DIPARTIMENTO DI SCIENZE DEL FARMACO

Direttore Chiar.ma Prof.ssa Simona Collina

LAUREA MAGISTRALE A CICLO UNICO IN

CHIMICA E TECNOLOGIA FARMACEUTICHE

Preparation and characterization of polymer-coated liposomes for the alleviation of dry mouth symptoms

Relatore:

Prof.ssa Maria Cristina Bonferoni

Correlatore:

Prof.ssa Marianne Hiorth
Farmasøytisk institutt, Universitetet i Oslo

Tesi di Laurea Magistrale a Ciclo Unico di

Chiara Maria Calloni

Anno Accademico 2024/2025

To my parents

Acknowledgements

This master thesis was conducted at the Section for Pharmaceutics and Social Pharmacy within the Department of Pharmacy, University of Oslo, thanks to the Erasmus programme.

My deepest thanks go to Professor Marianne Hiorth for her constant support and kindness. Beyond the professional teachings, her efficient and empathetic approach to every challenge has significantly contributed to my personal growth. Her mentorship has been an immense source of learning.

I am also deeply grateful to Tove Larsen for her patience and for the extensive advice regarding laboratory operations and instrumentation. Her constant attention and her ability to accompany every practical action with sound scientific reasoning were fundamental to my training in the lab.

I wish to thank Nisrin Doudouh for her emotional support, her reassuring presence, and the insightful advice she provided daily in the laboratory.

Furthermore, I would like to extend my gratitude to all the members of the SiteDel research group for their hospitality and for the stimulating environment they provided during my stay.

I also want to thank my fellow master student, Paolopio, for being a constant support and a reliable companion during the months of laboratory work.

I would like to express my sincere gratitude to Professor Maria Cristina Bonferoni for providing me with the opportunity to undertake this extraordinary experience. Her support, which began well before my departure and continued until the very end, has been invaluable. She successfully conveyed her passion and interest in pharmaceutical technology and provided essential guidance throughout my academic path.

TABLE OF CONTENT

ABSTRACT *6*

LIST OF ABBREVIATIONS *10*

1. INTRODUCTION *11*

1.1. ANATOMY OF THE ORAL CAVITY **11**

1.2. XEROSTOMIA AND CURRENTLY AVAILABLE TREATMENTS.............**12**

1.3. CHALLENGES IN LOCAL ORAL DRUG DELIVERY **13**

1.4. LIPOSOMES **14**

1.4.1. Structure 14
1.4.2. Theory of coating 15
1.4.3. Adhesion in the oral cavity 16
1.4.4. Alginate 17
1.4.5. Chitosan 18
1.4.6. Physical characterization of liposomes 19

2. AIM OF THE THESIS *21*

3. EXPERIMENTAL SECTION *22*

3.1. MATERIALS **22**

3.1.1. Phospholipids 22
3.1.2. Polysaccharides 23
3.1.3. Other substances 24
3.1.4. Solutions 25
3.1.5. Solvents 26

3.2. INSTRUMENTS AND EQUIPMENT **27**

3.2.1. Preparation of liposomes 27
3.2.2. Measuring particle size and Zeta potential 27
3.2.3. Assorted 27

3.3. METHODS **28**

3.3.1. Preparation of the liposomes 28
3.3.2. Coating 29
3.3.3. Additions of preservative and viscosity enhancer 30
3.3.4. Spray ability 31
3.3.5. Characterization 31
3.3.6. Viscosity measurement 32

3.4. EXPERIMENTAL DESIGN **33**

4. MAIN RESULTS AND DISCUSSION *35*

4.1. ALGINATE COATED LIPOSOMES 35
4.2. CHITOSAN COATED LIPOSOMES..37
4.3. PRESERVATIVE AND VISCOSITY MODIFIERS 40
4.4. COMPARISON BETWEEN HEC AND HPMC AS THICKENERS..........43
4.5. VISCOSITY OF THE SOLUTIONS..47
4.6. SPRAYABILITY 48
5. CONCLUSIONS ***50***
FUTURE PERSPECTIVES ***52***
REFERENCES.. ***53***

ABSTRACT

The oral cavity performs several essential functions, enabling nutrient intake and speech; furthermore, it represents a primary route for drug administration. Although this route is readily accessible and non-invasive, the treatment of oral diseases remains challenging due to the complex and highly dynamic environment. In this setting, saliva flow, the swallowing reflex, and continuous exposure to external factors, such as food and beverages, rapidly clear applied formulations, significantly limiting their residence time at the site of action.

In this study, on the basis of prior research performed in the same laboratory, liposomes were formulated and characterized with the aim of developing an oral spray for the treatment of xerostomia. Although xerostomia by itself is not a dangerous condition, it may lead to complications such as difficulty in swallowing, inflammation, infections, and dental caries.

Liposomes were prepared via the thin-film hydration method employing Soy-phosphatidylcholine (Soy-PC) in combination with either dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) or Egg-Phosphatidylglycerol (Egg-PG) to produce positively and negatively charged liposomes, respectively. The liposomes were then coated with varying concentrations of alginate or chitosan, to identify the most stable formulation with the potential to enhance mucoadhesive properties. Furthermore, the influence of the hydration medium was evaluated by comparing a standard phosphate buffer (PB, pH 6.8) with a phosphate buffer containing 15% glycerol (PBG, pH 6.8) to exploit the intrinsic moisturizing and humectant properties of the latter. Also, the effect of adding a viscosity enhancer, specifically Hydroxyethyl cellulose (HEC) and Hydroxypropyl methylcellulose (HPMC) at different concentrations (0.5% w/v or 1% w/v), was evaluated. The formulations were characterized in terms of hydrodynamic diameter, polydispersity index (PDI), and Zeta potential immediately after preparation and over time to evaluate their stability. Preliminary studies were also conducted to assess the effect of

viscosity changes on the sprayability and delivered dose of the final product at different temperature (4°C and 22°C).

The results demonstrated that both positively and negatively charged liposomes, when coated with alginate and chitosan respectively, remained stable over a two-month evaluation period in both PB and PBG media. In contrast, uncoated liposomes tended to destabilize after around 4 weeks. Chitosan was selected for further development due to its superior mucoadhesive and antibacterial properties. Regarding the hydration medium, PBG (pH 6.8) was preferred as it leveraged the intrinsic humectant and lubricating properties of glycerol, which are essential for treating xerostomia, without compromising the colloidal integrity of the vesicles. The addition of viscosity enhancers resulted in a concentration-dependent increase in viscosity; however, this addition also induced modifications in the physicochemical liposomal characteristics that compromised their stability. HPMC 1% w/v emerged as the optimal candidate, providing the maximum increase in viscosity while maintaining a stable, homogeneous, and monodisperse population. This significantly higher viscosity was directly reflected in the sprayability tests, where HPMC 1% produced a more concentrated and controlled spray pattern compared to all other formulations. Notably, the delivered dose remained practically unchanged despite temperature variations between 4°C and 22°C, demonstrating the robustness and reliability of the final formulation under different storage conditions.

RIASSUNTO

La cavità orale permette lo svolgimento di molteplici funzioni fisiologiche essenziali, tra cui l'assunzione dei nutrienti e l'articolazione del linguaggio, rappresentando al contempo una via preferenziale per la somministrazione di agenti terapeutici. Nonostante tale via risulti agevolmente accessibile e non invasiva, il trattamento delle patologie orali permane complesso. Ciò è dovuto principalmente alle peculiari condizioni dell'ambiente orale, dove il costante flusso salivare, il riflesso della deglutizione e l'esposizione a fattori esogeni determinano una rapida rimozione dei prodotti applicati, limitandone drasticamente il tempo di permanenza nel sito d'azione.

Nel presente studio, sulla base di evidenze pregresse ottenute presso il medesimo laboratorio, sono stati formulati e caratterizzati sistemi liposomiali volti allo sviluppo di uno spray orale per il trattamento della xerostomia. Sebbene quest'ultima non costituisca di per sé una condizione patologica critica, essa può favorire l'insorgenza di complicanze quali disfagia, processi infiammatori, infezioni e carie dentali. I liposomi sono stati prodotti mediante la tecnica thin-film hydration, impiegando fosfatidilcolina di soia (Soy-PC) in associazione a 1,2-dioleoil-3-trimetilammonio-propano (DOTAP) o fosfatidilglicerolo d'uovo (Egg-PG) per l'ottenimento di liposomi dotati, rispettivamente, di carica superficiale positiva e negativa. Al fine di studiarne la stabilità, i sistemi sono stati rivestiti con polimeri (alginato o chitosano) a diverse concentrazioni (0.5% p/v o 1% p/v).

Successivamente, è stata studiata l'influenza del mezzo di idratazione, confrontando un tampone fosfato standard (PB, pH 6.8) con un tampone fosfato addizionato di glicerolo al 15% (PBG, pH 6.8). Quest'ultimo è stato scelto per le sue proprietà umettanti. È stato altresì valutato l'impatto di agenti viscosizzanti, nello specifico idrossietilcellulosa (HEC) e idrossipropilmetilcellulosa (HPMC), a diverse concentrazioni (0,5% p/v o 1% p/v). La caratterizzazione ha previsto la determinazione del diametro idrodinamico, dell'indice di polidispersione (PDI) e del potenziale Zeta, sia in fase post-preparativa che durante studi di stabilità a lungo termine. Infine, sono state condotte indagini preliminari per

valutare l'influenza della viscosità sulle proprietà di nebulizzazione e sull’uniformità della dose erogata a diverse temperature (4°C e 22°C).
I risultati hanno evidenziato come i liposomi rivestiti con alginato o chitosano mantengano una stabilità ottimale per un arco temporale di due mesi in entrambi i mezzi di idratazione (PB e PBG). Al contrario, i sistemi non rivestiti hanno mostrato fenomeni di instabilità già dopo circa quattro settimane. Il chitosano è stato confermato come il polimero d'elezione per le finalità applicative del sistema, grazie alle sue proprietà mucoadesive e antibatteriche. Relativamente al mezzo di idratazione, il PBG è risultato preferibile, in quanto capace di coniugare l’azione lubrificante del glicerolo con il mantenimento dell’integrità colloidale dei sistemi liposomiali. L’incorporazione degli agenti viscosizzanti ha indotto un incremento della viscosità proporzionale alla concentrazione; tuttavia, tale processo ha comportato alterazioni strutturali che hanno compromesso la stabilità di alcune formulazioni. L'HPMC all'1% p/v è risultato il candidato ottimale, in quanto ha indotto il maggiore incremento della viscosità nella sospensione senza compromettere la stabilità colloidale del sistema, assicurando una distribuzione dimensionale omogenea dei liposomi. Tali proprietà si sono riflesse nei test di nebulizzazione, dove l'HPMC all'1% ha prodotto un pattern di spruzzo più concentrato ma comunque riproducibile. Inoltre, la dose erogata è risultata costante anche al variare della temperatura.

LIST OF ABBREVIATIONS

Alg: Alginate

API: Active Pharmaceutical Ingredient

Chit: Chitosan

DDS: Drug Delivery System

DLS: Dynamic Light Scattering

DOTAP: 1,2-DioleOyl-3-TrimethylAmmonium-Propane

Egg-PG: Egg Phosphatidylglycerol

HA: Hydroxyapatite

HEC: Hydroxyethyl Cellulose

HPMC: Hydroxypropyl Methylcellulose

MLV: Multilamellar Vesicles

M_W: Molecular weight

NP: Nanoparticle

PB: Phosphate buffer

PBG: Phosphate buffer with glycerol

PDI: Polydispersity index

RT: Room Temperature (25 °C)

Soy-PC: Soy phosphatidylcholine

ULV: Unilamellar Vesicles

1. INTRODUCTION

1.1. ANATOMY OF THE ORAL CAVITY

The oral cavity represents a highly specialized gateway to the human body, serving as a multifunctional environment essential for mastication, speech, and the initiation of the digestive process.[1] This anatomical space is characterized by hard tissues, including the teeth and alveolar processes, and soft tissues such as the tongue, lips, gingivae, and palatal structures.[2] (Figure 1.1.a)

The internal surface of the oral cavity is entirely lined by the oral mucosa, a mucous membrane that acts as a barrier against mechanical trauma, chemical irritants, and pathogenic microorganisms.[3] Histologically, the oral mucosa consists of several layers (Figure 1.1.b), with the outermost layer being a stratified squamous epithelium. This epithelium acts as a physical and immunological barrier, preventing the penetration of potentially harmful agents.[4] A highly viscoelastic and adhesive mucus layer coats the surface of the mucosa.[5]

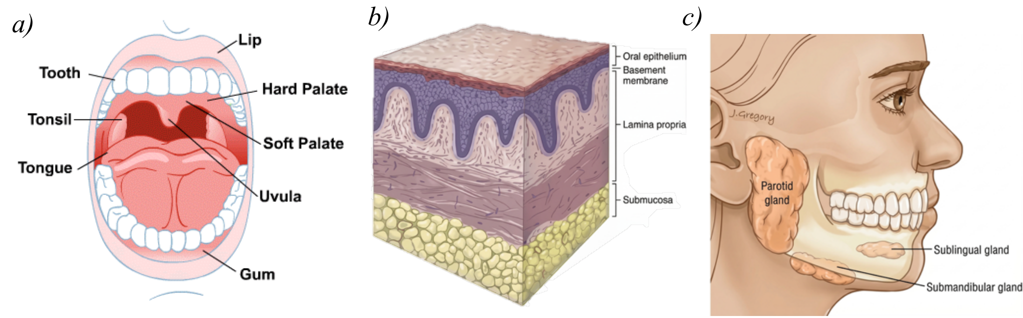


Figure 1.1. Schematic representation, for illustrative purposes, of a) the oral cavity and its key anatomical features[6]; b) the oral mucosa structure; c) the major salivary glands.

