UNIVERSITY OF TROMSØ UIT
FACULTY OF HEALTH SCIENCES
DEPARTMENT OF PHARMACY

Master Thesis for the degree Master of Pharmacy

DEVELOPEMENT OF MUCOADHESIVE POLYMER-COATED LIPOSOMES FOR HYDRATION OF THE ORAL MUCOSA

REBWAR SALAR NORI SALEH

2013

External Supervisors

Professor Gro Smistad

Associate Professor Marianne Hiorth

School of pharmacy

Faculty of Mathematics and Natural Sciences

University of Oslo

Internal Supervisor

Professor Natasa Skalko-Basnet

Department of Pharmacy

Faculty of Health Science

University of Tromsø

UiO : School of Pharmacy
University of Oslo

DEVELOPEMENT OF MUCOADHESIVE POLYMER-COATED LIPOSOMES FOR HYDRATION OF THE ORAL MUCOSA

By

REBWAR SALAR NORI SALEH

DEPARTMENT OF PHARMACY

FACULTY OF HEALTH SCIENCE

UNIVERSITY OF TROMSØ

NORWAY

2013

External Supervisors

Professor Gro Smistad

Associate Professor Marianne Hiorth

School of Pharmacy

Faculty of Mathematics and Natural Sciences

University of Oslo

Internal Supervisor

Professor Natasa Skalko-Basnet

Department of Pharmacy

Faculty of Health Science

University of Tromsø

UiO : School of Pharmacy
University of Oslo

UNIVERSITY OF TROMSØ UiT

Acknowledgements

This study was done at the University of Oslo, School of Pharmacy. I would like to express my deep gratitude to everyone working in the *SiteDel* group, especially Tove Larsen for her never stopping patience with me. Thanks to professors Gro Smistad and Marianne Hiorth for all the guidance, advices and support throughout the study, and for kindly letting me use the very much needed resources. I also want to thank Professor Ørjan Grøttem Martinsen from the Department of Physics for all the help, guidance and motivation.

Back to my home University which is the University of Tromsø, and Institute for Pharmacy. Thank you, Professor Natasa Skalko-Basnet for opening the door to my master thesis, without you I wouldn't be able to do this particular study in Oslo. I can't thank you enough for that and for your motivating e-mails when needed. I also want to thank all the professors, lecturers and lab supervisors from the Department of Pharmacy for all the challenges through these five years.

And of course, I don't think these five years would be the same without my class mates. Thank you every one for all the memories that I will bring with me. A few persons that I would like to give a special thanks to are Ben Tore Henriksen, Ingrid Albert, Kristian Mortensen, and my Norwegian big brother Morten Tranung. Without you guys these 5 years would be so boring. Thank you for all the good times spent in group rooms preparing for exams. I will never forget you.

Normally I joke about the fact that my parents, Salar Saleh and Samira Muhi-Aldin, brainwashed me when I was a kid because they always pushed me to do my best in school so that I could become either a doctor or a pharmacist. And now, I can't thank you both enough for all your hard work, sweat, yelling and sometimes nagging. I would not be here without you. My brother Range, and my sister Roza; I made it!

My dear, Ragnhild Osbak, I want to thank you for always being there for me, supporting and motivating, and challenging me all these years. You have been a true inspiration and source of motivation for me.

And finally I want to give thumbs up to myself for getting her and never giving up!

Abstract (Norwegian)

Tørre slimhinner i munnhulen er problematisk for pasienter og det kan resultere i karies og erosjon av tannemaljen. Reduksjon eller bortfall av spyttsekresjon kan være et resultat av sykdom, bivirkninger av legemidler, alder eller skade fra radioterapi. Et system som kan hydrere slimhinnen vil kunne redusere ubehaget og samtidig å forebygge relaterte problemer. Liposomer og polymer-dekkede liposomer har vist potensiale som et system for lokal applikasjon i munnhulen. Ved bruk av mukoadhessive polymerer kan man forlenge tiden systemet er til stedet etter applikasjon.

Hensikten med studiet var å tilberede og karakterisere polymer-dekkede liposomer, samt. undersøke sammenhengen mellom mukoadhessive- og potensielt hydreringsegenskapene deres. I første omgang ble polymerkonsentrasjoner nødvendig for fullstendig dekking av liposomene bestemt. Polymeren pektin (HM-, LM-, og AM-pektin) ble brukt til å dekke positivt ladet EggPC-DOTAP liposomer og polymeren chitosan ble brukt til å dekke negativt ladet EggPC-EggPG liposomer. For dette ble det lagt vekt på endring i størrelse (og størrelsesfordeling), turbiditet og zeta potensiale målt over 7 uker. Deretter ble det utviklet en metode basert på væskeopptak og masseendring som et resultat av væskeopptaket. Masseendringen ble målt av et Dynamic Vapour Sorption (DVS) Intrinsic instrument. For denne metoden ble mucin fra underkjeven på storfe i fosfatbuffer pH 6,8 brukt som en enkel modell for slimhinne. Liposomformuleringene ble testet med denne metoden. Til slutt ble liposomenes mukoadhessive egenskaper estimert ved å undersøke interaksjonen med mucin i løsning.

DVS Intrinsic metoden hadde potensiale for å bli brukt til å undersøke hydreringen av slimhinner, men det er fremdeles behov for justering av ulike parametere i analysen for å kunne sammenligne hydreringsevnen med mukoadhesiviteten til liposomer.

Abstract

Dry mucus in the oral cavity is uncomfortable for patients and can lead to other additional illnesses. Reduced or absence of saliva secretion, caused by the diseases, side effects of drugs, aging, or radiotherapy, can result in issues related to xerostomia and dental health. A system that could contribute to increased hydration of the mucus would reduce the discomfort for the patient. In addition, it would prevent the additional issues related to dry mucus. Liposomes and polymer-coated liposomes are proposed as promising systems for local application in the oral cavity. By using mucoadhesive polymers the residence time of the system at the site of administration in the oral cavity can be prolonged.

The overall aim of this thesis was to prepare and characterise different polymer-coated liposomes and investigate the correlation between the mucoadhesive and potential hydrating properties of the liposomal formulations. Firstly, the polymer concentration necessary for complete coating of positively charged EggPC-DOTAP liposomes was determined using negatively charged pectins (HM-, LM-, and AM-pectin). Negatively charged EggPC-EggPG liposomes were coated with chitosan. The determination of the optimal polymer concentrations was based on the measurements of changes in the size and size distribution, transmittance, and zeta potentials. The stability of the samples was followed for a period of 7 weeks. Also, a method using a Dynamic Vapour Sorption (DVS) Intrinsic instrument based on the dynamic sorption/desorption of water and the change in the mass was developed. Mucin from bovine submaxillary glands in phosphate buffer pH 6.8 was used as model for mucosa in this method, and was dried at room temperature in the aluminium pans. The liposomal formulations obtained from the first part of the study was analysed by the newly developed method. In addition the mucoadhesive properties of the formulations were estimated based on the interaction between the liposomal formulations and mucin in solution.

The DVS Intrinsic method showed potential in investigation of hydration abilities. However, there is still a need for more tuning of the instrument to be able to fully investigate the correlation.

Key words: oral mucosa; hydration; liposomes; polymer-coating; DVS Intrinsic

Table of content

List of Figures

List of Tables

1. Introduction

1.1 Background

The salivary secretion in the oral cavity is crucial for the wellbeing and oral health. Saliva has several important functions including providing the antimicrobial effect and containing the proteins needed for the protection of the teeth and mucus, and facilitating the extraction of the taste out of food. In addition, it shows buffering properties against the acids produced by the dental plaque (1)

The reduced salivary secretion leads to dry oral mucosa, which can be damaging both for the oral health and general life quality. It results in a huge discomfort, bad breath, and chewing and swallowing difficulties. In progression, the illness can result in the oral candida infection. It can be caused by the organic changes in salivary glands, different diseases or drug treatment (2, 3).

Some of the options in the rehydration of oral mucosa are the saliva replacement products such as spray products which contain artificial saliva and chewing or taste stimulus that can increase the saliva secretion. Acupuncture has also been suggested. Drug treatments include pilocarpine which has been for the stimulation of saliva secretion. The use is best documented in the case of Sjõgrens syndrome and damages related to radiotherapy. One of the downsides of this treatment is the frequency of the drug administration, as the effect is short lasting and the high cost of the treatment (2, 3).

The local treatment of the oral cavity exhibit some limitations such as involuntary swallowing and tearing at time of mastication which can lead to a short residence time of the drug/dosage form. Adhesive plasters and tablets have proved to prolong the residence time, but have the drawback of disturbance of the normal function of the cells in the oral cavity (4). In addition, from a patient perspective, the plasters can be too uncomfortable leading to the compliance related issues.

Liposomes are proposed to be a promising choice of a delivery system for the local treatment in the oral cavity as the system would spread out on a large area without affecting the normal cellular function. The delivery system is also considered to be safe, non-immunogenic, non-toxic, and biocompatible (5, 6). However, liposomes exhibit some limitations. They are prone to the chemical degradation by hydrolysis of ester bonds and peroxidation of unsaturated acyl chains, physical aggregation and fusion. They have a low shelf-life and coalescence problems because of the reduction of surface charge over time (5, 7, 8). Those named limitations could be overcome by the surface modification of liposomes.

The use of mucoadhesive polymers may prolong the residence time in the oral cavity and protect the delivery system from degradation and removal. The polymers such as chitosan and pectin have been shown to exhibit mucoadhesive properties (9-12).

A Dynamic Vapour Sorption (DVS) Intrinsic has been used to measure the water content in human skin through measuring the change of the skin mass when the skin was exposed to cycles of relative humidity (RH). The instrument provides some potential to be used in the investigation of the hydration ability of different substances (13).

1.2 Aim of the study

The overall aim of this project was to prepare and characterise different liposomal formulations with the mucoadhesive and potentially hydrating properties, focusing on the oral mucosa

More specifically the aim was divided into four goals;

1. Prepare and characterise uncoated- and polymer-coated liposomes

2. Investigate the mucoadhesive potential of the liposomal formulations by the mucin-coating model.

3. Develop a new method for measuring the hydrating potential of the liposomal formulations

4. Investigate the hydrating properties of the liposomal formulations

A hypothesis was that liposomes would provide lipids and polymers able to bind water, and water from the core. Liposomes may be expected to spread out on the surface to which it is applied, thereby hydrating the oral mucosa.

2 Theory

2.1 Oral mucosa

2.1.1 Anatomy and histology

Budowick et al. (14) defined the oral cavity as a hollow space between lips and pharynx (Figure 2.1.1). The oral mucosa is classified in three types according to Nanci et al. (15): (1) Masticatory, (2) lining, and (3) specialized where the masticatory covers gingiva and hard palate. This part is covered by keratinized epithelium due to the constant pounding and tearing of food during mastication (chewing). The lining mucosa (not keratinized) is flexible, and the specialized mucosa on the tongue contains papillae and taste buds.

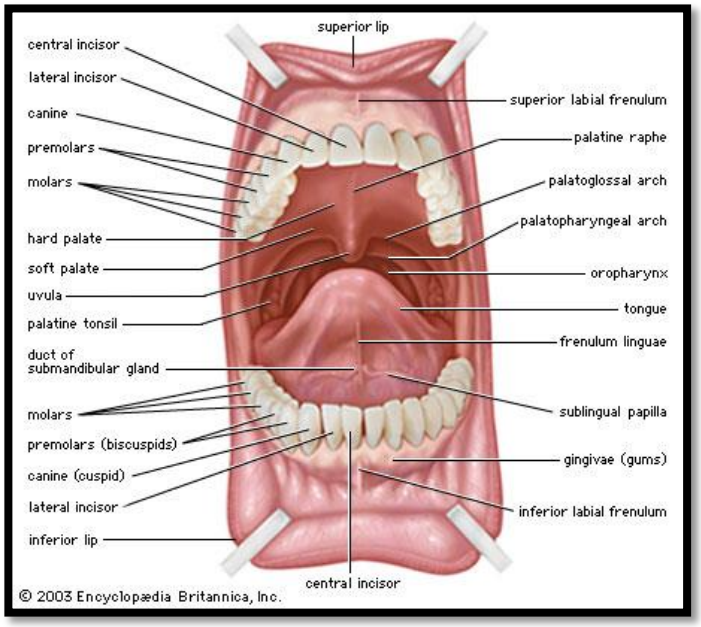

Figure 2.1.1 Anterior view of the oral cavity. © 2003 Encyclopædia Britannica Inc.

Salamat-Miller et al. described the three distinctive layers of the oral mucosa as the epithelium, basement membrane, and connective tissues (16). The epithelium lines the oral cavity, supported by the basement membrane, which in turn is supported by connective tissues (Figure 2.1.2).

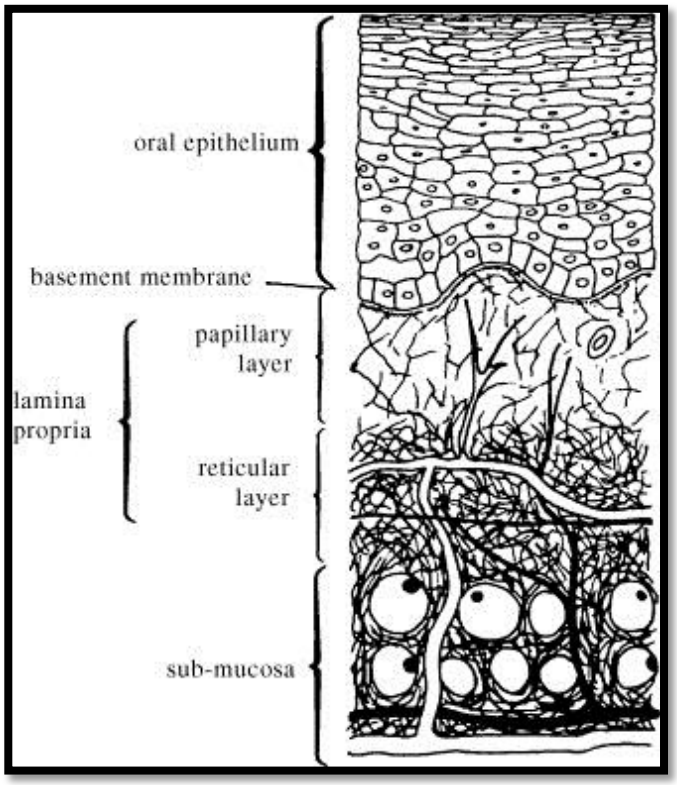

Figure 2.1.2 Anatomy of the oral mucosa (Salamat-Miller et al., 2005)

The oral cavity is covered entirely by the mucus, a gel-like secretion which contains mostly water-insoluble glycoproteins. The mucus is bound to the apical cell surface and acts as a protective layer to the cells below. Up to 5 % of its constituents are the water-insoluble glycoproteins; 95-99 % is water. However, this composition can vary based on the origin of the mucus secretion in the body. Other important components of the secretion are mucin which facilitates mastication and swallowing, enzymes, electrolytes, other proteins, and nucleic acids. In addition to small salivary glands in the oral cavity, there are other salivary glands. The secretions from the different salivary glands have different properties; the small salivary glands produce viscous and mucin-rich secretion, while the bigger salivary glands outside of the digestive system produce less viscous secretion which can reach the oral cavity through canals.

2.1.2 Oral mucosal dryness

"Dry mouth" can be categorized as xerostomia and hyposalivation. Xerostomia is perceived as the general feeling of dryness, while hyposalivation is a measurable reduction in the saliva secretion (3). For simplification, "oral mucus dryness" is defined as both conditions.

The oral mucus dryness is more prevalent among women than men. The illness can affect young people if they are using drugs exhibiting side effects related to oral dryness, but also elderly regardless of the medications. The prevalence is increasing with age (2). Table 2.1.1 summarises common causes of oral mucus dryness;

Table 2.1.1 Common causes of oral mucus dryness

Diseases	Drugs	Others
Salivary gland diseases	Antihypertensive drugs	Aging (Age dependent atrophy)
Autoimmune (Sjögrens syndrome)	Antihistamines	Dehydration
Infections (HIV, Hepatitis C)	Anticholinergic drugs	Radiotherapy dependent atrophy
Diabetes	Anti-Parkinson drugs	Environmental factors (dry air)
Psychogenic (Depression, anxiety)	Antidepressants	
	Analgesics	

Different formulations have been used for local treatment of the oral cavity, e.g. gargle products containing fluoride ions or disinfecting agents such as chlorhexidine, chewing gums, lozenge, sprays, dental gels etc. (Felleskatalogen.no).

The oral mucosa as the site of drug administration provides several advantages:

1. It is highly vascularized and easy reachable to administrate dosage forms,
2. Compared to other non-oral administration routes of drugs the oral cavity is an easier option for the patients and hence improve the compliance of the patients,
3. This route allows the avoidance of acidic hydrolysis in the gastrointestinal tract (GI) and the “first-pass” metabolism effect, and
4. The enzymatic activity in oral mucosa is lower as compared to mucosa in other sites of the body (16, 17).

However, the oral cavity does not have a uniform permeability property. The buccal membrane exhibits a relatively lower permeability as compared to other compartments of the oral cavity, i.e. the sublingual membrane (18, 19). This might cause a potential challenge in the choice of formulation. The sublingual membrane exhibits a higher permeability; in addition it is also highly vascularized. These properties make the site suitable for drugs with quick onset of therapeutic effect and are utilised in the sublingual melting tablets or spray in treatment of angina pectoris where quick onset of the effect is required (20).

In addition to the lower permeability, the buccal membrane has also a smaller surface area. Collins and Dawes et al. report on the total surface area available for drug absorption in the oral cavity to be 170 cm^2, of which approx. 50 cm^2 represents non-keratinized tissues, including the buccal membrane (21, 22). One must also consider the risk of loss of the dosage form when the patient is eating and drinking, hence the limits related to the administration of drug to the oral mucosa are both the risk of short residence time and involuntary removal.

Comparing the advantages and disadvantages, one can include the recent progress in delivery of a variety of compounds, i.e. peptides and proteins, development of delivery systems which can prolong residence time, and an increased potential for drug administration (17).

2.1.3 Mucoadhession

Mucoadhession is the adhesion of materials where at least one of them is a mucosal surface. Smart et al. (23) described the phenomena as the meeting of two surfaces, one of which is a mucus membrane, and adherence of these two to each other in a two stages event. The first stage is the contact stage, followed by an establishment of the adhesive interactions. Klemetsrud et al. (10) described mucoadhession as the attachment of particles to mucin e.g. natural or synthetic polymers. The authors described the different theories which have been proposed and include: the electronic theory, the adsorption theory, the wetting theory, the diffusive theory, and the fracture theory, respectively. A system able to adhere to the mucosal membrane and protect the drug from dilution, degradation and the "wash-off" effect would overcome the limits of short residence time.

For mucoadhession to take place there should be a close contact between the mucosal surface and the particle, followed by the connection between the two in the forms of entanglement of the surfaces and the particles chains, and ultimately the formation of bonds (24). As mentioned earlier, the mucus consists of, amongst other components, mucins which are responsible for the gel-like properties of mucus.

Mucin, large macromolecules with a complex and highly segregated structure exhibiting regions of heavy glycosylation interspersed with polypeptide regions with no or little glycosylation, has a molecular weight ranging from approx. 200 kDa to 20-40 MDa (25). At pH ~ 7 (physiological pH) mucin is negatively charged (10) and, due to the electrostatic interactions, the stronger interactions with positively charged molecules are to be expected.

Through investigation of a delivery system's ability to interact with mucin by a simple mucoadhession analysis method, one can estimate the mucoadhesive properties of the system. In some studies the interactions between mucin and other agents have been investigated through the determination of the changes in size and electrophoretic mobility (10), in other studies the adsorption of mucin to other particles has been investigated by studying depletion techniques, and the fraction remaining after exposure studies (26).

2.2 Liposomes

2.2.1 Introduction

Liposomes have been studied intensively since their discovery in the 1960s by Alec Bangham, who found that lipid vesicles were formed by hydration of a dry lipid film (27, 28). Until today over ten thousands of articles containing the word "*Liposomes"* have been published. The enthusiasm of scientist to explore new strategies around liposomes can be illustrated by the numerous patents that have been issued related to liposomes (29). The application of liposomes as a drug delivery system (DDS) started with the investigation of Gregoriadis and colleagues early in the 1970s, but the development of the first liposomal marketed product took longer time than expected (29, 30).

Liposomes are biodegradable, non-toxic, and can readily be prepared in a large scale. Because of their biocompatibility, liposomes were perceived as drug delivery systems for intravenous route, but they have shown to be useful for other routes of drug administration as well, i.e. ocular, (trans)dermal, and pulmonary route (31, 32). For dermal and transdermal applications, liposomes can be used as;

1- Solubilizing matrix for poorly soluble drugs,
2- Local depot for the sustained release of dermally active compounds,
3- Penetration enhancer via improved hydration of stratum corneum, and
4- Rate-limiting controlled transdermal delivery systems (29, 31)

Liposomes as DDS have shown promising potentials. They are applied in the diagnostic imaging of tumours, cancer chemotherapy, antimicrobial therapy, as adjuvants for vaccines, the vectors for gene transfer, enzyme and hormone replacement therapy, in the treatment of ophthalmic disorders and as cosmetic agents for the delivery of moisturizers and anti-aging agents to the skin (33, 34).

The advantages of liposomes can be summarised as following:

- They can provide controlled release of entrapped drugs, controlled vesicle residence in systemic circulation or other compartments of the body, and enhanced uptake by targeted cells.
- Their properties such as size, composition, surface charge, fluidity, ability to incorporate drug or carry ligands specific for the cell surface gives them the potential to be put in production of optimal formulations for clinical use (34).
- Those liposomes composed of natural lipids especially, are biodegradable, biologically inert, shown to have limited immunogenic and intrinsic toxicity as the main constituents are phospholipids (5, 35, 36).

Some products on the Norwegian market containing liposomal formulation are the antifungal amphotericin B in Ambisome®, the cytostatic doxorubicin Caelyx® and Myocet®, and hepatitis C vaccine Epaxal® (http://www.felleskatalogen.no/medisin/sok?sokord=liposome (21.02.2013, 16:48))(20).

2.2.2 Characteristics of liposomes

Liposomes are spherical vesicles which contain one or more lipid bilayers. They can be classified based on their size and number of bilayers; small unlilamellar vesicles (one bilayer) or oligolamellar vesicles, large unilammelar vesicles and multilamellar vesicles. In respect to their size, liposomes can vary from as low as 40 nm to up to 50 µm (37).

The properties of the lipids, the conditions in the environment used for their preparation, and the method of preparation, are all reported to determine their properties (8). Liposomes can be made from a wide range of phospholipids based on the desired properties for their destined administration.

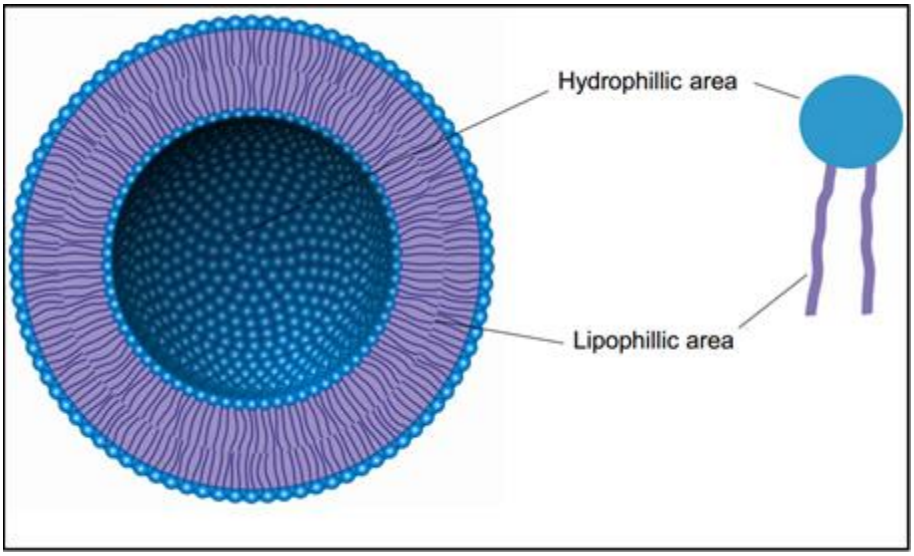

Figure 2.2.1 Illustration of liposome structure and phospholipid.

