POLYMERIC NANOCARRIERS FOR THE ENHANCEMENT OF ENZYME REPLACEMENT THERAPY IN LYSOSOMAL STORAGE DISEASES: A PROOF OF CONCEPT STUDY

By

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ABSTRACT

Lysosomal Storage Diseases (LSDs) are a group of inheritable genetic diseases caused by mutant lysosomal enzymes, leading to the accumulation of undigested macromolecules in the lysosomes and causing increases in lysosome size and number, cellular dysfunction, clinical abnormalities and premature death. These LSDs can be treated with Enzyme Replacement Therapy (ERT) through intravenous administration of a recombinant enzyme in replacement of the defective enzyme. However, this is an expensive and inefficient method with adverse side effects associated with the high enzyme amounts required for the treatment, the need of post-translational modification of the enzyme and the host immune system response.

Nanoparticle drug delivery systems (DDSs) are a promising alternative to enclose the therapeutic cargo, then overcoming the drawbacks associated with ERT. As building blocks of those DDSs, biodegradable synthetic polymers are considered an attractive alternative for protein delivery applications because they can be designed to obtain desirable properties like low immune response, stimuli response, specific circulation time and affinity to certain drugs and environments.

This research project aims to develop biodegradable and bioresponsive polymersomes, composed of amphiphilic block copolymers of Polyethylene glycol and Polycaprolactone (PEG-PCL), suitable for the encapsulation and delivery of protein therapeutics as proof of concept for ERT applications. This goal was achieved through the synthesis and optimization of protein loaded polymersomes using a Water in-Oil-in Water (WOW) double emulsion method, and the addition of a protein corona composed of Fetal Bovine Serum (FBS) components.

First, a group of copolymers were synthesized by Ring Opening Polymerization, using either Stannous Octoate (SO) or Hydrochloric Acid (HCl) as catalysts. Characterization was carried out by Proton Nuclear Magnetic Resonance (1H-NMR) and Gel Permeation Chromatography (GPC). Results demonstrated that SO was a better catalyst for achieving the synthesis of block copolymers of the desired molecular weight. This catalyst was used to synthesize the copolymers used in the formation of bioresponsive polymersomes.

The second part of the project was to prepare a series of Water-in-Oil in Water (WOW) emulsion formulations by varying copolymer molecular weight, solvent evaporation pressure, copolymer: stabilizer ratio, emulsification technique and protein concentration in the aqueous core. The *in*

vitro performance of the polymersomes was assessed in order to obtain ideal properties in terms of behavior under biologically relevant buffers, size, encapsulation efficiency and protein release profile. The optimal formulation consisted of using PEG–PCL with a molecular weight of the polymeric chain of 2000g/mol and 5000g/mol, respectively (PEG2000-PCL5000); a ratio of 1:1 of PEG–PCL:PVA, and reduced pressure (generated by a vacuum pump) for solvent evaporation to generate the desired polymersomes through the WOW emulsion method. In the aqueous core, the addition of model protein with a concentration of 10mg/mL (compared to concentrations of 25 and 50 mg/mL) gave the best entrapment efficiency. These polymersomes were found to be nontoxic at concentrations of up to 2mg/mL.

A protein corona was added to the polymersomes to further fine-tune the desired protein release behavior. This led to the formulation of a system that completely suppresses protein release in physiological conditions (Phosphate Buffer Saline Solution, PBS, pH 7.4) and shows sustained release in acidic lysosomal environment (Artificial Lysosomal Fluid, ALF, pH 4.5). The size of these particles was determined to be theoretically appropriate for cellular internalization through measurement by Dynamic Light Scattering techniques (221 ± 21 nm in PBS and 190 ± 66 nm in ALF).

We conclude that we have developed a system that warrants further investigation for the development of polymeric nanocarriers that are suitable for the enhancement of Enzyme Replacement Therapy for the treatment of Lysosomal Storage Diseases.

RESUMEN

Las Enfermedades del Almacenamiento en el Lisosoma (EAL) corresponden a un grupo de enfermedades genéticas hereditarias causadas por enzimas mutantes en los lisosomas. Eso provoca la acumulación de macromoléculas sin degradar en los lisosomas, causando efectos adversos en la célula como aumento en el tamaño y la cantidad de lisosomas, disfunción celular, anormalidades clínicas y muerte prematura. Estas EAL pueden tratarse con Terapia de Reemplazo Enzimático (TRE), a través de infusiones intravenosas de una enzima recombinante, la cual reemplaza la enzima defectiva en el paciente. Sin embargo, estos tratamientos son ineficientes, costosos y acarrean una serie de efectos adversos relacionados a las altas dosis requeridas para que sea efectivo, el hecho de que las enzimas recombinantes necesitan modificaciones post-translacionales y la posibilidad de respuesta inmunológica adversa por parte del paciente.

Sistemas de administración de fármacos (SAFs) utilizando nanopartículas son una alternativa prometedora para encapsular agentes terapéuticos de EAL, superando así las desventajas asociadas a las TRE. Como materia prima para estos SAFs, los polímeros sintéticos biocompatibles son considerados una alternativa atractiva para administración de proteínas terapéuticas porque estos pueden ser diseñados para obtener ciertas propiedades específicas como baja respuesta inmunológica, respuesta a estímulos como temperatura o pH, tiempos de circulación específicos y afinidad a ciertas drogas o ambientes.

Este proyecto de investigación busca desarrollar polimerisomas biodegradables y bioresponsivos, compuestos de copolímeros anfifílicos de bloque de Poliglicol de etileno y Policaprolactona (PGE-PCL), apropiados para la encapsulación y administración de proteínas terapéuticas como prueba de concepto para aplicaciones en TREs. Esto se logró sintetizando y optimizando polimerisomas cargados de proteína, preparados usando el método de dobles emulsiones con la metodológica conocida como “Agua – en Aceite – en Agua” (AAA) y la adición de una corona de proteínas en la superficie de la nanopartícula, utilizando componentes del Suero Fetal Bovino (SFB).

Primeramente, se sintetizó un grupo de copolímeros con la técnica de Polimerización de Apertura de Anillo, utilizando Octoato de Estaño (OE) o Ácido Hidroclorhídrico (AH) como catalizadores. La caracterización de los copolímeros se llevó a cabo utilizando Resonancia

Magnética Nuclear (RMN) y Cromatografía de Permeación de Gel (CPG). Los resultados demostraron que OE era un mejor catalizador que AH porque se obtuvieron copolímeros de bloque del peso molecular deseados. Este catalizador fue utilizado para sintetizar los copolímeros utilizados en la formación de polimerisomas bioresponsivos.

En la segunda parte del proyecto se prepararon diferentes formulaciones de emulsiones AAA variando el largo de las cadenas de los copolímeros, la presión del proceso de evaporación de solvente, la razón del copolímeros y su agente estabilizador, la técnica de emulsificación y la concentración de proteína modelo en el centro acuoso de las nanopartículas. El rendimiento *in vitro* de los polimerisomas se evaluó para obtener propiedades ideales en términos de comportamiento en amortiguadores biológicamente relevantes, tamaño hidrodinámico, eficiencia de encapsulación y perfil de liberación de la proteína modelo. La formulación óptima consistió de un copolímero de PGE-PCL con peso molecular de cadena de 2000g/mol para PGE y 5000g/mol para PCL (PGE2000-PCL5000); de una razón copolímero:estabilizador de 1:1 y de evaporación de solvente usando vacío. La concentración de 10mg/mL de proteína modelo en el centro acuoso de las nanopartículas resultó ser la candidata con mayor eficiencia de encapsulación (comparado con concentraciones de 25 y 50mg/mL). Estos polimerisomas resultaron no ser tóxicos hasta concentraciones de 2mg/mL.

La corona de proteínas de suero fue añadida con el propósito de afinar el comportamiento en los ensayos de liberación de proteína en ambientes biológicamente relevantes. Esto causó la generación de un sistema que suprimió completamente la liberación de proteína a condiciones fisiológicas (Amortiguador Salino de Fosfato, ASF, pH 7.4) y mostró liberación sostenida en ambiente lisosomal ácido (Fluido Artificial de Lisosoma, FAL, pH 4.5). El tamaño de las partículas fue determinado para que cumpliera con los requisitos teóricos para internalización de nanopartículas a nivel celular, usando la técnica de Dispersión Dinámica de Luz (DDL). Los resultaros dieron que el tamaño hidrodinámico de los polimerisomas fue 221 ± 21 en ASF (pH 7.4) y 190 ± 66 en FAL (pH 4.5).

Con estos hallazgos concluimos que se ha logrado obtener un sistema que permite el adelanto de investigaciones futuras para el desarrollo de nanovehículos poliméricos apropiados para el mejoramiento de la Terapia de Reemplazo Enzimático para el tratamiento de Enfermedades de Almacenamiento en el Lisosoma.

To Hilda Martinez, Jose Roman and Santa Sofia

Your unconditional love and support have made possible the fulfillment of my dreams…

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1. INTRODUCTION

1.1 Justification

Nanotechnology advances in drug delivery systems (DDSs), have been key to the development and improvement of a wide range of therapeutic agents, such as small molecules and biological structures. It opens the possibilities to therapies that otherwise couldn't be used due their toxicity or fragility, by isolating the cargo from the host and by targeting the therapy to specific sites in the organism. In general, DDSs aims to improve the safety and efficacy of therapies by increasing the specificity of the drugs and by lowering doses and side effects[1]. For example, Abraxane ® is a commercially available drug, consisting of an Albumin-Paclitaxel conjugation and is used as anticancer agent. The nanoparticle formulation help to avoid side effects like neuropathy and allergic reactions associated with solubilizing compounds like Cremophor and ethanol, founded in conventional formulations[2].

Even when a broad spectrum of bioactive structures appear to be promising therapeutic candidates, certain drawbacks like toxicity, short circulation half-life, poor stability and propensity to enzymatic degradation have to be overcome in order to achieve commercialization[3]. An extensive variety of nanocarriers have been currently established for drug delivery applications, such as liposomes (lipid-based vesicles), polymeric micelles, polymersomes (polymeric vesicles), polymer-drug conjugates, dendrimers, protein carriers and protein-drug conjugates[4,5]. Several examples can be found in literature were DDSs have been used for a wide variety of bioactive structures like small interfering ribonucleic acid (siRNA), proteins and enzymes[6].

- Synthetic siRNA can be designed to target specific genes responsible for diseases in different types of cells. Extensive research has been done to use siRNA as a therapy to overcome human genetic disorders by silencing target genes without disrupting the endogenous micro-RNA (miRNA) pathway[7]. Nanocarriers made of Polylactide-co-glycolide acid (PLGA) and Ciclodextrin-based polymers are some examples of what is currently being used to optimize siRNA therapy by increasing circulation time and diminishing toxicity by insulating the cargo from the host, thus improving the safety and efficacy of the therapy[8,9].

- Polyethylene glycol and Poly(2-methacryloyloxyethyl phosphorylcholine) have been used to conjugate with the therapeutic proteins like Interferon-R2a (Roferon-A), in order to increase stability, reduce aggregation, prolong circulation time and decrease toxicity[10,11]. Also, encapsulation of insulin in polymeric nanocarriers have been achieved[12]; thus, demonstrating various approaches to improve protein therapies.
- Protein-enzyme nanoparticles have been made using enhanced Green Fluorescent Protein (eGFP) to encapsulate β-Galactosidase, an enzyme necessary for the transformation of lactose into monosaccharides[13,14]. This enzyme therapy is an example of Enzyme Replacement Therapy (ERT), a treatment used for a group of genetic diseases called Lysosomal Storage Diseases (LDSs)[13].

Biodegradable synthetic polymers are considered an attractive alternative for drug delivery applications because they can be designed to obtain desirable properties like low immune response, stimuli response, specific circulation time and affinity to certain drugs and environments[15]. Polymersomes are polymeric vesicles that can be made of amphiphilic block copolymer, which can encapsulate an extensive variety of cargo of both hydrophilic and hydrophobic compounds. They have has special attention in research because of their tunable properties like molecular weight, stability under different environments, biodegradability, blood circulation time, permeability and responsiveness to stimuli[16]. Several studies have found that different enzymes and metalloproteins in polymersomes remain functional after encapsulation, making them a valuable option to enhance the effectiveness of therapeutic proteins and enzymes[6]. The combination of Polyethylene glycol (PEG) and Polycaprolactone (PCL) to form an amphiphilic block copolymer have grown attention because their physical and chemical properties can be tuned for specific applications in DDSs. PEG is a non-toxic hydrophobic polymer, while PCL is crystalline hydrophobic polymer, which is biodegradable at certain physiological conditions. This combination has been extensively studied for controlled drug delivery and has been shown to enhance the cargo performance compared to conventional dosage forms in terms of therapeutic effect, biological activity and dosage frequency[17]. These findings make them a promising tool to enhance existing therapies like Enzyme Replacement Therapy (ERT), the current inefficient and costly treatment for a group of rare diseases named Lysosomal Storage Diseases (LSDs). ERT consists in administration of the defective enzyme, usually as intravenous infusions. This treatment helps to increase the concentration of the

enzyme that is defective in the patient, improving then the digestion of the macromolecules that are building up as consequence of the enzyme deficiency[18–20].

Advantages of the use of polymersomes for ERT are: (1) Enhanced internalization of replacement enzyme per endocytic event, (2) Protection against host's immune system and proteases, (3) Elimination of the need of a Manosse-6-Phosphate tag, lowering the treatment cost and (4) Lower dosage. The enhancement of therapeutic cargo into the lysosome by delivery through polymersomes was assessed, thought the solvent evaporation technique of Water- in Oil-in Water (WOW) double emulsion method. To our knowledge, the combination of biodegradable nanocarriers and ERT using the WOW technique for the treatment of LSDs has not been reported.

We hypothesize that model proteins can be loaded into vesicular nanocarriers composed of PEG-PCL block copolymers, where this formulation will remain intact in biological conditions until intracellular delivery into the lysosome, which contains an acidic environment. PEG properties will help to protect the cargo until reaching the lysosome, where the low pH and high esterase content of this organelle must be sufficient to degrade ester bonds in PCL and release therapeutic cargo.

1.2 Objective

This research project aims to develop biodegradable and bioresponsive polymeric nanocarriers (PNs), specifically polymersomes, composed of amphiphilic block copolymers of Polyethylene glycol and Polycaprolactone (PEG-PCL), suitable for the encapsulation and delivery of protein therapeutics. The research strategy is focused on the design, synthesis and evaluation of the in vitro performance of the polymersomes, capable of encapsulating and delivering proteins into the lysosome, in order to enhance intralysosomal protein uptake. The general idea is to protect the therapeutic cargo until it reaches the lysosome and to control the release upon nanocarrier exposure to lysosomal esterases. This work will serve as a proof of concept to deliver defective enzymes in Lysosomal Storage Diseases (LSDs). These are group of around 50 genetic disorders and are categorized as an "orphan diseases" by the National Institute of Health (NIH). This project is part of an effort to enhance Enzyme Replacement Therapy (ERT), the only existing treatment for these diseases, an inefficient and costly treatment. We intend for this work to serve as platform for the potential advancement of the treatment of Lysosomal Storage Diseases.

In order to achieve this goal, the following tasks were performed:

- Synthesis and characterization of PEG-PCL amphiphilic block copolymers out of Polyethylene glycol and epsilon-Caprolactone monomers.
- Preparation and characterization of aqueous cored PEG-PCL nanocarriers, using the Water - in Oil – in Water double emulsion method, a type of solvent evaporation technique.
- Addition of a model protein into the aqueous core of the polymersomes.
- Addition of a protein corona on the aqueous cored PEG-PCL nanocarriers composed of fetal bovine serum components
- Study of nanoparticle behavior and release of model protein under biologically relevant buffers.
- Study of nanoparticles in cultured cells to determine toxicity.

1.3 Background

1.3.1 Rare Diseases and Orphan Drugs

In United States, a disease is categorized as "*rare*" if the prevalence of less than 200,000 patients, approximately 65 patients per 100,000 individuals[21]; while in Europe is defined by an incidence of less than 50 patients per 100,000 inhabitants [22]. Even when there are several definitions of the term, depending on the country, mostly refers to diseases that affects a small part of the population and are usually life-threatening[23]. There have been discovered around 7000 of *rare diseases*, which range from bacterial, fungal and viral infections to rare cancers and metabolic disorders [24]. These diseases are considered as "*orphans*" since the pharmaceutical industry doesn't invest in their development because of the small population that requires these drugs [25]. For this reason, government intervention has been necessary to increase the available treatments for those diseases.

Orphan Drug Act

In 1983, the United States Drug Act (ODA) law was implemented to provide incentives for the development and commercialization of drugs to treat rare diseases, also termed as *"orphan drugs"*. As an effort to lower the cost of drug development and extend the effective patent protection to drugs used to treat rare diseases, the ODA provides 7 years of exclusive marketing right on orphan drugs that manage to get approval before the competition. This means that, even if the drug exists as an over the counter medicine, no other company can market the product as treatment for that rare disease during this period of time. Additionally, this regulation provides a 50% tax credit from costs associated to research and development of the drug [26,27]. After this act, other countries adopted similar policies, as the European Union (1995 and revised in 2000), Singapore (1991), Japan (1960 and revised in 1993), Australia (1989 and revised in 1997) and Taiwan (2000), which resulted on an enormous increase in the research on the field [23]. Since 1983, the US Food and Drug Administration (FDA) have approved orphan drugs and biological products for more than 400 uses or indications. Also, more than 45 drugs have been pass to marketing approval through The Orphan Grants Program and more than 50 Humanitarian Device Exemption approvals have been possible thanks to The Humanitarian Use Device Program[28].

1.3.2 Lysosomes

Lysosomes are vesicular organelles with an acidic environment due to high proton concentration (pH < 5) and more than 50 hydrolases such as proteases, glucosidases and lipases. The targeting of those enzymes depends on the presence of mannose 6-phosphate (M6P) residues that are recognized by specific receptors in the vesicle. Their main function consists in the degradation of macromolecules contained in the cytoplasm (autophagy of old organelles) and materials taken up via endocytosis (extracellular space), by fusing with the lysosomes to form a secondary lysosome, also called endosome. They are also involved in membrane repair, pathogen defense, antigen presentation and cell death and signaling. [29–31]

Four receptors are involved in the enzyme targeting of lysosomal protein: MPR300/CI-MPR, MPR46/CD-MPR, LIMP-2 and Sortilin. The first two are mannose 6-phosphate (M6P) receptors with a variable amount of binding sites and are involved in most of the transport of the enzymes to the lysosome. The other two carry out trafficking of proteins in s M6P independent manner.[32]

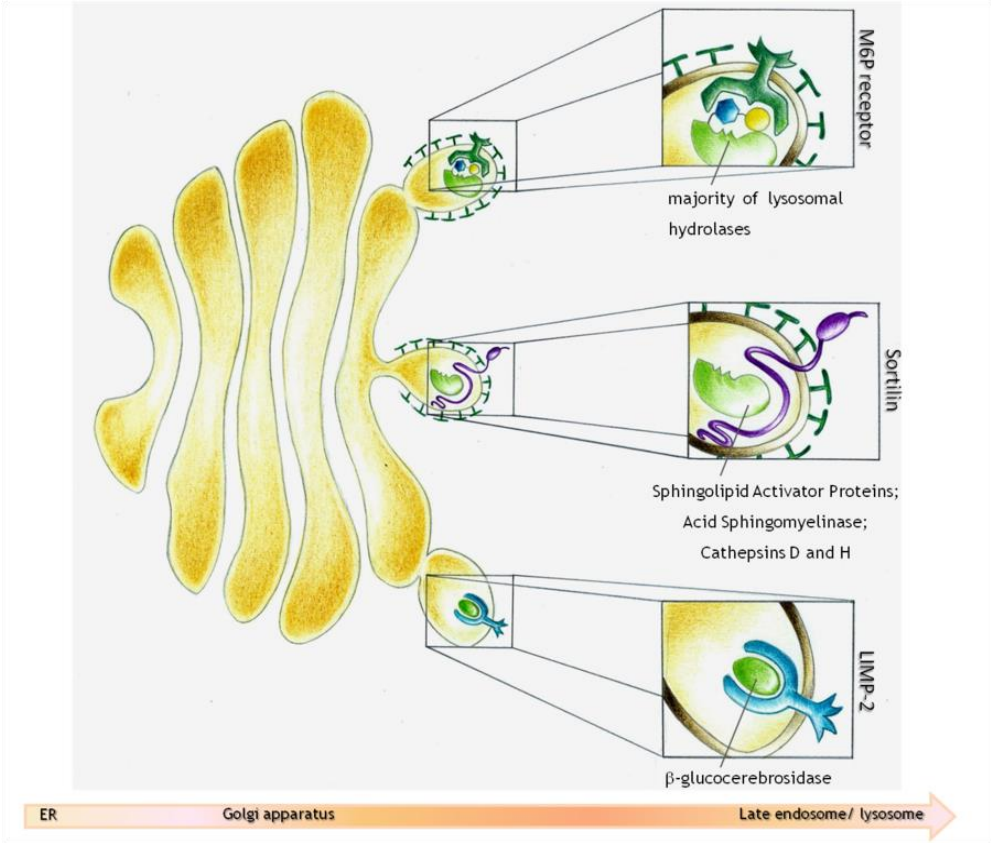


Figure 1.1: Receptors associated with lysosomal transport and their respective targets
(From: http://alexiellady.deviantart.com/art/Intracellular-Transport-of-Lysosomal-Enzymes-288722240)

Precursors of the lysosomal enzymes are synthesized in the Endoplasmic Reticulum. In the Golgi apparatus, theses precursors of lysosomal enzymes are posttranslational modified by the addition of mannose 6-phophate groups (M6P). Then, they are segregated by clathrin-coated vesicles present in the *trans* Golgi network, which concentrated M6P receptors and bind the modified enzymes, which are transported as Endosomes that acidify and eventually transforms into Lysosomes. At the low pH of the late endosome, the enzymes dissociate from the receptors. These receptors are transported back to the Golgi apparatus to recycle and the phosphate group is removed from the mannose in the enzymes the present in the late endosome, ensuring that these do not come back to the Golgi apparatus. A representation of this mechanism is shown in Figure 1.2[29,32]

