGA Microcapsules and (b) Treated PLGA - PLGA Microcapsules by HIFU.

3.1.1.2. CLSM Characterization

To be able to analyse the composition of the microcapsules, CLSM was used. The PLGA polymer was dyed with Nile red dye and catalase from bovine skin was not dyed but it appears blue/purple in the CLSM images.

Some issues were detected while analysing the microcapsules under the CLSM, polymer solution from the microcapsule seal was filling the inside of the microcapsule creating bulk microcapsules that were difficult to break under sonication **(Figure No. 3.1.1.2. – 1).**

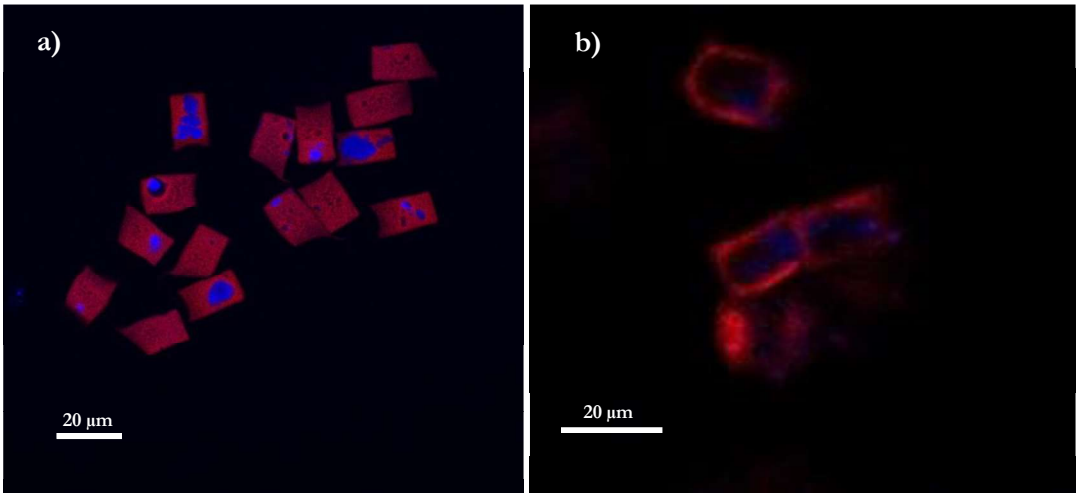

Figure 3.1.1.2. - 1 CLSM Micrographs of PLGA - PLGA Microcapsules (a) Bulk microcapsules filled with sealing polymer solution, and (b) Optimized Microcapsules.

In **Figure No. 3.1.1.2 – 1 (a)** shows microcapsules filled with the PLGA polymer solution used for the sealing process (red), this polymer solution was labelled with Nile Red dye, the area in blue is the catalase from bovine skin, this was unlabelled, and it is showed in blue under the CLSM. On the figure, some capsules are shown to be without catalase and only the polymer solution can be seen inside the microcapsule. Some other, show little catalase encapsulated, both encapsulation issues were related to the manufacturing process due to the lack of removal of the exceeding polymer after the sealing step. This caused many difficulties as well in the ultrasound release experiment.

On the other hand, in **Figure No. 3.1.1.2. – 1 (b),** microcapsules show a more defined shape without polymer solution (red) inside. The catalase is shown in blue, it shows with less intensity than **Figure No. 3.1.1.2 – 1 (a)** due to low cargo load inside. Nevertheless, significant changes can be observed.

This improvement displayed on **Figure No. 3.1.1.2. (b)** were achieved by optimizing the sealing process making very low pressure over the PDMS and by using a 1% PLGA/Acetone to remove the excess of polymer solution after the sealing process.

On the microcapsules displayed on **Figure No. 3.1.1.2. (a)**, too much pressure was applied in the sealing process and the excess of polymer solution after the sealing process was removed with a 10% PLGA/Acetone polymer solution, which ended up filling up the microcapsules and making the encapsulation a difficult process.

3.1.2. LBL (PAH/PSS) – PLGA Microcapsules

3.1.2.1 CLSM Characterization

The LBL methodology was a promising methodology used in this research, some challenges arise in the production of this type of microcapsules due to the coating time. Nevertheless, some micrographs in the CLSM were able to be taken to analyse the composition of the microcapsules both from the outside and the inside.