2.2.3 Preparation methods

The phospholipid that is most widely used in liposome preparation is phosphatidylcholine (PC)(38). It can be used alone or in a combination with other lipids, e.g. phosphatidylglycerol (PG), 1,2-dipalmitoyl-3-trimethyl-ammonium propane (DPTAP), phosphatidylserine (PS) etc. (7, 28, 38, 39).

The most common method to prepare liposomes is based on the hydration of the lipid followed by agitation using either a sonicator, high-shear propeller, or a homogenizer. To reduce the size, the process is followed by the filtration through a high-pressure membrane extruder (40, 41).

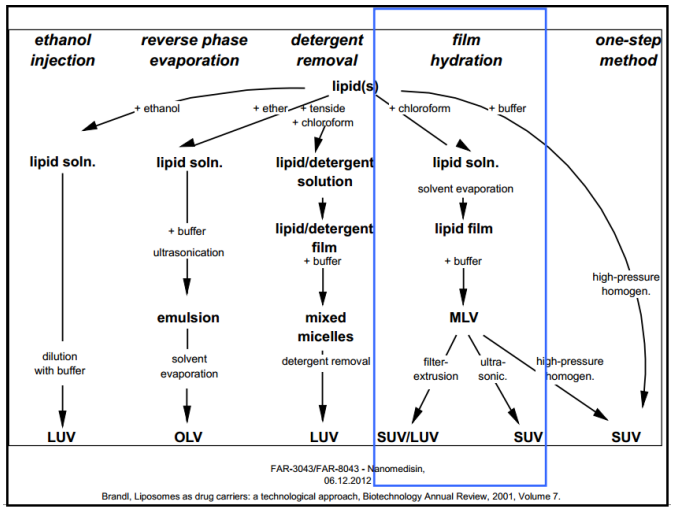

Figure 2.2.2 Overview of the preparation methods for liposomes. Marked with blue square: the approached conducted in this study (42).

As shown in Figure 2.2.2, the liposomes were prepared using the film hydration method, where the lipids were dissolved in chloroform, followed by the solvent evaporations step. A hydrations step of the lipid films facilitated the conformational change of the lipid films to create vesicles in different sizes. A finishing step of the filter extrusion controls the size and lamellarity of the liposomes. Entrapping the drug or agent in liposomes can be done in either a passive or active manner. Passive loading of the required component into the liposomes can be done during the formation of the liposome or in a stage where the liposome is at its structural weakest point (43). The active loading of the components into the liposomes can be based on the pH gradient (44) or metal ions (43).

2.2.4 Stability issues of liposomes

The stability of liposomes can possess a challenge in pharmaceutics. They are prone to the the chemical degradation, by hydrolysis of ester bonds and peroxidation of unsaturated acyl chains, physical aggregation and fusion depending of the bilayer composition (7). They have also been reported to have a relatively short shelf-life (5). Taylor et al. (8) highlighted the issues of the leakage of encapsulated drug over time; Gregoriadis et al. (45) discussed the possibilities of coalescence of liposomes because of the reduction of the surface charge. Liposomes are also prone to disruption or coalescence when put under mechanical forces such as mixing (37).

2.3 Coating of liposomes with polymers

The surface of liposomes could be modified in a way that would:

- Prevent the uptake through recognition and removal by the reticuloendothelial system (RES) and prolong the circulation time in blood (by the use of polyethylene glycol (PEG)) and increase *in vivo* protection from degradation
- Sterically stabilise liposomes to prevent coalescence and disruption and control the release of the entrapped drug so that the drug is released only after being triggered by a stimulus like pH, light, temperature, and enzyme
- Prolong the shelf-life (46).

For the purpose of improving the stability of liposomes, liposomes can be coated with polymers either during the formation of the liposomes or by the addition of polymers to already formed liposomes. Chemically, a polymer can be grafted onto the liposomal surface (e.g. PEGylation). Physically, a polymer can be added to the liposomal surface with electrostatic interactions (i.e. pectin, chitosan) or hydrophobic interactions (N-isopropyl acrylamide (*NIPAAm*) (47)). Fukui et al. (48) coated liposomes with polymers by the use of the layer-by-layer deposition technique, in their study a cationic polymer was deposited onto a negatively charged liposomal surface followed by addition of anionic dextran sulphate or deoxyribonucleic acid.

The result of polymer-coating of liposomes is the changes of the characteristics of the system. The size is expected to increase because of the addition of extra layer onto the liposome structure (39). Filipović-Grčić et al. (9) reported that coating of fluoresceinisothiocyanato-*dextran* (FICT-dextran)-containing liposomes originally in the size range of 250-280 nm, resulted in liposomes in the range of 300-330 nm

In addition, the surface charge of the system changes depending on the constituents of the liposome, their characteristics (charge) and the type of polymers used. A simple way to monitor this change is by measuring the zeta potential. Having a positively charged liposome, coated with

a negatively charged polymer would theoretically result in a change of the charge depending on the concentration and the ratio between the polymer and the liposome (5, 37, 39, 48, 49).

The coating of the liposomal surface with a hydrophobically modified polymer could potentially be detected by differential calorimetric scanning. A change in the phase-transition temperature could indicate the interaction/penetration of the hydrophobic moiety in the liposomal membrane (47).

Laye et al. (37) have shown that insufficient coating concentration of the polymer will lead to aggregation and sedimentation, while too high polymer concentration will lead to desorption of the polymer from the liposomal surface as a result of osmotic pressure. When a polymer is added to the liposomes, flocculation may occur. This might be due to the bridging or depletion flocculation. The surface interaction parameter Xs is a measure of the net interactions between the liposomal surface, the polymer, and the medium (solvent). When Xs has a positive value, the adsorption of the polymer onto the liposomal surface is favoured. However, if Xs is less than a certain critical value, the adsorption of the polymer onto the liposomes is unfavourable.

If too low concentration of the polymer is used and insufficient to yield full surface coverage, a polymer chain may adsorb onto the two discrete liposomes, causing aggregation. This is called the bridging flocculation. In the case of the Xs having a value lower than the critical value, the centre of mass of the polymer coil is displaced from the interface, leading to a polymer-depleted zone (50).

Advantages of polymer-coating of liposomes - Improved absorption with mucoadhesive polymers

Wu et al. (51) showed in a study on mice that chitosan-coated liposomal insulin after oral administration could enhance enteral administration of insulin. This was also shown by Takeuchi et al. (52) and attributed to chitosan's mucoadhesive properties. Other researchers have shown that Eudragit ®RS also improves the pharmacokinetics of insulin in rats (53). Karn et al. (5) showed in an *in vitro* study that Eudragit® S100 was superior to chitosan in respect to mucoadhession. Pectin has also been reported to exhibit mucoadhesive properties (11).

2.3.1 Pectin

Pectin, a polysaccharide, is the major component of cell walls in plants. It plays an important role in the cell walls in respect to the growth and development of the plant. Pectins are widely used in the food industry because of their gelling properties, hence they are regarded as safe, and are reported to have some positive physiological effects (54). In addition they are used as replacement for fat and sugar in food and beverages (55, 56).

The polysaccharides have also been used in pharmaceutics as an excipient, for nasal drug delivery and for specially designed drug release profiles (57). They are used as components of mucoadhesive plasters, and in preparation of biodegradable films and foams (11, 55, 58). The United State Food and Drug Administration (FDA) and World Health Organisation (WHO) consider pectin as safe and have approved its use in food and pharmaceutical formulations (56).

Pectins are in general water soluble, depending on the type of pectin and ambient pH as their pKa value is approx. 3. Their structure consists of 3 domains;

1. Homogalacturonan (HG) – this domain consist of polygalactunorin acids
2. Rhamnogalacturonan I (RGI) – this domain consist of repeating disaccharides of galacturonic acids and rhamnose to which different glycan chains are attached
3. Rhamnogalacturonan II (RGII) – this domain has a backbone of polygalacturonic acids and complex sugar side chains attached.(59)

The functional galacturonic acids can be methoxylated, or amidated (Figure 2.3.1). The degree of methoxylation (DM) or amidation (DA) is widely used to characterize pectin. In their natural form pectins are found as high methoxylated pectin (HM-pectin) with a DM around 70 %. Low methoxylated pectin (LM-pectin) has a DM of approx. 35 %, and amidated pectin (AM-pectin) has a DM around 30 % and DA around 20 % (11).

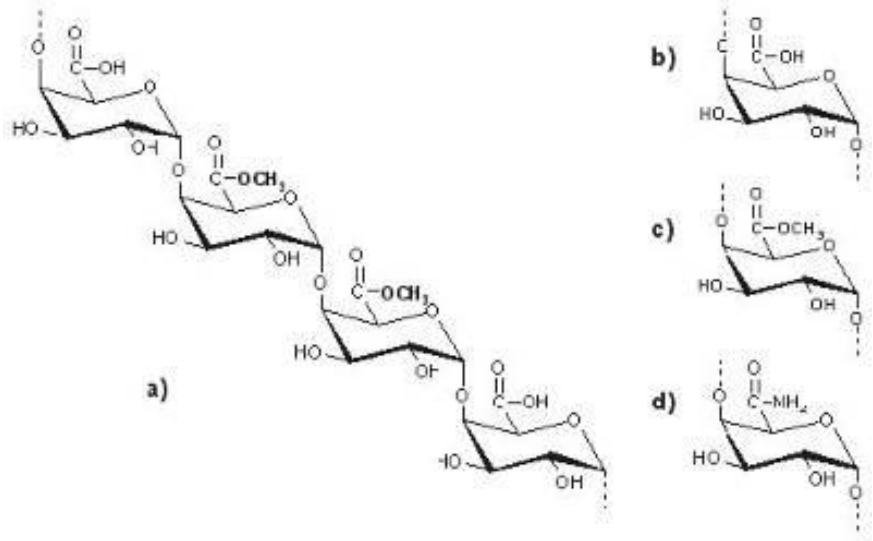

Figure 2.3.1 Structural formula of pectin; a) repeating fragment of pectin molecule and functional groups: b) unsusbstituted galacturonic acid; c) methoxylated; d) amidated (60)

Pectin has been shown to have a great potential in mucoadhession (10, 11). Researchers discussed the need for interacting functional groups i.e. carboxyl or hydroxyl groups, to exhibit the mucoadhesive properties. In addition the charge (anionic, cationic) is also reported to be important for mucoadhession (61). The mucoadhesive properties of pectin are somehow difficult to categorise as the results from several studies are conflicting. Hagesaether et al. (62) discussed the progress of the potential of pectin as mucoadhesive from its start to the confirmed stage. The authors also described the reasons for the conflicting results which might contributed to the different methods of analysis, and complexity of mucoadhession as the process including several parameters such as the flexibility of the polymer chains, ionic interactions, configuration of the polymers and optimal degree of hydration.

One can postulate that since HM-pectin has higher DM leading to less degree of free carboxylic acid functional groups, it would provide less electrostatic interaction with mucin than LM-pectin with a higher degree of the free acids, and that AM-pectin would be in the same area as LM-pectin when it comes to ionic strength and free acid groups.

2.3.2 Chitosan

Chitosan (β (1,4) 2-amino-2-deoxy-D-glucose) is a natural cationic polysaccharide derived from deacetylation of chitin. Chitin is the main component of shells of crabs, shrimps and krill. This makes chitosan one of the most abundant polymers found in nature, and regarded as safe because of their biocompatibility (non-toxicity, biodegradability) (9, 12). The use of chitosan is mostly in pharmaceuticals, cosmetics, agricultural materials and food products. Chitosan is extensively studied in the context of drug delivery, including as liposome-stabilising component (12, 52, 63, 64).

Chitosan is linearly structured with amino groups readily available for chemical reactions. The polymer consists of D-glucosamine and N-acetyl-D-glucosamine units linked together in a random manner (62). For the characterisation of chitosan, the degree of de-acetylation (DD) and molecular weight appear to be crucial. DD of commercial chitosan is between 70-95 %, and the molecular weight between 10 000 – 1.000 000 Da. The amino groups have a pKa value between 5.5 -7.0 depending on DD. The polymer is reported to be soluble at low pH (~4) (62).

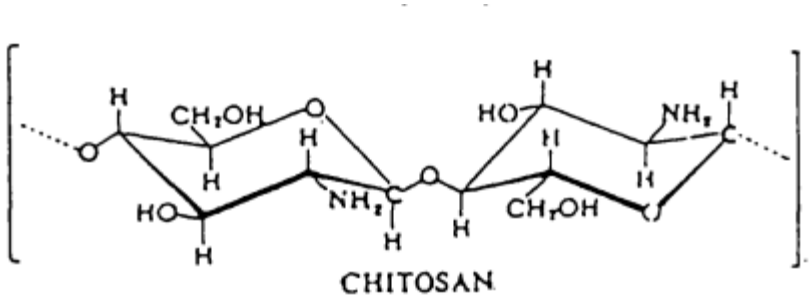

Figure 2.3.2 Structural formula of chitosan (12)

Chitosan is reported in several studies to have mucoadhesive properties and exhibiting huge potential for use in drug delivery. The polymer has been compared to other agents, such as carbopol, hyaluron, pectin etc. and has shown better mucoadhesive properties (5, 65). Its mucoadhesive properties might be result of its high charge density, and hence strong electrostatic interactions with negatively charged surfaces.

2.4 Liposomal formulations in topical application as moisturisers

Liposomes, as described in chapter 2.2, have been investigated for delivery of drugs in different areas. Also in topical application, liposomes have received increased attention for the delivery of drugs such as antibiotics and anti-inflammatory drugs. The ability to deliver their content into the superficial and deep layers of the skin has made them important in the cosmetic industry (6, 43, 66). Lasic et al. (66) argued that even without any active ingredients liposomes could deliver moisture in form of either lipids that could bind water, or water content in the liposome core. In addition, liposomes could be loaded with molecules that are known to bind water such as sugars.

Liposomes applied to the skin would also provide a barrier over the surface of the skin to hydrate skin layers and protect and inhibit water desorption from the skin. Egbaria et al. (31) studied the effect of liposomal treatment of skin prior to the topical administration of corticosteroids and showed a significant response in the liposomal pre-treated arm. The study suggested that the applied phospholipids either supplement the lipid content of the skin, or provide a film that may promote hydration of the stratum corneum.

Although liposomes have been investigated for topical application, few studies have explained the mechanism of the liposomal action as moisturisers. In addition, there is a lack of papers on liposomes' ability to hydrate mucus, although there are some to be found in the field of vaginal mucosa (68).

2.5 Dynamic Vapour Sorption (DVS) Intrinsic – Instrument description

The DVS Intrinsic measures accurate changes in the mass of the sample, while the sample sorbs controlled concentrations of water vapours in the surroundings air. The sample is loaded into a chamber where air with known percentage of relative humidity (RH %) is passed over the sample at a controlled and known flow rate and temperature. The mass readings then reveal the sorption/desorption behaviour of the sample. The basic principle of this method is that samples that absorb more water will weigh more; hence the higher mass readings, the higher amount of water in the sample. And when the RH % is decreased, leading to the evaporation of water, the higher mass readings during this time indicate the ability to hold on to water molecules.

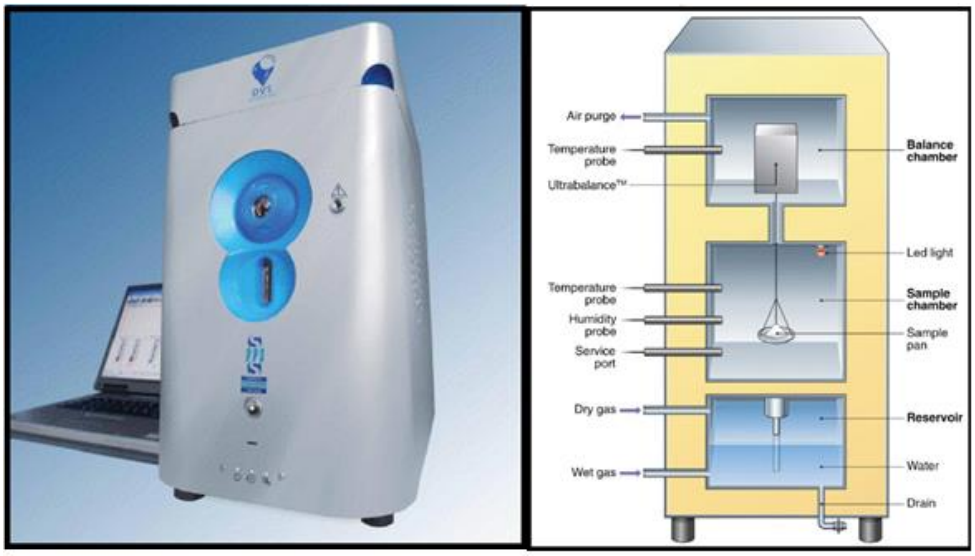

Figure 2.5.1 To the left: The Dynamic Vapour Sorption (DVS) Intrinsic, to the right; Schematic illustration of a DVS Intrinsic interior. Http://www.smsuk.co.uk

The instrument contains a microbalance capable of measuring the changes in mass lower than 1 part per million. This type of recording can provide suitable measurements of vapour sorption, and hence the hydration ability and can take from minutes to days.

Johnsen et al. and Martinsen et al. showed that the content of water in skin and also the time constant of desorption mechanism could be calculated by following the mass of the sample over

time, and from there calculate the time constant of the change (how long time it takes for a change to take place) (13, 69-71).

The time constant, in this area of interest, is calculated based on the mass change measurements that are done for a chosen sample. It reports the time a system uses to decrease/increase approx. 63 % of its total mass change given that this change follow an exponential pattern. This time constant, in the current study, might provide information on the ability of the liposomal formulations to induce the changes in RH %, and therefore their abilities to hold on to moist.

3 Materials and Instruments

3.1 Materials

3.1.2 Lipids

Phosphatidylcholine from egg (EggPC), LIPOID ®, Lipoid GmbH (Ludwigshafen, Germany)

Phosphatidylglycerol from egg (EggPG), LIPOID ®, Lipoid GmbH (Ludwigshafen, Germany)

1,2- Dioleoyl-3-trimetyl-ammonium-propan (DOTAP), AVANTI®, Avanti-Polar-Lipids Inc. (Alabaster, USA)

3.1.2 Polymers

Amidated pectin (AM-Pectin), Genu® pectin LM102 AS (Lille Skensved, Denmark) Mw = 9.6 $x10^4$ degree of methoxylation (DM) 30.0, degree of amidation (DA) 19.0

High methoxy pectin (HM-pectin) Genu® pectin LM150 USA-SAG, CPKelco (Grossenbrode, Germany), degree of methoxylation (DM) 70.2 %, Mw= 1.1 x 10^5

Low methoxy pectin (LM-Pectin), Genu® pectin LM12 CG-Z, CPKelco (Grossenbrode, Germany), degree of methoxylation (DM) 34.8 %, Mw = 7.6 x 10^4

Mw, DA, and DM of pectin is taken from the earlier paper by Smistad et al. (39)

Chitosan; Protosan UP Cl 213, Novamatrix, FMC Biopolymer AS (Sandvika, Norway) degree of deacetylation (DD) 83 %, Mw 3.1 x 10^5 (according to the producers).

Mucin from bovine sub maxillary glands (BSM), Type I-S, Sigma (Sigma-Aldrich, St. Louis, USA)

3.1.2 Buffer salts

Sodium di hydrogen phosphate monohydrate (NaH_2PO_4 x H_2O), analytical grade, Merck (Darmstadt, Germany)

Disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O), analytical grade, Merck (Darmstadt, Germany)

3.1.2 Solvents

Chloroform ($CHCl_3$), analytical grade, Merck kGaA (Darmstadt, Germany)

3.2 Solutions

3.2.1 Phosphate buffer 5mM, pH 6.8.

Sodium dihydrogen phosphate monohydrate (NaH_2PO_4 xH_2O) (I), 689. 95 mg, was dissolved in 1 L purified water. Disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O) (II), 889.95 mg, was dissolved in 1 L purified water. The two solutions (I+II) were mixed in the 2:1 ratio. The pH was adjusted to 6.8. The end solution was filtered through the 0.2 µm filters.

3.2.2 Lipid solutions

EggPC 10 mg/ml

EggPC, 250 mg was dissolved in 25 ml of chloroform.

EggPG 2 mg/ml

EggPG, 50 mg was dissolved in 25 ml of chloroform.

DOTAP 2 mg/ml

DOTAP, 50 mg was dissolved in 25 ml of chloroform.

3.2.3 Mucin solutions

BSM 3 % (w/w)

One gram of phosphate buffer 5 mM pH 6.8, and 30 mg of BSM was mixed. The mixture was stirred magnetically until the next day. On the second day the mixture was filtered 5 µm (Versapore® membrane filter).

BSM 2 % (w/w)

One gram of phosphate buffer 5 mM pH 6.8, and 20 mg of BSM was mixed. The mixture was magnetically stirred until the next day. On the second day the mixture was filtered 5 µm (Versapore® membrane filter).

BSM 1 % (w/w)

One gram of phosphate buffer 5 mM pH 6.8, and 10 mg of BSM was mixed. The mixture was magnetically stirred until the next day. On the second day the mixture was filtered 5 µm (Versapore® membrane filter).

BSM 0.2 % (w/w)

Twenty five g of phosphate buffer 5 mM pH 6.8, and 50 mg of BSM was mixed. The mixture was magnetically stirred until the next day. On the second day the mixture was filtered 5 µm (Versapore® membrane filter).

3.2.4 Polymer solutions

Pectin solutions

Mixtures of 70 ml phosphate buffer 5 mM, pH 6.8, and 350 mg of the three different types of pectins (HM-, LM-, and AM-pectin, respectively) were prepared. The mixtures were put on magnetic stirring overnight. The following day the solutions were filtered 2 µm (Nucleopore® membrane filter), and the pH was adjusted to 6.8 ± 0.1.

Series of dilutions with phosphate buffer pH 6.8 were done to obtain the following concentrations of pectin:

0.5, 0.2, 0.1, 0.05 and 0.01 % (w/v)

Chitosan solutions

A mixture of 20 ml phosphate buffer 5 mM pH 6.8, and 32 mg chitosan was prepared. The mixture was put on magnetic stirring overnight. The following day the solution was filtered 2 µm (Nucleopore® membrane filter), and the pH was adjusted to 6.8 ± 0.1.

Series of dilutions with phosphate buffer pH 6.8 were done to prepare the following concentrations of chitosan:

0.16, 0.12, 0.08, 0.04 and 0.01 % (w/v)

3.3 Instruments and Equipment

3.3.1 Instruments

In preparation

Heidolph W 2001 rotavapor, Heidolph Instruments GmbH & Co. KG (Kelheim, Germany)

LIPEX™ extruder, Lipex Biomembranes, Northern Lipids Inc. (Vancouver, Canada)

Watson-Marlow 520S IP3 pump (Cornwall, United Kingdom)

Christ Alpha 2-4 freeze drier, Christ (Osterode am Harz, Germany)

In characterisation

Mass change determination; Dynamic Vapour Sorption (DVS) Intrinsic, Surface Measurement Systems (SMS) Ltd, (London, United Kingdom)

Size measurements; Coulter® N4 Plus submicron Particle sizer, Coulter Corporation (Miami, USA)

Zetasizer Nano ZS, Malvern Instruments Ltd. (Worcestershire, United Kingdom)

Transmittance; Ultrospec II Spectrophotometer, LKB Biochrom

Lupe: Leica DFC 320 (Q Win) microsystem (Sollentuna, Sweden)

3.3.2 Miscellaneous

Analytical Weights

Analytical weight; Mettler-Toledo DeltaRange ® AG 204, and PB3002 DeltaRange ®

pH-Meter

Mettler Toledo MP 220 pH-meter, Mettler-Toledo, LLC (Columbus, USA)

Filters

200 nm membrane filter, Nucleopore®, Costar Corp. (Cambridge, USA)

2 µm membrane filter, Nucleopore® Costar Corp. (Cambridge, USA)

5 µm membrane filter, Versapore, PALL, Pall corp. (Exton, USA)

Surface substrates

Benchkote paper from Whatman ®

Perkin-ELMER DSC sample pan cover, (Waltham, USA)

Perkin-ELMER DSC sample pan (30 µL), (Waltham, USA)

Perkin-ELMER DSC sample pan (50 µL), (Waltham, USA)

Cuvettes for analyses

Plastic cuvettes, Polystyrol/polystyrene, SARSTEDT AG & Co. (Nümbrecht, Germany)

4 Methods

4.1 Preparation of liposomes

Egg-PC (90 mol %)-DOTAP (10 mol %), 3 mM, 50 ml

From the lipid solution prepared (See chapter 3.2.2): 10.395 ml of EggPC (10 mg/ml) solution was mixed with 5.239 ml of DOTAP (2 mg/ml) in a round flask. The mixture of the two lipid solutions was put on a rotary evaporator (Heidolph W 2001) to remove the solvent following a standard operation procedure (SOP) and obtain a lipid film. The film was left on a vacuum pump for additional evaporation of the solvent traces. On the following day, 10 ml of phosphate buffer 5 mM (pH 6.8) was added to the lipid film and the flask put on a rotary stirring for 10 minutes at 90 rpm, followed by the storage at room temperature protected from light for 2 hours. Afterwards, the flask was stored in a fridge at 4 °C. On the final day of the preparation, liposomes were extruded through 200 nm filter according to the SOP for the instrument (LIPEX™ extruder).