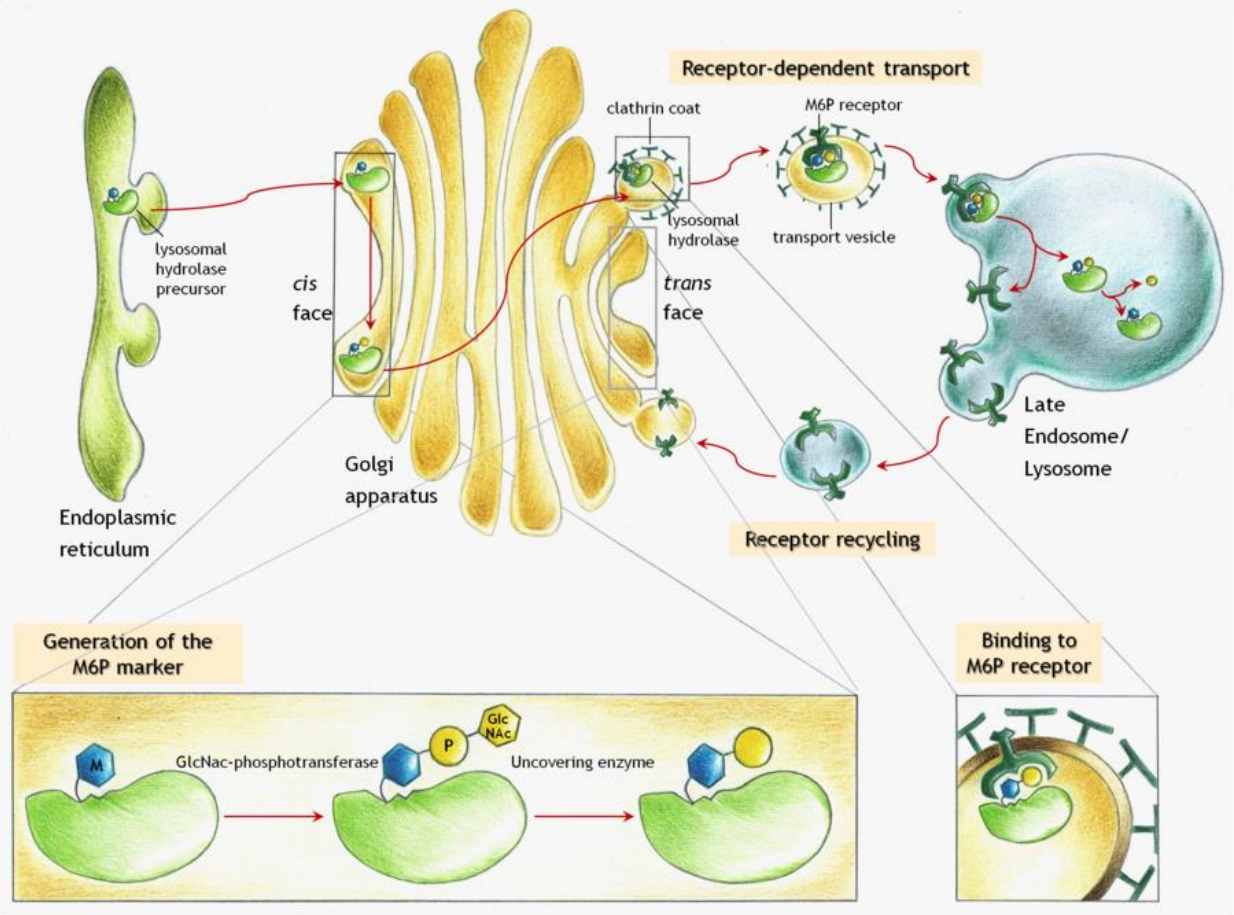


Figure 1.2 Transport of lysosomal enzymes from the Endoplasmic Reticulum to the lysosome using M6P receptor.
(From: http://alexiellady.deviantar t.com/art/The-Mannose-6-Phosphate-Pathway-274184235)

Lysosomal proteins tagged with M6P that escape the receptor recognition in the trans Golgi apparatus are transported to the cell surface and excreted from the cell. These proteins re-enter the cell by recognition and binding to the M6P receptor, thus reaching then the lysosome by receptor-mediated endocytosis. The diagram in Figure 1.3 shows the internalization mechanism and fusion with the lysosome of extracellular enzymes tagged with M6P. This phenomenon is currently used as the general mechanism for the internalization of defective enzymes of the lysosome (Enzyme Replacement Therapy for Lysosomal Storage Diseases)[33]

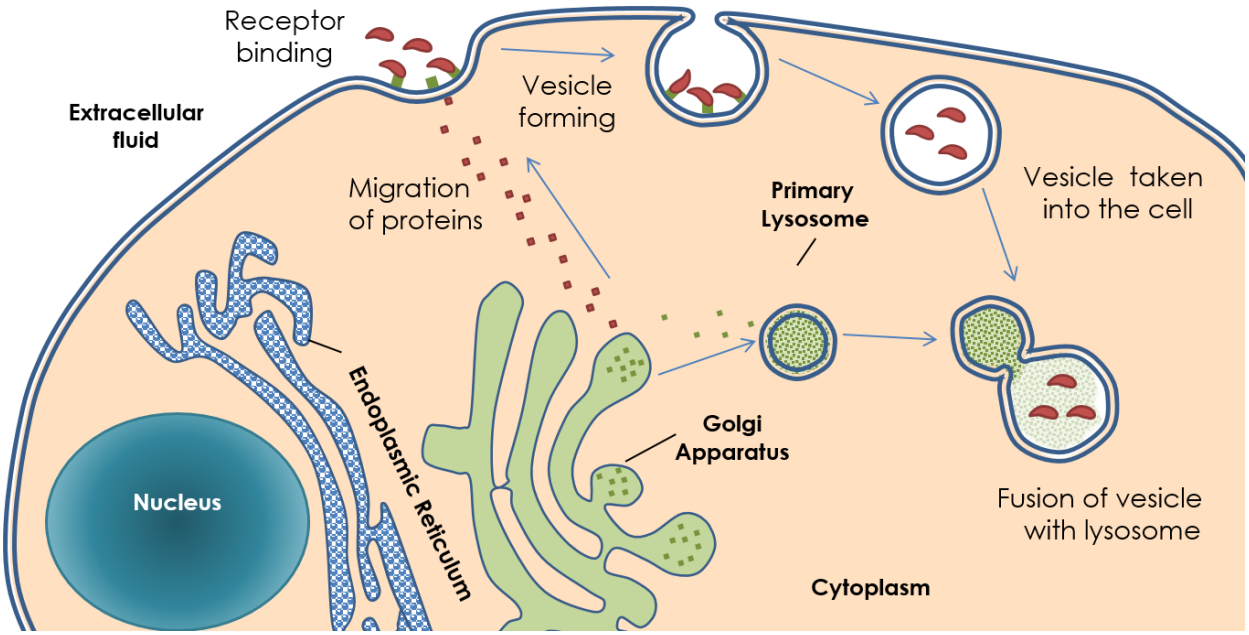


Figure 1.3: Receptor-dependent transport to the lysosome for extracellular materials. Enzymes with the M6P tag are recognized by receptors in the cellular membrane and are internalized into the cell.[33]

Different from the transport controlled by receptors, in order to reach the lysosomes, other materials do not necessarily need an M6P-tag in order to fuse with the lysosomes. Endocytosis is a mechanism to internalize extracellular macromolecules from the cell surface into the cytosol by endocytic vesicles. Macropinocytosis is a form of endocytosis for non-selective macromolecules in which large endocytic vacuoles, also called macropinosomes, form in an actin-dependent process. The primary lysosome fuses with a vesicle containing degradable material in order to form a secondary lysosome, where digestion takes place by degradation of macromolecules carried out by enzymes in the lysosomal sack.[25]

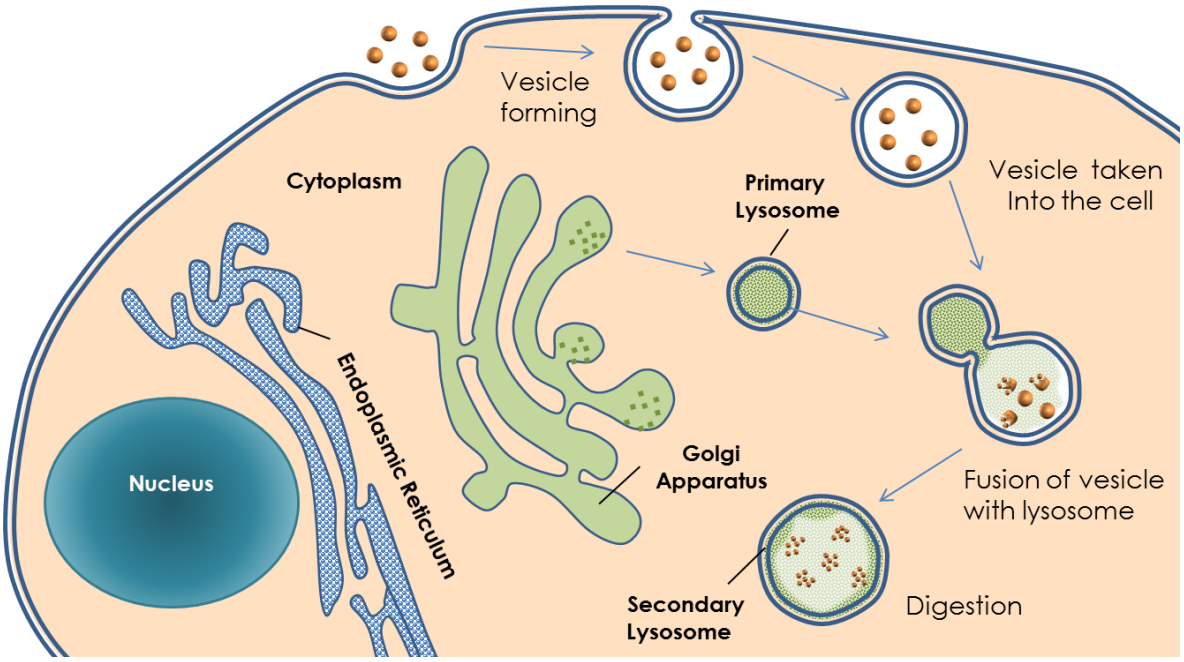


Figure 1.4: Non Receptor-dependent transport to the lysosome and metabolism for extracellular materials. Degradation of materials internalized by macropinocytosis takes place by lysosomal enzymes after the fusion of a clathrin-coated vesicle and a lysosome. [29,34]

1.3.3 Lysosomal Storage Diseases (LSDs)

Different cell types have different amount of lysosomes present in the cytosol, form 0.5% of cytoplasmic volume in normal fibroblasts, to a considerable larger amount in the macrophages. The size and frequency of the lysosomes can increase dramatically in any cell type when lysosomes accumulate non degraded materials. These accumulations can be cause by overloading of with non-physiological substrates (e.g., saccharose,), by application of enzyme inhibitors (e.g., acarbose inhibiting a-glucosidases), by certain drugs (e.g., cationic amphiphilic drugs) which interfere with the intralysosomal digestion of certain substrates and by problems in the biosynthesis of the lysosomal enzymes. Defects associated any of the proteins contained in the lysosomes cause a series of pathologies known as Lysosomal Storage Diseases (LSDs)[29,30]

The LSDs are a group of rare diseases, each characterized by the accumulation of specific substrates in the lysosomes. These inherited genetic disorders caused the formation of mutant lysosomal enzymes, which are misfiling or expressed on inappropriate levels, leading in a gross accumulation of waste products, such as carbohydrates, proteins and lipids that naturally accumulate in the lysosome. This will cause enlargement, collapsing and eventually leakage of

the lysosomal content into the cytosols, which will eventually cause cell death [34,35]. Since the discovery of Pompe's disease in 1932 by the Dutch pathologist JC Pompe (more than 20 years before the former discovery of lysosomes), around 50 different diseases have been discovered[29,36]. Figures 1.5 to 1.7 presents examples of accumulation of different substrates in the lysosome. The classification of these diseases is based on the primary stored materials and function of the defective protein. They are categorized into five groups: (1) Defects in glycan degradation, (2) Defects in lipid degradation, (3) Defects in protein degradation, (4) Defects in lysosomal transporters and (5) Defects in lysosomal trafficking[25]. Examples are provided in Table 1.1. Appendix 1.1 provides details about the diseases, defective proteins and storage materials for all known LSDs.

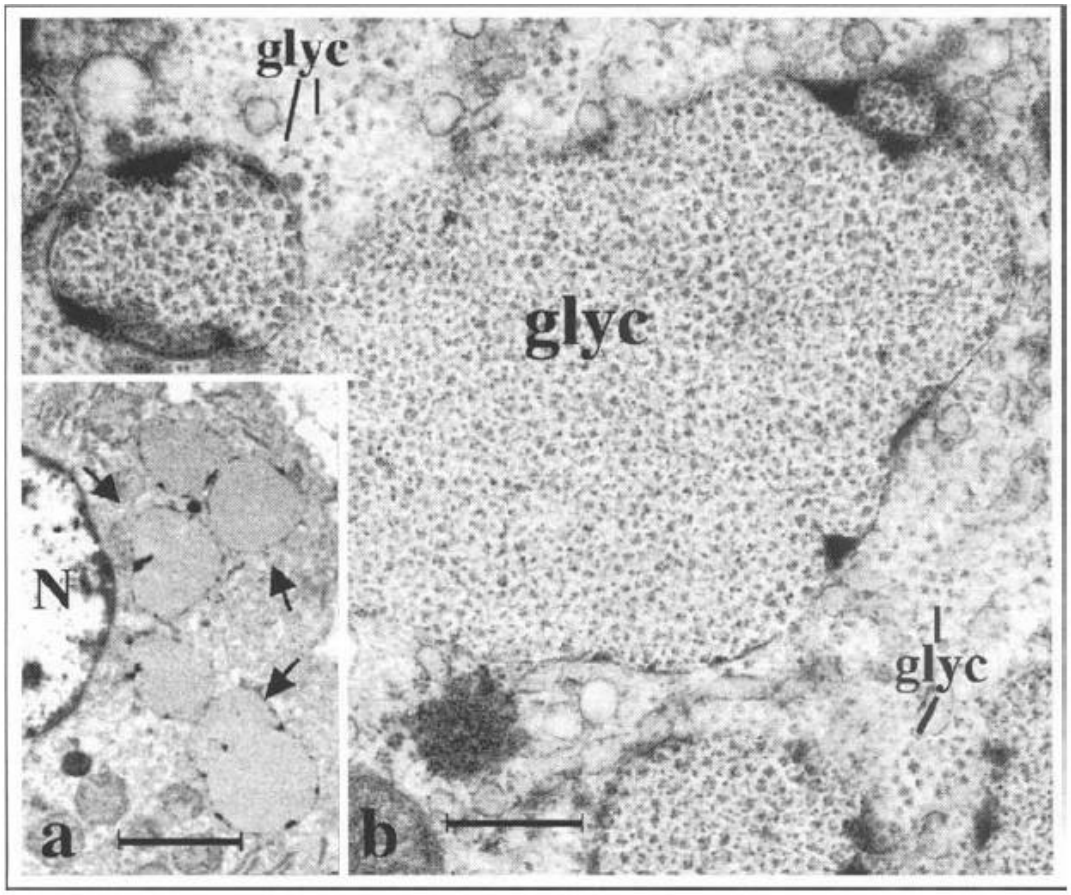


Figure 1.5: Lysosomal glycogen storage in a patient with Pompe disease. Glycogen agglomeration are present in cytoplasmic and membrane-limited storage organelles. Bars represent 2µm (a) and 0.5µm for (b)[29].

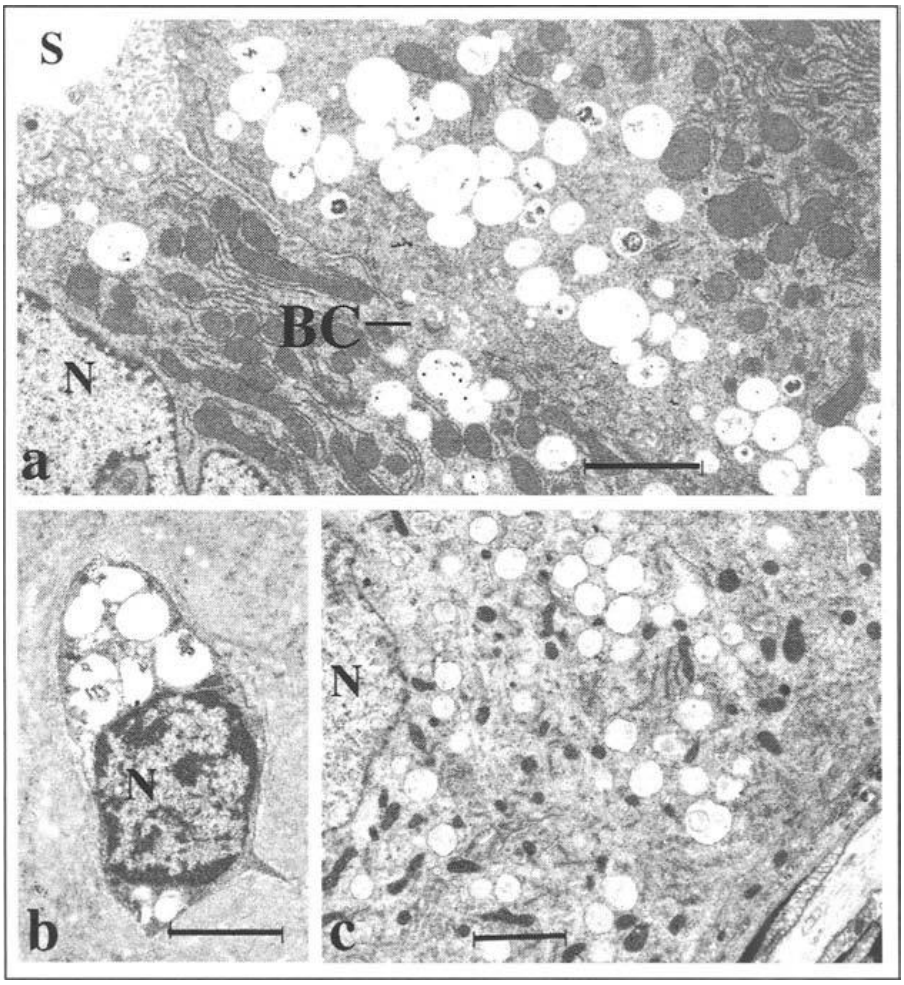


Figure 1.6. Animal model of alpha-mannosidose (mice). Accumulation of oligossacarides in hepatocytes (a), osteocytes (b), and vestibular ganglion cells (c). The lysosomes appear as empty vacuoles because the water-soluble storage material (mannose-containing oligosaccharides) are lost during tissue processing. BC, bile canaliculus. N, nucleus. S, sinusoid. Bars represent 1 µm (a) and .5 µm(b, c).[29]

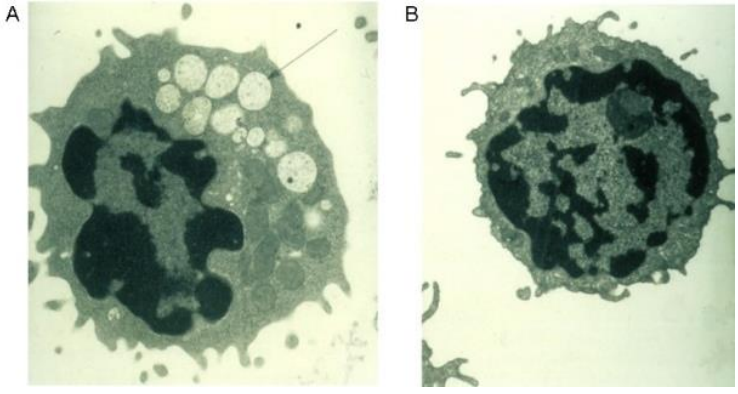


Figure 1.7: Comparison between the lymphocytes of an alpha-mannosidosis patient (a) and a normal control (b). In (a), an accumulation of oligosaccharides is presented in the form of granules.[37].

Table 1.1: Examples of Lysosomal Storage Disorders: Disease, Defective Protein and Storage Materials [25,34,38–49]

Disease	Defective Protein	Storage Material
DEFECTS IN GLYCAN DEGRADATION		
Defects in glycoprotein degradation		
Sialidosis (Mucolipidosis I)	α-Sialidase	Sialyloligosaccharides and sialylglycopeptides
Defects in glycolipid degradation		
A. GM1 Ganglioside		
GM1 gangliosidosis	β-Galactosidase	GM1, GA1, GM2, GM3, GD1A, lyso-GM1, glucosylceramide, lactosylceramide, oligosaccharides, keratan Sulphate
B. Defects in the degradation of sulfatide		
Krabbe (Globoid cell leukodystrophy)	β-Galactocerebrosidase	Galactosylceramide, psychosine lactosylceramide, globotriaosylceramide, globotetraosylceramide, fucosylneolactotetraosylceramide
C. Defects in degradation of globotriaosylceramide		
Fabry	α-Galactosidase A	Globotriaosylceramide, galabiosylceramide, globotriaosylsphingosine, blood-group-B glycolipids
Defects in degradation of Glycosaminoglycan (Mucopolysaccharidosis – MPS)		
A. Degradation of heparan Sulphate		
Hunter (MPS II)	α-Iduronate-2- sulfate sulfatase	Dermatan sulphate and heparan sulphate, GM2, GM3, SCMAS
B. Degradation of other mucopolysaccharides		
Maroteaux Lamy (MPS VI)	N-Acetylgalactosamine-4-sulfatase (arylsulphatase B)	Dermatan sulphate, GM2, GM3, unesterified cholesterol
Defects in degradation of glycogen		
Pompe	α-Glucosidase	Glycogen
DEFECTS IN LIPID DEGRADATION		
Defects in degradation of sphingomyelin		
Farber lipogranulomatosis	Acid ceramidase	Ceramide
Defects in degradation of triglycerides and cholesteryls ester		
Wolman and CESD (Cholesteryl ester storage disease)	Acid lipase	cholesteryl esters and triacylglycerols
DEFECTS IN PROTEIN DEGRADATION		
Pycnodystostosis	Cathepsin K	
DEFECTS IN LYSOSOMAL TRANSPORTERS		
Cystinosis	Cystinosin (cystine transport)	Cystine
DEFECTS IN LYSOSOMAL TRAFFICKING PROTEINS		
Danon	Membrane protein 2 (LAMP-2)	Unprocessed autophagosomes

Left column provides the name of the disease, middle column the deficient protein and the right column the stored compounds. GM1, GA1, GM2, GM3, GD1A are abbreviations for the respective gangliosides. SCMAS: subunit c of mitochondrial ATP synthase.

1.3.4 Enzyme Replacement Therapy (ERT)

The first treatment LSD was Cysteamine, used for the first time in 1976 to treat Cystinosis, caused by the deficiency of a lysosomal cystine transporter (cystinosin), which transports cystine that has been liberated in the process of lysosomal protein degradation back into the cytosol. Cysteamine is a weak base that reacts with cystine in lysosome, forming a disulfide linked cystine cysteamine compound, thus decreasing the cysteine concentration in the lysosome. This compound can exit the lysosome via a transport system for cationic amino acids, preserving the renal function, thus delaying its degenerative decay in patients [38,50]. Since then, several approaches have been investigated to treat LSD, such as stem cell therapy, allogeneic bone marrow transplantation, enzyme replacement therapy and small molecules (for enzyme stabilization or substrate reduction)[18].

