UiT
NORGES
ARKTISKE
UNIVERSITET

INSTITUTT FOR FARMASI

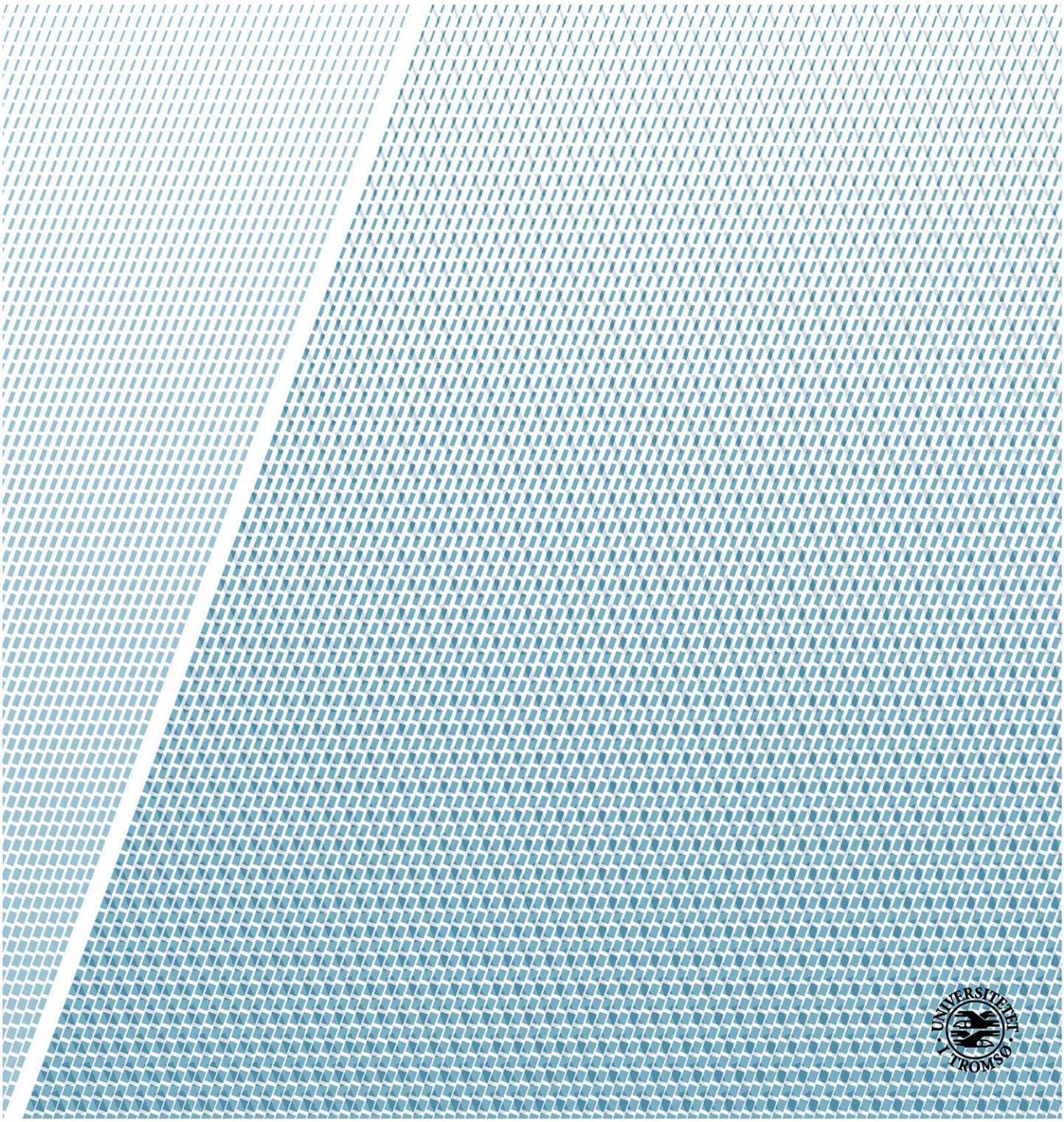
Polymer-coated liposomes for hydration of the oral mucosa

Investigation of water adsorption, retention, and release properties

—

Stina Sand Bowitz

Master thesis for the degree Master of Pharmacy, May 2014



Polymer-coated liposomes for hydration of the oral mucosa

Investigation of water adsorption, retention, and release properties

By

Stina Sand Bowitz

Department of Pharmacy

Faculty of Health Sciences

University of Tromsø

The Arctic University of Norway

2014

External Supervisors

Professor Gro Smistad

Associate Professor Marianne Hiorth

School of Pharmacy

Faculty of Mathematics and Natural Sciences

University of Oslo

Professor Ørjan Grøttem Martinsen

Department of Physics

Faculty of Mathematics and Natural Sciences

University of Oslo

Internal Supervisor

Professor Natasa Skalko-Basnet

Department of Pharmacy

Faculty of Health Sciences

University of Tromsø

The Arctic University of Norway

Acknowledgements

This master thesis was performed at the Department of Pharmacy, School of Pharmacy, University of Oslo, from October 2013 to May 2014.

My deep gratitude goes to Professor Gro Smistad for all the guidance, advice, help and support along the way. Encouragement always came when it was needed the most, and for that I will be forever grateful.

I would like to thank Professor Marianne Hiorth for her useful guidance and contribution early in the process. Professor Ørjan Grøttem Martinsen from the Department of Physics for his insights and knowledge, and for making me enjoy physics a bit more than first assumed. Also I would like to thank Head Engineer, Tove Larsen, for important training in the lab, her patience and help with all questions and problems.

Also I would like to express my gratitude to Professor Natasa Skalko-Basnet, University of Tromsø, Department of Pharmacy, for giving me the opportunity to carry out my thesis at the Department of Pharmacy, University of Oslo.

Of course I have to thank all my fellow students for making my years in Tromsø unforgettable, and a special thanks to Marthe Karoline Grønvold for carrying out some of the procedures I was prevented from doing due to my pregnancy.

I am forever grateful to my family and friends for all motivational support and love. Especially to my twin sister, Tonje Engebretsen Mortensen, who helped me a lot during this thesis and in other aspects of life.

To my boyfriend, and best friend, Jon Didrik Sand, I thank you for all your support, motivation and patience, and for always being there for me.

Finally I want to thank my daughter, Emma Sand Bowitz, for making this year the best year of my life!

Abstract

Ailments from a dehydrated mouth may result in problems with food intake, such as chewing and swallowing, with smiling and appearance, and some of them may cause pain. A system to increase hydration of oral mucosa will reduce patient ailments, or the frequency of them, and increase quality of life.

The focus of this thesis is to study the potential of polymer coated liposomal systems for hydration of the oral mucosa. To establish an *in vitro* method for determination of the water adsorption/retention capacity of liposomes and polymers by using the DVS method and to examine the release of a marker to determine different leakage profiles. Finally, to see if there is a possible correlation between the water adsorption/retention abilities and the release profiles.

The water adsorption/retention abilities of liposomes and polymers have been investigated with a DVS-Intrinsic apparatus. This was executed at 35 °C with a % RH ranging from 0-95 %. The release profiles of CF from EggPC/DOTAP coated with negative polymers and EggPC/EggPG and DPPC/DPPG coated with a positive polymer have been studied at 35 °C. Release from uncoated liposomes were determined as well.

The DVS Intrinsic studies showed that the uncoated liposomes display the same water adsorption/retention abilities. Of the different polymers LM Pectin had the highest adsorption and retention ability of water, although it was not significantly different. Alginate and Chitosan shared adsorption/retention properties of water. PNIPAAM had the lowest adsorption/retention ability, and was significantly different. The release studies showed that EggPC/DOTAP coated with LM Pectin had a higher release than uncoated or coated EggPC/DOTAP. EggPC/DOTAP coated with PNIPAAM had the lowest release profile, although never significantly different.

The liposome formulation that would be a good candidate for future xerostomia treatments is dependent on the release profile that is optimal for the purpose of hydrating a mouth. If a high release profile is desirable, EggPC/DOTAP coated with LM Pectin would be a good choice.

Abstract (Norwegian)

Plager fra en dehydrert munn kan føre til problemer med matinntak, som for eksempel å tygge og svelge, med smilet og utseendet, og noen av dem kan forårsake smerte. Et system for å øke hydreringen av slimhinnen i munnen vil redusere pasientens plager, eller frekvensen av dem, og øke livskvaliteten.

Fokuset i denne oppgaven er å studere potensialet til polymer coatede liposom systemer for hydrering av slimhinnen i munnen. Å etablere en in vitro metode for bestemmelse av liposomers og polymerers evne til å adsorbere og holde tilbake vann ved hjelp av DVS metoden, og å undersøke frigjøring av en markør for å bestemme forskjellige frigjøringsprofiler. Til slutt å se om det er en mulig korrelasjon mellom vanns adsorpsjon og retensjonsevner og disse frigjøringsprofilene.

Liposomers og polymerers evne til å adsorbere eller holde tilbake vann har vært undersøkt med et DVS-Intrinsic apparat. Dette ble utført ved 35 °C med en relativ fuktighetsprosent som spenner fra 0-95 %. Frigjøringsprofiler av CF fra EggPC/DOTAP coatet med negative polymerer og EggPC/EggPG og DPPC/DPPG coatet med en positiv polymer har blitt studert ved 35 °C. Frigjøring fra ucoatede liposomer ble i tillegg bestemt.

DVS Intrinsic studiene viste at ucoatede liposomer hadde samme adsorpsjons- og retensjonsevner når det gjelder vann. Av polymerene hadde LM pektin den høyeste absorpsjons- og retensjonsevnen av vann, selv om den ikke var signifikant forskjellig. Alginat og Kitosan har like vannadsorpsjons- og retensjonsegenskaper. PNIPAAM hadde den laveste vannadsorpsjons og retensjonsevnen, og var signifikant forskjellig. Frigjøringsstudiene viste at EggPC/DOTAP coatet med LM Pektin hadde høyere utslipp enn ucoatede eller coatede EggPC/DOTAP. EggPC/DOTAP coatet med PNIPAAM hadde lavest frigjøringsprofil, selv om den aldri var signifikant forskjellig.

Liposom formuleringen, som ville være en god kandidat for fremtidige xerostomia behandlinger, er avhengig av en frigjøringsprofil som er optimal for å hydrere en munn. Hvis en høy frigjøringsprofil er ønskelig, ville EggPC/DOTAP coatet med LM Pektin være et godt valg.

Table of content

1. Introduction

1.1 Background

Today we get 13 results on drugs containing liposomes in Norway when searching for the word "liposome" in Felleskatalogen (10th of December 2014). None of these drugs are for oral use, but to use as injections or infusions. There are limited drugs today, containing a liposome intraoral drug delivery system, but we have some liposome containing drugs that have affect in other parts of the human body (Barenholz 2012). We also have systems without liposomes but who has intraoral effect (Zamany et al. 2003).

One of the most common public health issues worldwide today is oral disease. These ailments can affect an individual in a day-to-day basis. Some of the ailments treated by local drug therapy are gingivitis, oral lesions, dental caries, oral candidacies, and xerostomia. Many of these ailments may result in problems with food ingestion, such as chewing and swallowing, with smiling and appearance, and some of them may even cause pain (Petersen et al. 2005).

Hydration of the oral mucosa is important due to serious consequences linked to the conditions of a dehydrated mouth. Our saliva has many tasks, and some of them important for maintenance of a healthy mouth. First of all saliva is needed to lubricate the mouth and to help with taste, chewing and swallowing the food. It's more important roles are to break down food and at the same time break down bacteria which is necessary for prevention of bad breath and oral health. It maintains oral health because it contains minerals, proteins and enzymes that protect our enamel and prevent gum disease and tooth decay (Benn & Thomson 2014; Nguyen 2011). That is why a system that can help increased hydration of oral mucosa will reduce patient ailments or the frequency of them.

1.2 Aim of the study

The main objective of this thesis was to study the potential of polymer coated liposomal systems for hydration of the oral mucosa.

More specifically the thesis was divided into four main objectives:

- To establish an *in vitro* method for determination of the water adsorption/retention capacity of nanoparticulate systems by using the DVS instrument.

- To determine the water adsorption/retention capacity of liposomes and polymers

- It was also to examine the release of a marker from the formulations to determine different release profiles.

- Finally, to see if there is a possible correlation between the water adsorption/retention abilities and the release profiles.

1.3 Abbreviations

AM Pectin	Amidated Pectin
CF	Carboxyfluorescein
DLS	Dynamic light scattering
DOTAP	1.2-dioleoyl-3-trimethylammonium-propane
DPPC	1.2-dipalmitoyl-sn-glycero-3-phosphocholine
DPPG	1.2-dipalmitoyl-sn-glycero-3-phospho - (1`-rac—glycerol)
EggPC	L-∝-phosphatidylcholine
EggPG	L-α-phosphatidylglycerol
HM Pectin	High - methoxylated pectin
LM Pectin	Low – methoxylated pectin
Mw	Molecular weight
PdI	Polydispersity index
PEG	Polyethylenglycol
PNIPAAM	Poly (N-isopropylacrylamide-co-methacrylic acid)
rpm	Revolutions per minute
T_c	Transition temperature

2. Theory

2.1 The oral cavity

2.1.1 General

The human oral cavity consists of lips, cheeks, tongue, hard palate, soft palate and the floor of the mouth. The inside of the mouth is covered with a layer of oral mucosa. The oral mucosa can again be divided into the buccal, sublingual, gingival, palatal and labial mucosa. Oral surfaces are continuously moisturized by our salivary glands, and are always covered in fluid consisting of saliva, bacteria, leukocytes, dead epithelial cells, residues from food and more (Rathbone et al. 1994; Gandhi & Robinson 1994; Anon n.d.). For the simplicity of it, the mixture of these substances will just be called saliva through the rest of this assignment.

2.1.2 Mucoadhesion

To overcome oral clearance, and be able to treat the disease both directly (the disease itself) and indirectly (symptom relief or prophylactic treatments), mucoadhesion has been looked at as good opportunities to achieve this. Mucoadhesion is defined as an interaction between two surfaces where at least one of the surfaces consists of a mucosal membrane (Khutoryanskiy 2011). Concerning oral health, methods where mucoadhesion to oral mucosa or adhesion to dental enamel occurs are researched (Nguyen, Hiorth, et al. 2011; Nguyen et al. 2010; Smistad et al. 2011; Nguyen et al. 2013). When it comes to problems regarding salivary hypofunction, mucoadhesion may improve a drugs effect on xerostomia (Andrews et al. 2009) even more than usual because it no longer has to overcome oral clearance.

2.1.3 Saliva

The main transportation device in human oral cavity is saliva and because of that, one cannot evade interactions with saliva and materials from outer systems. This constitutes an important role for the drug delivery to the oral cavity, as the saliva will affect the delivery system. The human clearance of saliva is efficient and can quickly remove or reduce the concentration of oral or exogenous substances, e.g. dead epithelial cells and pathogenic bacteria respectively. Because of this efficient mechanism, saliva will also wash away substances introduced externally to protect the oral cavity, and a slow clearance of the introduced substance by the saliva is preferable. People with dry mouth or salivary hypofunction will have a slower clearance than people with normal production of saliva, which again will make it easier to prolong a drugs presence within the oral cavity.

2.1.4 Dry mouth

Dry mouth, or xerostomia, is defined by a dry oral cavity (Mariotti 2008). Medication is a common cause of salivary hypofunction, and other typical reasons are systemic diseases (most commonly Sjögren`s syndrome), infections, dehydration, head and neck radiation, psychological disorders and old age (Ship 2004; Mariotti 2008; Turner & Ship 2007; Anon 1989; Anon 2014).

Xerostomia can lead to a series of ailments, mostly due to the lack of saliva and all its tasks. A person with salivary hypofunction may suffer from pain; have problems with chewing and swallowing, which again can lead to a change in the persons eating pattern and can result in bad nutrition. A dry mouth can also result in different oral diseases as dental caries, dental cavities, and infections (Ship 2004).

Luckily there are many treatments for xerostomia on today's market. One of them is an oral lubricant, which are substances created to relieve the discomfort as follows a dry mouth. An oral lubricant can for example be water, milk or olive oil. Another treatment consists of antimicrobial saliva substitutes. These agents exist in products as gels, liquids, toothpastes, gels, sprays and chewing gum, and contain mainly antimicrobial agents. Salivary stimulants are also widely used as treatment, and they are used on people who still have some function left in their salivary glands. They work by physical stimulation or by affecting the parasympathetic nervous system (Anon 2014). Sugar-free chewing gum or lozenges exercise local treatment by physical stimulation. Systemic treatment, affecting the parasympathetic nervous system, increases the secretion of bodily fluids, and pilocarpine is normally used (Johnson et al. 1993; Gil-Montoya et al. 2008; Vivino et al. 1999).

2.2 Liposomes

2.2.1 General

A typical liposome consists of phospholipids in a bilayer. A single phospholipid consists of a hydrophilic head group with two hydrophobic tails attached to it as illustrated in Fig. 2-1.