Ultrasound release experiments were performed using these microcapsules to evaluate the ability that this type of microcapsule has when it comes to protect sensitive cargoes. Using CLSM microcapsules before and after the sonication treatment were taken. This LBL microcapsules were sealed with a solution of PLGA, by doing this we combined soft lithography into this technique. The polyelectrolytes used were PAH and PSS **(Figure No. 3.1.2.1. – 1)**

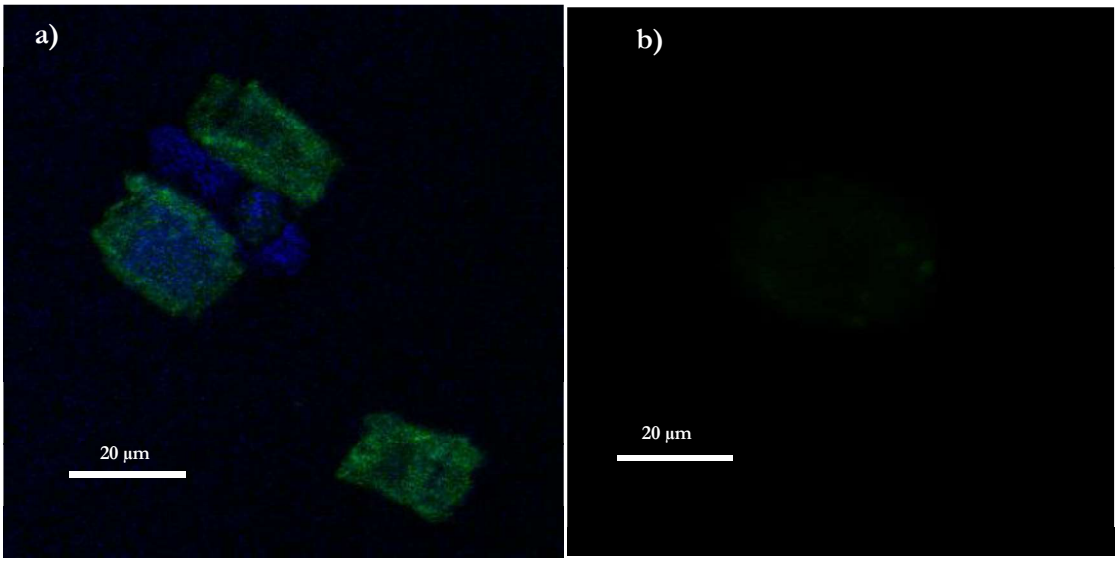

Figure 3.1.2.1 - 1 CLSM Micrographs of LBL - PLGA (PAH/PSS) Microcapsules with catalase as cargo (a) before ultrasound sonication treatment and (b) after sonication treatment.

In **Figure No. 3.1.2.1 – 1 (a)**, it is shown on green the LBl shell cover made from PAH/PSS polyelectrolytes and in blue it is shown the catalase from bovine skin. In this figure, it can also be observed a broken microcapsule, we can conclude this due to the LBL shell broken (green) and the catalase from bovine skin outside (blue).

On the other hand, on **Figure No. 3.1.2.1. – 1 (b),** it can be appreciated some slight green spots, this is related to the LBL shell that has been broken by the ultrasound sonication.

3.1.3. LBL (DS/PA) – PLGA Microcapsules

A different type of polyelectrolytes was used to optimize the shell of LBL by selecting polymers that are more biodegradable such as Dextran Sulphate (DS) and Poly Arginine (PA). To do this, the protocol from Section **2.2.2 Soft Lithography Microcapsules Manufacturing, Section 2.2.3. Layer – by – Layer Microcapsules Manufacturing, and Section 2.2.9. Microcapsules Characterization** were followed.

These microcapsules made were analysed on the CSLM. The LBL – PLGA microcapsules (DS/PAG) are displayed in **Figure No. 3.1.3. – 1.**

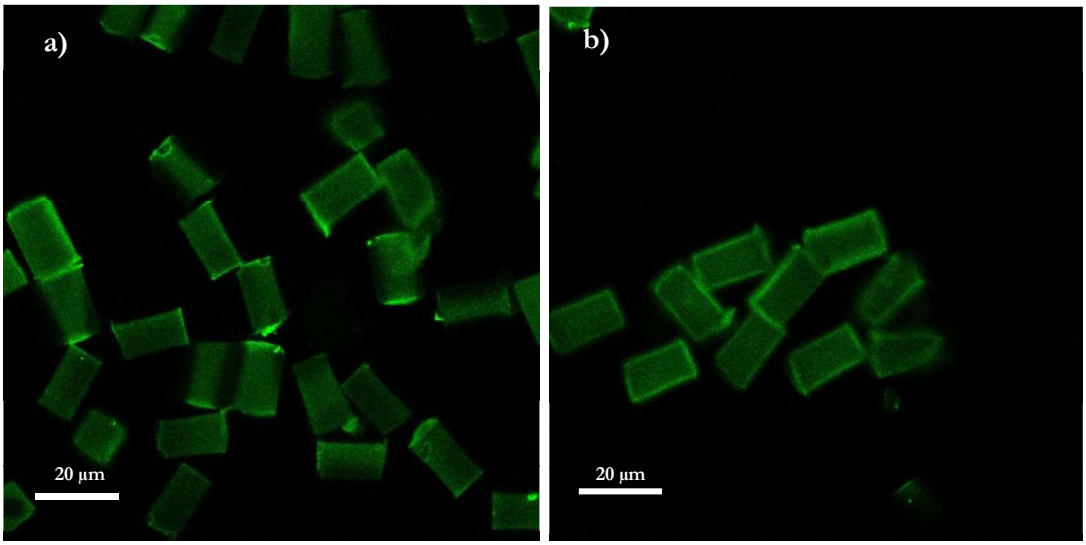

Figure 3.1.3. – 1 CLSM Micrographs of LBL - PLGA Microcapsules made from Dextran Sulphate and Poly Arginine polyelectrolytes.