The liposomes were extruded 10 times. After the extrusion, liposomes were diluted with phosphate buffer to gain 50 ml liposomes with the concentration of 3 mM.

EggPC (90 mol %)- Egg-PG (10 mol %), 3mM, 50 ml

For these liposomes, 10.395 ml of EggPC 10 mg/ml and 5.857 ml EggPG 2 mg/ml were mixed together in a round flask. The other preparation steps were the same as described above.

4.2 Coating of liposomes with polymers

Liposomes were coated with polymers by adding 1 ml liposomes to 4 ml polymer solution. The polymer solutions were put on magnetic stirring with such a speed which caused a swirl in the solution, and the liposomes were added in a drop wise manner with the aid of pump (Watson-Marlow 520S) and the pumping speed of 20 rpm (6.8 ml/min). After the addition of liposomes, the samples were stirred for 5 additional minutes, and stored in the fridge at 4 °C. For each of the polymer concentration, 3 parallels were prepared. The pectin solutions were used to coat the positively charged liposomes (EggPC-DOTAP), while chitosan was used to coat the negatively charged liposomes (EggPC-EggPG).

4.3 Liposome characterisation methods

4.3.1 Size and size distribution

Measurements of particle size were done on coulter N4 Plus Submicron Particle Sizer with the aid of photon correlation spectroscopy (PCS). The measurements were conducted at an angel of 90°, and a temperature of 25 °C. The viscosity and the dielectric coefficient of water were used, which had the respective values of 0.89 cP and 79 at 25 °C. The intensity interval in which the concentration of the sample should be was $5.0 \times 10^4 – 1.0 \times 10^6$.

The temperature equilibration time was set to 15 minutes on the beginning of the measurement and was changed to 10 minutes after the first sample. The results from the measurements were analysed by CONTIN-analysis, enabling us determine the size of both even distributions and polydisperse samples.

For size measurements the plastic cuvettes were prewashed with phosphate buffer. After flushing of cuvettes, 1 ml filtered (0.2 µm) phosphate buffer (pH 6.8) was added to the cuvettes followed by the addition of sample (0.1 ml). All samples were controlled visually for dust, contamination and precipitation before analysis.

4.3.2 Zeta potential

The zeta potential of the samples was determined as the electrophoretic mobility at 25 °C, using the Malvern Zetasizer. Five measurements were done for each sample. The Henry equation was applied for the determination from the electrophoretic mobility;

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

Where the viscosity (η) and the dielectric coefficient (ε) for pure water were used and the Smuluchowski approximation was applied ($f(Ka)$= 1.5).

For these measurements, plastic cuvettes were prewashed with phosphate buffer (pH 6.8), filled with 0.75 ml phosphate buffer (pH 6.8) and 75 µl of the test sample, respectively. All the samples were controlled visually for dust, contamination and precipitation before analysis.

4.3.3 Transmittance

The transmittance of the prepared samples was measured at 25 °C, λ = 550 nm, using the ULTROSPEC II Spectrophotometer. For this, 1 ml of the samples was analysed against phosphate buffer as blank solution. All samples were controlled visually for dust, contamination and precipitation before testing.

4.4 The interaction of liposomes with mucin in a solution

Both the uncoated- and the polymer-coated liposomes were mixed with the mucin solution (1:1, v/v) by adding 2 ml of mucin solution to 2 ml sample in a drop wise manner during magnetic stirring. The stirring was conducted with a rotation which caused a swirl which could be seen in the solution. The speed of the drop-wise addition of mucin was set to 20 rpm (6.8 ml/min). The procedure was performed by the same instrument as for coating of the liposomes with polymers (Watson-Marlow 520S). After the addition of BSM, the flask was stirred for 5 additional minutes before it was stored at room temperature for 2 hours before characterization. Three parallels of each sample were prepared.

4.5 Preparation of mucin films

On benchkote paper

Circles of paper were cut with a diameter of 0.9 mm. The BSM solution (3 %:10 µl) in phosphate buffer pH 6.8, were applied to the benchkote papers. The papers were dried overnight in room temperature, and examined the next day using the Leica DFC 320 (Q Win) lupe.

On aluminium foils

Circles of aluminium foils (0.9 mm in diameter) were prepared and treated in the same way as described above. The samples were dried in room temperature and visually examined the next day.

In aluminium pans (50 µl) and covers

The pans (50µl) and covers were filled with 50 µL and 10 µL, respectively, of 3 % (w/w) BSM in phosphate buffer pH 6.8. They were left to dry at room temperature and examined with Leica DFC 320 (Q Win) lupe the next day.

4.6 Estimation of hydration capacity

4.6.1 Mass change determination

For the purpose of measuring the mass change using the DVS Intrinsic, aliquots of 15 µl of the sample was applied to a premade mucin film. The mucin film was made by adding 100 of µl 3 % (w/w) mucin to an aluminium pan and dried overnight at room temperature.

The sample was put on the "hang-down" pan in the chamber of the instrument. Environment temperature in the chamber was set to 25 °C, gas inlet pressure on 2.03 bar, and the mass measurement frequency was on 1 minute.

The procedure of the experiment was categorised into stages, which was in turn based on RH %. The duration of each stage depends on the stability of the measured mass, meaning that when the instrument detected stability of mass the stage was finished and the instrument went on to the next stages. A demand was put on the instrument for detection of stable mass readings, which was mass change less than 20 parts per million/minute (ppm). In case a sample did not meet these requirements, a maximum stage time frame limit was put to 600 minutes per stage to ensure effectiveness of the analysis.

4.6.2 Time constant determination

A Programme Wolfram Mathematica 9.0 (Wolfram research, Oxfordshire, United Kingdom), which was kindly provided by prof. Ø.G. Martinsen, called "Time constant of desorption data" was used to analyse the data obtained from the mass change measurements.

The desorption time constants were determined by fitting an exponential time course to selected date, Y= $a + b * Exp\ [-c * t]$ utilizing Findfit, a "curve-fitting tool" in the programme. A course proportional to Exp [-c*t] would yield the time constant τ equal to 1/c. The desorption time

constant in this case is described as the decrease of mass of the samples from the beginning of the 30 % RH stage and 30 following minutes (Figure 4.6.1).

The programme finds numerical values (a, b, and c) that give the best fit to the selected data from the mass change measurements. For more closely details refer to wolfram home page → Mathematica -> Findfit (72) and (13, 73).

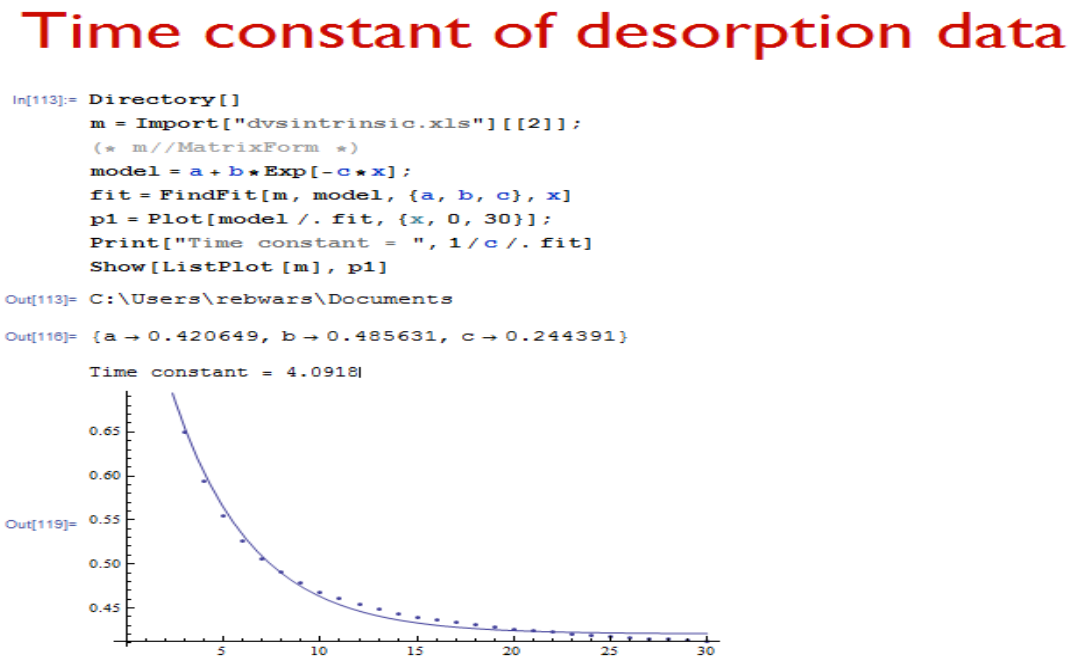

Figure 4.6.1 Print screen shot of the programme used for calculation of the time constant. The data shown are from 0.05 % HM-pectin-coated EggPC-DOTAP liposomes.

To calculate the time constant of the analysis, 30 specific mass measurements from each sample were elected. These 30 measurements were the first 30 after the change of the stage. The 30 measurements were ordered chronologically, and the lowest whole number was subtracted from them. A simple explanation is shown in Figure 4.6.2.

H	I	J	K	L	M	N	O	P	Q
1	38.6401	0.6401		Column H	1 to 30				
2	38.5589	0.5589		Column I	Measurements from the start of RH % stage of 30.				
3	38.4923	0.4923		Column J	The lowest whole number of these measurements is 38.				
4	38.4476	0.4476			Subtracting the number 38 from the measurements.				
5	38.4163	0.4163							
6	38.3942	0.3942							
7	38.377	0.377							
8	38.3637	0.3637							
9	38.3527	0.3527							
10	38.3443	0.3443							
11	38.3378	0.3378							
12	38.3323	0.3323							
13	38.3287	0.3287							
14	38.325	0.325							
15	38.3214	0.3214							
16	38.3183	0.3183							
17	38.3164	0.3164							
18	38.3146	0.3146							
19	38.3116	0.3116							
20	38.3096	0.3096							
21	38.3085	0.3085							
22	38.3067	0.3067							
23	38.3039	0.3039							
24	38.3028	0.3028							
25	38.3016	0.3016							
26	38.3007	0.3007							
27	38.3001	0.3001							
28	38.2978	0.2978							
29	38.2972	0.2972							
30	38.2968	0.2968							

Figure 4.6.2 Preparation of excel file for time constant calculation

4.7 Statistical analyses

ANOVA- one way between groups

In this analysis the difference between groups were investigated using the Minitab ® 16 statistical software (Minitab Inc., USA). A one way analysis of variance (ANOVA) was used followed by Tukey's Post hoc test using $p < 0.05$ as level of significance.

5. Experimental setup

5.1 Development of the method for determination of the hydration properties of liposomes

5.1.1 Choice of mucosa model

Prior to the analysis of the hydration abilities of the liposomal formulations, investigations were conducted in order to create a model resembling mucosa. For this purpose, the different surface substrates were analysed for their potential as a supporting bottom layer on which a mucin film could be applied (See chapter 4.5).

The next step in the study was to investigate which concentration of mucin solution had the best ability to form a film after evaporation of the solvent, which in this case was 5 mM phosphate buffer pH 6.8. Three mucin solutions, namely 3, 2, and 1 % (w/w) were prepared and tested. In the beginning, 50 µl of the mucin solutions were applied. After examination and in accordance to the user manual, the volume was increased to 100 µl, applied over two days to ensure suitable thickness of the film and full coverage of the surface substrate. Based on these experiments, the optimal combination of the surface substrates and the concentration of the mucin solution was obtained and utilized in the study.

5.1.2 Determination of experimental parameters for the DVS Intrinsic method

First approach

The aim was to investigate how the mass of a mucin film changes as a result of RH % in the instrument chamber and to estimate the time needed to reach the mass stability. For this purpose, a premade mucin film, pre-dried at room temperature the day earlier, was placed in the instrument chamber at 0 % RH. The RH % was changed stepwise (10 % at the time). This means that the analysis was performed as following: 0-10-20-30-40-50-60-70-80-90-80-70-60-50-40-30-20-10-0 (RH % stages)

Adjustments of the method

After taking into the consideration potential disruptions of the liposomal formulations when exposed to low RH %, the method was adjusted to include the RH % stages as shown below.

A. RH % was set to start at 90 %, and the next stage was put to 40 %. The rest of the factors were as mentioned in chapter 4.6.

B. RH % was set to start at 90 %, and the next stage was put to 30 %. In addition, the time frame limit was adjusted to maximum 800 minutes. The rest of the factors were as mentioned in chapter 4.6.

C. RH % was set to start at 80 %, and the next stage was put to 30 %. In addition, the time frame limit was adjusted to maximum 800 minutes. The rest of the factors were as mentioned in chapter 4.6.

In the methods A, B, and C, two different types of samples were analysed. These were 1- mucin film, 2- 15 µl phosphate buffer added to the mucin films.

5.2 Determination of the optimal coating concentration of the polymers

For determination of the optimal coating concentration of the chosen polymers, five different concentrations of the polymers were selected. Table 5.2.1 gives an overview of the type of polymers and their concentrations.

Table 5.2.1 Type of polymers and their concentrations for the study

Type of polymer	HM-pectin	LM-pectin	AM-pectin	Chitosan
Concentration of polymer	0.01 %	0.01 %	0.01 %	0.01 %
	0.05 %	0.05 %	0.05 %	0.04 %
	0.10 %	0.10 %	0.10 %	0.08 %
	0.20 %	0.20 %	0.20 %	0.12 %
	0.50 %	0.50 %	0.50 %	0.16 %

After the completion of coating, liposomal suspensions were analysed for size, zeta potential and turbidity. In addition, stability analysis was conducted through 7 weeks. The lowest polymer concentration resulting in the complete coating for each type of polymers were selected and further investigated in the context of the interaction with mucin in solution, as well as for estimation of the hydration abilities.

5.3 Estimation of liposomal mucoadhesive properties – the interaction with mucin in solution

For the estimation of mucoadhesive properties of the liposomal formulations, new batches of uncoated- and polymer-coated liposomes were prepared and mixed with 0.2 % (w/w) mucin solution as described in chapter 4.4. Size, zeta potential and transmittance were analysed prior to mixing and 2 hours after the mixing procedure. The ability of different liposomal formulations to interact with mucin was statistically evaluated using one way ANOVA-test followed by Tuckey's post hoc test by comparing both the size and zeta potential before and after the addition of mucin.

5.4 Estimation of the hydration properties of liposomal formulations

Hydration properties were estimated by applying the liposomal formulations to premade mucin films and measuring the mass change as described in Chapter 4.6.1. In Table 5.4.1 an overview of the tested samples is given.

Based on the results obtained from the mass change measurements, the time constants for the mass decrease was calculated, as described in Chapter 4.6.2, and compared as described Chapter 4.7.

Table 5.4.1 Overview of the conditions used to determine the hydration abilities of liposomes using the DVS Intrinsic

Sample	Volume added to mucin films	Number of parallels
Dried mucin film	-	2
Phosphate buffer (pH 6.8)	15 µl	2
EggPC-DOTAP liposomes	15 µl	2
EggPC-EggPG liposomes	15 µl	2
0.05 % HM-pectin-coated EggPC-DOTAP liposomes	15 µl	3
0.10 % LM-pectin-coated EggPC-DOTAP liposomes	15 µl	3
0.05 % AM-pectin-coated EggPC-DOTAP liposomes	15 µl	3
0.04 % Chitosan-coated EggPC-EggPG liposomes	15 µl	3

6 The method for determination of the hydration properties of liposomes

6.1 Choice of model for mucosa

Testing the surface substrates as support for mucin films

Benchkote paper

The paper was difficult to analyse both prior and after the application of the mucin solution. Also when the BSM solution was applied to the papers, the medium seemed to overflow the paper. Since the size of the papers could not be increased because of limited space of the pan within the DVS Intrinsic instrument (Figure 2.5.1), the Benchkote paper was not further used in the study.

Aluminium foils

Application of 3 % BSM solution on aluminium foils, and drying at room temperature overnight, resulted in a mucin film (Figure 6.1.1). However, the foils proved to be difficult to handle as they were flexible and not rigid enough, which lead to disruption of the film and were therefore not used in further study.

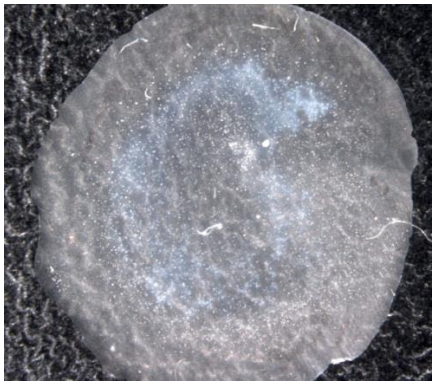

Figure 6.1.1 Picture of mucin films obtained from aluminium foil using 3 % BSM.

Aluminium pans and covers

After the application of 50 µl of the BSM solution to the covers and pans, one could clearly see a thin film in the pans and on the covers after drying (Figure 6.1.2). The 50 µl pan proved to be the most promising surface substrate for application as they had enclosing edge/wall which facilitated the application of the sample and transportation, in addition providing an appropriate area assuring that the applied sample stayed within the pan.

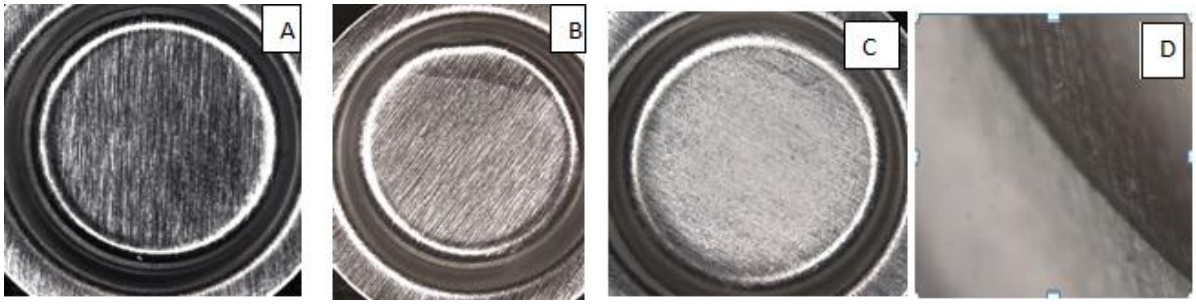

Figure 6.1.2 Photos of an empty 50 µl Perkin-ELMER pan A, with added buffer B, and with 3 % (w/v) mucin from bovine submaxillary glands C and D. (D is magnification (x10) of C.

Mucin film formation

Application of 3, 2 and 1 % (w/w) mucin solution to aluminium pans showed that both 3 and the 2 % mucin solutions left a film after evaporation of the solvent, but the 1 % BSM solution did not. It took approx. 1 day for the 3 % (w/w) BSM solution to dry, while it took approx. 2 days for the 2 % and the 1 % BSM solutions. Application of additional 50 µl 3 % BSM and subsequent drying resulted in a thicker film. Based on these results, 100 µl of 3 % (w/w) BSM solution was chosen as a model for mucosa in the further study.

The finding in the current study was similar to the ones of Kulichikhin et al. (74) who investigated the effect of salts on mucin films by preparing two types of mucin films; one containing NaCl and one salt free.

6.2 Determination of experimental parameters for the DVS Intrinsic Method

In Figure 6.2.1 the mass change of a mucin film in response to changing RH % is shown. The figure shows that the mass of the sample (mucin film) increases with increased RH % in the chamber. And when RH % is decreased, the mass of the mucin film decreases as well. The highest increase of mass was recorded at the increase of RH % stage from 80 to 90 RH %.

This is in agreement with the observations done by Johnsen et al. (73) who measured the amount of water in human skin using the DVS Intrinsic Method. This study showed that the amount of moist in the sample can be determined by measuring the weight of the sample.

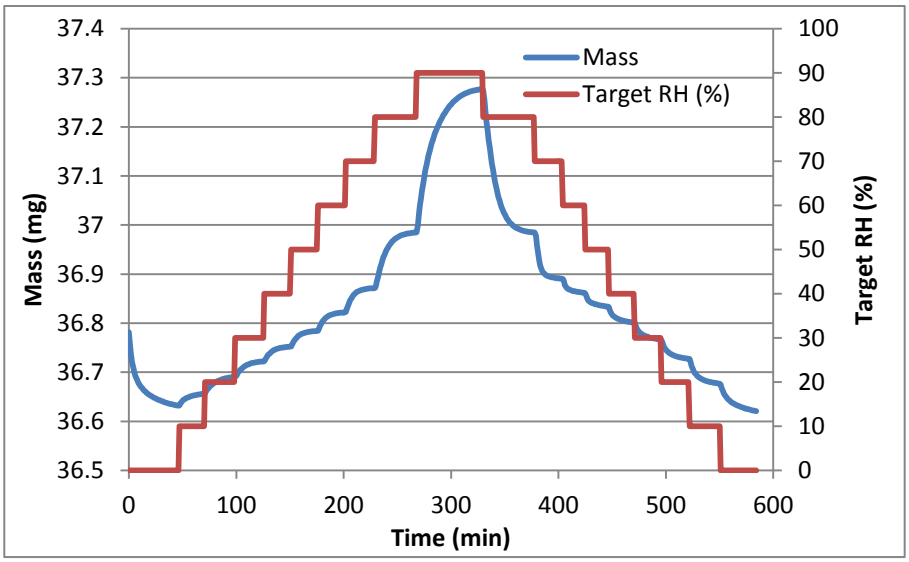

Figure 6.2.1 DVS Intrinsic analysis of dry mucin film; RH % stages from 0-90-0 changing with a factor of 10. The red line represents the target RH% for each stage, while the blue line represents the mass reading of the mucin film in the aluminium pan**.**

The RH % shown in Figure 6.2.1, at the beginning of the experiment would be too low and could lead to disruption of the liposomal structure.

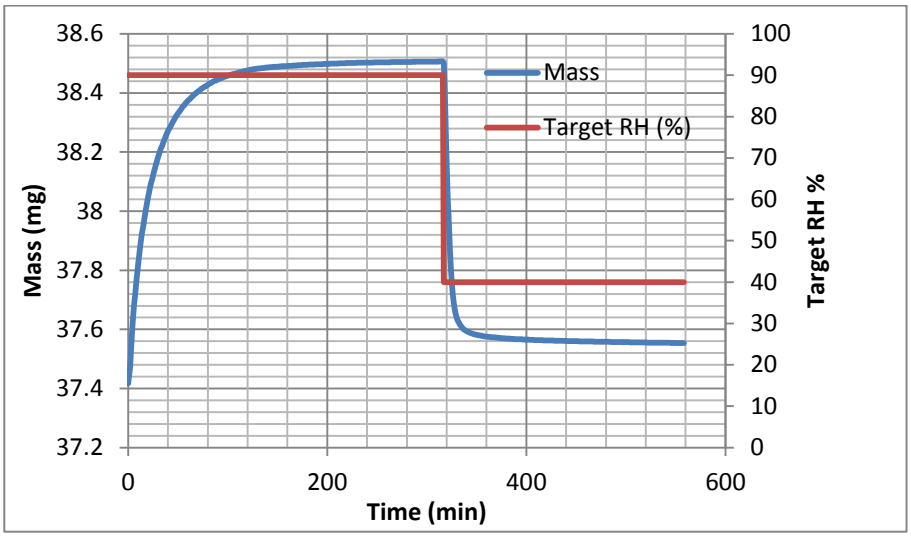

Figure 6.2.2 DVS Intrinsic analysis of dry mucin films; RH % stages from 90 – 40

In Figure 6.2.2 the mass measurements of a mucin film with the RH % stages 90-40 are shown. The mucin film was placed in to the instrument at 90 % RH. The mass of the mucin film increased to 38.5 mg at 90 % RH. After 317 minutes the first stage reached a stable mass reading and changed to 40 % RH; as a result of the decrease in RH % the mass of the mucin film also decreased.