All anatomical components of the oral cavity are continuously bathed in a complex biological fluid known as whole saliva. This fluid plays a crucial role in protecting and maintaining oral health, providing essential functions such as lubrication, enzymatic digestion, and antimicrobial defense.[6] Saliva also acts as a buffer, helping to maintain a stable oral pH through components such as bicarbonate, phosphate, urea, and sialin.[7] This fluid is a heterogeneous mixture primarily produced by the major salivary glands: parotid, submandibular, and sublingual (Figure 1.1.c); and numerous minor salivary

glands scattered throughout the oral mucosa. In addition, saliva contains non-glandular components, including gingival crevicular fluid, desquamated epithelial cells, and oral microorganisms, which collectively contribute to the stability of the oral ecosystem.[8]

Saliva is composed of approximately 99% water and contains a wide range of biologically active substances, including: electrolytes, glucose, hormones and vitamins, proteins and peptides, such as mucins or amylase, and immunoglobulins. Its pH, under normal condition, ranges from 6.2 to 7.6 (with an average of 6.8),[9] but both its composition and flow rate can vary significantly between individuals and within the same person depending on different conditions.

1.2. XEROSTOMIA AND CURRENTLY AVAILABLE TREATMENTS

Saliva plays a crucial role in maintaining oral health. However, several factors can alter its normal functions, leading to a condition known as “Dry mouth”, or “Xerostomia”. The factors include the use of certain medications (side effects) such as antidepressants, antihistamines, and antihypertensives; radiation therapy for head and neck cancers, which can damage the salivary glands and reduce saliva production; autoimmune diseases like Sjögren’s syndrome; and systemic conditions such as diabetes, dehydration, and aging.[10]

Xerostomia is the subjective feeling of oral dryness, a symptom often accompanied by hyposalivation, which is the objective reduction of salivary flow rates.[11] Xerostomia affects between 5.5% and 46% of the population, being most prevalent in older adults and more common in women than in men.[12] This condition significantly impairs quality of life, manifesting through difficulties in chewing, swallowing, or speaking, as well as altered taste, halitosis, oral candidiasis, and dental caries.[13]

Although there is no permanent cure for xerostomia, various therapeutic options are available to manage its symptoms, typically categorized into topical agents and systemic sialagogues. Topical medications are first-line therapies and include chewing gums or candies, salivary stimulants, and saliva substitutes.

Systemic treatments include pilocarpine and cevimeline. Pilocarpine, a muscarinic agonist, is particularly effective for patients who have undergone head and neck radiation, but it may cause side effects such as vision changes, nausea, and increased urination. It should be used with caution in patients with cardiovascular or pulmonary diseases and is contraindicated for those with iritis or narrow-angle glaucoma.[13]

1.3. CHALLENGES IN LOCAL ORAL DRUG DELIVERY

A big challenge for conventional pharmaceutical formulations in the treatment of oral diseases is their short residence time in the oral cavity due to the saliva clearance and to the oral muscular function.[14] This rapid clearance, further driven by swallowing, as well as food and drink intake, limits efficacy and necessitates frequent reapplication. To overcome these limitations, bioadhesive drug delivery systems have been developed[15] to adhere to oral surfaces, such as the mucosa or teeth. By prolonging residence time and enabling the sustained release of active agents, this approach improves therapeutic efficacy, reduces dosing frequency, and increases patient compliance. However, bioadhesive formulations require careful evaluation regarding toxicity, especially in compromised oral environments.

1.4. LIPOSOMES

1.4.1. Structure

Liposomes are spherical nanosized vesicles composed of phospholipids of natural or synthetic origin.[16] Phospholipids consist of a hydrophilic headgroup and two hydrophobic hydrocarbon tails. Once dispersed in an aqueous environment, they spontaneously arrange themselves into a bilayer structure to reach a thermodynamic equilibrium (as illustrated in Figure 1.4.1.).

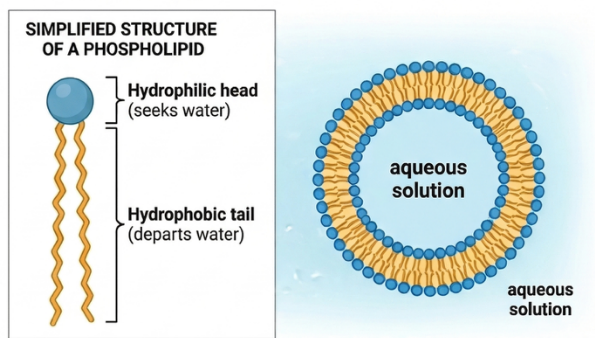


Figure 1.4.1. Schematic representation of an individual phospholipid unit (left) and a conventional liposome (right).

Specifically, the hydrophilic heads orient toward both the external aqueous phase and the internal aqueous cavity, while the hydrophobic tails remain sequestered within the bilayer, that spontaneously closes into a spherical form and lead to the formation of liposomes.[16]

Lipid composition strongly affects liposomes particle size, rigidity, fluidity, stability, and electrical charge. The hydrophilic group in the lipids may be negatively, positively charged, or zwitterionic and the charge provides stability through electrostatic repulsion. The hydrophobic group varies in the acyl chain length, symmetry, and saturation.

Liposomes offer significant advantages as drug delivery systems (DDS) due to their ability to encapsulate both hydrophilic and lipophilic drugs, allowing the transport of a diverse range of active compounds. They can protect the loaded drugs from degradation, enable controlled and sustained release, and improve the bioavailability of substances that would otherwise be poorly absorbed.[17]

Liposomes are generally considered biocompatible and biodegradable because they are primarily composed of phospholipids, the same molecules that constitute cell membranes in the human body. Due to this structural mimicry, they exhibit high biological compatibility with tissues and rarely cause irritation, toxicity, or immune responses, allowing them to interact gently with mucosal surfaces or even fuse with cells. Furthermore, the phospholipids are enzymatically degradable; once absorbed in the body, their components are metabolized through enzymatic pathways.[17] For example, phospholipases can degrade the lipid bilayer, ensuring that liposomes do not persist in the body or accumulate in tissues, which is a crucial criterion for a delivery system to be considered biodegradable and safe for repeated or long-term use. Moreover, liposomes, especially when coated with appropriate polymers, can interact with oral surfaces, which helps enhance their retention in the oral cavity. [18]

1.4.2. Theory of coating

The surface charge of uncoated liposomes is determined by the lipid composition used in their formulation. This surface potential plays a critical role in determining how liposomes interact with charged polymers in solution influencing the efficacy of coating. But this approach also improves colloidal stability and can enhance adhesion and targeting ability.

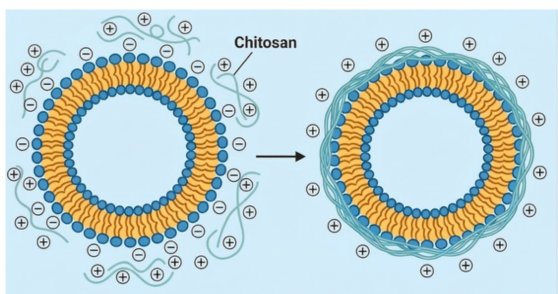


Figure 1.4.2. Schematic representation of a negatively charged uncoated liposome (left) and a chitosan-coated liposome (right).

When oppositely charged components are combined (Figure 1.4.2.), such as negatively charged liposomes and positively charged polymers (or vice versa), electrostatic interactions can lead to adsorption of the polymer onto the liposome surface.[19] This adsorption alters the surface charge, typically shifting the Zeta

potential toward neutrality. With sufficient polymer coverage, the surface charge may even invert, resulting in a reversal of the liposome's original charge. The effectiveness of the coating process and the resulting colloidal behaviour are governed by electrostatic interactions and the polymer conformation in the medium.[20] Polymer adsorption is intrinsically defined by a statistical distribution of chain conformations, specifically categorized as trains, loops and tails. Trains are segments in direct contact with the lipid bilayer maximising the attractive enthalpy and driving the charge neutralisation or inversion. Interspersing these contacts are loops, which are segments that extended into the solvent before re-attaching to the surface, and tails, representing the free ends of the polymer chain that project further into the bulk medium.[21] At high adsorption densities, the formation of extended loops and tails create a "brush-like" shell; this configuration is crucial for colloidal stability, as it provides a steric barrier that prevents liposome aggregation even when the Zeta potential approaches the point of zero charge.[22]

Various polymers are utilized for this purpose; among them, polysaccharides are particularly prominent due to their biocompatibility, biodegradability, and non-toxic nature, which make them ideal candidates for pharmaceutical formulations.[23]

1.4.3. Adhesion in the oral cavity

The oral mucosa represents the most extensively studied site for bioadhesive formulations within the oral cavity.[24] This bioadhesive capacity is fundamentally driven by the interaction with the mucus layer, a complex hydrogel primarily composed of mucins. These are large, highly glycosylated proteins that contain terminal sialic acid residues; having a pKa ≈ 2.6 [25] these residues are fully deprotonated at the physiological pH of the oral cavity (about 6.8), conferring a significant net negative charge to the mucosal surface. Consequently, the bioadhesive mechanism is largely governed by electrostatic attraction with cationic polymers.

However, recent research has also focused on the dental surface, demonstrating that liposomes possess a significant capacity for adsorption onto dental enamel. This has been established through in vitro studies using hydroxyapatite (HA) as a model substance for human enamel.[26]

The adsorption of liposomes is primarily driven by electrostatic interactions, a process that can be significantly influenced by the presence of saliva. Saliva is a critical factor in oral drug delivery, as it has been shown to affect not only the adsorption process but also the colloidal stability of liposomal suspensions.[27,28]

In artificial saliva, negatively charged liposomes exhibited the highest stability; furthermore, the stability of positively charged formulations was enhanced by coating them with a negatively charged polysaccharide. Conversely, while positively charged liposomes tend to aggregate when exposed to artificial saliva,[29] they demonstrated superior bioadhesive properties.[30]

This is consistent with the behaviour of positively charged chitosan nanoparticles, which interact strongly with negatively charged mucin in vitro, further highlighting the role of electrostatic attraction in oral bioadhesion.[31]

1.4.4. Alginate

Alginate is a natural, water-soluble linear polysaccharide extracted from the cell walls of brown algae (such as Ascophyllum nodosum) or produced by certain bacterial strains (e.g., Pseudomonas spp.). Structurally, it is a copolymer composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G) units linked by 1–4 glycosidic bonds. These monomers are arranged in a block-wise pattern along the polymer chain, forming homopolymeric segments of M-units and G-units (Figure 1.4.4.), interspersed with alternating heteropolymeric sequences (MG-blocks). Due to its high biocompatibility, biodegradability, and non-antigenic properties, alginate is extensively utilized in pharmaceutical and biomedical research. It serves as a versatile matrix for tissue engineering, a carrier for controlled drug delivery systems, and a primary component in advanced wound dressing formulations.[32]

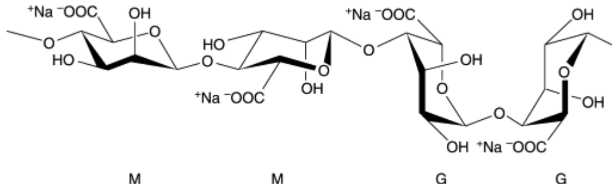


Figure 1.4.4 Representative chemical structure of alginate.

1.4.5. Chitosan

Chitosan is a natural polysaccharide derived from chitin, which is found in the exoskeletons of crustaceans, insects, and fungi. It is produced through a deacetylation process in an alkaline environment that removes acetyl groups from the chitin structure. This chemical structure (Figure 1.4.5.) consists of a variable number of repeating glucosamine units that carry positively charged amine groups at slightly acidic to neutral pH (pKa ~ 6–7). The repetition of these units gives chitosan its distinctive cationic character.[33]

Figure 1.4.5 Representative chemical structure of chitosan; n indicates that the number of repeating units can vary.

Due to this nature, chitosan is considered an excellent candidate for coating liposomes, offering a pathway to overcome the stability and retention issues typically encountered in dental applications.[14] Its positive charge allows it to interact electrostatically with negatively charged surfaces, such as mucosal membranes, thereby enhancing mucoadhesion and retention in the oral environment.[33] Beyond these adhesive properties, chitosan also exhibits inherent antibacterial activity.[34] Due to its safety, bioactivity, and biodegradability, chitosan stands out as a promising excipient for these formulations.

1.4.6. Physical characterization of liposomes

Particle Size and Polydispersity Index (PDI):

The Zetasizer performs measurements using dynamic light scattering (DLS) with the non-invasive backscatter (NIBS) technique at a scattering angle of 173°. The hydrodynamic diameter represents the apparent size of particles in suspension, including both the particle core and the surrounding solvent layer that moves with the particle.

The hydrodynamic diameters of the particles are calculated by the software using the Stokes–Einstein equation, which relates the translational diffusion coefficient derived from Brownian motion to particle size, with smaller particles exhibiting faster diffusion.

Stoks-Einstein equation:

$$d_H = \frac{k_B T}{3\pi\eta D}$$

d_H = hydrodynamic diameter
k_B =Boltzmann's constant
T = absolute temperature
η = viscosity
D = translational diffusion coefficient

The Zetasizer software analyzes the autocorrelation function via the cumulants method to derive the Z-average diameter (mean size) and the polydispersity index (PDI), the latter of which provides a dimensionless measure of the particle size distribution width.

The PDI is a dimensionless numerical value that measures the particle size distribution within a sample and ranges from 0 to 1, with lower values indicating a more uniform particle size distribution and higher values reflecting a broader distribution. According to the Zetasizer user manual, the Z-average value is considered reliable only when the PDI is below 0.5.[35]

Zeta Potential:

Zeta potential represents the electrokinetic potential at the slipping plane of a particle, reflecting the sign and magnitude of its net electrostatic surface charge. This value is a fundamental indicator of colloidal stability, as high Zeta potential values (whether positive or negative) provide sufficient electrostatic repulsion to prevent particle aggregation.

The Zetasizer measures this parameter using Electrophoretic Light Scattering (ELS). When an electric field is applied, particles migrate at a velocity proportional to their charge. The instrument determines their electrophoretic mobility (UE) by detecting the Doppler frequency shift in the scattered laser light.