Among the possibilities to treat the LSDs, ERT is the most widely used therapy, which consists of administering an enzyme intravenously to a patient whom this particular enzyme is deficient or absent in the lysosome. The replacement enzyme is often referred to as a "recombinant" enzyme and has an M6P tag, which it requires in order to reach the lysosome. ERT takes advantage of plasma M6P receptors for the cellular uptake of enzyme into cells after exogenous intravenous administration by internalizing via receptor-mediated endocytosis[33,51]. The production of lysosomal enzymes with the M6P tag is difficult since is it not possible to use enzymes purified from tissue, since these are already dephosphorylated. The phosphate group is required for targeting the enzyme to the lysosomes. Then, the enzyme is dephosphorylated by acid phosphatase since the M6P tag is not required for the activity of the enzyme itself once it reaches the lysosome. Chinese hamster ovary (CHO) cells, among other mammalian cells lines, are used to produce the replacement enzyme since they can overexpress soluble recombinant lysosomal enzymes with a highly phosphorylated form. Some examples of replacement enzymes being currently produced are α-glucosidase, α-l-iduronidase and α-galactosidase, among others[52–56].

ERT does not yet represent cure because it doesn't affect the underlying genetic defect, but it helps to increase the lysosomal concentration of the deficient enzyme. This therapy involves a life-long treatment with regular and frequent infusions of the enzyme, normally once or twice a month[57]. The safety and effectiveness of ERT for LSDs has been demonstrated in several

diseases, such as Gaucher disease type I, Fabry disease, Mucopolysaccharidosis (MPS) I, also known as Hurler syndrome, MPS II, MPS VI and Pompe's. Some of these drugs are currently commercially available throughout the world[58]. Unfortunately, this treatment has the following drawbacks: (1) the average yearly cost of the drug for ERT is $200,000-$300,000 in the United States, depending on the individual's weight, prescribed dose, and the average price of the drug. (2) it is time consuming, often requiring a day off of work or absence from school during every infusion, and often requires patients to travel great distances for access to infusion centers to receive the drug, (3) adverse infusion-related reactions ranges from mild reactions like itching and redness at the injection site, edema, allergic-like symptoms, fatigue to more severe consequences like chest tightness, respiratory failure and cardiac arrhythmia, which could lead to anaphylactic shock[55,57,59]. As developing therapies for some of these rare diseases becomes more financially feasible, we foresee that the work present in this research thesis could contribute to such development of more conveniently selective, safe, and cost-beneficial therapies that could help prevent the devastating complications of LSDs in patients.

1.3.5 Polymer-based nanocarriers as vehicles for drug delivery

Since the 1950s, numerous efforts have been made in order to develop nanocarriers for drug delivery, with the purpose of overcoming the drawbacks associated with the administration of "bare" drugs. The idea of combining therapeutic agents with polymers from both natural and synthetic origin to enhance their beneficial properties has been in development for more than sixty years, since Jatzkewitz used a dipeptide spacer to attach the drug mescaline to the polymer polyvinyl pyrrolidone back in 1955. After that, an extensive amount of polymers and formulations have been developed as carriers for therapeutic agents[60–62]. Nanocarriers are defined as colloidal particulate systems that range between 10 to 1000nm, which can be loaded or conjugated with an vast variety of therapeutic agents, such as anticancer agents, hormone proteins and enzymes[6]. Numerous polymer-based nanocarriers have been developed for biomedical applications, which can be categorized into: liposomes, polymeric micelles, polymersomes, polymer-drug conjugates, dendrimers and protein carriers, among others[4,5]. Figure 1.8 illustrates the formulation for these nanocarriers along with detailed information.

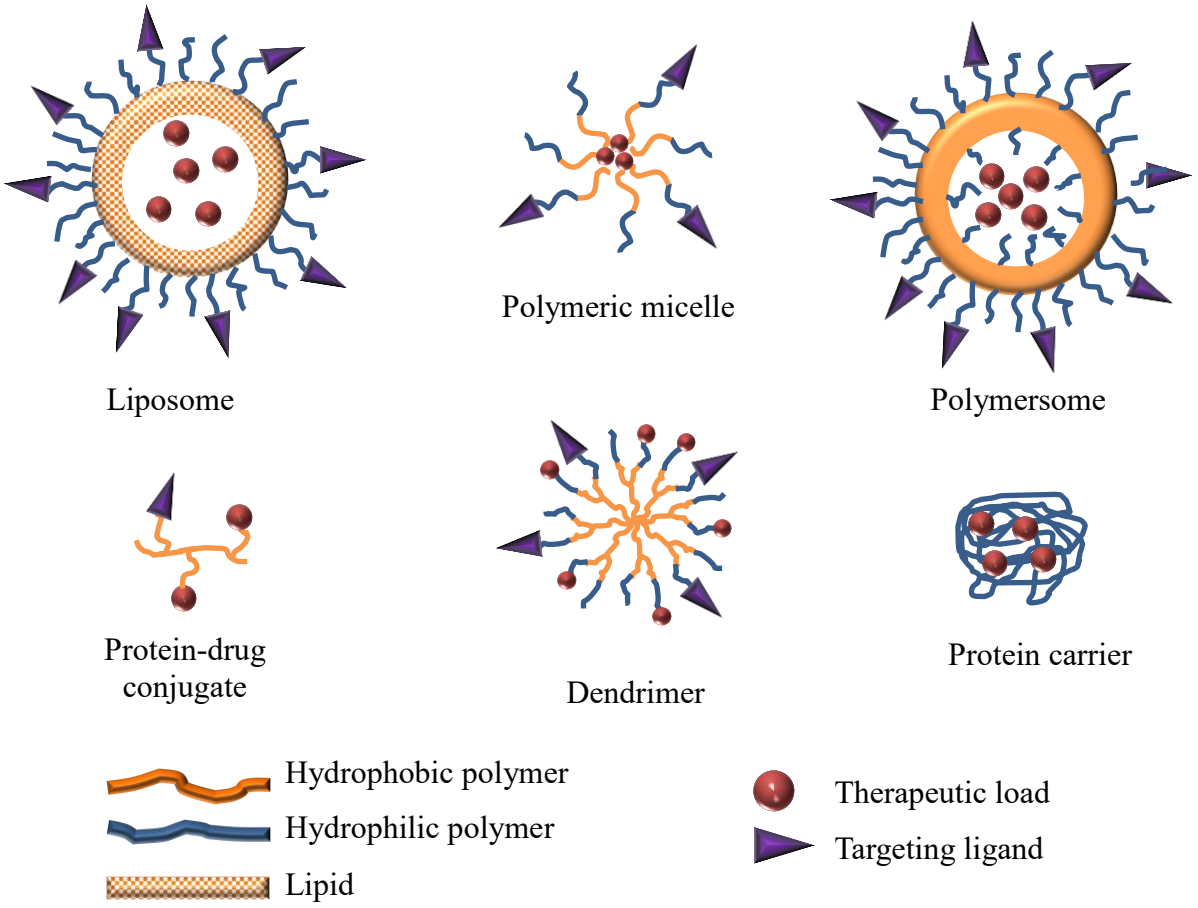


Figure: 1.8 Formulation of Nanocarriers for Drug Delivery Applications

Liposomes

Liposomes were discovered in the 1960s by Bangham, where he described "multilamellar smectic mesophases" or "banghasomes", which consisted of a phospholipid bilayer composed of lecithin and enclosed on a spherical shape[63]. Three decades later, the first commercially available formulation occurred with the approval of Doxil in 1995 for the treatment of various types of cancer. Doxil is a combination of doxorubicin, an anthracycline antibiotic with anti-cancer properties, enclosed in nanocarriers composed of hydrogenated soya phosphatidylcholine, cholesterol and phosphatidylethanolamine polyethylene modified with glycol (PEG)[64,65]. In general, liposomes are self–assembling colloidal structures composed of lipid bilayers with a spherical shape and enclosing an inner aqueous compartment. These structures can be obtained

in the microscale and nanoscale and have the ability to encapsulate both hydrophilic and hydrophobic drugs due to its composition and aqueous center. After Doxil, several drugs have been encapsulated in lipid–based systems such as Ambisome used to treat fungal infections, DaunoXome for certain types of cancer, Epaxal for hepatitis A and Inflexal V for influenza[64,66]. Even when liposomes have been widely used for several decades, pure lipid vesicles are generally cleared from the bloodstream after only a few hours of circulation. Also, even with the addition of PEG to modify the nanocarrier surface, liposomes generally lack mechanisms for controlled release. Therefore, an alternative to overcome this limitation needs to be developed for these lipid vesicles[67,68].

Polymeric micelles

Polymeric micelles are spherical structures, formed through the self-assembly of amphiphilic block copolymers in an aqueous environment, with a typical size of 5 to 150nm. Their structure encloses the hydrophobic chains in the inside, while the surface is covered by the hydrophilic chains of the copolymer. It is generally used to encapsulate drugs that are poorly soluble in water, opening the possibilities for drugs that could not be used in the past, by enhancing their aqueous solubility, in vivo stability, pharmacokinetics and biodistribution[69–71]. Some anti-cancer agents such as Ethaselen, Paclitaxel and Phorphyrin have improved their solubility and therapeutic properties using this type of DDSs[72].

Polymer-drug conjugates

The first DDS developed was composed by the conjugation of a drug and a polymer by Jatzkewitz in the early 1950s[60]. These polymer–drug conjugates, also called pro–drugs, are delivery systems in which the therapeutic agent is covalently bound to a polymeric carrier, in order to enhance properties like solubility, blood circulation time and release profile[62,71,73]. Opaxio, a combination of Paclitaxel and polyglutamic acid, is a prodrug which is in current clinical evaluation for the treatment of several cancers and expected to enter the market in the near future. For this case, the main benefits when compared to the free drug are passive tumor targeting due to enhanced permeability and retention (EPR) effect, a decrease in toxicity, an increase in solubility, the ability to overpass some mechanisms of drug resistance and the

avoidance of immune response[71]. Many other hydrophilic polymers have been used to form conjugates with drugs, such as polyvinyl alcohol, polyvinyl pyrrolidone, polyglutamic acid, polymalic acid, N-2-hydroxypropyl methacrylamide (HPMA) and polyethylene glycol (PEG). The last two have been widely investigated in the field[73]. HPMA has been used because of its degradability profile, crystalline nature and ability to be functionalized with a variety of ligands. As carrier, HPMA has been used for delivery of anti–cancer agents, such as doxorubicin, paclitaxel and platinates; and to treat musculoskeletal diseases such as prostaglandin E1, a bone anabolic agent used for the treatment of osteoporosis[61,73]. On the other hand, PEG–drug conjugates, which is the main polymer of used for this purpose, has been employed in a variety of molecules including insulin, daunorubicin, camptothecin and adenosine deaminase, among others. The main advantages of PEG–drug conjugates are reduced protein immunogenicity, increased residence time in the body and reduced enzymatic degradation. It is currently used in several commercially available drugs for cancer, immune diseases and rare syndromes, among others[73,74].

Protein carriers

A subgroup of the polymer–drug conjugates consists of proteins that serves as backbone for the delivery of therapeutic agents. Proteins are natural polymeric macromolecular structures with unique functionalities that can be advantageous in the biomedical field. Their amphiphilic nature allows them to effectively interact with both the drug and biological environment during drug delivery. Also, protein nanocarriers are biodegradable, biocompatible, and its surface can be easily modified to attach drugs and targeting ligands due to their defined primary structure. Moreover, they can be synthesized under mild conditions without the use of harmful chemicals, like organic solvents. These nanocarriers have been successfully synthesized from various proteins including water–soluble proteins like bovine and human serum albumin, and insoluble proteins like zein and gliadin. Currently, a combination of paclitaxel and human albumin (Abraxane) is commercially available for the treatment of breast and pancreatic cancer. On the other hand, even when protein nanoparticles have successfully been used for cancer therapy, research that compares its performance and therapeutic efficiency with other more widely used drug delivery systems is still needed in order to expand its use on other type of drugs[2,75,76].

Dendrimers

Polymeric structures with a hyper branched and well-defined spherical shape are known as dendrimers. This unique structure maximizes the potential for biological interactions by allowing the conjugation of drug, targeting ligands and imaging agents into the surface of nanocarriers. Also, drugs can be encapsulated in the empty spaces between adjacent polymeric branches, protecting the cargo when these biological interactions are not desired. Its major advantage over a polymer–drug conjugate is its very low polydispersity index, making it a great candidate for enhanced drug delivery. The application of these nanocarriers has been studied from small molecules for cancer treatment to bioactive compounds for gene therapy[73]. Studies of dendrimers as potential delivery agents have focused on their use for noncovalent encapsulation of drug molecules, which is based on physical entrapment, hydrophobic interactions, and ionic interactions. Nanocarriers composed of polyamidoamine (PAMAM) nanocarriers can selectively target cancer cells and have been used deliver anti–cancer medications, such as cisplatin and doxorubicin[77]. Positively charged dendrimers composed of chitosan and PAMAM, have been investigated as gene carriers for their ability to form a complex with DNA, which is a negatively charged macromolecule. Chitosan is an abundant natural polymer which is non–toxic, biodegradable and easy to modify; while PAMAM is a polymer with simple synthesis for a tunable structure and size, minimal cytotoxicity, biodegradability and high transfection efficiencies[78]. On the other hand, despite the advances in the development of dendrimers, these nanocarriers have several challenges that affect its performance and that must be overcome in order to achieve commercialization. Dendrimers have relatively small size, which are easily excreted through the renal route. Also, multiple steps involved in the synthesis can affect the homogeneity and integrity between nanoparticle batches. Finally, the conjugation with multiple molecules can result on heterogeneous populations, which could lead to inconsistent and sometimes biologically inactive batches of conjugates during large-scale manufacturing[79].

Polymersomes

Polymersomes are polymer–based vesicles, considered to be one of the most versatile and interesting structure among self-assembled DDSs. They are composed of amphiphilic copolymers and can carry therapeutic agents through the host circulatory system in both the core of the nanoparticle and the polymeric matrix, making them suitable for both hydrophilic and hydrophobic drugs[80]. Recently, Gastromark (ferumoxsil) was briefly marketed in the US and EU (Lumirem). This DDS was composed of superparamagnetic iron oxide with a silicone coating shell and was used as contrast imaging agents in the gastrointestinal track[81,82]. In general, the vesicular structures have demonstrated to achieve high drug loading capacity, stability in biologically relevant environments, prolonged circulation time in the bloodstream, controlled drug release and selective accumulation at the site of action. They have been used for the development for the encapsulation and controlled release of therapeutic agents ranging from small molecules for cancer treatment to biologically relevant macromolecules like genetic material and therapeutic proteins[6]. The efficient loading of significant quantities of doxorubicin into the aqueous core and paclitaxel into the matrix of polymersomes composed with PEG and polyester have made possible the use of a dual drug anti–cancer treatment in a single nanocarrier[83,84]. Concerning to gene therapy, short and long segments of DNA have been encapsulated in polymersomes composed of the diblock copolymer polybutadiene-b-N-methyl-4-vinyl pyridinium (PBD-P4VPQI). Assessment of these PBD-P4VPQI polymersomes demonstrated to be effective for in vitro release and transfection on HeLa cells[85,86]. On the other hand, protein drugs like cytochrome C have been successfully encapsulated in pH–responsive polymersomes and showed to have antitumor response on an in vitro prostate cancer cells model[87].

The popularity of using polymersomes over liposomes for biomedical applications is growing because of its tunable properties, which can be controlled by the design of amphiphilic polymers, opening the possibilities to adapt to a wide range of therapeutic purposes. Their demonstrated biocompatibility, biodegradability, stability and easy functionalization, among many other advantageous properties, would allow obtaining nanocarriers with high loading capacity, controlled release, higher uptake in the site of action and extended shelf and circulating time[6,80,88].

1.3.6 Background on polymers being used - PEG and PCL

Opsonization is the process in which a foreign organism or particle becomes covered with opsonin proteins, thereby making it more visible to phagocytic cells. This increases the efficacy of the phagocytic process, which consists in the engulfing and eventual destruction or removal of foreign materials located in the bloodstream. Together, these two processes form the main clearance mechanism for the removal of undesirable bloodstream components larger than 8nm, the renal threshold limit from the blood[89,90]. In the case of polymeric nanoparticles, which cannot normally be destroyed by the phagocytes, sequestration in the mononuclear phagocytic system (MPS) organs typically occurs[91–93]. Since the initial opsonization of particles is so critical to the process of phagocytic recognition and clearance from the bloodstream, most research in the area of stealth drug delivery has focused on trying to stop or block this stage of the process. There are no absolute rules or methods available to completely and effectively block the opsonization of nanoparticles, but the research carried out over the last 3 decades has found some trends and methods that can be effective at slowing down the process, thus increasing the blood circulation half-life and effectiveness of stealth devices[94–96]. A correlation between surface charge and opsonization has also been demonstrated in vitro, with research showing that neutrally charged particles have a much lower opsonization rate than charged particles[97]. Therefore, one widely used method to slow opsonization is the use of surface adsorbed or grafted shielding groups, which can block the electrostatic and hydrophobic interactions that help opsonins bind to particle surfaces. Some examples of polymer systems that have been reported in the literature as shielding groups include polysaccharides, polyacrylamide, polyvinyl alcohol, poly-Nvinyl-2-pyrrolidone, polyethylene glycol (PEG), and PEG-containing copolymers. PEG chains are always available even after the degradation of surface layers. The purpose of these PEG chains is to create a barrier layer to block the adhesion of opsonins present in the blood serum, so that nanoparticles remain invisible to the phagocytic cells[89].

<u>Polyethylene Glycol</u>

Polyethylene Glycol (PEG) is a synthetic hydrophilic polymer composed of repeating ethylene glycol units ($-[CH_2-CH_2-O]_n-$), where the integer *n* is the degree of polymerization (number of times that the unit is repeated in the polymeric chain)[98]. Of all the polymers tested to date, the most effective and widely used is PEG and the PEG-containing copolymers, which are typically

very flexible, highly hydrophilic and can help to protect even hydrophobic or charged particles from bloodstream proteins. Also, they are also commonly charge neutral, which lessens the effect of electrostatic interactions[89] Moreover, PEG have several ideal properties like very low toxicity, excellent solubility in aqueous solutions and extremely low immunogenicity and antigenicity. Even though it is known to be non-biodegradable, PEG is readily excretable after administration into living organisms and its presence in aqueous solutions doesn't showed deleterious effect on protein conformation or the activity of enzymes. PEG also exhibits excellent pharmacokinetic and biodistribution behavior. It shows high persistence in blood compartment and low accumulation in reticuloendothelial system (RES) organs, liver and spleen on experiments made on animals. It is commonly used to form bioconjugates because PEG has the propensity to exclude macromolecules, like proteins and particles, from its surroundings. These advantageous properties have been explained by the high mobility of its polymeric chains associated with their ability to bind with water and its conformational flexibility[74,99]. Also, the modification of nanocarriers by conjugating PEG on the surface reduces many undesirable side effects triggered by biological recognition mechanisms. For instance, they cause prolonged systemic circulation, which results in passive targeting of DDSs even in the areas with a compromised vasculature such as infarcts and tumors by the enhanced permeability and retention (EPR) effect[74,100]. Most of the DDSs with polymer-based stealth properties that have reached the market contains PEG (PEGylated products)[74].

Polycaprolactone

Polycaprolactone (PCL) is a synthetic hydrophobic polymer composed of repeating units of $[-(CO-(CH_2)_2-CH_2-(CH_2)_2-O)_m-]$, where the integer *m* is the degree of polymerization (number of times that the unit is repeated in the polymeric chain)[101]. It was one of the earliest polymers synthesized in the early 1930s and is has the ability to be degraded by microorganisms, which is the main reason that it became commercially available in the first place[102]. PCL has superior rheological and viscoelastic properties over many of its counterparts, which renders it easy to manufacture and manipulate into a large range of scaffolds[103–106]. PCL can be used in a wide range of scaffold fabrication technologies and it is relatively inexpensive compared with other aliphatic polyesters, being then, tremendously advantageous over its counterparts. Since, PCL can be degraded by lipases and esterases, it is ideal to use when the target is the lysosome,

which contains a high concentration of these hydrolytic enzymes. Even when this polymer is more susceptible to degradation by outdoor living organisms compared to animals, like bacteria and fungi, it is also bioresorbable, a process that process takes much longer than biodegradability, mainly degrading via hydrolytic degradation, making it a good candidate for long-term drug release[31,107–112]. A long-term study of the in vivo excretion PCL discovered that an implant made out of this polymer gradually lost strength and broke into pieces. Then, low molecular weight PCL pieces were metabolized and ultimately excreted from the body through urine and feces, eliminating the possibility of accumulation in any body organ[110].

Moreover, PCL is also suitable for controlled drug delivery due to a high permeability to many drugs, excellent biocompatibility and its ability to be fully excreted from the body once it is bioresorbed. Since, biodegradation of PCL is slow in comparison to other polymers, it is most suitable for long-term delivery extending over a period of more than 1 year. PCL also has the ability to form compatible blends with other polymers, which can affect the degradation kinetics, facilitating tailoring to fulfill desired release profile. It is currently used in a wide variety of applications like sutures to wound dressings, artificial blood vessels, nerve regeneration, drug-delivery devices and bone engineering applications[113,114].

PEG-PCL copolymers

Synthetic amphiphilic block copolymers from PEG (hydrophilic block) and PCL (hydrophobic block) have been extensively studied because they allow the modification of physical and chemical properties, leading to countless applications in the biomedical field. PCL–PEG takes advantage of the properties of both polymers in order to improve biodegradability and enhance the performance of the copolymer, compared with any of the homopolymers alone. This copolymer also showed higher hydrophilicity and better performance in the cell culture studies than the PCL homopolymers, being a better option for a wide variety of applications, like development of bioresponsive nanoparticles and thermosensitive hydrogels, among others[115].

PEG-PCL biocompatibility

In order to design new polymeric DDSs, is fundamental to understand the biocompatibility of the materials intended to be used in close contact to biological systems, especially at a cellular and tissue levels. The influence of PEG segments on the biocompatibility of PCL–PEG copolymer has showed improvement in the hydrophilicity and inhibition of bacterial adhesion on its surface. With an increase in PEG/PCL ratio, PCL–PEG had better cellular response as well as lower degree of platelet and monocyte activation[116,117]. It has been suggested that protein absorption (opsonin) could be prevented by the flexible and hydrophilic chains of PEG by forming dense conformational clouds in the surface of nanoparticles, thus avoiding capture by the mononuclear phagocyte system (MPS)[118]. The combination of PEG and PCL polymers have shown low toxicity on several cellular lines such as liver hepatocellular (HepG2) cells, human breast adenocarcinoma (MCF-7) cells and normal human fibroblast cells, to mention a few. These characteristics make them promising candidates for a wide variety of applications and has been tested as biofunctionalized nanofibers, semi-interpenetrated hydrogels and gene carriers with alpha-cyclodextrin molecules, to mention a few examples[119].

