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Nanomedicine

Development and Physicochemical Evaluation of Liposomal Formulations with Quercetin and Sulforhodamine B, including a Co-loaded system.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AFM	Atomic Force Microscopy
BSA	Bovine Serum Albumin
CHOL	Cholesterol
Cryo- TEM	Cryogenic Transmission Electron Microscopy
D_h	Hydrodynamic Diameter
DLS	Dynamic Light Scattering
DOL	1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine
DOPS	1,2-Dioleoyl-sn-glycero-3-phospho-L-serine
DPMC	1,2-Dimyristoyl-sn-glycero-3-phosphocholine
DPPG	1,2-Dipalmitoyl-sn-glycero-3-phospho-(1-rac-glycerol)
DSPC	1,2-Distearoyl-sn-glycero-3-phosphocholine
DSPE-PEG2000	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]
DSPG	1,2-Distearoyl-sn-glycero-3-phospho-(1-rac-glycerol)
ELS	Electrophoretic Light Scattering
EMA	European Medicines Agency
EPC	Egg Phosphatidylcholine
EPG	Egg Phosphatidylglycerol
FDA	Food and Drug Administration
HSPC	Hydrogenated Soy Phosphatidylcholine
MLVs	Multilamellar Vesicles
MVVs	Multivesicular vesicles
NP	Nanoparticle
OLVs	Oligolamellar Vesicles
PDI	Polydispersity Index
PEG	Polyethylene Glycol
POPC	1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPE	1-Palmitoyl-2oleoyl-sn-glycero-3-phosphoethanolamine
Que	Quercetin
RES	Reticuloendothelial System
SAXS	Small Angle X-ray Scattering
SDS	Sodium Dodecyl Sulfate
SRB	Sulforhodamine B

TEM	Transmission Electron Microscopy
ULVs	Unilamellar Vesicles

ABSTRACT

Quercetin is a flavonoid with antioxidant and anti-inflammatory properties. However, its poor water solubility and low permeability limit its in vivo efficacy. Liposomes have emerged as a drug delivery system that can overcome these pharmacokinetic problems. Simultaneously, they offer the possibility of combination therapy with other agents. Sulforhodamine B is a hydrophilic tracer that is mainly used for drug kinetics studies in biological systems. In the present study, it was used as a model compound to simulate hydrophilic drugs.

The aim of this thesis was to develop and evaluate liposomal formulations with quercetin and sulforhodamine B. For this purpose, four formulations were prepared with the same lipid ratio (DSPC:POPE:CHOL:DSPE-PEG2000, 60:20:15:5 mol%), differing only in the incorporated agent. Liposomal systems were prepared by the thin-film hydration method. A blank formulation, as well as formulations loaded only with quercetin or sulforhodamine B were prepared and compared with each other and with the co-loaded formulation.

The physicochemical characteristics of the prepared formulations were studied over a period of 33 days. Evaluation was conducted at storage (25ºC), physiological (37ºC) and elevated (40ºC) temperature, to identify potential thermosensitivity. Hydrodynamic diameter (D_h), polydispersity index (PDI) and the zeta potential of the liposomal systems were determined by dynamic and electrophoretic light scattering. Interactions with serum proteins and leakage were also assessed. The results showed that DSPC:POPE:CHOL:DSPE-PEG2000 liposomes exhibited acceptable physicochemical characteristics, good stability and were able to incorporate both agents with satisfactory encapsulation rates.

INTRODUCTION

1.1 Introduction to Nanotechnology and Drug Delivery

Nanotechnology refers to the design and development of devices and systems in the nanoscale, within the range of 1-100 nanometers. At these dimensions, materials present distinctive physicochemical properties arising from their high surface to volume ratio and quantum effects. These properties render them suitable for various applications in different scientific fields [1]. Nanomedicine is the field of medicine that uses nanostructured materials to diagnose and cure various diseases. Nanotechnology in medicine can lead to faster and more accurate diagnosis of diseases and improves treatment. One of the most important applications of nanotechnology in medicine is in the specialized area of drug delivery [2].

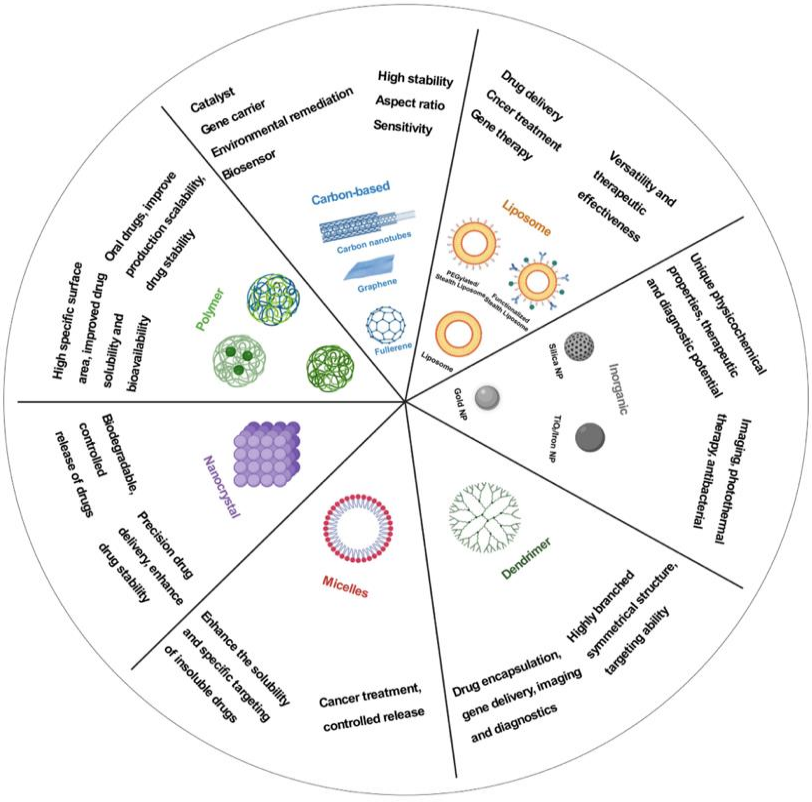


Figure 0.1 Properties and different applications of NPs in medical field.

Adapted from [1].

Drug delivery is the pharmaceutical technology field that focuses on the methods and systems used to introduce pharmaceutical compound into the body to produce a therapeutic outcome [3]. Drug delivery systems are defined as technological formulations that aim to improve therapeutic effectiveness and safety by controlling timing, kinetics and site of therapeutic agent's release [4]. Conventional drug delivery systems administered through the oral, nasal and inhaled mucosal, while effective, face

some limitations [5]. Poor solubility of drugs in water, rapid clearance and enzymatic degradation is some of them. These limitations may reduce treatment efficacy [5]. Furthermore, conventional drug formulations, especially chemotherapeutics, may increase the risk of adverse effects by damaging normal cells, when the active compound is systemically distributed rather than being specifically released to the diseased area [6]. Nanotechnology aims to address these issues.

Nanoscale drug delivery systems are designed to deliver the drug directly to the target site in the right time and amount. Nanocarriers surface can be functionalized to bind to specific ligands at the target site. This method enhances drug efficacy while decreases toxicity in adjacent healthy tissues. Additionally, nanocarriers can be engineered to respond and release drugs in specific physicochemical parameters including temperature and pH. Also, their circulation time in the bloodstream can be extended by attaching to their surface hydrophilic polymers such as polyethylene glycol (PEG) chains. This modification makes them stealth to cells of the mononuclear phagocyte system. As a result, prolonged action reduces dosing and therefore improve patient compliance [7].

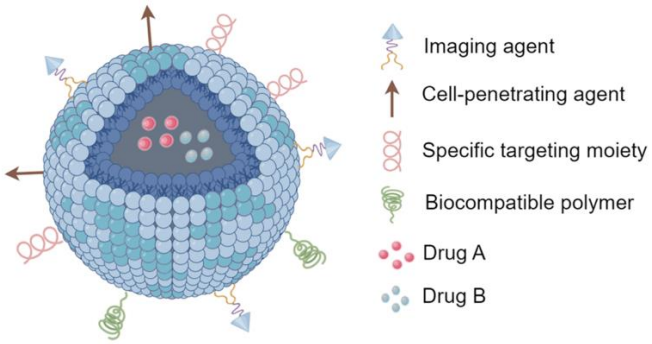


Figure 0.2 Multifunctional NP for Drug delivery.

Adapted from [8].

Furthermore, nanotechnology enables the development of systems that can deliver simultaneously multiple therapeutic agents. These molecules can be proteins, genes and imaging agents and their actions can be combined. Another important aspect is that nanocarriers due to their small size can penetrate into inaccessible biological barriers. An example consists blood brain barrier, which is otherwise difficult to penetrate. Different types of nanosized carriers have been developed for targeted drug delivery including liposomes, micelles, solid lipid nanoparticles, metallic, polymeric, carbon nanotubes and dendrimers **[Table 1.1]**, [9].

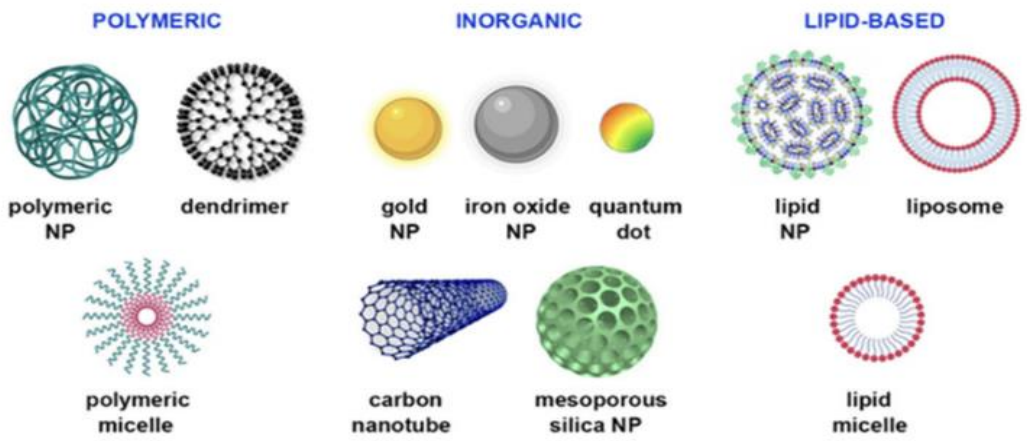


Figure 0.3 Types of NPs used as drug delivery systems.

Adapted from [1].

Table 0.1 Types of NPs.

Class of NP	Characteristics	Advantages	Applications	Ref.
Liposomes	Biocompatible, Easy entrapment	Active and passive targeting	Drug/ Gene delivery	[1]
Carbon Nanotubes	Electrical properties, unique strength	Cell specificity, Reduced toxicity	Drug/ Gene delivery	[1]
Fullerenes	Composed of 60 carbon atoms	Free radical scavenger, Stimulate host immune response	Drug/ Gene delivery, Imaging,	[1]
Polymeric NPs	Biocompatible, biodegradable	Different structures	Drug/ gene delivery Tissue engineering	[1]
Dendrimers	Branches emanating from a core	Uniform in shape, Easy penetration through cells	Drug/ Gene delivery Sensors	[10]

1.2 Liposomes

Liposomes are spherical vesicles, composed mainly from phospholipids and cholesterol. They possess a hydrophilic core surrounded by one or more lipid bilayers [11]. Phospholipids consist of a hydrophilic part containing a phosphate group and a hydrophobic one containing aliphatic chains. In aqueous environments, these parts naturally arrange themselves to create spherical structures with aqueous cores. This process can be controlled to achieve more homogenous dispersions through the application of sonic or thermal energy. Lipid composition strongly affects liposomal formulation characteristics including stability, membrane fluidity, particle size and surface charge [4]. The hydrophilic part of the phospholipid can be positively or negatively charged or zwitterionic and contributes to stability through electrostatic forces.

As drug delivery vehicles, liposomes present significant advantages that made them among the most commonly utilized nanoparticle for drug delivery purposes [4]. Liposomes can be used as carriers for both hydrophilic and hydrophobic molecules [12]. Lipophilic substances are entrapped inside the lipid bilayer, while hydrophilic ones are encapsulated in the inner aqueous compartment [13]. Especially, for drug molecules with low solubility in water, liposomal dosage form is a very effective alternative to conventional drug delivery methods [14].

Another important asset is its biocompatibility and biodegradability. Liposomes are composed of natural and synthetic phospholipids, which decreases the danger of acute and chronic toxicity [12]. This is due to the fact that, since in the composition of the cell membrane there are phospholipids, liposome's structure resembles to that of cell membranes [14]. Additionally, liposomes as drug delivery vehicles, protect the encapsulated drug from degradation. The lipids from which liposomes are composed are not susceptible to degradation from enzymes.

However, liposomal formulations face limitations related to their physical, chemical and biological instability. Liposomes can become physically unstable when exposed to biological fluids or when temperature changes. These interactions may disrupt lipid bilayer. As a result, the encapsulated drug may be released [15]. Additionally, liposomes may undergo fusion or form aggregates. Chemical instability is associated with lipid oxidation and hydrolysis. Hydrolysis of the ester bond in phospholipids releases fatty acids and thus, the membrane is disrupted [16]. Biological instability refers to the recognition and clearance of liposomes from the immune system. When liposomes enter to the bloodstream, they are detected and opsonized from the reticuloendothelial system (RES). This process leads to rapid clearance and consequently, the drug is eliminated before it reaches its target [17]. Lastly, the development of liposomal formulations in large scale presents many difficulties. These include difficulties in maintaining batch-to-batch uniformity, achieving high encapsulation efficiency and managing costs [18].

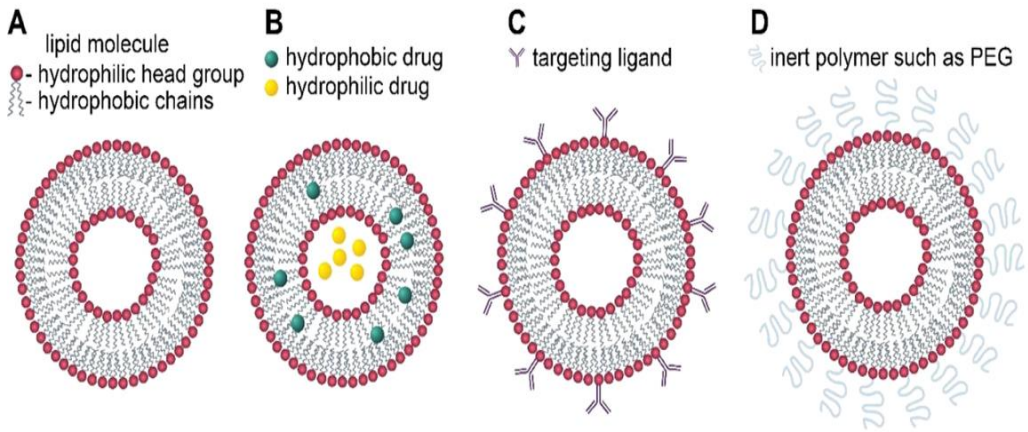


Figure 0.4 Schematic representation of: (A) liposome, (B) co-loaded liposome, (C) immunoliposome and (D) "stealth" liposome.

Adapted from [19].

1.3 Liposome Composition

Lipids are biological molecules, well soluble in organic solvents [20]. They are a diverse class that include many different natural compounds, such as triglycerides, phospholipids and sterols [20]. Lipids utilized in liposome development can be classified as natural lipids, synthetic lipids, steroids and surfactants [4].

Phospholipids are amphiphilic molecules composed of a hydrophilic head and hydrophobic tails attached to an alcohol. According to the alcohol, they are divided to glycerophospholipids and sphingomyelins. Glycerophospholipids contain glycerol in their main structure, while sphingomyelins contain sphingosine [21].

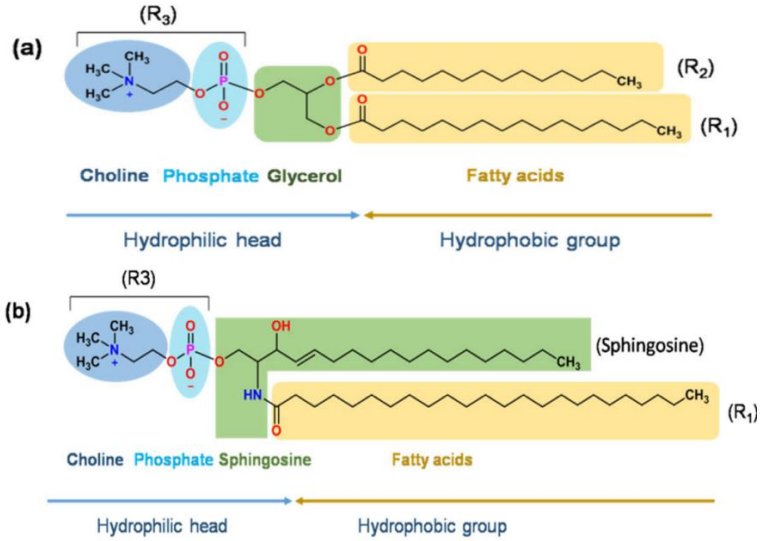


Figure 0.5 Structural illustrations of: (a) glycerophospholipids and (b) sphingomyelins.

Adapted from [22].

Phospholipids' phosphate group may be linked also with a polar molecule including choline, ethanolamine, serine or inositol. Phospholipids' hydrophobic tails contain two fatty acid chains, that can be saturated or unsaturated [4].

The type of glycerophospholipids in the formulation affects liposome characteristics including particle size, charge and stability [4]. Length, saturation degree and branching of the hydrocarbon chains influence physicochemical characteristics of the lipid bilayer including its rigidity. Lipid bilayers composed of glycerophospholipids with larger hydrocarbon chains present increased drug retention because glycerophospholipids are packed closer. In contrast, the high degree of saturation, lead to a less tight membrane packing due to the interaction between the saturated hydrocarbon chain and cholesterol [23]. The charge of the hydrophilic group may be positive, negative or zwitterionic. The

charge provides stability by creating electrostatic repels [24]. Based on the source, phospholipids can be classified to natural and synthetic [14].

1.3.1 Natural Phospholipids

Natural phospholipids are obtained from natural sources such as soya bean and egg yolk. Natural phospholipids are composed of a variety of fatty acids from which one is saturated. This can be palmitic acid or margaric acid [4]. In liposomes development, the use of natural lipids creates fewer stable preparations than the use of synthetic lipids. This happens due to the unsaturated characteristics of the hydrocarbon chain [25].