When 15 µl of 5 mM phosphate buffer was applied to the mucin film, the time limit for the first stage did not allow the sample mass to stabilise as required, and therefore the experiment was forced on by the system as each stage had a time limit of 600 minutes per stage (Figure 6.2.3). An explanation for this is that the closer the water content gets to the level of equilibrium the longer time will be needed for stabilisation of the water content, and hence the longer time will be needed to reach a predefined stable state.

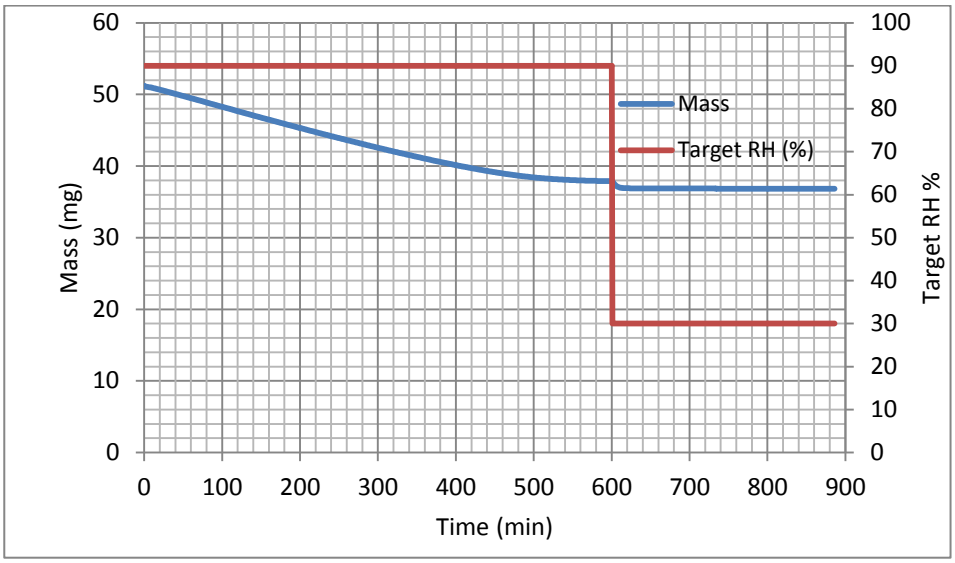

Figure 6.2.3 DVS Intrinsic analysis of buffer added to mucin film; RH % stages from 90-30. The stage was forced to shift after 600 minutes which was before the sample mass was stable.

When changing the RH % interval, starting at 80 % RH and shifting to 30 % RH with a time limit of 800 minutes, the analysis took less time. This shows that when starting at 80 % RH more water would evaporate in a faster manner than at 90 % RH. In Figure 6.2.4 the mass measurements of mucin film with RH % stages 80-30 are shown. Here the same behaviour was observed for mucin film as seen in Figure 6.2.2 where the mass of the mucin film increases and decreases as a result of the change of RH. When comparing Figures 6.2.3 and 6.2.5 it can be seen that the time needed for mass stabilisation was shorter when the RH stages were changed. With the new RH % stages (80 – 30 % RH) the mass measurements reached a stable state already after 361 minutes.

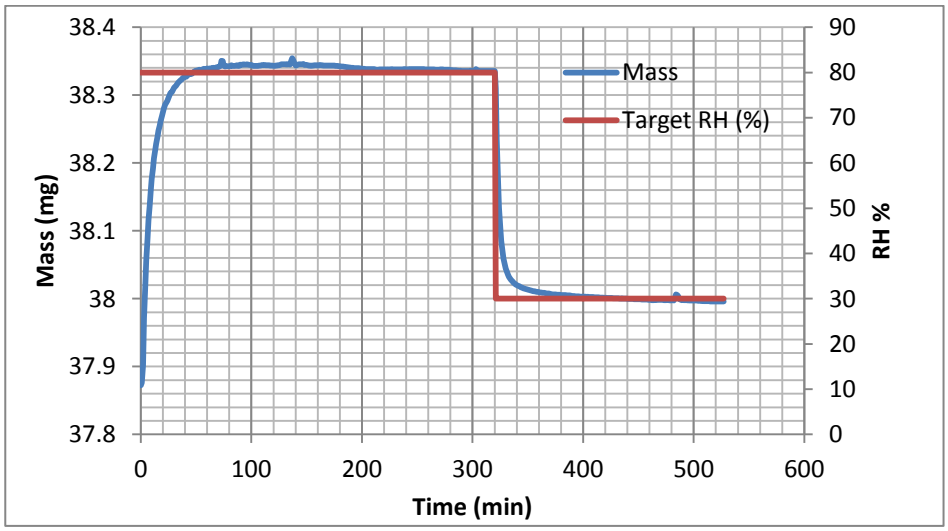

Figure 6.2.4 DVS Intrinsic analysis of mucin film; RH % stages from 80 - 30.

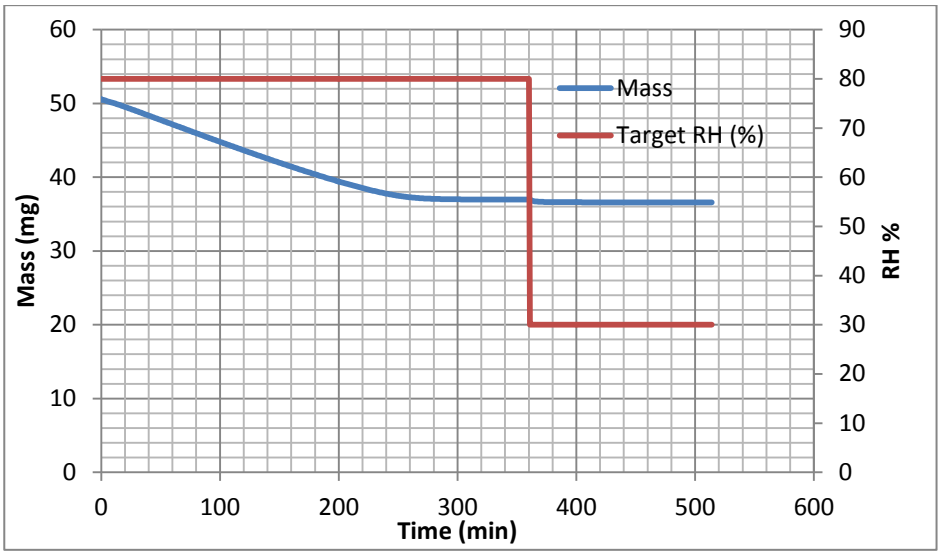

Figure 6.2.5 DVS Intrinsic analysis of phosphate buffer added to mucin film; RH % stages from 80 – 30

Based on these results, the stages from 80 – 30 % RH were chosen for the analysis of the liposomal formulations and the time limit per stage was kept at 800 minutes. The rest of the parameters of the method were as described in Chapter 4.6.1

7 Results and Discussion

7.1 Determination of the optimal coating concentration of the polymers

The purpose of this part of the study was to obtain the concentration of polymers which indicated complete coating of the liposomal surfaces. In the current study only the charged liposomes were used and they were coated with oppositely charged polymers. Therefore, electrostatic interaction was expected to be the main force behind the interaction between the liposomes and the polymers. Positively charged liposomes were coated with negatively charged pectin and negatively charged liposomes were coated with positively charged chitosan. For this means the minimum polymer concentration that gave the best results, indicating non-existence of aggregation or flocculation of particles, was selected as the optimal polymer concentration. It is known that too high polymer

concentration might lead to excess free polymers in the solution and depletion flocculation as described by Laye et al. (37). Therefore, in situations where there was no significant difference between polymer concentrations, the lower concentration was selected. Table 7.1.5 shows the concentration that was selected for each polymer.

7.1.1 Coating with HM-pectin

Positively charged EggPC-DOTAP liposomes were coated with the negatively charged HM-pectin. Two batches of each formulation was prepared and characterised. In Table 7.1.1 the unimodal size distribution analysis of the HM-pectin-coated liposomes is shown. The results showed that the size of the liposome-polymer complex increased with increasing concentration of the polymer. The 0.01 % and the 0.05 % HM-pectin-coated liposomes showed the lowest polydispersity indexes (PI) with the values of 0.098-0.130 and 0.182-0.288, respectively. The other concentrations had a higher PI.

Table 7.1.1 Unimodal distribution of size of the HM-pectin-coated liposomes

Concentration of polymer (% w/v)	Batch number	Mean size (nm)	Poly dispersity index (PI)
0.01	1	309	0.098
	2	307	0.130
0.05	1	312	0.288
	2	320	0.182
0.1	1	385	0.320
	2	336	0.353
0.2	1	415	0.387
	2	412	0.161
0.5	1	499	0.387
	2	489	0.170

Nguyen et al. (58) compared the undiluted pure pectin samples (0.05 and 0.2 %) with diluted pectin in pectin-liposome complexes and suggested that the size increase as can be seen in Figure 7.1.1 is due to liposomes/coated liposomes and the free pectin amount in the sample would be too low to be measured by the instrument.

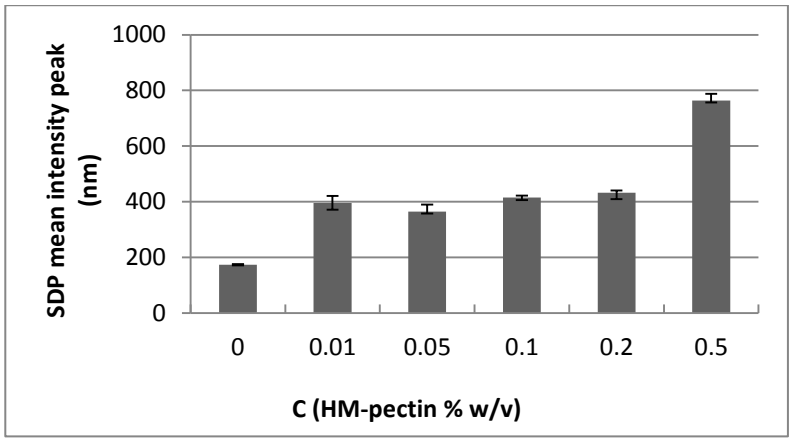

Figure 7.1.1 SDP Intensity size measurements (diameter) of the HM-pectin-coated liposomes. The measurements of naked EggPC-DOTAP liposomes are from a different batch prepared in the current study.

The SDP Intensity analysis of the HM-pectin-coated liposomes showed an undesirable size increase of liposomes as a result of coating with 0.5 % HM-pectin.

The transmittance measurements, shown in Figure 7.1.2, confirmed the results from the SDP size analysis, i.e. samples with the bigger particles were more turbid than samples with smaller particles. The highest polymer concentration (0.5 %) showed the lowest transmittance values, and was the most turbid. Even though the size of the 0.01 % HM-pectin-coated liposomes was small, the transmittance value was lower than the samples of 0.05 - 0.2 % HM-pectin-coated liposomes. When statistical analysis was done on the transmittance measurements, both 0.01 and 0.5 % HM-pectin-coated liposomes were significantly more turbid (95 % CL, p 0.001). The transmittance measurements showed that all the polymer-coated liposomes had an increased turbidity compared to uncoated liposomes, as seen in the SDP Intensity analysis where the size of the polymer-coated liposomes increased after the addition of the polymer.

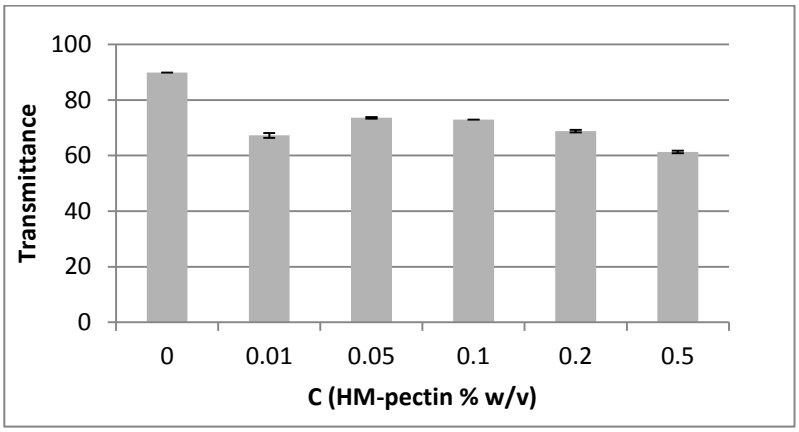

Figure 7.1.2 The transmittance value of the HM-pectin-coated liposomes. Uncoated liposomes were less turbid, indicating complexation/large structures in the polymer-coated liposomes samples.

The liposomes coated with HM-pectin were positively charged (EggPC-DOTAP) prior to coating; after the coating the liposomes exhibited a negative zeta potential. The coating of the EggPC-DOTAP liposomes with HM-pectin had an even effect on the zeta-potential as all of them exhibited a shift of zeta potential from a positive to a negative value (see Figure 71.3).

The main force behind the interaction between the liposomes and the polymer is electrostatic attraction of the oppositely charged particles as suggested by Guzey et al. who studied the effect polymer-coating of emulsions containing oil droplets had on the electrostatic interactions (75), and Thirawong et al. who investigated increased intestinal absorption of calcitonin entrapped in liposome-pectin complexes (76).

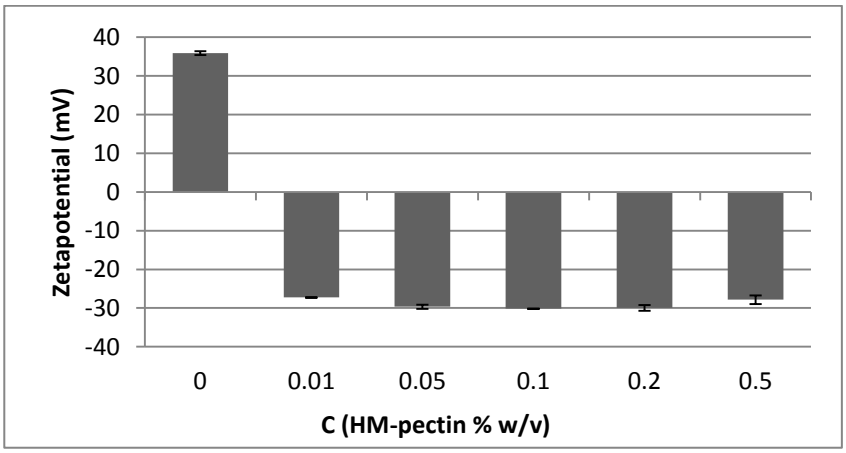

Figure 7.1.3 Zeta potential measurements of the HM-pectin-coated liposomes.

The stability of the HM-pectin-coated liposomes

The stability of the HM-pectin-coated liposomes was measured over a period of 7 weeks. The SDP analysis showed that the 0.01 - 0.2 % HM-pectin-coated liposomes were stable through the 4 weeks of investigation with little variation in size. On the other hand, the 0.5 % HM-pectin-coated liposomes seemed to decrease in size and increase again (Figure 7.1.4). This may be due to excessive free polymers as a result of the high coating concentration. Another factor that can have affected the size is the area in the container from which the sample was withdrawn from time to time during preparation of the samples.

As seen in the Figure 7.1.4, at week 7 the size of the 0.01 % and the 0.1 % HM-pectin-coated liposomes had increased more as compared of the 0.05 % HM-pectin-coated liposomes.

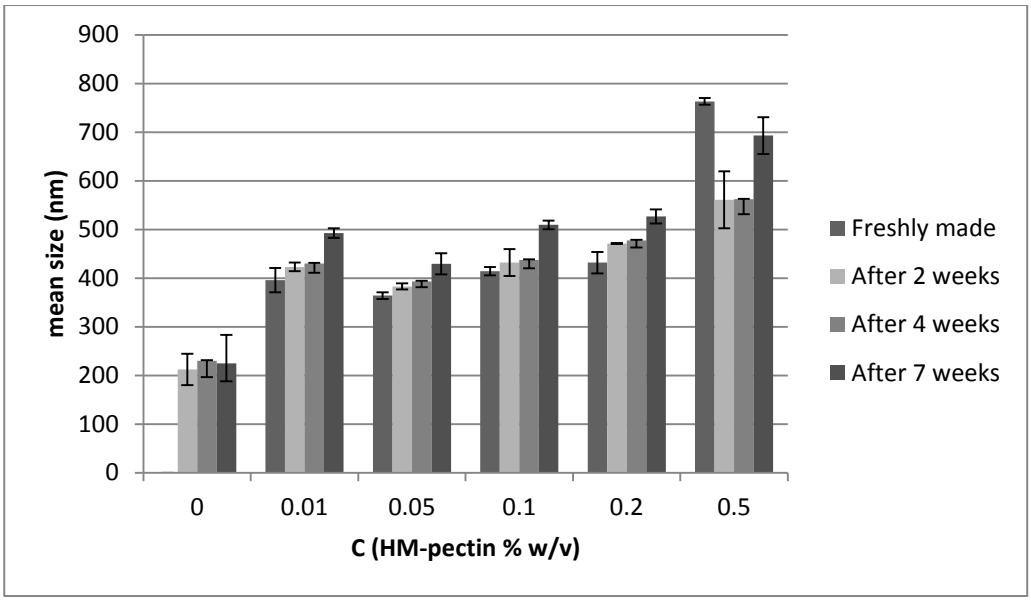

Figure 7.1.4 Stability analysis of the HM-pectin-coated liposomes; SDP Intensity peak.

The transmittance measurements over 7 weeks are shown in Figure 7.1.5. The pattern seen is similar to the result of the freshly prepared formulation, where the most turbid samples were the 0.01 and the 0.5 % HM-pectin-coated liposomes. The results also showed the same as seen in the

SDP Intensity peak analysis (size) of the samples, confirming the stability of the 0.05 % HM-pectin-coated liposome samples. The differences (slight decrease and increase) between the 0.1 and the 0.2 % HM-pectin-coated liposomes were not significant.

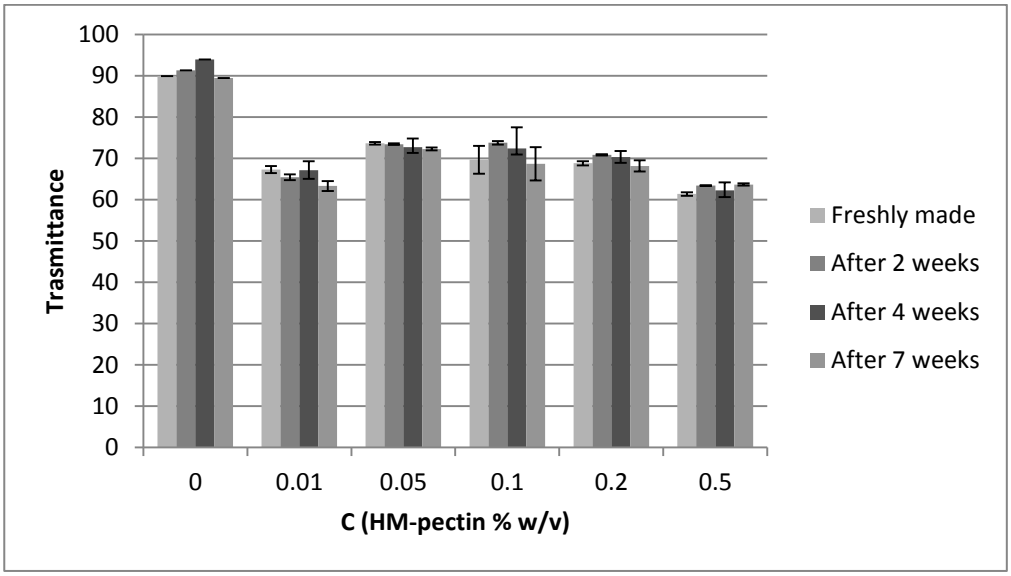

Figure 7.1.5 Stability analysis of the HM-pectin-coated liposomes; Transmittance.

The zeta potential of the HM-pectin-coated liposomes was followed for 4 weeks and all the samples maintained the negative surface charge, while the uncoated liposomes remained to be positively charged. As seen in the Figure 7.1.6 the HM-pectin-coated liposomes showed a small decrease in the negative charge leading to less negative zeta potential during the 4 weeks.

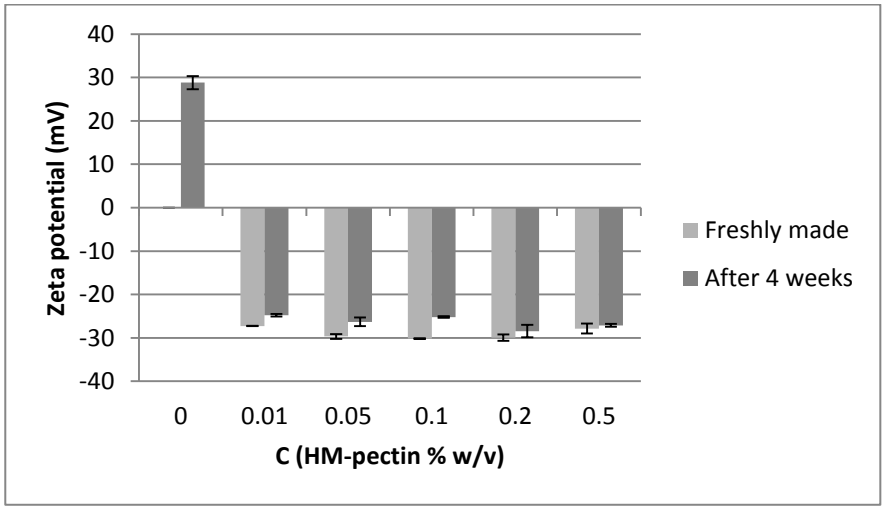

Figure 7.1.6 Stability analysis of the HM-pectin-coated liposomes; zeta potential.

From these result, the optimal coating concentration of the polymer seemed to be in the range of 0.05 - 0.2 % of HM-pectin. As mentioned earlier, when several concentrations of the polymer were proven to be sufficient for complete coating of liposomes, the lowest concentration was selected to avoid excess polymer in the solution, hence the 0.05 % HM-pectin-coated EggPC-DOTAP liposome solution was selected for the further studies.

7.1.2 Coating with LM-pectin

Positively charged EggPC-DOTAP liposomes were coated with negatively charged LM-pectin. As seen in the unimodal size distribution analysis in Table 7.1.2, all samples showed a relatively low PI ranging from 0.014-0.215. This means that the samples had a narrow size distribution. The SDP Intensity analysis showed that the size of the coated liposomes decreased when the concentration of LM-pectin was increased from 0.01 to 0.05 %. When the concentration of the polymer was further increased, the size of the coated liposomes also increased (Figure 7.1.7).

The 0.01, 0.2, and the 0.5 % LM-pectin-coated liposomes were significantly bigger than the 0.05 and 0.1 % LM-pectin-coated liposomes. The 0.01 % LM-pectin-coated liposomes had in addition larger error bars indicating large size distribution.

Table 7.1.2 Unimodal distribution in size of the LM-pectin-coated EggPC-DOTAP

Concentration of polymer (% w/v)	Batch number	Mean size (nm)	Poly dispersity index (P.I)
0.01	1	255	0.014
	2	230	0.091
0.05	1	223	0.175
	2	222	0.122
0.1	1	223	0.215
	2	220	0.171
0.2	1	253	0.137
	2	252	0.156
0.5	1	294	0.095
	2	301	0.151

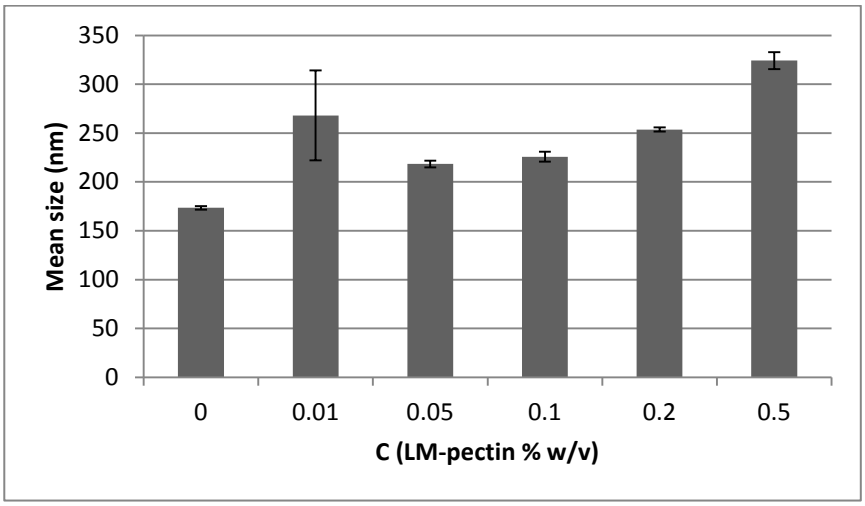

Figure 7.1.7 SDP Intensity analysis; size measurements of the LM-pectin-coated liposomes. The size of the naked liposomes is from another batch in the study.