PEG-PCL degradation

Usually, studies on degradation of PEG–PCL copolymers are focused on the degradation of PCL and metabolism, since PEG in non-degradable[120,121]. There are two mechanisms that polymers can undergo in order to break down into monomers. The process is called passive occur by hydrolysis and active when enzymes are responsible to cleave the polymeric bonds[122]. Both mechanisms play a role in the degradation of PEG–PCL copolymer, but it depends on the type of bond within the polymer backbone is what determines the type and rate of polymer erosion. In vitro experiments have showed that hydrolysis causes degradation of PCL in both the copolymers and the homopolymers, which is slow, as we have mentioned before[123]. On the other hand, in vivo studies have shown a more rapid degradation when compared with in vitro due to the presence of enzymes that can cleave the ester bonds in PCL[111]. Additional to enzymes, other factors like mechanical stress, temperature, pH can also affect the rate of degradation[112]. There have been found that the degradation of PCL at low molecular weight lead to a second stage of degradation mainly involving intracellular phagocytosis[124]. The final stage of degradation of PCL led to water (H_2O), carbon dioxide (CO_2) and 5-hexenoic acids as products[125]. The

combination of both PEG and PCL characteristic, biocompatibility and degradation profile makes them an excellent alternative for drug delivery applications.

1.3.7 Polymersomes as candidates to entrap and deliver protein therapeutics

Therapeutic proteins have emerged as an alternative to small molecules for their high potency, selectivity and low toxicity. These drugs are currently covering around 10% of the pharmaceutical market, with more than a 100 approved treatments and a fast growing when compared to small molecules[126,127]. Since protein drugs can be designed to target a wide range of molecules, they are suitable to use for diagnosis, as vaccines and to bind with a specific target in order to interfere, regulate or improve a specific biological activity, which includes the case of replacing absent or defective enzymes for LSDs[128]. These peptide and protein therapeutics have some disadvantages. They may have poor membrane permeability due to low lipophilicity and charged functional groups within the molecule. Also, they are highly susceptible to pH, proteases and opsonization, which cause rapid metabolism and loss of biological activity due to conformational changes, dissociation of subunits within the protein, complexation with blood products and destruction of functional groups[127,129].

A promising option to enhance the efficacy of ERT and other therapeutic proteins is the use of nanocarriers for drug encapsulation and delivery. The first strategy to solve setbacks associated with protein drugs was their conjugation with polymers in order to enhance its therapeutic properties. Protein drugs have been conjugated with PEG in order to achieve stability, increase blood circulation time and decrease immune response[74,130,131]. On the other hand, a major drawback associated with PEGylation is the covalent attachment of the PEG molecule to the protein, which is nonspecific and can lead to changes in the protein conformation and structure, thus limiting its therapeutic activity[132]. Encapsulation in liposomes has been also used as a route to carry and protect protein therapeutics and, even when they have demonstrated to prolong circulation time, this method also has disadvantages. Unfortunately, these DDSs have shown to be unstable, leaky, non–suitable for controlled release and can be metabolized quickly by the body, even if polymers were used to modify the surface of the nanocarrier[67,68,133,134].

Contrary to these methods, encapsulation of proteins in polymersomes has been successfully carried out without losing their functional conformation. Combining this with other proven

advantages, such as controlled release and tunable properties (molecular weight, stability under different environments, biodegradability, blood circulation time, permeability and responsiveness to stimuli, among others), protein therapeutics can be tremendously improved[6,16,80]. For example, in one study, polymersomes encapsulated model proteins using diblock copolymers without the need of further surface modification. Opposing to liposomes, they showed resistance to high temperatures, some of them even surviving autoclaving. Also, they were stable in blood plasma. Furthermore, phagocytosis was not stimulated and cultured cells were unaffected in the presence of the nanoparticles[135]. On another study, a fluorescent protein was used to demonstrated that proteins were indeed encapsulated in the aqueous core of the polymersomes[136]. Furthermore, polymersomes loaded with different enzymes and proteins like hemoglobin, myoglobin, glucose oxidase and β-lactamase have proven to remain functional after encapsulation[137–140]. Polymersomes prepared using PEG–PCL block copolymers and loaded with hemoglobin demonstrated to have similar oxygen affinities to human red blood cells, thus proving its functionality after encapsulation[141]. In another study, polymersomes functionalized by outer membrane proteins (OmpF), have proven to serve as nanoreactors by the hydrolysis of ampicillin into ampicillinoic acid throughout β-lactamase. In this case, the encapsulated enzyme reacts with the substrate, which enters into the polymersomes through the channels formed by OmpF on the polymeric matrix[140].

Numerous polymersomes have been designed to respond to external stimuli, such as pH, oxido-reductive conditions, temperature and enzyme degradation. This response is controlled by both the chemical structure of the individual polymer chains and their self-assembling process[142]. For example, oxidation-responsive polymersomes have been demonstrated by nanocarriers composed of PEG and polypropylene sulfide (PPS), in which the glucose oxidase was entrapped and served as oxidizing agent when exposed to glucose in order to form hydrogen peroxide. This oxidation product then helped to break down the polymeric matrix of the polymersome[137,143]. Also, polymersomes containing an ionizable membrane were prepared with PEG and polytrimethylene carbonate, demonstrated to respond to pH by significantly increasing release of the encapsulated protein in lysosomal pH compared to physiological pH[144].

For purpose of this research project, polymersomes were chosen since they are the best available option to encapsulate proteins on PEG–PCL block copolymers as proof of concept to

demonstrate that ERT can be enhanced. The general idea is to increase ERT efficiency and delivery into the lysosome, then reducing the required dosage, the costs and side effects associated with the treatment. Since they can enclose the hydrophilic therapeutic cargo into the nanoparticles inner core, there's no need to covalently bound the cargo, which will avoid conformational and structural changes that would negatively affect proteins functionality[70,132,135–141]. The entrapment in polymersomes composed of PEG–PCL will also allow enhanced protection from both host immune system and degradation while traveling throughout the circulatory system[1456,16,80,89,127,129,145]. PEG–PCL is an ideal copolymer for intralysosomal delivery because PEG can protect the cargo until reaching the lysosome, while PCL susceptibility to lysosomal environment and esterases, which will allow the release of the therapeutic protein in this action site[31,74,99,104,106,108]. These nanocarriers will be able to enhance the internalization of non–modified proteins, thus eliminating the current need of M6P tag in enzymes used for ERT, the diminishing cost and increasing the efficacy of the treatment[52,53,53–57,59].

2. OPTIMIZATION OF PEG-PCL BLOCK COPOLYMERS SYNTHESIS

Amphiphilic PEG–PCL block copolymers were synthesized using two different catalysts, in order to determine best option for further use in the formation of polymersomes for protein encapsulation and delivery.

2.1 Materials

Unless otherwise noted, all materials were used as received. In order to remove water from the reagents, monomethyl polyethylene glycol (mPEG, Sigma-Aldrich, molecular weight = 5000 g/mol and 2000 g/mol) was dried by azeotropic distillation in the presence of toluene (Fisher); epsilon-caprolactone (ε-CL, Acros) and dichloromethane (DCM, Acros) were dried by distillation in the presence of calcium hydride (CaH2, Alta Aesar). Stannous octoate (SO, MP Biomedicals) and hydrochloric acid (HCl, Acros) were used as catalysts for the polymerization reaction. Other solvents used were tetrahydrofuran (THF), hexane (Fisher-Scientific), diethyl ether (Acros) and deuterated chloroform (Sigma-Aldrich).

2.2 Synthesis of PEG-PCL block copolymers by Ring Opening Polymerization

Polyethylene glycol – polycaprolactone (PEG-PCL) block copolymers were synthesized, based on the techniques employed by Olayo et. al.[146], Azzam et. al.[147], Liu et. al.[148], Oh et. al.[149] and Hyun et. al.[150]., by the ring-opening polymerization (ROP) of epsilon-caprolactone (ε-CL), using polyethylene glycol methyl ether (mPEG) as the initiator (either 5000 g/mol or 2000 g/mol). Either stannous octoate (SO) or hydrochloric acid (HCl) were used as the monomer activator (catalyst) and dichloromethane (DCM) as solvent.

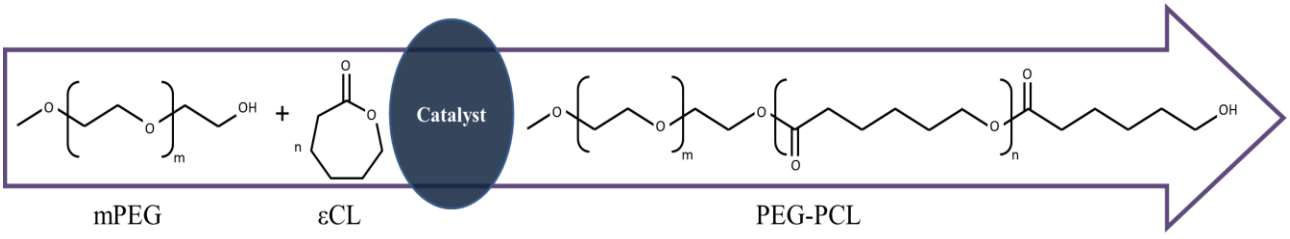


Figure 2.1: Chemical reaction for the synthesis of PEG-PCL block copolymers

2.2.1 Reagents distillation

Since the polymerization needed to be carried out on an environment free of oxygen and water, reagents were previously purified to be water-free and were stored on an inert environment (glovebox on N2 gas) for further use. Oven dried water-free flasks were used as mixers and reagent collectors. To ensure proper material transfer from the mixing flask to the collector flask, insulating materials were used throughout the pipelines of the system, an oil bath was used for heating and an ice bath was used under the collector flask, as presented in Figure 2.2

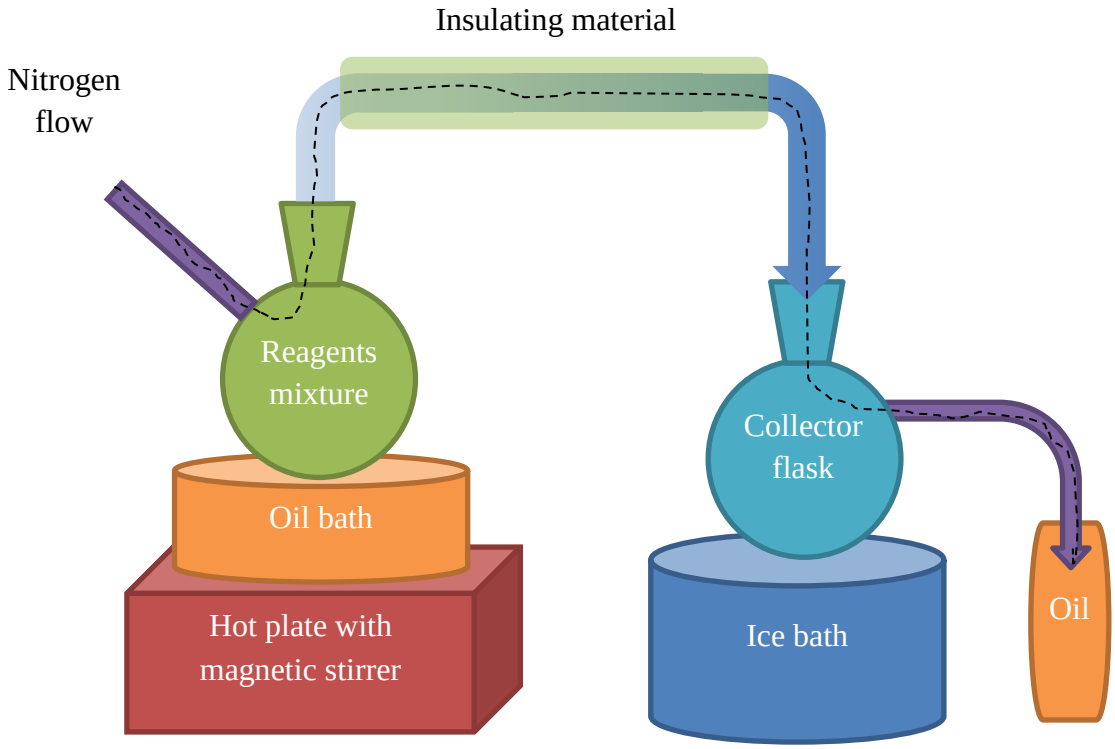


Figure 2.2: Distillation system to purify reagents for polymerization of PEG-PCL copolymer

Polyethylene glycol

Since water and toluene forms an azeotrope, an azeotropic distillation there was carried out to eliminate water absorbed on the PEG. Approximately 10g of PEG and 100 mL of toluene were mixed under magnetic stirring for 24 hours. Then, the mixture was heated to 130°C under a nitrogen atmosphere until all the water-toluene mixture was evaporated from the PEG (approximately 8 hours).

Dichloromethane

Dichloromethane (DCM) was distilled in presence of CaH_2 to eliminate its water traces. Approximately 200 mL of DCM were mixed with a tablespoon of CaH_2 under magnetic stirrer for 24 hours. Then, the mixture was heated at 90°C under a nitrogen environment for approximately one hour, until all the DCM was evaporated and collected on another water-free flask.

Epsilon-Caprolactone

Liquid ε-CL we also distilled in presence of CaH_2. Approximately 200mL of ε-CL were mixed with a tablespoon of CaH_2 under magnetic stirrer for 24 hours. Then, the mixture was heated to 110°C under reduced pressure (generated by a vacuum pump) until all the ε-CL was transferred to a water-free flask (approximately one hour).

2.2.2 Polymerization of PEG-PCL by ROP

All reagents were added in a glovebox under N_2 atmosphere in order to avoid contamination with water and oxygen.

Polymerization by SO

To prepare copolymers using SO as catalyst, an hermetic glass bottle was needed to serve as the reactor. For example, to prepare PEG2000-PCL5000 (Mw,PEG=2000 g/mol; Mw,PCL= 5000 g/mol), a mixture of 1.000 g of mPEG (0.500 mmol), 2.427 mL of ε-CL (21.9 mmol), and 5 μL of SO was added to the reactor, sealed and taken out of the glovebox to polymerize at 110-130°C for 20 hours using a bath oil. The product was dissolved in approximately 15mL of DCM and added to approximately 60mL of a mixture of 50/50 hexane/ether at 4°C (4x the volume of DCM) in order to precipitate the formed copolymer and was stored on the fridge. After 24 hours, the copolymer was isolated by centrifugation at a speed of 7000rpm for 15 minutes. Samples were then dried in a vacuum oven at 60°C in order to eliminate remaining solvents for a minimum of 24 hours.

Polymerization by HCl

For the polymerization using HCl as catalyst, addition of DCM to the polymerization mixture was required. For example, to prepare PEG5000-PCL5000 (Mw,PEG=5000 g/mol; Mw,PCL= 5000 g/mol) using HCl, with a conversion of 80%, a mixture of 1.000g of mPEG (0.200 mmol), 1.124 mL of ε-CL (11.0 mmol), 0.600mL of HCl (.6mmol) and 12.5mL of DCM was added to a glass vessel. The mixture was taken out of the glovebox and stirred at room temperature for 8 hours. The procedure for polymer isolation and drying was the same as the previous case.

2.3 Characterization of PEG-PCL copolymers

PEG-PCL copolymers were synthesized by ROP of ε-CL, using mPEG as initiator. They were characterized by gel permeation chromatography (GPC, Figures 2.2 to 2.3) and proton nuclear magnetic resonance (1H-NMR, Figure 2.4). Copolymer properties are shown in Table 2.1.

2.3.1 Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance (NMR) is a spectroscopic technique based on the interaction of magnetic moments of nuclei of certain atoms with magnetic fields in order to determine chemical and physical properties of the studied material. Proton nuclear magnetic resonance spectroscopy (1H-NMR) study specifically the hydrogen atoms in the molecules [151,152]. 1H-NMR was performed in a Bruker Avance 500 MHz 29 spectrometer, using tetramethylsilane (TMS) as internal reference standard. This technique was employed to determine the molecular weight of both polymeric chains in the copolymer. PEG-PCL samples were prepared by the dissolution of 10mg in 1mL of deuterated chloroform. The software used to analyze the samples was XWIN-NMR version 3.5 on BHI11604.

2.3.2 Gel Permeation Chromatography

Gel permeation chromatography (GPC), also called size-exclusion chromatography (SEC), is a technique used to separate and characterize molecules based on their molecular weight. This chromatographic technique consists in a stationary phase composed of polymer beads with pores of different sizes, which separate the sample by size, by retarding the exit of smaller molecules

that have to go through the pores in the beads, while larger molecules will continue to move forward. Then, molecules of the same size will elute together, larger molecules elute first and smaller molecules will elute later [153].

GPC was performed on a Waters GPC system equipped with a BI-DNDC differential refractometer (Brookhaven Instruments) and a mixed pore size column (PLgel 5 µm MIXED C, Agilent Technologies). The GPC calibration curve was prepared with polystyrene standards and the mobile phase flow was set to 0.5 mL/min. THF was used as mobile phase and approximately 10mg of PEG-PCL sample was dissolved in order to have a polymer concentration of 5 to 10mg/mL. Each sample took around 20 minutes to completely elute and three washes of the injection syringe with fresh THF were made before each sample injection. Covalent attachment of ε-CL to PEG and the Polydispersity index of the copolymer were determined by this technique.

2.4 Results and Discussion

2.4.1 Nuclear Magnetic Resonance

Proton Nuclear Magnetic Resonance (1H-NMR) was used to determine the molecular weight of the two chains of the PEG-PCL block copolymer in order to determine whether SO or HCl are better catalysts for the polymerization process. Four copolymers were initially synthesized, two with SO and two with HCl as catalyst, by using PEG5000 and two different of theoretical molecular weight for PCL (2000 and 5000 g/moL). This apparatus used the hydrogen atoms of the molecules to determine the identity of the monomer units and the amount of repetitions of these monomers. The spectrum presented in Figures 2.2 shows the peaks corresponding to PEG and PCL monomers and the area under the curve of each peak is representative of the amount of times that the monomer is repeated in the chain. In this case, PCL peak shows at 4.1 ppm and PEG shows at 3.6 ppm approximately. Although PCL can be found in other parts of the spectra, this specific peak was selected to eliminate any water interference in the measurements. Molecular weight results are presented in Table 2.1. Calculations for the determination of the molecular weight are presented in Appendix 2.2 and all data to generate Figures 2.2 to 2.4 is presented in Appendix 2.3.

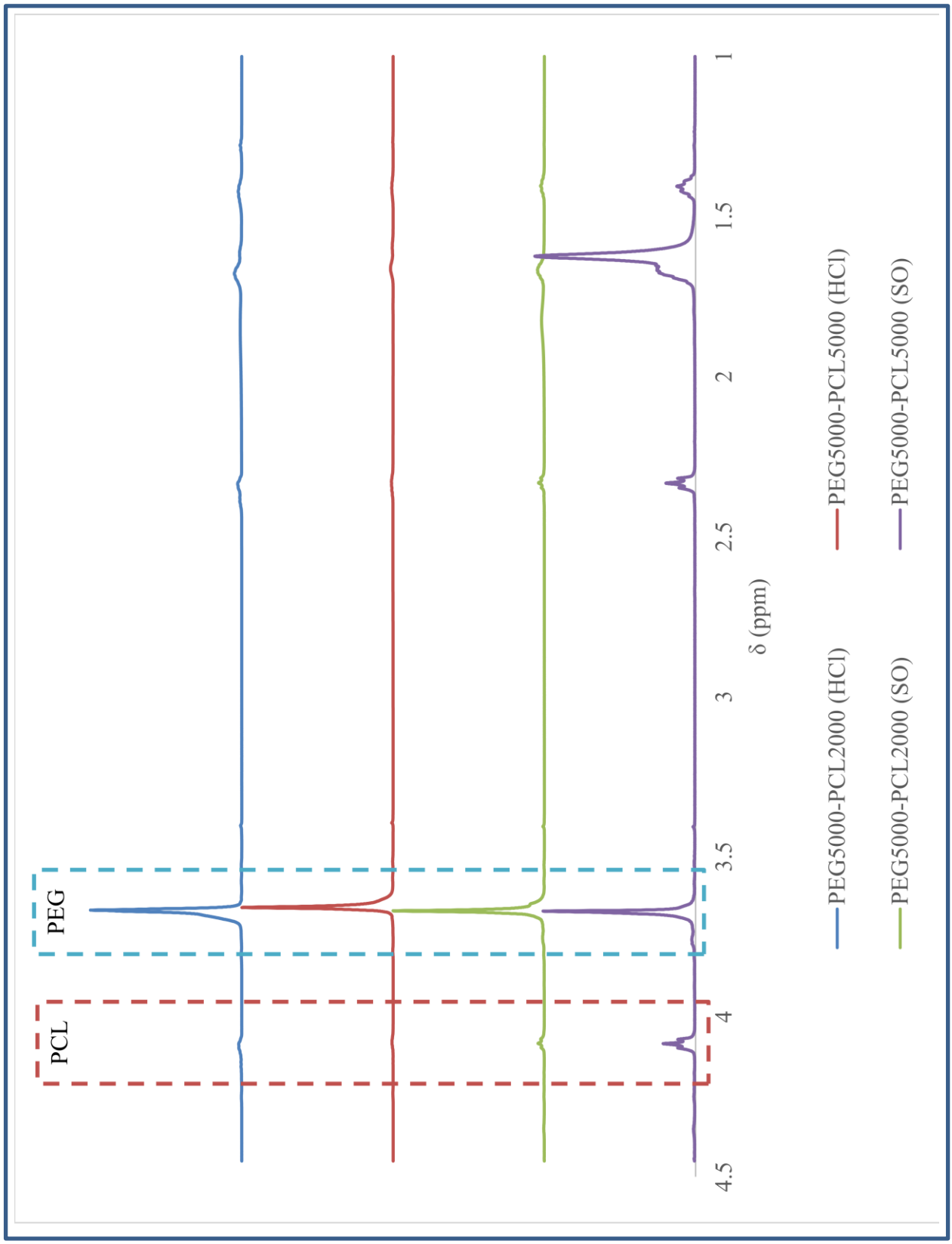


Figure 2.3: Nuclear Magnetic Resonance (H-NMR) Spectrum of PEG-PCL Block Copolymer – Initial approach to determine the optimal catalyst for the polymerization process