Finally, the software calculates the Zeta potential (ζ) from the electrophoretic mobility using the Henry equation:

$$U_E = \frac{2\varepsilon z f(ka)}{3\eta}$$

U_E = electrophoretic mobility,
z = Zeta potential,
ε = dielectric constant of the dispersant at RT
μ = viscosity at RT
f(ka) = Henry's function

In aqueous systems, the Smoluchowski approximation is typically used (f(ka)=1.5) to convert the measured mobility into the final Zeta potential value.

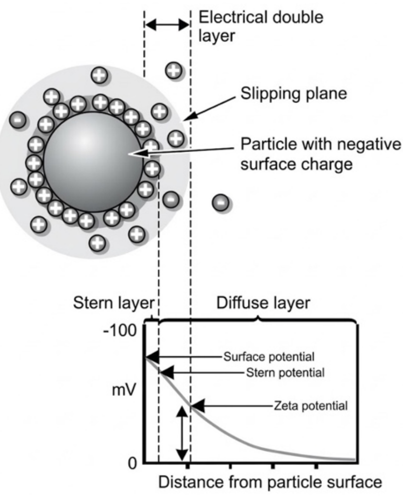


Figure 1.4.6. Schematic representation of a negatively charged particle and its Electrical Double Layer.[35] *The graphic shows the location of the slipping plane where the Zeta Potential is determined.*

2. AIM OF THE THESIS

The overall aim of this work was to investigate different polymer-coated liposome formulations as pharmaceutical dosage forms with intrinsic therapeutic potential for alleviation of dry mouth symptoms.

The first objective was to evaluate the coating efficiency of different polymers, specifically alginate (at various concentrations) and chitosan.

The second objective focused on assessing the influence of the medium by comparing phosphate buffer (PB) and phosphate buffer with glycerol (PBG) on the formation and stability of coated liposomes.

The third objective involved the incorporation of two different viscosity enhancers, Hydroxyethylcellulose (HEC) and Hydroxypropylmethylcellulose (HPMC) at various concentrations to evaluate their influence on the formulation stability.

A common objective across these three phases was to evaluate the in vitro stability of the prepared formulations over a relevant period of time by measuring particle size, Polydispersity Index (PDI), and Zeta potential.

The fourth objective involved investigating the changes in viscosity and sprayability of the formulations resulting from the addition of the viscosity enhancer HEC and HPMC.

3. EXPERIMENTAL SECTION

3.1. MATERIALS

3.1.1. Phospholipids

Soy-phosphatidylcholine (Soy-PC) was used as the neutral lipid for all liposome formulations. Depending on the desired liposome charge, either 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP), for positive charge or Egg-Phosphatidylglycerol (Egg-PG), for negative charge was employed, maintaining a constant 90:10 ratio between the neutral and charged lipids.

Table 3.1.1 Phospholipids

LIPIDS	CHEMICAL STRUCTURE	TYPE OF CHARGE	PROPERTIES
DOTAP:		Positive	M_W ~ 699 Da Tc = -12 °C
Egg-PG:		Negative	M_W ~ 782 Da Tc = < 0
Soy-PC: (S100)		Neutral	M_W ~ 775 Da Tc = < 0

3.1.2. Polysaccharides

For surface coating, the negatively charged polymer alginate and the positively charged chitosan were utilized. Hydroxyethyl cellulose (HEC) and HPMC was used as a viscosity modifier.

Table 3.1.2. Polysaccarides

POLYMERS	CHEMICAL STRUCTURE	PROPERTIES
Alginate (sodium salt) LFR 5/60 20.01.25 TK)		Negatively charged Mw ~ 147 kDa
Chitosan PROTOSAN UP CL-213		Positively charged Mw ~ 307 kDa
Hydroxyethyl-cellulose (HEC) Natrosol 250LPHARM-Ashland	$R = H$ or CH_2CH_2OH	Neutral Mw ~ 90 kDa
Hydroxypropyl methylcellulose (HPMC) Sigma H8384	$R = –H, –CH_3, –CH_2–CHOH–CH_3$	Neutral Mw ~ 10 kDa

3.1.3. Other substances

Glycerol (85%) was incorporated into the phosphate buffer medium. Methyl 4-hydroxybenzoate (methyl para-hydroxybenzoate) was employed as a preservative to ensure microbiological stability, while Erythrosine B was added as a colouring agent to facilitate visual characterization.

Table 3.1.3. Other substances

OTHER SUBSTANCES	CHEMICAL STRUCTURE	PROPERTIES
Glycerol 85% Lot: 22J314115, CAS: 56-81-5	OH, HO, OH	Water soluble, Igroscopic, Density: 1.23 kg/L (RT) Mw: 92.09 g/mol
Methyl para-hydroxybenzoate K:110008	O, OCH_3, HO	Slightly soluble in water (dissolves better in warm water)
Erythrosine B Certistain, K91422436 Merck KGaA Darmstadt, Germany	COONa, I, I, NaO, O, O, I, I	Organoleptic/Colouring agent Water-soluble

3.1.4. Solutions

Preparation of stock solutions

- Soya-PC 10 mg/ml in chloroform
- DOTAP 2 mg/ml in chloroform
- Egg-PG 2 mg/ml in chloroform

Dry lipids were stored in glass vials at -20 °C. Due to their hygroscopic nature, the vials were allowed to reach room temperature before opening to prevent moisture condensation. The lipids were weighed using glass weighing boats and quantitatively transferred into volumetric flasks by rinsing the boats with chloroform. Solutions were pre-prepared at the desired concentrations and stored in injection vials, flushed with nitrogen, and sealed with Teflon-lined stoppers at -20 °C. In the case of mono- or polyunsaturated lipids, the vials were flushed with nitrogen to prevent oxidation.

Phosphate buffer, 5 mM, pH 6.8 (PB)

The 5 mM phosphate buffer was prepared by mixing two stock solutions in a 2:1 ratio (700 mL of Solution A and 350 mL of Solution B).

Solution A: A 5 mM sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) solution was prepared by weighing 690 mg of the salt in a plastic weighing boat using an analytical balance. The substance was then transferred into a volumetric flask and diluted to 1 L with Milli-Q water.

Solution B: A 5 mM disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) solution was prepared following the same procedure, by weighing 445 mg of the salt and dissolving it in Milli-Q water up to a final volume of 500 mL in a volumetric flask.

The pH was measured using a pH meter at RT and, if necessary, adjusted to 6.8 ± 0.04 by adding Solution A or B. Since the expected pH is 6.8, the instrument was calibrated between pH 4.0 and 7.0 using standards. The resulting buffer was filtered through a 0.2 µm polycarbonate membrane into a glass bottle, sealed with a plastic cap, and stored in the refrigerator. After one month, the pH remained unchanged.

Phosphate buffer with Glycerol 15%, 5mM, pH 6.8 (PBG)

The calculated amount of an 85% glycerol solution was weighed on an analytical balance and diluted with Milli-Q water to the appropriate volume to obtain a 15% glycerol solution. Phosphate buffer containing 15% glycerol was prepared following the same procedure described for the PB, using the 15% glycerol solution as the dissolving medium instead of Milli-Q water.

Polysaccharide solution at different concentrations

A specific amount of polysaccharide was weighed into a glass bottle using an analytical balance and dispersed in the appropriate volume of hydration medium to achieve the target concentration. The bottle was sealed with a plastic cap, and the solution was left to stir overnight; the magnetic stirring speed was adjusted to maintain a slight vortex in the center, ensuring complete dissolution of the polymer. This solution was always prepared fresh and used the following day. The solution was filtered through a 5 µm syringe filter immediately before use.

3.1.5. Solvents

For the preparation of liposomes and polymer-coating solutions the following materials were used:

Ultrapure Water (Milli-Q): Obtained from a Milli-Q® integral system (Merck Millipore) and used for all aqueous solutions and cleaning procedures.

Chloroform (CHCl3): Analytical grade, used as the primary organic solvent to dissolve the lipid components during the thin-film hydration process.

Phosphate Buffer (PB, pH 6.8): Prepared using sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$: batch AM0863421632) and disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$: batch K29977180147). This buffer served as the hydration medium and solvent for the coating process.

3.2. INSTRUMENTS AND EQUIPMENT

3.2.1. Preparation of liposomes

Instrument / equipment	Model	Producer
Rotary evaporator	Rotavapor Hei-VAP Advantage Associated vacuum pump PC 511	Heidolph, Germany Vacuubrand, Germany
Freeze-dryer	Christ-Alpha 2-4 LOC- 1M Associated vacuum pump RV 8	Martin Christ, Germany Edwards High Vacuum International, England
Extruder	Lipex™ Thermobarrel Extruder, 10 ml	Lipex Biomembranes INC, Canada
Peristaltic pump	Peristaltic Pump P-1	General Electric

3.2.2. Measuring particle size and Zeta potential

Instrument / equipment	Model	Producer
Particle size and Zeta potential	Zetasizer Nano - ZS	Malvern Instruments, England
Cell	Disposable Folded Capillary Cell. DTS1070	Malvern Instruments, England
Standard	Zeta potential transfer standard -40 mV ± 5.8 mV	Malvern Instruments, England

3.2.3. Assorted

Instrument / equipment	Model	Producer
Analytical balance	AG 204 Delta Range	Mettler Toledo, USA
Analytical balance (LIP-006)	EP2255M-DR	Swiss Made
Balance	PB 30002 Delta Range	Mettler Toledo, USA
Finnpipette® F1, 20-200 µm	U31661	Thermo Electron Corporation, USA
Finnpipette® F1, 200-1000 µm	U27916	Thermo Electron Corporation, USA
Finnpipette® F1, 1-5 ml	EH23517	Thermo Scientific Inc, USA

pH-meter	Seven Compact PH/Ion meter S220, FiveEasy pH/mV F20	Mettler Toledo, USA/Switzerland
Calibration solutions	Buffer solution pH 4, Buffer solution pH 7	Prolabo, France
Magnetic stirrer	RO 10 power	IKA-Werke GmbH & Co, Germany
Filter	25 mm Syringe filter, 5 µm Versapor® membrane, Ref 6094184	Pall Corporation, USA
N_2	Grade 5.0	
Parafilm ″M″		Bemis
Polycarbonate membrane for filtration of hydration medium	Nucleopore Track-Etch Membrane, Whatman, 0,2 µm, 47 mm, Lot No. A29708963	GE Healthcare, England
Polycarbonate membrane for extrusion	Nucleopore Track-Etch Membrane, Whatman, 0,2 µm, 25 mm, Lot No. A29708965	GE Healthcare, England
Supportive Drain disc	Whatman Drain disc PE 25 mm, Lot No. 7068947	GE Healthcare, England
Viscometer	DV2T Touch Screen Viscometer	AMETEK Brookfield, USA

3.3. METHODS

3.3.1. Preparation of the liposomes

Liposome preparation followed the thin-film hydration method. Standard procedures and specific quantities were established based on prior experimental protocols and existing laboratory expertise.

Initially, 10 mL of a 3 mM liposome suspension was prepared. The liposomes consisted of 90 mol% Soy-PC and 10 mol% of either DOTAP or Egg-PG to obtain positively or negatively charged particles, respectively. The initial total lipid concentration was either 3 mM or 6 mM; in the latter case, the solution was diluted using the buffer to a final concentration of 3 mM after extrusion, prior to coating. The lipids were first dissolved in chloroform, and the organic solvent was removed via rotary evaporation. This step was followed by overnight

freeze-drying to eliminate residual solvent and ensure the formation of a thin lipid film.
The following day, the lipid film was hydrated at room temperature with the appropriate medium, either phosphate buffer (PB) or glycerol phosphate buffer (PBG) under manual stirring. The suspension was flushed with nitrogen and rotated at 90 rpm for 10 minutes. Subsequently, the sample was placed in a closed cabinet to ensure protection from light and was manually stirred every 30 minutes for 2 hours. The flask was then stored in the refrigerator (4 °C) overnight to allow the lipids to become fully hydrated.
On the day after production, the resulting liposomal suspension was extruded 10 times through two 200 nm polycarbonate membranes to convert multilamellar vesicles (MLVs) into unilamellar vesicles (ULVs) and to reduce the particle size. As the process must be conducted above the lipid phase transition temperature (Tc), and considering the Tc values of the lipids used, the extrusion was successfully performed at room temperature.
N_2 was employed during the extrusion process and for flushing the vial headspace to displace residual oxygen, thereby preventing lipid oxidation during storage.

3.3.2. Coating

After extrusion, liposomes were coated by adding one part of the liposomal suspension to four parts of the coating solution (e.g., 5 mL of liposomes added to 20 mL of coating solution) using a peristaltic pump at room temperature. The liposomes were coated with either alginate or chitosan at various concentrations. To prepare the coating solutions, the polymer was dissolved in the buffer (PB or PBG, 5 mM, pH 6.8) at the target concentrations, stirred overnight, and filtered through a 5 µm pore-size membrane filter before use.

The liposomal suspension was transferred to a particle-free Eppendorf tube, while the coating solution was placed in a glass vial with a magnetic stir bar and maintained under constant stirring. The coating was performed by adding the liposomes dropwise to the polymer solution using a peristaltic pump at a

controlled flow rate of 1.6 mL/min to ensure reproducibility and prevent aggregation.The coating procedure was performed in triplicate.

To confirm a successful production of liposomes, the formulations were characterized using a Zetasizer by measuring particle size, polydispersity index (PDI), and Zeta potential one day after coating (see chapter 2.3.5 for detailed method). To evaluate the long-term stability, these parameters were monitored at 7, 14, 28, and 60 days. Throughout the analysis period, all liposomal formulations were flushed with nitrogen and stored at 4 °C. Each time a vial was opened, it was re-flushed with nitrogen, sealed with a Teflon-lined cap, and returned to 4 °C storage.