The transmittance of the LM-pectin-coated liposomes was very similar for all coating concentrations, as shown in Figure 7.1.8. All samples were more turbid than the uncoated EggPC-DOTAP liposomes. The transmittance measurements were not in agreement with the SDP Intensity analysis. The transmittance of the 0.01 and the 0.5 % were expected to be lower than

shown in Figure 7.1.8 as the decreased transmittance is often a result of increased size. The unexpected result might be due to small drifts in the instrument that was used for these measurements and the instrument limitations such as relatively long time needed to stabilise.

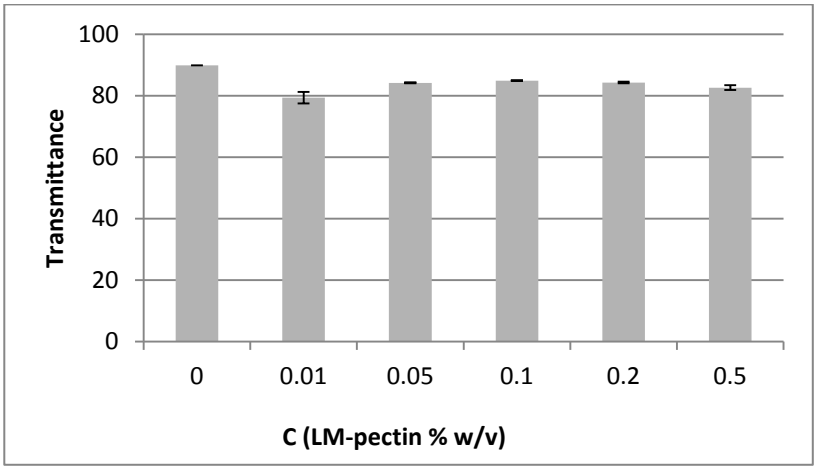

Figure 7.1.8 Transmittance measurements of the LM-pectin-coated liposomes.

As was already observed for the HM-pectin-coated liposomes, the LM-pectin-coated liposomes also exhibited the opposite surface charge after the coating. The zeta potential of the LM-pectin-coated liposomes was more negative as compared to HM-pectin-coated liposomes. This may be due to a lower degree of methoxylation in LM-pectin resulting in a higher amount of free carboxylic acid side groups contributing to the negative charge. In addition, due to the higher concentration of the LM-pectin, more carboxylic groups can be deprotonated, thus having an impact on the zeta potential. As seen in Figure 7.1.9 the negativity of the LM-pectin-coated liposomes increases when the concentration of the polymer is increased from 0.01 to 0.1 %.

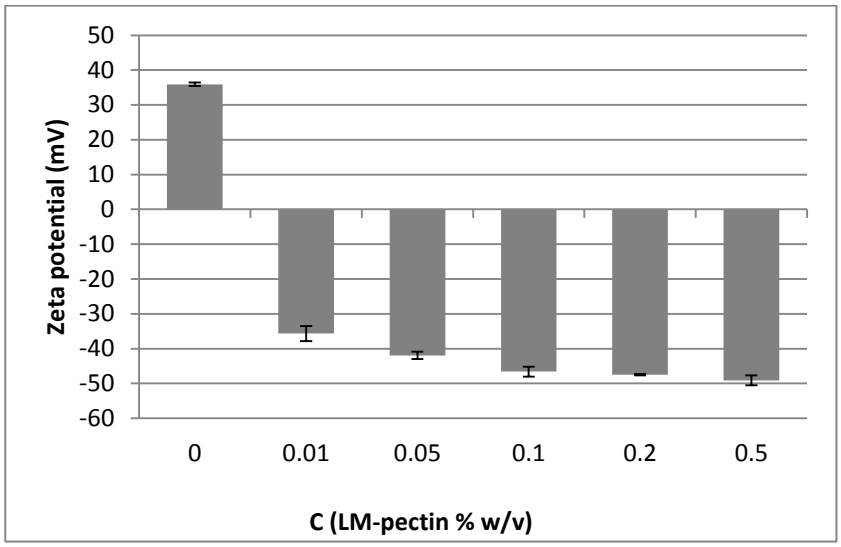

Figure 7.1.9 Zeta potential measurements of the LM-pectin-coated liposomes

The stability of the LM-pectin-coated liposomes

As can be seen in Figure 7.1.10 the 0.01 % LM-pectin-coated liposomes showed variation in the size, based on the SDP Intensity analyses, from week to week, while the 0.05 and the 0.1 % LM-pectin-coated liposomes were stable throughout the 7 weeks. This indicates an optimal coating concentration range. The 0.2 % LM-pectin-coated liposomes were significantly ($p < 0.05$) larger in size than the 0.05 - 0.1 % samples. This concentration was therefore discarded.

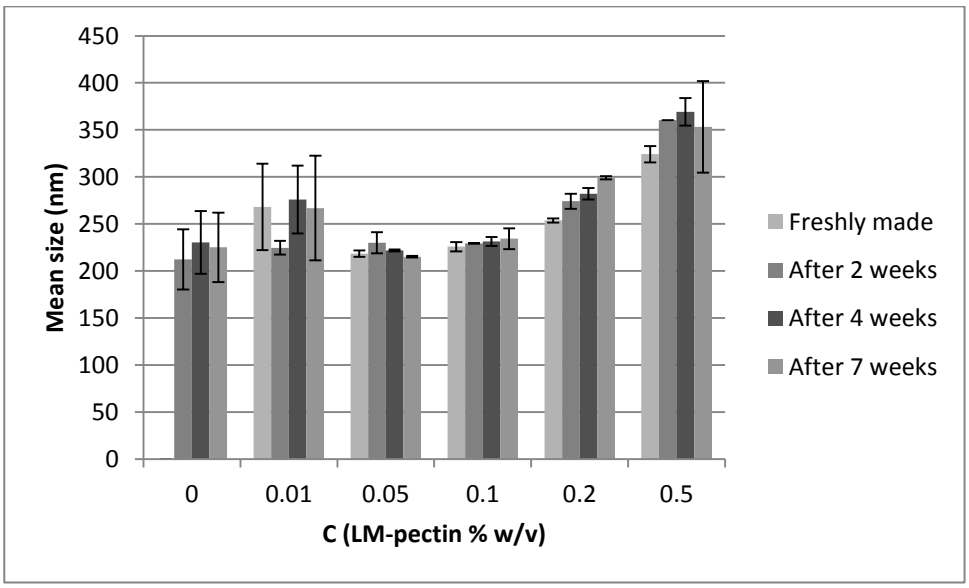

Figure 7.1.10 Stability analyses of the LM-pectin-coated EggPC-DOTAP; SDP Intensity peak.

The transmittance measurements, as shown in Figure 7.1.11, done over the 7 weeks showed a stable pattern for the 0.05 and the 0.1 % LM-pectin-coated liposome samples.

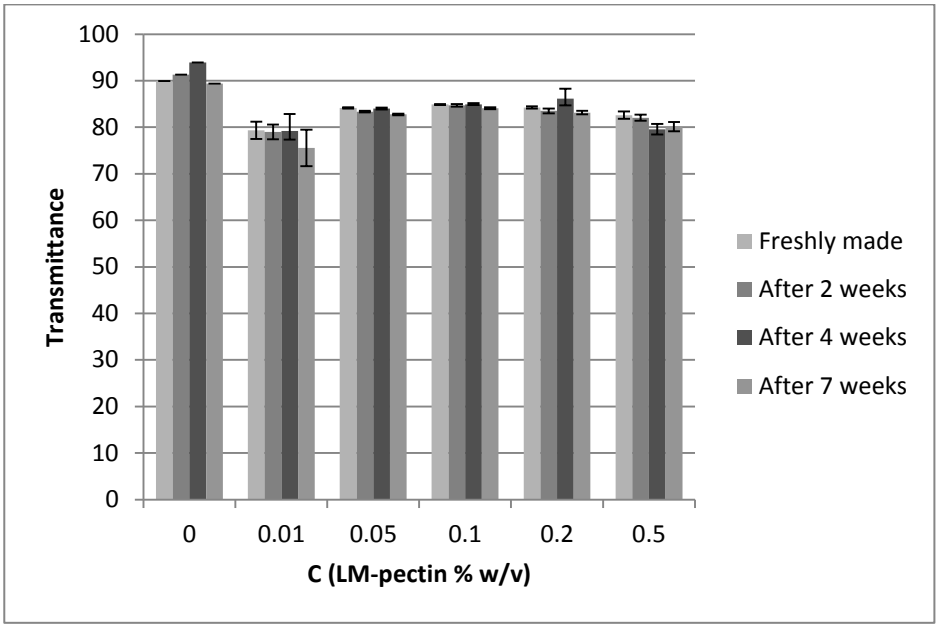

Figure 7.1.11 Stability analysis of the LM-pectin-coated EggPC-DOTAP; Transmittance

The zeta potentials of the uncoated and the LM-pectin-coated EggPC-DOTAP liposomes are shown in Figure 7.1.12. LM-pectin-coated liposomes were all negatively charged in comparison to the uncoated liposomes. The zeta potential of the polymer-coated liposomes decreased (i.e. became less negative) during the 4 weeks of storage. Smistad et al. (39) observed a decrease of zeta potential of HM-pectin-coated liposomes over time. This was not observed for LM-pectin-coated liposomes. For HM-pectin-coated liposomes, one could expect an increase in the negative charge due to hydrolysis of the methyl esters leading to free carboxylic groups and consequently more negative charge at pH 6.8. LM-pectin was reported (39) to be more stable due to its lower degree of methoxylation. The observed decrease of the negative charge as seen for the LM-pectin-coated liposomes, in the current study, may be due to the conformational changes of polymer structure.

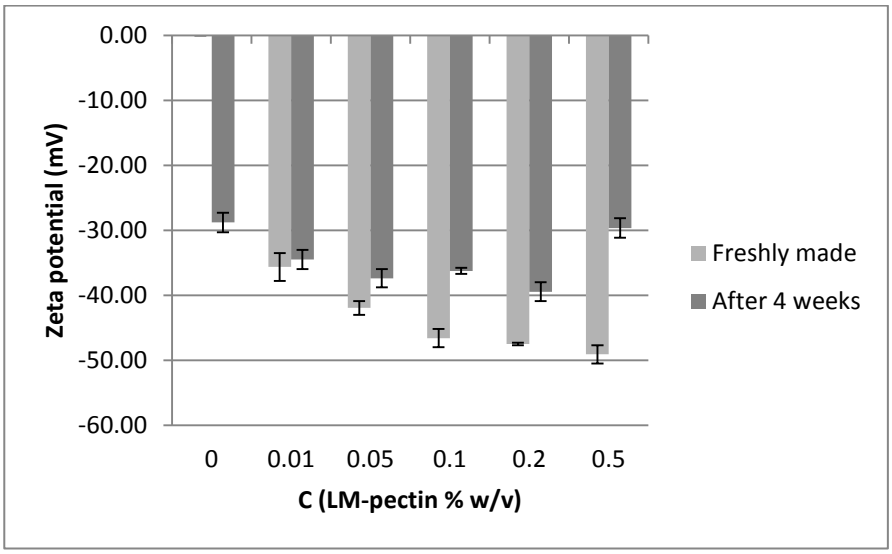

Figure 7.1.12 Stability analysis of the LM-pectin-coated EggPC-DOTAP liposomes; Zeta potential

Based on these findings, the optimal coating concentration of the LM-pectin seemed to be in the range 0.05 - 0.1 %. A coating concentration of 0.05 % LM-pectin has been reported by Alund et al. (77) to be insufficient for complete coating of liposomes. This may be due to the molecular weight and the size of LM-pectin compared to HM-pectin. Therefore, 0.1 % LM-pectin-coated EggPC-DOTAP liposome solution was elected for further studies.

7.1.3 Coating with AM-pectin

The unimodal size distribution of the AM-pectin-coated liposomes showed higher PI when the coating concentration of AM-pectin was increased. The 0.5 % AM-pectin-coated liposomes showed unsatisfactory size distribution (PI 0.377-0.410) while the 0.01 - 0.1% samples showed more satisfactory results (Table 7.1.3)

Table 7.1.3 Unimodal distribution of size measurements of the AM-pectin-coated liposomes

Concentration of polymer (% w/v)	Batch number	Mean size (nm)	Poly dispersity index (PI)
0.01	1	277	0.130
	2	288	0.183
0.05	1	259	0.235
	2	268	0.142
0.1	1	269	0.163
	2	262	0.245
0.2	1	279	0.356
	2	284	0.299
0.5	1	350	0.410
	2	340	0.377

The SDP Intensity analysis confirmed the results obtained from the unimodal size distribution analysis where the 0.5 % AM-pectin-coated liposomes had large error bars and were significantly ($p < 0.05$) larger than the other AM-pectin-coated liposomes (Figure 7.1.13). The 0.01 % AM-pectin-coated liposomes also showed large error bars indicating large size distribution. Statistically, no difference between the 0.01 – 0.2 % AM-pectin-coated liposomes in size was seen. Due to the large error bars in the SDP Intensity analyses the 0.01 and the 0.5 % AM-pectin-coated liposomes were discarded in the evaluation of the optimal concentration for complete coating.

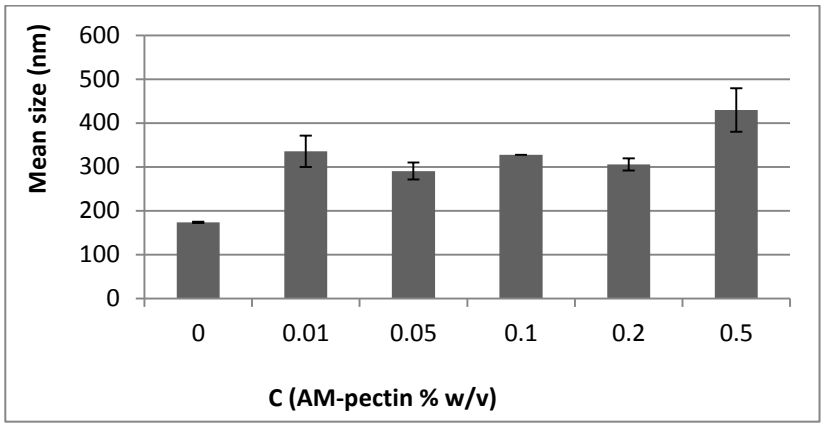

Figure 7.1.13 SDP Intensity analysis, size measurements of the AM-pectin-coated liposomes.

The transmittance measurements of the AM-pectin-coated liposomes are shown in Figure 7.1.14. The 0.01 and the 0.5 % AM-pectin-coated liposomes were significantly ($p < 0.05$) more turbid than the 0.05 - 0.2 % AM-pectin-coated liposomes. The lower transmittance of the samples, especially for the 0.5 % AM-pectin-coated liposomes, was in agreement with the SDP Intensity analysis. The coating concentration range between 0.05 - 0.2 % resulted in the equal transmittances of the coated liposomes.

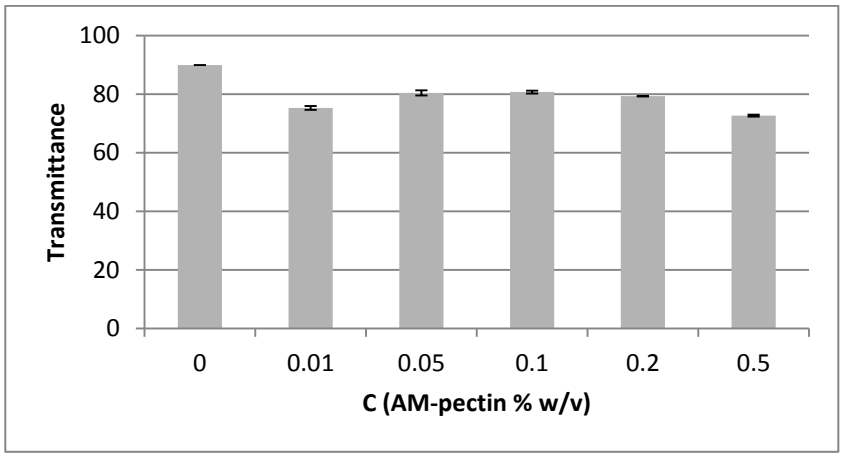

Figure 7.1.14 Turbidity measurements of the AM-pectin-coated liposomes.

All AM-pectin-coated liposomes exhibited a negative surface charge. This was in agreement with the results obtained for the HM- and LM-pectin-coated liposomes. The AM-pectin-coated liposomes had a greater negative surface charge than HM-pectin-coated liposomes, however less than LM-pectin-coated liposomes (Figure 7.1.15). The reason for the higher negative charge of the AM-pectin-coated liposomes as compared to the HM-pectin-coated liposomes may be due to the higher degree of free carboxylic acid groups in the structure of AM-pectin. The less negative

charge of AM-pectin when compared to LM-pectin-coated liposomes may be due to the presence of the amide groups in AM-pectin that can decrease the negative charge of the polymer

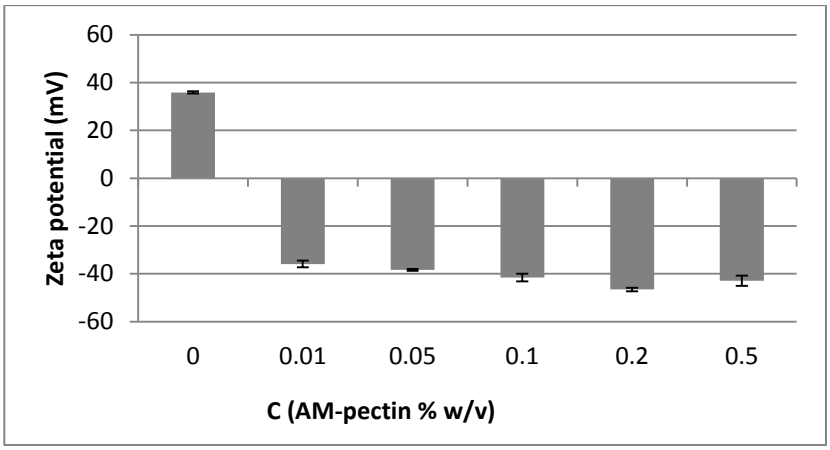

Figure 7.1.15 Zeta potential of the AM-pectin-coated liposomes.

The stability of the AM-pectin-coated liposomes

In the SDP Intensity analysis, the variations were observed for all AM-pectin-coated liposomes in respect to the size measurements. The size of the 0.01 % AM-pectin-coated liposomes increased, decreased and again over the last 2 measurements. This resulted in the elimination of the coating concentration for complete coating of the liposomes.

The 0.05 and the 0.1 % AM-pectin-coated liposomes showed a stable behaviour over 4 weeks, followed by a change in the size on week 7. For the samples of 0.2 and 0.5 % AM-pectin the sizes showed large variations (Figure 7.1.16). Despite the high initial PI, the coating concentration 0.2 % of AM-pectin which initially showed potential for complete coating of liposomes had a size increase over 100 nm after 2 weeks; the size decreased to a level similar to freshly prepared polymer-coated liposomes after 4 weeks.

After 7 weeks, the size of the 0.2 % AM-pectin-coated liposomes was similar to uncoated liposomes indicating instability of the formulation. The same pattern was observed for the 0.5 % AM-pectin-coated liposomes, although not as clear as for the 0.2 % AM-pectin-coated liposomes.

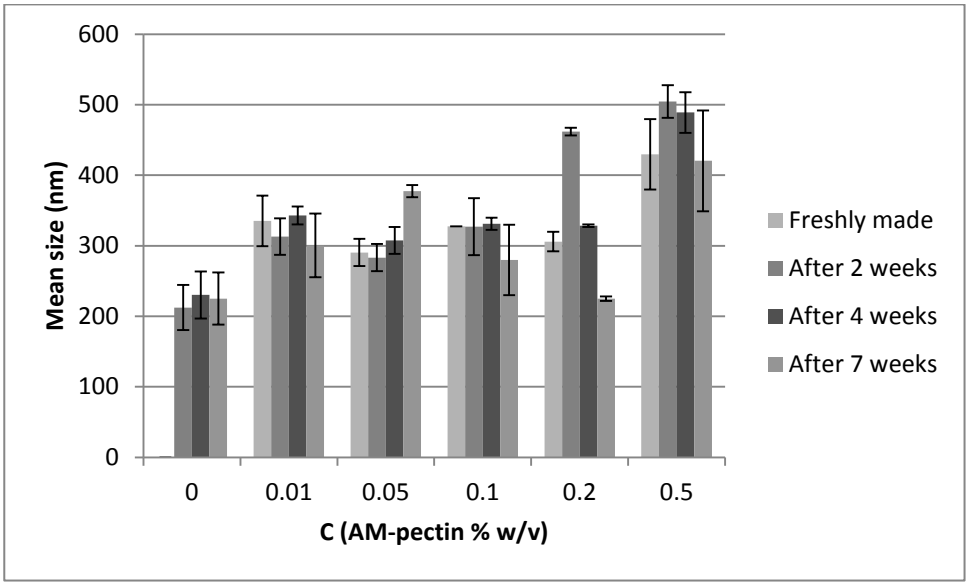

Figure 7.1.16 Stability analysis of the AM-pectin-coated liposomes; Size.

The transmittance measurements of AM-pectin-coated liposomes throughout the 7 weeks of investigation showed a similar pattern to the SDP intensity size measurements.

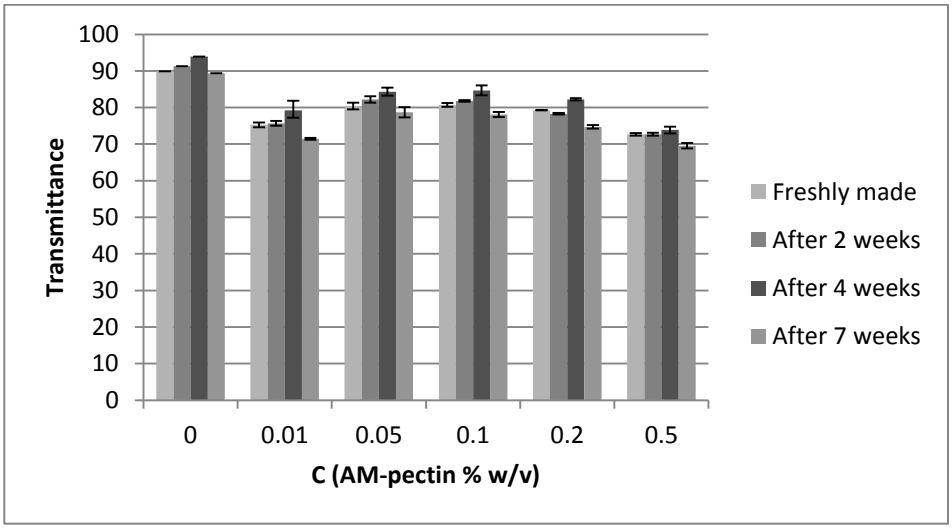

Figure 7.1.17 Stability analysis of the AM-pectin-coated liposomes; Transmittance.

Similar to the LM-pectin-coated liposomes, the AM-pectin-coated liposomes also showed a decrease in the negative charge after 4 weeks of storage. One exception was the 0.05 % AM-pectin-coated liposomes exhibited the least decrease in the negative surface charge.