The microcapsules under the CLSM showed a very good morphology and shape, none of them showed to be broken demonstrating the good interactions among the chosen polyelectrolytes. Nevertheless, all of them were completely fill inside with the sealing polymer (green). The shell made out from LBL DS/PA was labelled with DA-FITC which displays green under the CLSM. Therefore, on **Figure No. 3.1.3. – 1** showed completely green images.

Further research needs to be done to optimize the encapsulation of these microcapsules and demonstrate the applicability of these combination of polyelectrolytes. Also, there performance under ultrasound sonication and their ability to protect sensitive cargoes needs to be assessed.

3.2. Amplex Red ® Standard Curve

Following the protocol of **Section 2.2.5 Amplex Red ® Catalase Assay**, fluorescence intensity parameters were obtained. On **Table No. 3.2. – 1** the catalase activity concentrations used for the standard curve are shown.

Table No. 3.2. - 1 Catalase activity concentrations

Label	Catalase Activity U/mL
A	9978.2
B	4989.1
C	2494.6
D	1247.3
E	623.6
F	311.8
G	155.9
H	0.0

The above concentrations were placed in a 96 well plate, after the addition of the **Amplex Red ®** reagent, they showed a pink colour palette degradation from dark prink to light pink as it's shown in **Figure No. 3.2. – 1.** The darker the colour, the higher the fluorescence intensity and the lower the catalase activity will be.

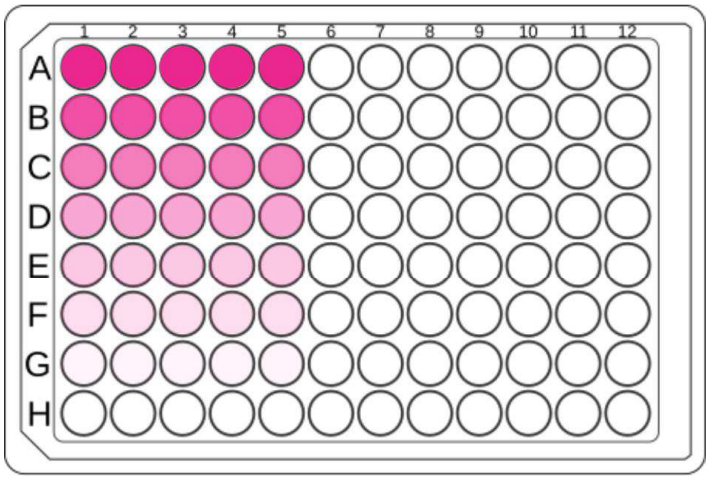

Figure 3.2. – 1 Amplex (R) Red Catalase Standard Curve in CoStar 96 well plate.

The concentration display above were used by five replicates in a CoStar 96 micro well plate. On **Table No. 3.2. – 2** the fluorescence intensity results obtained are shown.

Table No. 3.2. – 2 Fluorescence intensity measurements for Amplex Red ® standard curve.

	Fluorescence Intensity (Arbitrary Units)					
	1	**2**	**3**	**4**	**5**	**6**
A	725	633	931	605	773	
B	3631	3795	3322	3997	3451	
C	25942	31757	27200	26626	26372	
D	45400	45419	41795	42543	44635	
E	52640	51541	51736	52183	52422	
F	53946	52675	54411	54087	54047	
G	56819	54830	55652	55984	55388	
H	55483	56388	57417	57461	57922	

From the data shown on **Table No. 3.2. – 2,** the average was taken and used as the value for the standard curve calculations like it is shown in **Table No. 3.2. – 3.**

Table No. 3.2. – 3 Catalase Activity concentration data and transformation.

	Catalase Activity U/mL	**Real Cat. Act. mU/mL**	**F.I. (A.U.)**	**Transformation**
A	9978.2	2494.55	733.4	56200.8
B	4989.1	1247.28	3639.2	53295.0
C	2494.6	623.64	27579.4	29354.8
D	1247.3	311.82	43958.4	12975.8
E	623.6	155.91	52104.4	4829.8
F	311.8	77.95	53833.2	3101.0
G	155.9	38.98	55734.6	1199.6
H	0.0	0.00	56934.2	0.0

From the data below display on the column "Catalase Activity U/mL" on **Table No. 3.2. – 3,** a calculation of the real catalase activity concentration was performed. This is done because on the micro well plate CoStar 96 the solution of catalase is placed along with other solutions such as H_2O_2 40 µM (25 µL) and the Amplex Red ® Reagent (50 µL) four folding the concentration. The real concentration is displayed in the column "Real Cat. Act. mU/mL" on **Table No. 3.2. – 3**.