For Zetasizer measurements, samples were diluted with the hydration medium (phosphate buffer, pH 6.8). Special care was taken during sample preparation to avoid contamination, as dust particles are typically larger than the liposomes and can significantly interfere with the results.

3.3.3. Additions of preservative and viscosity enhancer

Methylparaben was employed as a preservative and added to 50 mL of PBG in the amount required to reach a final concentration of 0.1% w/v. Due to its limited aqueous solubility, the mixture was heated to 50°C and stirred until complete dissolution was achieved. This solution then served as the medium for the preparation of the coating solution, into which the polymer was subsequently dissolved. It was crucial to allow the solution to cool to room temperature (RT) before the addition of the polymer.

Following the addition of the preservative, either HEC or HPMC used as viscosity enhancers was added at a concentration of 0.5% w/v or 1.0% w/v. The polymer powder was weighed and added directly to the liposome dispersion.

3.3.4. Spray ability

To evaluate the sprayability of the liposomal formulations, a spraying test was performed. For each formulation with HEC or HPMC, a volume of 12 mL was loaded into a spray bottle. To facilitate visual assessment of the spray pattern and characterization, 4 mg of Erythrosin B was added to each sample as a colouring agent. Each sample was sprayed three times onto a target sheet, maintaining the bottle fixed at a constant distance of 7 cm between the nozzle and the collection surface. To determine the mass of the delivered dose, the spray device was weighed before and after the series of three sprays using an analytical balance. The value of a single dose was defined as the amount delivered in one spray, calculated as the average of the three consecutive sprays. To determine the spray diameter for each formulation, the dimensions of the resulting coloured spot were recorded. The diameter was measured three times for each spray and he final spray diameter for each formulation was calculated as the average of these three measurements.

3.3.5. Characterization

Particle size, polydispersity index (PDI), and Zeta potential were measured using a Malvern Zetasizer Nano ZS (ZEN3600, Malvern Instruments, UK) equipped with a red laser (λ=632.8 nm). All measurements were performed at 25 °C. For the calculations, the viscosity of the dispersant at 25 °C was used as a constant. For samples prepared in PBG (containing 15% glycerol), the instrument settings were specifically adjusted to account for the increased viscosity and the higher refractive index (1.347) compared to that of pure water (1.330).
Samples were diluted using the same buffer employed during liposome preparation and the coating process (either PB or PBG). To ensure a comparable particle concentration in the measurement volume, different dilution ratios were applied. Uncoated liposomes were diluted at a 1:9 (v/v) ratio (100 µL of suspension in 900 µL of buffer). Coated liposomes, which already undergo dilution during the coating procedure, were diluted at a 1:1 (v/v) ratio (500 µL

of suspension in 500 μL of buffer). In all cases, the final volume in the cuvette was maintained at 1mL.

Prior to loading each sample, the electrophoresis cuvette was sequentially rinsed with ethanol, Milli-Q water, and the appropriate buffer to pre-condition the cell with the sample medium. Before starting the analysis, samples were allowed to equilibrate in the cuvette for 5 min for size and PDI measurements, and for 2 min for Zeta potential measurements.

Size and PDI measurements were performed in triplicate using disposable cuvettes (Sarstedt AG & Co. KG, Nümbrecht, Germany). Zeta potential measurements were carried out in quintuplicate using disposable folded capillary cells (Malvern Instruments Ltd., Worcestershire, UK).

3.3.6. Viscosity measurement

Viscosity was measured using a DV2T Touch Screen Viscometer (Brookfield Engineering, MA, USA), a rotational viscometer that quantifies the torque required to rotate a spindle in a fluid sample and converts this into a viscosity value in centipoise (cP, equivalent to mPa·s).[36]

Viscosity represents a fluid's resistance to flow under an applied force and can be interpreted as the internal friction arising when adjacent layers of fluid move relative to each other. In rheological terms, viscosity (η) is defined as the ratio of shear stress (force per unit area) to shear rate (velocity gradient) and describes how a fluid resists deformation under shear.

The dynamic viscosity depends on both temperature and shear rate: temperature generally influences molecular interactions and thus the ease of flow, while shear rate can affect apparent viscosity in complex fluids.[36]

Prior to the measurements, the viscometer was calibrated using Milli-Q water as a standard reference material, confirming its viscosity at 25°C (approximately 0.89 mPa·s) to ensure the accuracy of the instrument. All measurements were carried out at a controlled temperature of 25.4 ± 0.1 °C. The rotational speed was set at 15 or 30 RPM.

3.4. EXPERIMENTAL DESIGN

The experimental design was structured into four phases to evaluate the influence of polymer concentration, buffer composition, and the addition of viscosity-enhancing agents on liposomal properties. All liposomes were prepared using a fixed lipid ratio, determined by preliminary internal research, while other components were varied across the different formulations (as shown in Table 3.4).

In the first phase, positively charged liposomes were prepared and subsequently coated with the anionic polymer alginate. Two different concentrations (0.125 wt% and 0.0625 wt%) were utilized to assess how variations in coating concentration affect the physicochemical properties of the vesicles.

The second phase focused on negatively charged liposomes coated with a fixed concentration of the cationic polymer chitosan 0.1 wt% (selected based on preliminary optimization studies that ensured effective surface coverage while maintaining colloidal stability), while varying the hydration medium between PB and PBG in order to assess the influence of buffer composition on the physicochemical characteristics of the liposomes

In the third phase, liposomes coated with 0.1 wt% chitosan in PBG were formulated with addition of the preservative methyl p-hydroxybenzoate (0.1% w/v) and either HEC or HPMC at two different concentrations (0.5% w/v and 1.0% w/v). This was done to investigate strategies for increasing formulation viscosity and to identify the optimal formulation that would not alter the physicochemical properties of the liposomes.

In the fourth phase, the formulations containing HEC or HPMC were compared by evaluating their viscosity and sprayability. Viscosity was measured once for the solution without viscosity enhancer, while the formulations containing HEC and HPMC (at both 0.5% w/v and 1.0% w/v) were prepared in triplicate, with viscosity measured for each independent replicate.

The sprayability of the liposomal formulations containing HEC or HPMC was evaluated through a spray test. For each formulation, three consecutive actuations were performed onto a collection surface, maintaining a constant distance and orientation between the nozzle and the target. The dispensed mass

per dose was quantified gravimetrically by weighing the delivery device before and after the spraying cycle. Simultaneously, the spray pattern was characterized by measuring the diameters of the resulting deposition spots to evaluate the spreading capacity.

For the first two phases of the study, the coating procedure was performed in independent triplicates, as the primary objective was to optimize the polymer-vesicle interaction. In the third phase, however, the coating process was conducted as a single replicate since the core formulation parameters had already been established. In this stage, the experimental focus shifted to the incorporation of viscosity-modifying agents; consequently, the addition of these polymers (HEC and HPMC) was performed in triplicate for each specific concentration and polymer type.

To ensure successful production and assess long-term stability, all formulations were characterized in terms of: Particle Size, Polydispersity Index (PDI) and Zeta Potential. Measurements were performed 24 hours after production to verify correct synthesis and repeated at specific intervals over time to evaluate the physical stability of the systems.

Table 3.4. Composition of the different formulations

NEUTRAL LIPID	CHARGED LIPID	HYDRATION MEDIUM	COATING POLIMER	PRESERVATIVE	VISCOSITY MODIFIERS
SOY-PC 90%	DOTAP 10%	PB 5 mM pH 6,8	Alg 0.125 wt%		
SOY-PC 90%	DOTAP 10%	PB 5 mM pH 6,8	Alg 0.0625 wt%		
SOY-PC 90%	EGG-PG 10%	PB 5 mM pH 6,8	Chitosan 0.1 wt%		
SOY-PC 90%	EGG-PG 10%	PBG 5 mM pH 6.8	Chitosan 0.1 wt%		
SOY-PC 90%	EGG-PG 10%	PBG 5 mM pH 6.8	Chitosan 0.1 wt%	Methyl p-hydroxybenzoate 0.1%w/v	HEC 0.5% w/v
SOY-PC 90%	EGG-PG 10%	PBG 5 mM pH 6.8	Chitosan 0.1 wt%	Methyl p-hydroxybenzoate 0.1%w/v	HEC 1% w/v
SOY-PC 90%	EGG-PG 10%	PBG 5 mM pH 6.8	Chitosan 0.1 wt%	Methyl p-hydroxybenzoate 0.1%w/v	HPMC 0.5% w/v
SOY-PC 90%	EGG-PG 10%	PBG 5 mM pH 6.8	Chitosan 0.1 wt%	Methyl p-hydroxybenzoate 0.1%w/v	HPMC 1% w/v

4. MAIN RESULTS AND DISCUSSION

In this thesis, liposomes were coated with the polysaccharides alginate or chitosan; subsequently, the addition of a preservative and viscosity modifiers was studied. Line and scatter plots were employed to evaluate the temporal trends of particle size and Zeta potential, thereby assessing the stability of the formulations. Conversely, bar charts facilitated a direct assessment of the Polydispersity Index (PDI), which should ideally remain minimal and constant over time, and strictly below the 0.5 threshold. In drug delivery applications using lipid-based carriers a PDI of 0.3 and below is considered to be acceptable and indicates a homogenous population of phospholipid vesicles. [37]

As a general rule, absolute Zeta potential values equal to or higher than 30 mV are considered indicative of good colloidal stability[38],whereas values closer to zero indicate a greater tendency for aggregation. Moreover, in a combined electrostatic and steric stabilisation, ±20 mV is sufficient.[39]

4.1. ALGINATE COATED LIPOSOMES – investigating different polymer concentration

Positively charged liposomes were prepared and subsequently coated with alginate at two different concentrations (0.125% and 0.0625% w/v). For full composition see table 4.1.

Table 4.1 Composition of the formulations

NEUTRAL LIPID	CHARGED LIPID	HYDRATION MEDIUM	COATING POLIMER
SOY-PC 90%	DOTAP 10%	PB 5 mM pH 6,8	Alg 0.125 wt%
SOY-PC 90%	DOTAP 10%	PB 5 mM pH 6,8	Alg 0.0625 wt%

Regarding particle size, it was observed that the uncoated liposomes were smaller (135 nm) than their coated counterparts in all instances (172 nm Alg 0.125 wt% and 183 nm Alg 0.0625 wt%); see Figure 4.1.1.a. Liposomes coated

with the lower alginate concentration appeared slightly larger. Since the size difference is only 11 nm (approx. 6%), it could be explained by a conformational shift of the adsorbed polymer. A lower alginate concentration (0.0625%) may lead to a less compact loops and tails arrangement, extending the hydrodynamic diameter. Conversely, a higher concentration (0.125%) might force the chains into a denser, flatter train configuration that results in a smaller overall size.

The size of the uncoated vesicles remained nearly unchanged over a two-month storage period, whereas the coated formulations exhibited just an increase of approximately 10 nm.

For all formulations, the Polydispersity Index (PDI) remained around 0.1, which is considered an optimal value. This indicates a narrow size distribution and a high degree of homogeneity within the liposomal populations. While the PDI for the coated liposomes was slightly higher than that of the uncoated ones, it remained well within the acceptable range for stable colloidal systems (Figure 4.1.1.b).

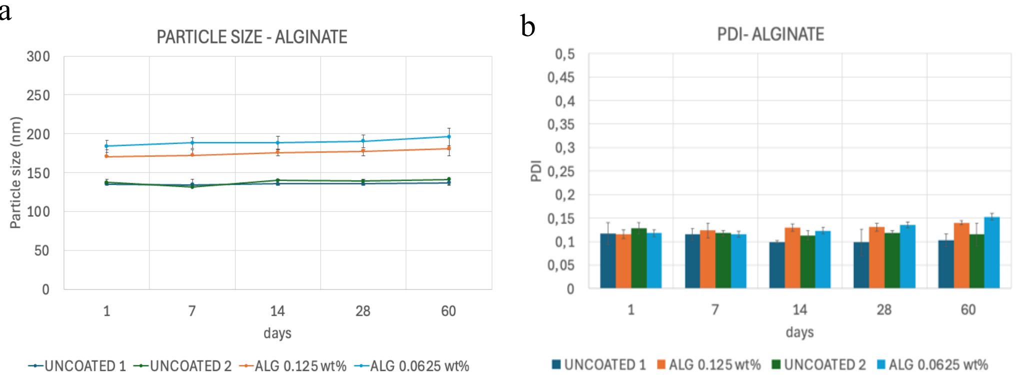


Figure 4.1.1. Physical characterization and 60-day storage stability of Soy-PC - DOTAP 10 mol% liposomes. (a) Particle size (nm) and (b) Polydispersity Index (PDI) of liposomes hydrated in PB. Data are presented for uncoated and alginate-coated liposomes (0.125 wt% or 0.0625 wt%). For uncoated liposomes, results correspond to a single sample. For coated liposomes, results represent the mean of three independent parallel samples. In all cases, the value for each individual sample is the mean of three technical replicates automatically performed by the Zetasizer instrument (n=3). Error bars represent the standard deviation; where not visible, they are equal to or smaller than the symbol size. Connection lines are provided as a visual guide.

A clear inversion of the Zeta potential was observed following the coating process, transitioning from positive values (approximately +33 mV) for the uncoated liposomes to negative values (approximately -43 mV) for the coated formulations (Figure 4.1.2). This charge reversal confirms the successful deposition of the anionic alginate layer onto the cationic liposomal surface. In conclusion, no significant differences were observed between the two

formulations, suggesting that both alginate concentrations can be effectively employed for liposome coating.