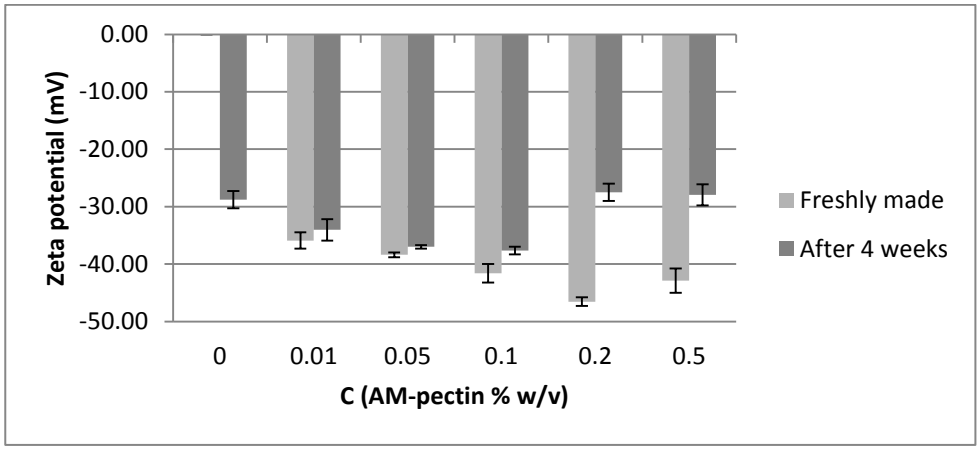

Figure 7.1.18 Stability analysis of AM-pectin-coated liposomes; Zeta potentials

Smistad et al. reported that AM-pectin-coated liposomes were the least stabile among the three types of pectin studied (39). The authors explained the phenomenon by the amide groups of AM-pectin interfering in the electrostatic interaction leading to loosely bound polymer on the surface of the liposomes. This ass also seen in the current study where the size, zeta potential and transmittance slightly increased and decreased at the time of measurements.

Based on these results, the optimal coating concentration of the polymer seemed to be in the range between 0.05 - 0.1 % of AM-pectin as there was no significant difference between these two concentrations. To obtain complete coating and avoid the excess polymer in the solution, 0.05 % AM-pectin-coated EggPC-DOTAP liposome solution was elected for further studies.

7.1.4 Coating with chitosan

Negatively charged EggPC-EggPG liposomes were coated with positively charged chitosan. In Table 7.1.4 the unimodal size distribution of the chitosan-coated liposomes is shown. As can be seen in the table, one batch of the 0.01 % chitosan-coated liposomes had a large size (802) as compared to the other batches. The size of the chitosan-coated liposomes decreased when the coating concentration of the polymer was increased to 0.04 %.

Further increase of the polymer concentration did not lead to any significant increase of the size of the chitosan-coated liposomes. One observation, which is rather difficult to explain (Table 7.1.4), is the PI of the 0.01, 0.04, and 0.08 % where the PI differed between the batches for each coating concentration.

The SDP Intensity analysis (shown in Figure 7.1.19) of the chitosan-coated liposomes showed the same outcomes in terms of size of the samples. The 0.01 % chitosan-coated liposomes had a mean size of 591 nm and larger error bar with the upper limit of the bar at 782 nm. This led to the elimination of this coating concentration in further investigation. The 0.04 - 0.16 % chitosan-coated liposomes exhibited similar size distributions.

The size of liposomes increased more than 100 % after the coating of the liposomes with chitosan. Filipović-Grčić et al. (9) described chitosan to adsorb to the liposomal surface in a flat manner when the solvent pH was low. However, when the solvent pH was increased above 6.2 (in the current study pH was 6.8) the manner of adsorption would change. The adsorption pattern will be in the form of loops and tails.

These new manners of adsorption could explain the larger size of the chitosan-coated liposomes in the our study as compared to the study by Filipović-Grčić et al. where the size of the chitosan-coated liposomes was below 400 nm. It might also be due to a combination of factors explained by Henriksen et al. who discussed that adsorption coagulation and bridging between liposomes could affect their size (64).

Table 7.1.4 Unimodal Distribution of size measurements of the chitosan coated EggPC-EggPG liposomes

C (chitosan % w/v)	Batch number	Mean size (nm)	PI	C (chitosan % w/v)	Batch number	Mean Size (nm)	PI
0.01	1	802	0.059	**0.12**	1	428	0.328
	2	516	0.057		2	434	0.132
	3	529	0.208		3	419	0.340
0.04	1	389	0.010	**0.16**	1	393	0.214
	2	375	-0.146		2	406	0.370
	3	405	0.040		3	521	0.278
0.08	1	415	0.270				
	2	395	0.104				
	3	395	-0.051				

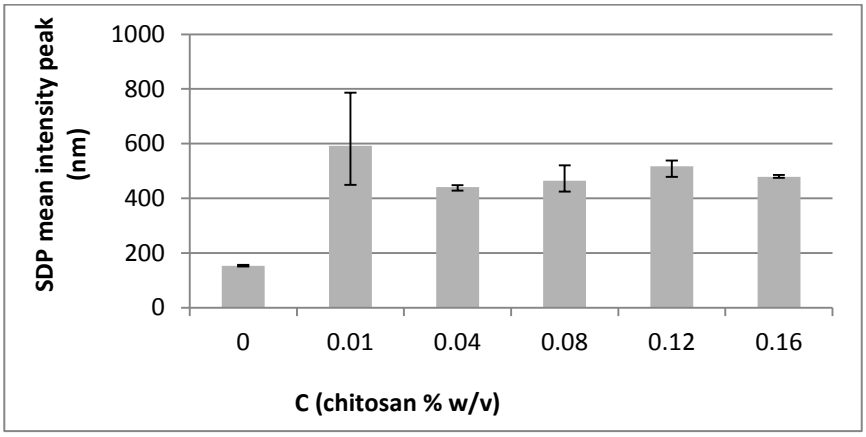

Figure 7.1.19 SDP Intensity analysis, size measurements of the chitosan-coated liposomes.

The transmittance measurements of chitosan-coated liposomes are shown in Figure 7.1.20. The results revealed that the 0.01 % chitosan-coated liposomes had the significantly ($P < 0.05$) lowest transmittance (95% CI). This confirms the existence of larger particles and is in agreement with the observations obtained from the SDP Intensity analysis. When the coating concentration of the polymer was increased, the transmittance of the coated liposomes was increased and stayed at the same level until the coating concentration reached 0.12 %.

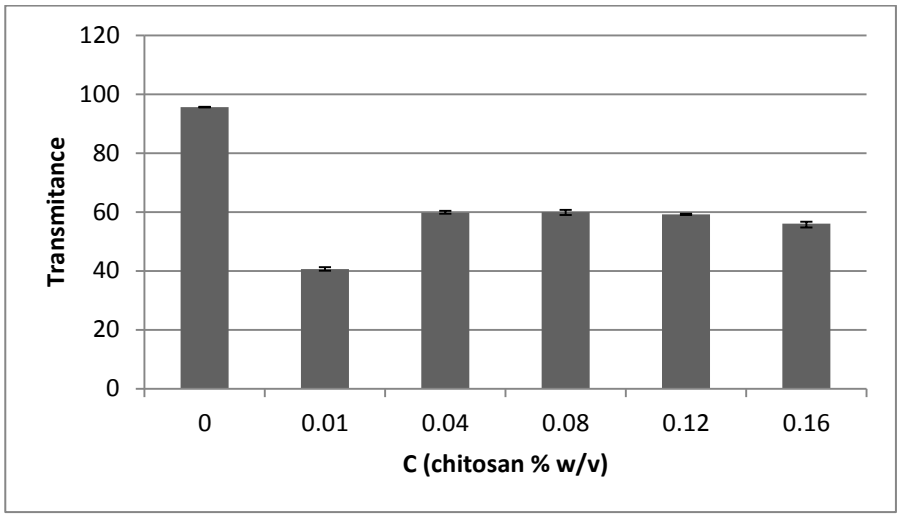

Figure 7.1.20 Transmittance of the chitosan-coated liposomes.

The zeta potential of the chitosan-coated liposomes is shown in Figure 7.1.21. The liposomes coated with chitosan were negatively charged (EggPC-EggPG) prior to coating, after the coating the liposomes exhibited a positive zeta potential. The coating of liposomes resulted in a shift of zeta potential from a negative to a positive value. The main force behind the interaction between the liposomes and the polymer is electrostatic attraction of the oppositely charged particles as described for pectin-coated liposomes. There was no significant difference between the samples regarding zeta potential when statistically analysed.

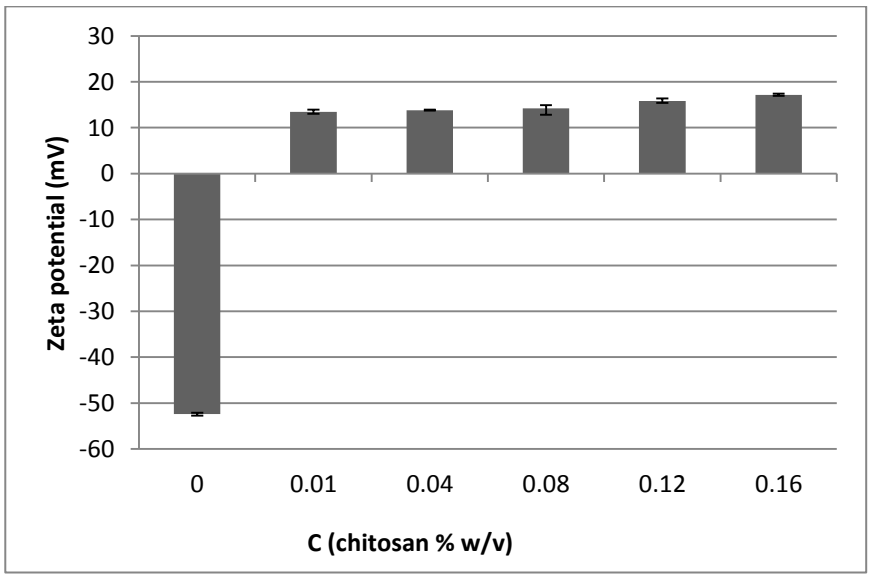

Figure 7.1.21 Zeta potential of chitosan-coated liposomes.

The stability of the chitosan-coated liposomes

The stability of the chitosan-coated liposomes was followed for 7 weeks and the results are shown in Figures 7.1.22-7.1.24. The 0.01 % chitosan-coated liposomes changed the size over the weeks indicating instability issues (Figure 7.1.22). The 0.16 % chitosan-coated liposomes exhibited aggregation.

The 0.04 % chitosan-coated EggPC-EggPG liposomes maintained their initial size and were stable during the time of investigation. The 0.08 and the 0.12 % showed small changes in size over time.

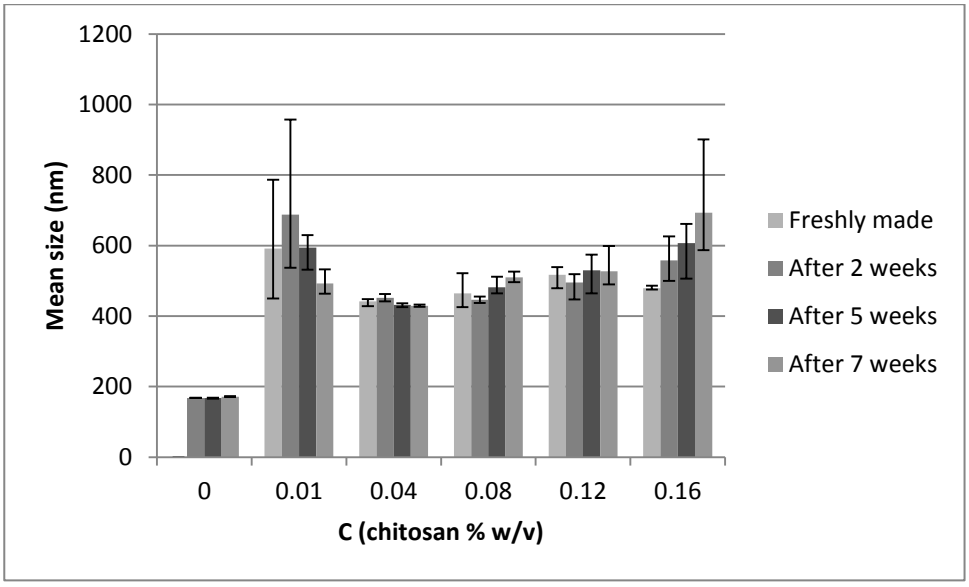

Figure 7.1.22 Stability of the chitosan-coated liposomes; size.

The transmittance measurements of the chitosan-coated liposomes (shown in Figure 7.1.23) were in agreement with the SDP Intensity analysis where the 0.01 % chitosan-coated liposomes showed significantly ($p < 0.05$) lower transmittance (thus more turbid) and the 0.16 % chitosan-coated liposomes showed a small decrease of transmittance over time of investigation. The coating concentration range of 0.04 - 0.12 % were similar. The uncoated EggPC-EggPG liposomes exhibited a dramatic decrease of transmittance from week 2 to week 4. The decrease of

transmittance indicated larger particles, which was not observed in the SDP Intensity analysis (Figure 7.1.22). This may be explained by the aggregated liposomes that were present in the sample. For the transmittance measurements the samples were not diluted. On the other hand, for the size measurements, the samples were diluted in phosphate buffer. This dilution might have had an effect on the aggregated liposomes.

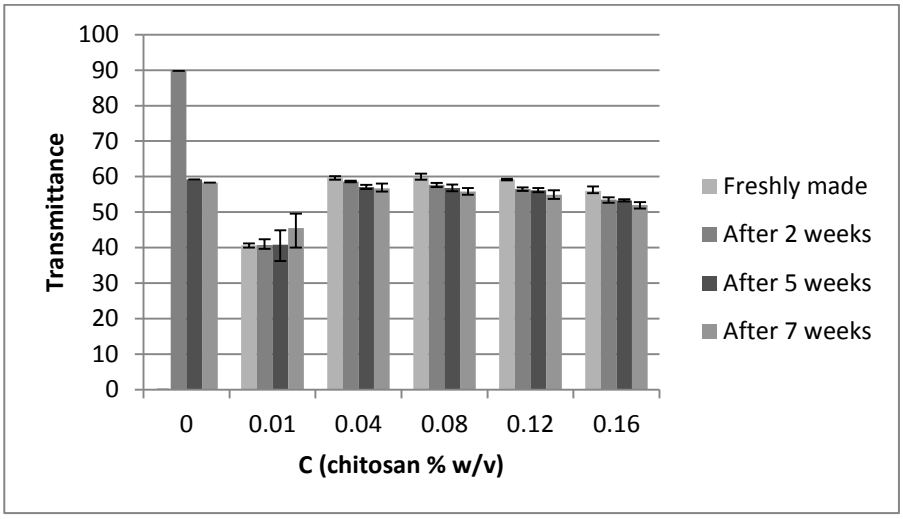

Figure 7.1.23 Stability analysis of the chitosan-coated liposomes; Transmittance

The zeta potential of the chitosan-coated liposomes, as shown in Figure 7.1.24 did not change during the 7 weeks of storage. This indicated that the chitosan layer remained stable on the liposomal surface. The uncoated EggPC-EggPG liposomes showed a decrease in negative charge from week 4 to week 7.

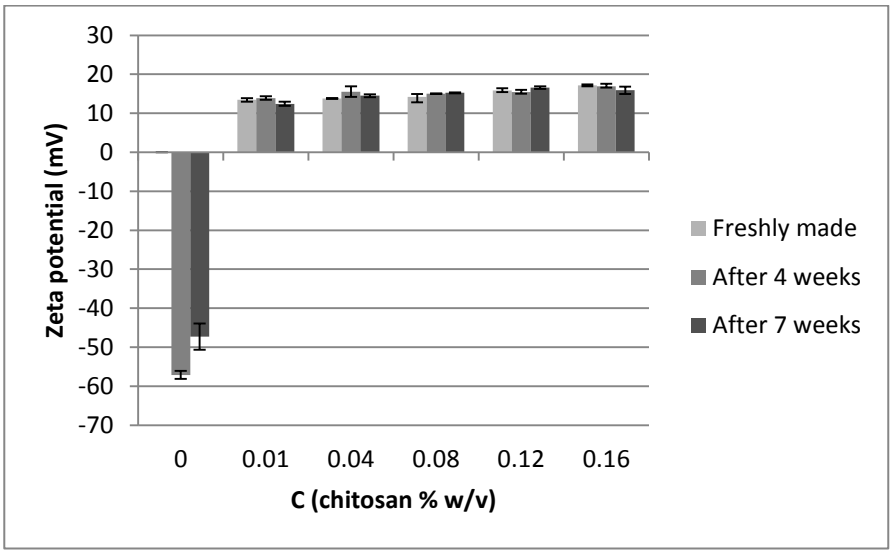

Figure 7.1.24 Stability analysis of the chitosan-coated liposomes; Zeta potentials

Therefore, the 0.04 % chitosan was selected as the optimal concentration for complete coating of liposomes.

Summarising the coating experiment

Based on the results in Chapter 7.1 the optimal concentration of each polymer was chosen and used in the further investigation of mucoadhesive and mucus hydration properties of the liposomes. Table 7.1.5 summarises the compositions of coated liposomes.

Table 7.1.5 Concentrations of the polymers used in preparation of new batches of polymer-coated liposomes.

Type of liposomes	Type of polymers	Concentration of polymer (% w/v)
EggPC-DOTAP	HM-pectin	0.05
EggPC-DOTAP	LM-pectin	0.10
EggPC-DOTAP	AM-pectin	0.05
EggPC-EggPG	Chitosan	0.04

7.2 Estimation of the mucoadhesive properties of the liposomal formulations

The unimodal size distribution and the mean size change are shown in Table 7.2.1. The size measurements done before and after mixing with 0.2 % mucin showed that naked EggPC-DOTAP liposomes and the 0.04 % chitosan-coated EggPC-EggPG liposomes mixed with mucin obtained the largest size (Increase of size: 96 nm and 115 nm, respectively) compared to the other samples. The 0.05 % HM-pectin-coated liposomes and the uncoated negatively charged EggPC-EggPG liposomes had the least size (Increase of size: 6 and 10 nm, respectively). The size increase observed after the mixing with mucin indicated that there was an interaction between the samples and mucin. The larger size increase for EggPC-DOTAP liposomes and chitosan-coated liposomes indicated that there was a higher degree of interaction with mucin as compared to the negatively charged formulations.

The 0.01 % LM-pectin-coated liposomes and the 0.05 % AM-pectin-coated liposomes showed a small increase in size after the mixing with mucin in phosphate buffer. This increase in size was not significantly different from the size increase observed for the HM-pectin-coated liposomes and the uncoated EggPC-EggPG liposomes.

Table 7.2.1 Change of unimodal size after the mixing of liposomal formulation with 0.2 % (w/w) mucin in phosphate buffer pH 6.8.

	Before mixing with mucin		After mixing with mucin		
Sample	Unimodal size (nm)	P.I	Unimodal size (nm)	P.I	Mean unimodal size change (nm)
EggPC-DOTAP liposomes	150	0.188	242	0.144	
	151	0.134	264	0.178	96
	150	0.185	234	0.209	
HM-pectin-coated liposomes	352	0.279	345	0.236	
	357	0.245	365	0.284	6
	351	0.172	369	0.212	
LM-pectin-coated liposomes	233	0.196	262	0.212	
	230	0.123	262	0.235	27
	234	0.199	255	0.208	
AM-pectin-coated liposome	275	0.262	265	0.189	
	284	0.266	316	0.286	21
	276	0.305	318	0.279	
EggPC-EggPG liposomes	131	0.127	142	0.265	
	132	0.130	141	0.302	10
	133	0.093	142	0.272	
Chitosan-coated liposomes	376	0.295	479	0.697	
	382	0.294	504	0.798	115
	380	0.264	501	0.733	

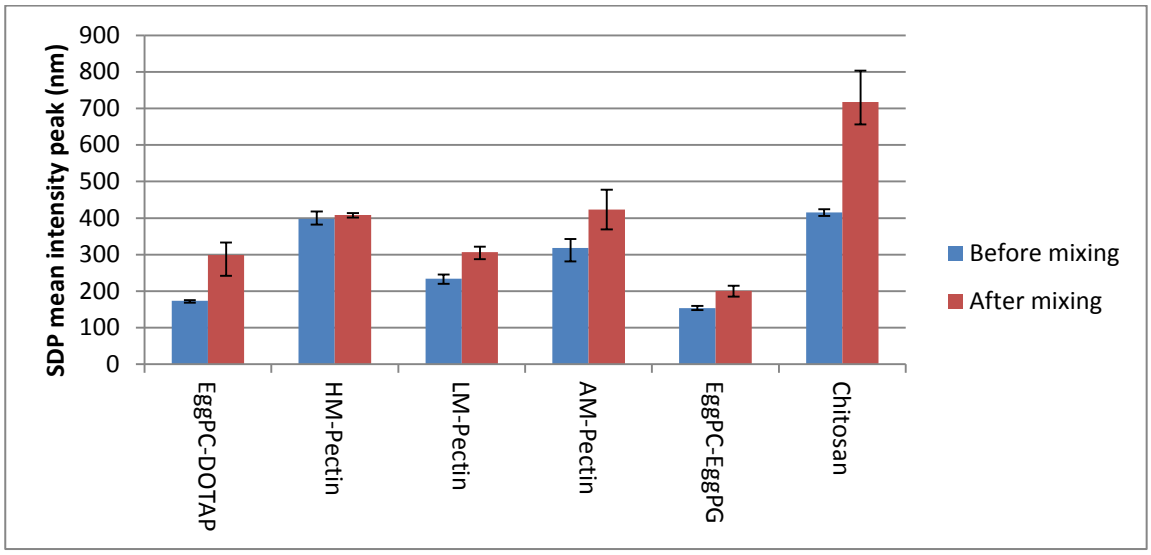

Figure 7.2.1 SDP Intensity analysis of the interaction of liposomes with mucin in phosphate buffer.

The strong interaction between the chitosan-coated liposomes and the positively charged uncoated EggPC-DOTAP liposomes with negatively charged mucin is due to strong electrostatic interactions. The change of size of the chitosan-coated liposomes after the mixing with mucin in phosphate buffer pH 6.8 was significant ($p < 0.05$). This significant change of size as a result of mixing with mucin was also observed for the positively charged, uncoated EggPC-DOTAP liposomes. Both these samples also became visually turbid after mixing with mucin, in contrast to all the other formulations which remained visually transparent.

The zeta potential of the pure mucin, the uncoated liposomes, and the polymer-coated liposomes was measured before and after mixing with mucin in phosphate buffer pH 6.8 (Figure 7.2.3). The 0.2 % mucin in phosphate buffer pH 6.8 had a negative charge and all the liposomal formulations mixed with mucin had a negative charge resembling that of pure mucin. It was interesting to investigate if the zeta potential of the liposomes and polymer-coated liposomes after mixing with mucin were the same as for pure mucin. When statistically analysed only the LM-pectin-coated liposomes and the uncoated EggPC-DOTAP liposomes had a significant difference in zeta potential from the zeta potential of pure mucin. The rest of the samples were all similar in zeta potential to pure 0.2 % mucin (95 % CL; $p < 0.05$). For the LM-pectin the zeta potential curves were not uniform, containing several peaks and indicating low mucoadhesive properties.

The uncoated EggPC-DOTAP liposomes, after coating with mucin, were expected to have approx. equal zeta potential as the unmixed mucin. Although this was not observed, one can still conclude that a mucin layer exists on the surfaces of liposomes as the surface charge changed from a positive to a negative value.