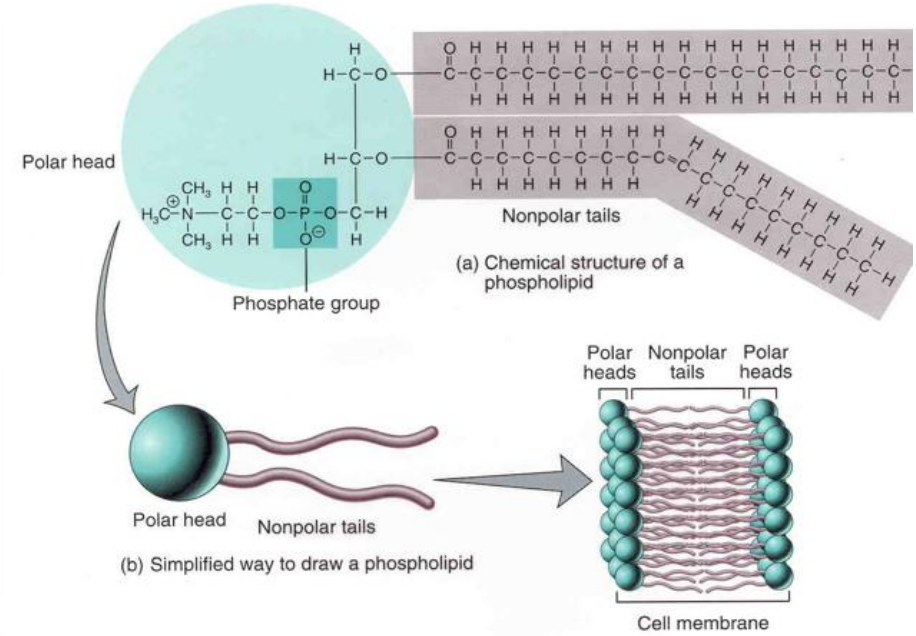


Fig. 2-1. An illustration of a single phospholipid and how they assemble into a bilayer. Http://www.homepage.smc.edu

In the presence of an aqueous phase these lipid molecules tend to spontaneously, self assemble into spherical vesicles, where the polar head group tend to be in contact with the water phase as shown in Fig. 2-2.

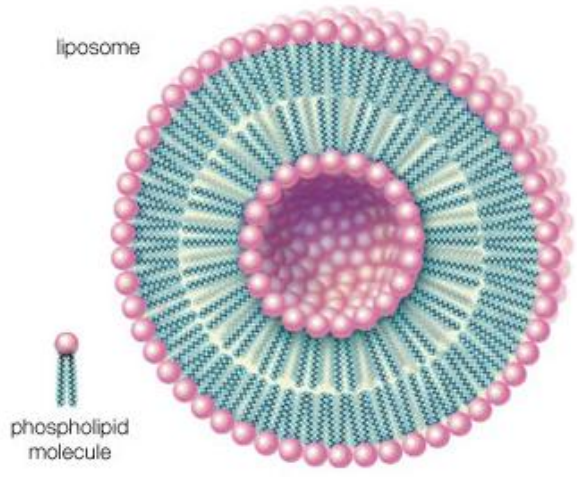


Fig. 2-2. Structure of a unilamellar liposome. © 2007 Encyclopædia Britannica, Inc.

The vesicles can vary in size, contain one or more lipid bilayer with different morphologies, and can be classified according to the basis of their structural properties as listed in Table 2-1.

Table 2-1. Liposome classification based on structural properties (Kreuter 1994)

MLV	Multilamellar large vesicles	> 0.5 µm
OLV	Oligolamellar vesicles	0.1 - 1 µm
UV	Unilamellar vesicles	(all sizes)
SUV	Small unilamellar vesicles	20 - 100 nm
MUV	Medium sized unilamellar vesicles	
LUV	Large unilamellar vesicles	> 100 nm
GUV	Giant unilamellar vesicles	(vesicles with diameter > 1 µm)
MVV	Multivesicular vesicles	(usually large > 1 µm)

As can be seen from Table 1, the size distribution of liposomes varies from 20 nm to over 1 µm. They can also have many different number and positions of lamellae and their bilayer liquid-crystalline versus gel state are dependent on the lipids involved. The rigidity of the membrane is an important factor in the formation of liposomes. The phospholipid bilayer can exist in a liquid-crystalline state (the "fluid" state) or in a "gel" state. A gel state bilayer will, with increasing temperature, "melt" at its specific transition temperature (T_c) and go from the gel state into a liquid-crystalline state. The bilayers are more rigid and usually less permeable when in their gel state. Hydrophilic molecules can be incorporated in the aqueous core of the liposome and the lipophilic molecules in the lipid bilayer.

The raw material used for the preparation of liposomes can be divided into five main groups of phospholipids (Kreuter 1994).

1. The natural ones
    i. Mainly from egg yolks and soybeans
2. The modified natural ones
    i. Are highly unsaturated and are therefore prone to oxidation, hence the modifications.
3. The semi synthetic
    i. Replacement of the unsaturated acyl chains with a chosen new acyl chain (can be done within certain limits), to make it more stable
4. The fully synthetic
    i. Chemical preparation of phospholipids
5. The ones with modified head groups (non-natural head groups)
    i. E.g. adding proteins or polyethylenglycol chains (PEG) to the membrane with the purpose of manipulating the liposomes fate in the human body.

2.2.2 Preparation of liposomes

Today there are many different techniques on how to prepare a liposome. A few main steps recur in most of them. First the lipid needs to hydrate, second they have to reform into desirable size, and third the drug that is not encapsulated in the vesicle must be removed.

Since there are many ways to prepare a liposome, only one is described in more detail here. The lipid film method starts with diluting the lipids in an organic solution, e.g. chloroform, to the desired concentrations, then removing the organic solution thereby creating a thin lipid film. After this, the lipid film rehydrates with the chosen hydration medium with simultaneously stirring to mix the two phases. This method usually creates a mixture of MLVs and SUVs, and a way to make the mixture more homogenous is to use low-pressure extrusion with selected polycarbonate membranes with pores of wanted size. The last step is to remove the solute, which is not encapsulated, and one way to do this is by using gel permeation chromatography. Here the formed liposomes will go through the column, while the gel will retain the non-encapsulated material (Poole 2013).

2.3 Characterization of liposomes

Dynamic light scattering (DLS) is a way of determining a liposomes size. The Tyndall effect, time variations of scattered light from a particle (liposome) in a buffer solution, and Brownian motions are the basis of obtaining liposomes hydrodynamic size distribution (Xu 2008; Xu et al. 2014; Hassan et al. 2014).

Light from a laser illuminates the particle suspended in the buffer solution and the light will scatter with certain intensity. This intensity creates a diffusion coefficient, measured by DLS, which makes it possible to calculate the liposomes size by using the Stoke-Einstein equation (Equation 2-1).

$$D = \frac{kT}{(6\pi\eta \mathrm{Rh})}$$ Equation 2-1

Where D is the translational diffusion coefficient, k is the Boltzmann´s constant, T is the absolute temperature, η is the solvent viscosity and Rh is the apparent hydrodynamic radius.

The polydispersity index (PdI) measures the broadness of distribution to the particle sizes. A high PdI value signifies a broad distribution in particle size, and a low PdI a small distribution (Hassan et al. 2014).

2.4 Zeta potential

Figure 2-3 below illustrates an ionic distribution close to a positively charged surface (Burns 2000). A particle in a solution has a net charge and therefore ions bound to its surface. These ions form a layer that is called the Stern layer. Outside of this layer a diffuse layer of ions will occur. When they move against the Stern layer a shear will appear between them, due to adhesion of the particles to the surface, and it is in this shear surface the zeta potential is measured (Clogston & Patri 2011; Xu 2008).

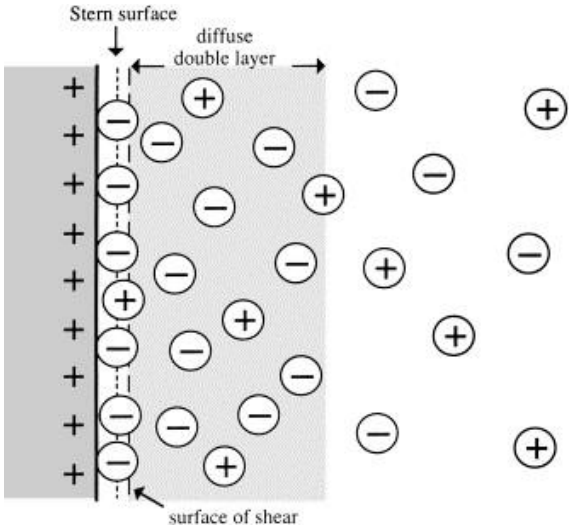


Fig. 2-3. An ionic distribution close to a positively charged surface.

A laser Doppler velocimetry measures the zeta potential by applying an electrical field across the sample. When this is done the movement of the particle is registered. In the end the zeta potential, z, is calculated by using the Henry equation (Equation 2-2).

$$Ue = \frac{2\varepsilon z f\,(\kappa a)}{3\eta}$$ Equation 2-2

Where Ue is the electrophoretic mobility, ε is the dielectric constant, z is the zeta potential, η is the solvent/medium viscosity and f (κa) is the Henry constant.

2.5 *Dynamic Vapor Sorption (DVS) Intrinsic*

The DVS Intrinsic can measure mass changes of samples as they take up or loose moisture. The sample is placed in a sample pan in a closed chamber. Inside the chamber a flow of nitrogen gas, with known percentage of relative humidity (% RH), passes over the sample at a controlled flow rate and temperature, as illustrated in Figure 2-3.

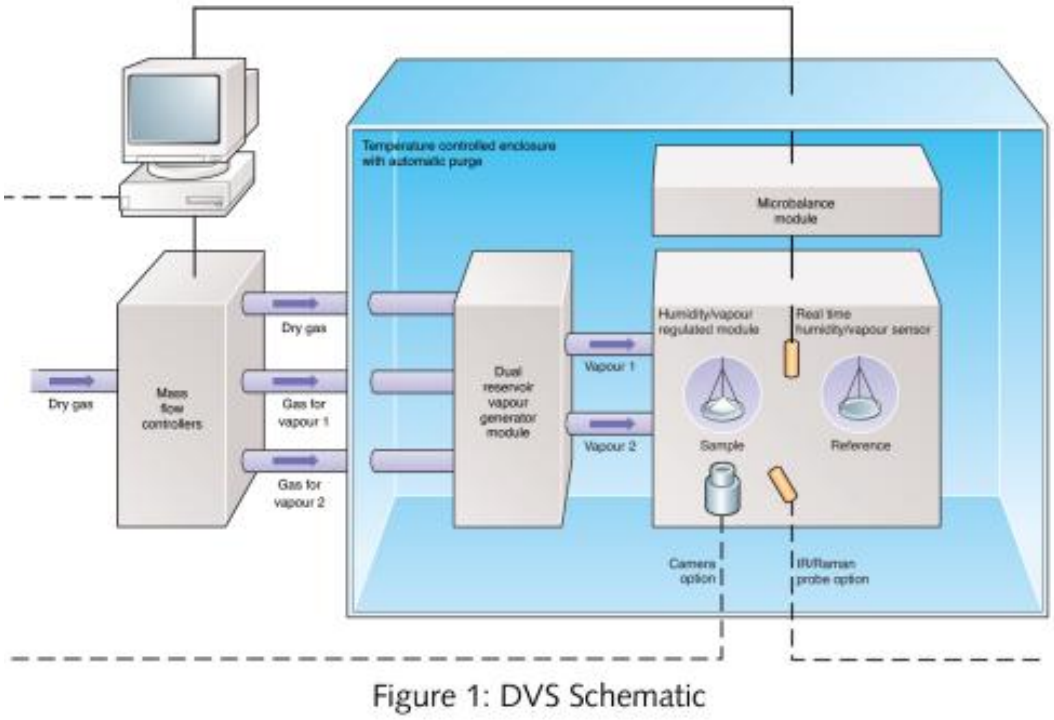


Fig. 2-3. Schematic of the main components of the DVS Intrinsic. Http://www.smsuk.co.uk

As the sample then sorbs/desorbs water vapour from the surroundings inside the chamber, mass readings reveal the sorption/desorption behaviour of the sample. If a sample absorbs a lot of water from the surrounding air at a given % RH, it will weigh more than a sample that does not. Also, a samples ability to hold on to water can be tested. For example, if a sample absorbs a lot of water with a specific % RH the weight will increase. Then, if the % RH is decreased, the weight will change accordingly. How much water that evaporates over a specific period, or how much weight loss the sample has will indicate its ability to hold on to water molecules. The instrument is capable of measuring mass changes lower than 1 part per million because it contains an ultra-sensitive recording microbalance. A computer runs and controls all parameters during an experiment.

3. Materials and instruments

3.1 Materials

3.1.1 Lipids

Lipids	Abbreviations	Mw (g/mol)	K-number	Manufacturer
L-∝-phosphatidylcholine	EggPC	770.123	108030-1/911 510800-04/921	Lipoid GmbH, Germany
1.2-dipalmitoyl-sn-glycero-3-phosphocholine	DPPC	734.039	563119-01/017	Lipoid GmbH, Germany
1.2-dioleoyl-3-trimethylammonium-propane	DOTAP	698.542	181TAP-150	Avanti Polar Lipids Inc., USA
L-α-phosphatidylglycerol	EggPG	782.284	841138 P	Avanti Polar Lipids Inc., USA
1.2-dipalmitoyl-sn-glycero-3-phospho - (1`-rac—glycerol)	DPPG	744.952	94H8377	Sigma, USA

3.1.2 Polymers

Polymers	Abbreviations	Mw (g/mol)	K-number	Manufacturer
High - methoxylated pectin	HM Pectin	$1.1x10^{5}$[a]	GR81611	CP Kelco, Germany GmbH
Amidated pectin	AM Pectin	$9.6x10^{4}$[a]	SK32079	CP Kelco, Germany GmbH
Low – methoxylated pectin	LM Pectin	$7.6x10^{4}$[a]	G74476	CP Kelco, Germany GmbH
Chitosan (Protasan UP CL 213)	Chitosan	150 000 – 400 000[b]	BP-0805-04	FMC Biopolymer AS, Norway
Alginate		75 000 – 200 000[b]	S12281	FMC BioPolymer AS, Norway
Poly (N-isopropylacrylamide-co-methacrylic acid)	PNIPAAM	≤ 2.5 (Mw/Mn, Mn=60 000)[b]	MKBF2188V	Sigma-Aldrich, USA

[a] (Smistad et al. 2012)
[b] Information provided by the manufacturer.

3.1.3 Other chemicals

Other chemicals	Abbreviations	Mw (g/mol)	K-number	Manufacturer
5(6)-carboxyfluorescein(s)	CF	376.32	BCBJ4360V 10H9062	Sigma, USA
Chloroform		119.38	13C260521	Merck, Germany
Sodium dihydrogen phosphate monohydrate(s)	NaH_2PO_4 x H_2O	137.99	K25001880	Merck, Germany
Disodium hydrogenphosphate dihydrate (s)	Na_2HPO_4 x $2H_2O$	177.99	97352	Merck, Germany
Ethanol 96 %		46.07	203031	Merck, Germany
Sodium hydroxide 0,1 M	NaOH 0,1 M	39.99	70800424070 C04	Merck, Germany
Hydrochloric acid 37 %	HCl	36.46	K33616217 432	Merck, Germany
t-octylphenoxy-polyethoxyethanol	Triton – X 100	250.38	10K0192	Sigma, USA
Zeta potential transfer standard			261209	Malvern instruments Ltd, UK

3.1.4 Solutions

Lipid stock solutions (w/v)

Dissolving the lipids in chloroform made lipid stock solutions. The amounts used are displayed below. All solutions were stored in a freezer.

Lipid	Concentration mg/ml	Amount lipid (g)	Chloroform volume (ml)
Egg-PC	10 mg/ml	1.00 g	Ad 100 ml
	20 mg/ml	2.00 g	Ad 100 ml
DOTAP	2 mg/ml	0.20 g	Ad 100 ml
DPPC	2 mg/ml	0.20 g	Ad 100 ml
Egg-PG	2 mg/ml	0.20 g	Ad 100 ml
DPPG	2 mg/ml	0.20 g	Ad 100 ml

Phosphate buffer 5 mM pH 6.8 (w/v)

A buffer solution, consisting of NaH_2PO_4 x H_2O (s) and Na_2HPO_4 x $2H_2O$ (s), was made by mixing the two substances in two different containers with MilliQ water. NaH_2PO_4 x H_2O (I), 690 mg was dissolved in MilliQ water ad 1000 ml in a volumetric flask. Na_2HPO_4 x $2H_2O$ (II) 890 mg was dissolved in MilliQ ad 1000 ml in another flask.

The two solutions, I and II, were mixed together in the 2:1 ratio. The pH of the final solution was measured and adjusted to 6.8±0.1. Finally the solution was filtered, using vacuum, through a 200 nm filter.

Polymer solutions 0.1 % (w/v)

15 mg (or 20 mg) of polymer was dissolved in 15 ml (or 20 ml) 5 mM phosphate buffer pH 6.8. Left under magnetic stirring overnight (room temperature) and adjusted to pH 6.8 by either 0.1 M NaOH or 1 M HCl.

Polymer solutions 0.5 % (w/v)

25 mg of polymer was dissolved in 5 ml 5 mM phosphate buffer pH 6.8. Left under magnetic stirring overnight (room temperature) and adjusted to pH 6.8 by either 0.1 M NaOH or 1 M HCl.

Polymer solutions 1.0 % (w/v)

50 mg of polymer was dissolved in 5 ml 5 mM phosphate buffer pH 6.8. Left under magnetic stirring overnight (room temperature) and adjusted to pH 6.8 by either 0.1 M NaOH or 1.0 M HCl.

Triton X-100 2 % (w/w)

Triton in a 2 % solution was made by weighing in 2 g of Triton X-100 and 98 g of 5 mM phosphate buffer pH 6.8 in a glass flask. The pH was adjusted to 6.8 by either 0.1 M NaOH or 1 M HCl.

1 M sodium hydroxide

4 g sodium hydroxide (NaOH) was dissolved in 100 ml of MilliQ water and stored at room temperature (20 °C).

1 M hydrochloric acid

3.46 g hydrochloric acid (37 %) (HCl) was dissolved in 100 ml of MilliQ water and stored at room temperature (20 °C).

3.1.5 Solution applied in the preliminary tests

Stock solution of 5(6) – carboxyfluorescein 1.5 mM in 5 mM phosphate buffer pH 6.8

CF (11.30 mg) was dissolved in 20 ml of 5mM phosphate pH 6.8.
To get the CF fully dissolved 1 M NaOH was added. The pH of the solution was adjusted to 6.8 – 7.2 by adding 1 M HCl. A polycarbonate membrane filter (Nucleopore, 200 nm) was used to filter the solution, and it was protected from light at 4°C.