To calculate the real catalase activity from the samples, the media (environment) where the experiment was conducted need to be subtracted, in this case, the analyse was performed in DI water. Therefore, the average value of the "H" row was subtracted from all the other values, this is displayed as "Transformation" on **Table 3.2. – 3.**

Using the results from the columns "Real Cat. Act. mU/mL" and "Transformation" the standard curve plot was performed as shown in **Figure No. 3.2. – 2.**

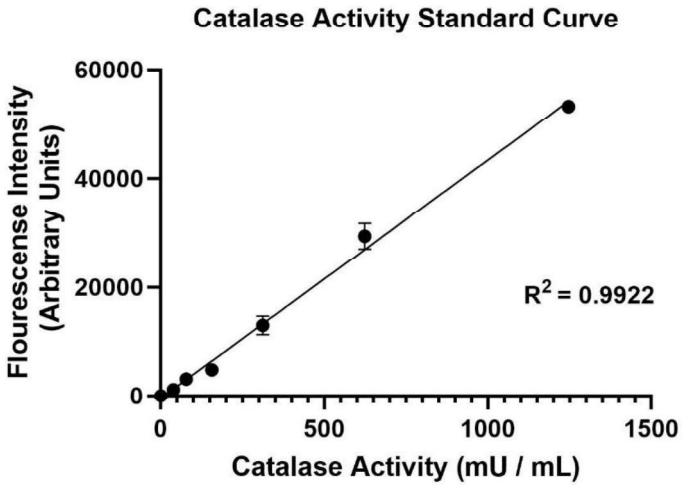

Figure 3.2. – 2 Catalase Activity standard curve from Amplex Red ® Catalase Assay. Plot made with GraphPad.

Only the values from row B to H were considered in the standard curve, due to the low Fluorescence Intensity measurement obtained in row A. This indicates that the values from row A are out of range for the limitations of the FluoStar Spectrophotometer.
As it is shown on **Figure 3.2. – 1,** the standard curve shows a **0.9922** coefficient of determination. This indicates that the values have a good fit into a linear regression, which allowed us to use this correlation to determine the catalase activity concentration from the microcapsules in the release experiments.

3.3. Ultrasound Release

Since the main goal of this research is the assessment of the performance of the PLGA – PLGA and LBL – PLGA in protecting highly sensitive cargoes such as catalase, an ultrasound release experiment was performed.

The more relevant challenges overcome in the ultrasound release experiment was the heat control when sonicating the sample, since the catalase enzyme is sensitive to temperature as well as pH, the microcapsules protection has been studied to prove that these microcapsules can protect the cargo. Following the protocol from **Section 2.2.4 Microcapsules count with Haemocytometer**, the following results were obtained.

Table No. 3.3. -1 Microcapsules count for Ultrasound Release Assay

Microcapsule Type	Microcapsule count per mL
PLGA – PLGA	100 x 10^4
LBL – PLGA	100 x 10^4

Following the protocol from **Section 2.2.6 Ultrasound Release**, the following parameters were used to release the cargo from microcapsules.

Table No. 3.3. – 2. Sonication Parameters for Ultrasound Triggered release.

Parameter	Value
Amplitude	40%
Sonication Time	1: 30 min
Pulse	3 sec. In / 3 sec. Off.
Frequency	120 kHz

To determine the sonication parameters mentioned above, some trials were performed to evaluate the survival of catalase under the sonication. The sonication creates cavitation and elevates the temperature of the sample, these are factors that affect the catalase activity. Different amplitudes and timings were assessed to determine the optimal parameters for the release that doesn't degrade or denature the catalase activity. Also, an ice shell was used to control the temperature created by the Sonicator.

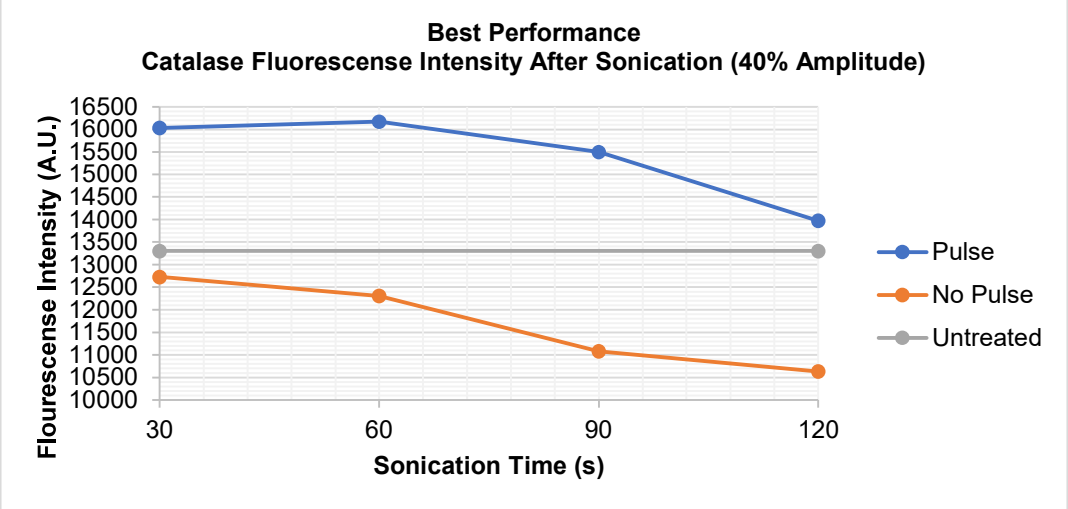

Figure 3.3. -1 Catalase fluorescence intensity performance after sonication with 40% amplitude with and without pulse.