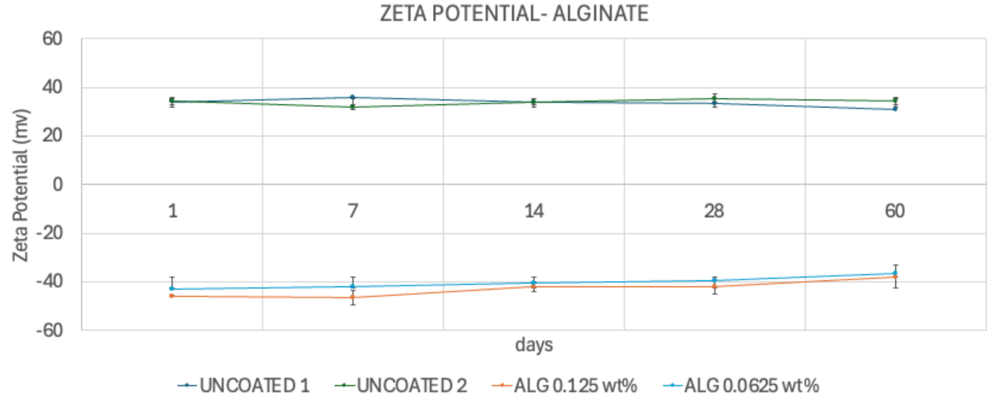


Figure 4.1.2. Zeta potential of Soy-PC - DOTAP 10 mol% liposomes, hydrated in PB, before and after coating with 0.125 wt% or 0.0625 wt% alginate. For uncoated liposomes, results correspond to a single sample. For coated liposomes, results represent the mean of three independent parallel samples. In all cases, the value for each individual sample is the mean of five technical replicates automatically performed by the Zetasizer instrument (n=5). Error bars represent the standard deviation; where not visible, they are equal to or smaller than the symbol size. Connection lines are provided as a visual guide.

4.2. CHITOSAN COATED LIPOSOMES – investigating different hydration mediums

Negatively charged liposomes were prepared and subsequently coated with chitosan at a constant concentration. The focus was on evaluating the impact of different hydration media, phosphate buffer (PB) and phosphate buffered with 15% glycerol (PBG), on the formulations. For full composition see table 4.2.

Table 4.2 Composition of the formulations

NEUTRAL LIPID	CHARGED LIPID	HYDRATION MEDIUM	COATING POLIMER
SOY-PC 90%	EGG-PG 10%	PB 5 mM pH 6,8	Chit 0.1 wt%
SOY-PC 90%	EGG-PG 10%	PBG 5 mM pH 6,8	Chit 0.1 wt%

The initial characterization confirmed that Chitosan coated liposomes were larger than their uncoated counterparts (approximately 180 nm versus approximately 140 nm), as expected. In PB, the uncoated liposomes remained stable for the first 28 days; however, a significant increase in particle size was

recorded after two months (Figure 4.2.1a), likely due to aggregation phenomena. This trend was mirrored by the Polydispersity Index (PDI) (Figure 4.2.1b), which indicated a broadening of the size distribution over time. Additionally, the attenuator value increased from 7 to 11 during this period, even when the dilution was maintained, suggesting a higher presence of large aggregates in the sample. In contrast, the uncoated liposomes hydrated with PBG showed stability up to 60 days.

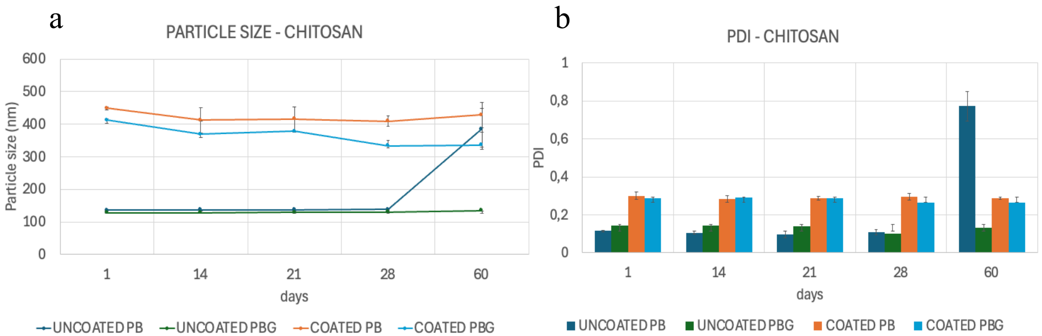


Figure 4.2.1. Physical characterization and 60-day storage stability of Soy-PC – Egg-PG 10 mol% liposomes. (a) Particle size (nm) and (b) Polydispersity Index (PDI) of uncoated and chitosan 0.1 wt% coated liposomes hydrated in PB or PBG. For uncoated liposomes, results correspond to a single sample. For coated liposomes, results represent the mean of three independent parallel samples. In all cases, the value for each individual sample is the mean of three technical replicates automatically performed by the Zetasizer instrument (n=3). Error bars represent the standard deviation; where not visible, they are equal to or smaller than the symbol size. Connection lines are provided as a visual guide.

When comparing the coated liposomes, those prepared in PB were larger than those in PBG. Specifically, after one day, the mean diameter for the three parallel PBG samples was 412 nm, compared to 448 nm for the PB samples. This difference of approximately 30 nm was not a transient fluctuation; rather, it was maintained throughout the entire 60-day monitoring period. This suggests that the presence of 15% glycerol in the PBG medium may promote a more compact arrangement of the Chitosan chains on the liposomal surface. Interestingly, a slight decrease in the size of the coated liposomes was observed over the weeks, particularly in the PBG-hydrated samples. With the exception of the uncoated samples at the 60-day point, the PDI remained consistently below 0.3, indicating a homogeneous and monodisperse population (Figure 4.2.1b).

The Zeta Potential data are the most telling evidence of the coating's success. The inversion of charge from approximately -50 mV to +22 mV confirmed that the Chitosan (a polycation) had masked the negative charge of the Egg-PG.

While a Zeta potential ≥ |30| mV is generally required for purely electrostatic stabilization, the recorded value of +22 mV is considered highly stable for Chitosan-coated systems (Figure 4.2.2.). This is because Chitosan provides electrostatic and steric stabilization. The presence of the polymer chains creates a steric barrier that prevents the liposomal cores from approaching closely enough for Van der Waals forces to cause aggregation. In both PB and PBG, the Zeta potential decreased within 60 days for the uncoated liposomes. The drastic drop in Zeta potential for uncoated liposomes in PB (from -50 mV to -9 mV) aligns with the observed increase in particle size. This suggests that perhaps some lipid degradation products are screening the surface charge, leading to a loss of electrostatic repulsion and subsequent flocculation. On the contrary, the chitosan-coated liposomes in both media maintained a stable Zeta potential of approximately +22 mV. These results suggest that the chitosan layer effectively stabilizes the liposomal surface, thereby preventing charge variations and potential subsequent aggregation. Consistent with these observations, studies on similar systems have demonstrated that a pectin coating significantly improves the colloidal stability of liposomes compared to uncoated formulations. The coating stabilizes the vesicles against aggregation during storage through both electrostatic repulsion and steric hindrance. While uncoated liposomes exhibit poor size stability, often increasing in size by 100–200% due to aggregation, pectin-coated vesicles remain stable for up to 12 weeks.[40]

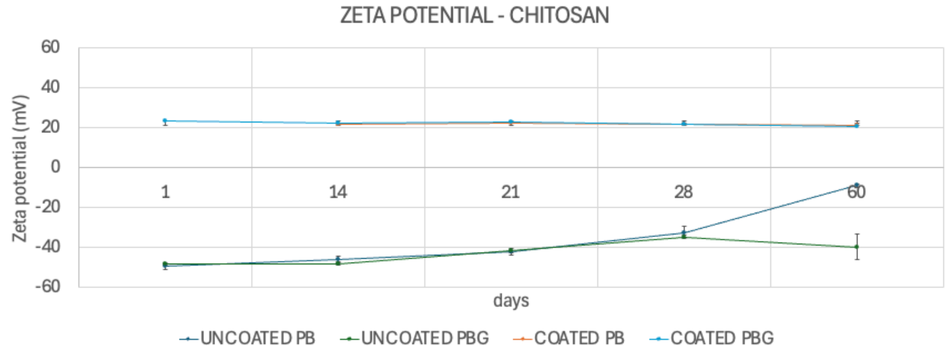


Figure 4.2.2. Zeta potential of Soy-PC – Egg-PG 10 mol% Chitosan coated liposomes, hydrated in PB or PBG. For uncoated liposomes, results correspond to a single sample. For coated liposomes, results represent the mean of three independent parallel samples. In all cases, the value for each individual sample is the mean of five technical replicates automatically performed by the Zetasizer instrument (n=5). Error bars represent the standard deviation; where not visible, they are equal to or smaller than the symbol size. Connection lines are provided as a visual guide.

4.3. PRESERVATIVE AND VISCOSITY MODIFIERS

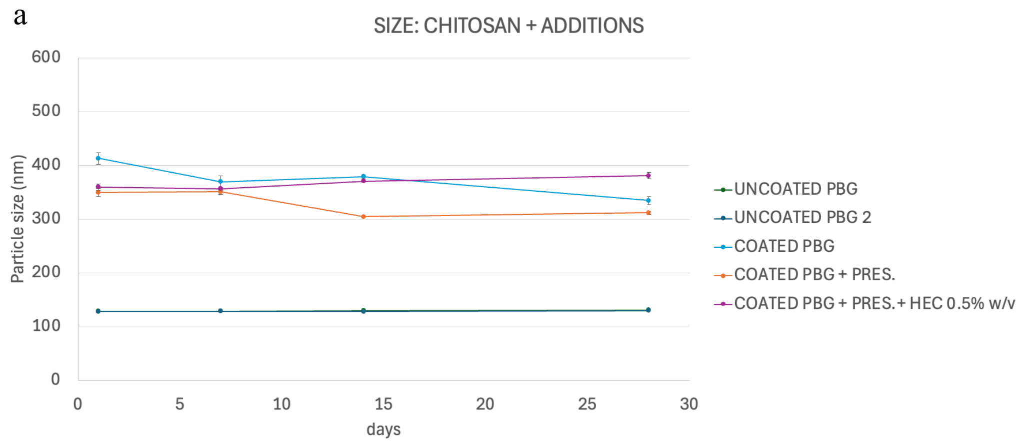
Negatively charged liposomes were prepared in PBG and subsequently coated with chitosan at a constant concentration. In this part, the focus was on evaluating the impact of the adding 0.1%w/v Methyl p-hydroxybenzoate and 0.5% w/v Hydroxyethyl cellulose (HEC) on the formulations. For full composition see table 4.3.

Table 4.3 Composition of the formulations

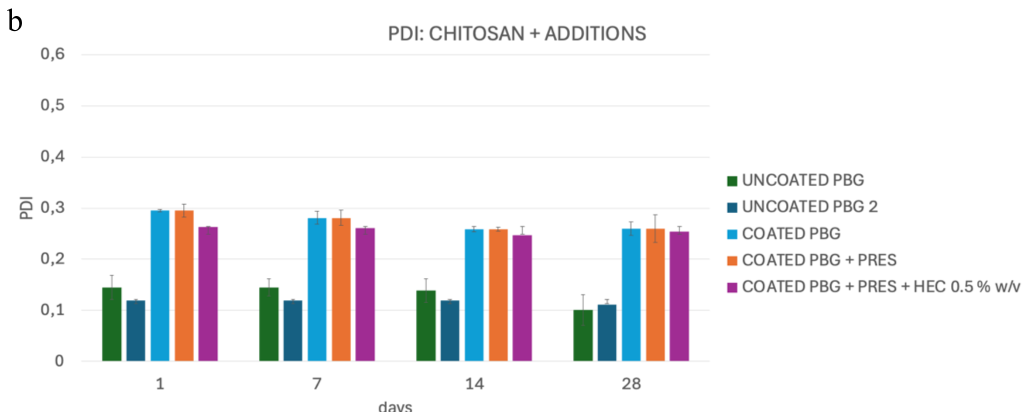
NEUTRAL LIPID	CHARGED LIPID	HYDRATION MEDIUM	COATING POLIMER	PRESERVATIVE	VISCOSITY MODIFIERS
SOY-PC 90%	EGG-PG 10%	PBG 5 mM pH 6.8	Chitosan 0.1 wt%		
SOY-PC 90%	EGG-PG 10%	PBG 5 mM pH 6.8	Chitosan 0.1 wt%	Methyl p-hydroxybenzoate 0.1%w/v	HEC 0.5% w/v

The uncoated liposomes exhibited high stability, maintaining a constant hydrodynamic diameter (size) of approximately 130 nm throughout the 28-day monitoring period (Figure 4.3a). The identical size trends observed across two independent preparation batches (Uncoated PBG and Uncoated PBG 2) confirmed the high reproducibility of the experimental protocol. The application of the coating resulted in a significant increase in particle size, with values ranging between 350 nm and 450 nm. However, the incorporation of additives influenced not only the initial particle size (measured one day after preparation) but also the dimensional evolution over 28 days. The “Coated PBG” formulation, which doesn’t contain any additional additives, showed the largest initial size (approximately 412 nm), followed by a progressive decrease to approximately 335 nm by day 28. This contraction, consistent with the observations reported in Section 4.2., can be explained by a structural rearrangement of the chitosan layer around the liposome.

a



b



c

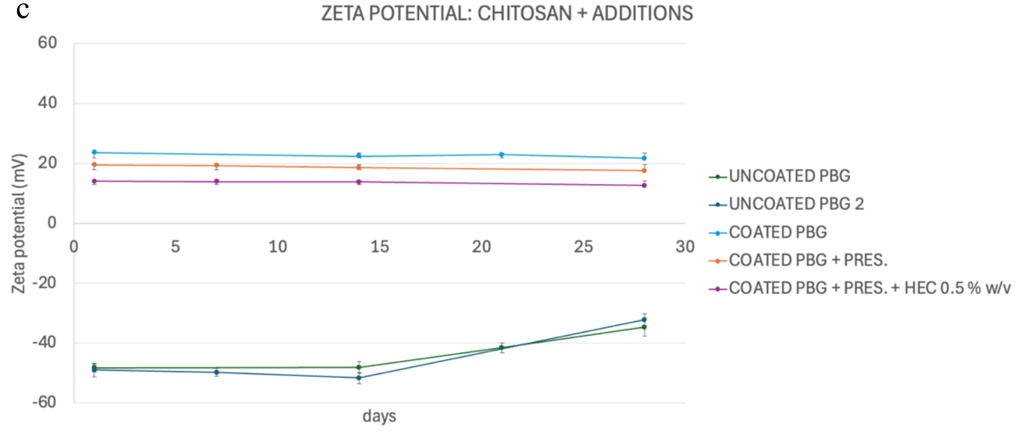


Figure 4.3 Physical characterization and 28-day storage stability of Soy-PC/Egg-PG (10 mol%) liposomes. (a) Particle size, (b) Polydispersity Index (PDI), and (c) Zeta potential of uncoated and chitosan-coated (0.1 wt%) liposomes hydrated in PBG. Selected formulations were supplemented with 0.1% w/v methyl p-hydroxybenzoate and 0.5% w/v HEC. Results for uncoated liposomes correspond to a single sample, while those for coated formulations represent the mean of three independent parallel samples. For each individual sample, the reported value is the average of automated technical replicates performed by the Zetasizer instrument: three for particle size (n=3) and five for Zeta potential (n=5). Error bars represent the standard deviation; where not visible, they are smaller than the symbol size. Connection lines are provided as a visual guide.