3.1.6 Solutions applied in release and leakage measurements

CF – solution 10 μM

0.1 ml CF 1.5 mM was mixed with 14.9 ml of 5mM phosphate buffer pH 6.8.

3.2 Instruments

3.2.1 Preparation of liposomes

Instrument	Model	Manufacturer
Analytical weight	AG204 DeltaRange®	Mettler Toledo GmbH, Switzerland
Rotary evaporator	Vacuum pump, Mz2C, serial number 23911722	Vaacubrand GmbH, Germany
	Heidolph VV 2001	Heidolph, Germany
Freeze drier	Christ Alpha 2 - 4	Martin Christ Gefriertrocknunganlagen GmbH, Germany
	Vacuum pump RV8	Edwards High Vacuum International, UK
Extruder	Lipex Thermobarrell 10 ml	Northern Lipids, Canada
Circulating refrigerating and heated water bath	MGW RC 6	Brinkman Lauda, USA

3.2.2 Coating of liposomes

Instrument	Model	Manufacturer
Peristaltic pump	520 S	Watson-Marlow, Great Britain
Magnetic stirrer	RO10	IKA Werke, Germany

3.2.3 Other instruments

Instrument	Model	Manufacturer
Zeta sizer	Nano SZ	Malvern Instruments, UK
Plate reader	Wallac Victor^3 1420	Perkin Elmer, USA
PH meter	MP 220	Mettler Toledo, Switzerland
Heating cabinet	TS8056, serial number 3-2461	Termaks, Norway
Whirlmixer	Reax top	Heidolph, Germany
Centrifuge	Eppendorf Centrifuge 5430 R	Eppendorf AG, Germany
DVS - Intrinsic		Surface Measurement Systems (SMS) Ltd, London UK

3.2.4 Other equipment

Equipment	Model	Manufacturer
Desalting column	PD 10	GE Healthcare Biosciences AB, Sweden
Centrifuge tube 6 ml	Spin-X® UF 6	
Sample pan (30 µl)	Perkin-ELMER DSC	Waltham, USA
Polycarbonate membrane (200 nm)	Nucleopore Track-Etch Membrane	Whatman, UK

4. Methods

4.1 DVS – Intrinsic mass change determination

The instrument was first adjusted to desirable parameters as shown in Table 4-1. Then the weight was tarred including an empty sample pan. 15 µl of the sample was applied in the pan, which was then hung up in a chamber within the instrument. As soon as the sample was put in the chamber, the desired sample procedure was selected.

The experiments procedures was set in stages based on percentage of relative humidity (% RH), and there was a fixed time per stage. The parameters used in the set ups are listed in Table 4-1.

Table 4-1. Parameters used when measuring mass changes with the DVS–Intrinsic.

Temperature	35°C
Inlet pressure	2.03 bar
Mass measurement frequency	1 minute
Solvent	Water
Relative vapour pressure units	RH

4.2 Time constant determination

A mathematical programme, Origin, was used to calculate the different liposomes and polymers time constants. This was done by exponentially fitting the data to Equation 4-1.

$$y = y_0 + A_1 e^{-x/t_1} \qquad \text{Equation 4-1}$$

Where y_0 = end of stage weight, A_1 = amount of mg lost (water loss), x = time and t_1 = time constant.

4.3 Preparation of liposomes (thin film method)

Lipids, from the stock solution, and chloroform are added to a 250 ml round flask. The organic solvent was evaporated with a "Rotavapor" with a water bath and vacuum pump. The temperature of the bath was 40 °C, the rotation speed 90 rounds per minute (rpm) and the pressure 200 mbar. The pressure was slowly lowered to 200 mbar. When the content was dry, the pressure was lowered to 60-69 mbar and held there for 20 minutes. To remove all traces of chloroform from the lipid film the flask was set on a vacuum pump overnight.

While stirring the mixture, the lipid film was hydrated by adding the hydration medium (5mM phosphate buffer pH 6.8 or 1.5 mM CF solution pH 6.8-7.2) above the Tc temperature. Then it was fastened to the "Rotavapor" for 10 minutes, with a bath temperature for 40 °C (or room temperature) and a rotation speed of 90 rpm. After this it was set in a dark space, in 40 °C (or room temperature), for 2 hours with intermittent stirring. At last, the solution was stored in a refrigerator (4 °C) over night.

Before the extrusion of the liposome suspension, 2x10 ml of 5 mM phosphate buffer pH 6.8 was taken through the apparatus.
The liposome suspension was extruded above Tc 10 times through two stacked 200 nm polycarbonate filters and transferred to 20 ml brown glass vials. Dispersions containing unsaturated lipids were flushed with nitrogen gas. The finished product was stored in a refrigerator (4 °C).

4.4 Removal of non – encapsulated CF by gel filtration

4.4.1 Column preparation

The PD-10 desalting column was equilibrated 5 x 3.5 ml with 5 mM phosphate buffer pH 6.8.

4.4.2 Column saturation

The prepared column was added 2.5 ml of the naked 3 mM liposome suspension (without CF). When the suspension had entered the packed bed completely, the column was again washed with 3.5 ml of 5 mM phosphate buffer pH 6.8. In the first rinse, it was double-checked that the eluate from the column was turbid. Then the column was washed four more times with the same amount of the same buffer. The column was set for storage in 5 mM phosphate buffer pH 6.8, to avoid it from drying out, until usage.

4.4.3 Removal of non – encapsulated carboxyfluorescein before coating

The 5 mM phosphate buffer pH 6.8 in the column was discarded. The needed volume of liposome in question was added. Maximum sample volume that could be added to the column was 2.5 ml. When less than this volume of liposomes was added, an equilibrium buffer had to be added so that total sample volume was 2.5 ml. The eluate from these additions was discarded. Then 3.5 ml of 5 mM phosphate buffer pH 6.8 was added, and the eluate was collected in a dark vial. The collected sample was then diluted to 3 mM or 0.6 mM with 5 mM phosphate buffer pH 6.8.

Directly after sample collection, the 3 mM batch was divided in three equal parts and coated with a polymer as described in chapter 4.3.

4.5 Coating of liposomes with a polymer

First 4 ml of polymer was added to a prewashed dark vial containing a clean magnet. Then, using a peristaltic pump, 1 ml of 3 mM liposome was added to 4 ml of a polymeric solution one drop at a time, with a pump speed of 20 rpm (6.8 ml/min). The polymeric solution was kept under magnetic stirring during the process and furthermore for 5 minutes after coating. The unsaturated liposomes` were flushed with nitrogen before they were sealed.

4.6 Sample collections of released carboxyfluorescein

First 1 ml of each sample was pipetted into four centrifuge tubes. Then they were set in a refrigerator (4 °C) for 10 minutes before they were centrifuged for 5 minutes, in room temperature (20 °C), at 7000 rpm. 50 µl of the supernatant from the tubes were pipetted into the wells of a microtiter plate as described in chapter 4.5.2. In addition to this, 50 µl of non-centrifuged samples were pipetted into separate wells.

4.7 Fluorescence measurements

4.7.1 Preparations of known standard concentrations

From the 1.5 mM CF solution (chapter 3.1.5) 0.1 ml was taken out and added 14.9 ml of 5 mM phosphate buffer pH 6.8, and mixed thoroughly. The new solution is referred to as a 10 micro molar stock solution. 0.5 ml of the stock solution was transferred to an Eppendorf tube and added 0.5 ml of 5 mM phosphate buffer pH 6.8 (standard 1). Of this standard 0.5 ml was taken over into a new Eppendorf tube and 0.5 ml of the same phosphate buffer was added. The solutions were mixed with vibrations using a whirl mixer. This procedure was followed until eight dilution steps were created.

4.7.2 Pipetting to microtiter plates.

Liposomes encapsulated with carboxyfluorescein were pipetted into the pre-filled wells with either 5 mM phosphate buffer pH 6.8 or Triton X–100 2% (total volume 100 µl). There were used two different set ups on two different plates, shown in Figure 4-1 and 4-2. Fifty µl of 5 mM phosphate buffer pH 6.8 were pipetted into 90 wells. In 42 other wells 50 µl of Triton X-100 2% were added by a pipette and bubbles of air were avoided as much as possible. 100 µl of 5 mM phosphate buffer were pipetted into 6 wells, and 100 µl of each standard solution were pipetted into three different wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	St.curve 2,5µM	St.curve 2,5µM	St.curve 2,5µM	P1+B 0 min	P2+B 0 min	P3+B 0 min	P4+B 0 min	P1+B 20 min	P2+B 20 min	P3+B 20 min	P4+B 20 min	
B	St.curve 1,25µM	St.curve 1,25µM	St.curve 1,25µM	P1+B 0 min	P2+B 0 min	P3+B 0 min	P4+B 0 min	P1+B 20 min	P2+B 20 min	P3+B 20 min	P4+B 20 min	
C	St.curve 0,63µM	St.curve 0,63µM	St.curve 0,63µM	P1+B 0 min	P2+B 0 min	P3+B 0 min	P4+B 0 min	P1+B 20 min	P2+B 20 min	P3+B 20 min	P4+B 20 min	
D	St.curve 0,31µM	St.curve 0,31µM	St.curve 0,31µM	P1+T 0 min	P2+T 0 min	P3+T 0 min	P4+T 0 min	P1+B 40 min	P2+B 40 min	P3+B 40 min	P4+B 40 min	
E	St.curve 0,16µM	St.curve 0,16µM	St.curve 0,16µM	P1+T 0 min	P2+T 0 min	P3+T 0 min	P4+T 0 min	P1+B 40 min	P2+B 40 min	P3+B 40 min	P4+B 40 min	
F	St.curve 0,078µM	St.curve 0,078µM	St.curve 0,078µM	P1+T 0 min	P2+T 0 min	P3+T 0 min	P4+T 0 min	P1+B 40 min	P2+B 40 min	P3+B 40 min	P4+B 40 min	
G	St.curve 0,039µM	St.curve 0,039µM	St.curve 0,039µM	B+T	B+T	B+T	P1+B 60 min	P1+B 60 min	P1+B 60 min	P2+B 60 min	P2+B 60 min	P2+B 60 min
H	Buffer	Buffer	Buffer				P3+B 60 min	P3+B 60 min	P3+B 60 min	P4+B 60 min	P4+B 60 min	P4+B 60 min

Fig. 4-1. The first plate filled with different solutions. St. curve = Standard curve, B = 5 mM phosphate buffer pH 6.8, T = Triton X-100 2 %, P1 = sample parallel uncoated liposome, P2 = sample parallel 1 coated liposome, P3 = sample parallel 2 coated liposome and p4 = sample parallel 3 coated liposome.

	1	2	3	4	5	6	7	8	9	10	11	12
A	St.curve 2,5µM	St.curve 2,5µM	St.curve 2,5µM	P1+B 2 hours	P2+B 2 hours	P3+B 2 hours	P4+B 2 hours	P1+B 4 hours	P2+B 4 hours	P3+B 4 hours	P4+B 4 hours	
B	St.curve 1,25µM	St.curve 1,25µM	St.curve 1,25µM	P1+B 2 hours	P2+B 2 hours	P3+B 2 hours	P4+B 2 hours	P1+B 4 hours	P2+B 4 hours	P3+B 4 hours	P4+B 4 hours	
C	St.curve 0,63µM	St.curve 0,63µM	St.curve 0,63µM	P1+B 2 hours	P2+B 2 hours	P3+B 2 hours	P4+B 2 hours	P1+B 4 hours	P2+B 4 hours	P3+B 4 hours	P4+B 4 hours	
D	St.curve 0,31µM	St.curve 0,31µM	St.curve 0,31µM	P1+B 24 hours	P2+B 24 hours	P3+B 24 hours	P4+B 24 hours	P1+T 4 hours	P2+T 4 hours	P3+T 4 hours	P4+T 4 hours	
E	St.curve 0,16µM	St.curve 0,16µM	St.curve 0,16µM	P1+B 24 hours	P2+B 24 hours	P3+B 24 hours	P4+B 24 hours	P1+T 4 hours	P2+T 4 hours	P3+T 4 hours	P4+T 4 hours	
F	St.curve 0,078µM	St.curve 0,078µM	St.curve 0,078µM	P1+B 24 hours	P2+B 24 hours	P3+B 24 hours	P4+B 24 hours	P1+T 4 hours	P2+T 4 hours	P3+T 4 hours	P4+T 4 hours	
G	St.curve 0,039µM	St.curve 0,039µM	St.curve 0,039µM	B+T	B+T	B+T	P1+T 24 hours	P1+T 24 hours	P1+T 24 hours	P1+T 24 hours	P1+T 24 hours	P1+T 24 hours
H	Buffer	Buffer	Buffer				P1+T 24 hours	P1+T 24 hours	P1+T 24 hours	P1+T 24 hours	P1+T 24 hours	P1+T 24 hours

Fig. 4-2. The second plate filled with different solutions. St. curve = Standard curve, B = 5 mM phosphate buffer pH 6.8, T = Triton X-100 2 %, P1 = sample parallel uncoated liposome, P2 = sample parallel 1 coated liposome, P3 = sample parallel 2 coated liposome and p4 = sample parallel 3 coated liposome.

4.7.3 Measurement and quantification of leaked carboxyfluorescein

As soon as a set of samples were added to the microtiter plate it was placed in the Wallac Victor^3 1420 Multilabel Counter plate reader. The plate reader was adjusted to read the parameters listed in table 4-2, and the measurements took place at room temperature (20°C).

Table 4-2. Parameters used when measuring sample fluorescence with Wallac Victor^3 1420 Multilabel Counter plate reader.

Label technology	Prompt fluorometry
Microtiter plate	Generic 8x12 size plate
Measurement height	Default
Shaking duration	1.0 s
Shaking speed	Fast
Shaking diameter	0.10 mm
Shaking type	Linear
CW-lamp filter name	F485
CW-lamp filter slot	A5
CW-lamp energy	2000
Emission filter name	F535
Emission filter slot	A5
Emission aperture	Normal
Emission side	Above
Measurement time	0.1 s

4.8 Particle size measurements

The Zetasizer Nano ZS was used to measure the different liposomes sizes. Each disposable cell was cleaned with 5 mM phosphate buffer pH 6.8, and then 1 ml of the same buffer was added to the pre cleaned cell. Before 100 µl of liposome was added and mixed, the cell was inspected for any visible dust particles.

The parameters used for the liposome size measurements are shown in Table 4-3.

Table 4-3. Parameters used when measuring liposome sizes with the Zetasizer Nano ZS.

Attenuator	Automatic
Duration of each measurement	Automatic
Measurement angle	173°
Dispersion medium and viscosity	Water, 0.8872 cP
Approximation	Mark-Houwink parameters
Measurement temperature	25.0 °C
Cell type	Disposable cell
Equilibration time	300 seconds
Number of runs	3

4.9 Zeta potential measurements

The zeta potential was measured in the same cell and with the same apparatus. A zeta potential transfer standard (- 42 mV ± 10 %) had to equilibrate the apparatus before use. A dip cell, pre cleaned with distilled water, was added to the cell.

The parameters used for the liposome zeta potential measurements are shown in Table 4-4.

Table 4-4. Parameters used when measuring liposome zeta potentials with the Zetasizer Nano-ZS.

Attenuator	Automatic
Duration of each measurement	Automatic
Approximation	Smoluchowski
Dispersion medium and viscosity	Water, 0.8872 cP
Measurement temperature	25.0 °C
Refractive index	1.330
Dielectric constant	78.5
Cell type	Zeta dip cell
Equilibration time	120 seconds
Number of runs	5

4.10 pH measurements

The MP220 pH meter was calibrated before use by choosing two buffer solutions suitable for the specific sample measurements (pH 4 and 7 for the samples based on 5 mM phosphate buffer pH 6.8). The sample was added to a 1 ml Eppendorf tube and measured at room temperature (20 °C).

4.11 Statistical analysis

The differences between studied groups were examined by using the Minitab statistical software (Minitab Inc., USA). A one – way ANOVA analysis, a variance analysis, were carried out followed by a Tukey`s Post hoc test. The level of significance was $p < 0.05$.

5. Experimental setup

5.1 DVS - Intrinsic

5.1.1 Preliminary tests – Determination of experimental parameters

The preliminary testing were used to determine how long the different stages should last and which percentage of RH were to be tested. The initial testing was also used to find out which concentration, of liposomes and polymers, that was desirable to test on the DVS – Intrinsic instrument.

First 5 mM phosphate buffer pH 6.8 was tested in different setup procedures, and then liposomes and polymers were tested using the same setup. By doing this the final method to find the water adsorption/retention abilities for the DVS – Intrinsic instrument was determined. The different setups used are listed below.

1) Liposome collapse humidity (%) – 5 mM phosphate buffer pH 6.8 and 15 mM EggPC/DOTAP
    a. 80-70-60 % RH, each step 120 min.
    b. 94-92-90-88-86-84-82-80 % RH, first step 90 min and remaining steps 120 min.

2) Determination of equilibrium times – 5 mM phosphate buffer pH 6.8 and 15 mM EggPC/DOTAP
    a) 80-70-60 % RH, each step 120 min.
    b) 80-70 % RH, first step 90 min and second step 120 min.
    c) 80-70 % RH, first step 90 min and second step 150 min.
    d) 0-90-0 % RH, first step 90 min, remaining steps 30 min and last step 90 min. The steps changes with a factor of 10.

3) Investigation of different concentrations – 0.1 % and 1.0 % LM Pectin

    a) 0-90-95-90-0 % RH, first step 240 min (0%), step 0-60 % 30 min, step 70-95-70 % 60 min, step 60-0 % 30 min and last step 120 min (0%). The steps changes with a factor of 10 except from stage 90-95-90 were it changes with a factor of 5.

5.1.2 DVS – Intrinsic hydration / dehydration studies

All liposomes and polymers were in the end set to the same sample method and made in the same concentrations for better comparison of results. 0.5 % solutions were made and investigated using the method as described in Chapter 4.2.1.