Specific peaks of PEG and PCL studied

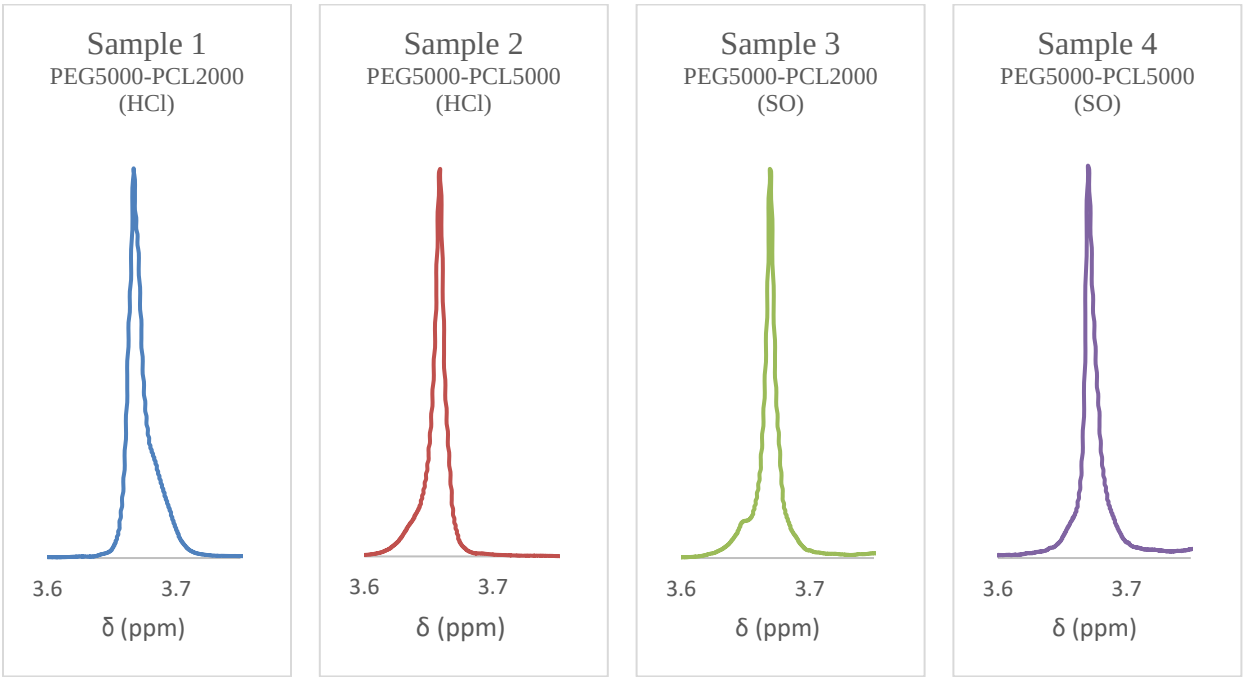


Figure 2.4: 1H-NMR Spectrum for PEG Peak in all Copolymer Samples

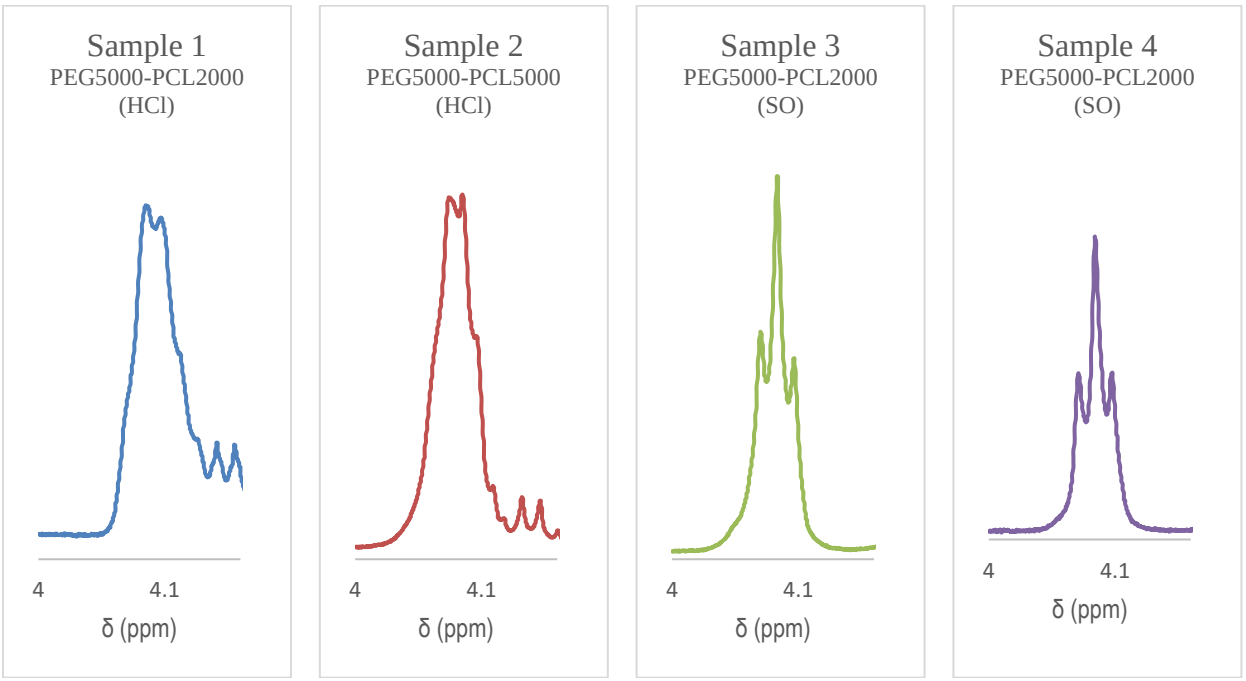


Figure 2.5: 1H-NMR Spectrum for studied PCL Peak in all Copolymer Samples

Table 2.1: Theoretical and experimental PEG-PCL copolymers molecular weight using HCl or SO as catalyst determined by Nuclear Magnetic Resonance

Polymer ID (Sample #)	1	2	3	4
PEG MW theoretical (g/moL)	5000	5000	5000	5000
PCL MW theoretical (g/moL)	2000	5000	2000	5000
Catalyst	HCl	HCl	SO	SO
PEG MW experimental (g/moL)	**5420.8**	**5374.6**	**4407.7**	**4147**
PCL MW experimental (g/moL)	**1624.5**	**655.5**	**1596**	**6509.4**

Table 2.1 shows that the molecular weight of the PEG5000-PCL5000 was much lower than expected. Overall, the molecular weights of PEG:PCL in the case of SO were much closer to the expected values, than those prepared with SO. This led us to the determination that HCl is not as reliable a catalyst for the polymerization of our copolymer of interest. The decision was made that all future copolymers must be synthesized with SO.

2.4.1 Gel Permeation Chromatography

PEG-PCL block copolymers were synthesized by ROP of ε-CL, using mPEG as initiator. Four different versions of PEG-PCL copolymers using SO as catalyst were prepared and characterized by GPC in order to confirm PCL had been covalently attached to PEG. (Figures 2.6 and 2.7). The polydispersity of the copolymers and the initiator monomer are shown in Table 2.2.

Gel permeation chromatography is a technique used to separate molecules by their molecular weight. The stationary phase of the equipment is made of polymer beads with different pore sizes. The smaller molecules will enter the pores, while the larger molecules will continue to move forward. Thus, larger molecules will elute before the smaller ones.

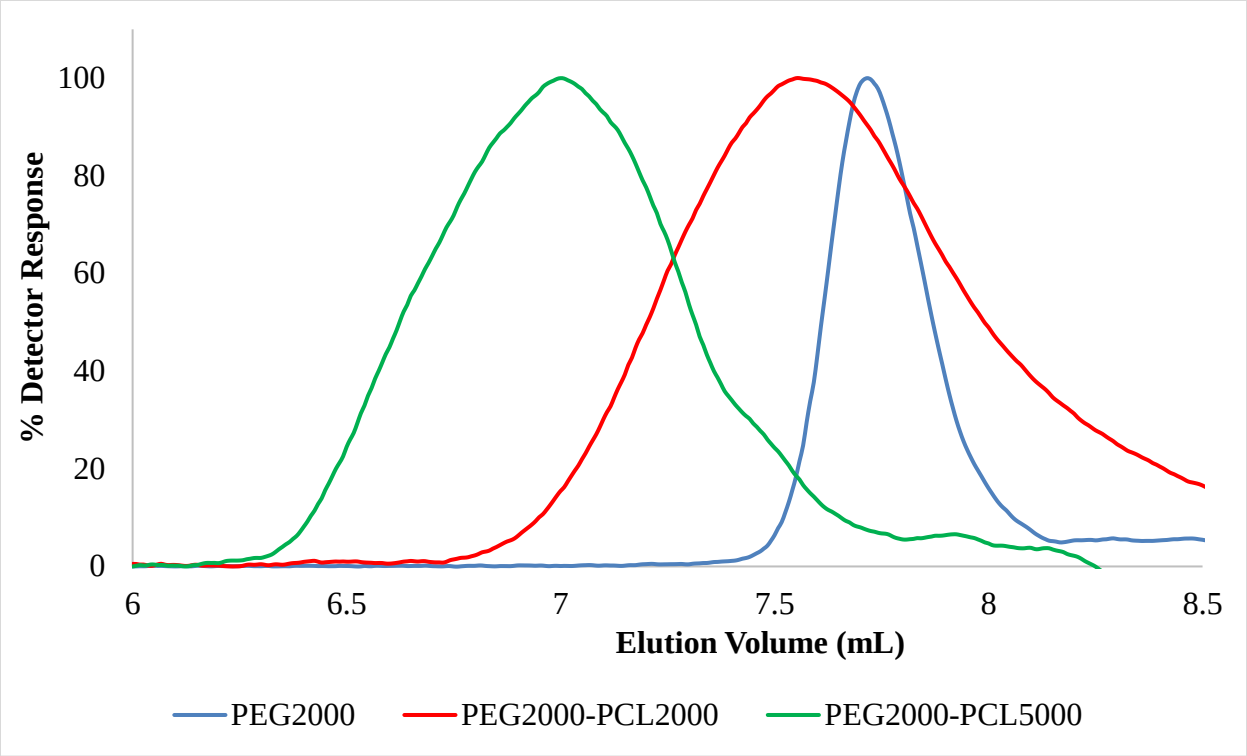


Figure 2.6: Gel Permeation Chromatography of mPEG2000 and PEG-PCL Copolymers

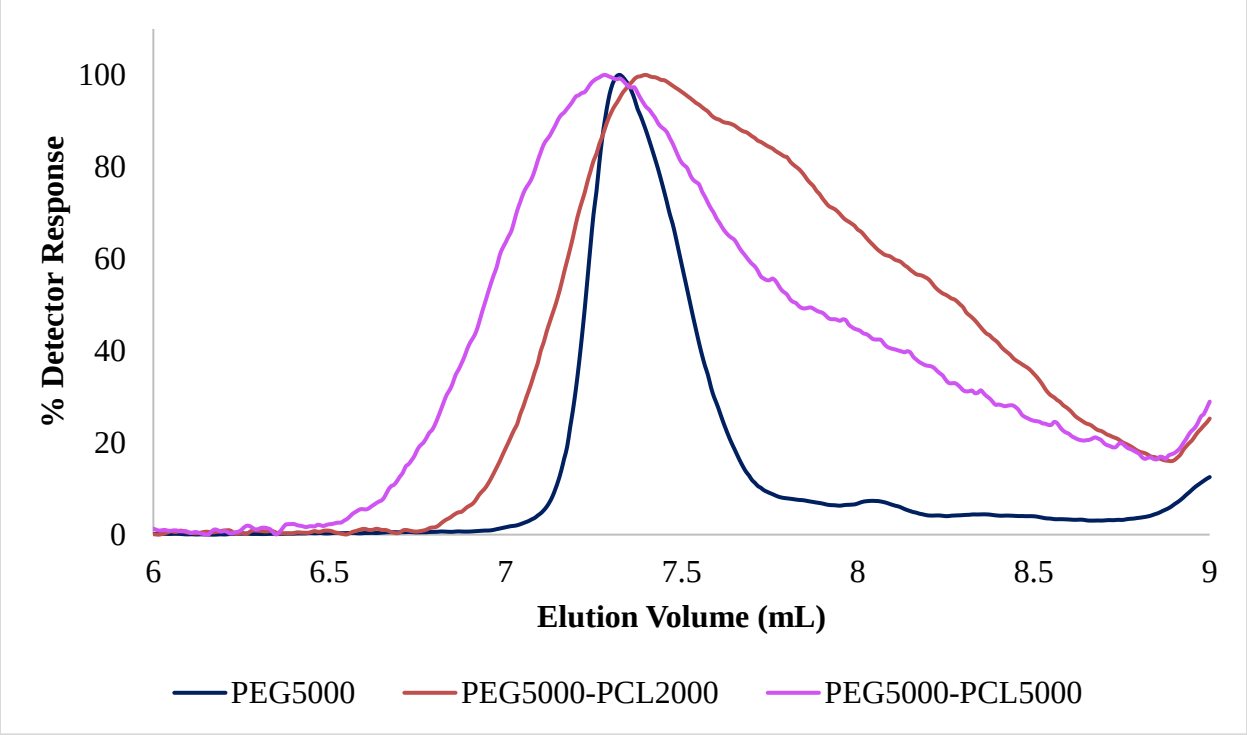


Figure 2.7: Gel Permeation Chromatography of mPEG2000 and PEG-PCL Copolymers

Table 2.2: Elution Volume, Relative Molecular Weight and Polydispersity Results from Gel Permeation Chromatography

Sample Identification	Range of Elution Volume for the Peak (mL)	MWn Number-average molecular-weight	MWw Weight-average molecular-weight	Polydispersity (D)
PEG2000	7.34-8.22	2197	2394	1.09
PEG2000-PCL2000	6.50-8.90	1704	4114	2.41
PEG2000-PCL5000	5.77-7.27	7336	13550	1.85
PEG5000	6.84-7.99	5380	5973	1.11
PEG5000-PCL2000	6.71-8.82	1147	3688	3.22
PEG5000-PCL5000	6.51-8.86	1987	7126	3.59

The polydispersity parameter is obtained from a comparison of the two types of molecular weight calculated by the apparatus and based on the polystyrene standards used for calibration: MWn (Number-average molecular-weight) and MWw (Weight-average molecular-weight). This parameter indicates the heterogeneity of size of the block copolymer chains (size distribution of the copolymers).

In Table 2.2, there can be observed an increase in polydispersity was observed in PEG-PCL compared to mPEG. The polydispersity in the PEG-initiator was approximately 1.1 for both PEGs molecular weights, corresponding to a narrow distribution. For the case of the copolymers, the values of D were in the range of 1.4 to 3.6, corresponding from a medium to a wide distribution. This increase in polydispersity makes sense because we are attaching Caprolactone monomers to PEG, which will cause an increase in the heterogeneity of the sample.

In this table, we can observe that the initial elution volumes in all copolymers started before their respective PEG comparisons, indicating that they are larger than the initial PEG polymer. Also, there is observed that, the bigger the theoretical molecular weight, the earlier the material started to elute, then assuring that bigger polymers were obtained after the polymerization. This confirms that covalent attachment of ε-CL monomers to PEG took place during the synthesis. This behavior is also observed in Figures 2.3 and 2.4, in which we can observe a displacement towards the right for the curves corresponding to the copolymer when compared with PEG alone.

3. OPTIMIZATION OF EXPERIMENTAL CONDITIONS TO OBTAIN POLYMERSOMES WITH DESIRED BEHAVIOR

PEG–PCL polymersomes were prepared varying several experimental conditions in order to characterize and study their behavior under biologically relevant buffers. Our purpose was to achieve the creation of polymersomes that remain stable until they reach the acidic environment of the lysosomal cellular compartment. Based on literature, the expected behavior was that polymersomes prepared with PEG–PCL copolymer were more stable in physiological conditions than in lysosomal conditions. Two parameters were studied to determine if the nanoparticles were having the expected behavior: size over time and protein release in biologically relevant buffers.

3.1 Materials

For polymersomes syntheses, previously synthesized PEG-PCL block copolymers were used as described in Chapter 2. Reagents were used as received unless otherwise indicated. Sodium chloride (NaCl, Fisher), Polyvinyl Alcohol (PVA, Sigma-Aldrich), Sorbitan monooleate (Span 80, Fluka) and PEG-20 Sorbitan monolaureate (Tween, Sigma-Aldrich) were used as received and served as stabilizers for the emulsions. Deionized water was filtered with a .2μm filter, while Dichloromethane (DCM, Acros) was filtered with a .1μm filter. For loaded polymersomes, two versions of Albumin were used as a model protein: Bovine Serum Albumin (BSA, Sigma-Aldrich), and Fluorescein isothiocyanate labelled BSA (FTIC-BSA, Sigma-Aldrich)). Fetal bovine serum (FBS, Life Technologies) was used to form a protein corona for polymersomes and as a cell culture component. Protein loading and release profile were studied by spectrofluorometry using FITC-BSA.

Several buffers were prepared and filtered with a .2μm filter. Sodium Hydroxide (NaOH, Fisher) and Hydrochloric acid (HCl 37%, Sigma-Aldrich) were used to prepare buffers and to regulate the pH of solutions. Phosphate Buffered Saline pH 7.4 (PBS, Sigma-Aldrich) with Sodium Chloride (NaCl), Potassium Chloride (KCl), Disodium Phosphate (Na2HPO4) and Dipotassium Phosphate (K2HPO4) was commercially obtained and mixed with 1L of deionized water. Acetate buffer with pH 4.6 was prepared by mixing 0.1N solutions of Acetic acid and Sodium

acetate in proportions of 102:98. Artificial Lysosomal Fluid with pH 4.5 was prepared by mixing the following reagents in 1L of deionized water: 0.50g of Magnesium Chloride (Sigma-Aldrich), 3.21g of Sodium Chloride, 0.071g of Disodium Phosphate (Fisher), 0.039g of Sodium Sulphate (Fisher), 0.128g of Calcium Chloride Dihydrated (Sigma-Aldrich), 0.077g of Sodium Citrate (Sigma-Aldrich), 6.00g of Sodium Hydroxide, 20.8g of Sodium Citrate (Sigma-Aldrich), 0.059g of Glycine (Sigma-Aldrich), 0.090g of Sodium Tartrate (Sigma-Aldrich), 0.085g of Sodium Lactate (Sigma-Aldrich) and 0.086g of Sodium Pyruvate. These buffers were used to study the polymersome behavior under biologically relevant conditions and to explore model protein release profile.

Culture media was composed of Minimum Essential Medium (MEM, Gibco), Sodium Bicarbonate (Sigma-Aldrich), Ethylenediaminetetraacetic acid 0.02% Solution (EDTA, Sigma-Aldrich), Trypsin (Sigma-Aldrich), and 10% Fetal Bovine Serum (FBS, Life Technologies). Cytotoxicity was measured using the Cell Titer Blue® assay (Resazurin, Promega) diluted in Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich). Cell Titer Blue® was used to measure the metabolic activity of the cultured cells and HBSS was used as a substitute of culture media to maintain pH, osmotic balance and to provide cultured cells with water and essential inorganic ions.

3.2 Preparation of WOW Emulsions with PEG-PCL Copolymers

Empty polymersomes were created to determine the optimal copolymer molecular weight to obtain polymersomes of adequate size, which would remain stable in PBS and show decreased stability in an acetate buffer with a pH 4.6. Afterwards, a model protein was added to continue the optimization process, in order to obtain polymersomes with the desired protein release properties. Finally, we added a protein corona to our polymeric nanocarriers to test the effects it would have on protein release behavior. Figure 3.1 presents a general schematic of the formation of polymersomes and Figure 3.2 offer details of the different variations of the process, respectively.

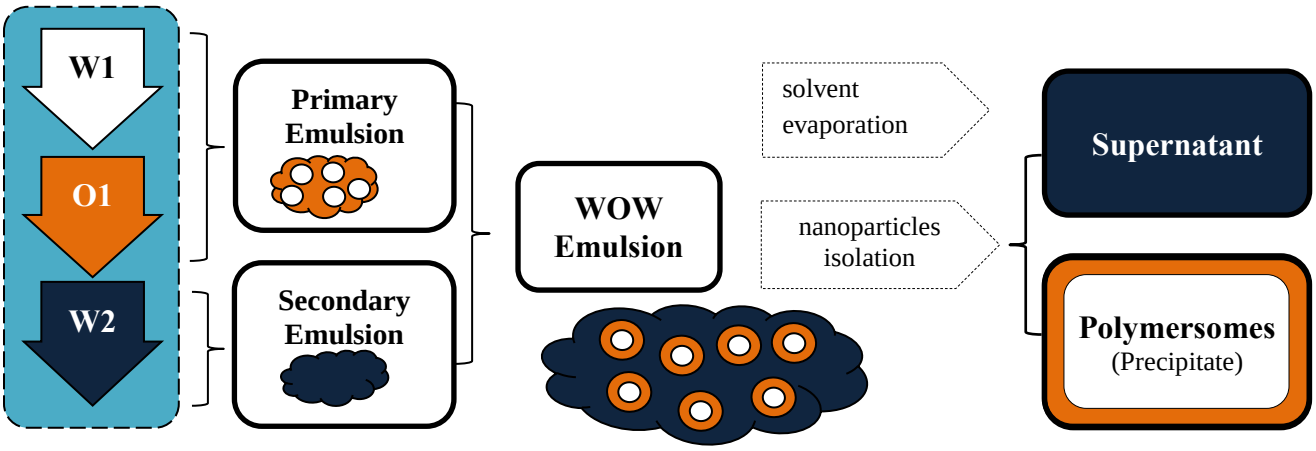


Figure 3.1: Diagram of the Synthesis of Polymersomes by Water-in Oil-in Water (WOW) Double Emulsion Technique

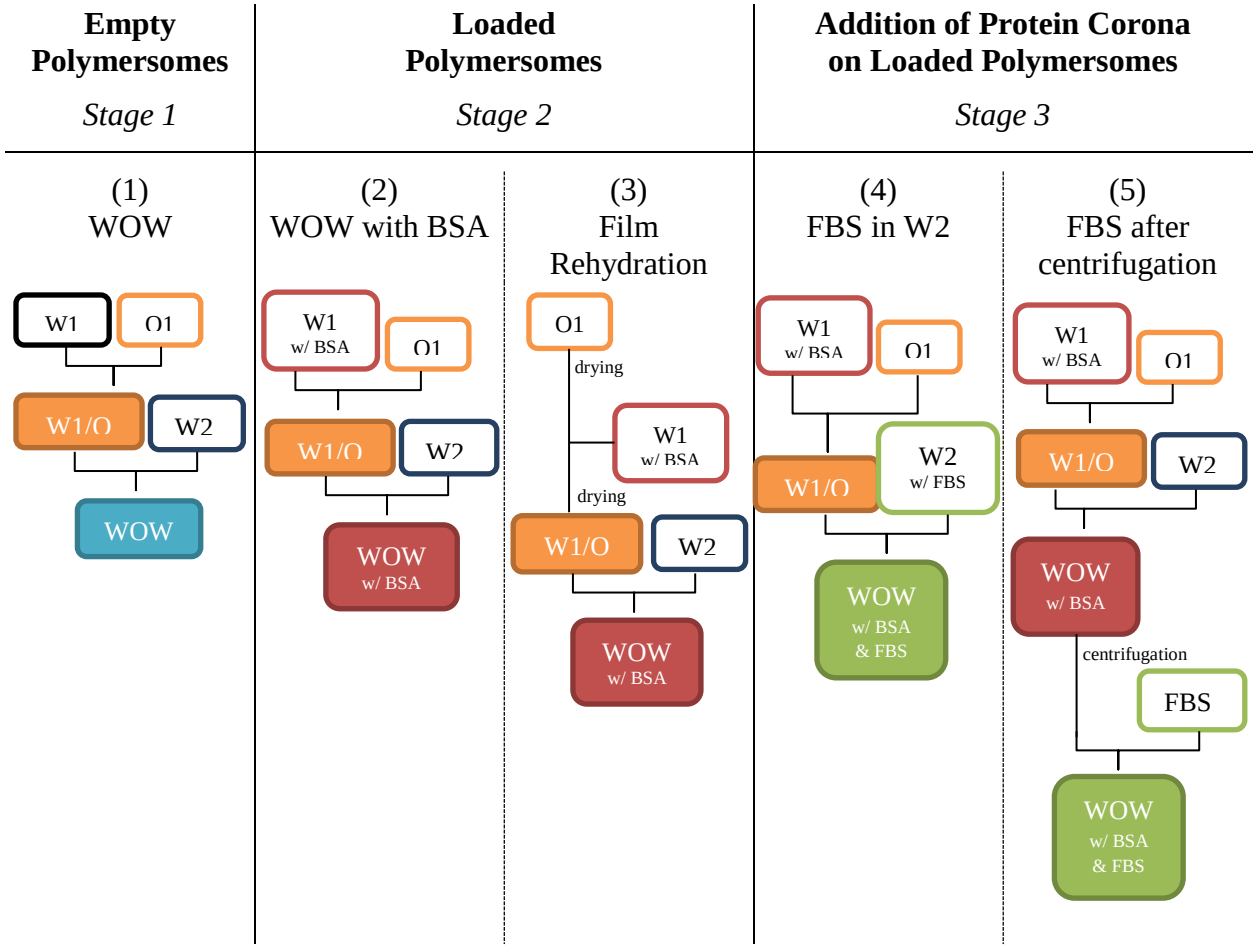


Figure 3.2: Variations of Syntheses of Polymersomes

3.2.1 Preparation and characterization of aqueous cored PEG-PCL nanocarriers, using the Water - in Oil – in Water (WOW) double emulsion method

Aqueous-cored vesicular nanocarriers were prepared by WOW double emulsion method, a type of solvent evaporation method. PEG-PCL polymeric nanocarriers (polymersomes) were prepared by a water/oil/water (WOW) double emulsion method, using an adaptation of the protocols established by Kim et. al.[154] and Rastogi et, al [12]. As an initial approach for the optimization of the process, a PEG-PCL/PVA ratio of 1:2 was selected and non-loaded nanoparticles were made using various copolymer molecular weights. In the aqueous core (W1), 1mL of water was used. The polymeric outer layer was made by dissolving PEG-PCL into DCM and Span (O1). These two solutions were mixed by sonication (Branson Ultasonic Corporation Model 450) for one minute at a 50% amp to form the primary emulsion (W1/O1). The secondary emulsion (W2) was made by dissolving PVA into a saline solution containing 0.5% w/v of NaCl and 0.1% v/v of Tween by sonication for 5 minutes. Both emulsions were mixed by sonication for 1 minute at 30%Amp to form the WOW emulsion. The final product was mixed for 24 hours at atmospheric pressure to let the solvent evaporate. The emulsion was dialyzed and freeze dried, in order to obtain water-free polymersomes for further studies. Samples were frozen at -80°C, freeze-dried at -49°C and a pressure less than 0.800mBar for 48 hours. Finally, they were stored at 4°C, in vacuum bags containing drierite to avoid moisture.