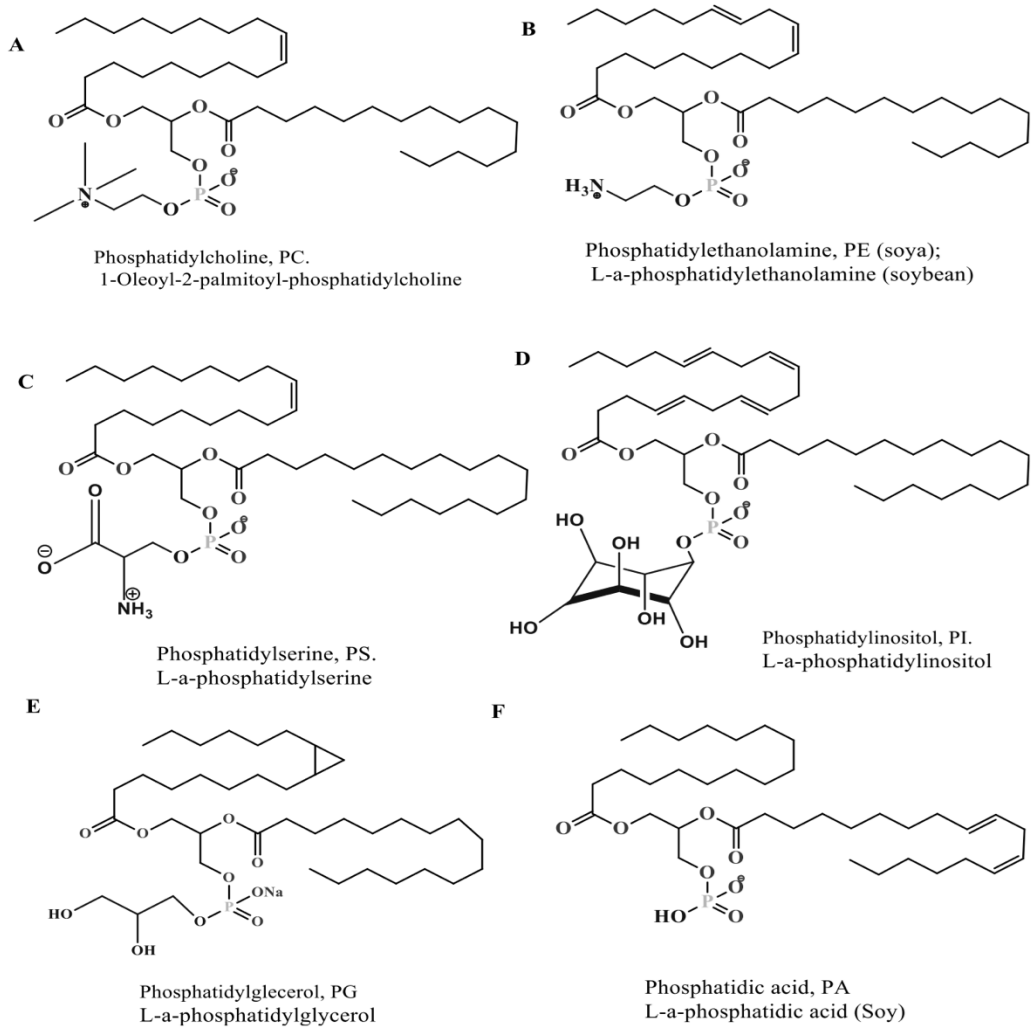


Figure 0.6 Various natural phospholipids used as components in liposomal formulations.

Adapted from [4].

1.3.2 Synthetic Phospholipids

Synthetic phospholipids are made by chemical modification to the hydrophilic and hydrophobic parts of the natural phospholipids. Synthetic phospholipids can be made from different fatty acids, unsaturated in both hydrocarbons or only one hydrocarbon chain [4]. **Figure 1.7** present different synthetic lipids used in liposomal formulations development.

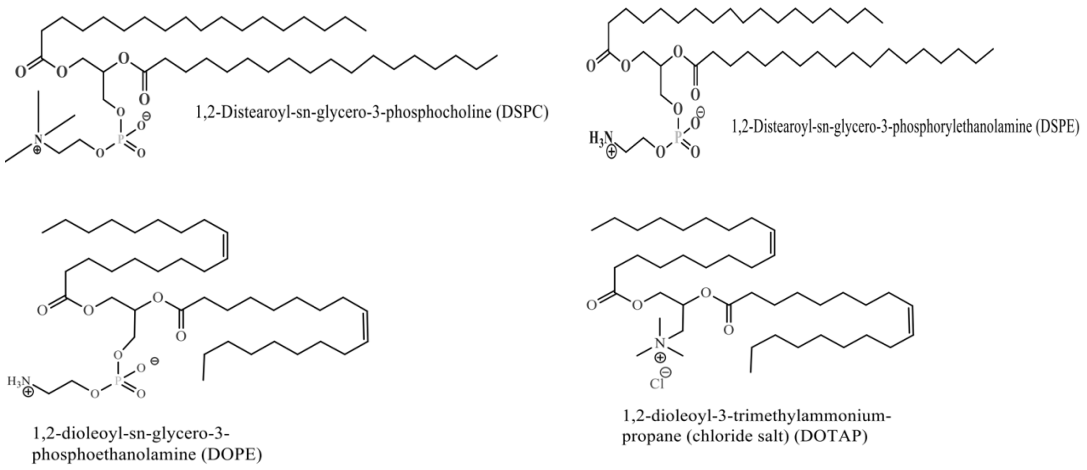


Figure 0.7 Different synthetic phospholipids used in liposome development.

Adapted from [4].

1.4 Cholesterol

Cholesterol is a steroid commonly used in the development of liposomes because it improves lipid bilayer fluidity and stability. Cholesterol possesses a tetracyclic ring and a hydroxyl functional group, in which is attributed its amphiphilic nature. In the lipid bilayer, hydroxyl groups orient towards the aqueous phase, while steroid rings align with phospholipids' hydrocarbon chains. The effect of cholesterol on the lipid bilayer is associated with its ability to promote phospholipid packing; it functions as a modulator of membrane fluidity [23]. However, a study demonstrated that higher concentrations (>40-50%) of cholesterol in the formulation decreased encapsulation efficiency and modified drug release behavior [26]. In the same study, the optimal formulation was achieved for 70:30 lipid to cholesterol ratio [26].

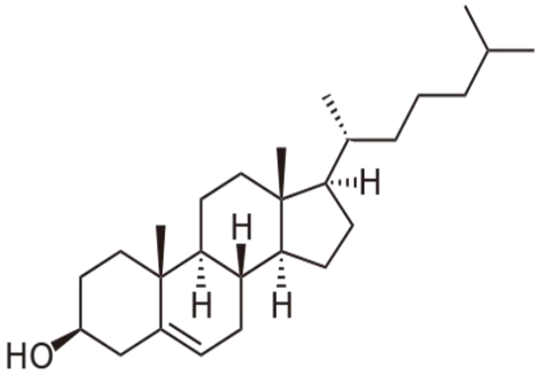


Figure 0.8 Chemical structure of Cholesterol.

Adapted from [22].

1.5 Surfactants

Surfactants are amphiphilic molecules that reduce surface tension between different phases including solids, liquids and gases. They can be used as emulsifiers, foaming agents and detergents. Depending on their charge, they can be classified in four groups; anionic, cationic, non-ionic and amphoteric surfactants. In pharmaceutics, non-ionic surfactants are mostly used. In liposomes, surfactants influence release properties through surface tension reduction and the consequent destabilization of the membrane. Most commonly used surfactants in liposomal formulations are: sodium cholate, Span 60, Span 80, Tween 60 and Tween 80 [4].

Surfactants affect physicochemical properties of nanoparticle system such as particle size, PDI and zeta potential. As a result, they can influence cellular uptake and therapeutic effect [27]. Furthermore, surfactants may improve drug loading, while preserving formulation's stability [28]. More specifically, matching formulation components with drug's charge and hydrophobicity enhances loading effectiveness [29].

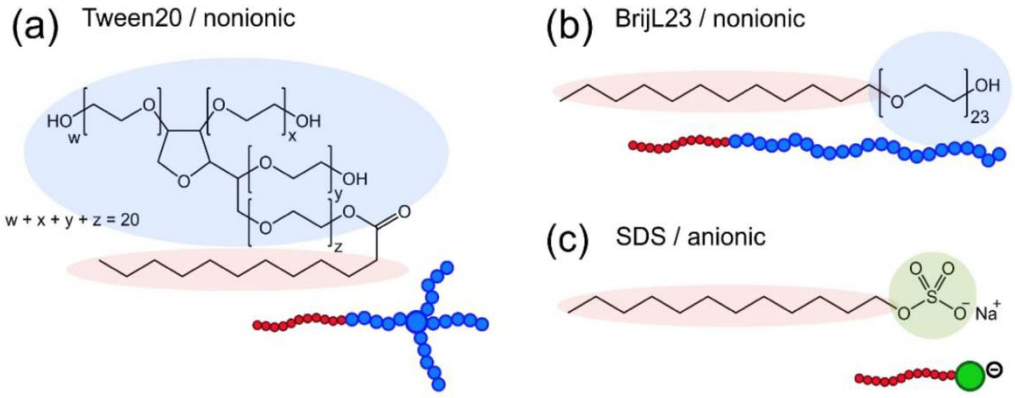


Figure 0.9 Chemical structure of surfactants: (a) Tween 20, (b) Brij23 and (c) sodium dodecyl sulfate (SDS).

Adapted from [30].

1.6 Liposomes classification based on their Size and Number of Bilayers

Based on their size and number of bilayers, liposomes can be classified in four categories:

- Small Unilamellar Vesicles (SUVs)
- Oligolamellar Vesicles (OLVs)
- Multilamellar Vesicle (MLV)
- Multivesicular Vesicles (MVV)

Unilamellar structures have only one phospholipid bilayer, while oligolamellar and multilamellar have more. MVLs contain multiple aqueous compartments that are irregularly distributed, surrounded by a lipid bilayer. Based on their size, ULVs can be further categorized as small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs) and giant unilamellar vesicles (GUVs) [23]. Size and number of bilayers influence encapsulation efficiency of drugs. As the size grows encapsulation efficiency increases. In contrast, an increase in the number of bilayers lead to lower encapsulation rates for hydrophilic drugs [4].

Table 0.2 Classification of liposomes based on size and lamellarity.

Category	Size	Lamellarity
SUV	20-100 nm	1
LUV	>100 nm	1
GUV	>1-100 µm	1
OLV	100-1000 nm	2-5
MLV	>500 nm	>5
MVV	>1 µm	Honeycomb like

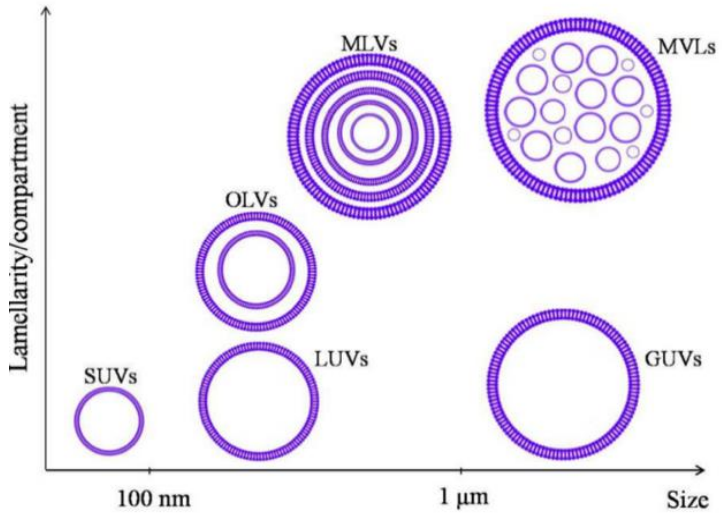


Figure 0.10 Various types of liposomes based on size and lamellarity

Adapted from [31].

1.7 Liposomes classification based on their Composition and Applications

Based on their composition and applications they can be classified as:

- Conventional liposomes
- Stimuli responsive liposomes
- Sterically stabilized (stealth) liposomes

1.7.1 Conventional Liposomes

Conventional liposomes are composed of natural or synthetic phospholipids such as 1,2-distearoryl-sn-glycero-3-phosphatidylcholine (DSPC), sphingomyelin, egg phosphatidylcholine. Cholesterol may also be included in the formulation. Conventional liposomes showed a short blood circulation time due to their elimination by the RES [4].

1.7.2 Stimuli Responsive Liposomes

Stimuli responsive liposomes are smart liposomal formulation that can release its content under the regulation of specific stimuli. The idea arose from the physiological process of living cells to respond to external environmental stimuli [32]. Stimuli responsive liposomes are modified with chemical moieties that are triggered to release bioactive components to pathological tissues. The stimuli can be internal, such as PH changes or enzymes activity or applied from the outside, including temperature, light or ultrasound. This approach gives the benefit of a more precise delivery of the pharmaceutical agent concerning time and location [33].

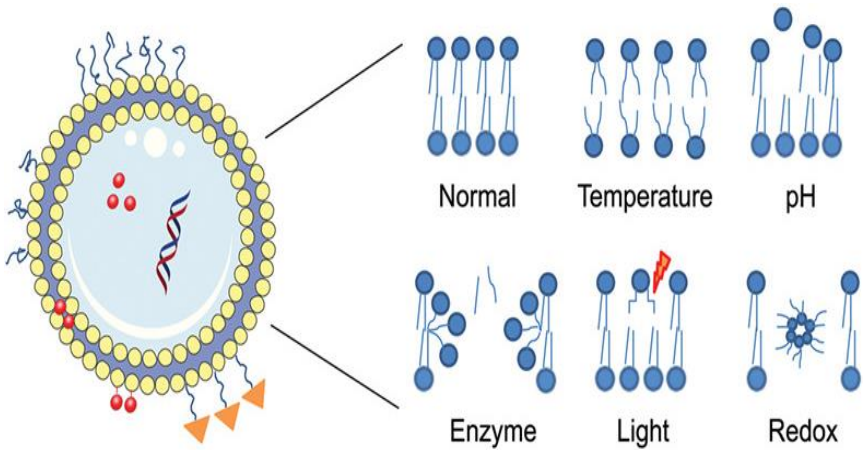


Figure 0.11 Different types of stimuli used to trigger drug release.

Adapted from [33].

pH Sensitive Liposomes

pH sensitive liposomes are designed to release the encapsulated agent in response to changes in pH. These liposomes are generally composed of a natural lipid component sensitive to pH changes [33]. This approach is mainly applied to target cancer, as solid tumors present an acidic pH. The mechanism of pH-responsive liposomes is based on the destabilization of the liposomal lipid bilayer in acidic conditions [34]. As a result,

their content is released. Additionally, drug release can also happen inside endosomes and lysosomes, where the environment is acidic [34].

Temperature Responsive Liposomes

Liposomes' lipid bilayers undergo phase changes at specific temperatures. These changes can disrupt liposomes membrane. Phospholipids' thermodynamic properties like the phase transition temperature, can serve for modulatory controlled drug delivery. Additionally, to enhance drug delivery in cancer therapies, medicines can be released when the tumor is heated externally. One method to introduce thermosensitive characteristics into liposomal systems is with the incorporation of thermosensitive polymers. These polymers chain can aggregate when passing a precise temperature threshold, which may disrupt liposome membrane. This interference can change membrane stability, permeability and fluidity [35].

1.7.3 Sterically Stabilized Liposomes "Stealth"

One widely used method to prolong circulation time of liposomes is by coating the surface with polyethylene glycol (PEG) chains. PEG is an inert hydrophilic polymer. PEG chains create steric hindrance in the surface of liposomes that prevents adsorption of opsonin and recognition by the mononuclear phagocyte system that led to clearance. As a result, circulation time is prolonged and therapeutic efficacy is improved. These liposomes are called "stealth". Additionally, steric hindrance in the surface of liposomes prevent aggregation and therefore improves stability during storage [36].

1.8 Methods of Preparation

1.8.1 Thin-Film Hydration Method (Bangham Method)

This method utilizes an organic solvent to dissolve lipids, which is then removed by evaporation under vacuum at a temperature of 45-60°C. A dry thin lipidic film is created on the flask wall. In the next step, the thin film is hydrated by adding an aqueous solution under continuous agitation for 1-2 hours. Temperatures must be above the lipid transition temperature. This technique produces heterogenous both in size and in shape dispersions. Size reduction and uniformity can be achieved by probe sonication or by extrusion through polycarbonate membrane filters. This method is simple and suitable for all lipid mixtures. However, it is confined to lab scale production due to scale up challenges [37].

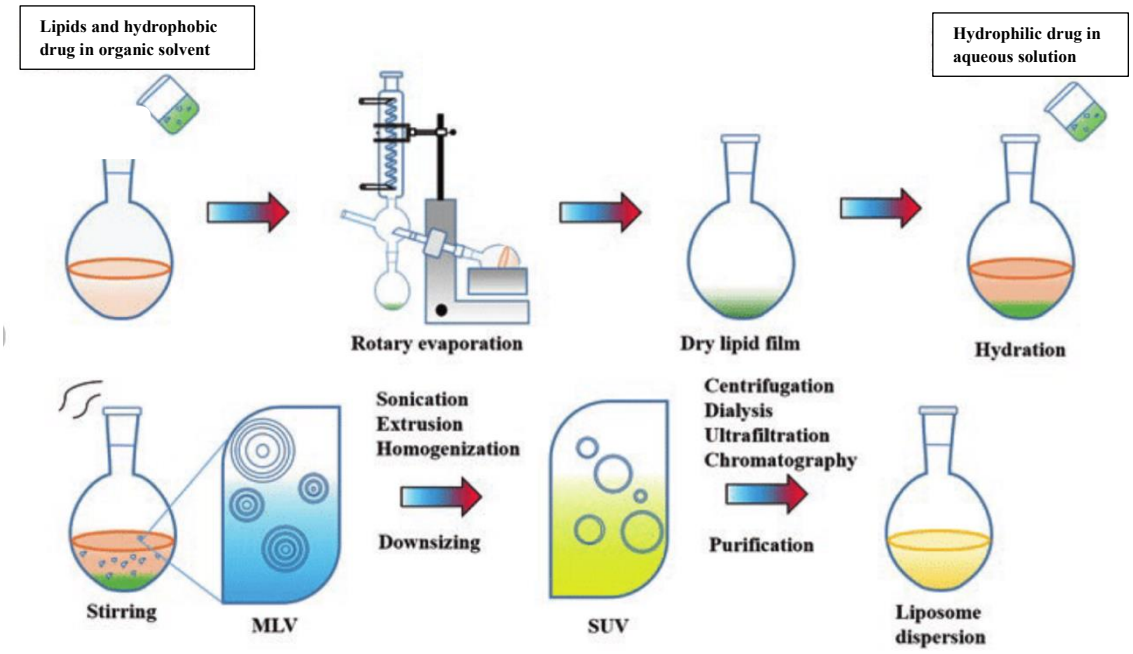


Figure 0.12 Schematic representation of thin-film hydration method.

Adapted from [38].

1.8.2 Reverse- Phase Evaporation

Reverse-phase evaporation involves dissolving the lipids in an organic solvent. This solution is mixed with an aqueous media which might contains the drug molecule. Subsequently, the mixture is sonicated to produce a water in oil emulsion. Organic solvent is then slowly removed using a rotary evaporator. As the solvent evaporates, a viscous gel is formed and at a critical point, the inverted micelles transition into lipid bilayer particles [39].

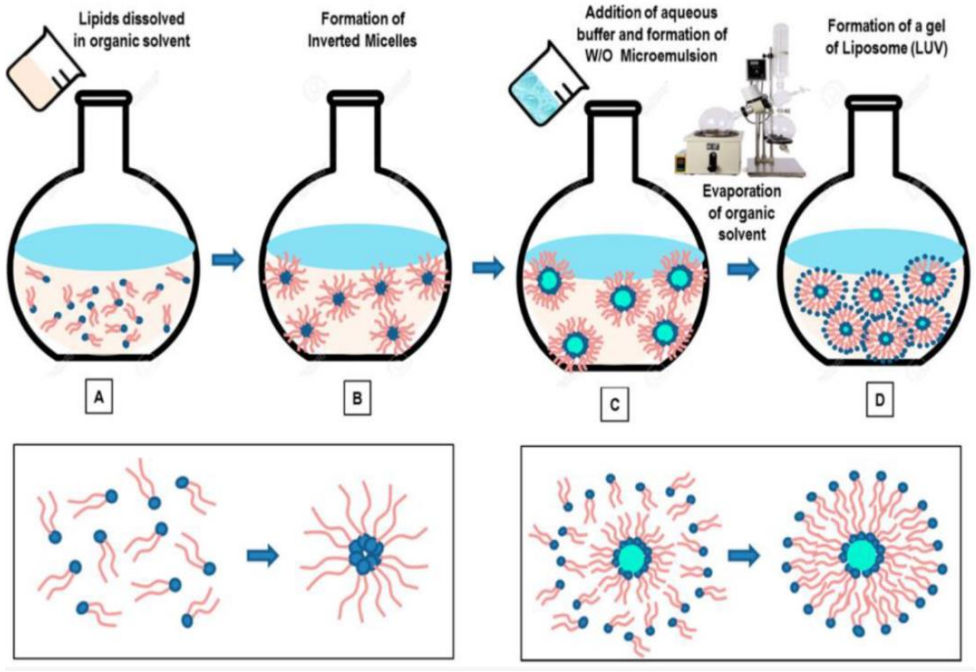


Figure 0.13 Schematic representation of reverse phase evaporation method.