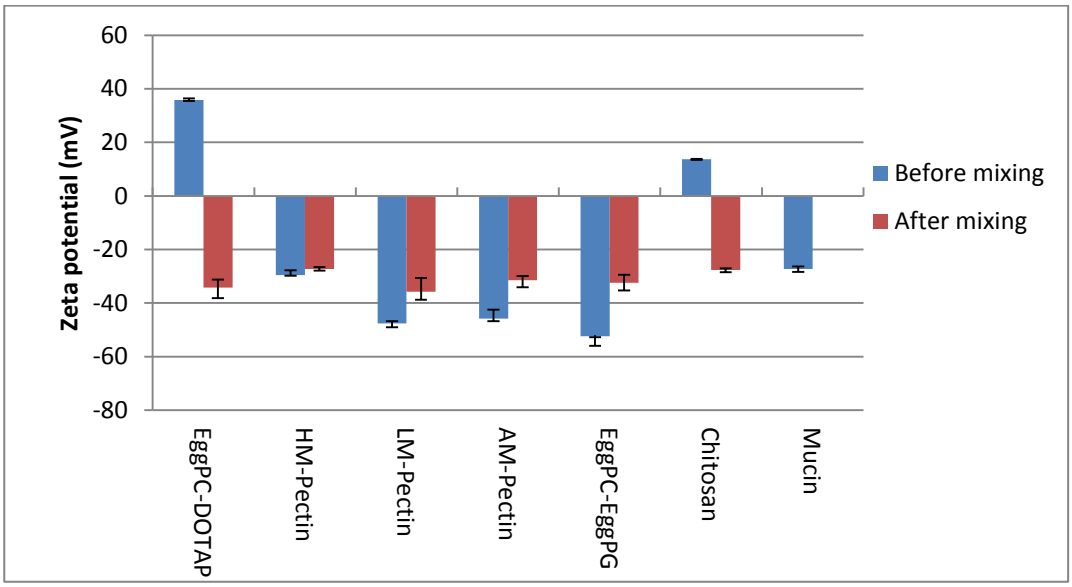

Figure 7.2.2 Measurements of zeta potential of samples prior and following mixture with mucin in solution.

Positively charged chitosan-coated liposomes and positively charged uncoated EggPC-DOTAP liposomes interacted the most with the negatively charged mucin. This was probably due to electrostatic interactions. Thus, these positively charged formulations seem to have the greatest potential for attachment to the mucosa. The mucoadhesive properties of chitosan have also been reported earlier (5, 9, 26, 37).

The ability of pectin to interact with mucus is conflicting, as mentioned earlier. As pectin is a hydrophilic polymer containing H-bonding groups (in form of carboxyl groups), one could expect formation of H-bond with mucin. This has been proposed as a mechanism for adhesion to mucus (22). Several studies (10, 11) reported conflicting results demonstrating mucoadhesiveness of pectin and no adhesion ability. In order to be able to conclude on the mucoadhesive properties of pectin one must consider several important factors as described by Sriamornsak et al. (78) such as the degree of hydration and physical properties of the pectin types, including DM and Mw. A positive change of rheological parameters (increased viscosity) after mixing of pectin with mucin is proposed to indicate physical entanglement between pectin and mucin.

In the current study, a small but not significant, difference was observed between HM-pectin and LM- and AM-pectin. The HM-pectin showed less mucoadhesive properties than the other two types. This has also been reported by Scmidgall and Hensel (79), who studied the mucoadhesive properties of pectin on colonic mucus. This might be due to the difference in the degree of methoxylation. HM-pectin has less free carboxylic acid functional groups that can facilitate the interaction with mucin, compared to the other two types of pectin.

For future studies, the following issues should be taken into account;

- Investigation of the mucoadhesive properties of pectin in different environment (i.e. pH of the medium, ionic strength) and compare these to the findings reported by Sriamornsak et al. (78).

- Investigation of other polymers (i.e. Eudragit) for mucoadhesive properties. Karn et al. (5) showed that Eudragit, dependant of type, had good mucoadhesive properties. The author used a different method for the preparation of the liposomes, the coating of liposomes and for the determination of the interaction abilities with mucin.

- In the current study only charged particles were investigated, hence only electrostatic forces were set to determine the grade of interaction. Including neutral particles might help understand other mechanisms that are required for interaction between liposomal formulations and mucin.

7.3 Estimation of the hydration properties of liposomal formulations

The liposomal formulations (Table 7.1.5), both positively charged uncoated EggPC-DOTAP liposomes and negatively charged uncoated EggPC-EggPG liposomes, were analysed for their mucin hydrating properties with the newly developed method described in Chapter 6. In Figures 7.3.1-7.3.6, the mass change measurements of uncoated liposomes and polymer-coated liposomes are shown. One representative curve for each formulation is included in the figures. As seen in the figures the same pattern of the mass decrease was seen for all samples. However, the time frames of the mass changes were varying. Some of the samples reached a stable mass in less than 400 minutes, while some took longer time. These differences suggest that there was a difference in the hydration properties of the formulations.

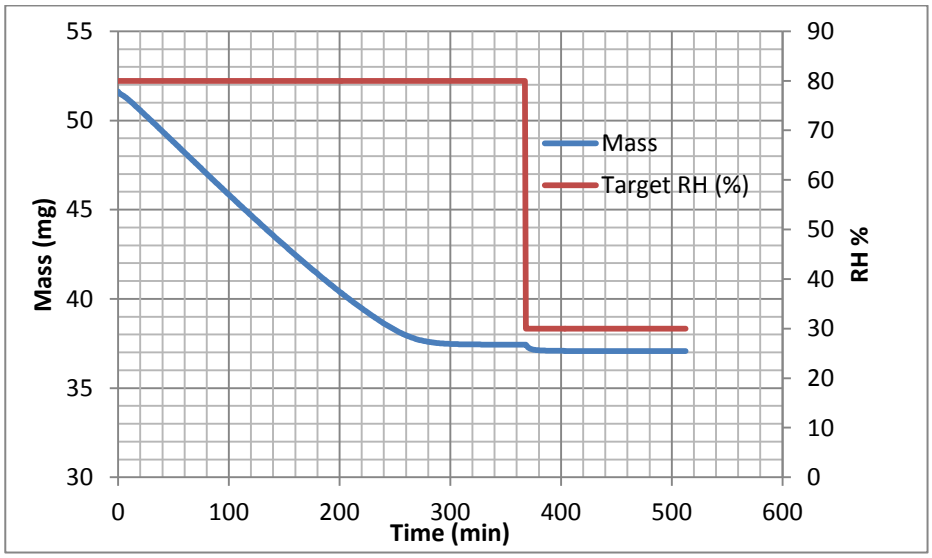

Figure 7.3.1 DVS Intrinsic analysis of uncoated EggPC-DOTAP liposomes, RH stages from 80 – 30 RH %.

In Figure 7.3.1 one can see that after 368 minutes the mass of uncoated EggPC-DOTAP liposomes reached a stable state. When the RH was decreased to 30 %, the mass decreased and the new mass stability was reached after 512 minutes leading to the end of the experiment.

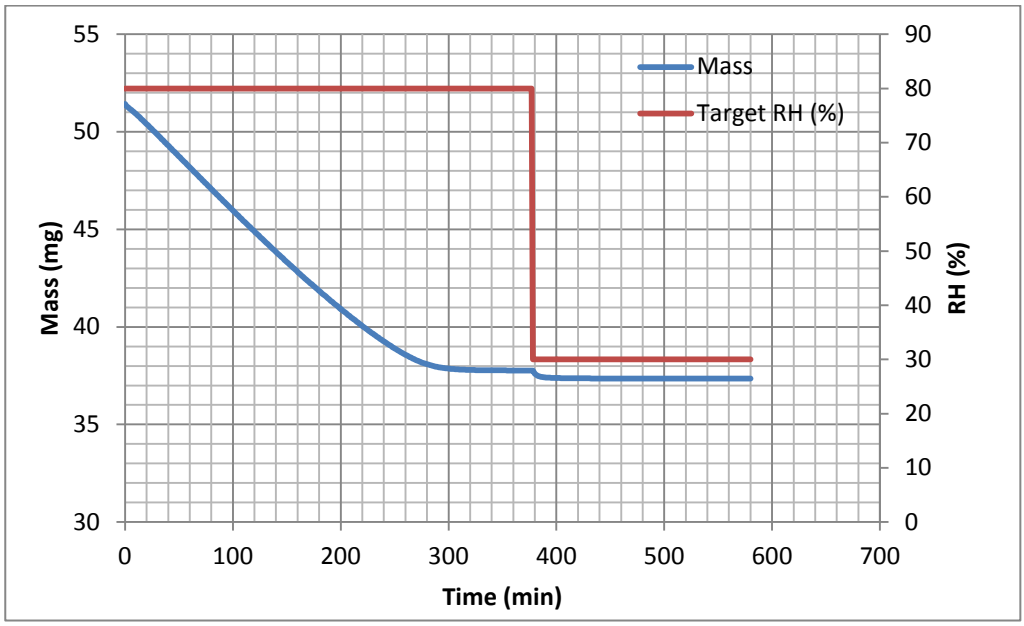

Figure 7.3.2 DVS Intrinsic analysis of the HM-pectin-coated EggPC-DOTAP, RH stages from 80-30 RH %.

For HM-pectin-coated EggPC-DOTAP liposomes, as shown in Figure 7.3.2, the first stage was finished after 378 minutes, and the whole experiment ended after 580 minutes. Compared to the mass change measurements of uncoated EggPC-DOTAP, the HM-pectin-coated liposomes required longer time to stabilise after the change of the RH % stage.

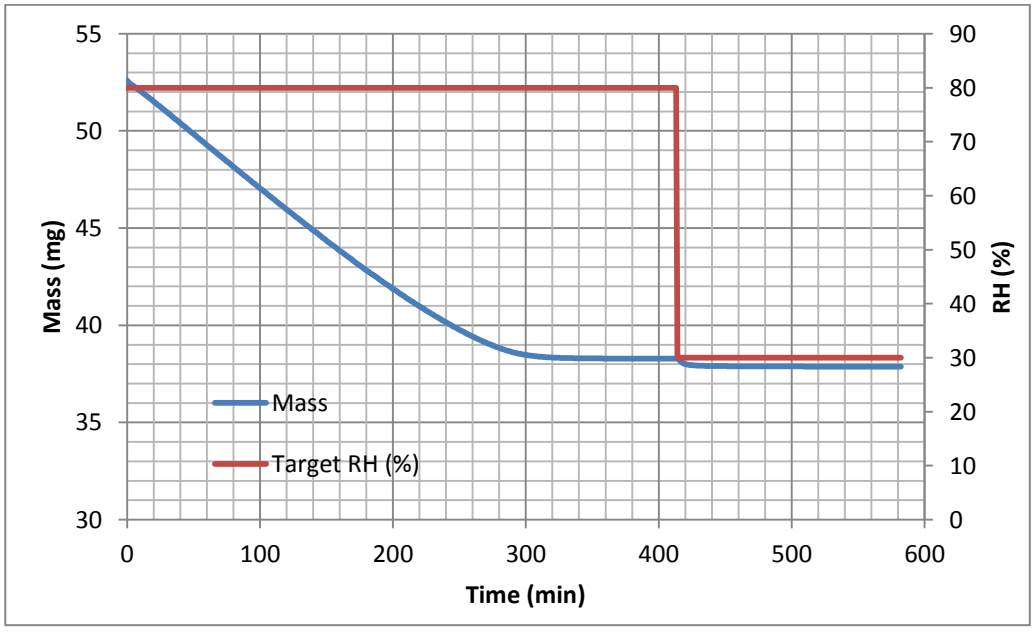

Figure 7.3.3 DVS Intrinsic analysis of the LM-pectin-coated EggPC-DOTAP, RH stages from 80-30 RH %

The LM-pectin-coated EggPC-DOTAP liposomes (Figure 7.3.3) also required longer time before the mass was stabilised. The RH % stage was changed from 80 to 30 % RH after 414 minutes and the whole experiment ended after 582 minutes.

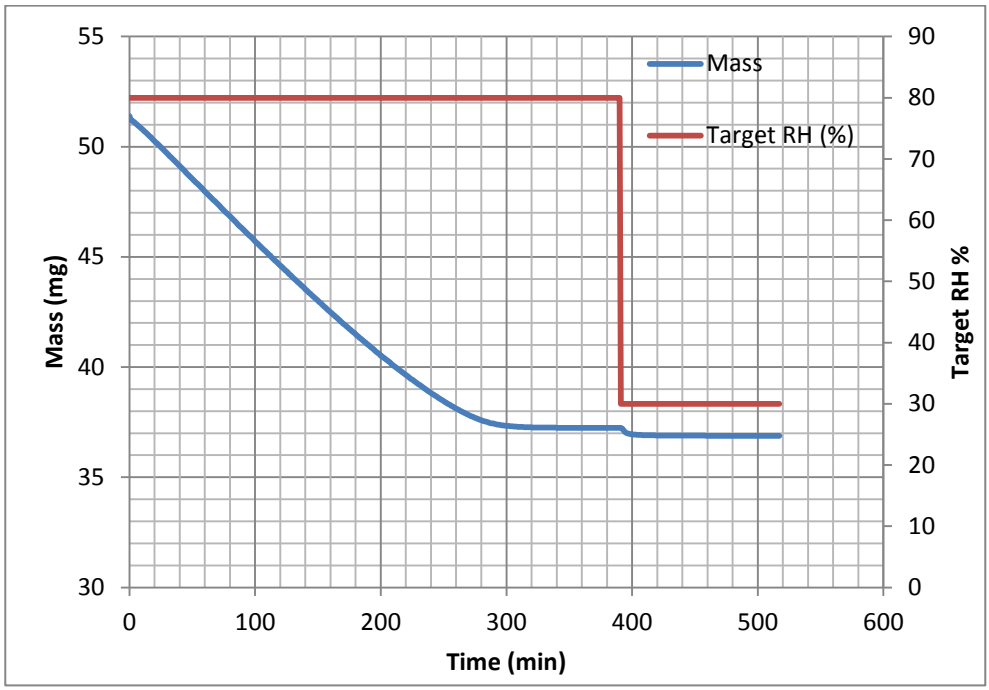

Figure 7.3.4 DVS Intrinsic analysis of the AM-pectin-coated EggPC-DOTAP; RH stages from 80-30 RH %

In Figure 7.3.4 the mass change measurements of AM-pectin-coated EggPC-DOTAP liposomes are shown. The first RH stage was finished after 391 minutes as the mass readings reached a stable state. The experiment as whole ended after 517, similar to the experiment with uncoated EggPC-DOTAP liposomes.

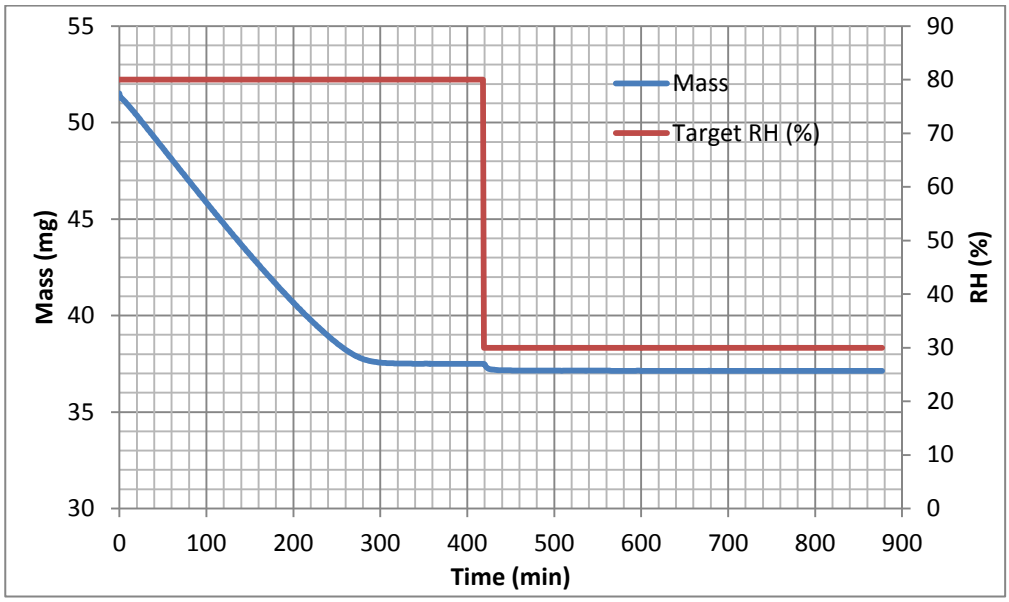

Figure 7.3.5 DVS Intrinsic analysis of uncoated EggPC-EggPG liposomes, RH stages from 80-30 RH %

The uncoated EggPC-EggPG liposomes as seen in Figure 7.3.5, reached a stable mass reading after 418 minutes, leading to the change of RH stage. After the change of RH %, the sample used longer time to stabilise again at 30 % RH. The experiment was finally ended after 877 minutes. The chitosan-coated liposomes, shown in Figure 7.3.6, reached the mass stability demand after 428 minutes and the experiment ended after total of 653 minutes.

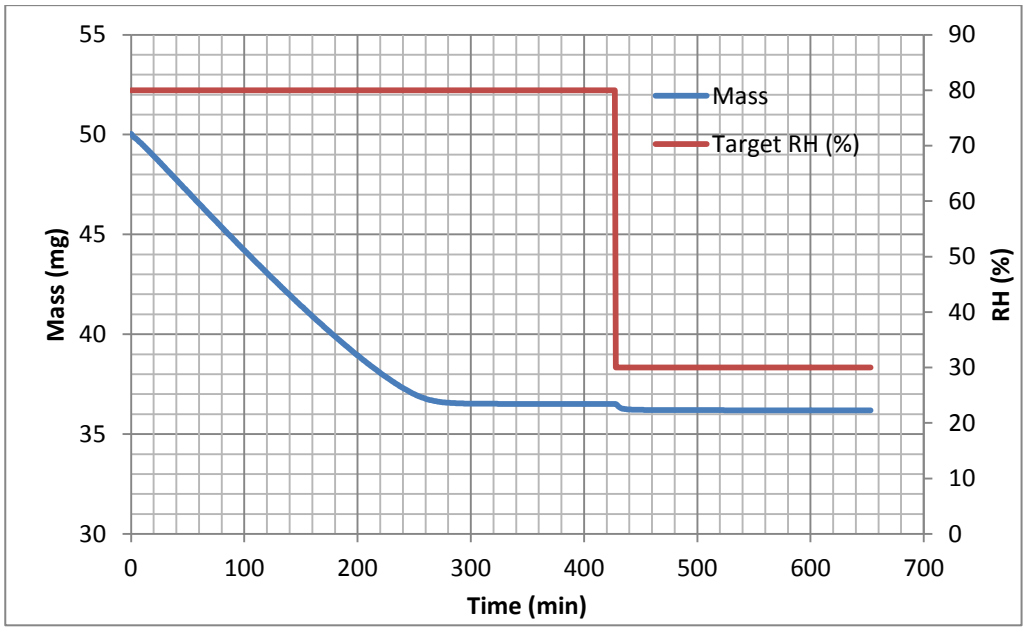

Figure 7.3.6 DVS Intrinsic analysis of the chitosan-coated EggPC-EggPG, RH stages from 80-30 RH %.

Some of the observed differences may be due to disturbances from the external environment, such as vibration of the table on which the instrument was situated. The instrument has high analytical sensitivity and thus is prone to external influence/stimulus that can lead to disturbances. Another factor that can affect the time it takes to stabilise includes the time it took for an experiment to start. At the beginning of each experiment the instrument had to be calibrated. The calibration of the instrument required stable mass readings of an empty pan and maintenance of the stable state for at least five minutes together with stable RH % and temperature measurements. It was observed that if a new sample was examined a short time after the previous sample, the calibration time was shorter as compared to the situation when there was a time gap between the sample measurements.

Despite the mentioned external factors that could potentially affect and mislead in the investigation, there is clear indication that the abilities of the different liposomal formulations play an important role in the time frame differences. Looking at the time needed for each sample to reach stable mass readings, both prior to and after the shift of RH %, one can see that the time frame is different for each sample.

Comparison of the time constant of the mass decrease for the different liposomal formulations

The time constant for each formulation was calculated at the dip appearing in the mass curve when the RH % was changed from 80 to 30 % RH as shown in Figure 7.3.7.

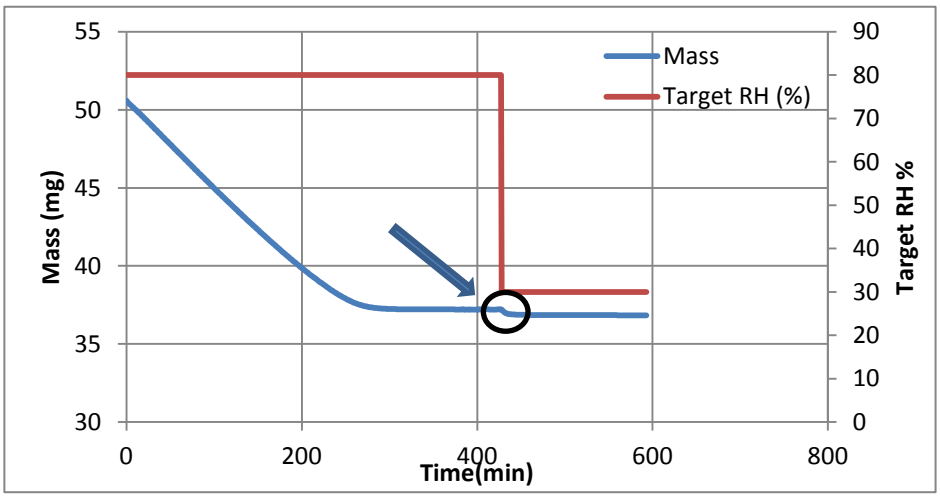

Figure 7.3.7 Illustration of which time interval that the time constant is calculated from. The measurements are from phosphate buffer added to mucin film

The results for the time constant calculations showed no significant difference between the samples, except that the time constant for the LM-pectin-coated liposomes was significantly ($p < 0.05$) higher than the time constant for the chitosan-coated liposomes. A higher value of the time constant in this scenario means that the LM-pectin-coated liposomes have a higher ability to bind and maintain the bond with water molecules. This might be due to the difference between the coating concentrations of the polymers used to coat the liposomes. The EggPC-DOTAP liposomes were coated with 0.1 % LM-pectin and the EggPC-EggPG liposomes were coated with 0.04 % chitosan. Another factor is the structural formula of LM-pectin and chitosan where amount of groups able to bind water molecules through H-bonds might play a role. However, with this theory one would expect a significant difference between all pectin types and chitosan and not only for the LM-pectin.

As seen in Figure 7.3.8, phosphate buffer seemed to have a time constant equal to the polymer-coated liposomes, indicating no difference in the impact of the mass change, hence no increased hydration compared to phosphate buffer.

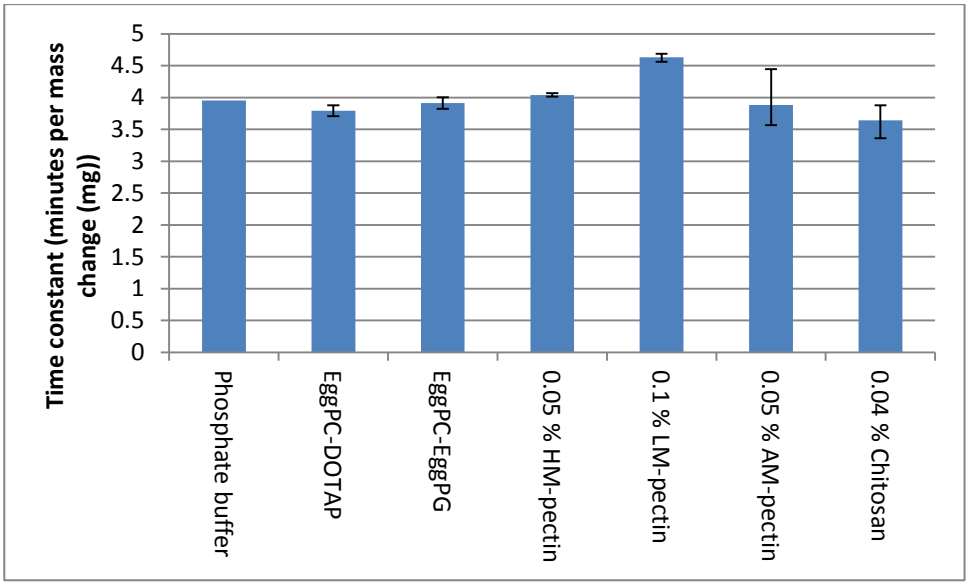

Figure 7.3.8 Time constant of mass change from the DVS Intrinsic analysis

As described in Chapters 4.6.2 and 5.4 the time constant of the mass change was calculated based on the 30 first measurements after the shift of the RH % stage. This means that only 30 minutes of

the event course is analysed. Including only these 30 minutes provides an idea on what is happening to the sample during that time.

Analysing the experiment outcomes in a different way might give different results. Including 50 measurements after the stage shift gave a different time constant for the samples (result not shown). This suggests that the time constant alone is not sufficient for the investigation of the ability to hold on to water molecules during a decrease in the RH %. One must consider the whole course of the experiment to understand the behaviour of the samples.