The variations in molecular weight were as follows: PEG2000-PCL2000, PEG2000-PCL5000, PEG5000-PCL2000 and PEG5000-PCL5000. Size, behavior under biologically relevant buffers and literature were used to determine the optimal chain length in order to obtain a model polymersome to carry out protein loading and release experiments.

3.2.2 Addition of model protein into the aqueous core of the polymersomes

After determining the appropriate PEG-PCL copolymer chain size, the model protein BSA and other variables were incorporated into the emulsion process. Most of the procedure remained the same, with a few changes. Throughout the syntheses, an ice bath was used to avoid protein denaturation during the mixing and sonication processes. In the aqueous core, water was replaced for PBS in order to avoid agglomeration and misfolding of the model protein. 50mg of BSA was dissolved into 1mL of PBS in order to form W1. To form O1, 1mL of Span was

dissolved into 10mL of DCM and a variable amount of PEG-PCL. As in the previous case, O1 and W1 were mixed by sonication for 1 min at 50%amp. For W2, fixed values were used: 500mg of PVA, 50µL of Tween and 50mL of a saline solution with 1mg/mL of NaCl. The dissolution of W2 was replaced to mixing and heating at 70°C in a water bath for 1 hour in order to completely dissolve the PVA. Before further processing, W2 was allowed to cool down. Then, W1/O1 was added to W2 and mixed by sonication at 50%amp. Solvent was left to evaporate for 4 hours under either reduced pressured (generated by a vacuum pump) or atmospheric pressure. Samples were centrifuged at 14, 000 rpms for 1 hour at 4°C. Both precipitate (polymersomes) and supernatant were conserved. A procedure known as film rehydration technique was also tested to incorporate model protein into the aqueous core of the polymersome, and will be discussed below.

Experimental variations

- 3.2.2.1. *PEG-PCL/PVA ratio*: According to the suggested composition for preparation of nanocapsules by the double emulsification method founded by Mora-Huertas et. al.[145], the polymer in the organic phase must be between 5 to 10 percent of the organic phase solvent. PEG-PCL/PVA proportions of 1:2, 1:1 and 2:1 were used. This was achieved but keeping the amount of PVA constant and varying the amounts of the PEG-PCL copolymers. Amounts of 250mg, 500mg and 1000mg of PEG-PCL were used.
- 3.2.2.2. *Solvent evaporation pressure*: In order to make a transition between the parameters used for empty and loaded polymersomes, pressures of 1atm and 0atm (vacuum) were used for the solvent evaporation process.
- 3.3.3.3. *Incorporation of Film Rehydration Technique*: An alternative procedure of polymersome synthesis was carried out. This is an adaptation from the technique proposed by Martin et al. [155]. This technique is a hybrid of film rehydration technique [83,156] and the Water-in Oil-in Water double emulsion method[17]. A polymeric layer was made by mixing reagents from O1 (500mg PEG-PCL, 10mL of DCM and 1mL of Span) and transferring then to a 600mL glass beaker. The solvent was left to evaporate at room temperature in the fume hood extractor for 24 hours, after which a thin film of polymer was formed. When O1 was completely dry, the inner aqueous phase (W1), consisting of 1mL of PBS and 50mg of BSA, was added to the surface of the polymeric film and was left to dry for another 24 hours at

room temperature. The polymeric film was then suspended in 10mL of DCM and transferred to a centrifuge tube for full dissolution. This constitutes the equivalent of the Primary Emulsion (W1/O1).

An outer aqueous phase (W2) was prepared as usual. 500mg of Polyvinyl Alcohol (PVA), 50mL of a 1mg/mL saline solution, and 50µL of Tween were mixed by magnetic stirring at 70°C, in a water bath for one hour. This step is necessary in order to completely dissolve the PVA in the mixture. When the W2 was cool, the Primary Emulsion was added to W2 and sonicated for 1 minute at 30% amp. The organic solvent was evaporated by magnetic stirring, under vacuum and an ice bath for 4 hours. Then, the polymersomes were centrifuged and freeze dried as usual.

A variant of the incorporation of film rehydration technique was made by eliminating Span from O1. This sub-part of the experimental variables ere added because, during the initial process of forming the thin film of polymer, the addition of the surfactant Span caused agglomeration of the O1 and the thin film didn't form adequately. Then, the vessel used to form the thin film was replaced by another option, causing that the polymeric film could be done in both presence and absence of Span, then making possible a comparison of both scenarios.

3.2.3 Addition of a Protein Corona to the polymeric nanocarriers

After the optimization of the protocol to obtain our protein-loaded polymersomes, Fetal Bovine Serum (FBS), a protein-rich serum, was incorporated into the nanoparticles formulation by two different methods.

- 3.3.3.1. Addition of 10% v/v of FBS to the components of W2 during the dissolution process that forms the secondary emulsion. In this case, 5mL of the 50mL of water used to form the secondary emulsion were substituted with 5mL of FBS. The rest of the components (PVA, Tween and NaCl) remained the same. In this case, the emulsion had contact with the components of protein corona during the 4 hours established for the solvent evaporation. After that, unbound protein was removed by centrifugation.
- 3.3.3.2 Addition of 10% of the corresponding volume of an aliquot of WOW emulsion subjected to centrifugation. In this case, the WOW emulsion was prepared a usual. After centrifugation, a volume corresponding to 10% of the original volume of the emulsion was

added and the sample was mixed in the vortex to allow the polymeric nanocarriers to be covered with serum proteins. In this case, centrifuged samples were placed in contact with the protein corona for 24 hours prior to being transfer to the freezer for further use.

3.3 Characterization Techniques and Behavior Assays

Polymersomes composed of PEG–PCL were synthesized by different versions of the solvent evaporation technique. Characterization of the nanoparticles was carried out by Dynamic light scattering (DLS, hydrodynamic size), Zeta-potential (surface charge) and Spectrofluorometry (protein encapsulation and cytotoxicity). Behavior under biologically relevant buffers over time was carried out by DLS (changes in hydrodynamic size) and Spectrofluorometry (protein release).

3.3.1 Polymersome size and behavior in biologically relevant buffers over time by Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS), is a non-invasive and non-destructive technique commonly used to measure hydrodynamic size, polydispersity and aggregation effects of nanoparticles in liquid dispersions. This technique monitors fluctuations in the intensity of light scattered by the Brownian motion of nanoparticles over short periods of time and correlates the data using the Stokes-Einstein equation to calculate the nanoparticle hydrodynamic diameter. During size analysis, four kinds of distribution index can be obtained from the apparatus: Number distribution, Surface Area distribution, Volume distribution and Intensity distribution, although some apparatuses don't calculate Surface Area distribution. These distributions depend on different n-power relationships, ranging from $n=0$ for Number distribution to $n=6$ for Intensity distribution[157–160].

DLS was carried out in a Brookhaven Instruments BI-90Plus Particle Size Analyzer at a scattering angle of 90°C and was used to determine the hydrodynamic size of nanoparticles at different conditions. Using DLS, hydrodynamic size of the polymersomes was measured at different times in order to observe behavior of the nanoparticles and determine their stability in acidic and neutral environments. Stability of empty polymersomes over time was tested in Acetate Buffer (AcB), pH 4.6 and PBS, pH 7.4.

Once empty polymersomes were evaluated for the desired characteristics, protein-loaded polymersomes were also tested for their behavior over time in biologically relevant buffers. Loaded polymersome behavior was examined (i) when resuspended in PBS (pH 7.4) in order to simulate the conditions the polymeric nanoparticle would find itself in before reaching the lysosome, and (ii) when resuspended in Artificial Lysosomal Fluid (ALF), in order to closely resemble the environment that the loaded polymersome would encounter inside the lysosome.

Samples were prepared by adding a drop of suspended polymersome solution to 3mL of buffer into a disposable cuvette. Contents were mixed before reading the hydrodynamic diameter in the various conditions tested. Samples were run for 5 minutes, with a measurement taken by the apparatus each minute. The equipment software, then combined these 5 measurements and generated a set of data from the average results of the entire run. The data sets were processed and analyzed using Igor® software.

3.3.2 Surface charge measurement

The Zeta-potential describes the nature of the electrostatic potential near the surface of a particle. It is determined by measuring the velocity of the particles in a direct current electric field[161]. Zeta-potential was carried out in a Brookhaven Instruments BI-90Plus Particle Size Analyzer at a scattering angle of 90°C and was used to determine the surface charge of the nanoparticles and the model protein under PBS and ALF. Samples were prepared by adding a drop of suspended polymersomes or bare model protein in solution into 3mL of the buffer of interest into a disposable cuvette. Contents were mixed before reading the zeta-potential in the conditions tested. Samples were measured for 5 minutes, with a measurement taken by the apparatus each minute.

3.3.3 Cytotoxicity assay for PEG-PCL polymersomes in model cells

The cytotoxicity of the polymersomes was determined by measuring the metabolic activity of human epithelial colorectal adenocarcinoma (CaCo2) cells after being exposed to various polymersome concentrations. For potential drug and drug delivery candidates, CaCo2 cells have been extensively used as *in vitro* model to study several aspects, such as cellular permeability, pathways of drug transport, toxicity, metabolism, physicochemical characteristics for passive diffusion and to improve drug formulations[162]. Since CaCo2 and blood vessels belongs to the

same type of the four basic types of animal cells (CaCo2 cells are epithelial cells while blood vessels are endothelial cells, a subtype of epithelial cells) and our formulation is intended for parenteral administration, this is the best available option to simulate polymersomes behavior in the circulatory system[163].

Preparation of Cell Culture Media

Approximately 95mL of deionized water was added to a graduated cylinder. Powdered MEM was slowly added and mixed by magnetic stirring at room temperature. Then 2.2g of sodium bicarbonate and antibiotics were added. The pH was adjusted to have a final value between 7.0 and 7.4, using either 1 N NaOH or 1N HCl. The water level in the cylinder was filled to 1000 mL and the media was filtered by membrane filtration with a 0.22-μm filter using a positive-pressure system and stored in sterile containers.

Cytotoxicity Studies

Sample Preparation

A stock solution of previously synthesized PEG-PCL freeze-dried polymersomes was prepared and used to prepare several dilutions in order to study different polymersomes concentrations ranging from 0.01 to 5mg/mL.

Cell Culture and Cytotoxicity Assay

Approximately 10,000 CaCo2 cells were seeded into a 96-well plate and incubated for 48 hours at 37˚C with 5% of CO2. After allowing the cells to grow, culture media was removed and replaced by 200μL of the corresponding polymersomes suspension concentration. Two controls were also prepared: A positive control containing culture media without polymersomes, while the negative control was deprived of culture media. Cells were exposed to polymersomes for 48 hours. Solution was removed and cells were washed twice with Hank's Balanced Salt Solution (HBSS), used to maintain pH, osmotic balance and to provide cultured cells with water and essential inorganic ions. A solution of Cell Titer Blue® (Resazurin) diluted in HBSS in proportions 1:5 was prepared and 120μL of the solution were added to each well. The essential function of HBSS is to maintain pH and osmotic balance as well as provide cultured cells with water and essential inorganic ions. Cells were incubated for 2 hours 37˚C with 5% of CO2.

Immediately after the incubation period, emission was measured by spectrofluorometry (ex 560/em 590).

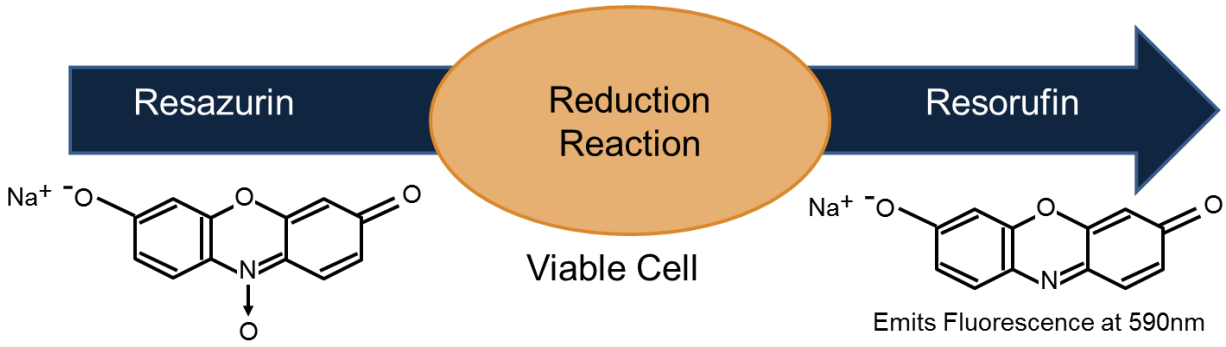


Figure 3.3: Chemical Reaction for Cell Titer Blue®

3.3.4 Determination of Protein Loading, Nanoparticle Yield and Release Profile into Polymersomes

Fluorescence assay of FITC-BSA polymersomes

Protein encapsulation was determined for polymersomes loaded with fluorescent BSA, aliquots used were from the supernatant obtained from the centrifugation of the sample after the solvent evaporation of the WOW emulsion. Aliquots of 200 uL of the supernatant of the samples were transferred to a 96-well plate and fluorescence was measured at 495 nm excitation and 515nm emission wavelengths on a Gemini EM ROM v3.0.22 spectrofluorometer. A calibration curve was made and an equation was obtained using a quadratic curve fitting in order to determine the concentration of protein in the supernatant of the samples.

Entrapment Efficiency (EE) and Nanoparticle Yield (NPY)

Entrapment efficiency (EE) or protein loading was determined by determining the amount of protein present in the samples of polymersomes by the supernatant collected after centrifugation, using a spectrofluorometric technique. Nanoparticle yield was determined by comparing the Polymersomes weight after freeze drying with the maximum possible weight of the solid components of the WOW emulsion. Calculations are presented in Appendix 3.4 and 3.6, respectively.

$$EE\ (\%) = \frac{Weight\ of\ Encapulated\ Protein}{Total\ Weight\ of\ Protein} \times 100$$

$$NPY\ (\%) = \frac{Weight\ of\ Polymersomes}{Total\ Weight\ of Solids\ in\ the\ WOW\ Emulsion} \times 100$$

Release profile of selected polymersomes under biologically relevant buffers

This process was an adaptation of several publications concerning drug release (Du et.[164] al., Sood et. al.[165], Sanson et. al.[166] and Liu et. al[167]). Freeze-dried FITC-BSA loaded polymersomes were dispersed in either PBS or ALF buffer in order to obtain a nanoparticle concentration of 15mg/mL. Then, aliquots of 1mL were added to Float-A-Lyzer (R) dialysis tubes (see Figure 3.5) and 5mL of their respective buffer was added as an outer layer for the release. Samples were incubated at 37°C and 100rpm. After pre-determined periods of time (0.5, 1, 2, 6, 24, 48 and 168 hours), the outer buffer volume was collected and replaced with fresh media. Time points were chosen taking into account literature short-term and long-term release and cumulative release was determined for each variable, referring to the total amount of protein released up to a specific period of time. In other words, is the sum of all the protein mass accumulated from the first sample collection to a certain time point within the graph. Fluorescence of collected samples was analyzed by Gemini EM ROM v3.0.22 spectrofluorometer and the cumulative release of the model protein was calculated. Detection limit of the detector could be stated to be the lowest concentration used on the calibration curve for ALF (0.1µg/mL), since at this concentration, result of fluorescence of the sample didn't overlap the fluorescence obtained at 0 concentration.

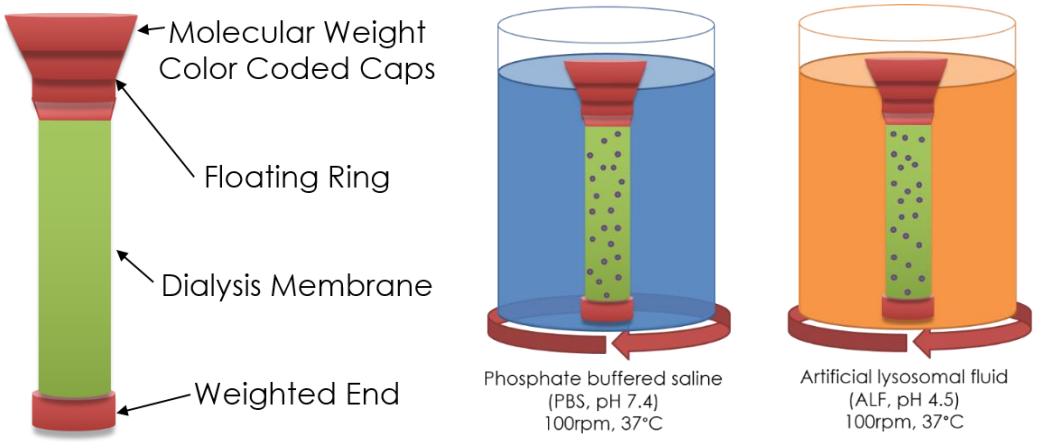


Figure 3.4: Float-a-Lyzer ® Structure and Setup for the Release Experiments

3.4 Results and discussion

3.4.1 Empty Polymersomes

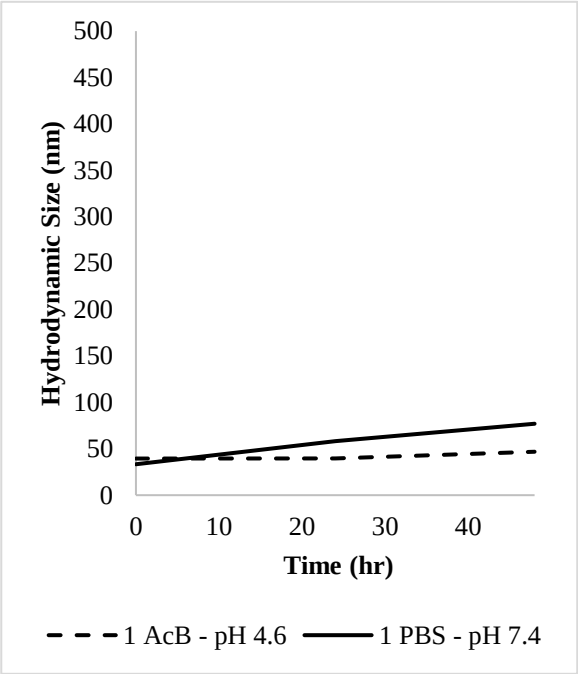
Size and behavior under biologically relevant buffers by Dynamic Light Scattering (DLS)

As a first approach to determine the optimal PEG-PCL polymersomes for our desired application, nanoparticles were synthesized with four different copolymer chain lengths. Hydrodynamic size and behavior over time in biologically relevant buffers was analyzed in order to determine if the desired behavior took place. A critical feature necessary for our system was to obtain nanoparticles that can travel through the circulatory system (neutral pH) and remain stable until reaching the acidic lysosome, where the particle would destabilize and release therapeutic cargo.

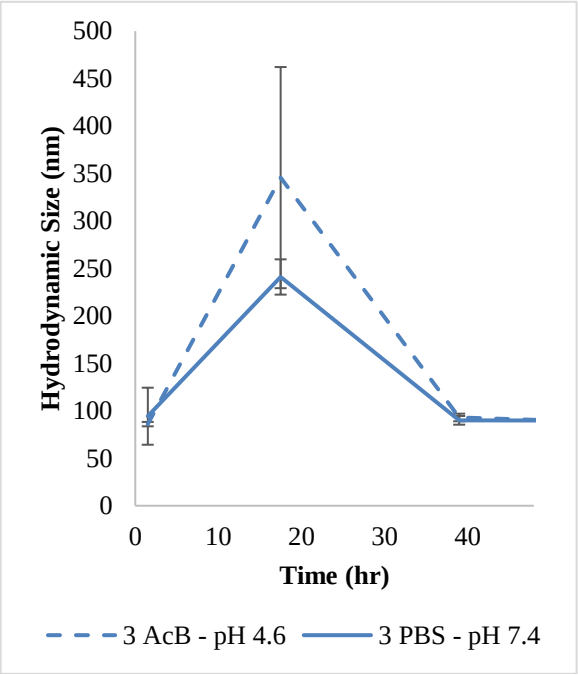
The hydrodynamic diameter size of the polymersomes was determined using a Brookhaven Instruments BI-90 Plus Particle Size Analyzer. Data was interpreted using intensity, volume, surface area and number distribution functions of the apparatus, which provides a histogram of particle size and their corresponding relative intensity at every point. Results were summarized in lognormal distribution graphs generated by Igor Software®, using a special template (Macros) added to the Igor® software, which is provided in Appendix 3.1. The key parameter to determine size and mainly study behavior was based on the number distribution function. Since this parameter accounts for the majority of the nanoparticles, size results from number distribution allows to have information of the smaller nanoparticles that can be overlapped by a very small amount of large particles or by agglomerations that can be present in the nanoparticle dispersion. Figure 3.5 presents changes in size over time for the two buffers studied, using the number distribution, while more details are available in Appendix 3.2

Note from Figure 3.5: Samples 1 and 2 were carried out in a different experimental set than Sample 3 and 4. In Samples 1 and 2 only one replicate was made, while in Samples 3 and 4, three replicates where made. Even when 5 runs were made for each replicate, separate data cannot be currently obtained from the apparatus. From the apparatus, only a mean value of particle size can be obtained with a standard deviation. Then, a statistical analysis cannot be made in order to determine the relevance of the results. Even so, literature was used to determine the best option of polymeric chain for further experiments.