The first stage, 0 %, was set to 240 minutes. Then the following stages from 0 - 60 % were set to 30 minutes, and stages 70 – 95 % to 60 minutes. The downwards steps were identical as the up going steps, except from the last step of 0 % which lasted for 120 minutes.

5.2 Release studies

5.2.1 Determination of centrifuge speed, time and temperature

The liposome batch was diluted to 0.6 mM, so the concentration for the uncoated liposomes was the same as for the coated ones.

Liposomes, room temperate, was taken out (1 ml) and over to a centrifuge tube. This was done twice, since a centrifuge tube needs a weight partner. The samples were taken over and in to the centrifuge, and the parameters were adjusted a few times to find the right ones.

5.2.2 Release studies

The release at 35 °C of carboxyfluorescein from both uncoated and coated liposomes in 5 mM phosphate buffer pH 6.8 was investigated. The samples were stored in dark glass vials at 35 °C during the study. Three parallel extractions of each sample were measured at t = 0, t = 20 minutes, t = 40 minutes, t = 1 hour, t = 2 hours, t = 4 hours and t = 24 hours. The fluorescence was measured according to chapter 4.7 at each time point, and the concentrations and per cent of release were determined by using the standard curves.

An overview of liposomes studied is listed in figure 5-1 below.

Table. 5-1. The different liposomes used in studying the leakage of CF, both naked and coated formulations.

Liposome	Polymer coating
EggPC / DOTAP (10 mol %)	Pectin (HM, LM, AM), alginate and PNIPAAM
EggPC / EggPG (10 mol %)	Chitosan
DPPC / DPPG (10 mol %)	

6. Results and discussion

6.1 DVS – Intrinsic

6.1.1 Preliminary tests – Determination of experimental parameters

Liposome collapse humidity (%)

Initially, before the water adsorption/retention method was intended, the humidity of which the liposomes collapsed was investigated. In Figure 6-1 5 mM phosphate buffer pH 6.8 is illustrated. The % RH was set to run from 80-70-60 %, and each run lasted for 120 minutes.

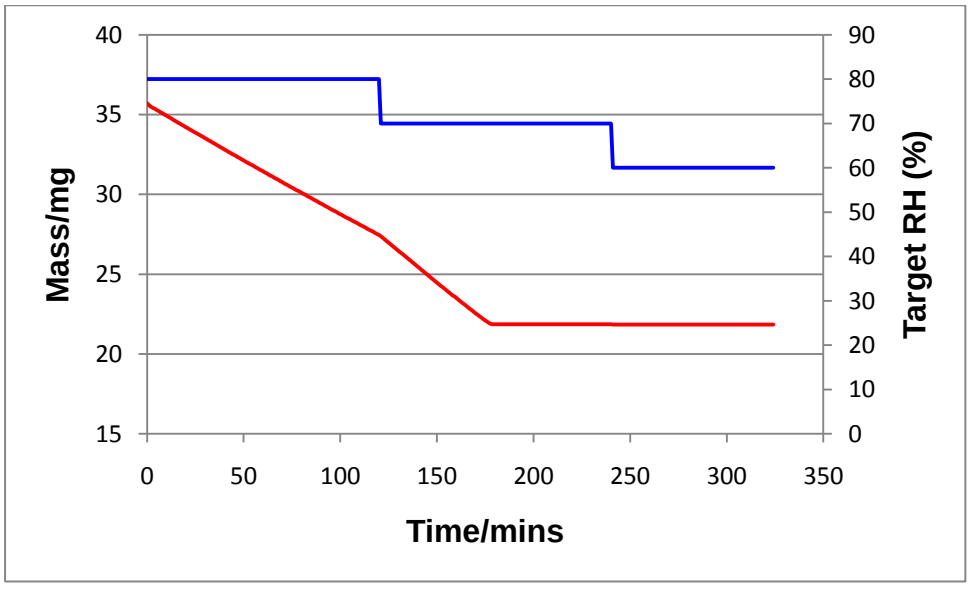


Fig. 6-1. DVS-Intrinsic analysis of 5 mM phosphate buffer pH 6.8: % RH stages set from 80-60 with a changing factor of 10. The blue line represents the target % RH for each stage and the red line represents mass readings of 5mM phosphate buffer pH 6.8 as it changes with the % RH.

A liposome, 15 mM EggPC/DOTAP, was tested to see if the % RH where the liposomes breaks could be found. In Figure 6-2 a method run from 94-80 % RH is shown, and a better setup description can be located in Chapter 5.1.1. From the mass readings relative to % RH no data for detection of a specific % RH, where the liposome gets destroyed, could be located.

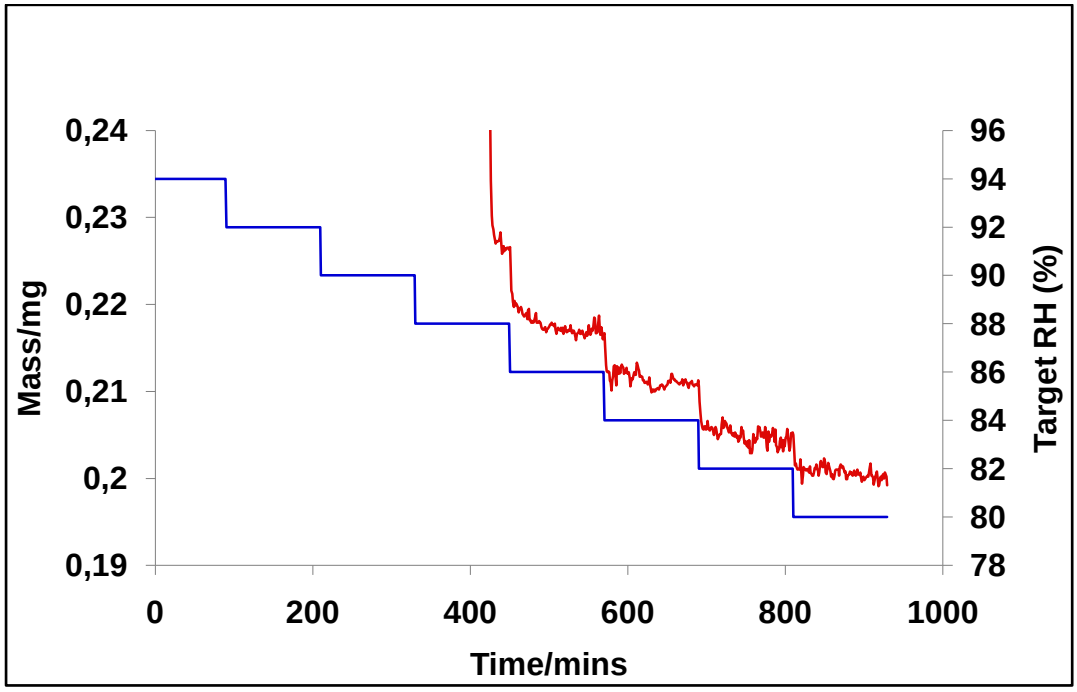


Fig. 6-2. DVS-Intrinsic analysis of 15 mM EggPC/DOTAP: % RH stages set from 94-80 with a changing factor of 2. The blue line represents the target % RH for each stage and the red line represents mass readings of 15mM EggPC/DOTAP as it changes with the % RH.

Since it was not possible to identify a collapse % RH, it was decided that the adsorption/retention abilities of liposomes and polymers was to be investigated instead. To find a suitable method to determine these abilities different procedures were carried out. It is important to be aware of the fact that the sample was dried out at first by running the % RH of 0 % for a certain amount of time. Because of the small amount applied at each test a pipette was used to apply 15 µl instead of weighing in 15 µg, to make the experiment more reproducible. After dehydration the sample was rehydrated in a stepwise manner and then dehydrated once more.

The amount of water in a sample can be determined by measuring the weight of it as observed by using the DVS-Intrinsic method (Johnsen et al. 2011). This is equivalent to the observations made after the runs with liposomes and polymers (all data not shown), as presented in Figure 6-3, where the sample mass increases if the % RH increases and the sample mass decreases if the % RH decreases.

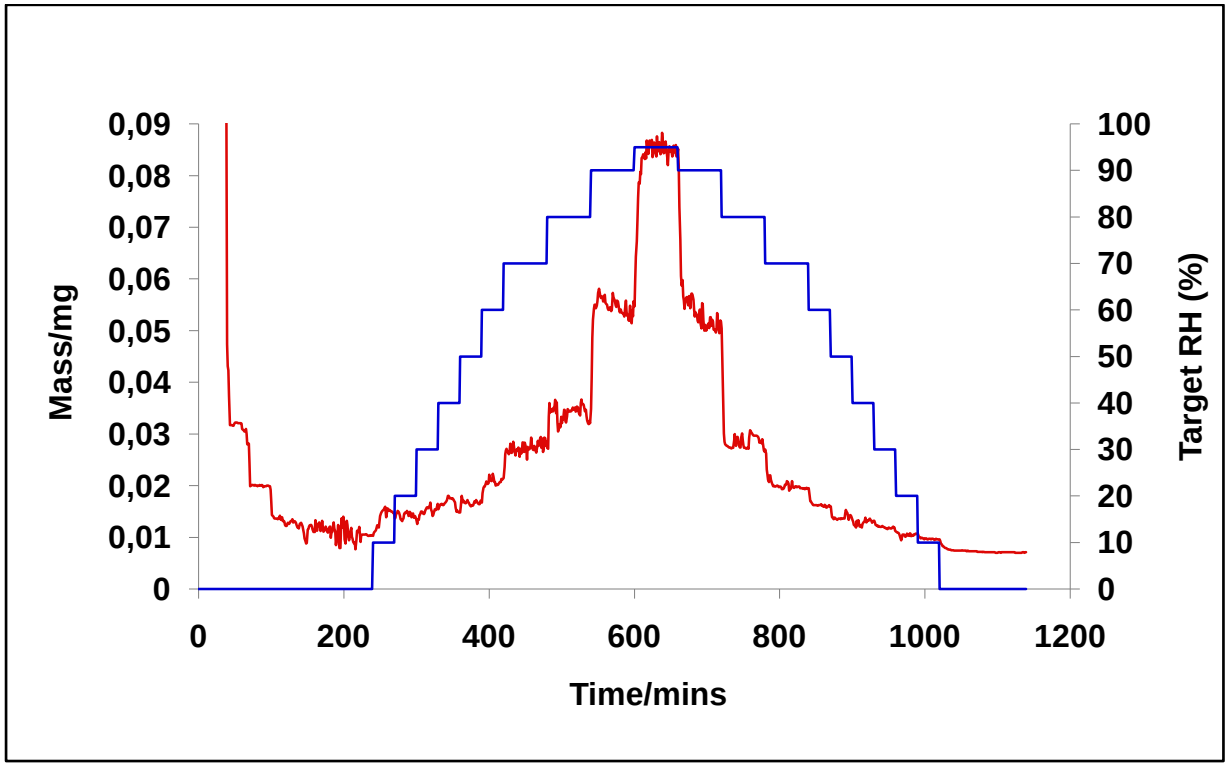


Fig. 6-3. DVS-Intrinsic analysis of 0.1 % LM Pectin: % RH stages set from 0-95-0 with a changing factor of 10. The blue line represents the target % RH for each stage and the red line represents mass readings of 0.1 % LM Pectin as it changes with the % RH.

Temperature equilibration time after sample loading

To determine how long (minutes) the first step needed to be for the temperature to stabilize, 5 mM phosphate buffer pH 6.8 was run from 80-70 % RH. As shown in Figure 6-4 the temperature stabilizes after approximately 150 minutes after loading the sample.

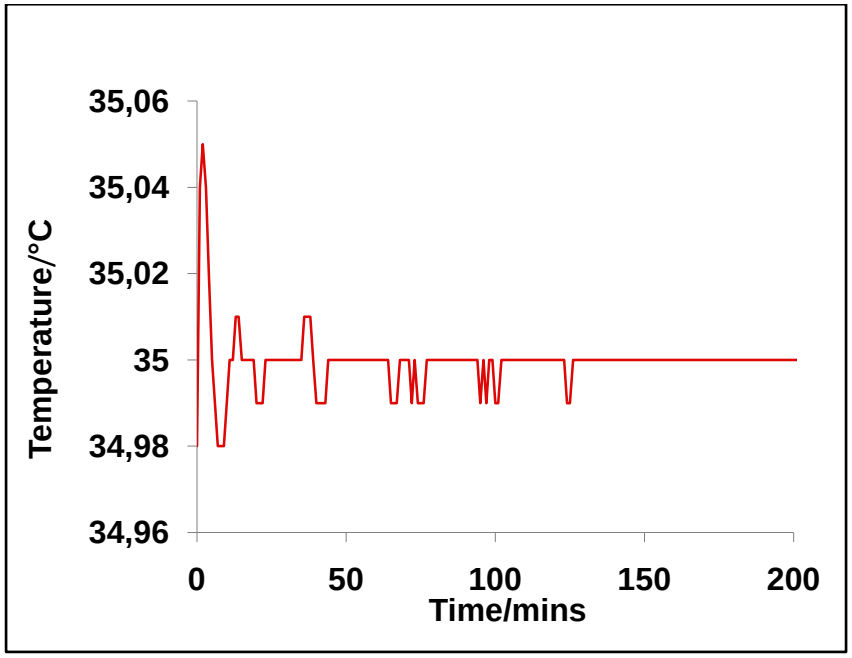


Fig.6-4. DVS-Intrinsic analysis of 5 mM phosphate buffer pH 6.8: % RH stages set from 80-70 with a changing factor of 10. The red line represents the sample temperature during the whole sample run.

Sample dehydration time after loading

Another aspect of timing the first step is the samples dehydration time. A volume of 15 µl was applied and all the samples water content had to be dry before the sorption/desorption testing could commence. Figure 6.5 shows a magnification of the first step (0 % RH), which lasted for 90 minutes. The sample mass decreases with time, and if it is compared with the dehydration step after 690 minutes (data not shown) it is higher than the last sample mass recorded at 0 % RH.

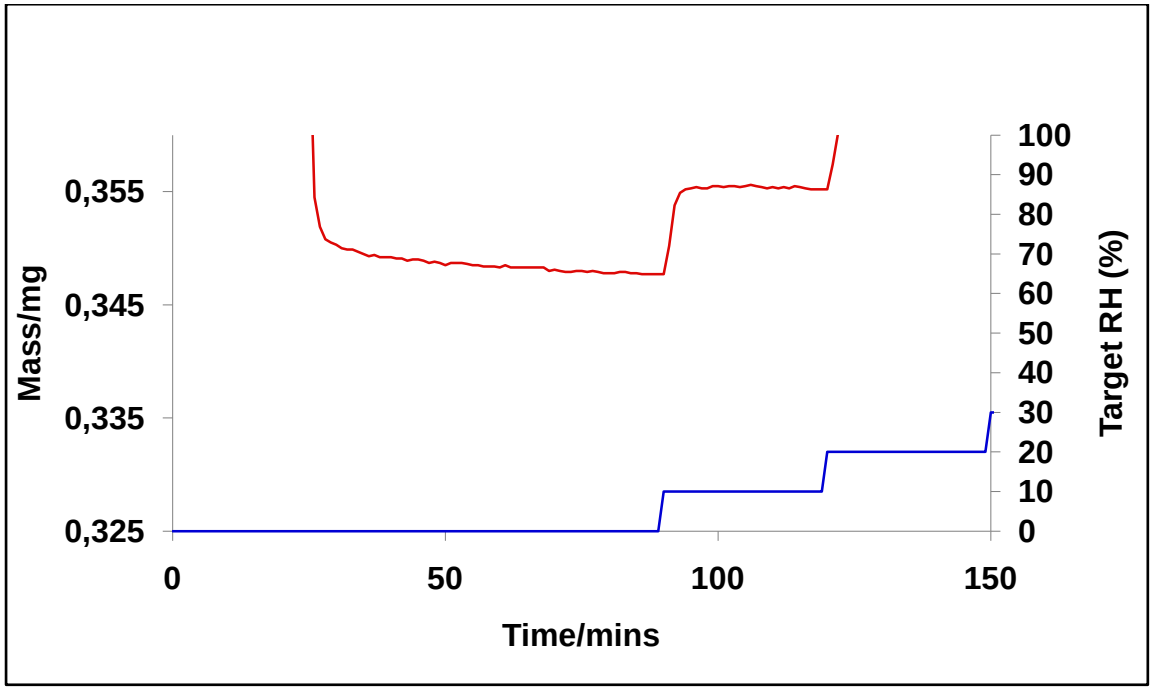


Fig. 6-5. Magnification of DVS-Intrinsic analysis of 15 mM EggPC/DOTAP: % RH stages set from 0-95-0 with a changing factor of 10. The blue line represents the target % RH for each stage and the red line represents mass readings of 15 mM EggPC/DOTAP as it changes with the % RH.

Step equilibration time

To determine the duration of each step from 10 % RH up to 95 % and down again to 0 % RH, a series of different tests were run on both 5 mM phosphate buffer pH 6.8 and 15 mM and 30 mM EggPC/DOTAP. First each step factor lasted for 90 minutes, and then it was reduced to last for only 30 minutes to save time because no major differences between the mass changes were observed. This is illustrated in Figure 6-6 below.