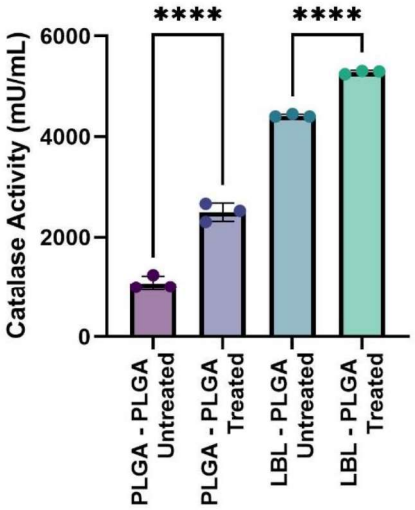

Figure 3.3. – 2 Catalase activity before and after ultrasound sonication treatment for both types of microcapsules (PLGA - PLGA, LBL - PLGA).

Due to the high number of microcapsules count present in both samples, some of the microcapsules broke before treatment in both situations. In the case of PLGA - PLGA show less microcapsules broken before treatment. This is interpreted from the catalase activity that it exists before the treatment (untreated microcapsules).

On the other hand, LBL – PLGA microcapsules have a higher catalase activity before the sonication treatment, meaning that a high number of Microcapsules were broken and

promoting permeability and early release of the microcapsule. Or it can also indicate that LBL are just more permeable due to the nature of such polymer shell.

Even though, both due the high concentration of microcapsules presented issues, with an optimal amount of microcapsule count both can protect catalase from degradation and release can be stimulated by ultrasound.

An ordinary one – way ANOVA analysis was performed stating that in multiple comparisons both microcapsule types are statistically significant with a p – value < 0.0001.

Another study regarding the microcapsule properties was performed. In this occasion, it was to study the effect of the Nile Red dye used to label microcapsules for CSLM analysis. In this study, the main interest was to analyse if the polymer releases something else than the cargo or if there's a clear interference. For this, Microcapsules of PLGA – PLGA were manufactured and leaved empty. A group of microcapsules were labelled, and another group was left unlabelled.

The same Amplex Red ® assay was performed to analyse the fluorescence intensity and the same wavelength of emission and excitation (544/590). The reason of this is to be able to analyse and see if there is some interference in the same wavelength as the Amplex ® Red assay. There results obtained can be observed in **Figure No. 3.3. – 3.**

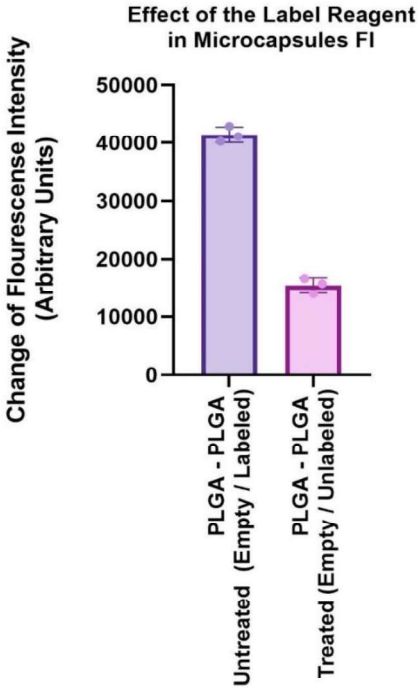

Figure 3.3. - 3 Effect of the Label Reagent (Nile red) in the microcapsules Fluorescence Intensity.

As in can be appreciated in **Figure No. 3.3. - 3** the Label dye affects and interferes with the **Amplex ® Red Catalase Assay** because it produces higher Fluorescence Intensity. In the situation of the unlabelled microcapsules, it has shown to have less impact and the reasons of this slight fluorescence intensity its unknown, it might be for something related to the polymer, it might be that some of them is release. Further studies need to be done to make a more complete conclusion.

3.4. Kinetics Release Assay

In drug delivery systems, the release kinetics of therapeutic agents plays a crucial role in determining the effectiveness and safety of the treatment. Understanding how a drug is released from its delivery vehicle over time is essential for designing systems that offer controlled, sustained, or targeted drug delivery.

These kinetics release assay was performed to measure and evaluate the rate of catalase release from the microcapsules without an external stimulus (**Figure No. 3.4. – 1**). A total of 30 x 10^4 MC´s /mL made from PLGA – PLGA were used for this assay.