What can be seen (Figure 7.3.6) from the mass change measurements is that in the beginning there is a large loss of mass. This is thought to be due to the unbound water in the sample (i.e. solvent), which is in the surrounding of the polymer-coated liposomes. When the mass measurements reach a stable state, one can hypothesise that in the aluminium pan there are mucin and the polymer-coated liposomes containing the phosphate buffer that was entrapped during preparation of the liposomes. When the RH % stage is changed from 80 to 30 %, a new loss of mass is recorded. This loss is quite small and the amount is thought to be partly the phosphate buffer which is entrapped within the liposomal structure and the phosphate buffer trapped between the liposomal surface and the polymeric layer, as the dehydration of polymers can lead to change in the structural conformation of the polymers (80).

An additional issue that also has to be taken into consideration is the concentration of liposomes in which the phosphate buffer is entrapped. In the current study 15 µl sample was added to the mucin films. The samples contained 3 mM liposomes were diluted 1 to 4 with polymers. This means that, in the aluminium pans very small amount of lipids were present. Increasing the liposome concentration might result in better results in terms of being able to determine a difference between liposomal formulations and pure phosphate buffer.

For future investigations, the following issues are recommended to be examined in more details:

- Calculate the time constant for each event and include them in a multi time constant analysis as suggested for human skin (13, 69, 73)

- Increase the concentration of liposomes, which can be done either prior to the coating procedure, or the already prepared polymer-coated liposomes can be up-concentrated by centrifugation and resolving in in a small amount of buffer.

Summary of discussed observations from Chapter 7

Understanding the different properties of liposomal formulations facilitates the further development towards a formulation that can be applied for the hydration of the oral mucosa. As a step towards this purpose one must first consider the preparation of liposomes. Complete polymer coating of liposomes a prerequisite for further investigations of the liposomal properties in the current study. The purpose of the first part of this study was to stipulate the polymer concentration necessary for complete coating of the liposomal surfaces. As the study aimed to investigate the properties of the liposome-polymer complexes, excess polymer in the formulation was undesirable as it can result in destabilisation of the complexes (37).

As shown in other studies (9, 39) uncoated liposomes tend to aggregate. Coating of the liposomes with correct concentration of polymer showed to stabilise liposomes. Smistad et al. (39) showed that the pectin types HM-, LM-, and AM-pectin increased the stability of the liposomes, with HM- and LM-pectin as the better option. In the SDP intensity analysis all pectin types showed the same pattern in respect to size; too low concentration of the polymer (0 – 0.01 %) lead to a bigger size and size distribution as compared to the other polymer concentrations (0.05 - 0.2 %) reaching a plateau phase. Continuing the increase of the polymer concentration lead again to bigger size of

the particles as a result of desorption of excess polymer and flocculation as also described by Laye et al.(37).

The turbidity measurements confirmed the SDP size analysis, where the samples with the biggest particles were more turbid than the samples containing smaller particles. The lowest polymer concentrations showed less transmitting ability, hence more turbid samples. The coating of the liposomes with polymers led to a shift of charge indicating the existence of a polymeric layer on the liposomal surface (Figures in Chapter 7.1).

Chitosan-coated liposomes behaved similarly to pectin-coated liposomes. The lowest concentration used for coating (0.01 %) resulted in large size distribution (broad error bar) due to the aggregation of the particles. As seen for the pectin, increasing the concentration of chitosan resulted in more acceptable size and size distribution. The size was about the same for the polymer concentration range between 0.04 - 0.16 % when the samples were freshly prepared (Figure 7.1.19). Transmittance measurements confirmed the SDP size analysis and the zeta potential of all chitosan-containing liposomes was positive indicating presence of a polymeric layer on the liposomal surface. Therefore, the lower concentration of polymer was selected (Table 7.1.5).

The use of mucoadhesive polymers in the modification of the liposomal surface has shown to increase the liposomal ability to attach to the mucosal surface and increase the interaction and prolong the residence time at the site of administration (9, 17). Positively charged uncoated liposomes and chitosan-coated liposomes had stronger interaction with mucin in phosphate buffer pH 6.8 as compared to other liposomes. This was probably due to the electrostatic interaction between the positively charged liposomes and polymer-coated liposomes, and the negatively charged mucin at pH 6.8. The mucoadhesive properties of the pectin are difficult to categorise as some studies showed a mucoadhesive potential while others failed to do so. Despite that, as the zeta potential measurements after mixing with mucin was similar to those obtained for pure mucin, it could be concluded that the pectin might have a potential and needs to be investigated more thoroughly as mucoadhesive polymer.

Establishing a method that can facilitate the investigation of the hydration abilities of liposomal formulation could provide the information needed to move one step further in the development of

liposomal formulations for hydration of the oral mucosa. In this study, a method based on mass change measurements of a model mucosa when exposed to the changes in relative humidity, was investigated. The results indicate that the method has a potential in the determination of the hydrating properties of liposomal formulations. However, the optimisation of the method and the way of analysis of the results need be continued. Increasing the amount of lipids in the sample might be one way to increase the sensitivity of the method. Another amendment that can be suggested is the change in the number of measurements included in the calculation of the time constant. Other researchers have been able to calculate the time constant of larger time frame in order to explain the events during the sorption/desorption of water vapour using human skin (73).

Once the method is optimised to lead to satisfactory results, the correlation between the mucoadhesive properties, estimated by the interaction with mucin, and the hydration abilities can be investigated. In the current study a correlation was limited due to the aforementioned reasons. In addition, the concentration of the lipids in the experiments was probably too low to enable to differentiate between the different liposomal formulations.

8 Conclusions

Uncoated and polymer-coated liposomes with positive and negative charge were successfully prepared. The formulations with the optimal coating concentration of the polymers were determined to be further investigated for their mucoadhesive and potential hydrating properties. For the HM-pectin and AM-pectin types 0.05 % (w/v) showed to be the optimal concentration that gave reproducible and complete coating of the positively charged liposomes while for LM-pectin 0.1 % (w/v) was sufficient. For chitosan, 0.04 % (w/v) was found to be the optimal for the complete coating of the negatively charged liposomes.

Based on the estimation of the mucoadhesive properties of liposomal formulations, the uncoated positively charged EggPC-DOTAP liposomes and chitosan-coated EggPC-EggPG liposomes showed to interact more strongly with mucin in solution. These formulations seem to have a greater potential for adhesion to the oral mucosa than the negatively charged liposomal formulations (EggPC-EggPG- and pectin-coated EggPC-DOTAP).

The new method, based on the measuring of the mass changes of mucin films when exposed to changes of the relative humidity, was developed. With this method the sorption and desorption of water, during the changes of the relative humidity, was successfully measured in form of the mass change of the mucin film. However, to be able to investigate the correlation between the mucoadhesiveness and the hydrating properties of the liposomal formulations further optimisation of the method (i.e. increase of the concentration of lipids in the formulations) is needed.

9 Future aspects

In the current study, only the charged particles were investigated, hence only electrostatic forces were set to determine the grade of interaction between the charged liposomal formulations and mucin. Including the neutral particles might help understand other mechanisms that are required for the interaction between liposomal formulations and mucin.

Studies performed on the mucoadhesive properties of pectin are to a certain degree conflicting. This is probably caused by the difference in the methodology of the studies. Therefore, investigating the mucoadhesive properties of pectin in a different environments (i.e. pH of the medium, ionic strength) and compare these in a manner proposed by Sriamornsak et al. (78) would improve the understanding of the ability of pectins to attach to mucus.

Based on the observations and experiences obtained from this project and literature study, the next steps in order to develop a new method for investigating the hydration properties of liposomal formulations would be to optimize the conditions. The most interesting parameters to investigate are the concentration of lipids in the liposomal formulations and the determination of the time constant of the mass change. Once the method and the conditions of the experiment are optimised, other liposomal formulations can be included in respect to the hydration properties.

10 List of references

1. 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* [Internet]. 2013. Available from: http://www.sciencedirect.com/science/article/pii/S0928098713000997.

2. Legemiddelhandoka.no. http://legemiddelhandboka.no/terapi/16055 2013. Visited 20.10.2012].

3. Tidsskriftet.no. http://tidsskriftet.no/article/1156293 2013. visited 20.10.2012].

4. Hoogstraate J. L. Oral Transmucosal Drug Delivery. New York: Taylor & Francis Group; 2001.

5. Karn PR, Vanic Z, Pepic I, Skalko-Basnet N. Mucoadhesive liposomal delivery systems: the choice of coating material. *Drug Development and Industrial Pharmacy*. 2011 Apr;**37**(4):482-8.22.

6. Lasic DD. Liposomes: From physics to applications. 1 ed. Amsterdam: Elsevier Science B.V; 1993. 28 p.

7. Grit M, Crommelin DJ. Chemical stability of liposomes: implications for their physical stability. *Chemistry and Physics of Lipids*. 1993 Sep;**64**(1-3):3-18.

8. Taylor TM, Davidson PM, Bruce BD, Weiss J. Liposomal nanocapsules in food science and agriculture. *Critical Reviews in Food Science and Nutrition*. 2005;**45**(7-8):587-605.

9. Filipovic´-Grcic´ J, Škalko-Basnet N, Jalsenjak I. Mucoadhesive chitosan-coated liposomes: characteristics and stability. *Journal of Microencapsulation*. 2001;**18**(1):3-12.

10. Klemetsrud T, Jonassen H, Hiorth M, Kjøniksen A-L, Smistad G. Studies on pectin-coated liposomes and their interaction with mucin. *Colloids and Surfaces B: Biointerfaces*. 2013 3/1/;**103**(0):158-65.

11. Sande SA. Pectin-based oral drug delivery to the colon. *Expert Opinion on Drug Delivery*. 2005 May;**2**(3):441-50.

12. Kas HS. Chitosan: Properties, preparations and application to microparticulate systems. *Journal of Microencapsulation*. 1997;**14**(6):689-711.

13. Johnsen GK, Haugsnes AB, Martinsen OG, Grimnes S. A new approach for an estimation of the equilibrium stratum corneum water content. *Skin Research and Technology* : official journal of International Society for Bioengineering and the Skin (ISBS) [and] International Society for Digital Imaging of Skin (ISDIS) [and] International Society for Skin Imaging (ISSI). 2010 May;**16**(2):142-5.

14. Budowick M, Bjålie JG, Rolstad B, Toverud KC. Anatomisk atlas. 7. Finland: WS Boodwell; 2003. p. 230-1.

15. Nanci A. Ten Cate's Oral Histology: Developement, Structure, and Function: Elsevier Health Sciences; 2007. 432 p.

16. Salamat-Miller N, Chittchang M, Johnston TP. The use of mucoadhesive polymers in buccal drug delivery. *Advanced Drug Delivery Reviews*. 2005 11/3/;**57**(11):1666-91.

17. de Vries ME, Boddé HE, Verhoef JC, Junginger HE. Developments in buccal drug delivery. *Critical Reviews in Therapeutic Drug Carrier Systems*. 1991 /;**8**(3):271-303.

18. Rojanasakul Y, Wang L-Y, Bhat M, Glover DD, Malanga CJ, Ma JKH. The Transport Barrier of Epithelia: A Comparative Study on Membrane Permeability an Charge Selectivity in the Rabbit. *Pharmaceutical Research*. 1992;**9**(8):5.

19. Gandhi RB, Robinson JR. Oral cavity as a site for bioadhesive drug delivery. *Advanced Drug Delivery Reviews*. 1994 1//;**13**(1–2):43-74.

20. Felleskatalogen.no. http://www.felleskatalogen.no/medisin/ 2013. Visited 09.2.2013].

21. Collins LMC, Dawes C. The Surface Area of the Adult Human Mouth and Thickness of the Salivary Film Covering the Teeth and Oral Mucosa. *Journal of Dental Research*. 1987 August 1, 1987;**66**(8):1300-2.

22. Lee JW, Park JH, Robinson JR. Bioadhesive-based dosage forms: the next generation. *Journal of pharmaceutical sciences*. 2000 Jul;**89**(7):850-66.

23. Smart JD. The basics and underlying mechanisms of mucoadhesion. *Advanced Drug Delivery Reviews*. 2005 11/3/;**57**(11):1556-68.

24. Serra L, Doménech J, Peppas NA. Engineering design and molecular dynamics of mucoadhesive drug delivery systems as targeting agents. *European Journal of Pharmaceutics and Biopharmaceutics*. 2009 3//;**71**(3):519-28.

25. Svensson O, Arnebrant T. Mucin layers and multilayers — Physicochemical properties and applications. *Current Opinion in Colloid & Interface Science*. 2010 12//;**15**(6):395-405.

26. Svensson O, Thuresson K, Arnebrant T. Interactions between chitosan-modified particles and mucin-coated surfaces. *Journal of Colloid and Interface Science*. 2008 9/15/;**325**(2):346-50.

27. Allen TM. Long-circulating (sterically stabilized) liposomes for targeted drug delivery. *Trends in Pharmacological Sciences*. 1994 7//;**15**(7):215-20.

28. Anderson M, Omri A. The effect of different lipid components on the in vitro stability and release kinetics of liposome formulations. *Drug Delivery*. 2004 Jan-Feb;**11**(1):33-9.

29. Storm G, Crommelin DJA. Liposomes: quo vadis? *Pharmaceutical Science & Technology Today*. 1998 4/1/;**1**(1):19-31.

30. Subramaniam R, Xiao Y, Li Y, Qian SY, Sun W, Mallik S. Light-mediated and H-bond facilitated liposomal release: the role of lipid head groups in release efficiency. *Tetrahedron Letters*. 2010 1/20/;**51**(3):529-32.

31. Egbaria K, Weiner N. Liposomes as topical drug delivery system. *Advanced Drug Delivery Reviews*. 1990 (5):**13**.

32. Crommelin, Bos GW, Storm G. Liposomes - Successful Carrier Systems for Targeted Delivery of Drugs. *Drug Delivery* - Peptides, Proteins & Liposomes. 2003.

33. Sagristá ML, Mora M, Madariaga MAD. surface modified liposomes by coating with charged hydrophilic molecules. *Cellular & Molecular Biology Letters*. 1999;**5**:14.

34. Gregoriadis G, Florence AT. Liposomes in drug delivery. Clinical, diagnostic and ophthalmic potential. *Drugs*. 1993 01/;**45**(1):15-28.

35. Campbell PI. Toxicity of some charged lipids used in liposome preparations. *Cytobios*. 1983;**37**(145):21-6.

36. van Rooijen N, van Nieuwmegen R. Liposomes in immunology: multilamellar phosphatidylcholine liposomes as a simple, biodegradable and harmless adjuvant without any immunogenic activity of its own. *Immunological Communications*. 1980;**9**(3):243-56.

37. Laye C, McClements DJ, Weiss J. Formation of Biopolymer-Coated Liposomes by Electrostatic Deposition of Chitosan. *Journal of Food Science*. 2008;**73**(5):N7-N15.

38. Scherphof G, Morselt H, Regts J, Wilschut JC. The involvement of the lipid phase transition in the plasma-induced dissolution of multilamellar phosphatidylcholine vesicles. *Biochimica et Biophysica Acta (BBA) - Biomembranes*. 1979 9/21/;**556**(2):196-207.

39. Smistad G, Boyum S, Alund SJ, Samuelsen ABC, Hiorth M. The potential of pectin as a stabilizer for liposomal drug delivery systems. *Carbohydrate Polymers*. 2012 Oct 15;**90**(3):1337-44.

40. Lichtenberg D, Barenholz Y. Liposomes: Preparation, Characterization, and Preservation. *Methods of Biochemical Analysis*: John Wiley & Sons, Inc.; 2006. p. 337-462.

41. Olson F, Hunt CA, Szoka FC, Vail WJ, Papahadjopoulos D. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. *Biochimica et Biophysica Acta*. 1979 10/;**557**(1):9-23.

42. Brandl M. Liposomes as drug carriers: a technological approach. *Biotechnology Annual Review*. Volume 7: Elsevier; 2001. p. 59-85.

43. Crommelin DJ, H. S. Liposomes. In: Kreuter J, editor. *Drug Delivery Systems*. New york, Basel, Hong Kong: Marcel Dekker Inc; 1994.

44. Barea MJ, Jenkins MJ, Lee YS, Johnson P, Bridson RH. Encapsulation of Liposomes within pH Responsive Microspheres for Oral Colonic Drug Delivery. *International Journal of Biomaterials*. 2012;2012:**8.**

45. Gregoriadis G. Drug entrapment in liposomes. *Federation of European Biochemical Societies letters*. 1973 Nov 1;**36**(3):292-6.

46. Lemarchand C, Gref R, Couvreur P. Polysaccharide-decorated nanoparticles. *European Journal of Pharmaceutics and Biopharmaceutics*. 2004 9//;**58**(2):327-41.

47. Wei H, Zhang X, Cheng C, Cheng S-X, Zhuo R-X. Self-assembled, thermosensitive micelles of a star block copolymer based on PMMA and PNIPAAm for controlled drug delivery. *Biomaterials*. 2007 1//;**28**(1):99-107.

48. Fukui Y, Fujimoto K. The preparation of sugar polymer-coated nanocapsules by the layer-by-layer deposition on the liposome. *Langmuir : the ACS Journal of Surfaces and Colloids*. 2009 Sep 1;**25**(17):10020-5.

49. Takeuchi H, Thongborisute J, Matsui Y, Sugihara H, Yamamoto H, Kawashima Y. Novel mucoadhesion tests for polymers and polymer-coated particles to design optimal mucoadhesive drug delivery systems. *Advanced Drug Delivery Reviews*. 2005 11/3/;**57**(11):1583-94.

50. Jenkins P, Sowden M. Depletion flocculation in colloidal dispersions. *Advances in Colloid and Interface Science*. 1996;68:39.

51. Wu ZH, Ping QN, Wei Y, Lai JM. Hypoglycemic efficacy of chitosan-coated insulin liposomes after oral administration in mice. *Acta pharmacologica Sinica*. 2004 Jul;**25**(7):966-72.

52. Takeuchi H, Yamamoto H, Niwa T, Hino T, Kawashima Y. Enteral Absorption of Insulin in Rats from Mucoadhesive Chitosan-Coated Liposomes. *Pharmaceutical Research*. 1996 1996/06/01;**13**(6):896-901.

53. Damgé C, Maincent P, Ubrich N. Oral delivery of insulin associated to polymeric nanoparticles in diabetic rats. *Journal of Controlled Release*. 2007 2/12/;**117**(2):163-70.

54. Sørensen I, Pedersen HL, Willats WG. An array of possibilities for pectin. *Carbohydrate Research*. 2009 09/01;**344**(14):1872-8.

55. Mohnen D. Pectin structure and biosynthesis. *Current Opinion in Plant Biology*. 2008 Jun;**11**(3):266-77.

56. Thakur BR, Singh RK, Handa AK. Chemistry and uses of pectin--a review. *Critical Reviews in Food Science and Nutrition*. 1997 Feb;**37**(1):47-73.

57. Tho I, Anderssen E, Dyrstad K, Kleinebudde P, Sande SA. Quantum chemical descriptors in the formulation of pectin pellets produced by extrusion/spheronisation. *European Journal of Pharmaceutical Sciences*. 2002 Aug;**16**(3):143-9.

58. Nguyen S, Alund SJ, Hiorth M, Kjoniksen AL, Smistad G. Studies on pectin coating of liposomes for drug delivery. *Colloids and Surfaces: Biointerfaces*. 2011 Dec 1;**88**(2):664-73.

59. Willats WG, Paul KJ, Dalgaard MJ, Pectin: newinsight into an old polymer are starting to gel. Kidlington, Royaume -uni: Elsevier; 2006.

60. Sriamornsak P. Chemistry of Pectin and Its Pharmaceutical Uses: A Review. *Silpakorn University International Journal. 2003*. (3):(1-2)

61. Bøyum S. Stabilitet av pektindekkede liposomer for lokal behandling av munnhulen: University of Oslo; 2010.

62. Hagesaether E, Hiorth M, Sande SA. Mucoadhesion and drug permeability of free mixed films of pectin and chitosan: An in vitro and ex vivo study. *European Journal of Pharmaceutics and Biopharmaceutics*. 2009 2//;**71**(2):325-31.

63. Henriksen I, Smistad G, Karlsen J. Interactions between liposomes and chitosan. *International Journal of Pharmaceutics*. 1994 1/25/;**101**(3):227-36.

64. Henriksen I, Vagen SR, Sande SA, Smistad G, Karlsen J. Interactions between liposomes and chitosan II: effect of selected parameters on aggregation and leakage. *International Journal of Pharmaceutics*. 1997 //;**146**(2):193-204.

65. Goncalves MC, Mertins O, Pohlmann AR, Silveira NP, Guterres SS. Chitosan coated liposomes as an innovative nanocarrier for drugs. *Journal of Biomedical Nanotechnology*. 2012 Apr;8(2):240-50.

66. Lasic D. Liposomes. American Scientist. 1992;**80**(1):20-31.

67. Egbaria K, Weiner N. Liposomes as a topical drug delivery system. *Advanced Drug Delivery Reviews*. 1990 9//;**5(**3):287-300.

68. Pavelić Ž, Škalko-Basnet N, Filipović-Grčić J, Martinac A, Jalšenjak I. Development and in vitro evaluation of a liposomal vaginal delivery system for acyclovir. *Journal of Controlled Release*. 2005 8/18/;**106**(1–2):34-43.

69. Martinsen OG, Grimnes S, Nilsen JK, Tronstad C, Jang W, Kim H, et al. Gravimetric method for in vitro calibration of skin hydration measurements. *IEEE Transactions on Biomedical Engineering*. 2008 Feb;**55**(2 Pt 1):728-32.

70. Martinsen OG, Grimnes S, Nilsen SH. Water sorption and electrical properties of a human nail. *Skin research and technology* : official journal of International Society for Bioengineering and the Skin (ISBS) [and] International Society for Digital Imaging of Skin (ISDIS) [and] International Society for Skin Imaging (ISSI). 2008 May;**14**(2):142-6.

71. Johnsen GK, Haugsnes AB, Martinsen OG, Grimnes S. Stratum corneum in vivo water content from TEWL-measurements. *Conference proceedings* : Annual International Conference of the IEEE Engineering in Medicine and Biology Society IEEE Engineering in Medicine and Biology Society Conference. 2008;2008:3166-9.

72. Mathematica W. http://reference.wolfram.com/mathematica/ref/FindFit.html 2013.

73. Johnsen GK, Norlen L, Martinsen ØG, Grimnes S. Sorption Properties of the Human Stratum Corneum. *Skin Pharmacology and Physiology*. 2011;**24**(4):190-8.

74. Kulichikhin VG, Elenskii AA, Kharlov AE, Shabanov MP, Yampol'skaya GP. Peculiarities of the surface crystallization of sodium chloride on mucin films. *Colloid Journal*. 2012 2012/04/01;**74**(2):207-14.

75. Guzey D, McClements DJ. Impact of Electrostatic Interactions on Formation and Stability of Emulsions Containing Oil Droplets Coated by β-Lactoglobulin−Pectin Complexes. *Journal of Agricultural and Food Chemistry*. 2006 2007/01/01;**55**(2):475-85.

76. Thirawong N, Thongborisute J, Takeuchi H, Sriamornsak P. Improved intestinal absorption of calcitonin by mucoadhesive delivery of novel pectin–liposome nanocomplexes. *Journal of Controlled Release*. 2008 2/11/;**125**(3):236-45.

77. Alund SJ, Smistad G, Hiorth M. A multivariate analysis investigating different factors important for the interaction between liposomes and pectin. *Colloids and Surfaces A*: Physicochemical and Engineering Aspects. 2013 3/5/;**420**(0):1-9.

78. Sriamornsak P. Application of pectin in oral drug delivery. *Expert Opinion on Drug Delivery*. 2011;**8**(8):1009-23.

79. Schmidgall J, Hensel A. Bioadhesive properties of polygalacturonides against colonic epithelial membranes. *International Journal of Biological Macromolecules*. 2002 Oct 1;**30**(5):217-25.

80. Zhang D, Ward RS, Shen YR, Somorjai GA. Environment-Induced Surface Structural Changes of a Polymer: An in Situ IR + Visible Sum-Frequency Spectroscopic Study. *The Journal of Physical Chemistry B*. 1997 1997/10/01;**101**(44):9060-4.