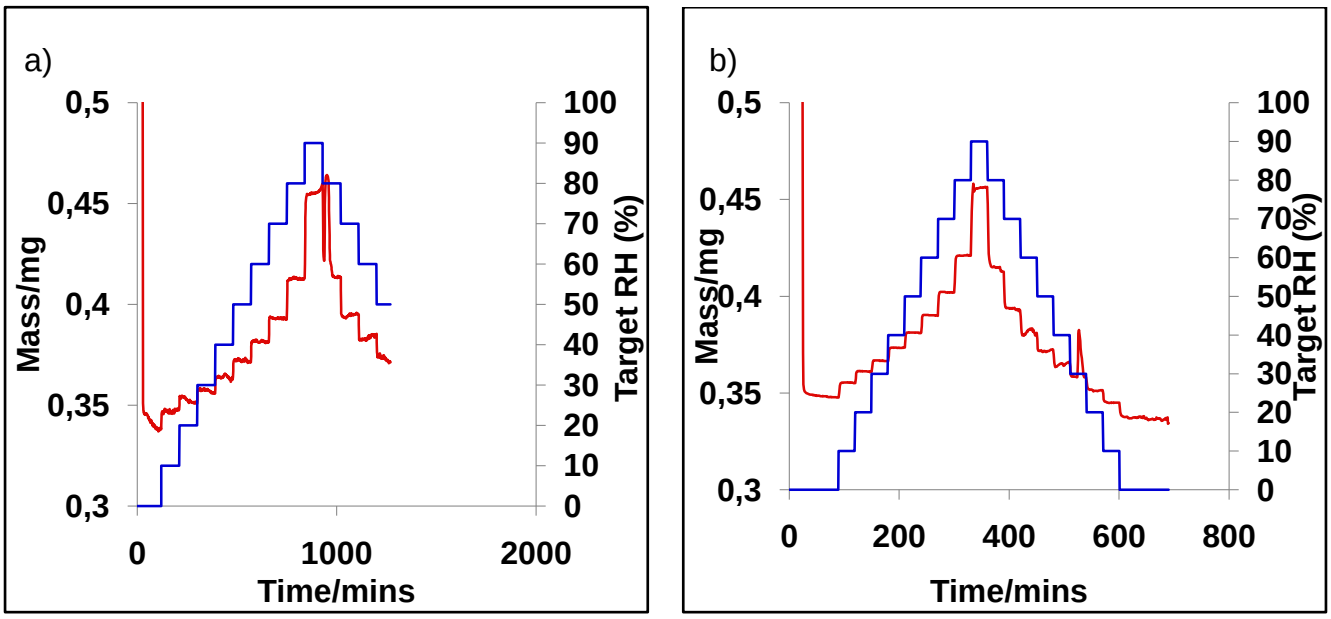


Fig. 6-6. DVS-Intrinsic analysis of 30 mM EggPC/DOTAP: a) % RH stages set from 0-90-0 with a changing factor of 10. Each step was set to last for 90 minutes, and the experiment was stopped at 50 % RH because of time efficiency. b) % RH stages set from 0-90-0 with a changing factor of 10. Each step was set to last for 30 minutes. The blue line represents the target % RH for each stage and the red line represents mass readings of 30 mM EggPC/DOTAP as it changes with the % RH.

The last and final step of the DVS-Intrinsic measurement was also determined by testing 5 mM phosphate buffer pH 6.8 and 15mM and 30 mM EggPC/DOTAP with the same procedure as described in Figure 6-3. From Figure 6-7 below, as seen, where the last step of 0 % RH is presented, the sample mass decreases slowly with time.

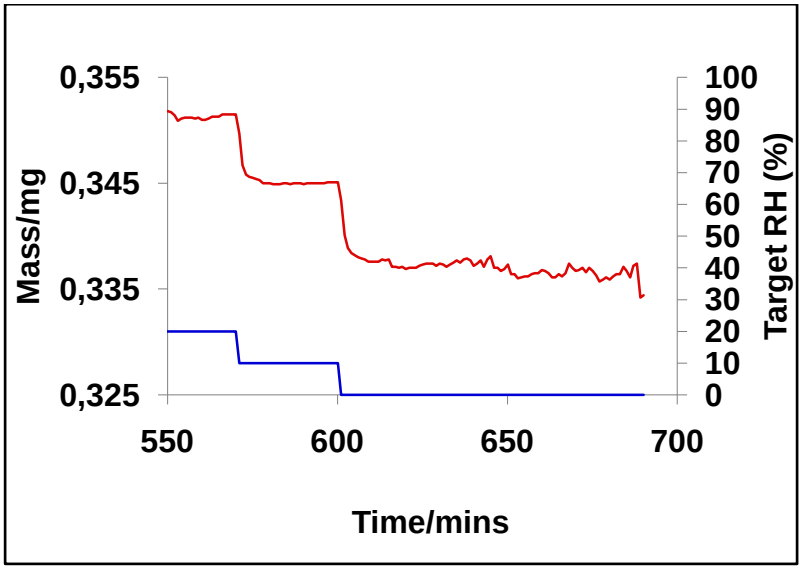


Fig. 6-7. DVS-Intrinsic analysis of 30 mM EggPC/DOTAP: % RH stages set from 0-90-0 with a changing factor of 10. The last step, 0 % RH, lasted for 90 minutes. The blue line represents the target % RH for each stage and the red line represents mass readings of 30 mM EggPC/DOTAP as it changes with the decreasing % RH.

Sample concentration

When the analysis of 0.1 % and 1.0 % LM Pectin is compared it is clear that a higher percentage of polymer solution gives a higher mass reading. By looking at Figure 6-3 and 6-8 the mass readings can be correlated with polymer concentration.

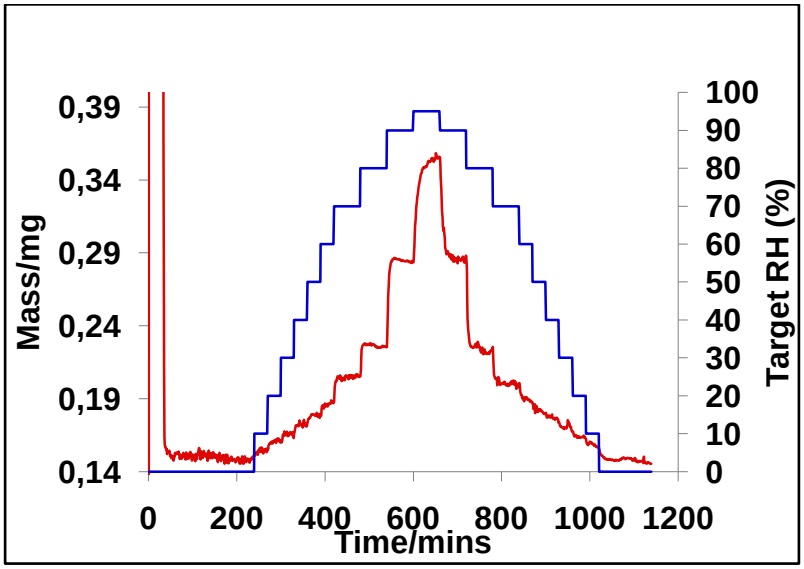


Fig. 6-8. DVS-Intrinsic analysis of 1,0 % LM Pectin: % RH stages set from 0-90-95-90-0 with a changing factor of 10, except from a changing factor of 5 from 90-95-90 % RH. The blue line represents the target % RH for each stage and the red line represents mass readings of 1.0 % LM Pectin as it changes with the % RH.

6.1.2 DVS – Intrinsic hydration/dehydration studies

Both liposomes and polymers were investigated with the DVS – Intrinsic study. Positively charged uncoated EggPC/DOTAP liposomes, negatively charged uncoated EggPC/EggPG and DPPC/DPPG liposomes, and Pectin- (HM, AM and LM), Alginate-, Chitosan- and PNIPAAM- polymers, were investigated for their water adsorption/retention abilities. The characteristics of the liposome samples are shown in Table 6-1 below, including characteristics of coated liposomes regarding the release studies.

Table 6-1. The zeta potential, the size and corresponding PdI of uncoated and coated liposomes with 1.5 mM carboxyfluorescein encapsulated.

Liposome uncoated/coated	Size (nm)	PdI	Zeta potential (mV)
EggPC/DOTAP	147.1	0.092	35.32
EggPC/DOTAP coated with AM Pectin	277.9	0.176	-36.66
EggPC/DOTAP coated with HM Pectin	350.9	0.233	-28.26
EggPC/DOTAP coated with LM Pectin	233.1	0.144	-30.20
EggPC/DOTAP coated with Alginate	192.2	0.149	-50.21
EggPC/DOTAP coated with PNIPAAM	282.3	0.237	-27.70
EggPC/EggPG	167.0	0.084	-39.08
EggPC/EggPG coated with Chitosan	1159.3	0.724	10.52
DPPC/DPPG	157.6	0.118	-42.00
DPPC/DPPG coated with Chitosan	3050.1	0.613	14.26

To measure the water sorption isotherms of liposomes and polymers, a specific setup for the DVS-Intrinsic analysis had to be established. The final procedure was in the end determined based on all the preliminary experiments. Originally the thought was to start the procedure from 0 % RH and from there go up to 100 % RH, and back down again with a factor of 10. But since the DVS-Intrinsic only had a humidity range from 0-98 % RH, the highest percentage examined was set to 95.

The first step (0% RH) was set to last for 240 minutes to make sure that the temperature and mass had stabilized completely before the next step was set in motion, and as shown in Chapter 6.1.1 the temperature stabilizes after approximately 150 minutes. The mass needs a bit more time to stabilize as seen in Figure 6-8, hence the final procedure time of 240 minutes. Even though 240 minutes is enough time for the sample mass to stabilize this amount of time does not seem to be enough to evaporate all water content within the sample. This can be seen from Figure 6-3 and 6-6 b), where at the last 0 % RH step, the sample mass is lower than at the first 0 % RH step. Another possible reason for differences between sample masses is tuning by the DVS-Intrinsic.

Each step from 0 - 60 % RH with a changing factor of 10 were set to last for 30 minutes, and the steps from 70 – 95 % RH were set to 60 minutes. Observations made from Figure 6.8 shows that the highest mass increase happens from 60 – 95 % RH, and therefore these steps lasts longer then the steps with lower percentage of RH.

The last step of 0 % RH was set to 120 minutes instead of 90 minutes as the last step shows in Figure 6.7. To let the sample mass stabilize even more 30 minutes was added to the last step. Although it most likely would continue to lose weight as time passed, it was considered to be enough since the last step was not of high interest.

6.1.2.1 Water adsorption

In Figures 6-1, the stepwise water uptake (mg), from 0 – 95 %, for EggPC/DOTAP, EggPC/EggPG and DPPC/DPPG are presented. As can be seen from the figures, water is adsorbed in each step, and it is the steps with highest % RH that adsorbs water the most. From approximately 60 – 95 % RH, the highest steps, the samples take up more water than the rest of the steps, and they are therefore the % RH that is most interesting.

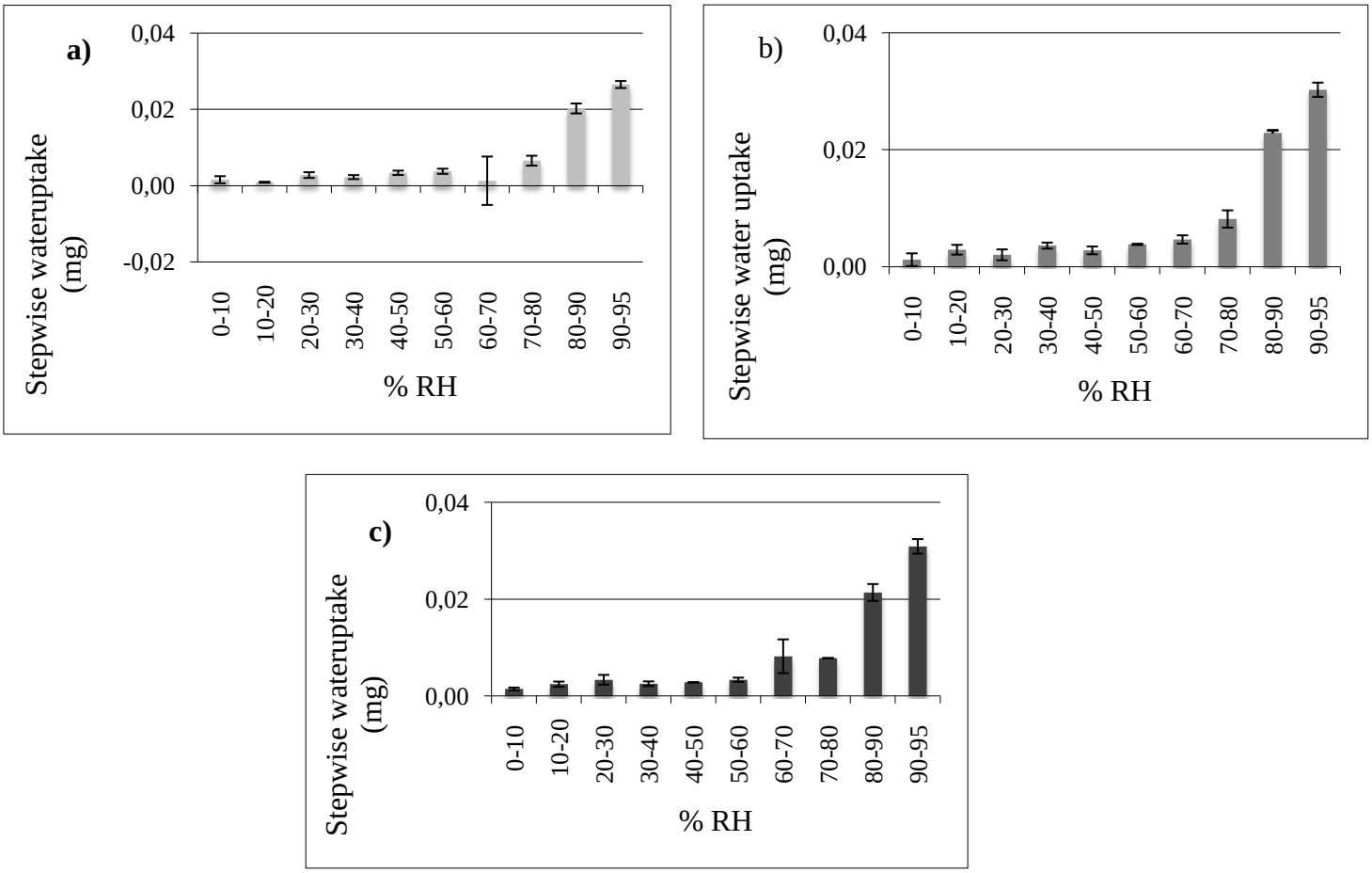


Fig. 6-9. Stepwise water uptake (mg) for liposomes. The average weight (of the 10 last measurements) of a % RH was deducted from the average weight (of the 10 last measurements) of the lower % RH. The error bars represent the highest and lowest sample values (n=2) a) EggPC/DOTAP, b) Egg/PC/EggPG, c) DPPC/DPPG.

In Figure 6-10 the stepwise water uptake for all the liposomes are compared in the % RH range 60 - 95. From this plot it is apparent that the humidity from 80-95 % is the two steps that adsorb most water.

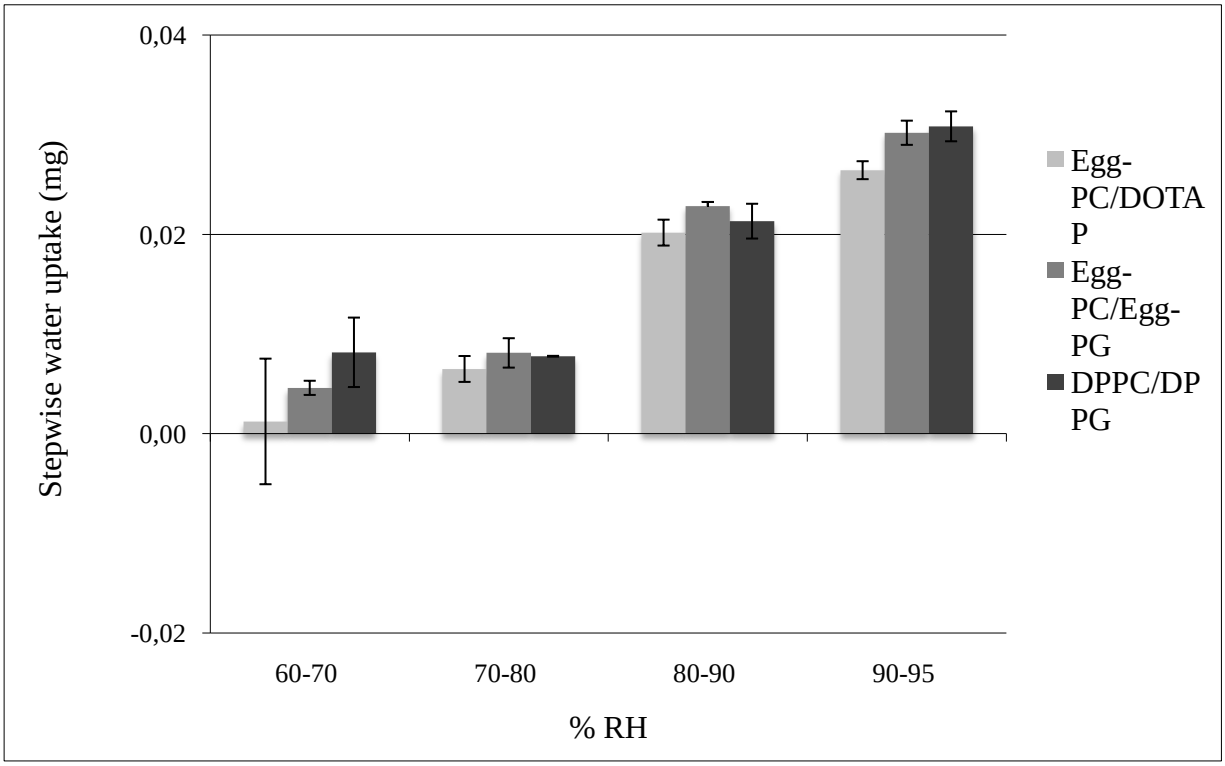


Fig. 6-10. Comparison of the stepwise water uptake (mg) for all liposome suspensions. The error bars represent the highest and lowest sample values (n=2)

In addition of studying the stepwise water uptake of liposomes, the total amount of water adsorbed at each % RH was investigated (Figure 6-11), and the same trend as in Figure 6-9 can be seen here.