Adapted from [39].

1.8.3 Double - Emulsification Method

This technique involves creating a double emulsion system and it has been used to produce MLVs. The first step is the formation of a water in oil emulsion, where the drug dispersed in an aqueous phase is emulsified in lipids diluted in an organic solvent. This emulsion is further emulsified in an aqueous solution creating a water-in-oil-in-water emulsion. Then, organic solvent is removed with the aid of vacuum pressure. This result in liposome formation as lipids self-assemble into lipid bilayers. Finally, the sample is filtered to remove free drug and exchange of external solution. This method is mainly used to create liposomes that encapsulate hydrophilic drugs [22].

1.8.4 Solvent Injection Techniques

In this method, lipids are dissolved in an organic solvent usually ethanol or diethyl ether. A syringe is used to inject the lipid solution in an aqueous phase at 55-65ºC or under reduced pressure. When lipids come into contact with water molecules, self-assemble to form liposomes. Organic solvent is removed by evaporation. This methos is simple and reproducible. Using ether as a solvent lead to the creation of liposomes with greater entrapment efficiency. However, ethanol removal is complicated since it creates an azeotrope mixture with water [22].

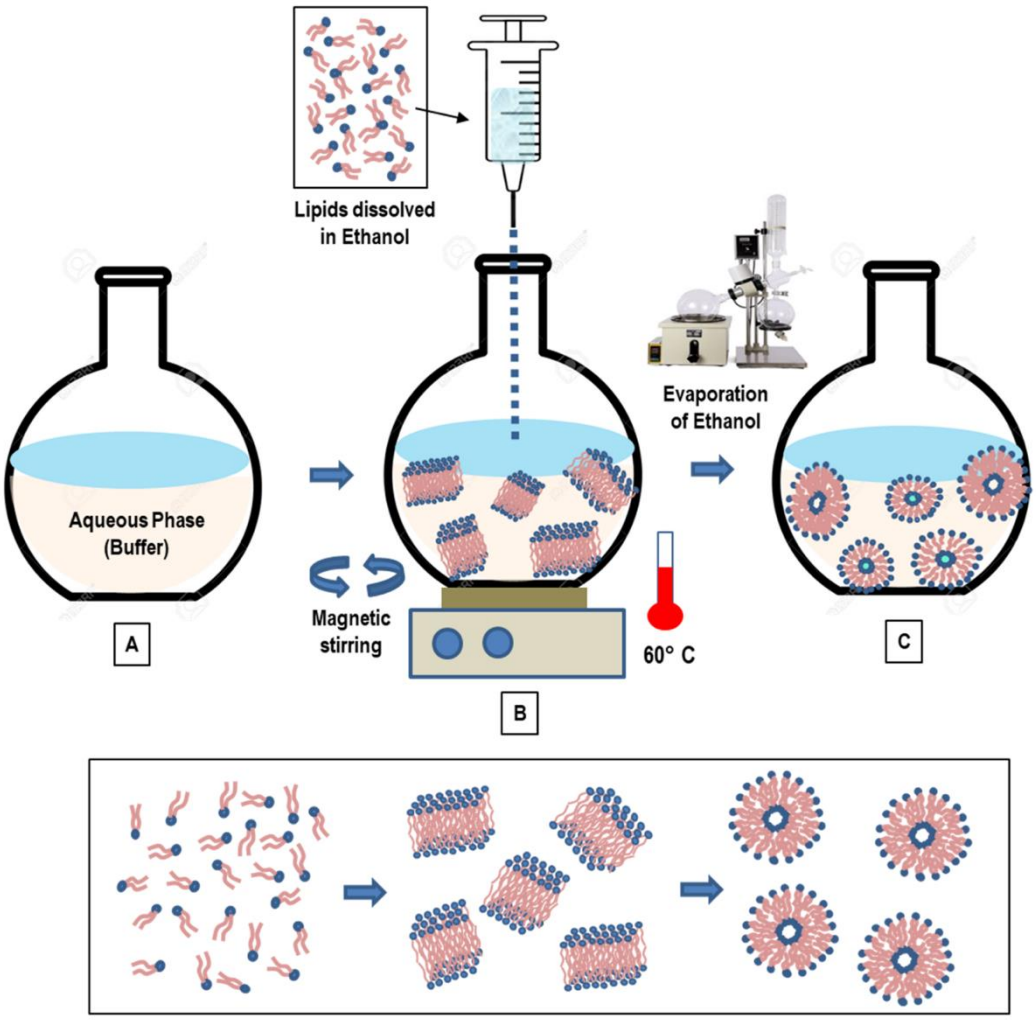


Figure 0.14 Schematic representation of solvent injection technique.

Adapted from [39].

1.8.5 Microfluidic Channel Method

Lipids are dissolved in an organic solvent, usually ethanol or isopropyl alcohol, which then passes through a device (microfluidic) with very small channels. In one channel, the organic solvent flows, and in the other, the aqueous phase. At a junction, the two channels meet, allowing the phases to mix. When lipids come into contact with water, they self-assemble into liposomes to protect their hydrophobic parts. Liposome size and size distribution can be controlled by changing the flow rate ratio and the total flow rate. For instance, higher flow rate leads to the production of smaller liposomes [40].

1.9 Size Reduction Techniques- Downsizing

Liposomes produced using the above techniques have variable sizes. However, size and size distribution are critical parameters in clinical use for the safety of liposomal formulations. The liposomes size must be well-defined based on use for parenteral, topical or respiratory route of administration. Downsizing techniques are used to covert MLVs to SUVs and LUVs. These techniques improve homogeneity and stability of the liposomal formulation. Several methods are available for size reduction including bath or probe sonication, extrusion, homogenization or a combination of them.

1.9.1 Sonication

There are two types of sonicators used to create SUVs from MLVs: probe sonicators and bath sonicators. In probe sonication, tip is directly inserted into the dispersion and high energy is applied to reduce liposome size. However, due to the increase in temperature at the tip, the vessel should be placed in a water-ice bath throughout the process. A potential disadvantage of this method, is that titanium particles from the tip may contaminate the liposome dispersion [41]. In bath sonication, liposome dispersion is placed into a bath sonicator and temperature is controlled during the process [42].

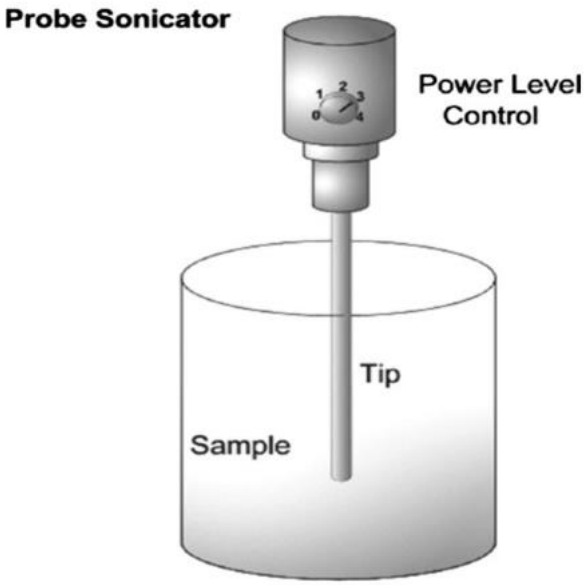


Figure 0.15 Illustration of a Probe-type Sonicator.

Adapted from [42].

1.9.2 Extrusion

Extrusion technique includes passing the liposome suspension repeatedly through polycarbonate membranes with defined pore sizes (50nm- 5μm). Number of passage cycles, the pore size of the polycarbonate membranes, pressure and flow rate can influence liposomes size and lamellarity [43].

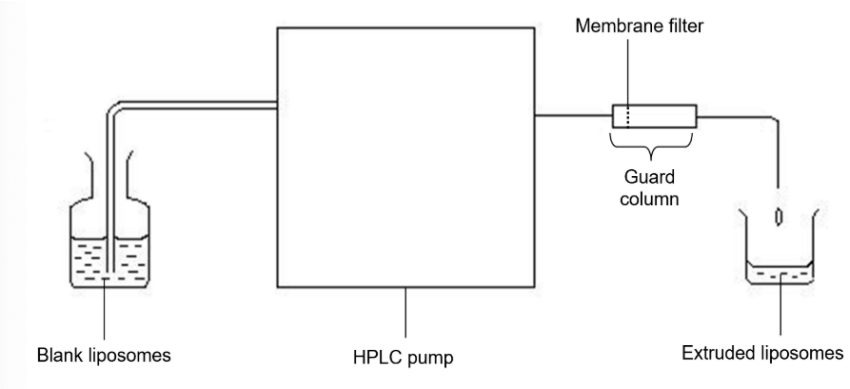


Figure 0.16 Instruments used for the extrusion process.

Adapted from [43]

1.9.3 High Pressure Homogenization

High pressure homogenization technique entails passing of the liposomal suspension through a narrow gap at a high pressure. This method is suitable for large scale pharmaceutical production. The increasing pressure decreases liposomes size and size distribution; however, it decreases encapsulation efficiency [44].

1.10 Drug Loading Methods

Liposomes can encapsulate both hydrophilic and hydrophobic molecules. Drug loading efficiency is important for the therapeutic efficacy of the liposomal formulation. Drug loading can be achieved through active or passive method.

In active loading, liposomes are already formed when the therapeutic agent is introduced into them through transmembrane gradient such as pH or ion concentration. Active loading method achieves high encapsulation efficiency especially for ionizable pharmaceutical compounds. Ionizable groups may be ammonium sulfate, chloride ammonium, calcium acetate and polyanions. These groups bind to pharmaceutical molecules and the resulting complex can control drug release [35].

Passive drug loading involves the incorporation of drug agent during the liposome formation process. More specifically, hydrophilic drugs are dissolved in the aqueous

phase and lipophilic drugs in the organic solvents. The main disadvantage of this technique is the low entrapment efficiency and, therefore, the step of removing the free drug [23].

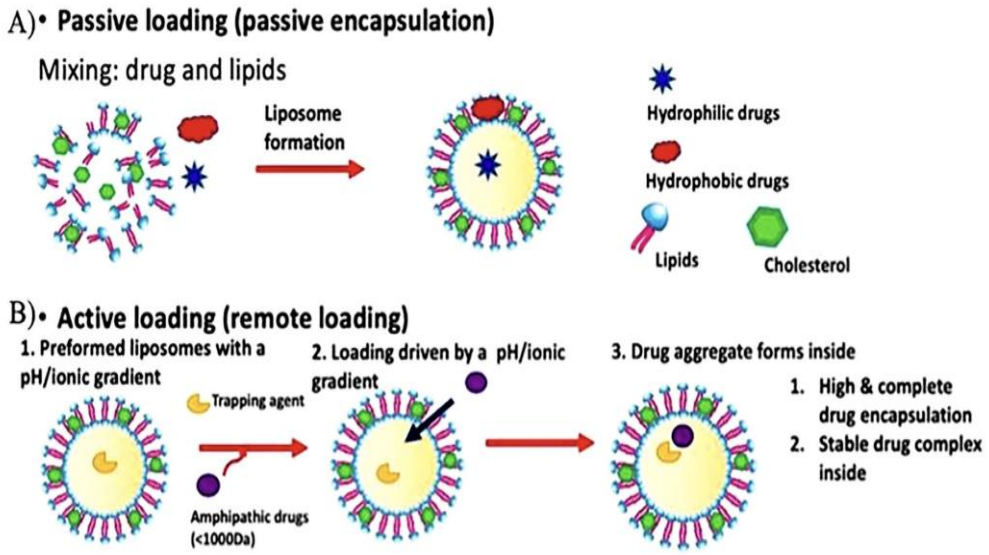


Figure 0.17 Drug loading methods: (A) passive and (B) active.

Adapted from [23].

1.11 Commercially Available Liposomal Formulation

According to FDA and EMA data, available liposomal formulation for drug delivery purposes are presented in **[Table 1.3]**.

Table 0.3 Approved liposomal formulations.

Name	Description	Composition	Indication	Route of administration	Approval
AmBisome®	Amphotericin B encapsulated in liposomes (60-70nm)	HSPC Cholesterol DSPG (2/0,8/1 molar)	Systemic Fungal infections	Parenteral	FDA 1997
Doxil®	Doxorubicin hydrochloride encapsulated in Stealth® liposomes (100nm)	DSPE-PEG2000 HSPC Cholesterol	AIDS- related Kaposi Sarcoma, multiple myeloma, ovarian cancer	Parenteral	FDA 1995
Myocet®	Doxorubicin encapsulated in oligolamellar liposomes (180nm)	EPC Cholesterol (1/1 molar)	Metastatic breast cancer	Parenteral	Europe 2000
Marqibo®	Vincristine sulfate encapsulated in liposomes (100nm)	Sphingomyelin Cholesterol (60/40 molar)	Acute lymphoid leukemia	Parenteral	FDA 2012
Mepact™	Mifamurtide incorporated into large multilamellar liposomes	POPC DOPS	Non-metastasizing resectable osteosarcoma	Parenteral	Europe 2009
Visudyne®	Verteporfin encapsulatedin liposomes	DPMC EPG	Photodynamic Therapy of ocular syndromes	Parenteral	FDA 2000
DaunoXome®	Daunorubicin citrate encapsulated in liposomes (45nm)	DSPC Cholesterol (2/1 molar)	HIV-related Kaposi Sarcoma	Parenteral	FDA 1996
DepoDur®	Morphine sulfate encapsulated in multivesicular liposomes	DOL Cholesterol DPPG Tricaprylin Triolein	Chronic pain	Parenteral	FDA 2004

Data adapted from [11].

1.12 Other Applications of Liposomes

1.12.1 Gene delivery

Gene therapy can improve the treatment of genetic disorders by delivering nucleic acids to replace deleted or mutated genes [45]. The safety of gene therapy relies on the ability of the delivery system to reach its target, without accumulating to other tissues, causing toxicity. Gene delivery systems are classified in viral and non-viral vectors. Non-viral vectors include, but are not limited to, cationic liposomes and polymers. Cationic liposomes are used because of their positive charge can interact with the negatively charged phosphate group of nucleic acids [45]. The incorporation of biologically active complexes such as genes and proteins to liposomes provides the possibility of targeted delivery by modifying liposomal surface with appropriate molecules, such as sugars or hydrophilic compounds [46]. However, cationic liposomes are often related to cytotoxicity problems, reduced stability during storage and undesirable interaction with serum proteins, which may cause severe clotting [47].

1.12.2 Vaccines

Liposomes act as adjuvant and carrier of co-adjuvants and potentiate an immune response to the vaccine antigen [48]. One authorized influenza vaccine using liposomal technology is Inflexal V®. It incorporates influenza virus antigens, hemagglutinin and neuraminidase, on the surface of liposomes. Liposomes mimic the structure of the virus, thus allow for cellular entry and membrane fusion [11]. Epaxal® is an aluminum free hepatitis A vaccine based on formalin inactivated hepatitis A antigen incorporated in liposomes, with superior efficacy in comparison to conventional aluminum containing vaccines [49].

1.12.3 Cancer therapy

Conventional cytotoxic drugs damage both cancerous and healthy tissues, causing many severe side effects to patients. Liposomes loaded with cytotoxic drugs can be modified with ligands that recognize tumor-specific antigens. These modifications enhance the accumulation in tumor tissues while reducing uptake in healthy organs. Furthermore, liposomes exploit EPR effect and they can pass through leaky blood vessels of tumor cells and deliver cytotoxic drugs directly to the cancer site [34]. In **Figure 1.18** is depicted the mechanism of action of pH responsive liposomes in a solid tumor. The liposome penetrates through the leaky vessels due to the EPR effect and accumulates at the tumor site. Then, the liposome is engulfed by the cancer cell and form the endosome. The acidic pH of the endosome destabilizes the liposomal membrane and leads to drug release into the cytosol of the cancer cell [34]. Liposomal formulations can also enhance photodynamic therapy through the delivery of photosensitive agents for longer circulation times [50]. Overall, liposomes can improve safety and effectiveness in cancer treatment.

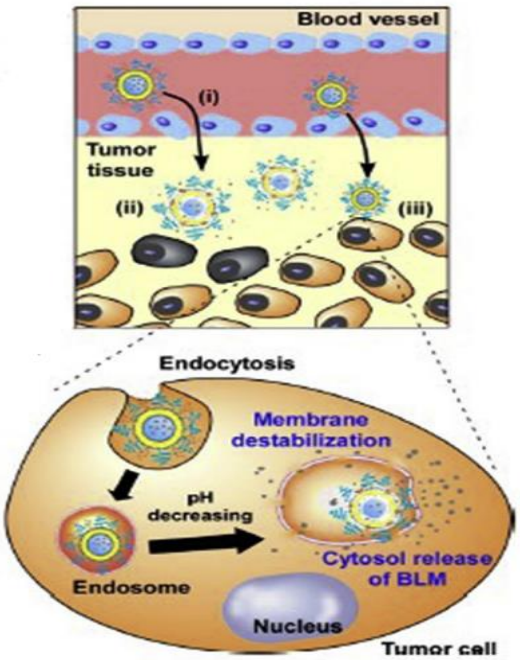


Figure 0.18 Mechanism of action of pH-Responsive liposomes in cancer therapy.

Adapted from [34].

1.13 Quercetin

Quercetin (3,3',4',5,7-pentahydroxyflavone) is a natural flavonoid with antioxidant, anti-inflammatory and antimicrobial properties. Quercetin is sensitive in pH changes and hydrophobic [51]. Quercetin helps reduce the effects of oxidative stress from free radicals and reactive oxidative species [52]Additionally, studies have demonstrated that quercetin can reduce the growth of cancer cells through mechanisms that involve induction of apoptosis [53].

However, quercetin is classified as a BCS Class IV compound, characterized by low permeability and poor solubility [54]. Due to its low water solubility, quercetin is mainly designed for oral administration but, its bioavailability remains low. After ingestion, quercetin is mainly absorbed by the small intestine. In this area, digestive enzymes activity is milder and surface is larger compared to stomach [54]. However, the combination of poor solubility and first pass metabolism reduce significantly the amount of the molecule that reaches systemic circulation. Drug delivery systems such as liposomes, have been explored to surpass these limitations. They enhance quercetin solubility, protect from enzyme degradation and help deliver quercetin in the intestine area [54].

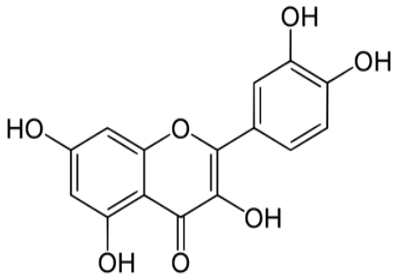


Figure 0.19 Chemical structure of Quercetin.

1.14 Sulforhodamine B (SRB)

Sulforhodamine B is a hydrophilic fluorescent dye that belongs to the Rhodamines family. It is used as a fluorescent tracer for drug kinetics studies in biological systems. In liposome, SRB is encapsulated within the aqueous core and it can be used as detection molecule for release studies. Another use of this molecule is in SRB assay, a test for quantification of living cells in a population. This test is based on its ability to bind to cellular proteins. Incorporating SRB in liposomal systems co-loaded with other therapeutic agents allow in vitro and in vivo imaging capabilities to monitor biodistribution [55].

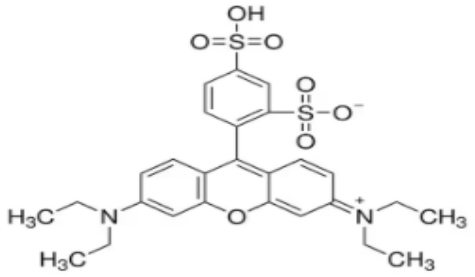


Figure 0.20 Chemical structure of Sulforhodamine B.