The incorporation of 0.1% w/v Methyl p-hydroxybenzoate (indicated as '+ PRES' in Figure 4.3) resulted in smaller initial dimensions (approximately 350 nm) compared to the preservative-free control. This indicates that Methyl p-hydroxybenzoate may promote a more condensed coating from the moment of production. This effect is likely due to hydrogen bonding with chitosan or because the preservative, given its lipophilic nature (logP ≈ 2)[41] intercalates into the phospholipid bilayer, increasing lipid packing density and leading to a more compact vesicular structure.

The formulation containing 0.5% w/v HEC showed the highest stability among the coated system. After an initial size of approximately 360 nm, it exhibited a slight upward trend, reaching approximately 380 nm at day 28. The neutral hydrophilic polymer (HEC) seems to prevent the chitosan layer from contracting, phenomenon observed in HEC-free samples, by providing a more voluminous and hydrated steric shield.

The Polydispersity Index (PDI) (Figure 4.3.b) remained lower than 0.3 for all formulations. Uncoated liposomes showed the lowest PDI values (≈0.12), whereas coated systems exhibited slightly higher but consistent values between 0.25 and 0.30. Importantly, the addition of HEC and preservatives did not compromise the homogeneity of the suspension, which maintained a monodisperse distribution.

Zeta potential analysis (Figure 4.3c) provided evidence of the coating's success, showing a clear charge inversion from the negative values of uncoated liposomes (averaging -48 mV) to positive values for all coated variants. A clear downward trend in the magnitude of the positive surface charge was observed as the formulation complexity increased. Specifically, the coated PBG systems exhibited the highest Zeta potential, averaging +23 mV. The addition of the preservative led to a slight reduction (approximately +20 mV), while the inclusion of 0.5% HEC resulted in a further decrease to a mean value of +14 mV. This progressive decrease is attributed to the shielding effects of the additives. As a non-ionic polymer, HEC interpenetrates the cationic chitosan layer, shifting the shear plane further from the particle surface and physically masking the

positive amino groups. Similarly, methylparaben contributes to this trend through its intercalation within the lipid-polymer interface, where potential hydrogen bonding with chitosan leads to a partial screening of the surface charge. Despite these lower absolute values, the Zeta potential remained notable constant over the 28-day period. This suggests that the formulations, particularly those containing HEC, do not rely solely on electrostatic repulsion for stability. Instead, they are stabilized via a synergistic electrostatic and steric mechanism, where the physical bulk of the polymer chains creates a robust steric barrier that prevents vesicle aggregation even at a reduced surface charge of +14 mV.

4.4. COMPARISON BETWEEN HEC AND HPMC AS THICKENERS

Negatively charged liposomes were prepared in PBG and subsequently coated with chitosan at a constant concentration. This part focused on comparing the effects of Hydroxyethyl cellulose (HEC) and Hydroxypropyl Methylcellulose (HPMC); therefore, 0.5% w/v or 1% w/v of either HEC or HPMC was added to the same base formulation. For full composition see table 4.4.

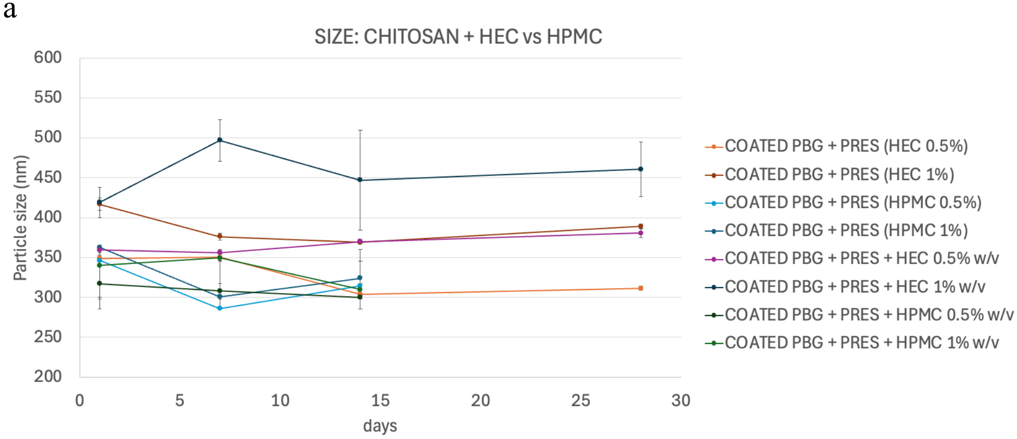
Table 4.4 Composition of the formulations

NEUTRAL LIPID	CHARGED LIPID	HYDRATION MEDIUM	COATING POLIMER	PRESERVATIVE	THICKENERS
SOY-PC 90%	EGG-PG 10%	PBG 5 mM pH 6.8	Chitosan 0.100 wt%	Methyl p-hydroxybenzoate 0.1%w/v	HEC 0.5% w/v
SOY-PC 90%	EGG-PG 10%	PBG 5 mM pH 6.8	Chitosan 0.100 wt%	Methyl p-hydroxybenzoate 0.1%w/v	HEC 1% w/v
SOY-PC 90%	EGG-PG 10%	PBG 5 mM pH 6.8	Chitosan 0.100 wt%	Methyl p-hydroxybenzoate 0.1%w/v	HPMC 0.5% w/v
SOY-PC 90%	EGG-PG 10%	PBG 5 mM pH 6.8	Chitosan 0.100 wt%	Methyl p-hydroxybenzoate 0.1%w/v	HPMC 1% w/v

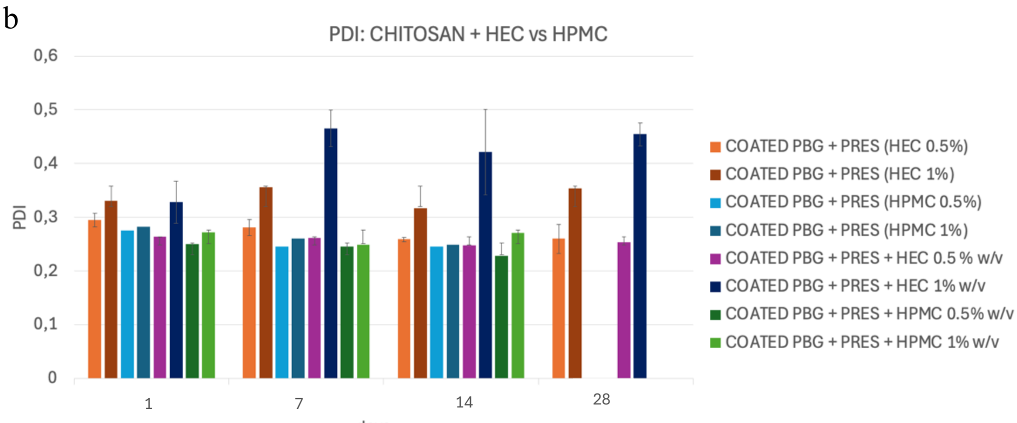
A discrepancy was observed in the base formulations (COATED PBG + PRES) prior to the addition of cellulose derivatives (HEC or HPMC). While all other preparations exhibited initial sizes of approximately 350 nm at day 1 (consistent with Section 4.3), the specific batch used for the + HEC 1% w/v formulation deviated, starting at approximately 420 nm. This initial variance might be attributed to the incomplete solubilization of the preservative or variations during the coating phase.

Particle size analysis over 28 days (Figure 4.4a) showed that the HPMC formulations (0.5% and 1%) maintained more constant liposome dimensions throughout the study. In contrast, the HEC series, particularly at 1% w/v, exhibited a more complex and unstable profile. A critical finding regarding the HEC 1% w/v formulation is the significant increase in size recorded after one week, followed by a subsequent decrease. Notably, on Day 1, the corresponding base formulation (COATED PBG + PRES without HEC) used as a reference point and the HEC-supplemented sample exhibited the same particle size (420 nm). This indicates that the instability was not immediate upon addition, but rather a time-dependent phenomenon occurring during the first week of storage. This difference in behaviour suggests that HPMC, being more hydrophobic than HEC, interacts more effectively with the chitosan-coated surface, forming a compact and hydrated steric shield that anchors firmly to the vesicle. Conversely, the more hydrophilic nature of HEC may prevent such robust anchoring, leading to destabilizing phenomena, as polymer bridging and depletion flocculation. Excessive HEC chains can act as "bridges" between multiple liposomes, linking separate vesicles into a temporary network or macro-clusters. The subsequent decrease in size after the first week likely represents a structural rearrangement or the eventual collapse of these unstable bridged networks. At higher concentrations, non-adsorbing HEC polymers can interact with the liposomes together, causing them to aggregate into larger clusters. This effect provides an explanation for the initial spike in particle size, as the "free" polymer in the bulk phase drives the vesicles into aggregate formations to minimize the system's energy.

a



b



c

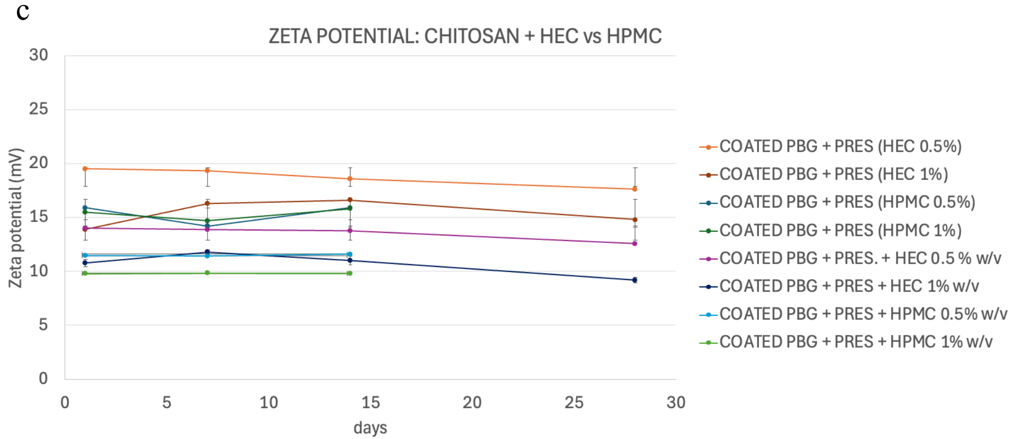


Figure 4.4 Physical characterization and 28-day storage stability of Soy-PC/Egg-PG (10 mol%) liposomes. (a) Particle size, (b) Polydispersity Index (PDI), and (c) Zeta potential of chitosan-coated liposomes in PBG, following the addition of 0.1% w/v methyl p-hydroxybenzoate and either HEC or HPMC (at 0.5% or 1% w/v) to the base formulation Note that data for HEC-containing formulations are limited to 14 days due to the conclusion of the laboratory period. The label "COATED PBG + PRES (...)" refers to the base formulation (chitosan-coated liposomes in PBG with 0.1% w/v methyl p-hydroxybenzoate); suffixes in parentheses, such as (+ HEC 0.5% w/v) or (+ HPMC 1% w/v), indicate the specific polymer and concentration added. Results for uncoated liposomes correspond to a single sample, while those for coated formulations represent the mean of three independent parallel samples. Each data point reflects the average of automated technical replicates: three for particle size (n=3) and five for Zeta potential (n=5). Error bars represent the standard deviation; where not visible, they are smaller than the symbol size. Connection lines are provided as a visual guide.

The PDI data (Figure 4.4b) confirms the superior homogeneity of the HPMC systems, which maintained values consistently below 0.3. The HEC 1% w/v formulation, however, exhibited a sharp increase in PDI (exceeding 0.4), reflecting a highly polydisperse population. This trend is consistent with the formation of irregular aggregates, discussed in the prior paragraph. Notably, even before the addition of the cellulose derivative, the specific COATED PBG + PRES (HEC 1%) formulation exhibited a higher PDI (> 0.3) compared to the other base preparations, which remained consistently below 0.3.

All formulations maintained a positive surface charge (Figure 4.4c), confirming that the chitosan coating remained intact. The addition of either HEC or HPMC to the base formulation led to a decrease in Zeta potential that was directly proportional to the polymer concentration. Specifically, the higher the polymer concentration, the greater the observed decrease in Zeta potential. This phenomenon can be attributed to the shielding effect of the non-ionic cellulosic polymers. At identical concentrations (0.5% and 1%), HPMC led to a slightly greater reduction in Zeta potential compared to HEC. This reinforces the hypothesis that HPMC, due to its higher hydrophobicity, achieves a more efficient and dense anchoring to the liposomal surface. A more compact and robust coating layer by HPMC provides superior masking of the surface charges compared to the more hydrophilic and HEC chains, thus shifting the shear plane more effectively. For the first two weeks, the Zeta potential remained remarkably stable for all formulations. However, after 28 weeks, both HEC-containing formulations showed a slight downward trend in surface charge, that likely reflects the structural instability previously discussed in particle size analysis. No data were collected for HPMC at 28 days.