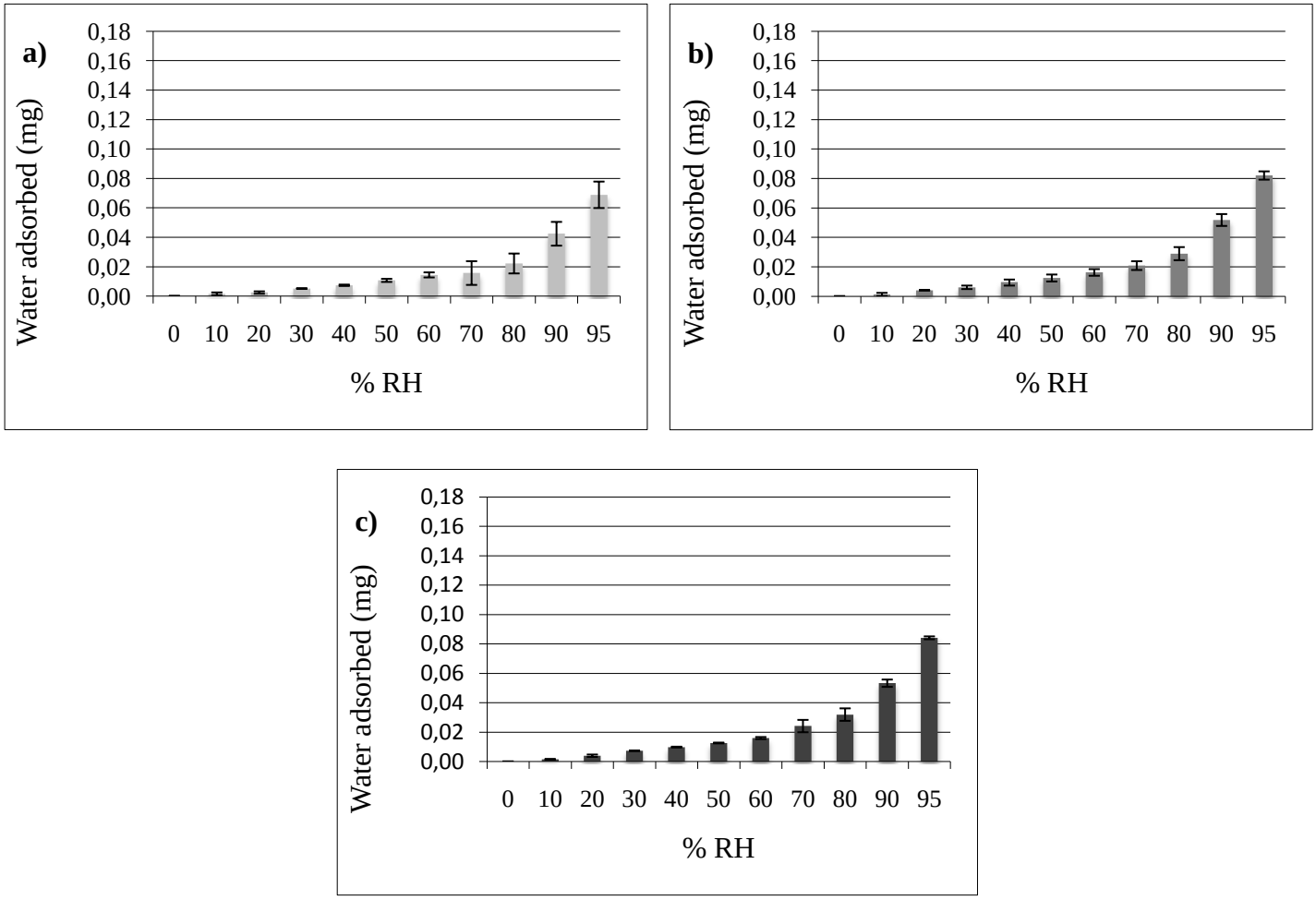


Fig. 6-11. Total amount of water adsorbed (mg at the different % RH for liposomes. The average mass (of the 10 last measurements) of 0 % RH was deducted from the average mass (of the 10 last measurements) of the different % RH. The error bars represent the highest and lowest sample values (n=2). a) EggPC/DOTAP, b) EggPC/EggPG, c) DPPC/DPPG

The water uptake increases simultaneously as the % RH, and it is still the percentages from the last steps of % RH that displays the highest uptake of water as can be seen in Figure 6-11. For comparison the values in the most interesting range (60-95 %RH) are plotted together in Figure 6-12.

There is no significant difference between the amounts of water adsorbed by the different liposomes at 95 % RH ($p < 0.05$).

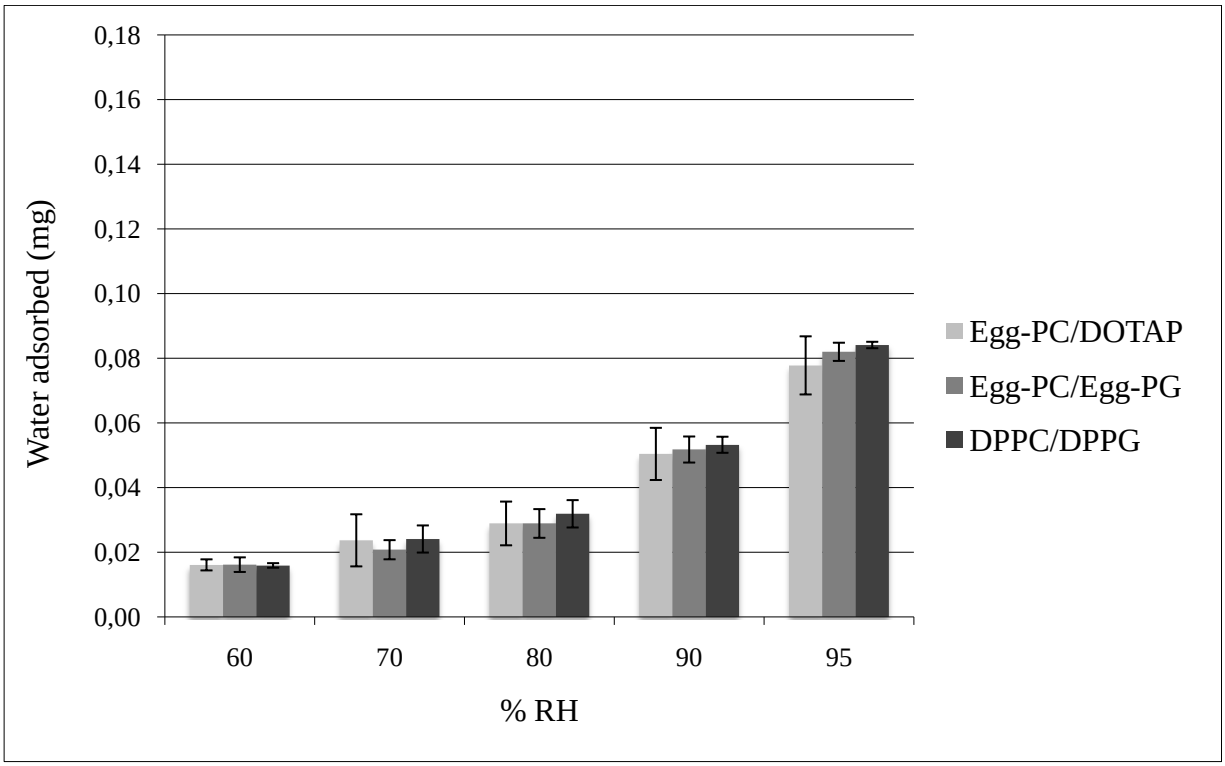


Fig. 6-12. Comparison of the total amount of water adsorbed (mg) at each % RH for all liposome suspensions. The error bars represent the highest and lowest sample values (n=2)

As seen in Figures 6-13 and 6-14, PNIPAAM, adsorbed more water in mg and more water percentage wise from about 60 – 95 %, which is the same trend as for the liposomes presented in Figure 6.10 and 6.12. All polymers (data not shown) show the same trend, as PNIPAAM, and the water adsorbing properties from 60-95 % are therefore the range, which are more closely looked at.

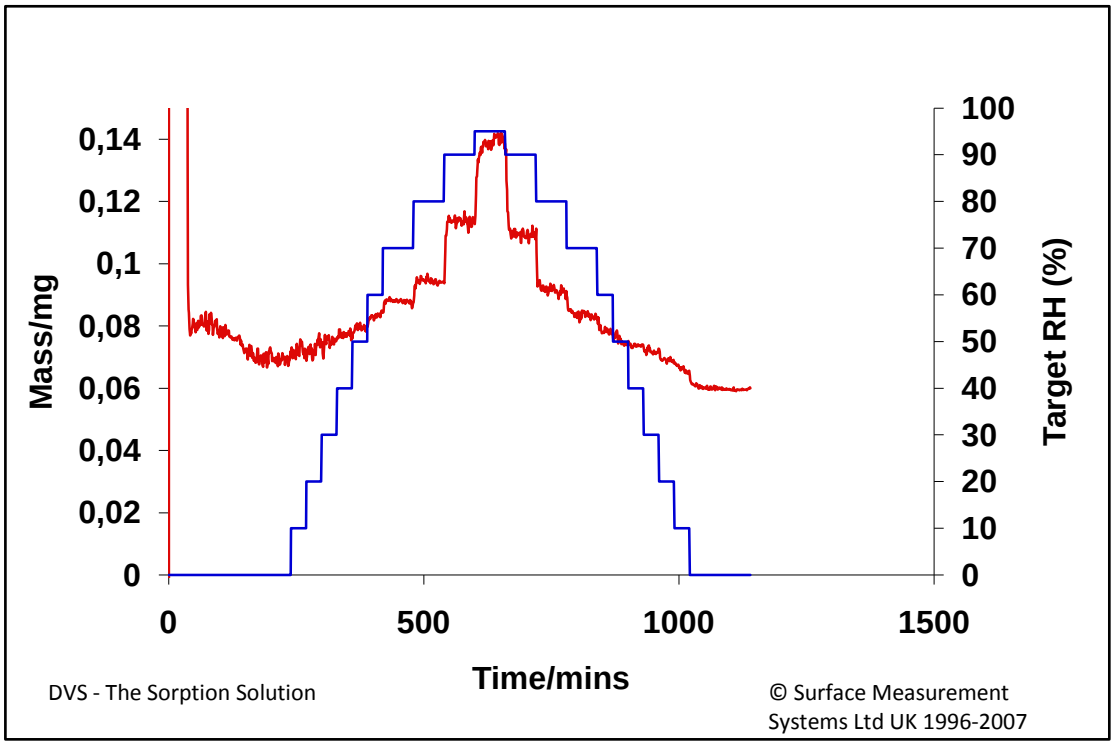


Fig. 6-13. DVS-Intrinsic mass (mg) plot of 0.5 % PNIPAAM: % RH stages set from 0-90-95-90-0 with a changing factor of 10, except from a changing factor of 5 from 90-95-90 % RH. The blue line represents the target % RH for each stage and the red line represents mass readings of 0.5 % PNIPAAM as it changes with the % RH.

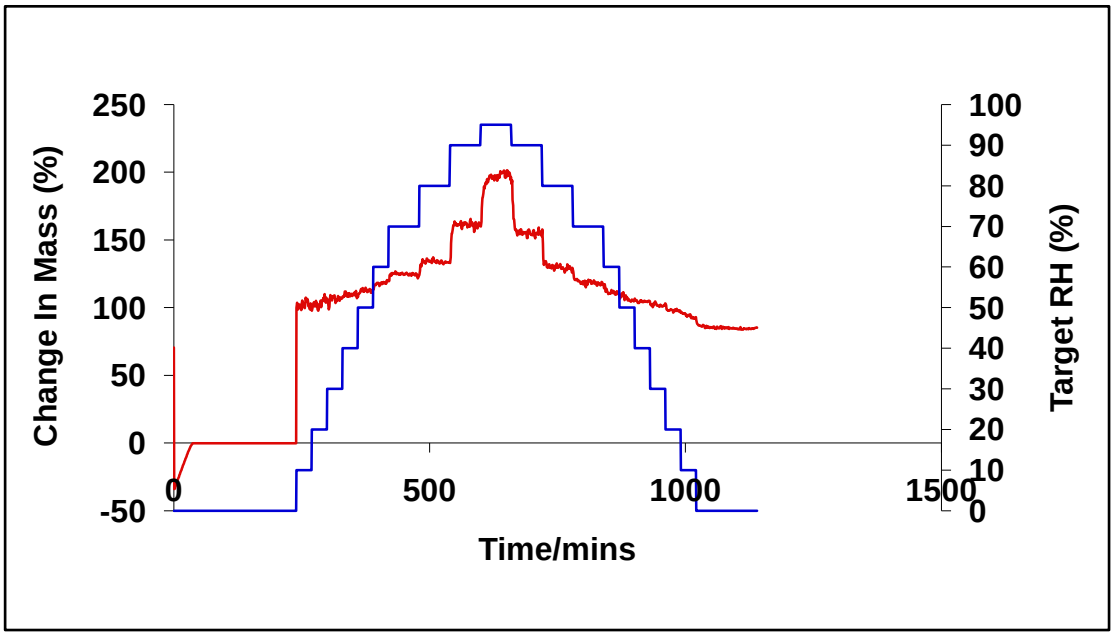


Fig. 6-14. DVS-Intrinsic change in mass (%) plot of 0.5 % PNIPAAM: % RH stages set from 0-90-95-90-0 with a changing factor of 10, except from a changing factor of 5 from 90-95-90 % RH. The blue line represents the target % RH for each stage and the red line represents mass readings of 0.5 % PNIPAAM as it changes with the % RH.

From Figure 6-15 and 6-16 the water adsorbing abilities of the different polymers, including PNIPAAM, can be seen. LM Pectin adsorbs more water than the other polymers, and PNIPAAM is the polymer that takes up the least amount of water.

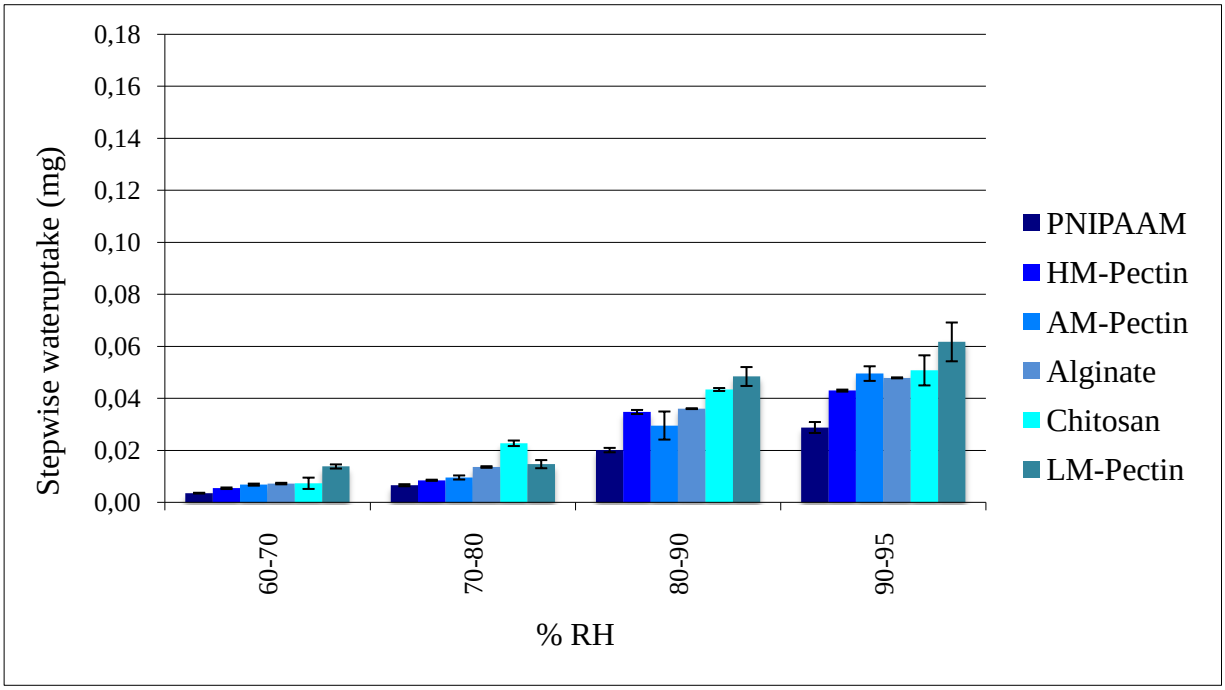


Fig. 6-15. Compared stepwise water uptake (mg) for polymers. The error bars represent the highest and lowest sample values (n=2).