1.15 Co-delivery Strategies using Liposomal carriers

Co-delivery refers to the simultaneous encapsulation of two or more therapeutic agents within the same liposome. This approach, enables combined therapy when the action of encapsulated agents is complementary [56]. Additionally, the incorporation of both therapeutic and diagnostic agents allows real time monitoring of drug distribution. Vyxeos^{R} is an authorized medicinal product produced from Jazz Pharmaceuticals Ireland Limited., intended for the treatment of acute myeloid leukemia. It is a liposomal formulation of two antineoplastic agents, daunorubicin and cytarabine, that presents better results compared to standard chemotherapy [57].

1.16 In Vitro Liposomes Characterization

To assess quality of liposomes, several physicochemical parameters must be measured. This is also important to facilitate batch-to-batch comparison. For liposome applications in drug delivery, main parameters include the average mean diameter, the polydispersity index, zeta- potential, encapsulation efficiency and lamellarity determination [58].

1.16.1 Size and Size distribution

Liposome size affects in vivo release of drug loaded liposomes. Additionally, the average size and size distribution are critical when liposomes are designed for parenteral administration or inhalation [58]. The average size of liposomal formulation is influenced from the method of preparation and the type of phospholipids used in the formulation [37]. Various methods have been developed to determine the size and size distribution including: Dynamic light scattering and microscopic methods.

Dynamic Light Scattering (DLS)

This technique measures the Brownian motion of spherical particles in a liquid by analyzing the scattered light, after focusing a laser beam on the nanoparticle solution. The Doppler shift of the light is calculated through a photon detector. The shift changes over time because of fluctuations in the solution. The Stokes-Einstein equation is used to calculate particle size technique needs small samples with minimal preparation [59]. However, it does not give information about particle shape [58]. Additionally, results may be inaccurate due to the presence of dust or due to agglomerates.

DLS is used to evaluate:

- The stability of a formulation according to time/ temperature
- Aggregates in formulations developed by different methods
- Particle size and polydispersity of systems

Microscopy

Different types of electron microscopy techniques have been applied to acquire information about liposomes size and morphology. Two of the most commonly implemented are transmission electron microscopy (TEM) and freeze fracture (cryo-TEM). However, in TEM, the complex sample preparation and their requirement to

remove liposomes from their environment limit its routine use. The main limitation is the high cost [60]. In contrast, atomic force microscopy (AFM) provides information about morphology, size, and liposome aggregation with minimal sample preparation [61].

1.16.2 Lamellarity Determination

Lamellarity refers to the number of lipid bilayers in a liposome. Lipid bilayers number affect the encapsulation efficiency and the drug release kinetics. Several techniques are used to evaluate liposomes lamellarity, including Cryogenic Transmission Electron microscopy (Cryo-TEM), phosphorus nuclear magnetic Resonance (31P-NMR), small-angle X-ray Scattering (SAXS), and fluorescent markers [58].

1.16.3 Zeta potential

Zeta potential (ζ- potential) measurement is a useful tool to determine the electrostatic effects in charged liposomes [39]. It is related with the nature and distribution of the surface charge of liposomes [39]. It is measured using electrophoresis light scattering (ELS) technique which is based on laser Doppler electrophoresis. Zeta potential is important to predict liposomal formulation stability. Factors such as pH, ionic strength and particle concentration can influence zeta potential values. High negative or positive values indicates strong electrostatic repulsion between particles, which helps to prevent aggregation [43].

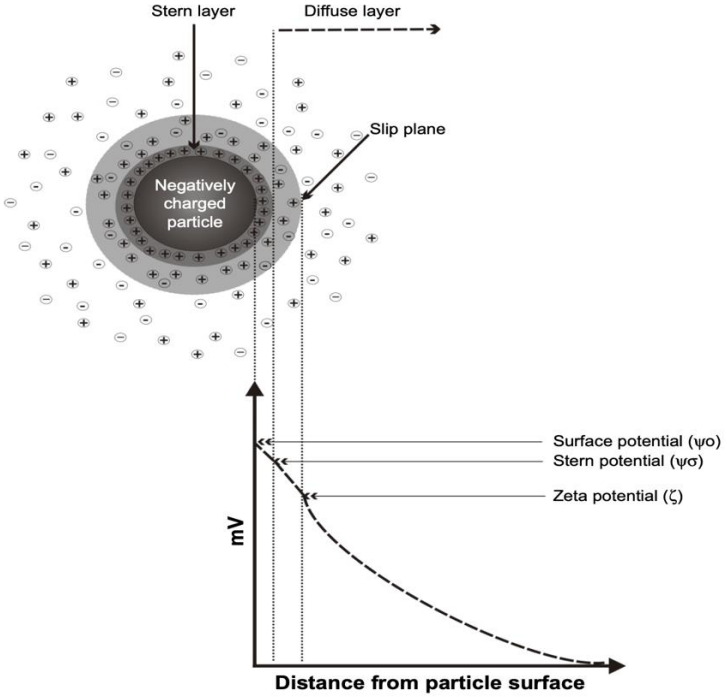


Figure 0.21 Illustration of the electrical charge around a NP in an ionic solution.

Adapted from [61].

In **[Figure 1.21]** is demonstrated how electrical charge is distribute around the nanoparticle in a liquid related to distance from particle surface. The zeta potential is the difference in electrical potential between the particle's surface and the solution. Near the surface the potential decreases linearly and as the distance increases, it decreases exponentially [61].

Table 0.4 ζ-potential value and its impact in colloidal stability.

ζ-potential	Colloidal Stability
0-3 mV	Rapid agglomeration, aggregation and precipitation
5 mV	Strong agglomeration and precipitation
10-15 mV	Threshold of agglomeration
15-30 mV	Threshold of delicate dispersion/ suspension
30-40 mV	Moderate stability
40-60 mV	Good stability
60-80 mV	Very good stability
80-100 mV	Extremely good stability

Data adapted from [62].

1.16.4 Encapsulation Efficiency/ Entrapment Efficiency

Encapsulation efficiency refers to the percentage of drug incorporated into the liposomes relative to the total amount of drug initially added to the formulation [63]. Following their preparation, liposomal dispersions contain a certain amount of drug that remains unencapsulated. To calculate encapsulation efficiency, it is necessary to separate the free drug. This can be achieved through ultracentrifugation or by the use of a dialysis membrane. To calculate encapsulation efficiency there is an indirect and a direct method. Indirect method measures the concentration of free drug in the external medium. Concentration of free drug can be quantified through UV-Vis spectroscopy or HPLC. In contrast, direct method, measures the concentration of drug inside the liposomes after disrupting them with Triton X-100 or other non-ionic surfactants [42]. Entrapment Efficiency (EE)% is calculated according to the equation **(1.1)**.

$$EE\% = \left(\frac{Encapsulated\ drug}{Total\ drug\ added}\right) x\ 100 \qquad (1.1)$$

1.16.5 In Vitro Drug Release Studies

These kinds of studies assess drug release at a particular temperature through in vitro diffusion cell or by using dialysis bag. The cell or bag must have conditions similar to those of the human body, including medium pH at 7.4 and continuous stirring [64]. At specific time points, a volume of the medium is collected and its concentration is determined using UV-Vis spectrophotometry. An equal volume of medium is added to maintain the total volume [65].

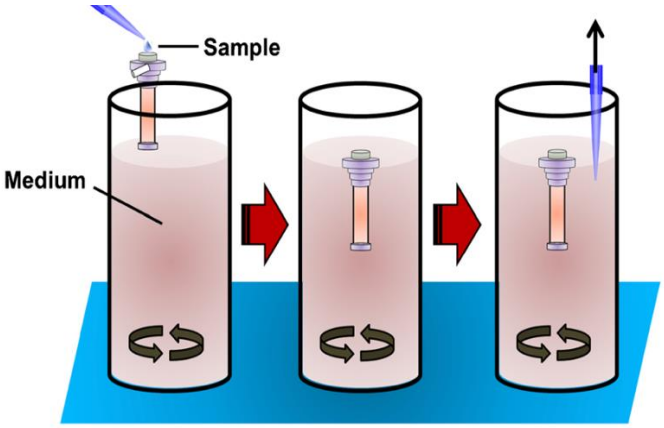


Figure 0.22 Illustration of dialysis.

Adapted from [64].

1.17 Stability Studies

A stable pharmaceutical dosage form maintains its structure and does not affect the active ingredient. Stability studies are conducted to assess physical chemical and microbial properties over time. These studies include product characterization and storage testing [65]. Liposomal formulations also need stability assessment. Liposomes are heterogenous in size and can change over time. During storage, liposomes tend to fuse and become larger. This happens because larger particles are more thermodynamically stable [66]. Additionally, liposomes are made of phospholipids which contains unsaturated fatty acids chains. These chains are sensitive to oxidation, that can change the structure of the lipid bilayer. Furthermore, the drug may interact with the lipids and affect the chemical stability over time [61].

AIM OF THE PRESENT STUDY

Although liposomes have been extensively studied as carriers of bioactive molecules, incorporating more than one bioactive molecule in them is challenging because these molecules may differ in physicochemical properties. The majority of existing studies focus either on the biological action of quercetin or to the use of sulforodamine B as a tracer. To the best of author's knowledge, no studies exist in the literature that examine the incorporation of these specific molecules into the same liposomal system.

This thesis aims to fill this gap, by developing liposomal systems containing quercetin and SRB. Additionally, it aims to investigate their physicochemical characteristics, the impact of co-encapsulation in liposomal stability, as well as, the possible interactions between the two agents and the lipid components.

Specifically, lipid bilayers were composed of DSPC:POPE:CHOL:DSPE-PEG2000 in a molar ratio: 60:20:15:5. Four different formulations were developed with the same lipid ratio, through the thin film hydration method.

Formulation F1: Liposomes without Quercetin and SRB.

Formulation F2: Liposomes with Quercetin.

Formulation F3: Liposomes with SRB.

Formulation F4: Liposomes co- loaded with Quercetin and SRB.

In these formulations, physicochemical characteristics including $D_{h,}$ PDI, ζ-potential and entrapment efficiency were calculated. Furthermore, stability of these systems was evaluated over one month period and in three different temperatures (25ºC, 37ºC and 40ºC). Additionally, these formulations were assessed for possible interactions with serum proteins and for possible leakage of quercetin and sulforhodamine B during storage conditions.

2. MATERIALS AND METHODS

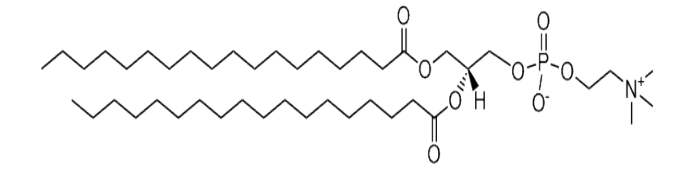
2.1 Materials

Materials used in this study:

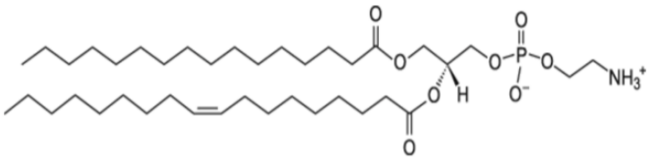
1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) lipids were obtained from Avanti Polar Lipids Inc (Albaster, AL, USA).

Quercetin was purchased from Fluka, BioChemica (Buchs, Switzerland). Sulforhodamine B was obtained from Sigma- Aldrich Chemical Co. (St. Louis, MO, USA).

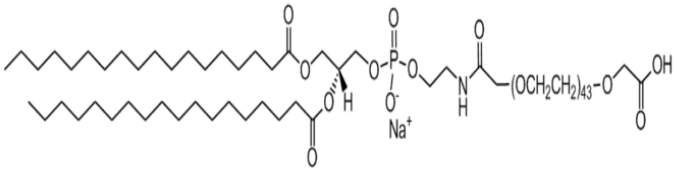
The organic solvent chloroform used in this experiment was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Phosphate Buffered Saline (PBS) tablets were purchased from Sigma- Aldrich Chemical Co. (St. Louis, MO, USA).



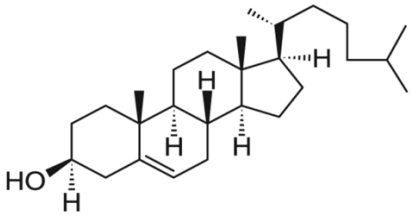
DSPC



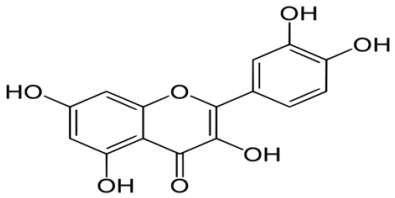
POPE



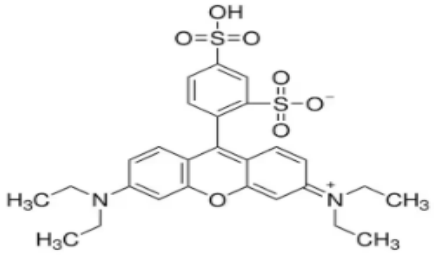
DSPE-PEG2000



Cholesterol



Quercetin



Sulforhodamine-B

Figure 2.1 Chemical structures of materials used in the liposomal formulations.

Instruments used in this study:

Rotary evaporator (Rota vapor R-114, Buchi, Switzerland) was used to remove organic solvent during the thin lipid film preparation.

Probe Sonicator (UP 200S, dr. hielsher GmbH, Berlin, Germany) used to decrease liposomes size.

Centrifuge (Hettich rotanta 460, Berlin, Germany), was used to separate free drug and lipid aggregates from the liposomal dispersion.

The hydrodynamic diameter and z-potential were determined by DLS (Zetasizer 3000 HAS, Malvern, UK).

2.2 Methods

2.2.1 Preparation of Liposomal Formulations

In this study, our aim was to prepare four different liposomal formulation as follows:

Formulation F1: Liposomes without Quercetin and SRB.

Formulation F2: Liposomes with Quercetin in 1.000μM.

Formulation F3: Liposomes with SRB in 40μM.

Formulation F4: Liposomes co-loaded with Quercetin in 1.000μM and SRB in 40μM.

To do so we created a lipid system with the following lipids: DSPC:POPE:Chol:DSPE-PEG2000 in a %molar ratio of 60:20:15:5., which was the same in each formulation. The lipid system was prepared by the thin film hydration method. The main steps are summarized in **[Figure 2.2]**. Lipids were dissolved in chloroform, so as to create stock solutions of 10mg/ml. The appropriate proportions of these were obtained to create the four formulations in round flasks. In formulations F2 and F4, Quercetin, due to its hydrophobic nature, was added at this organic stage, along with the lipids. The concentration of quercetin in these formulations was 1.000μM.

As follows, the organic solvent was evaporated using the apparatus Rotary evaporator, at a temperature of 40^{o}C for 30 minutes under continuous rotation (260 rpm). The lipid film was finally formed on the walls of the round flask.

In the next stage, the systems were hydrated with PBS in a rotary evaporator. Hydration was carried out at a temperature of 60^{o}C, under continuous rotation of 20rpm for 1 hour. At this stage, in solutions F3 and F4, SRB was added because of its hydrophilic nature.

After hydration, the liposomes were large in size and the dispersions were inhomogeneous. With the Probe Sonicator device, the size was optimized and the homogeneity of the nanoparticles was increased. Two five-minute sonication cycles were performed with five-minute interval between them.

However, during sonication small amounts of titanium appeared in the dispersions due to the age and wear of the Probe Sonicator's tip. Additionally, in the dispersions there were lipid aggregates, free quercetin and free SRB molecules that were not entrapped within the liposomes. To eliminate them, the dispersions were centrifuged for 30 minutes at 4000rpm.

Figure 2.2 Flow chart of the main steps applied to prepare liposomal formulations.

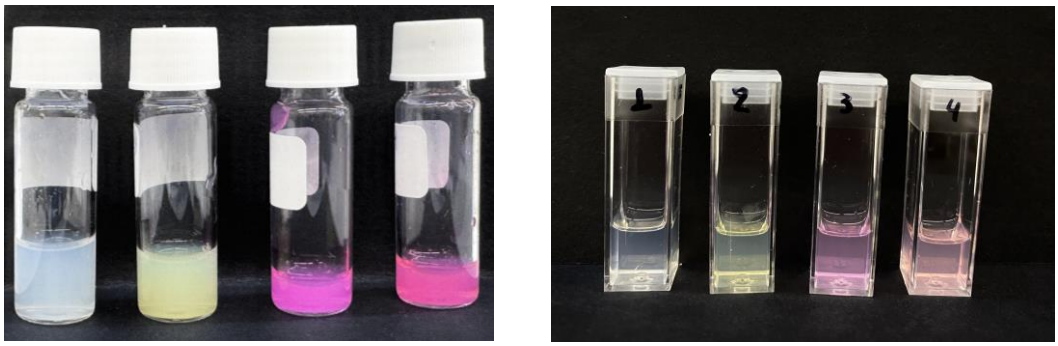


Figure 2.1 Photographs of the prepared liposomal formulations (F1, F2, F3, F4), shown from the left to the right.

2.2.2 Characterization of Liposomal Formulations

Determination of Physicochemical Characteristics

Dynamic Light Scattering (DLS) was used to determine the hydrodynamic diameter and polydispersity index (PDI) of the liposomal formulations. Electrophoretic Light Scattering (ELS) was used to measure ζ-potential. Samples were prepared in cuvettes by diluting 50μlt of liposomal dispersion in 1000μlt of HPLC-grade water. For ζ-potential determination, disposable folded capillary cuvettes were used. Measurements were conducted in a photon correlation spectrometer (Zetasizer 3000 HAS, Malvern, UK) at a detection angle of 90º and analyzed by the CONTIN method (MALVERN software). Each sample was measured in triplicate in three different temperatures 25ºC, 37ºC and 40ºC. The measurements were repeated in days 7, 14, 23 and 33 in order to evaluate the stability of the dispersions over time. The results were presented as mean± SD.

Calibration Curves

Calibration curves were prepared to convert absorbance measurements into drug concentration. This was necessary to quantify quercetin and sulforhodamine B so that we could use them in determination of Entrapment Efficiency and Drug Leakage. Quercetin calibration curve was created at the wavelength λ=376nm, in which quercetin exhibits maximum absorption [67]. Solution of different known quercetin concentration with a constant concentration of Sulforhodamine B was measured using UV-Vis spectrophotometry. The equations derived from the analysis of the results were used for the quantification of quercetin. A similar process was followed for the SRB calibration curve, which was created at λ=570nm. The measurement of the absorption of one compound in the presence of the other, was carried out to determine whether there is interference in the spectrum.

Calibration curves were prepared in medium without lipids. However, spectral scanning of blank liposomes (F1) revealed baseline absorbance around 370nm, which is the λmax of quercetin. To correct UV-Vis results, values of all formulations were corrected by subtracting the signal of F1 at the same wavelength.

Entrapment Efficiency (EE%)

Entrapment efficiency of Quercetin and SRB in the formulations was calculated by both the direct and indirect methods. Liposomes were separated from free drug by centrifugation at 4.000rpm for 30 minutes. Free Quercetin and free SRB sedimented in the pellet, while the liposomes remained in the supernatant. The concentration of drugs was calculated by UV-Vis spectrophotometry. Quantification was based on calibration curves that was prepared in advance for quercetin and SRB. In the indirect method, the concentration of free drug in the pellet was measured and subtracted from concentration of the total drug initially added to the formulation. The difference corresponded to the entrapped amount of drug. In the direct method, the supernatant was lysed with Triton X-100 to release the entrapped drug molecules, which were quantified. In the direct method, Entrapment Efficiency (EE)% was calculated according to the following equation.

$$EE\% = \left(\frac{Encapsulated\ drug}{Total\ drug\ added}\right) x\ 100 \qquad (1.1)$$

Iinteractions with Serum Proteins

Samples were prepared by diluting 50μlt of liposomal dispersion with 1.000μlt of BSA (1% w/v) in a cuvette. The samples were maintained at 37ºC for 30 minutes to simulate

human body conditions. Dynamic Light Scattering (DLS) was used to determine the hydrodynamic diameter and polydispersity index (PDI) of the samples.