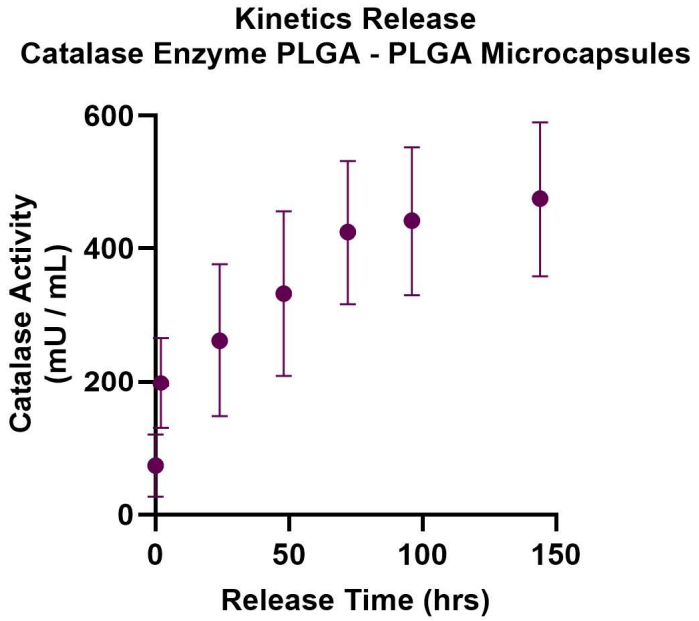

Figure 3.4. - 1 Catalase enzyme kinetics release without external stimulus.

As we can see on the graph, the catalase is successfully released, and around 150 hours it takes to release all the catalase from the microcapsules. It's not always convenient a slow release and over time other factors such as denaturation, degradation, and temperature can affect the cargo. Therefore, ultrasound stimulation is a promising alternative and effective approach to protect sensitive cargoes with these types of microcapsules and it is faster.

3.5. Ultrasound Imaging

Ultrasound imaging, also known as sonography, is a widely used medical imaging technique that employs high-frequency sound waves to produce images of structures within the body.

In this study a different version of Ultrasound imaging was used to study how the ultrasound makes a change in a substrate or microcapsules. The goal of this experiment was to try to prove the existence of air chamber inside the microcapsule. Many types of microcapsules made out from soft lithography were evaluated to see the performance under the Ultrasound Imaging Equipment. The types of microcapsules and films evaluated are listed in **Table No. 3.5. – 1.**

Table No. 3.5. – 1 Microcapsules and film types used in Ultrasound Imaging Assay.

Microcapsule or Film Type	Cargo
MC PLGA – PLGA	Catalase
	Empty
MC PLA – PLA	Catalase
	Empty
Film PLGA – PLGA	Catalase
	Empty
Film PLA - PLA	Catalase
	Empty

Following the protocol from **Section 2.2.2 Soft Lithography Microcapsules Manufacturing,** and **Section 2.2.8 Ultrasound Imaging** was followed, and the results were the following:

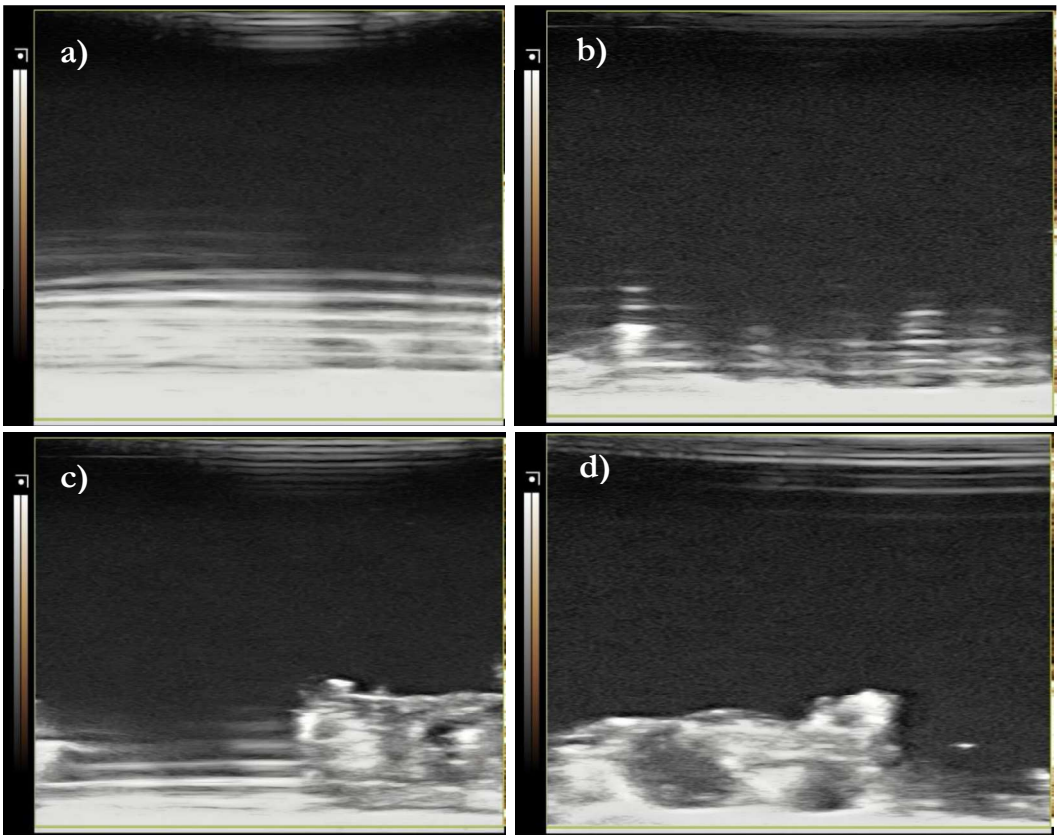

Figure 3.5. – 1 Ultrasound Imaging (a) PLGA - PLGA Microcapsules with catalase as cargo, (b) PLGA - PLGA Microcapsules empty, (c) PLGA - PLGA Film with catalase as cargo, and (d) PLGA - PLGA Film empty.

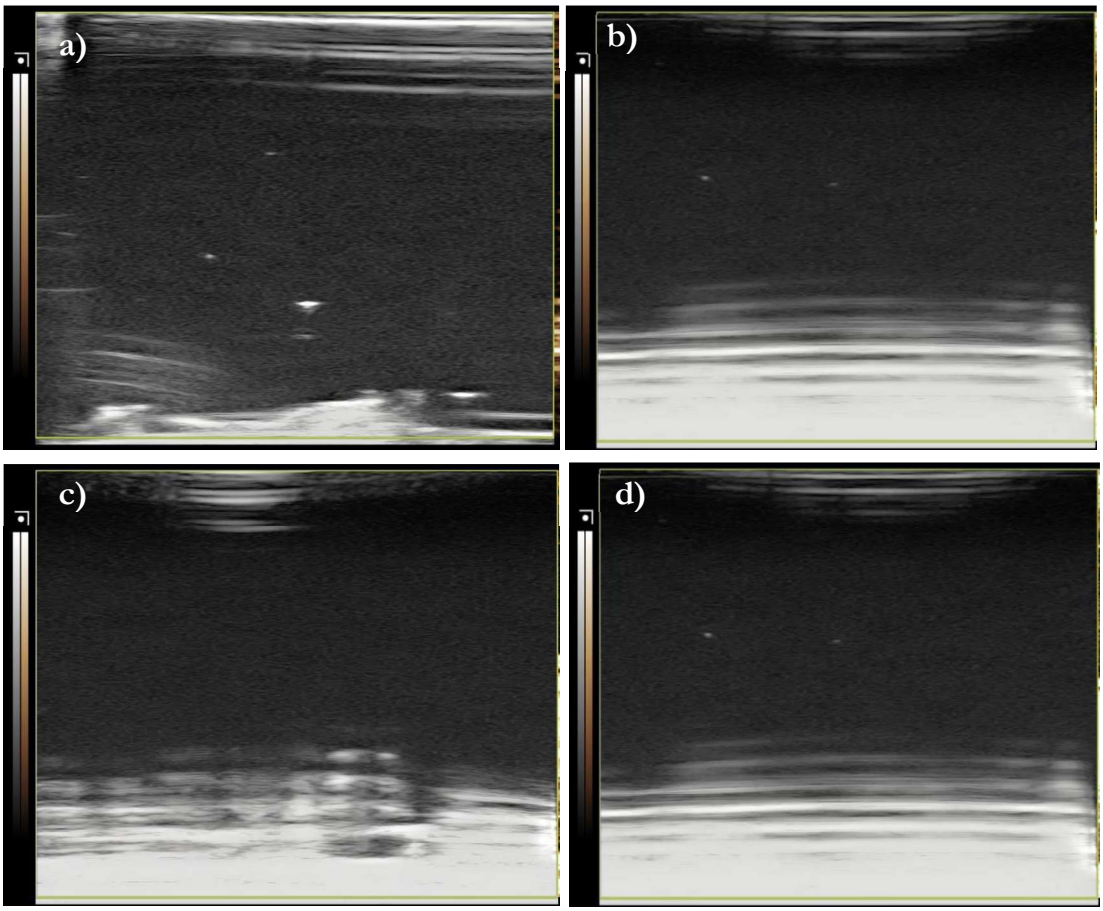

Figure 3.5. - 2 Ultrasound Imaging (a) PLA - PLA Microcapsules with catalase as cargo, (b) PLA - PLA Microcapsules empty, (c) PLA - PLA Film with catalase as cargo, and (d) PLA - PLA Film empty.

On **Figure No. 3.5. – 1** and **No. 3.5. – 2** are displayed the ultrasound imaging of two types of polymers in both microcapsules and films (PLGA and PLA), they were both evaluated empty and with catalase. The goal was to prove the existence of air chamber by comparing the contrast between both images. Unfortunately, significant issues arise making too many interferences in the imaging making it difficult to make a contrast and determine or distinguish the microcapsules and their internal composition.