While HPMC appears more promising, it cannot be excluded that the observed instability in the HEC 1% system is partially linked to the initial state of the liposomes, potentially exacerbating the effects of the polymer concentration.

4.5. VISCOSITY OF THE SOLUTIONS

In this section, the viscosity of the solutions discussed in Section 4.4 was compared to evaluate how the addition of HEC or HPMC at different concentrations (0.5% w/v or 1% w/v) modifies the rheological behaviour of the chitosan-coated liposome suspension.

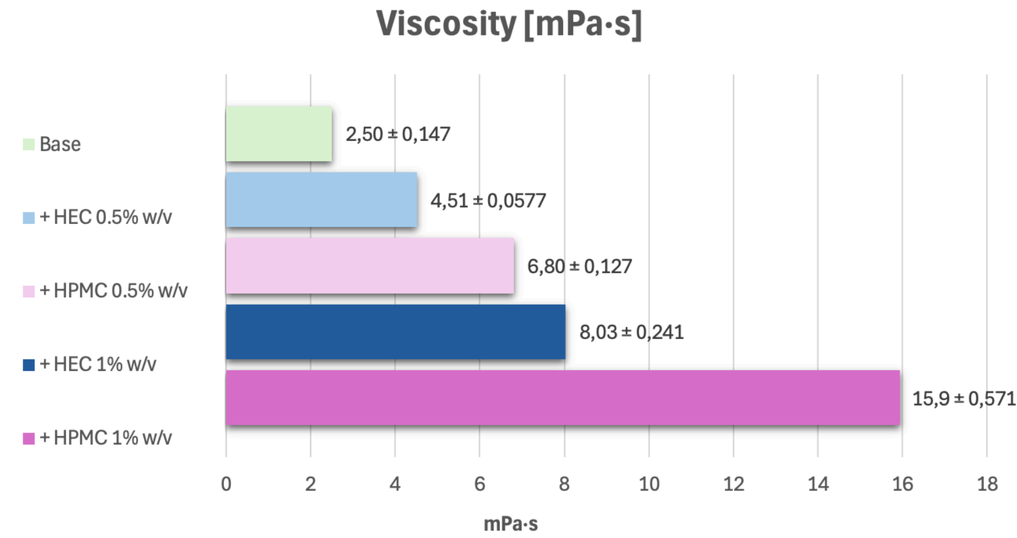


Figure 4.5 Apparent viscosity (mPa·s) of chitosan-coated liposomes in PBG, before and after the addition of HEC or HPMC (0.5% w/v and 1% w/v). Each value represents the mean ± standard deviation of three independent measurements performed on different samples prepared under identical conditions. All measurements were conducted at a constant rotational speed of 15 rpm and a controlled temperature of 25.4 ± 0.1 °C. The Base formulation consists of 90% SOY-PC and 10% Egg-PG in a 5 mM PBG buffer (pH 6.8), coated with 0.1 wt% chitosan and 0.1% w/v methyl p-hydroxybenzoate.

The results indicated that the addition of both polymers increased the viscosity of the system compared to the Base formulation (Figure 4.5). In both cases, the 1% w/v concentration yielded a significantly higher viscosity than the 0.5% w/v concentration, confirming a dose-dependent relationship between polymer concentration and viscosity. The primary molecular difference between HEC and HPMC lies in their substituent groups. HEC is characterized by hydroxyethyl side chains, which impart a highly hydrophilic and non-ionic character to the polymer. Conversely, HPMC contains both methyl and hydroxypropyl groups; the presence of the hydrophobic methyl substituents provides HPMC with an amphiphilic nature and surface-active properties that are absent in HEC. These structural variations directly influence the polymer-liposome interaction. HEC was found to increase viscosity to a lesser extent than

HPMC. While HEC relies primarily on hydrogen bonding, the amphiphilic nature of HPMC allows for stronger hydrophobic interactions with the lipid bilayers and the formation of a more complex intermolecular network. This leads to a more robust increase in the viscosity of the liposomal suspension.

4.6. SPRAYABILITY

In this section, a spray test was conducted to evaluate how the increase in solution viscosity could affect the sprayability of the formulation. Measurements were performed on the base formulation (without the addition of viscosity enhancers) and on the four formulations discussed in Sections 4.4 and 4.5, containing either HEC or HPMC at 0.5% w/v or 1% w/v.

Table 4.6 Delivered dose per spray (g) and spray diameter (cm) for the tested formulations at different storage temperatures (4 °C and 22 °C). Each sample was weighed before and after a series of three consecutive sprays. The single dose value corresponds to one spray and was calculated as the average of the three sprays. The spray diameter was measured at three different points of the sprayed solution pattern; therefore, the reported result corresponds to the mean of three measurements ± *standard deviation*. A distance of 7 cm was selected, considering that the spray is intended for oral administration. The Base* formulation consists of 90% SOY-PC and 10% Egg-PG in a 5 mM PBG buffer (pH 6.8), coated with 0.1 wt% chitosan and 0.1% w/v methyl p-hydroxybenzoate.

Base* Formulation				
Temperature	4°C	22°C		
Delivered dose / spray (g)	0,181	0,193		
Spray diameter (cm)	9,23 ± 0,371	9,02 ± 0,367		
+ HEC	0.5% w/v		1% w/v	
Temperature	4°C	22°C	4°C	22°C
Delivered dose / spray (g)	0,196	0,204	0,205	0,204
Spray diameter (cm)	6,14 ± 0,126	6,34 ± 0,107	6,33 ± 0,185	6,31 ± 0,038
+ HPMC	0.5% w/v		1% w/v	
Temperature	4°C	22°C	4°C	22°C
Delivered dose / spray (g)	0,176	0,195	0,205	0,205
Spray diameter (cm)	6,23 ± 0,152	6,38 ± 0,117	5,44 ± 0,107	5,09 ± 0,019

The delivered dose per spray remained consistent across all formulations. A very slight increase in the delivered mass was observed in the more viscous systems (0,205 g for 1% HPMC) compared to the base formulation (approximately 0,187 g). Regarding the effect of temperature, the data shows that for almost all formulations (base formulation and those containing 0.5% w/v HEC or HPMC), the dose delivered at 22°C was marginally higher than at 4°C. This can be attributed to the temperature-dependent viscosity; at higher temperatures, the viscosity slightly decreases, reducing the internal friction during the actuation and allowing for a more efficient discharge of the liquid.

The liposomes without a viscosity modifier had the largest spray diameter (approximately 9 cm). With the addition of a viscosity modifier at a concentration of 0.5%, the diameter decreases to around 6 cm, with no significant differences observed between HEC and HPMC. Adding a concentration of 1.0% HEC (double the amount) does not appear to influence the spray diameter; however, the same concentration of HPMC results in a further reduction to about 5.2 cm. This is consistent with the viscosity data (Section 4.5), where a significant increase in viscosity is observed with the addition of 1.0% HPMC. No trend regarding temperature could be identified, as no significant differences were detected when measuring sprayability at 4°C and 22°C.

5. CONCLUSIONS

The study focused on the development and characterization of polymer-coated liposomal formulations for the alleviation of dry mouth symptoms, evaluating the impact of different coating agents, hydration media, and thickening modifiers on their physical stability and rheological properties.

The initial phase of the research confirmed that both alginate and chitosan can be employed to coat liposomes. In both cases, a successful coating was demonstrated by a significant increase in hydrodynamic diameter and a complete inversion of the Zeta potential, indicating the deposition of the polymer layer onto the liposomal surface. Both systems exhibited physical stability over the monitoring period. However, chitosan-coated liposomes were selected for further development due to the mucoadhesive potential[33] and antibacterial properties of chitosan, both of which are important for enhancing the residence time and efficacy of the formulation on the oral mucosa.

Regarding the hydration medium, the incorporation of 15% glycerol into the phosphate buffer (PB, pH 6.8) did not compromise the stability of the liposomal suspension. On the contrary, the phosphate buffer with glycerol (PBG, pH 6.8) medium was preferred for the final formulations to leverage the intrinsic humectant and lubricating[42] properties of glycerol, which are beneficial for oral applications. Furthermore, glycerol's known ability to lower water activity can contribute to the overall preservative system of the formulation. The pH of 6.8 mimics that of natural saliva, ensuring that liposomes do not undergo environmental stress upon administration; this physiological alignment prevents pH-induced instability once the vesicles are sprayed into the oral cavity.

The addition of 0.1% w/v methyl p-hydroxybenzoate as a preservative was also successfully integrated. The results showed no significant effects on the size, PDI, or surface charge of the coated liposomes, confirming its suitability for maintaining the microbiological stability of the system without altering its physical integrity.

The comparison between viscosity modifiers provided crucial insights for the optimization of the formulation. While both HEC and HPMC increased the viscosity in a concentration-dependent manner, HPMC 1% w/v emerged as the most effective thickening agent. This higher viscosity directly influenced the mechanical performance of the system; indeed, it was the only formulation to exhibit a distinct change in sprayability, producing a more concentrated pattern compared to the other samples. By synthesizing the physicochemical and rheological data, it can be concluded that 1% HPMC provides the maximum viscosity enhancement while maintaining a stable, homogeneous, and monodisperse population of coated liposomes. Increasing the consistency can be used as a strategy to improve sensory perception and mouthfeel during oral administration, avoiding an overly watery consistency that could negatively impact patient compliance. Furthermore, temperature variations between 4°C and 22°C did not result in significant changes to either the spray diameter or the delivered dose. Since the therapeutic goal is the alleviation of dry mouth symptoms, the stability of the spray at lower temperatures is advantageous because it allows for the potential administration of the formulation at 4°C, which would provide an additional cooling and refreshing sensation for the patient without compromising the dose uniformity or the spray quality.

In summary, the optimized formulation consisting of Soy-PC/Egg-PG liposomes coated with 0.1% chitosan, hydrated in PBG, and supplemented with 0.1% methyl p-hydroxybenzoate and 1% HPMC, represents a stable and high-viscosity system suitable for further investigation in mucosal drug delivery.

FUTURE PERSPECTIVES

Building upon the promising results of this study, several directions for future research can be identified to further validate and enhance the clinical potential of the optimized formulation:

While this work focused on the structural and rheological properties of the "empty" formulation, the next step involves exploiting the liposomes' versatility as a delivery system. Their bilayer structure allows for the encapsulation of both hydrophilic and lipophilic active ingredients. The primary focus would be the encapsulation of humectants or hydrating agents (such as hyaluronic acid) to provide immediate and long-lasting relief from xerostomia symptoms. Furthermore, the encapsulation of anti-inflammatory agents, analgesics, or antioxidants could be performed to treat secondary complications, such as oral infections, which frequently affect patients with compromised salivary flow.

To better predict the formulation's behaviour in vivo, future studies should evaluate the stability and mucoadhesive strength of the chitosan-coated liposomes in Simulated Salivary Fluid.

To further improve patient compliance, the integration of food-grade flavouring agents should be explored. Developing a pleasant, "candy-like" taste profile is particularly important for patients undergoing chemotherapy or radiotherapy, who often suffer from severe dysgeusia (altered taste). A refreshing flavour would encourage patients to retain the formulation in the oral cavity for longer periods, avoiding immediate rinsing or swallowing, thereby maximizing the residence time and therapeutic efficacy.

REFERENCES

1. Gray's Anatomy, 39th Edition: The Anatomical Basis of Clinical Practice. *AJNR Am J Neuroradiol* **26**, 2703–2704 (2005).
2. Anatomy, Head and Neck, Oral Cavity (Mouth). *StatPearls* [Internet] (2023).
3. Ptasiewicz, M. *et al.* Armed to the Teeth—The Oral Mucosa Immunity System and Microbiota. *Int J Mol Sci* **23**, 882 (2022).
4. Brizuela, M. & Winters, R. Histology, Oral Mucosa. in *StatPearls* (StatPearls Publishing, Treasure Island (FL), 2025).
5. Frenkel, E. S. & Ribbeck, K. Salivary mucins in host defense and disease prevention. *J Oral Microbiol* **7**, 10.3402/jom.v7.29759 (2015).
6. Vila, T., Rizk, A. M., Sultan, A. S. & Jabra-Rizk, M. A. The power of saliva: Antimicrobial and beyond. *PLoS Pathog* **15**, e1008058 (2019).
7. Bardow, A., Moe, D., Nyvad, B. & Nauntofte, B. The buffer capacity and buffer systems of human whole saliva measured without loss of CO2. *Archives of Oral Biology* **45**, 1–12 (2000).
8. Humphrey, S. P. & Williamson, R. T. A review of saliva: Normal composition, flow, and function. *The Journal of Prosthetic Dentistry* **85**, 162–169 (2001).
9. Kurien, V. T. V., Shanker, R. K., Hegde, S., Onisha, V. & Pillai, S. A. S. Evaluation of Salivary Parameters and Oral Health Status in Periodontally Healthy Subjects and Chronic Periodontitis Subjects. *Advances in Human Biology* **14**, 203 (2024).

10. Kapourani, A. *et al.* A Review on Xerostomia and Its Various Management Strategies: The Role of Advanced Polymeric Materials in the Treatment Approaches. *Polymers (Basel)* **14**, 850 (2022).
11. Roblegg, E., Coughran, A. & Sirjani, D. Saliva: An all-rounder of our body. *European Journal of Pharmaceutics and Biopharmaceutics* **142**, 133–141 (2019).
12. Han, P., Suarez-Durall, P. & Mulligan, R. Dry mouth: A critical topic for older adult patients. *Journal of Prosthodontic Research* **59**, 6–19 (2015).
13. Millsop, J. W., Wang, E. A. & Fazel, N. Etiology, evaluation, and management of xerostomia. *Clinics in Dermatology* **35**, 468–476 (2017).
14. Pistone, S., Goycoolea, F. M., Young, A., Smistad, G. & Hiorth, M. Formulation of polysaccharide-based nanoparticles for local administration into the oral cavity. *European Journal of Pharmaceutical Sciences* **96**, 381–389 (2017).
15. Roque, L. *et al.* Bioadhesive polymeric nanoparticles as strategy to improve the treatment of yeast infections in oral cavity: *in-vitro* and *ex-vivo* studies. *European Polymer Journal* **104**, 19–31 (2018).
16. Nsairat, H. *et al.* Liposomes: structure, composition, types, and clinical applications. *Heliyon* **8**, 93–94 (2022).
17. Nsairat, H. *et al.* Liposomes: structure, composition, types, and clinical applications. *Heliyon* **8**, e09394 (2022).
18. Rahma, M. N. *et al.* The Role and Advancement of Liposomes for Oral Diseases Therapy. *Int J Nanomedicine* **20**, 1865–1880 (2025).