Drug Leakage

Drug leakage was evaluated at day 15 after preparation. Samples of the formulations were centrifuged at 4000rpm for 30 minutes to separate the free drug. Free drug was sedimented at the pellet and was subsequently quantified by UV-Vis spectrophotometry. Drug leakage was calculated as the difference between the initial entrapped drug and the entrapped drug remaining at the time of measurement as a percentage to the initially entrapped drug.

3. RESULTS AND DISCUSSION

3.1 UV-Vis Analysis

3.1.1 Calibrations Curves for Quercetin and Sulforhodamine B

Calibration curves were constructed for the quantification of quercetin and sulforhodamine-B by UV-Vis spectrophotometry. The curve for each molecule was obtained in the presence of the other, to investigate possible spectral overlap. More specifically, quercetin calibration curve was constructed at 376nm in the presence of 23μM SRB. While, SRB calibration curve was obtained at 570nm in the presence of 100μM quercetin. For the quercetin calibration curve, SRB in PBS/Tween-80 (0.5% w/v) was measured as a control. Similarly, for the SRB calibration curve, quercetin in PBS/Tween-80 was used as a control. Calibration curves are presented in **[Figures 3.1, 3.2]**. Similar methods are reported in the literature, for the spectrophotometric analysis of conventional co-formulated dosage forms [68], [69], [70]. The calibration curve construction remains the same although in this study the application is intended for liposomal formulations.

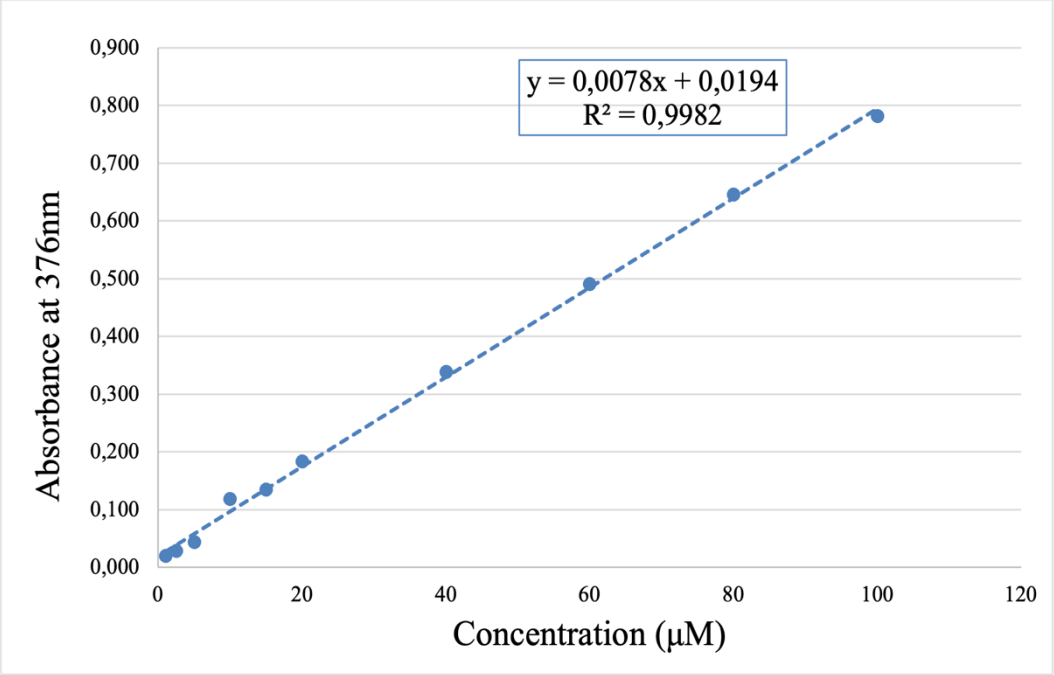


Figure 3.1 Calibration curve of Quercetin in presence of 23μM SRB at 376nm.

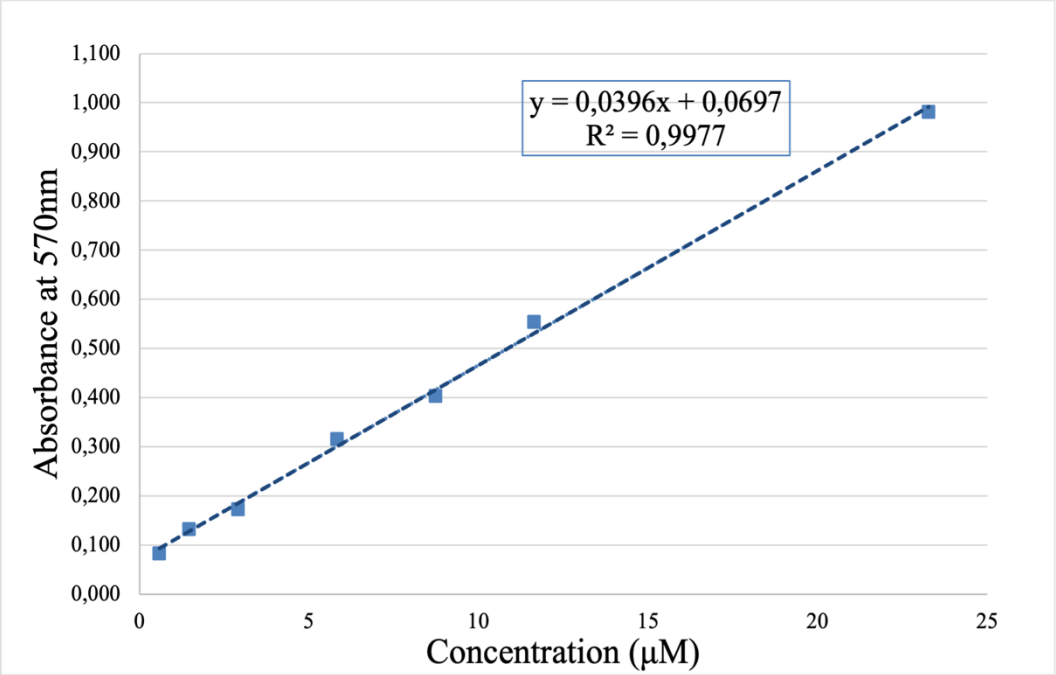


Figure 3.2 Calibration curve of SRB in presence of 100μM quercetin at 570nm.

The results showed that the presence of one molecule did not affect the linearity of the curve of the other. Thus, in these wavelengths there is no significant overlap between quercetin and SRB. The regression equations of quercetin and SRB were: y= 0.0078x + 0.0194 (R^2= 0.9982) and y=0.0396x + 0.0697 (R^2= 0.9977) respectively. In both of them, regression coefficients (R^2) were greater than 0.99. This, confirms the reliability of the quantification method. The regression equations were used for quantification of drug encapsulation, leakage and release. Similar approaches have been reported in related studies to ensure accuracy in the simultaneous analysis of two molecules [71].

It should be mentioned that the initial concentrations used in the formulations were 1.000μM for quercetin and 40μM for SRB. The range in which calibration curves were constructed was lower, up to 100μM for quercetin and 23μM for SRB. However, the concentrations measured during encapsulation and release studies were within the calibration range.

3.1.2 Effect of Lipids in UV-Vis spectrum

Spectral scanning of drug-free formulation F1 (DSPC:POPE:Chol:DSPE-PEG2000, 60:20:15:5 mol%) revealed absorbance attributed to lipids. Since, the calibration curves were constructed in 0.5% PBS/Tween 80 buffer, this effect was not visible in them **[Figures 3.1, 3.2].**

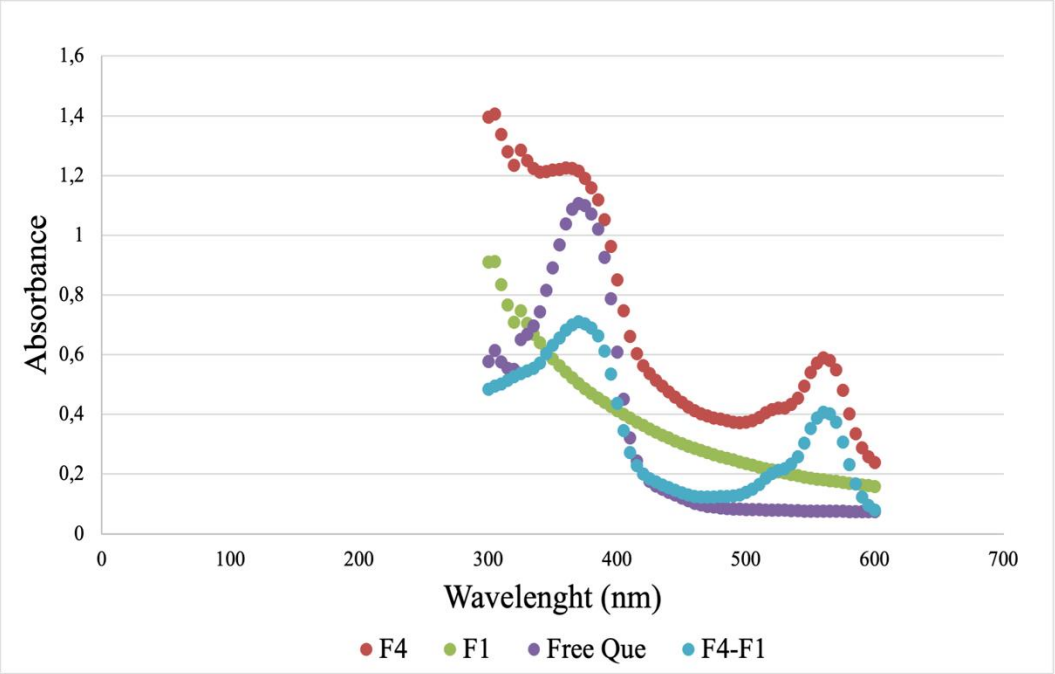


Figure 3.3 UV-Vis spectral scanning of drug free formulation (F1), co-loaded (F4), free quercetin and corrected F4 spectrum (F4-F1).

[Figure 3.3] shows the Uv-Vis spectra of drug-free formulation F1, the double loaded formulation F4, a free quercetin solution (1.000μM) and the corrected spectrum of formulation F4. It can be observed that F1 absorbs in the UV region, close to the maximum peak of quercetin at 376nm. As a result, the quercetin peak in F4 is overlapped by the lipids. Thus, to correct this interference, the absorbance of F1 was subtracted from all formulations. F4-F1 blue line shows the corrected spectrum of F4. This correction was applied to all UV-Vis measurements in the following studies, to ensure accurate quantification of quercetin and SRB. According to literature, phospholipids exhibit strong absorption in the UV region of 200-210nm [72], [73]. Therefore, the observed increased absorption in the region 300-350nm, may not be due to lipids absorption. It may be attributed to scattering effects by the liposomal suspensions [74], [75].

3.2 Physicochemical Characteristics of Liposomal Formulations at Day 0

Physicochemical characteristics of the four liposomal systems were evaluated on the day of their preparation. All formulations had the same lipid composition: DSPC:POPE:Chol:DSPE-PEG2000, 60:20:15:5 mol%. Measurements were carried out in 25, 37 and 40°C. The parameters that were measured include hydrodynamic diameter (D_h) **[Table 3.1, Figure 3.4]**, polydispersity index (PDI) **[Table 3.2, Figure 3.5]**, ζ-potential **[Table 3.3, Figure 3.6]** and entrapment efficiency (EE%) **[Table 3.4, Figure 3.7]**.

Table 3.1 Hydrodynamic diameter (D_h) of all formulations at Day 0 in 25, 37 and 40°C.

Liposomal Formulation	D_h 25°C (nm)	D_h 37°C (nm)	D_h 40°C (nm)
F1	103.5 ± 1.2	105.3 ± 0.4	106.7 ± 0.6
F2	139.7 ± 1.0	156.3 ± 9.5	152.3 ± 8.0
F3	118.8 ± 2.2	135.1 ± 5.8	131.9 ± 5.3
F4	136.7 ± 1.0	150.2 ± 9.0	139.2 ± 5.7

Note. Data presented as mean ± SD, n=3

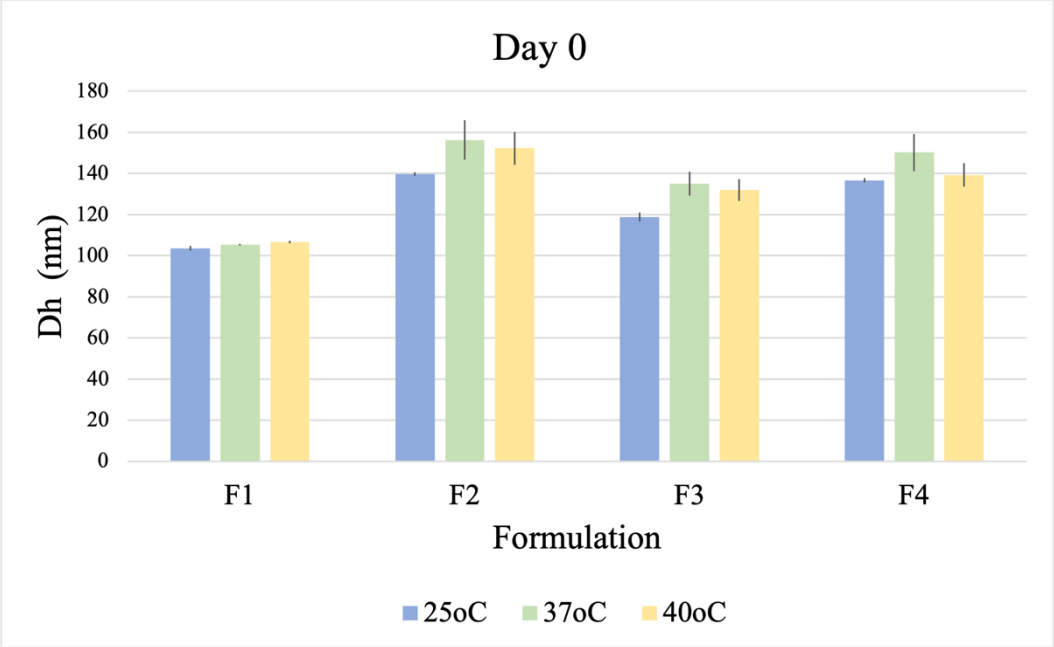


Figure 3.4 Hydrodynamic diameter (D_h) at day 0 in 25, 37 and 40°C.

All four formulations exhibited D_h values in the range of 100-160nm with minimal differences between them. Formulation F1 (blank) showed the smallest mean sizes (100-107nm), while F2 that was loaded with quercetin, exhibited the largest (140-152nm). F3 and F4 exhibited intermediate sizes, with F4 (double loaded) being slightly larger than F3. These results imply that the incorporated drug may have an impact on liposomal size.

The fact that quercetin-loaded formulation exhibited slightly larger D_h values compared to SRB-loaded formulation, may indicate that the incorporation of quercetin in the bilayer affects its packing and results to a slight expansion on the size of the liposome. Similar results have been reported in other studies, where the incorporation of hydrophobic drugs increased slightly hydrodynamic diameter. Zhang et al. (2022) showed that pegylated liposomes loaded with the hydrophobic agents docetaxel and resveratrol had slightly larger sizes compared to blank [76]. Sesarman et al. (2018) demonstrated that pegylated liposomes loaded with the hydrophobic molecule curcumin and the hydrophilic compound doxorubicin, had sizes close to blank liposomes, with a slight increase in the formulations with curcumin [77].

However, these findings are in contrast to the study of Romero-Arriera et al. (2020), who reported that in liposomes consisting of DSPC:Chol, the entrapment of the hydrophilic molecule resazurin increased D_h, while the incorporation of the hydrophobic pinocembrin maintained D_h values close to those of empty liposomes [75]. Therefore, the effect of the drug loading on liposome size varies and depends on the drug-lipid interaction and the composition of the formulation.

To further evaluate the physical properties of the liposomes on the day of their preparation, all formulations were also measured at human body (37ºC) and at slightly elevated (40ºC) temperatures. Results demonstrated minor changes, about 10nm, with temperature increase, indicating that formulations were not temperature-sensitive at day 0. Additionally, results showed that relative size differences between formulation remained the same at all tested temperatures. F2 was the largest, and F1 the smallest across all temperatures. These results confirm that at day 0 all formulations were stable and not sensitive in heat-induced changes.

Table 3.2 Polydispersity index (PDI) of all formulations at day 0 in 25, 37 and 40°C.

Liposomal Formulation	PDI 25°C	PDI 37°C	PDI 40°C
F1	0.166 ± 0.027	0.180 ± 0.022	0.179 ± 0.030
F2	0.128 ± 0.020	0.153 ± 0.013	0.140 ± 0.013
F3	0.116 ± 0.008	0.109 ± 0.016	0.116 ± 0.025
F4	0.162 ± 0.016	0.184 ± 0.033	0.180 ± 0.010

Note. Data presented as mean ± SD, n=3.

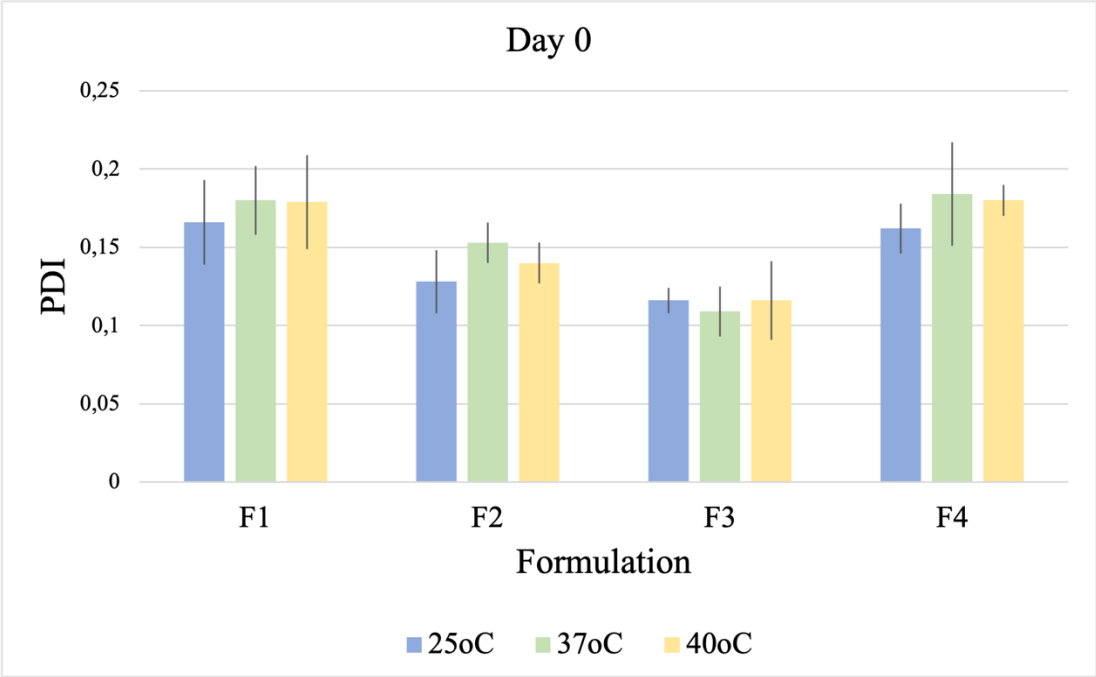


Figure 3.5 Polydispersity index (PDI) at day 0 in 25, 37 and 40°C.