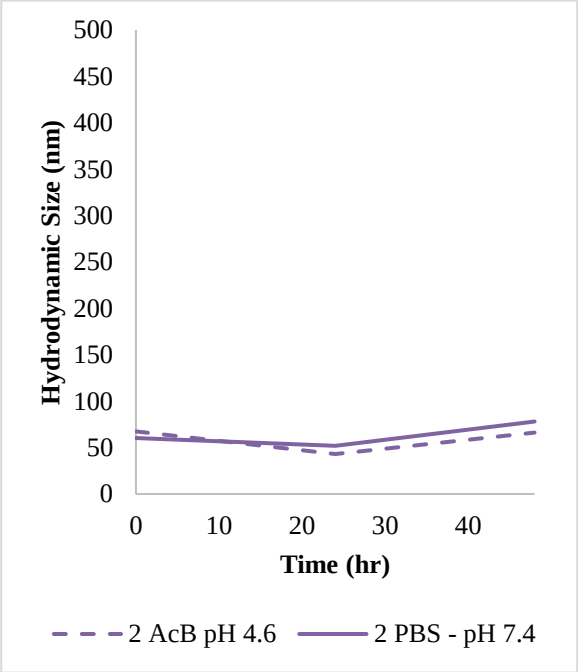
(a) Sample 1 (PEG2000-PCL2000) – N = 1



(c) Sample 3 (PEG5000-PCL2000) – N = 3



(b) Sample 2 (PEG2000-PCL5000) – N = 1



(d) Sample 4 (PEG5000-PCL5000) – N = 3

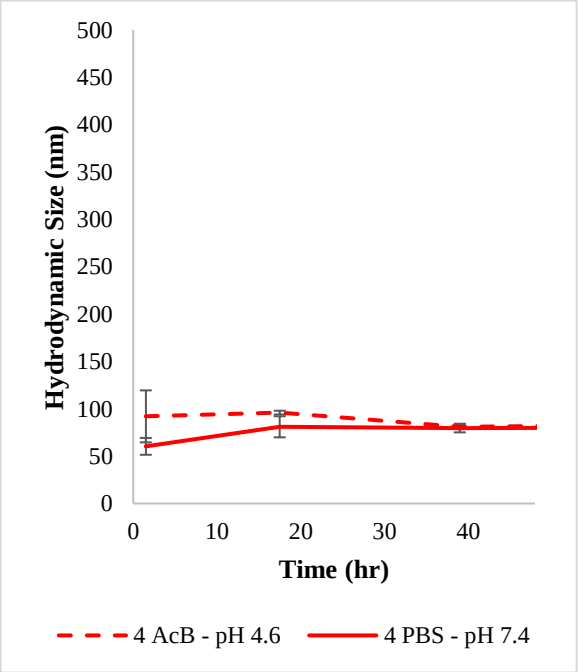


Figure 3.5: Behavior over time of empty polymersomes in physiological environment (PBS, pH 7.4) vs acidic environment (AcB, pH 4.6)

Table 3.1: Size and behavior under physiological relevant buffers of Empty Polymersomes – Summary of Results

Sample	PEG-PCL MW	Polymersomes Size	Behavior under biologically relevant buffers	
ID	(g/mol)	(nm)	Acetate Buffer (pH 4.6)	Phosphate Buffered Saline (pH 7.4)
1	2000-2000	37 N = 1	Stable – Slight change on size over time, about 8nm (39 to 47nm)	Unstable - Size increase over time (33 to 76nm)
2	2000-5000	84 N = 1	Slightly stable - Size decrease and the increase with a variation of 24nm (43 to 67nm)	Slightly Stable - Size change a little bit over time, ranging from 53 to 78nm with a variation of 25nm
3	5000-2000	122 N = 1	Unstable - Size drastically increase and the decrease to almost the same initial value (82 to 346 size range)	Unstable - Size drastically increase and the decrease to almost the same initial value (89 to 241 size range)
4	5000-5000	113 N = 1	Slightly Stable – Slight Changes in size over time. Size variation was around 15nm (96 to 81nm)	Slightly Stable - Slight increase in size and then remain stable. Size variation of 21nm (60 to 81nm)

In the four variations of PEG-PCL chains, we could observe polymersomes with particle sizes between 37 and 122nm. Considering that these nanoparticles must have an aqueous core, we consider the nanoparticles made with PEG2000-PCL2000 (Sample 1) be too small for the application, so this variation was eliminated for consideration as an optimal option. Concerning behavior under the biologically relevant buffers for our desired application, polymersomes synthesized with PEG2000-PCL2000 (Sample 1) and PEG5000-PCL2000 (Sample 3) were unstable at physiological pH, demonstrating these formulations as unsuitable for our desired application. PEG5000-PCL2000 nanoparticles weren't stable at any pH and PEG2000-PCL2000 nanoparticles had the contrary effect of what we were looking for. With these behaviors we cannot expect efficient intravenous circulation and cellular internalization, which make them unsuitable for our application.

These findings leave us with polymeric chains with PCL molecular weight of 5000 (PEG2000-PCL5000 and PEG5000-PCL5000). Even when, both copolymers show roughly the same

stability in Figure 3.5, since literature establish that vesicle formation is ensured when the copolymer contain a hydrophobic chain larger than the hydrophilic one[70], Sample 2 was selected as the best option for further experimentation.

Cytotoxicity of unloaded polymersomes synthesized with the selected block copolymer: PEG2000-PCL5000

The toxicity of unloaded polymersomes was evaluated in Human colorectal adenocarcinoma (CaCo2) cells, using Cell Titer Blue ® as viability reagent to measure metabolic activity after exposure to the nanoparticles for a period of 48 hours.

Viability of CaCo2 cells exposed to polymersomes of interest was assessed using several nanoparticle concentrations, ranging from 0.1 to 5mg/mL. A positive control consisting of cell culture without polymersomes was used to compare viability. Figure 3.7 presents viability results of the cells exposed to the polymersomes. The data obtained shows that the polymersomes were nontoxic up to 5mg/mL.

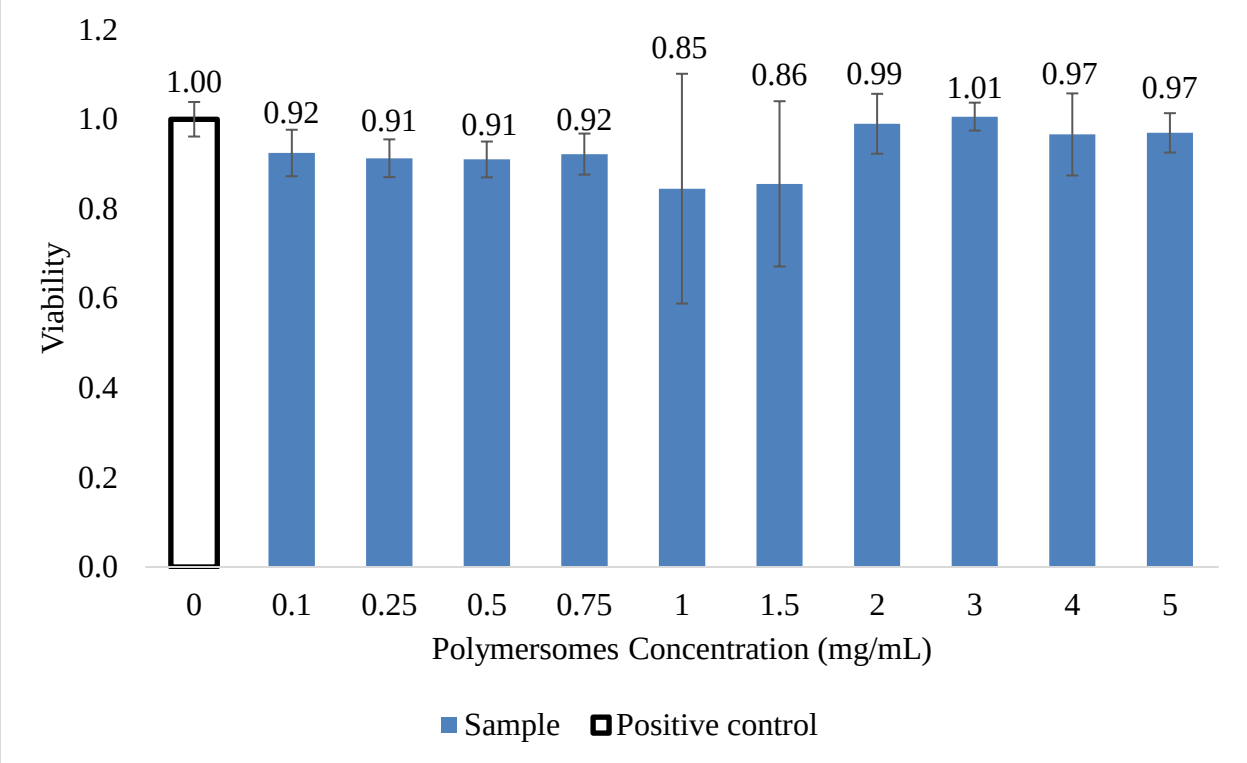


Figure 3.6: Toxicity Profile of Empty Polymersomes made with PEG2000-PCL5000

3.4.2 Protein Loaded Polymersomes

Size and behavior over time (DLS)

Optimization of the process to obtain loaded PEG2000-PCL5000 polymersomes was carried using BSA as cargo for encapsulation. From this point forward, Artificial Lysosomal Fluid (ALF, pH 4.5) was used to mimic chemical composition of the lysosomal sack, which is the final destination of our nanoparticles for our desired application. Phosphate Buffered Saline (PBS, pH 7.4) remained as the buffer to be used for physiological environment. Tables 3.2 and 3.4 present the variables tested on this part and the results of size and behavior under biologically relevant buffers. A vacuum pump was used to evaporate the solvent of the emulsion under a reduced pressure environment, generated by a vacuum pump.

Table 3.2 Experimental variables tested on protein loaded polymersomes

Sample ID	PEG-PCL:PVA Ratio	Pressure of solvent evaporation	Emulsification Technique	Span Concentration (%v/v)
5	1:2	Reduced pressure	WOW	1.6
6	1:1	Reduced pressure	WOW	1.6
7	2:1	Reduced pressure	WOW	1.6
8	1:1	1atm	WOW	1.6
9	1:1	Reduced pressure	Film rehydration	1.6
10	1:1	Reduced pressure	Film rehydration	0

Table 3.3 Behavior of protein loaded polymersomes under biologically relevant buffers

ID	Size (nm)	Behavior on ALF (pH 4.5)	Behavior on PBS (pH 7.4)
5	78	Slightly more unstable: Increase in a small range (40-90nm)	Slightly more stable: Erratic Change in size in a small range (60 to 80nm)
6	105	Unstable: Increase over time (150 to 300nm)	Stable: Not change in size over time
7	55	Same Stability: Erratic Change in size in a small range (50 to 70nm)	Same Stability: Erratic Change in size in a small range (50 to 70nm)
8	614	No further experiments were made with this formulation	No further experiments were made with this formulation
9	74	Same Stability: Erratic Change in size in a small range (50 to 100nm)	Same Stability: Erratic Change in size in a small range (50 to 100nm)
10	37	Same stability: Erratic Change in size in a small range (30 to 40nm)	Same Stability: Steady at around 30nm

Tables 3.2 and 3.3 summarizes the experimental variables and the effects these variables have on size and behavior in ALF and PBS. In Sample 8 we observed that the size was more than 6 times the size of the other nanoparticles due to the lack of vacuum to accelerate the solvent evaporation process. This sample was discarded and behavior studies weren't carried out. From the film rehydration technique (Samples 9 and 10), the obtained nanoparticles didn't present a distinguishable difference in stability over time in the two buffers, demonstrating that, at these conditions, this technique is not superior to form nanoparticles for the desired application. From the three remaining Samples (5, 6 and 7), Sample 6 presented the more dramatic change in size over time, demonstrating that this is the best option for our application. Figure 3.8 shows the behavior of the polymersomes in PBS and ALF buffers over time.

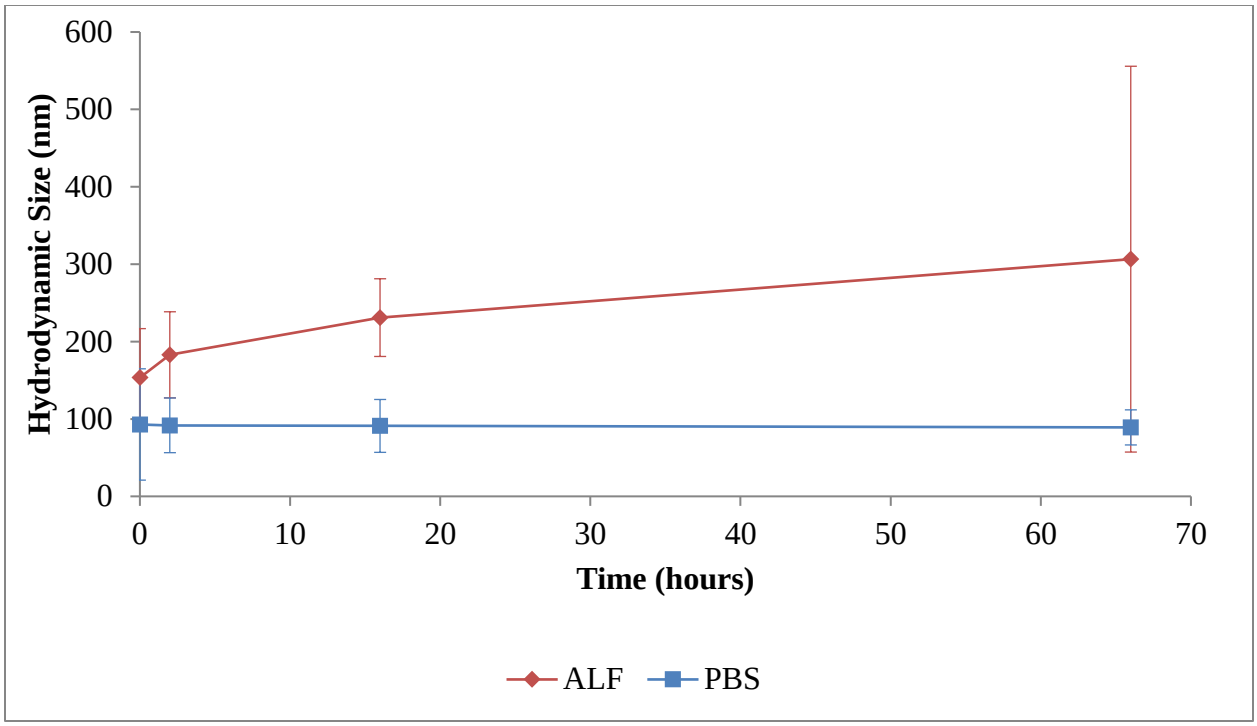


Figure 3.7: Behavior under Biologically Relevant Buffers – Hydrodynamic Particle Size (nm) Change over Time in ALF (pH = 4.5) and PBS (pH = 7.4) of Sample # 6

Cytotoxicity of BSA-loaded Polymersomes

As in the case of empty polymersomes, the toxicity of loaded polymersomes was evaluated in Human colorectal adenocarcinoma (CaCo2) cells, using Cell Titer Blue ® as viability reagent to measure metabolic activity after exposure to the polymeric nanocarriers.

Viability of CaCo2 cells was assessed using several nanoparticle concentrations. A positive control consisting of cell culture without polymersomes was used to compare viability. The negative control consisted of cells that had not been fed. Figure 3.8 presents viability results of cells exposed to the loaded polymersomes. These results demonstrate that the polymersomes are nontoxic at concentrations of up to 2mg/mL.

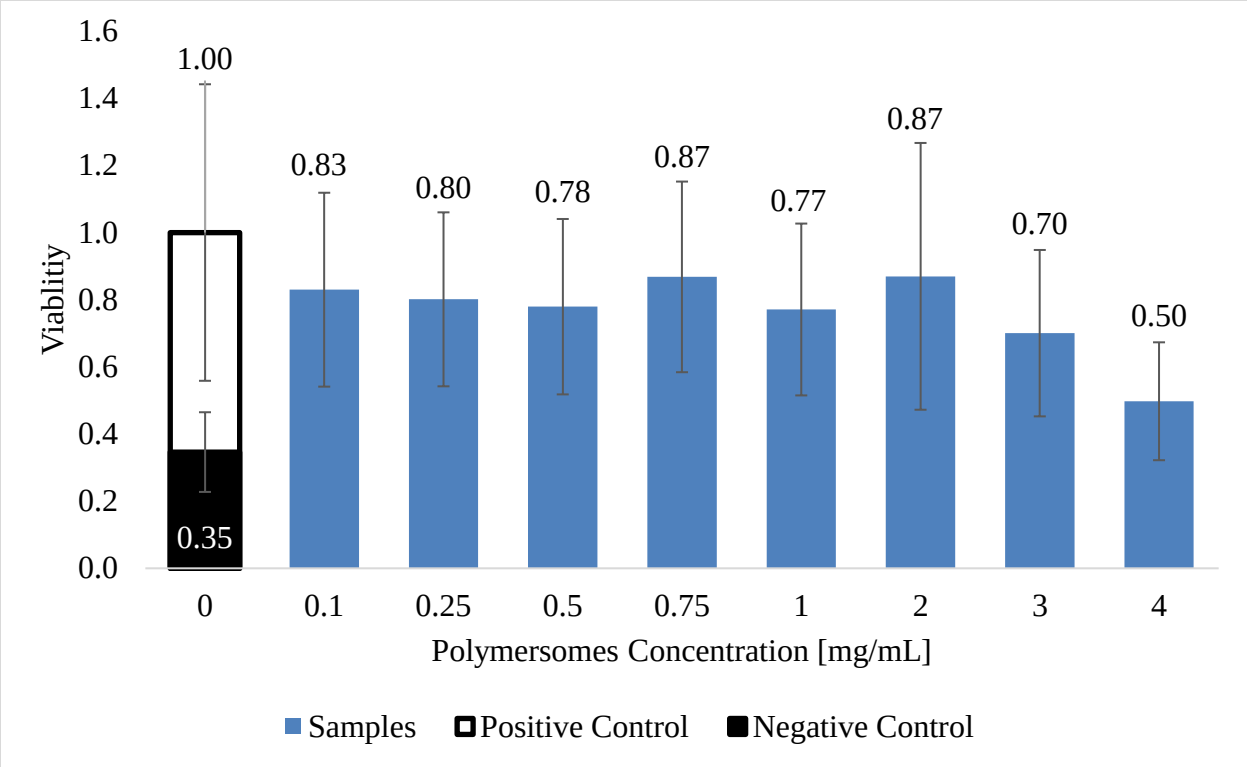


Figure 3.8: Toxicity Profile of Protein Loaded Polymersomes

3.4.3 Protein release in biologically relevant conditions

Even when the small amount of replicates cannot allow us to carry out a statistical analysis of the results, behavior have been observed in order to give suggestions of what could have happen with the system under the studied conditions. With these results of the protein release profiles, there was sustained protein release in both PBS and ALF buffers. However, the result obtained were not expected. Data suggests that there could have been higher rate of release in PBS than in ALF buffers (See Figure 3.9). From the nanoparticle hydrodynamic size stability over time assays we had previously performed, we had observed that the polymersomes were unstable at acidic conditions and stable in PBS buffer (Figure 3.7). This led us to predict that there would be more protein release in ALF than in PBS buffer, and this was not the case.