For further studies are need it to have better images and do a analyse by comparing the contrasts. Since the current phantom is made from agarose is suggested to use a gelatine phantom, so a blank can be run previous the experiment and a proper contrast can be obtained, and more relevant conclusions could be made.

Conclusions

Based on the results of this study, both PLGA-PLGA and LbL-PLGA microcapsules demonstrated significant potential in protecting the catalase enzyme from environmental degradation, especially under sonication conditions. Although some microcapsules were found to be broken before treatment—more notably in the LbL-PLGA samples, which showed higher premature catalase release compared to the PLGA-PLGA microcapsules—both systems effectively safeguarded the enzyme and allowed for ultrasound-triggered release. The premature rupture and release of the catalase cargo may be attributed to the high concentration of microcapsules in the sample. Both experiments used an average of 100 x 10^4 microcapsules per millilitre, a high concentration that likely caused microcapsule collisions, leading to breakage. In the case of the LbL-PLGA microcapsules, this collision also appeared to increase permeability, promoting early cargo release.

The results from the Amplex Red ® Assay demonstrated that the catalase enzyme retained its activity after the ultrasound treatment. The post-treatment fluorescence intensity was lower than the ones that were on the untreated sample, which, as discussed in Section 3.2, indicates a higher catalase concentration. This suggests that the catalase encapsulated within the microcapsules was successfully released and remained intact, without significant degradation or denaturation. The analysis of catalase release from microcapsules, both stimulated with ultrasound and those left to release naturally in a kinetic release assay, demonstrated that ultrasound stimulation significantly accelerates the release process. However, the elevated temperature generated by sonication poses a challenge. To mitigate this, an ice shell was employed to regulate temperature and prevent damage to the catalase.

Also, an analyse was performed to evaluate the potential interference caused by the Nile Red dye used for labelling of PLGA microcapsules in the CLSM. Results indicated that the labelled microcapsules exhibited a greater fluorescence intensity compared to the unlabelled ones, suggesting that the label reagent might interfere with the Amplex Red ® assay. On the other, the unlabelled microcapsules showed a low fluorescence intensity, the exact cause of the slight fluorescence in unlabelled microcapsules remains unclear, it could be related to polymer degradation or release. More comprehensive investigations are needed to clarify this issue. Due to this, microcapsule for ultrasound release assay were manufactured without Nile red dye for the ultrasound release experiment and it was used only for characterization samples.

An Ultrasound Imaging experiment was performed to detect the presence of air chambers within microcapsules. PLA and PLGA – both biodegradable biomaterials - were use in the manufacturing of microcapsules for this experiment, and two type of encapsulation methods were used: microcapsules and microfilms to see which of them show better contrast in proving the existence of air chamber within the microcapsule.

Both approaches of microcapsules and films were performed comparing a batch of empty units and another one filled with catalase enzyme as cargo. Both provided inconclusive images due to interference from the agarose phantom used in the experiment. None of the microcapsules or films tested provided clear enough images to draw a definitive conclusion. To improve the accuracy and relevance of future studies, it is recommended that a gelatine phantom be used instead, enabling better contrast and clearer imaging results. By doing this, a trial can be performed using only water as the sample, and then have an image which it can be subtracted from other assay, reducing the interference and having a better contrast to make a better approach on the imaging analysis.

The current Layer-by-Layer (LbL) approach utilized conventional polyelectrolytes, such as poly (allylamine hydrochloride) (PAH) and poly (sodium styrene sulfonate) (PSS), both are known for their biodegradability and biocompatibility. However, a new combination of polyelectrolytes was proposed to improve the performance and biodegradability of the LbL system. This novel combination, consisting of Dextran Sulphate (DS) and Poly-arginine (PAG), was used to fabricate microcapsules, which were initially analysed using Confocal Scanning Laser Microscopy (CSLM). The preliminary results showed promising morphology and structural integrity. Nevertheless, further research and optimization are required to unlock the full potential of these microcapsules. The LbL-PLGA microcapsules with DS/PAG need more comprehensive evaluations, including the encapsulation of active cargo, stability testing, and assessments of their release profiles and biocompatibility under various conditions. These additional studies will be crucial for fine-tuning their properties and assessing their effectiveness in drug delivery systems.

Future Research

- Optimizing the use of soft lithography for microcapsule fabrication, focusing on refining the structural integrity of capsules to minimize premature cargo release and improve the mechanical stability of microcapsules during high-intensity focused ultrasound (HIFU) treatments.
- Research on LbL-PLGA microcapsules with Dextran Sulphate (DS) and Poly-arginine (PA) polyelectrolytes, encapsulating active cargo, and evaluating their release profile through both kinetic release assays and ultrasound-triggered stimuli.
- Comparing the performance of LbL-PLGA microcapsules formed with PAH/PSS polyelectrolytes against those made with DS/PA, focusing on their biodegradability, stability, and release profiles under various environmental conditions such as pH, ultrasound, and temperature fluctuations.
- Conducting an Ultrasound Imaging using a gelatine phantom to enhance contrast and verify the presence of air chambers within microcapsules, addressing previous challenges faced using agarose phantoms.

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