The polydispersity index values at day 0, were measured in the range between 0.109-0.184. According to literature, PDI values below 0.2, indicate a narrow size distribution and good homogeneity. Such systems are considered as monodisperse and acceptable for parenteral administration, while values up to 0.3 may still be acceptable for other routes of administration [78]. Formulation F3 (loaded with SRB) showed the lowest PDI values, across all temperatures, indicating excellent homogeneity. However, the differences between the four formulations were very small and all the samples could be considered homogenous and well within the accepted range. Similar values, below 0.2 have been reported for quercetin-loaded stealth liposomes [79].

The ζ-Potential of the liposomal formulations was measured at day 7 to evaluate surface charge. The results are presented in the following Table.

Table 3.3 Zeta-potential of all formulations.

Liposomal Formulation	Z- potential (mv)
F1	-6.65 ± 0.50
F2	-7.12 ± 0.80
F3	-8.37 ± 0.05
F4	-8.06 ± 1.20

Note. Data presented as mean± SD, n=3.

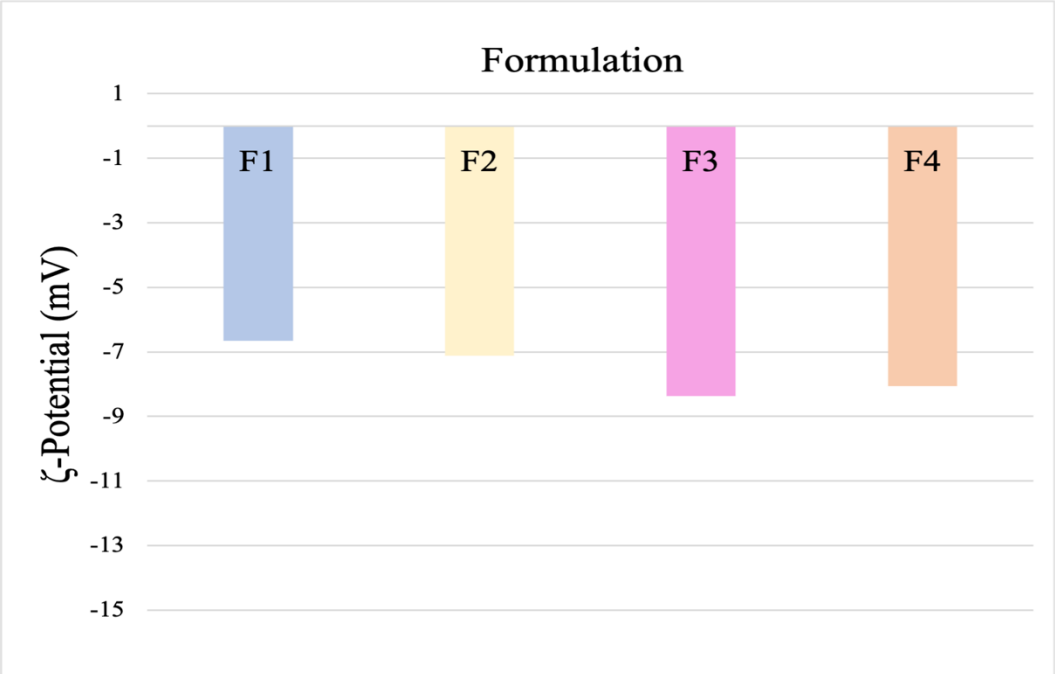


Figure 3.6 Zeta-potential of all formulations.

All formulations exhibited slightly negative ζ potential values in the range of -6 to -8mV. These near zero results were expected, since the lipids used in the composition do not have a positive or a negative charge.

The slight negative values may be attributed to the ionization of the phosphate groups of the phospholipids. Similar negative values were reported in studies with pegylated liposomes of similar composition, where ζ- potential was shown to vary with pH and

ionic strength [80]. Veiko et al. (2020) reported that the incorporation of quercetin into liposomal membranes increased ζ- potential, due to electrostatic interactions with the bilayer surface [81].

According to the literature, ζ-potential values at the range 10-15mV are in the threshold of agglomeration or aggregation [62]. However, the liposomal dispersion in this study remained stable. This is attributed to the presence of DSPC-PEG2000. The PEG chains of this phospholipid provide steric stabilization to liposomes through repulsive forces between polymer chains. Thus, liposomal aggregation was prevented [82].

Additionally, it can be observed that among the four formulations, those loaded with quercetin and/or SRB have shown a slight increase in ζ- potential (~2mV). This may indicate that drug load affects minimally the interfacial charge distribution. Similar minor changes in ζ-potential were reported in pegylated liposomes loaded with curcumin and doxorubicin [77].

3.3 Entrapment Efficiency Determination

The entrapment efficiency of quercetin and SRB of the liposomal formulations was evaluated on the day of their preparation. The results are presented in **[Table 3.4]**.

Table 3.4 Entrapment Efficiency (EE%) for quercetin and SRB in formulations F2, F3 and F4 at day 0.

Liposomal Formulation	EE (%) of Quercetin	EE (%) of SRB
F2	37.8	-
F3	-	40.1
F4	36.0	61.0

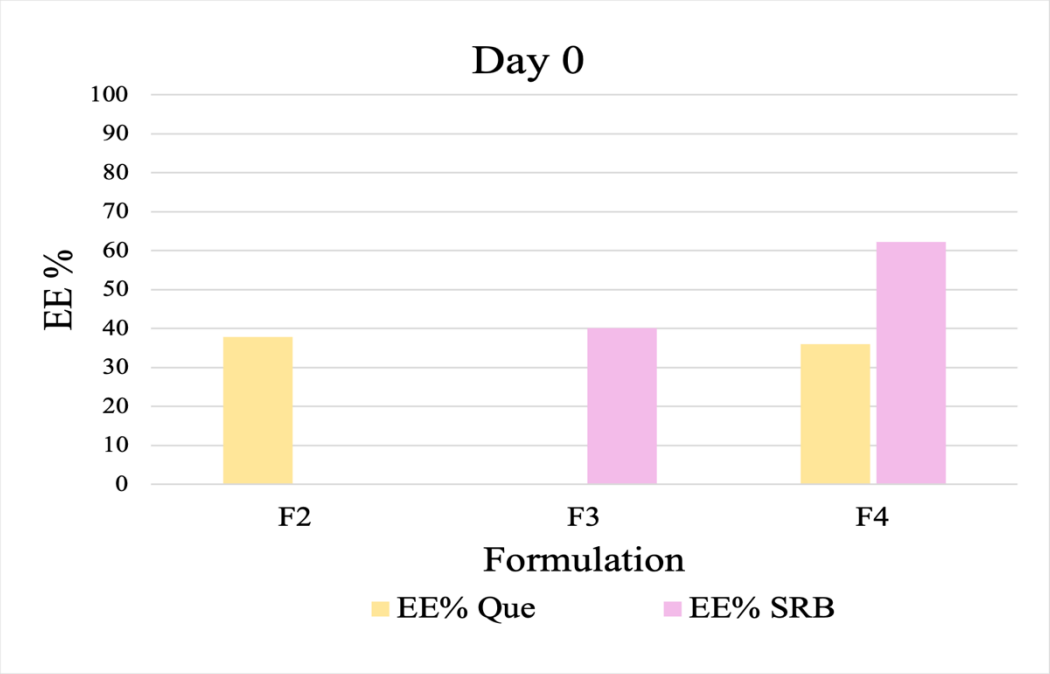


Figure 3.7 Entrapment Efficiency (EE%) for quercetin and SRB in formulations F2, F3 and F4 at day 0.

The initial concentration of quercetin added to the liposomal systems was 1.000μM. The concentration of encapsulated drug in F2 (loaded only with quercetin) was estimated at 378.0μM, while in F4 (double loaded) it was 360.0μM. The corresponding encapsulation percentages were calculated at 37.8% for F2 and at 36.0% for F4. Comparing the percentages between F2 and F4, similar values were observed. Therefore, the presence of SRB in F4 did not affect the encapsulation of quercetin. This result was expected, since quercetin is hydrophobic and during preparation, quercetin was added at the organic phase during the lipid-film formation stage before the addition of SRB to the hydration stage. Furthermore, it is observed that despite the high initial load of quercetin (1.000μM), the systems in both cases encapsulated up to only ~37%. This result may imply that loading is limited in this percentage.

According to the literature, similar systems loaded with quercetin showed higher encapsulation percentages, ~80% [83], [84]. The difference may be attributed to the very high initial concentration of quercetin loaded to the system. It is possible that lipid bilayer was saturated and the rest amount of quercetin was lost during the preparation process. Similar to these findings, Tefas et al. (2015) reported that EE% was affected from lipid composition and initial drug concentration. Very high initial quercetin concentrations were related to lower entrapment percentages [85]. In another study, Kulkarni et al. (1995) reported that encapsulation efficiency increased with rising initial drug concentration, up to a certain level, and then a further increase in initial concentration led to decrease in encapsulation efficiency [86]. Loading capacity refers

to the maximum amount of drug that can be accommodated in the bilayer at a specific lipid concentration. It is expressed as drug-to-lipid ratio. Chountoulesi et al. (2017) highlighted the importance of the drug-to-lipid ratio as a key parameter to optimize liposomal formulations [87]. Differences in this ratio may affect encapsulation efficiency, drug release and stability.

As for SRB, the initial concentration added to the formulations was 40μM. The concentration of the encapsulated drug in F3 (loaded with SRB) was estimated at ~16.0μM, while in F4 it was at ~24.0μM. The encapsulation percentages derived from these data were 40.1% for F3 and 61.0% for F4. Comparing these percentages, double loaded F4 showed greater SRB encapsulation than F3. Therefore, it appears that the presence of quercetin in F4 did not hinder, but instead may had favored SRB encapsulation. This may be explained by the fact that SRB is water-soluble and is encapsulated in the aqueous core of the liposome. The composition of the lipid bilayer affects leakage and stabilization of SRB. The entrapment of quercetin in the bilayer may had changed its physicochemical characteristics and may had made it less permeable. Thus, SRB retention in the core was enhanced.

Leite et al. (2022) reported that in lipid bilayers with high cholesterol concentration, quercetin increased bilayer rigidity and decreased permeability. In contrast, in heterogenous domains of the membrane, quercetin induced reorganization and enhanced leakage [88]. In our study, formulations contained a significant amount of cholesterol and thus, quercetin may have contributed to a less permeable bilayer. Furthermore, the calculated encapsulation efficiencies are consistent with the literature. More specifically, hydrophilic molecules show lower EE% because of the limited space of the aqueous core of SUVs [75].

Overall, the results showed that hydrophilicity plays an important role in drug encapsulation in liposomal systems. Hydrophobic quercetin exhibited a stable entrapment rate that was limited by bilayer saturation due to high initial load. In contrast, SRB encapsulation was affected more by the composition of the lipid bilayer.

3.4 Physicochemical characteristics of Liposomal Formulations in BSA medium

D_h and PDI was measured after the incubation of liposomal formulations in 1% w/v BSA for 30 minutes at 37ºC to evaluate the interaction with the serum proteins. The results are presented in **[Table 3.5]** and **[Figure 3.8]**.

Table 3.5 Physicochemical characteristics of all formulations in water vs in 1%w/v BSA at day 23 at 37ºC.

Liposomal Formulation	D_h Water (nm)	D_h BSA (nm)	PDI Water	PDI BSA
F1	119.8 ± 6.0	119.5 ± 4.0	0.206 ± 0.036	0.223 ± 0.010
F2	151.1 ± 0.8	161.0 ± 7.7	0.261 ± 0.010	0.237 ± 0.010
F3	132.8 ± 6.5	137.4 ± 5.8	0.164 ± 0.046	0.151 ± 0.021
F4	146.1 ± 6.2	156.9 ± 5.5	0.198 ± 0.008	0.176 ± 0.022

Note. Data presented as mean± SD, n=3.

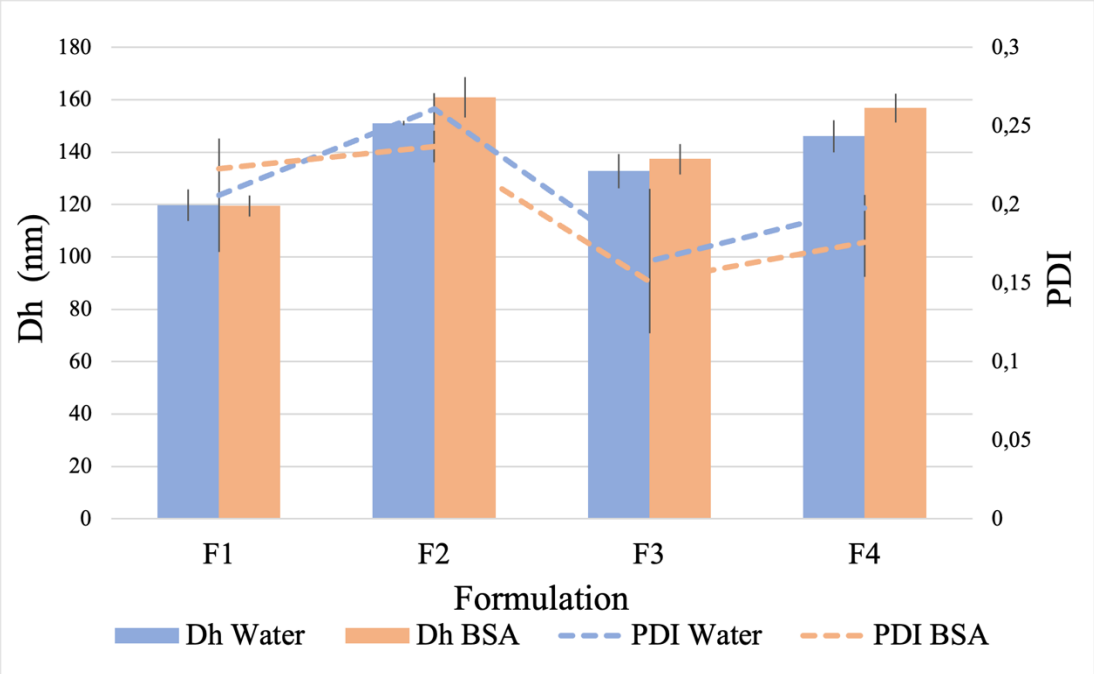


Figure 3.8 Physicochemical characteristics of all formulations in water vs in 1%w/v BSA.

Incubation of liposomal systems in 1% w/v BSA at body temperature (37ºC) did not result in significant changes in D_h and PDI values. More specifically, in F1 D_h values remained stable while PDI values increased slightly. The pattern was similar in F3, which was loaded with SRB. This was expected as SRB is encapsulated in the aqueous core of the liposomes and therefore does not affect the surface of the liposomes. In contrast, formulations F2 and F4, which both had quercetin entrapped in the bilayer, showed a negligible increase in D_h (10nm), along with a small decrease in PDI. These changes may be related to a very mild interaction of the proteins with the surface of the liposomes. This may be due to the changes caused by the incorporation of quercetin into the lipid bilayer [81]. However, the change is too small to reach a clear conclusion.

The constant values of D_h and PDI indicates the absence of protein adsorption in the surface of liposomes (absence of hard corona). This result may be related to the presence of DSPE- PEG2000 lipid in the formulations [89].

The results are consistent with literature, where in similar systems including DSPE-PEG lipid, pegylated liposomes showed limited protein adsorption. Du et al. (1997), demonstrated that increasing the amount of pegylated lipids in lipid bilayers, the adsorption of serum proteins in the bilayer was reduced. The study also showed that the use of pegylated lipids in bilayers inhibited the recognition from macrophages and lymphocytes, giving shield properties to the liposomes [90]. This behavior can be explained by the steric stabilization provided by polymer chains in colloidal systems [91].

Overall, the results in 1% w/v BSA support that protein adsorption was very limited in all formulations, demonstrating good colloidal stability and stealth properties.

3.5 Physicochemical characteristics of Liposomal Formulations over Time

Hydrodynamic Diameter (D_h, nm) and Polydispersity index (PDI) were measured at 25, 37 and 40ºC, at days 0, 7, 14, 23 and 33 to assess the stability of all formulation. The following Tables and Figures present the stability data for each formulation.

Table 3.6 Formulation F1: Hydrodynamic diameter ($D_{h)}$ and polydispersity index (PDI) over 33 days at three different temperatures.

t (days)	D_h 25ºC (nm)	D_h 37ºC (nm)	D_h 40ºC (nm)	PDI 25ºC	PDI 37ºC	PDI 40ºC
0	103.5±1.2	105.3±0.4	106.7±0.6	0.166±0.027	0.180±0.022	0.179±0.030
7	105.9±0.7	114.5±2.4	106.0±1.8	0.168±0.015	0.201±0.023	0.196±0.035
14	108.6±0.7	121.6±6.2	115.7±1.6	0.184±0.024	0.204±0.005	0.218±0.046
23	106.7±0.1	119.8±6.0	115.0±2.6	0.212±0.045	0.206±0.036	0.227±0.015
33	111.9±1.6	127.1±6.6	127.7±5.7	0.211±0.012	0.241±0.012	0.249±0.009

Note. Data presented as mean± SD, n=3.

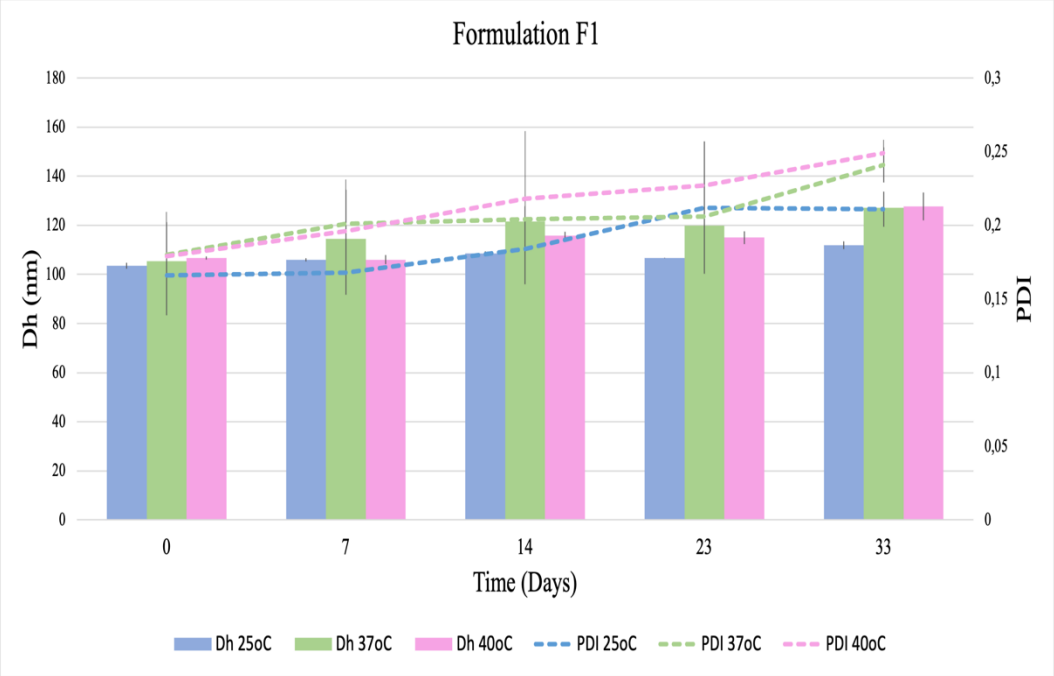


Figure 3.9 Formulation F1 stability results over 33 days in different temperatures.

Formulation 1, the drug-free formulation, exhibited satisfactory stability. At 25ºC, only a minor increase in D_h was observed, from 103,5nm at day 0 to 111,9nm at day 33 (+7.5%). PDI values were also relative stable and were maintained at low levels below 0.21. These results indicate good stability at storage temperature. At 37ºC and 40ºC the results were similar. At these temperatures, instability was greater compared to 25ºC. The hydrodynamic diameter of liposomes increased from 105,3 at day 0, to 127,1 at day 33. PDI also increased from 0,180 to 0,240. According to the literature, these values are still considered within the acceptable range for monodisperse systems [78]. Therefore, formulation F1 remained stable at elevated temperatures, although there was a small tendency of increasing instability over time.