Table 3.4: Cumulative release of protein loaded polymersomes under PBS and ALF

Time(hours)	0.5	1	2	6	24	168
Loaded Polymersomes in PBS	0.000	0.000	0.000	0.147	0.921	1.289
Loaded Polymersomes in ALF	0.044	0.044	0.044	0.044	0.119	0.580

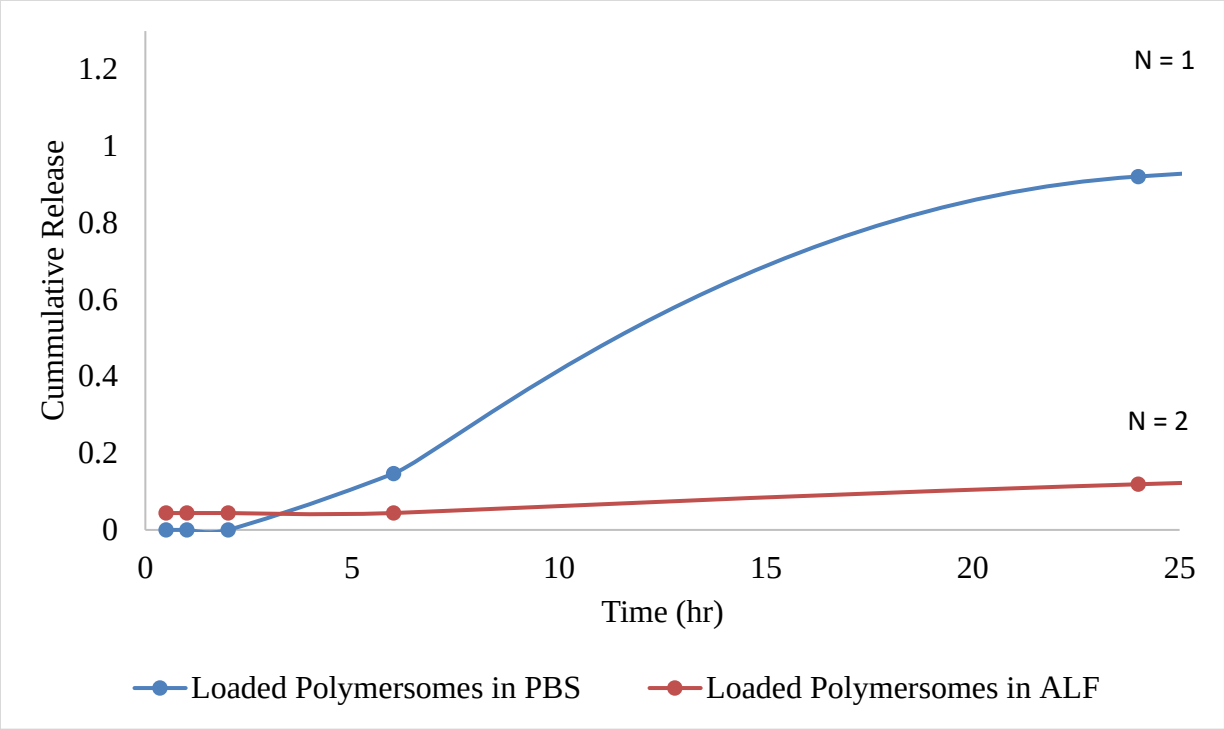


Figure 3.9: Protein Release of Polymersomes Loaded with Model Protein on the first 24 hours of the Experiment

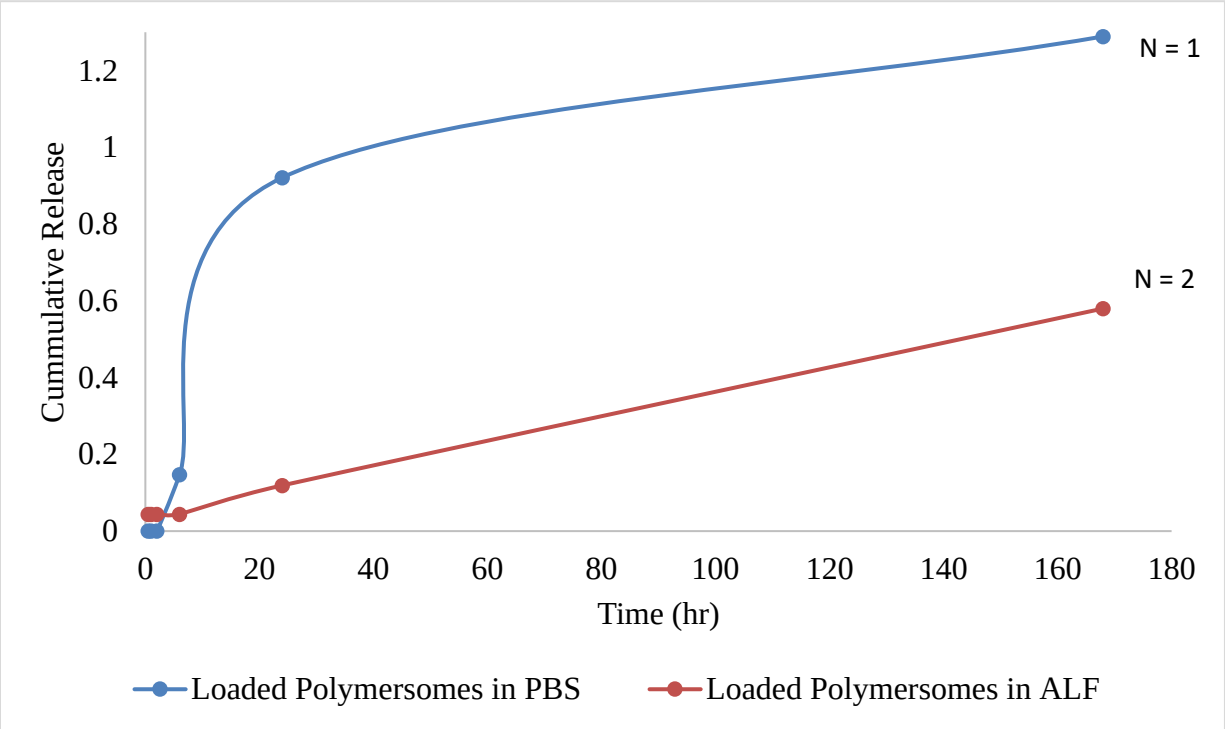


Figure 3.10: Protein Release of Polymersomes Loaded with Model Protein

3.4.4 Addition of a protein corona for tuning of protein release

There is an increasing amount of literature that suggests that a protein corona has effects on biocompatibility, cell/nanoparticle interactions, and payload release profile, among others. [168–171] We were particularly interested in the modulation of the payload release profile. The Vroman Effect postulates that when a nanoparticle comes into contact with a mixture of proteins, the most abundant proteins will first bind to the nanoparticle surface. However, as time passes, the nanoparticle will bind to the proteins that have the highest affinity[172]. This effect has been experimentally shown to occur in a matter of minutes up a few hours [171,173,174] (See Figure 3.11).

We devised a formulation where our polymersome would be incubated with Fetal Bovine Serum proteins several hours. This incubation took place in a neutral pH, so the proteins with the highest affinity to the polymersome would be in an environment similar to that of PBS. The incubation step took place for several hours (24 hours for Formulation B and 4 hours for formulation C), to ensure that there was enough time for the Vroman Effect to take place. We hypothesized that while in PBS, the protein corona would add stability to the protein loaded polymersome, and suppress protein release, and that when in contact with ALF, protein/nanoparticle interactions would change and the protein corona could lose its effect (See Figure 3.12). Fetal Bovine Serum (FBS), was incorporated either in the secondary emulsion (W2) or at the end of the emulsification process (See section 3.3.3.). Appendix 3.5 provides the components of FBS.

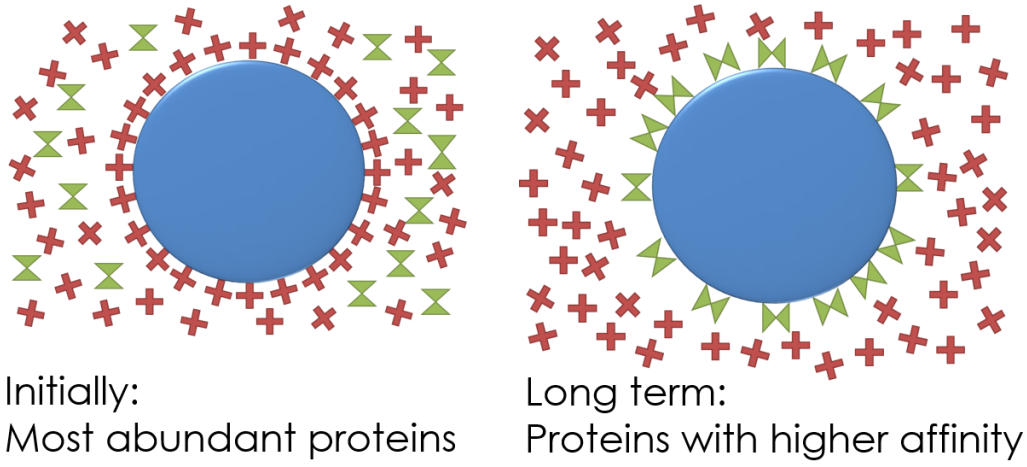


Figure 3.11: Vroman Effect for Protein Attachment to Nanoparticles at Different Times

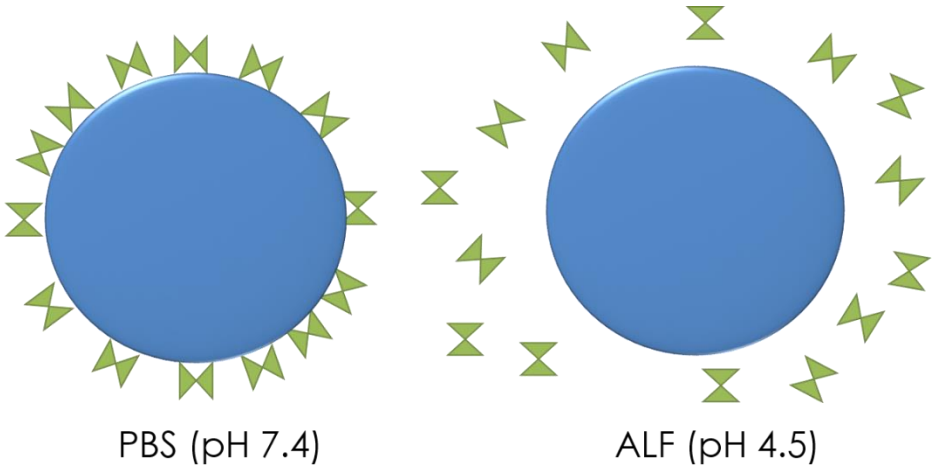


Figure 3.12: Expected Behavior of Protein Corona on Nanoparticles based on the Vroman Effect

From this point forward we will refer to the three different polymersomes created as "Formulation A", "Formulation B" and "Formulation C". We were not able to measure the hydrodynamic diameter or zeta potential of Formulation C.

Table 3.5: Characteristics of Polymersomes Formulations in Terms of Protein Loading, Entrapment Efficiency and Nanoparticle Yield

Formulation	Protein loading (µg/mL)	Entrapment Efficiency (%)	Nanoparticle Yield (%)
A	146.6 ± 1.2	76.3 ± 0.6	24.1 ± 14.7
B	146.6 ± 1.2	76.3 ± 0.6	47.8 ± 10.2
C	128.6 ± 1.2	66.9 ± 0.6	43.4 ± 6.1

Table 3.6: Characteristics of Polymersomes Formulations in Terms of Hydrodynamic Diameter and Zeta Potential under PBS and ALF

	Size (nm)		Zeta-Potential (mV)	
Formulation	PBS	ALF	PBS	ALF
A (bare)	170 ± 84	145 ± 36	-2 ± 3	0 ± 1
B (protein corona added)	221 ± 21	190 ± 66	-6 ± .4	-1 ± 1

We can see that in both ALF and PBS, there was a slight increase in hydrodynamic size in Formulation B compared to Formulation A, suggesting that there is an additional component attached to the polymersome surface that can be attributed to the presence of a protein corona.

In Figures 3.13 and 3.14 we can observe how in Particle Formulations B and C the protein corona seems to completely suppressed protein release when incubated in PBS. These results are in agreement with our hypothesis: If a high affinity protein corona is formed under certain conditions, it will remain surrounding the nanoparticle if these conditions are not changed. However, if these conditions are altered, as in our experiment, where the particles were incubated in an acidic buffer (ALF), the conformation of the proteins in the corona will change and polymersome/protein affinity can be altered. Protein release in Formulations B and C also seems to be suppressed in ALF, to a slighter degree. The obtained results suggest that Formulations B and C release more protein in ALF than in PBS, which is the effect we wanted to obtain. Then, with this data, we can conclude that Formulations B and Formulations C are meritorious for further investigation in the development of polymeric nanocarriers that are suitable for the enhancement of Enzyme Replacement Therapy for the treatment of Lysosomal Storage Diseases. More replicates need to be carried out in order to reinforce our findings and confirm these results.

Table 3.7: Cumulative Release of Formulation B under PBS and ALF

Time(hours)	0.5	1	2	6	24	168
Loaded Polymersomes in PBS	0.000	0.000	0.000	0.000	0.000	0.000
Loaded Polymersomes in ALF	0.000	0.000	0.000	0.000	0.000	0.121

Table 3.8: Cumulative Release of Formulation C under PBS and ALF

Time(hours)	0.5	1	2	6	24	168
Loaded Polymersomes in PBS	0.000	0.000	0.000	0.000	0.000	0.000
Loaded Polymersomes in ALF	0.000	0.000	0.000	0.000	0.000	0.000

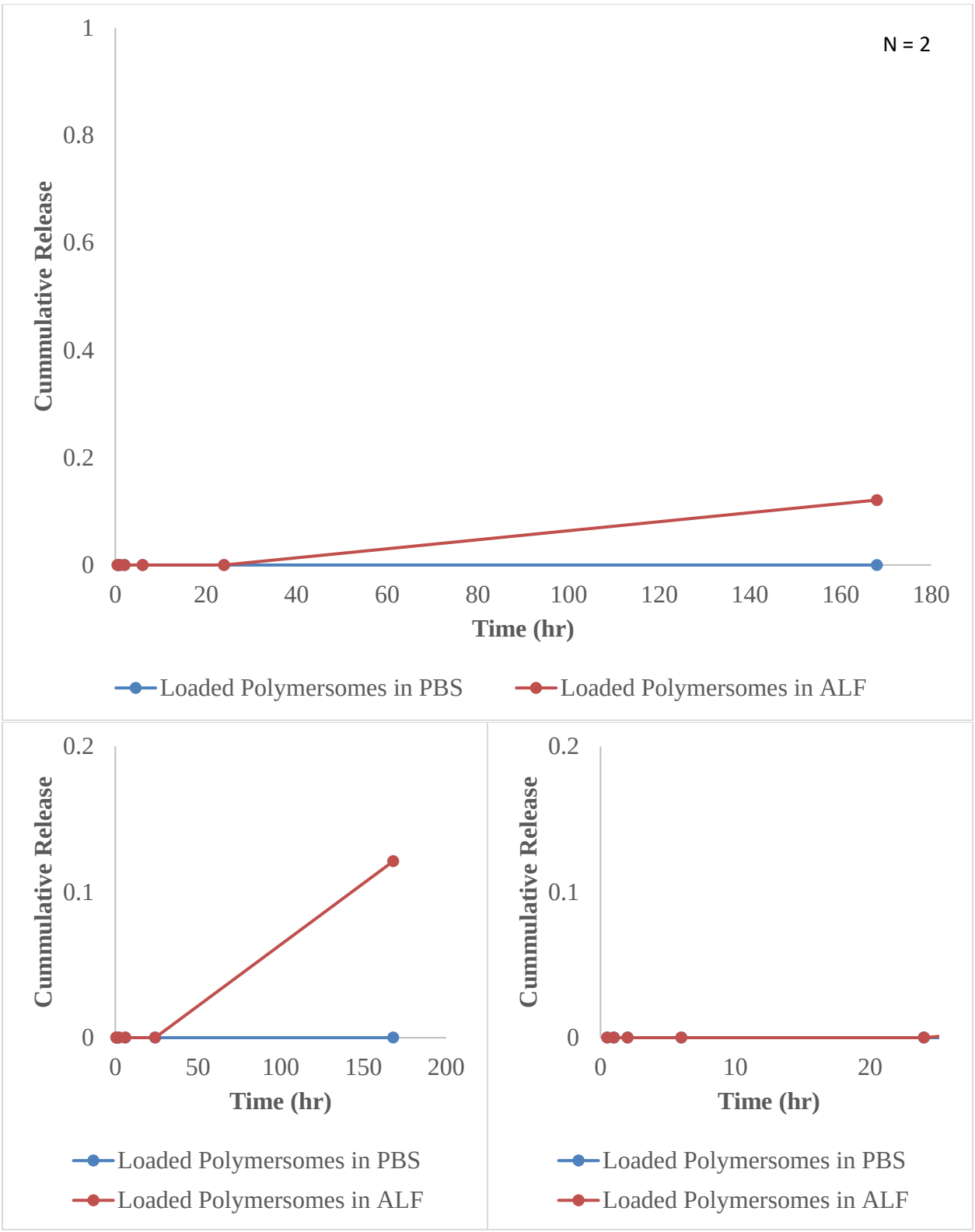


Figure 3.13: Protein release profile of Formulation B in PBS and ALF

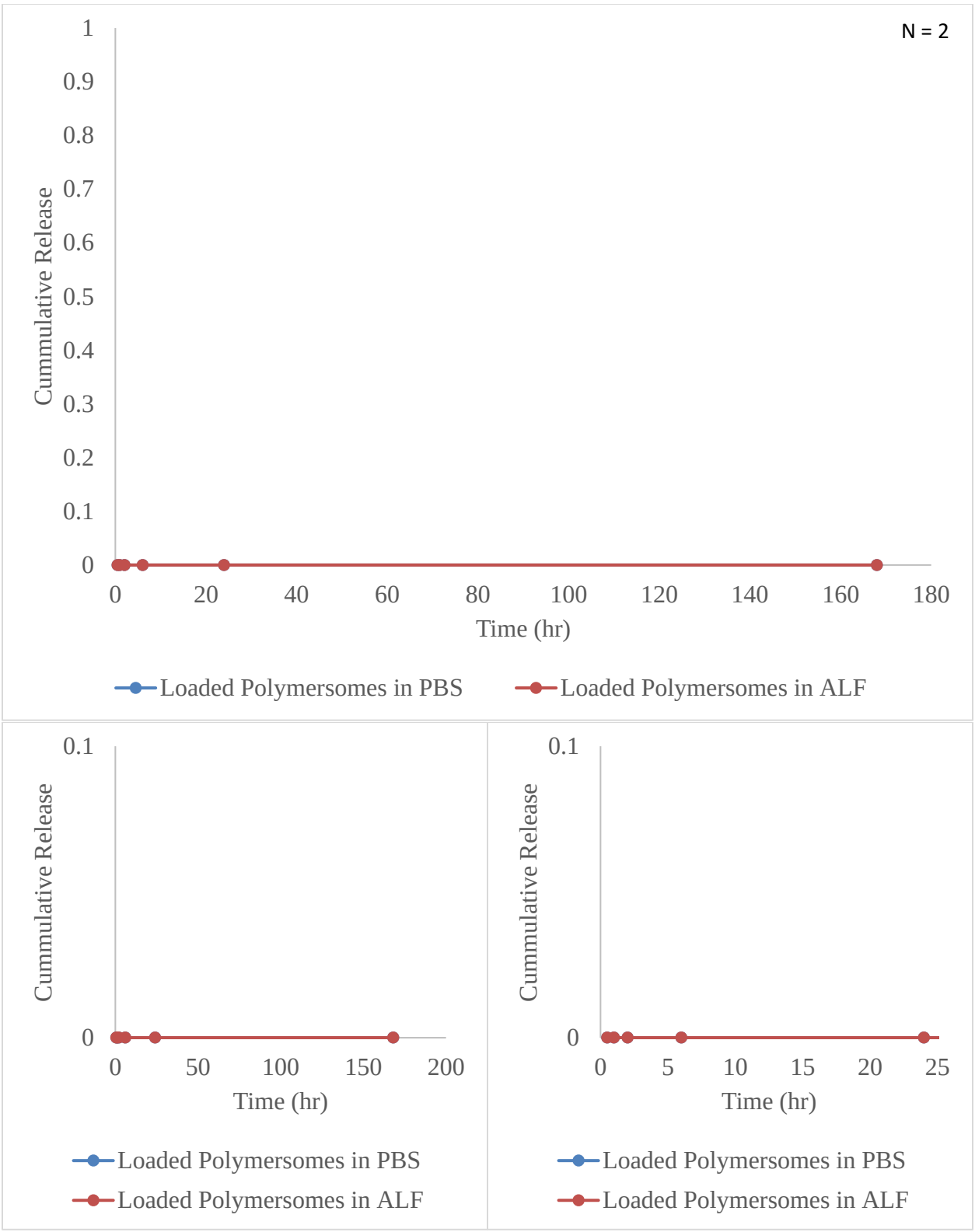


Figure 3.14: Protein release profile of Formulation C in PBS and ALF

4. CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

The main goal of this research project was to develop polymersomes composed from biocompatible amphiphilic block copolymers made from Polyethylene glycol and Polycaprolactone, suitable for protein encapsulation and responsive to pH in terms of nanoparticle stability and protein release profile. This is an effort to enhance the Enzyme Replacement Therapy, used to treat a group of pathologies called Lysosomal Storage Diseases.

To achieve this goal, PEG-PCL copolymers were synthesised and studied in order to determine the best formulation for polymersomes synthesis. Several variation of polymersomes were synthesized and studied in terms of size, behavior under biologically relevant buffers, toxicity, entrapment efficiency and protein release profile.

4.1.1 Optimization of PEG-PCL Block Copolymers Synthesis

PEG-PCL block copolymers were synthesized by Ring Opening Polymerization in the presence of Stannous Octoate (SO) or Hydrochloric Acid (HCl). Characterization tools demonstrated that PEG-PCL copolymers were successfully synthesized with SO. Nuclear Magnetic Resonance (NMR) results demonstrated that SO is a better catalyst than HCl in terms of obtaining copolymers with the desired molecular weight. We found that the block copolymers formed with SO were closer to the theoretical molecular weight than those synthesized with HCl. The GPC results demonstrated that Caprolactone monomers were attached in all of the syntheses, in agreement with the results obtained by NMR. We conclude that the synthesis of PEG-PCL copolymers using SO as catalyst should be suitable for the formulation of protein-loaded polymersomes for the purpose of this study.

4.1.2 Optimization of experimental conditions to obtain polymersomes with desired properties

In an initial approach to optimize the emulsification process, empty polymersomes were synthesized. From the four copolymers used (with four different combinations on the molecular weight of the PEG and PCL chains), polymersomes prepared with PEG2000-PCL5000 had the

desired size, were stable at phisiological pH and meet the requirements found in literature for the formation of vesicles. Empty nanoparticles were nontoxic in model cells in the range of concentrations studied (.1 to 5mg/mL).

After the selection of the adequate copolymer, loaded polymersomes were formulated and optimized by varying synthesis technique, pressure of solvent evaporation, concentration of protein added to the aqueous core and the ratio between PEG-PCL and the stabilizer of the secondary emulsion (PVA). Results suggests an optimal combination of parameters that are as follows: polymersomes generated by WOW emulsion method, use of reduced pressure for the solvent evaporation and a PEG-PCL:PVA ratio of 1:1. This combination showed to be the best option because they were stable at in PBS and unstable in ALF. This formulation demonstrated to be non-cytotoxic to model cells up to a concentration of 2mg/mL. A concentration of protein of 10mg/mL in the aqueous core gave the most efficient entrapment. The incorporation of the Film Rehydration technique didn't show any improvement in terms of the desired properties.

The Vroman effect was used to design improvements on our system. Polymersomes were incubated in FBS at a neutral pH. We expected that if left in contact for sufficient time, the proteins with the highest affinity to the particle would bind to the particle. We hypothesized that while in PBS, which has a neutral pH, the protein corona would add stability to the protein loaded polymersomes, and suppress protein release, and that when in contact with ALF, protein/nanoparticle interactions would change and the protein corona would lose its effect.

The presence of the protein corona was initially demonstrated by the slight increase in size of the polymersomes and the considerable difference in release profile compared to bare polymersomes. Results suggests that the addition of protein corona can dramatically reduce release of the model protein. Even when the reduction was observed in both PBS and ALF buffers, release was completely suppressed in PBS, which was desired for our application. Therefore, even when final conclusions cannot be given due to the lack of enough replicates in the release experiments, we can preliminarily conclude that the system we have formulated is meritorious for further investigation in the development of polymeric nanocarriers that are suitable for the enhancement of Enzyme Replacement Therapy for the treatment of Lysosomal Storage Diseases.

4.2 Future Work

Future work in this research line can be focused on several areas:

- Repeat behavior experiments in order to increase the amount of replicas and confirm the results obtained throughout this project.
- Carry out experiments in order to prove our theory of adsorption of serum proteins in the surface of the nanoparticles and the proposed effect in a range of pHs in terms of affinity of the proteins and release of the cargo in the nanocarriers.
- Use diseased cell models (eg., fibroblasts obtained from patients suffering from LSDs, which can be obtained commercially) to study the effect of the nanocarriers in terms of toxicity, internalization and other parameters toward the enhancement of the Enzyme Replacement Therapy.
- Encapsulation of therapeutic enzymes and comparison of the substrate degradation of the replaced enzyme that is encapsulated versus the actual treatment (non-encapsulated).
- Try other encapsulation techniques to simplify the process and the hazards associated with it.

5. REFERENCES

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