19. Zagana, P. & Paxinou, A. Advanced Liposomal Systems for Cancer Therapy with Focus on Lipid–Polymer Hybrids and Cell Membrane-Coated Liposomes. *Future Pharmacology* **6**, (2026).

20. Ahualli, S., Orozco-Barrera, S., Medina Castillo, A. L. & Delgado, A. V. Effect of coating nanostructure on the electrokinetics of polyelectrolyte-coated particles. Grafted vs adsorbed polymer. *Journal of Molecular Liquids* **375**, 121384 (2023).

21. Manciu, M. & Ruckenstein, E. Loops, tails and trains: A simple model for structural transformations of grafted adsorbing neutral polymer brushes. *Journal of Colloid and Interface Science* **354**, 61–69 (2011).

22. Martinez, J., Ingram, N., Kapur, N., Jayne, D. G. & Beales, P. A. Composition-dependent tunability of the cell interactions of hybrid lipid – block copolymer vesicles. *Journal of Colloid and Interface Science* **694**, 137664 (2025).

23. Arif, Z. U. The role of polysaccharide-based biodegradable soft polymers in the healthcare sector. *Advanced Industrial and Engineering Polymer Research* **8**, 132–156 (2025).

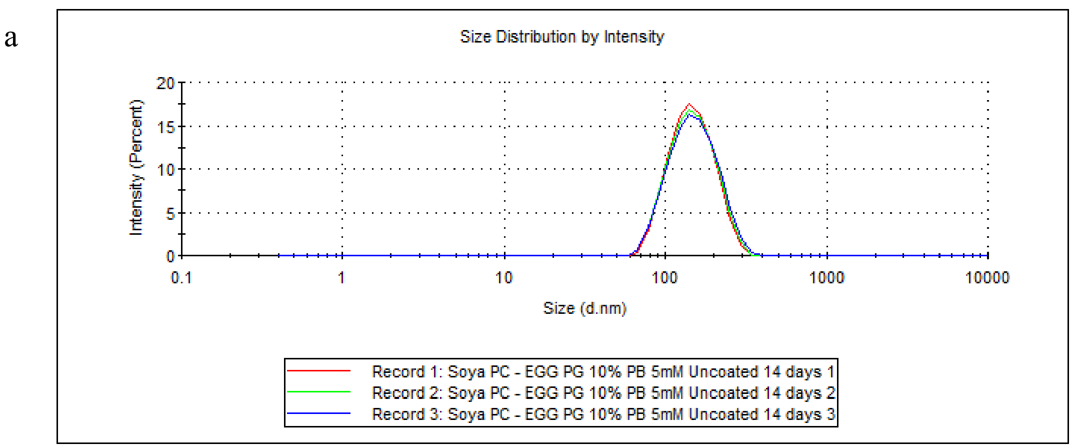
24. Paderni, C., Compilato, D., Giannola, L. I. & Campisi, G. Oral local drug delivery and new perspectives in oral drug formulation. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology* **114**, e25–e34 (2012).

25. Sogias, I. A., Williams, A. C. & Khutoryanskiy, V. V. Why is Chitosan Mucoadhesive? *Biomacromolecules* **9**, 1837–1842 (2008).

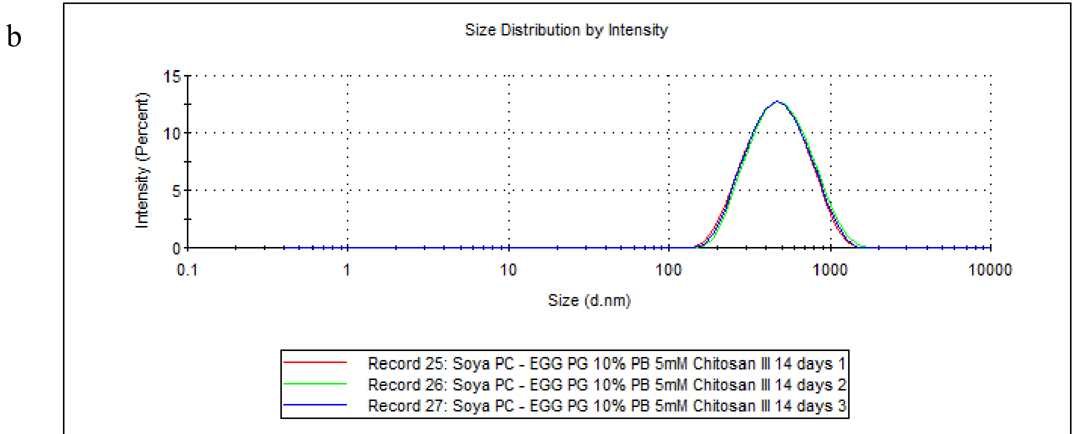
26. Nguyen, S. *et al.* The influence of liposomal formulation factors on the interactions between liposomes and hydroxyapatite. *Colloids and Surfaces B: Biointerfaces* **76**, 354–361 (2010).
27. Nguyen, S., Hiorth, M., Rykke, M. & Smistad, G. Polymer coated liposomes for dental drug delivery – Interactions with parotid saliva and dental enamel. *European Journal of Pharmaceutical Sciences* **50**, 78–85 (2013).
28. Nguyen, S., Hiorth, M., Rykke, M. & Smistad, G. The potential of liposomes as dental drug delivery systems. *Eur J Pharm Biopharm* **77**, 75–83 (2011).
29. Pistone, S., Rykke, M., Smistad, G. & Hiorth, M. Polysaccharide-coated liposomal formulations for dental targeting. *International Journal of Pharmaceutics* **516**, 106–115 (2017).
30. Pistone, S., Rykke, M., Smistad, G. & Hiorth, M. Polysaccharide-coated liposomal formulations for dental targeting. *International Journal of Pharmaceutics* **516**, 106–115 (2017).
31. Jonassen, H. Polysaccharide based nanoparticles for drug delivery applications.
32. Deng, Y., Shavandi, A., Okoro, O. V. & Nie, L. Alginate modification via click chemistry for biomedical applications. *Carbohydrate Polymers* **270**, 118360 (2021).
33. Kumar, A., Vimal, A. & Kumar, A. Why Chitosan? From properties to perspective of mucosal drug delivery. *International Journal of Biological Macromolecules* **91**, 615–622 (2016).

34. Kumar, A., Vimal, A. & Kumar, A. Why Chitosan? From properties to perspective of mucosal drug delivery. *International Journal of Biological Macromolecules* **91**, 615–622 (2016).
35. Zetasizer Nano Series User Manual. *(NANO317 Issue)* **12**, 1–256 (2004).
36. Brookfield DV2T Viscosimeter Manual.
37. Danaei, M. *et al.* Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems. *Pharmaceutics* **10**, 57 (2018).
38. Németh, Z. *et al.* Quality by Design-Driven Zeta Potential Optimisation Study of Liposomes with Charge Imparting Membrane Additives. *Pharmaceutics* **14**, 1798 (2022).
39. Faried, M. *et al.* Synthesis of silver nanoparticles via green method using ultrasound irradiation in seaweed Kappaphycus alvarezii media. *Res Chem Intermed* **42**, 7991–8004 (2016).
40. Smistad, G., Bøyum, S., Alund, S. J., Samuelsen, A. B. C. & Hiorth, M. The potential of pectin as a stabilizer for liposomal drug delivery systems. *Carbohydrate Polymers* **90**, 1337–1344 (2012).
41. PubChem. Compound Summary for CID 7456, Methylparaben. https://pubchem.ncbi.nlm.nih.gov/compound/7456.
42. Frigaard, J. M. Development of an innovative product to relieve dry mouth. Considerations of ingredients, toxicity, stability, and patient selection. (2024).

a



b



c

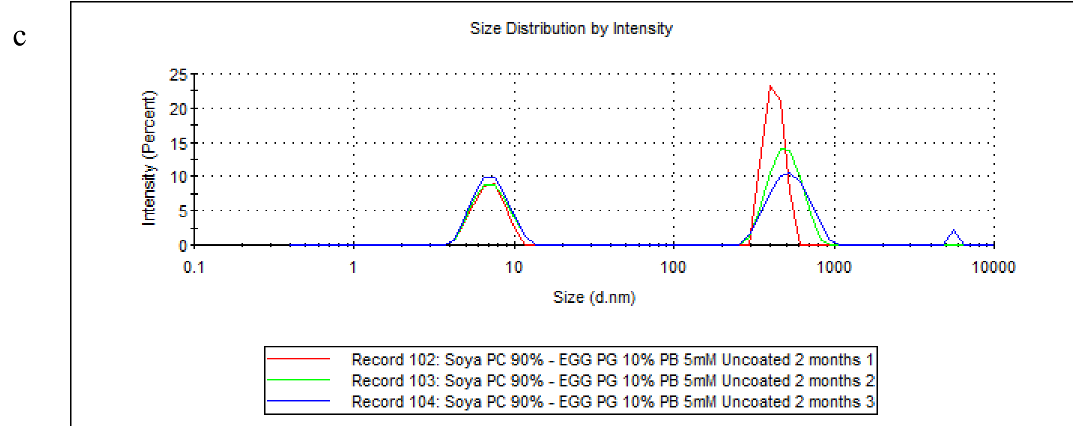
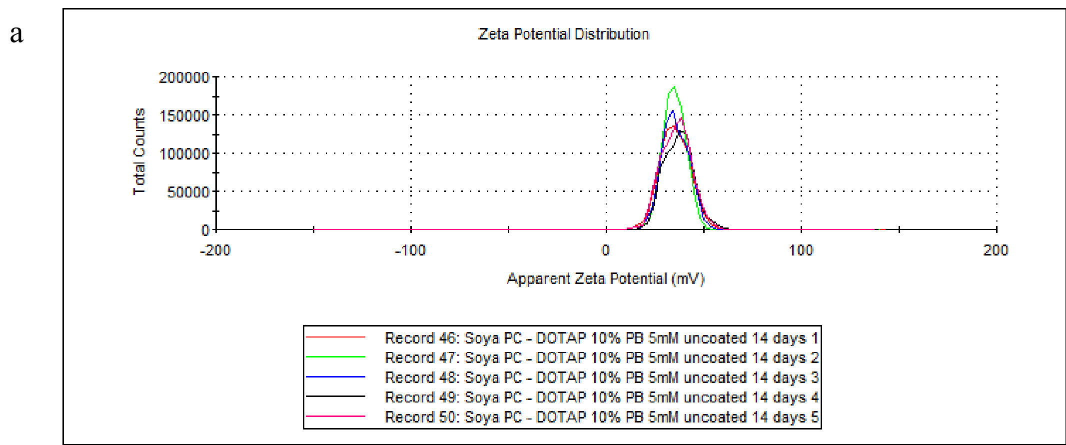


Figure 1. Representative DLS size distribution graphs obtained from Zetasizer measurements. (a) Uncoated liposomes (90% soy-PC, 10% egg-PG) and (b) 0.1% wt chitosan-coated liposomes, both analyzed after 14 days of storage. The results show an increase in particle size following the coating process; however, both samples exhibit a monomodal distribution with overlapping curves across the three replicates, indicating high measurement reproducibility and physical stability. In contrast, (c) the same uncoated liposomes measured after 2 months show a loss of stability, as discussed in Section 4.2.

a



b

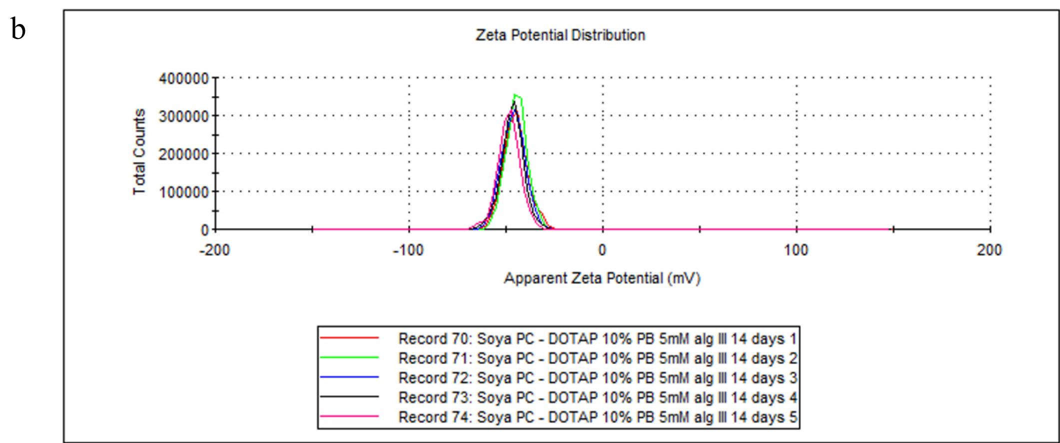


Figure 2. Representative zeta potential graphs obtained from Zetasizer measurements. (a) Uncoated liposomes (90% soy-PC, 10% egg-PG) and (b) 0.125% wt alginate-coated liposomes, both analyzed after 14 days of storage. The results show a clear shift of the curve from positive to negative values, highlighting the successful coating process. Both samples exhibit a monomodal distribution with overlapping curves across the three replicates, indicating high measurement reproducibility and physical stability, as discussed in Section 4.1.