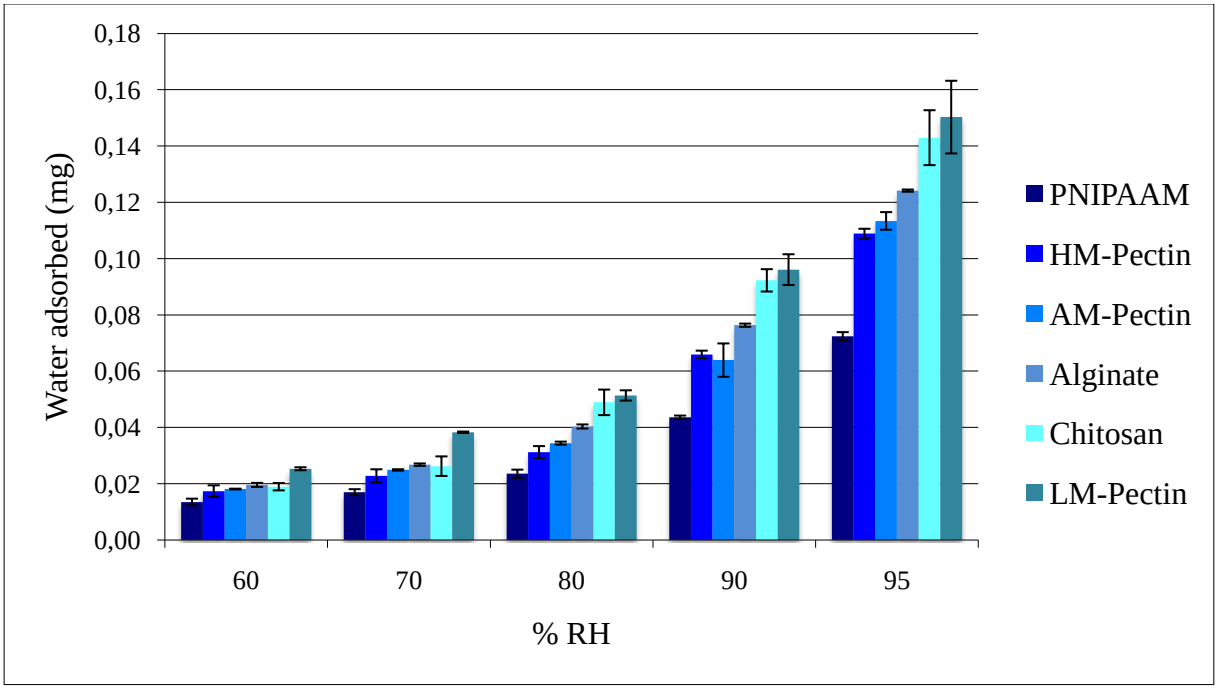


Fig. 6-16. Comparison of water adsorbed (mg) per % RH for polymers. The error bars represent the highest and lowest sample values (n=2).

At 95 % RH PNIPAAM is significantly different ($p < 0.05$) from all other polymers except from HM Pectin, and these are the two polymers that adsorbs the least water. In Figure 6-15 it looks like PNIPAAM and HM Pectin are significantly different. Calculations show that they in fact are different, but only when the negative polymers are taken into account.

Alginate and Chitosan are only significantly different ($p < 0.05$) from PNIPAAM at 95 % RH, and otherwise they show the same water adsorbing properties as the other polymers.

The Pectin formulations vary in their water adsorption properties. LM Pectin is significantly different ($p < 0.05$) from HM pectin, and takes up most water. That HM Pectin is the one that takes up the least amount of water and LM Pectin the one that takes up the most could be correlated to the Mw and zeta potential of LM, AM and HM Pectin. The Mw of the different polymers are LM Pectin < AM Pectin < HM Pectin, and the zeta potential for LM Pectin is most negative and least negative for HM Pectin (Nguyen, Alund, et al. 2011). It can seem that the smallest Pectin polymer, and most negative, takes up more water than larger and less negative Pectin polymers.

Then all samples at 95 % RH, liposomes and polymers, can be compared with each other ($p < 0.05$%). LM Pectin is different from all samples except from Alginate and Chitosan. PNIPAAM is different from all the polymer samples, and all the liposomes are different from the polymers except from PNIPAAM. Other samples that differ from each other are Chitosan and HM Pectin.

6.1.2.2 Water desorption

To determine the water desorption properties of both liposomes and polymers the % RH were decreased in a stepwise manner from humidity of 95 % and down to 0 %. After the experiment had reached 95 % RH, the step was set to last for 60 minutes. Then each step from 95 % to 70 % RH lasted for 60 minutes, and the steps leading to 0 % RH lasted for 30 minutes each. The last and final step, 0 % RH, lasted for 120 minutes.

Using the equation as described in Chapter 4.3.1 the time constants for all the samples were calculated in the range RH 95-90%. A time constant were used as a parameter to determine the water desorption and the constants for liposomes and polymers are shown in Figure 6-18. The time constants tell us how long time it takes for a sample to change 63 % towards it new value. In this case it tells us how long it takes for a liposome, or polymer, to go from a certain weight to fall 63 % from its original value to the next step (Ø. G. Martinsen et al. 2008; O. G. Martinsen et al. 2008; Johnsen et al. 2008). A longer time constant represents a better ability to hold on to water. Figure 6-17 shows a curve fitted of the data of 0.5 % EggPC/DOTAP in the step from 95-90 % RH as an example.

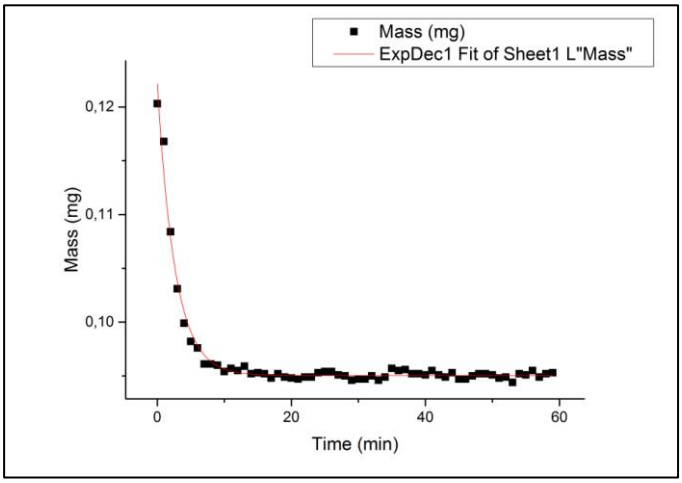


Fig. 6-17. A curved fit of the mass (mg) data of 0.5 % EggPC/DOTAP in the step from 95-90 % RH.

The liposomes have shorter time constants than the polymers from 95-90 % RH as seen in Figure 6-18. All liposomes exhibit the same water retention properties, and they differ significantly ($p < 0.05$) from LM-pectin, Chitosan and AM-Pectin. LM Pectin is significantly different from all samples except from Chitosan. Chitosan are different from PNIPAAM, HM-Pectin and Alginate.

Initially the time constant from each step was going to be calculated, but from 90-80 % RH and down the adjustment of the curves turned out to be bad for many of them. Therefore they were not suitable for comparison.

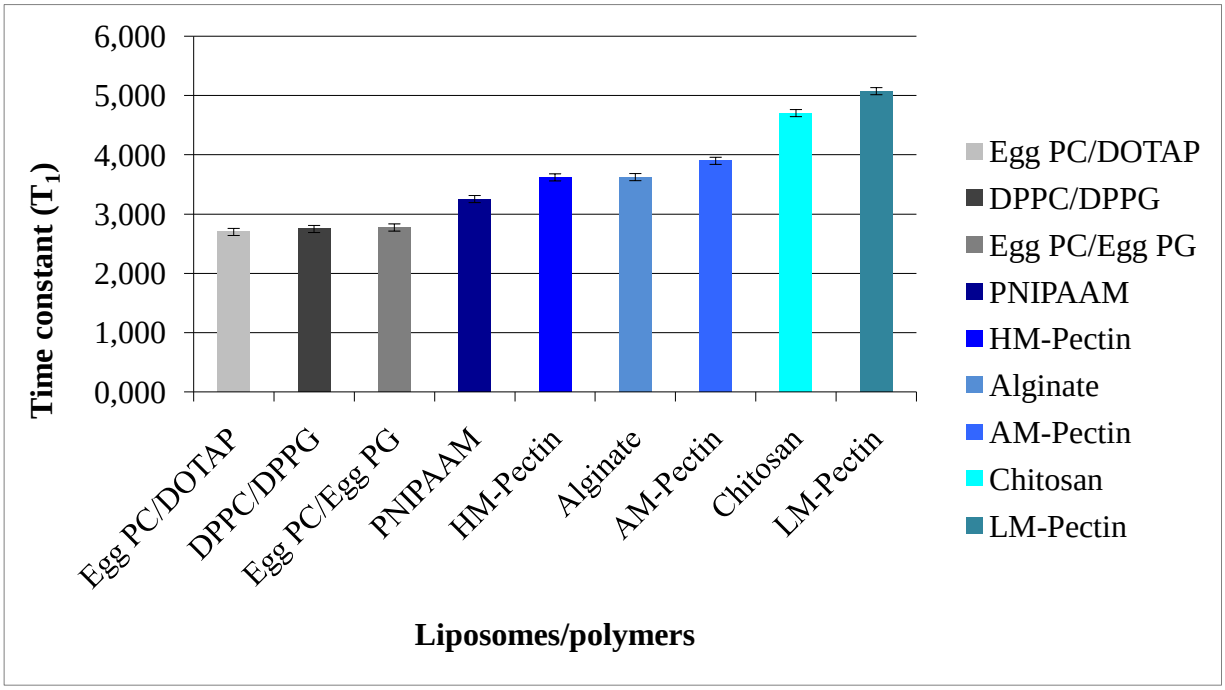


Fig. 6-18. Time constants for liposomes and polymers. The error bars represent the highest and lowest sample values (n=2).

Comparison of water adsorption and water retention ability

If the data from all the DVS-Intrinsic study is compared there are certain connections between them. The different liposomes are not significantly different ($p < 0.05$) from each other when it comes to their water adsorbing or water retention abilities. They are also the ones that display the lowest values when it comes to their water uptake, except from PNIPAAM, and when it comes to their time constants. All polymers, except PNIPAAM, exhibit better water adsorbing and water retention properties than the liposomes alone.

The time constant data, i.e. the water retention data seems to correlate with the results from the water adsorbing studies. LM Pectin is the polymer that adsorbs the most water and that holds on to most water, and it is significantly different ($p < 0.05$) from all other samples except from Chitosan.

When it comes to a drug formulation to treat xerostomia, this could indicate that the liposomes should be coated with a polymer to increase its water adsorbing/retention properties, and that LM Pectin or Chitosan are one of the better polymer choices. Studies have shown that both LM Pectin and Chitosan have mucoadhesive properties (Hagesaether & Sande 2007; Jøraholmen et al. 2014; Khutoryanskiy 2011), although Chitosan is more extensively researched. They also stabilize the liposomes after production (Smistad et al. 2012; Chen et al. 2014).

6.2 Release studies

6.2.1 Determination of centrifuge speed, time and temperature

The centrifugation speed, time and temperature needed for separating the supernatant from the liposomes in the release studies were determined. The first test was run with room tempered 0.6 mM EggPC/DOTAP, for 5 minutes with a temperature of 4 °C and an rpm of 7000. Five minutes were enough time to centrifuge the sample. However, since the centrifuge used approximately five minutes to achieve 4 °C, a new almost identical test, with a temperature adjustment to 20 °C was run. Also this time it was enough with 5 minutes to isolate enough supernatant for further testing.

6.2.2 Release studies

The per cent releases of carboxyfluorescein from uncoated liposomes, at 35 °C over a time period of 24 hours, are shown in Figure 6-19. The release from 20 min to 4 hours is most interesting because of the fact that the formulations are destined to be a drug that helps with xerostomia. And the instant effect, as well as the continued effect, will be central.

DPPC/DPPG released significantly less carboxyfluorescein compared to EggPC/DOTAP and EggPC/PG in the time period 20 min – 2 h ($p < 0.05$). At 24 hours all the uncoated liposomes showed similar release profiles. The release was, even after 24 hours, very low (<10 %) for all formulations.

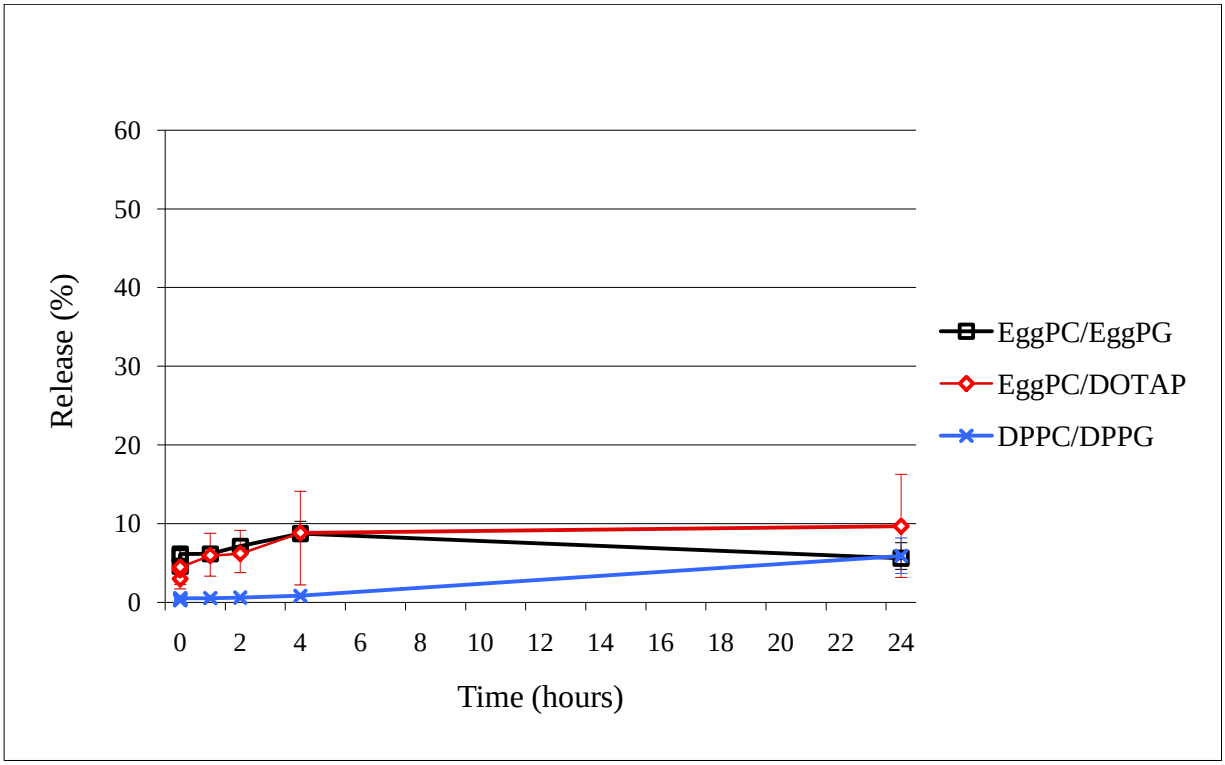


Fig. 6-19. Comparison of uncoated EggPC/DOTAP, EggPC/EggPG and DPPC/DPPG and their release profiles over a time period of 24 hours. The error bars represent the highest and lowest sample values (n=3). When invisible they are equal to or smaller than the size of the symbols.

Coated EggPC/DOTAP with negative polymers is shown in Figure 6-20. EggPC/DOTAP coated with PNIPAAM releases the least carboxyfluorescein after 24 hours, as low as just below 7% and EggPC/DOTAP coated with HM Pectin releases as much as about 50%.

After 20 minutes there are no significant difference ($p < 0.05$) between the EggPC/DOTAP coated with different negatively loaded polymers and the uncoated EggPC/DOTAP. However after 40 minutes there is some dissimilarity among the samples. The release from EggPC/DOTAP coated with LM-Pectin is significantly higher ($p < 0.05$) than the other coated EggPC/DOTAP liposomes and the uncoated EggPC/DOTAP.

Two hours into the 24-hour time period there are more differences between the samples. LM Pectin coated EggPC/DOTAP still releases the most carboxyfluorescein, but now the same amount ($p < 0.05$) as if coated with HM Pectin. The release from AM Pectin and Alginate coated liposomes are significantly higher than PNIPAAM coated EggPC/DOTAP.

After 4 hours EggPC/DOTAP coated with HM Pectin and LM Pectin are significantly different ($p < 0.05$) from uncoated EggPC/DOTAP and EggPC/DOTAP coated with PNIPAAM. The results regarding a liposome coated with different Pectin polymers are different from a former study (Smistad et al. 2012). Here there were no significant differences between an uncoated liposome and any Pectin coated liposome at 35 °C. Since a different liposome was used in the experiment no resolute conclusion can be drawn.

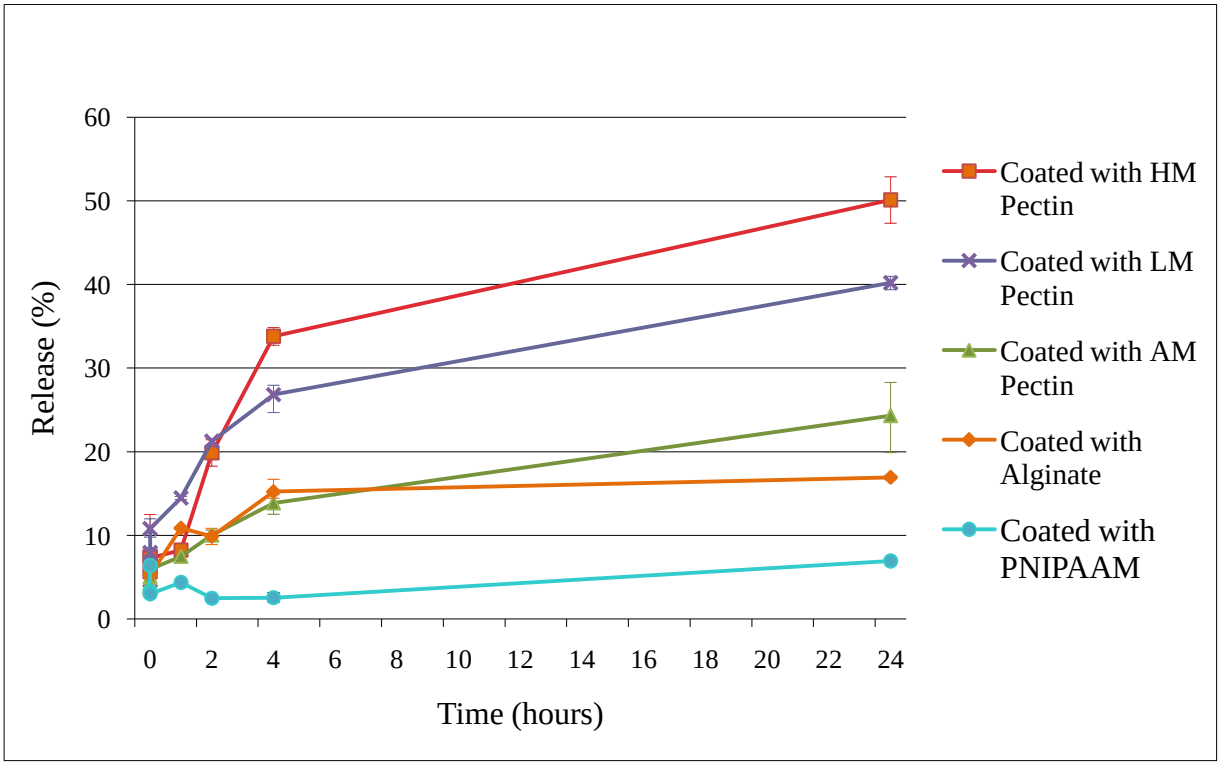


Fig. 6-20. Comparison of EggPC/DOTAP coated with HM-, LM- or AM-Pectin and Alginate or PNIPAAM and their release profiles over a time period of 24 hours. The error bars represent the highest and lowest sample values (n=3). When invisible they are equal to or smaller than the size of the symbols.