The stability of formulation F1 over time even in higher temperatures, may be attributed to the lipids used in the composition (DSPC:POPE:CHOL:DSPE-PEG2000). According to the literature, DSPC lipid has a high phase transition temperature (T_c= 55ºC), above the temperatures tested in this study. When included in the liposome composition, DSPC provides rigidity in the bilayer and improves drug retention, compared to lipids with lower transition temperatures [92]. Cholesterol plays also an important role in the stability of the liposomal dispersion. Kirby et al. (1980) reported that cholesterol-rich liposomes exhibited improved stability in vitro and in vivo condition compared to cholesterol-poor or free liposomes [93]. Additionally, the presence of DSPE-PEG2000 in the system enhances further stability. It provides steric stabilization by reducing interactions between liposomes, preventing aggregation over time [94].

However, POPE belong to the phosphatidylethanolamines which destabilize when reaching the hexagonal face transition temperature. When approaching this temperature, it occurs a structural rearrangement that destabilizes the bilayer and increases its permeability [95]. According to the literature, this temperature for POPE is near 71ºC [96]. The temperatures tested in this study are below this point, thus, the destabilizing effect of POPE might not be obvious.

Overall, formulation F1 was stable for one month under storage conditions (25ºC). At physiological (37ºC) and elevated (40ºC) temperatures, showed a small increase in D_h and PDI but remained within the acceptable range for monodisperse liposomal systems.

Table 3.7 Formulation F2: Hydrodynamic diameter (D_h) and polydispersity index (PDI) over 33 days at three different temperatures.

t (days)	D_h 25ºC (nm)	D_h 37ºC (nm)	D_h 40ºC (nm)	PDI 25ºC	PDI 37ºC	PDI 40ºC
0	139.7±1,0	156.3±0.5	152.3±8.0	0.128±0.02	0.153±0.013	0.140±0.013
7	143.0±2.2	150.8±5.2	129.5±3.7	0.162±0.053	0.150±0.041	0.105±0.066
14	151.4±1.6	168.0±6.0	158.6±1.7	0.260±0.018	0.274±0.002	0.261±0.012
23	147.0±0.5	151.1±0.8	157.6±4.0	0.225±0.013	0.261±0.010	0.249±0.014
33	149.3±0.8	170.7±8.6	164.4±5.1	0.258±0.027	0.266±0.023	0.277±0.008

Note. Data presented as mean± SD, n=3.

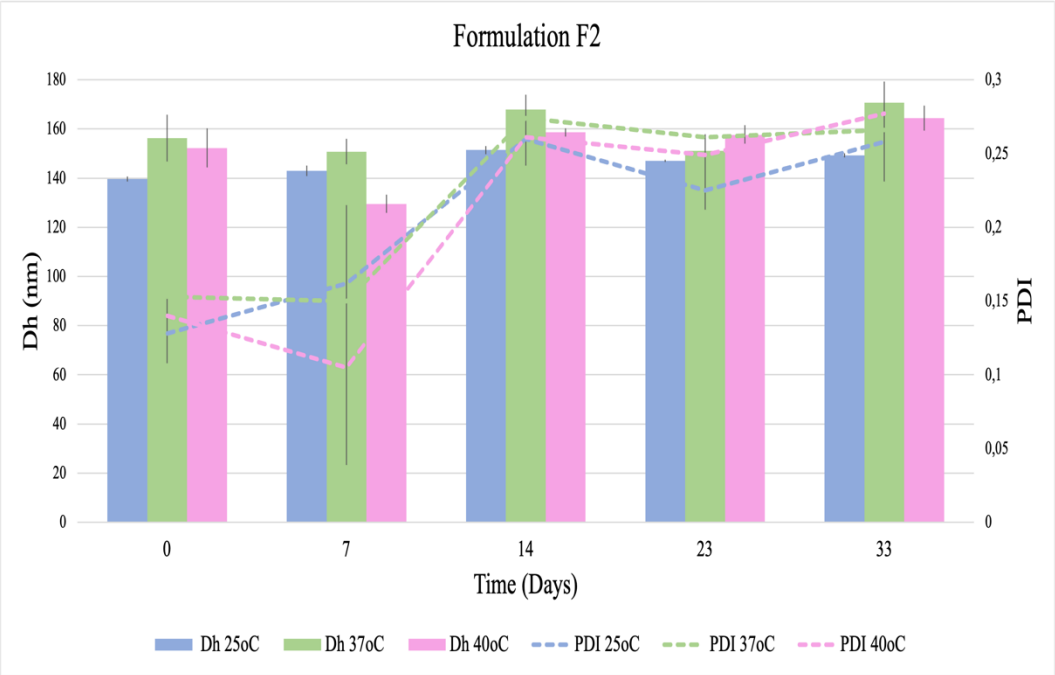


Figure 3.10 Formulation F2 stability results over 33 days in different temperatures.

At 25ºC, the hydrodynamic diameter of Formulation F2 increased moderately from 139.7nm to 149,3nm (+6.4 %) at day 33. However, an important increase in polydispersity index was observed from day 14, reaching 0.26. At 37ºC, the increase in D_h was a little higher (+8,43%) than at 25ºC. PDI values rose from 0.153 to 0.266,

indicating reduced homogeneity. At 40ºC, D_h had a similar increase as at 37ºC and PDI rose to 0.277 at day 33. Of note, at 40ºC at day 7, a temporary decrease in size (129.5nm) was observed.

These findings may be related to the influence of quercetin entrapment in the bilayer. In the literature, it is reported that flavonoids interact with lipid membranes and alter their organization, fluidity and permeability [81]. A study by Sanver et al. (2020) showed that entrapment of quercetin in DOPC bilayers led to bilayer thinning, increased fluctuations and expansion of interbilayer spacing [97]. Such findings may explain the fluctuation in D_h and the increase in PDI observed in the current study.

Overall, formulation F2 exhibited good stability for up to two weeks, but beyond this period PDI increased, indicating loss of colloidal stability. The entrapment of quercetin in the lipid bilayer, may affected long term stability of the bilayer, especially in higher temperatures.

Table 3.8 Formulation F3: Hydrodynamic diameter ($D_{h)}$ and polydispersity index (PDI) over 33 days at three different temperatures.

t (days)	D_h 25ºC (nm)	D_h 37ºC (nm)	D_h 40ºC (nm)	PDI 25ºC	PDI 37ºC	PDI 40ºC
0	118.8±2.2	135.1±5.8	131.9±5.3	0.116±0.008	0.109±0.002	0.116±0.025
7	119.8±0.2	130.1±4.9	118.7±1.9	0.121±0.020	0.137±0.035	0.147±0.021
14	123.5±2.3	134.3±5.0	129.3±4.0	0.109±0.022	0.095±0.024	0.143±0.033
23	121.4±1.0	132.8±6.5	128.5±4.1	0.124±0.010	0.164±0.046	0.128±0.027
33	124.0±0.9	136.3±6.5	132.0±2.1	0.143±0.011	0.199±0.039	0.191±0.023

Note. Data presented as mean± SD, n=3.

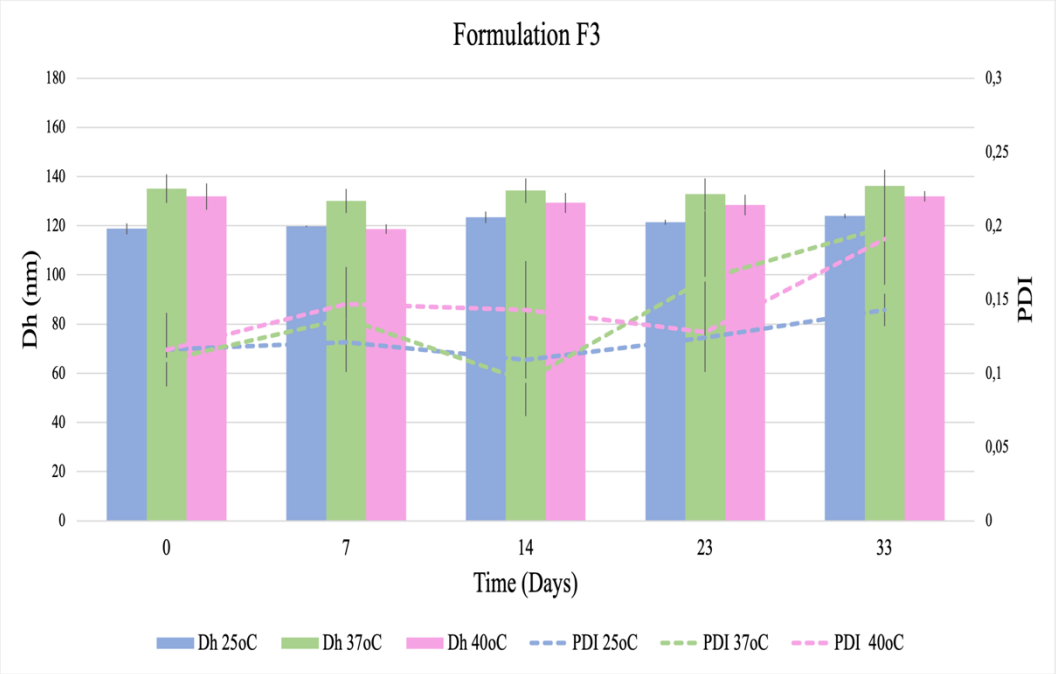


Figure 3.11 Formulation F3 stability results over 33 days in different temperatures.

Formulation F3, loaded only with SRB, exhibited excellent stability characteristics. At 25ºC, D_h increased slightly from 118,8nm to 124.0nm (+4%) after 33 days. PDI values remained low, 0.143 at day 33, indicating very good colloidal stability under storage conditions. At 37ºC, the size ranged between 130 to 135nm and PDI reached 0.199 at day 33, indicating some broadening but still values remained low, under 0.3 aggregation threshold [78]. At 40ºC, the values of hydrodynamic diameter and polydispersity index ranged at the same levels as at 37ºC, indicating very good stability even in higher temperatures.

Overall, formulation F3 exhibited excellent stability under all tested conditions for one month, with minimal changes in size and PDI. The incorporation of SRB did not affect liposomal stability, indicating that the molecule did not interact with the lipids within the bilayer.

Table 3.9 Formulation F4: Hydrodynamic diameter (D_h) and polydispersity index (PDI) over 33 days at three different temperatures.

t (days)	D_h 25°C (nm)	D_h 37°C (nm)	D_h 40°C (nm)	PDI 25°C	PDI 37°C	PDI 40°C
0	136.7±1.0	150.2±9.0	139.2±5.7	0.162±0.016	0.184±0.033	0.180±0.010
7	138.9±1.2	148.1±6.2	128.0±2.3	0.174±0.018	0.160±0.022	0.182±0.014
14	134.2±0.4	150.1±7.7	135.6±5.5	0.145±0.036	0.192±0.006	0.194±0.012
23	132.3±0.8	146.1±6.2	137.4±2.9	0.158±0.011	0.198±0.008	0.200±0.035
33	133.9±1.1	153.2±10.8	149.7±6.7	0.178±0.004	0.270±0.061	0.250±0.037

Note. Data presented as mean± SD, n=3.

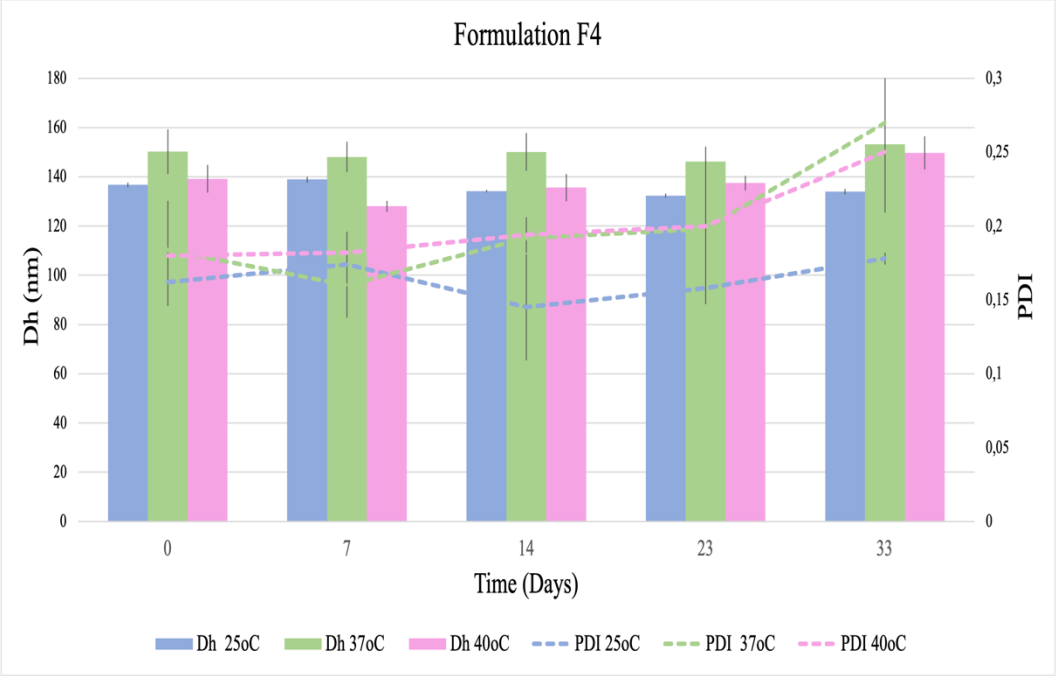


Figure 3.12 Formulation F4 stability results over 33 days in different temperatures.

Formulation F4 was loaded both with quercetin and SRB. At 25°C, sizes remained stable over the period of 33 days. PDI values were consistently low reaching 0.178 at day 33, indicating excellent stability under storage condition. At 37°C, D_h values

fluctuated between 146-153nm, showing a minimal change. However, PDI increased from 0.184 to 0.270 at day 33, indicating loss of homogeneity after one month in physiological temperature. At 40ºC, D_h values fluctuated between 128.0-149.7nm, while PDI values were similar with results at 37ºC. At day 33, PDI reached 0.250, which indicates reduced colloidal stability.

Since lipid composition remained the same across all formulation and formulation F3 exhibited excellent stability, the increase in PDI in the co-loaded system may be attributed to quercetin interaction with the lipid bilayer. As discussed in stability results for formulation F2, quercetin disturb membrane organization and reduce long term homogeneity.

Overall, stability results for formulation F4 were excellent at storage temperature over a month. However, at higher temperatures PDI values gradually increased over a 33-day period, suggesting the onset of aggregation and heterogeneity in the liposomal dispersion.

3.6 Comparative Stability study between all Formulations

Based on the data in **[Tables 3.6- 3.9]** comparative diagrams of D_h and PDI at 25, 37 and 40°C are presented in **[Figures 3.13-3.19]**. These figures allow for direct comparison of the stability results of all formulations under similar temperature conditions.

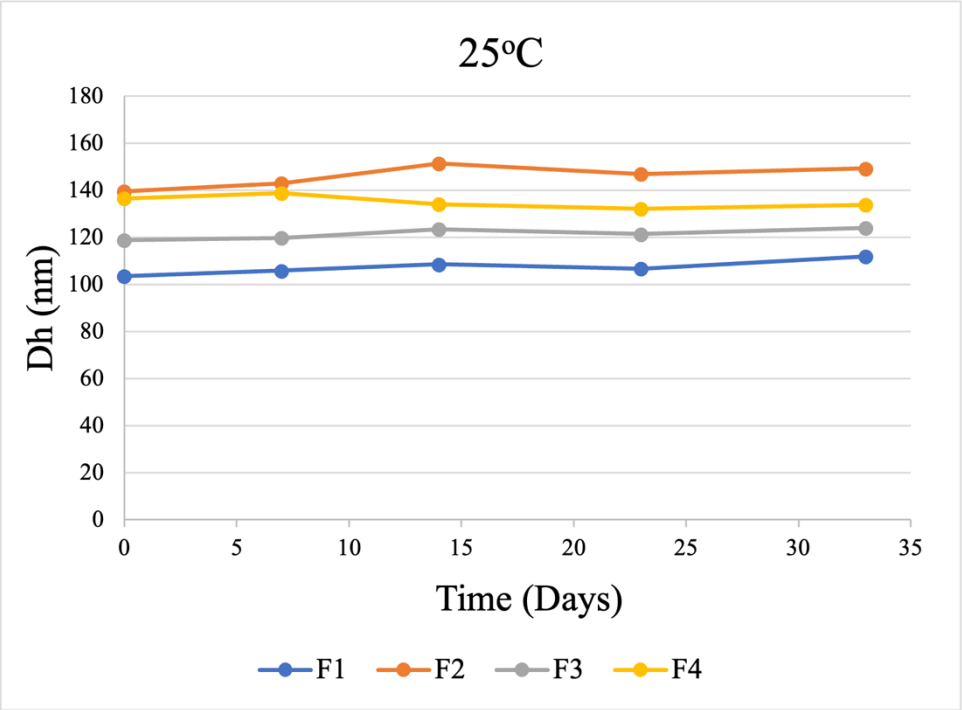


Figure 3.13 Comparative diagram of Hydrodynamic diameter (D_h) at 25°C for all formulations over 33 days.

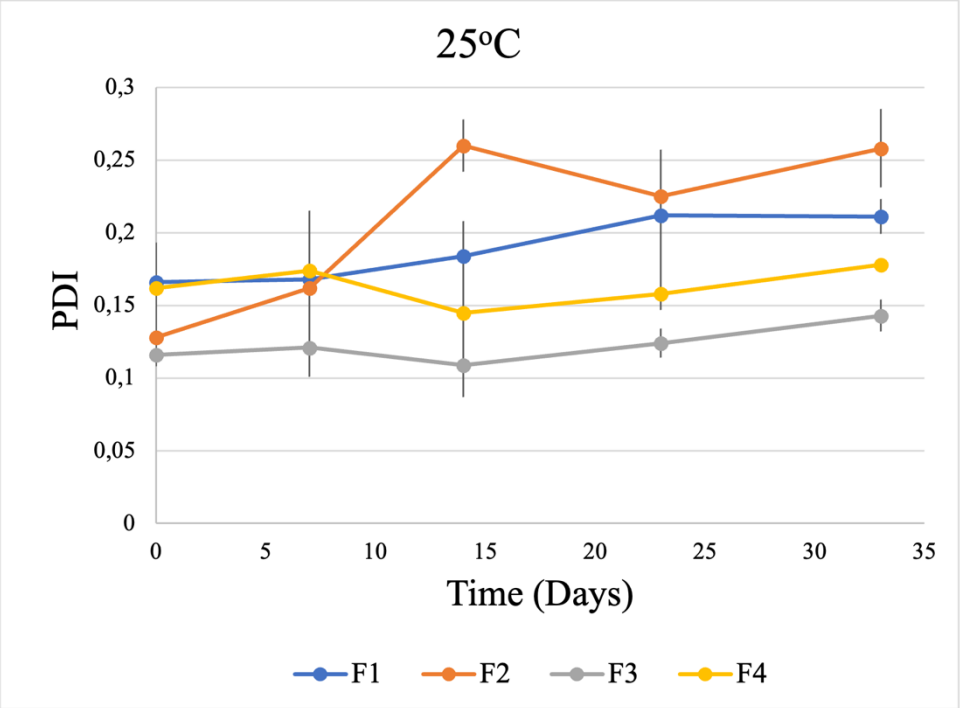


Figure 3.14 Comparative diagram of Polydispersity index (PDI) at 25ºC for all formulations over 33 days.