The results from the liposomes coated with Chitosan could not be compared statistically due to high variations within the sample measurements. This may be because of inconsistency of the sample collections as described in Chapter 4.2.5.

At t = 0 minutes the Chitosan coated liposomes varies a lot as shown in Figure 6-21. DPPC/DPPG coated with Chitosan releases a very high amount of carboxyfluorescein compared with EggPC/EggPG coated with Chitosan. From 20 minutes and up to 4 hours both Chitosan-coated liposomes increase their release of carboxyfluorescein, but as Chitosan-coated EggPC/EggPG continues its increasing release, DPPC/DPPG coated with Chitosan decreases with time up to t = 24 hours, but this is not a reality.

Since the samples were taken out of the same solution over time the concentration of carboxyfluorescein in it could not have decreased. Uncertainty in the method itself could be the source of error in the results. Examples of uncertainties are irregularities with sample collections, differences in sample temperatures during determination of carboxyfluorescein concentrations, inaccuracy of sample application to the microtiter plates and the like. The total release of carboxyfluorescein for Chitosan-coated EggPC/EggPG and DPPC/DPPG are low, and at the same level as for the uncoated liposomes (Figure 6-19).

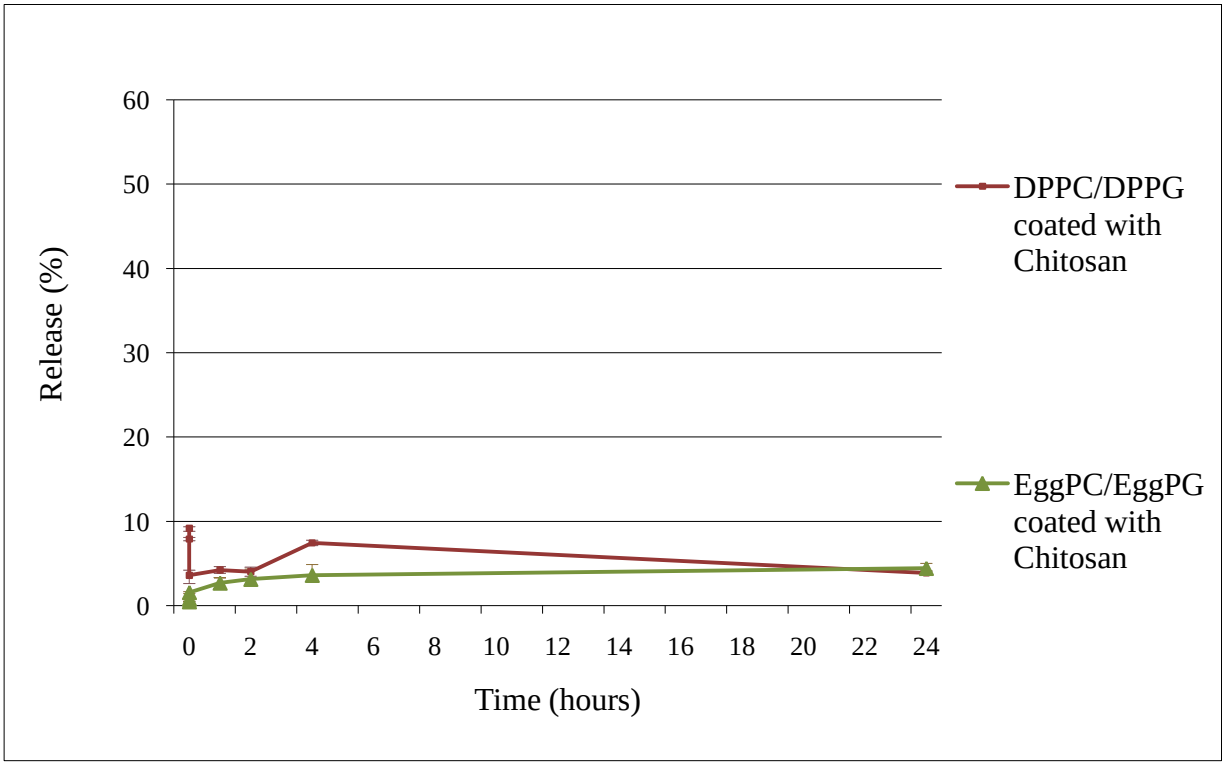


Fig. 6-21. Comparison of Chitosan coated EggPC/EggPG and DPPC/DPPG. The error bars represent the highest and lowest sample values (n=3). When invisible they are equal to or smaller than the size of the symbols.

6.3 Comparison of DVS – Intrinsic and release studies

When the results from the DVS-Intrinsic studies and release studies are compared LM-Pectin is the polymer that stands out. In the water adsorption/retention tests it takes up and holds on to water better than all other samples except a couple of polymers (Alginate and Chitosan). In the release studies EggPC/DOTAP coated with LM-Pectin are significantly different ($p < 0.05$) from all other samples (EggPC/DOTAP coated with negative polymers) after 40 minutes. Two hours into the experiment EggPC/DOTAP coated with LM-Pectin are different from almost all samples except from HM-Pectin coated EggPC/DOTAP.

HM-Pectin exhibits better water adsorption/retention properties than PNIPAAM and uncoated liposomes. In the release study this is also true after 40 minutes and 4 hours. EggPC/DOTAP coated with PNIPAAM has the smallest release of carboxyfluorescein after 2 hours. PNIPAAM is also the polymer that takes up the least amount of water in the water sorption tests, although it is only different from LM-Pectin, Chitosan and AM-Pectin.

Uncoated DPPC/DPPG is significantly different ($p < 0.05$) from uncoated EggPC/DOTAP and EggPC/EggPG in the release profiles after 20 minutes, 40 minutes and 2 hours. In the water sorption/desorption studies there are no differences between the uncoated liposomes. Uncoated EggPC/DOTAP and EggPC/EggPG would therefore be better to choose over uncoated DPPC/DPPG when it comes to drug formulation.

7. Conclusion

In this study the water adsorption/retention abilities of liposomes (EggPC/DOTAP, EggPC/EggPG and DPPC/DPPG) and polymers (HM Pectin, AM Pectin, LM Pectin, alginate, Chitosan and PNIPAAM) has been investigated with a DVS-Intrinsic apparatus. The release profile of carboxyfluorescein of EggPC/DOTAP coated with negative polymers, and EggPC/EggPG and DPPC/DPPG coated with a positive polymer (Chitosan) has been studied as well.

In this study a successful *in vitro* method for determination of the water adsorption/retention capacity of liposomes and polymers by using the DVS instrument was developed.

The water adsorption/retention capacity of liposomes and polymers at 35 °C was determined by the new DVS Intrinsic method. The liposomes were not significantly different from each other and displayed the same water adsorption/retention abilities. Of the different polymers LM Pectin exhibited the highest water adsorption/retention abilities, although it was not significantly higher than all of them. PNIPAAM displayed the lowest water adsorption/retention ability and was significantly different from all the other polymers.

The release studies of uncoated liposomes at 35 °C showed the same trend as the DVS Intrinsic studies. Uncoated liposomes had similar release profiles, with no significant difference between them. EggPC/DOTAP coated with LM Pectin had a higher release profile than the other coated or uncoated EggPC/DOTAP, although it was only significantly different after 40 minutes. EggPC/DOTAP coated with PNIPAAM had the slowest release profile, although it was never significantly different from all other EggPC/DOTAP coated with a polymer.

LM Pectin as a polymer shows good water adsorption/retention abilities and EggPC/DOTAP coated with LM Pectin has a high release profile from 0 minutes to 4 hours. The liposome formulation of choice for future xerostomia treatments is dependent on the release profile that is optimal for the purpose of hydrating a mouth. If a high release profile were desirable, EggPC/DOTAP coated with LM Pectin would be a good choice.

An optimal release profile for a liposomal drug destined for oral treatment therefore needs to be investigated in the future.

8. List of references

Andrews, G.P., Laverty, T.P. & Jones, D.S., 2009. Mucoadhesive polymeric platforms for controlled drug delivery. *European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V*, 71(3), pp.505–18. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18984051 [Accessed November 12, 2014].

Anon, mouth (anatomy) -- Encyclopedia Britannica. Available at: http://global.britannica.com/EBchecked/topic/395124/mouth [Accessed December 1, 2014].

Anon, 1989. XEROSTOMIA. *The Lancet*, 333(8643), p.884. Available at: http://www.sciencedirect.com/science/article/pii/S0140673689928730 [Accessed December 12, 2014].

Anon, 2014. Xerostomia treatments. *Dental Abstracts*, 59(2), pp.e51–e52. Available at: http://www.sciencedirect.com/science/article/pii/S0011848613003312 [Accessed December 12, 2014].

Barenholz, Y., 2012. Doxil®--the first FDA-approved nano-drug: lessons learned. *Journal of controlled release : official journal of the Controlled Release Society*, 160(2), pp.117–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22484195 [Accessed July 16, 2014].

Benn, A.M.L. & Thomson, W.M., 2014. Saliva: an overview. *The New Zealand dental journal*, 110(3), pp.92–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25265747 [Accessed November 18, 2014].

Burns, D., 2000. Buffer effects on the zeta potential of ultrafiltration membranes. *Journal of Membrane Science*, 172(1-2), pp.39–48. Available at: http://www.sciencedirect.com/science/article/pii/S037673880000315X [Accessed December 14, 2014].

Chen, M.-X. et al., 2014. Layer-by-layer assembly of chitosan stabilized multilayered liposomes for paclitaxel delivery. *Carbohydrate polymers*, 111, pp.298–304. Available at: http://www.sciencedirect.com/science/article/pii/S0144861714003956 [Accessed December 14, 2014].

Clogston, J.D. & Patri, A.K., 2011. Zeta potential measurement. *Methods in molecular biology (Clifton, N.J.)*, 697, pp.63–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21116954 [Accessed December 14, 2014].

Gandhi, R.B. & Robinson, J.R., 1994. Oral cavity as a site for bioadhesive drug delivery. *Advanced Drug Delivery Reviews*, 13(1-2), pp.43–74. Available at: http://www.sciencedirect.com/science/article/pii/0169409X94900264 [Accessed December 12, 2014].

Gil-Montoya, J.A., Guardia-López, I. & González-Moles, M.A., 2008. Evaluation of the clinical efficacy of a mouthwash and oral gel containing the antimicrobial proteins lactoperoxidase, lysozyme and lactoferrin in elderly patients with dry mouth--a pilot study. *Gerodontology*, 25(1), pp.3–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18194332 [Accessed November 11, 2014].

Hagesaether, E. & Sande, S.A., 2007. In vitro measurements of mucoadhesive properties of six types of pectin. *Drug development and industrial pharmacy*, 33(4), pp.417–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17523006 [Accessed December 14, 2014].

Hassan, P.A., Rana, S. & Verma, G., 2014. Making Sense of Brownian Motion: Colloid Characterization by Dynamic Light Scattering. *Langmuir : the ACS journal of surfaces and colloids*. Available at: http://dx.doi.org/10.1021/la501789z [Accessed December 12, 2014].

Johnsen, G.K. et al., 2011. Sorption properties of the human stratum corneum. *Skin pharmacology and physiology*, 24(4), pp.190–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21346399 [Accessed December 4, 2014].

Johnsen, G.K. et al., 2008. 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. Annual Conference*, 2008, pp.3166–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19163379 [Accessed December 4, 2014].

Johnson, J.T. et al., 1993. Oral pilocarpine for post-irradiation xerostomia in patients with head and neck cancer. *The New England journal of medicine*, 329(6), pp.390–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8326972 [Accessed December 1, 2014].

Jøraholmen, M.W. et al., 2014. Chitosan-coated liposomes for topical vaginal therapy: assuring localized drug effect. *International journal of pharmaceutics*, 472(1-2), pp.94–101. Available at: http://www.sciencedirect.com/science/article/pii/S0378517314004360 [Accessed December 9, 2014].

Khutoryanskiy, V. V, 2011. Advances in mucoadhesion and mucoadhesive polymers. *Macromolecular bioscience*, 11(6), pp.748–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21188688 [Accessed November 9, 2014].

Kreuter, J., 1994. *Colloidal Drug Delivery Systems*, Taylor & Francis. Available at: http://books.google.no/books?id=aH_6WI0OHWoC.

Mariotti, A., 2008. *xPharm: The Comprehensive Pharmacology Reference*, Elsevier. Available at: http://www.sciencedirect.com/science/article/pii/B9780080552323606993 [Accessed December 12, 2014].

Martinsen, O.G., Grimnes, S. & Nilsen, S.H., 2008. 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)*, 14(2), pp.142–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18412555 [Accessed December 4, 2014].

Martinsen, Ø.G. et al., 2008. Gravimetric method for in vitro calibration of skin hydration measurements. *IEEE transactions on bio-medical engineering*, 55(2 Pt 1), pp.728–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18270010 [Accessed November 28, 2014].

Nguyen, S., 2011. *Formulation of liposomes for protective functions for the human dental enamel*. University of Oslo - Norway.

Nguyen, S. et al., 2013. Polymer coated liposomes for dental drug delivery--interactions with parotid saliva and dental enamel. *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences*, 50(1), pp.78–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23524255 [Accessed November 25, 2014].

Nguyen, S., Alund, S.J., et al., 2011. Studies on pectin coating of liposomes for drug delivery. *Colloids and surfaces. B, Biointerfaces*, 88(2), pp.664–73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21862293 [Accessed November 25, 2014].

Nguyen, S. et al., 2010. The influence of liposomal formulation factors on the interactions between liposomes and hydroxyapatite. *Colloids and surfaces. B, Biointerfaces*, 76(1), pp.354–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20022224 [Accessed November 25, 2014].

Nguyen, S., Hiorth, M., et al., 2011. The potential of liposomes as dental drug delivery systems. *European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V*, 77(1), pp.75–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20875853 [Accessed November 25, 2014].

Petersen, P.E. et al., 2005. The global burden of oral diseases and risks to oral health. *Bulletin of the World Health Organization*, 83(9), pp.661–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2626328&tool=pmcentrez&rendertype=abstract [Accessed November 25, 2014].

Poole, C.F., 2013. *Reference Module in Chemistry, Molecular Sciences and Chemical Engineering*, Elsevier. Available at: http://www.sciencedirect.com/science/article/pii/B9780124095472043961 [Accessed December 13, 2014].

Rathbone, M.J., Drummond, B.K. & Tucker, I.G., 1994. The oral cavity as a site for systemic drug delivery. *Advanced Drug Delivery Reviews*, 13(1-2), pp.1–22. Available at: http://www.sciencedirect.com/science/article/pii/0169409X94900248 [Accessed December 12, 2014].

Ship, J.A., 2004. *Encyclopedia of Gastroenterology*, Elsevier. Available at: http://www.sciencedirect.com/science/article/pii/B0123868602007322 [Accessed December 12, 2014].

Smistad, G. et al., 2011. Influence of liposomal formulation variables on the interaction with Candida albicans in biofilm; a multivariate approach. *Journal of liposome research*, 21(1), pp.9–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20196625 [Accessed November 25, 2014].

Smistad, G. et al., 2012. The potential of pectin as a stabilizer for liposomal drug delivery systems. *Carbohydrate polymers*, 90(3), pp.1337–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22939349 [Accessed November 30, 2014].

Turner, M.D. & Ship, J. a., 2007. Dry Mouth and Its Effects on the Oral Health of Elderly People. *Journal of the American Dental Association*, 138(1), p.15S–20S. Available at: http://jada.ada.org/cgi/doi/10.14219/jada.archive.2007.0358.

Vivino, F.B. et al., 1999. Pilocarpine tablets for the treatment of dry mouth and dry eye symptoms in patients with Sjögren syndrome: a randomized, placebo-controlled, fixed-dose, multicenter trial. P92-01 Study Group. *Archives of internal medicine*, 159(2), pp.174–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9927101 [Accessed December 1, 2014].

Wick, J.Y., 2007. Xerostomia: causes and treatment. *The Consultant pharmacist : the journal of the American Society of Consultant Pharmacists*, 22(12), pp.985–92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18198958 [Accessed December 1, 2014].

Xu, C. et al., 2014. Fast nanoparticle sizing by image dynamic light scattering. *Particuology*. Available at: http://www.sciencedirect.com/science/article/pii/S167420011400128X [Accessed December 10, 2014].

Xu, R., 2008. Progress in nanoparticles characterization: Sizing and zeta potential measurement. *Particuology*, 6(2), pp.112–115. Available at: http://www.sciencedirect.com/science/article/pii/S1674200108000357 [Accessed November 6, 2014].

Zamany, A., Safavi, K. & Spångberg, L.S.W., 2003. The effect of chlorhexidine as an endodontic disinfectant. *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics*, 96(5), pp.578–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14600693 [Accessed November 25, 2014].