At 25ºC, all formulations remained stable during the 33-day period at storage conditions. Among them, F1 (drug-free) and F3 (loaded with SRB) exhibited the smallest sizes and the most stable PDI values. The co-loaded formulation (F4) also retained good stability with slightly higher D_h values. In contrast, the quercetin-loaded formulation (F2) demonstrated an important increase in PDI after two weeks, indicating reduced homogeneity compared to the other three formulations.

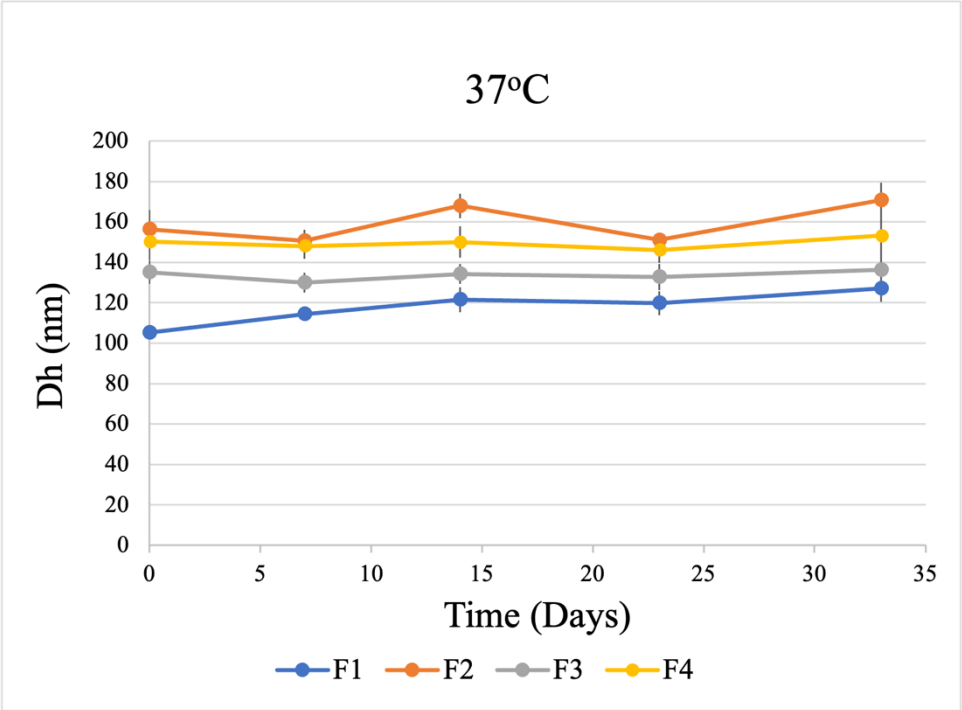


Figure 3.15 Comparative diagram of Hydrodynamic Diameter (D_h) at 37ºC for all formulations over 33 days.

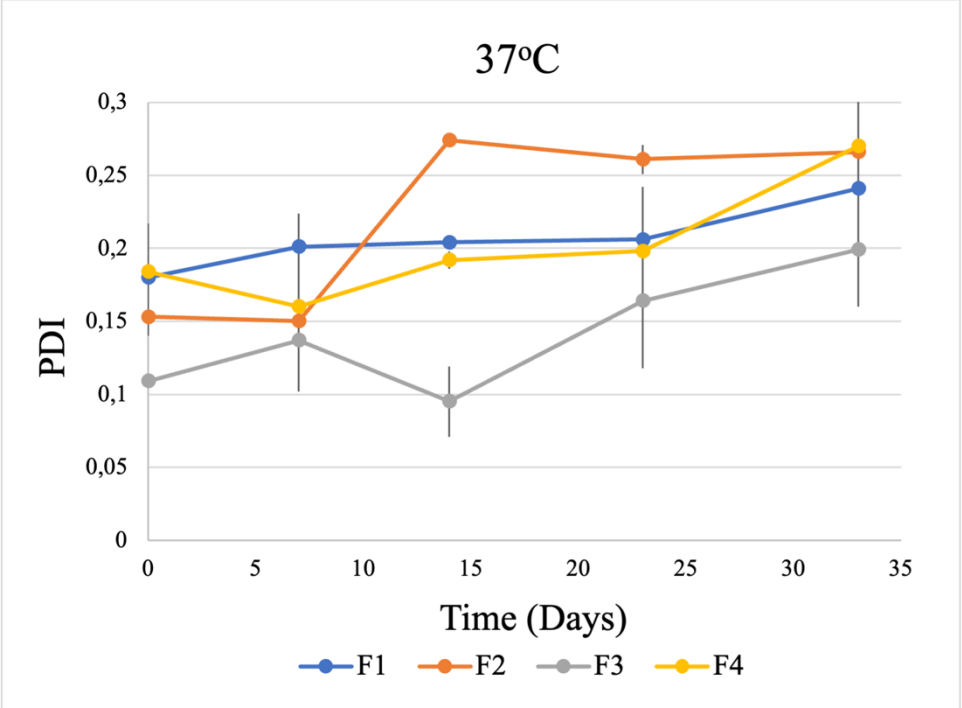


Figure 3.16 Comparative diagram of Polydispersity index (PDI) at 37ºC for all formulations over 33 days.

At 37ºC, the differences were similar to those observed at 25ºC but more pronounced. More specifically, F1 and F3 maintained acceptable stability, with stable D_h values and low PDI values. F4 displayed an increase in PDI values, showing a partial loss of homogeneity. F2 was the least stable with higher D_h and PDI values, indicating destabilization compared to the other three formulations.

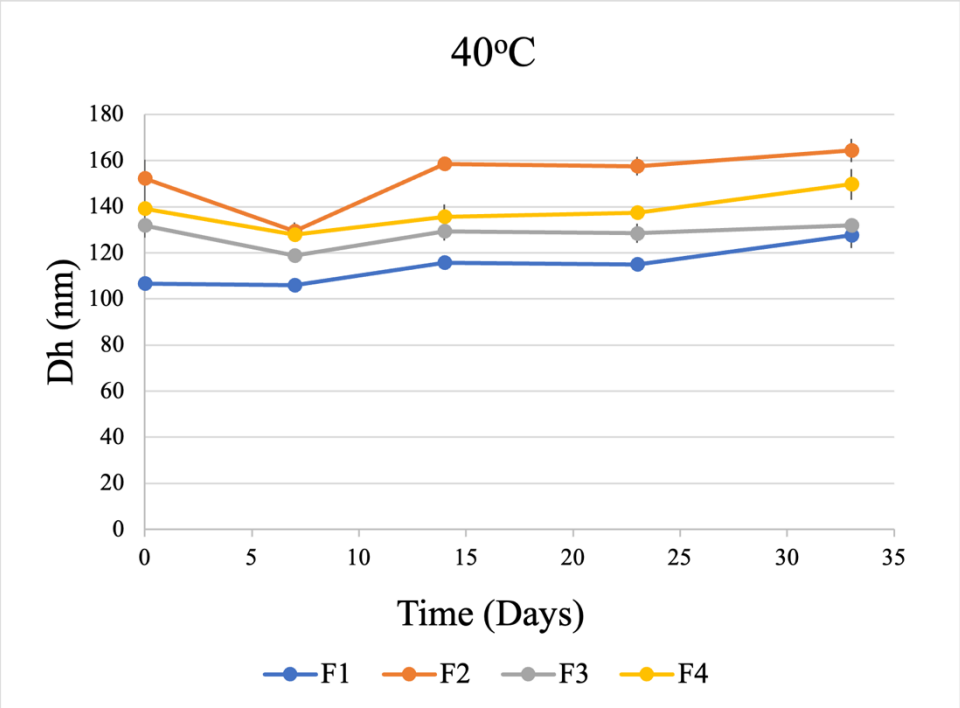


Figure 3.17 Comparative diagram of Hydrodynamic Diameter (D_h) at 40ºC for all formulations over 33 days.

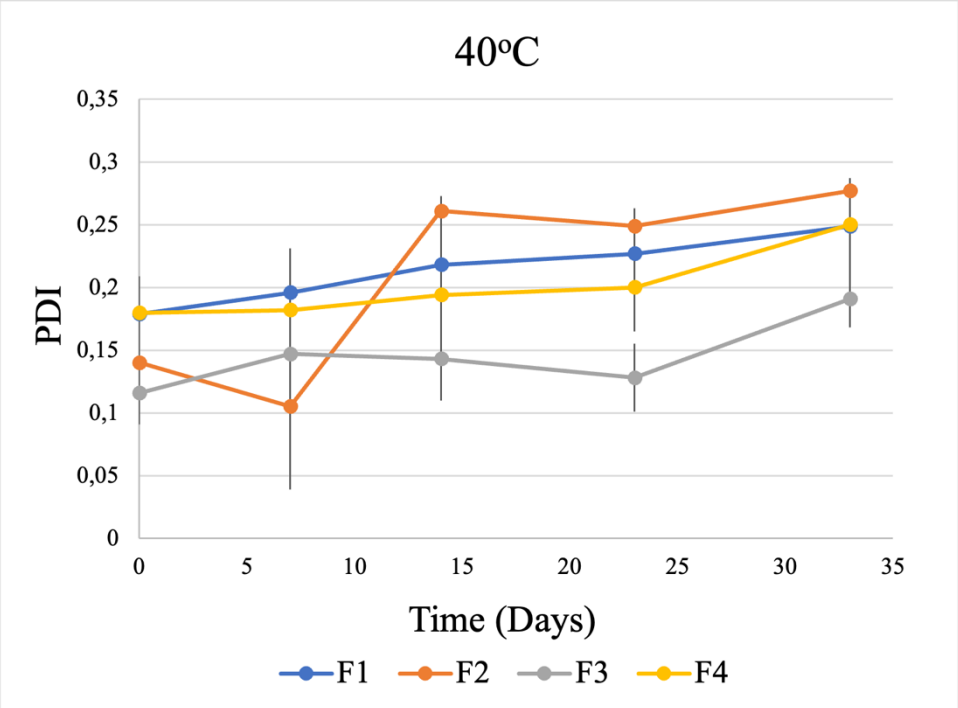


Figure 3.18 Comparative diagram of Polydispersity index (PDI) at 40ºC for all formulations over 33 days.

At 40ºC, stability results were comparable to those at 37ºC, with some formulations showing more noticeable changes. F1 and F3 remained the most stable, while F4 showed slightly more elevated values in D_h and a gradual increase in PDI. F2 was again the least stable, with sizes exceeding 160nm and PDI values greater than 0.25 after two weeks, confirming reduced homogeneity across all temperatures among the four formulations.

From the comparative diagrams results, comparing the four formulations, F1(drug-free) and F3 (SRB-loaded) demonstrated better stability across all temperatures, maintaining low PDI values and stable sizes. F4 (co-loaded) showed intermediate stability, with significant increase in D_h and PDI values in higher temperatures. F2 (quercetin- loaded) was the least stable, particularly after day 14.

From these results, it can be assumed that the long-term stability of blank formulation F1 demonstrates that the lipid ratio used in this study (DSPC:POPE:CHOL:DSPE-PEG2000, 60:20:15:5) creates a very stable platform for drug delivery even in higher temperatures. In the literature, different studies revealed that even a small change in the lipid composition can give totally different characteristics to the system. Tacechi-Haraya et al. (2016) study demonstrated that small alterations in lipid composition affects membrane rigidity which determines stability and cellular uptake [98]. In our formulation, the balance between DSPC, POPE and Cholesterol appear to play a key role in membrane fluidity and temperature sensitivity [92], [93], [94], [99].

Furthermore, the similar stability results of formulation F3 and F1, indicate that the incorporation of SRB did not affect liposomal stability. Due to its hydrophilic nature, SRB is encapsulated in the aqueous core of the liposomes and does not importantly affect physicochemical properties of the lipid bilayer. These findings are in accordance with the literature, where SRB is used as tracer in liposomal studies due to its minimal impact on properties. In Kurtz et al. (2019) study, SRB was used as a model hydrophilic tracer to study liposomal penetration in porcine tissue [100].

The observed differences in D_h and PDI values between formulation F2 and F3 indicate that the entrapment of hydrophobic quercetin influenced negatively the long-term stability, in contrast to hydrophilic SRB. This effect was more evident at higher temperatures, highlighting that both the hydrophobicity of the encapsulated agent and temperature may affect stability.

The co-loaded formulation F4 presented intermediate sizes and PDI values between those of formulation F3 and formulation F2. That may imply that the presence of two agents in the system may contribute to further interactions within the system. However, the overall differences between formulations remained small and D_h and PDI values remained at acceptable levels. Therefore, co-loaded of quercetin and SRB in our systems, did not compromise stability.

3.7 Drug Leakage Determination

Drug leakage percentages for quercetin and SRB were calculated at day 14. The results are presented in **[Table 3.10]**.

Table 3.10 %Drug leakage of quercetin and SRB in F2, F3 and F4 at day 15.

Liposomal Formulation	%Leakage of Quercetin	%Leakage of SRB
F2	31.7	-
F3	-	14.3
F4	39.0	~ 0

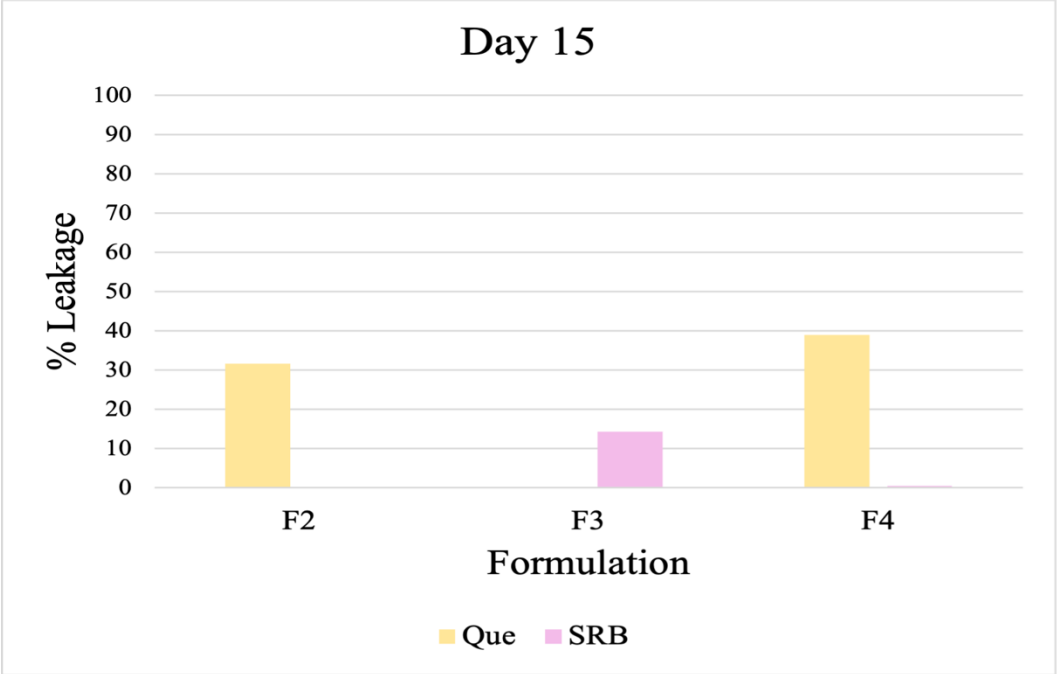


Figure 3.19 %Drug leakage for quercetin and SRB in formulations F2, F3 and F4 at day 15.

The leakage of the systems was evaluated on the 15th day after their preparation. For quercetin, leakage percentage was estimated at 31.7% in F2 and at 39.0% in F4. The slightly increased percentage in the double-loaded system may indicate that perhaps the entrapment of SRB inside the liposomes core affects the retention of quercetin in the membrane and contributes to leakage. However, this difference is relatively small and may not necessarily indicate that SRB affected quercetin leakage. Further studies would be required to confirm this relation.

In the literature, different liposomal formulations loaded with quercetin exhibited small losses through leakage. Huang et al. (2017) constructed EYPC/ Tween 80 liposomes loaded with quercetin and other flavonoids. It was reported that 15,5% of quercetin leaked after 26 days at 4ºC [101]. Bonechi et al. (2012) loaded quercetin in liposomes composed of DOPC/DOPE and DOTAP/DOPE. These systems demonstrated high stability with minimal quercetin leakage [102]. Other studies with flavonoids including quercetin, reported low leakage percentages during storage [103].

However, in our study, leakage percentages of quercetin were relatively high (~38%). This may be attributed to the high initial load of quercetin, that exceeded bilayer loading capacity. Additionally, differences in physicochemical properties of lipids used in the composition or co-encapsulation with SRB may have contributed.

In contrast to the increased leakage of quercetin, SRB leakage was significantly lower. More specifically, the %leakage of quercetin in F3 was 14.3%, while in F4 it was near zero. This difference aligns with the higher EE% observed in F4 and implies that co-

encapsulation of quercetin stabilizes the lipid bilayer. Therefore, the leakage of hydrophilic SRB from the aqueous core is limited [88]. Veiko et al. (2020) reported that quercetin interacts with liposomal membrane, modifying bilayer packing and hydration and leading to increased rigidity of membranes [81]. Additionally, in the literature, Silvander et al. (2002) reported that lipid compositions containing PEG2000 retain better hydrophilic cargo than composition without PEG lipid [82]. In our formulations, DSPE-PEG2000 was utilized and this may also explain the very low leakage percentage in F3 and F4.

Overall, the results indicate that hydrophilic molecule SRB show lower drug leakage percentages compared to hydrophobic quercetin, which is in agreement with previous studies [104]. Furthermore, the results suggest that the lipid composition, the physical properties of molecules co-encapsulated the interactions with the lipid bilayer may affect the retention of the drug [13].

4. CONCLUSION

Overall, concerning the physicochemical characteristics of the four liposomal systems at day 0, we observed that all formulations had sizes between 100-160nm and PDI below 0.2. These results indicated narrow size distribution and homogenous populations. Furthermore, the results confirmed that at day 0 all formulations were stable at storage (25ºC), physiological (37ºC) and elevated (40ºC) temperatures and thus, they were not sensitive in heat changes.

Zeta potential values were near zero, which is considered the threshold of aggregation. However, the liposomal dispersions remained stable due to the presence of DSPE-PEG2000 that was included in the lipid composition.

Entrapment efficiency percentages for quercetin were similar in both F2 and F4, indicating that co-loading did not affect quercetin entrapment. These percentages were low compared to the literature. However, this difference was attributed to the high initial load of quercetin. In contrast, entrapment efficiency percentages for SRB were higher. Additionally, they were increased in co-loaded formulation, suggesting that the entrapment of quercetin in the bilayer may had affected its organization and thus, SRB entrapment.

As for the interaction of the liposomal dispersions with serum proteins, it was demonstrated that adsorption in the liposomes was very limited, which is indicative of good colloidal stability and stealth properties.

During the stability study, the blank (F1) and the formulation loaded with SRB (F3) were the most stable across all temperatures. In contrast, it was observed that formulation loaded with quercetin (F2) showed signs of destabilization from day 14. This effect was more obvious in elevated temperatures. Co-loaded formulation (F4) was stable under storage temperature, but under elevated temperature an increase in PDI was observed.

Regarding drug leakage, hydrophilic compound SRB demonstrated much lower percentages compared to the hydrophobic quercetin. The findings highlighted that the physical properties of the incorporated molecules and their interactions affect the retention of the drug.

In conclusion, the selected lipid composition (DSPC:POPE:CHOL:DSPE-PEG2000) provides a stable platform, suitable for the simultaneous delivery of quercetin and SRB. The observed destabilization at elevated temperatures indicates a degree of thermosinsitivity. This characteristic could be exploited to introduce thermoresponsive properties to the system. This may be achieved by optimizing the composition of the lipid bilayer and the drug-to-lipid ratio. Additionally, the physical properties of the incorporated molecules, the interactions between them and with the lipid bilayer should

be further evaluated to improve the performance of this system. Finally, future studies in cellular *in vitro* models should be conducted to evaluate their potential *in vivo* action.

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