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Coating of neutral liposomes with hydrophobically modified hydroxyethyl cellulose

The influence of hydrophobic chain length on the liposomes' properties and stability

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Coating of neutral liposomes with hydrophobically modified hydroxyethyl cellulose. The influence of hydrophobic chain length on the liposomes' properties and stability

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Abstract

In this study, gel phase (DPPC) and liquid phase (soy PC) neutral liposomes were coated with hydrophobically modified hydroxyethyl cellulose (HM-HEC). The chain lengths of the hydrophobic moieties of HM-HEC were C8 and C16.

The influence of the hydrophobic chain length of the polymer coating on the release of a fluorescence marker was studied at both 35 °C and 4 °C. I addition the size, zeta potential, transmittance and pH were investigated during storage at 4 °C during a period of 12 weeks.

Differential scanning calorimetry was used to study the interaction between the liposomal membrane and the hydrophobically modified polymers. Egg PC, DMPC and DOPC liposomes were included in these studies.

The release studies at 35 °C showed that the HM-HEC coating protected against release, however, with no significant difference between the HM-HEC with C8 and C16 chain lenght. No difference in leakage was found between the HM-HEC C8 and HM-HEC C16 coated liposomes at 4 °C. The HM-HEC C8 coated liposomes were larger than the liposomes coated with HM-HEC C16. The zeta potential was found to be slightly negative, with no differences between the HM-HEC coated liposomes with different chain lengths. The transmittance was slightly lower for the HM-HEC C8 than the HM-HEC C16 coated liposomes, however, with no significant difference.

Although it was shown that the liposomes were stabilized by the polymer coating, no interactions of HM-HEC alkyl chains with the liposome membrane could be verified by DSC. The HM-HEC coated liposomes with C8 and C16 chain lengths showed no differences in the stabilization properties.

Abstract (Norwegian)

I denne studien har nøytrale gelfase (DPPC) og flytende fase (soya PC) liposomer blitt dekket med hydrofobt modifisert hydroksyetylcellulose (HM-HEC). Lengden på de hydrofobe kjedene på HM-HEC var C8 og C16.

Påvirkningen de hydrofobe kjedelengdene på polymerdekkingene hadde på frigjøringen av en fluorescensmarkør ble undersøkt ved 35 °C og 4 °C. I tillegg ble størrelse, zetapotensiale, tranmittans og pH undersøkt ved oppbevaring ved 4 °C i løpet av en 12-ukers periode.

Diffential scanning calorimetry (DSC) ble brukt til å undersøke interaksjonen mellom liposommembranen og de hydrofobt modifiserte polymerene. Egg PC-, DMPC- og DOPC-liposomer ble inkludert i disse studiene.

Frigjøringsstudiene ved 35 °C viste at dekking med HM-HEC beskyttet mot frigjøring, men med ingen signifikant forskjell mellom HM-HEC med C8 og C16 kjedelengde. Det ble ikke funnet noen forskjell i lekkasje mellom de HM-HEC C8- og HM-HEC C16-dekkede liposomene ved 4 °C. De HM-HEC C8-dekkede liposomene var større enn de HM-HEC C16-dekkede. Zetapotensialet var svakt negativt, uten noen merkbare forskjeller mellom de HM-HEC C16- og HM-HEC C8-dekkede liposomene. Transmittansen var noe lavere for de HM-HEC C8-dekkede enn de HM-HEC C16-dekkede, men uten noen signifikant forskjell.

Selv om det ble vist at liposomene ble stabilisert av polymerdekkingen, ble ingen interaksjoner mellom HM-HEC alkylkjedene og liposommembranen verifisert ved DSC. De HM-HEC-dekkede liposomene med C8 og C16 kjedelengder viste ingen forskjeller i forhold til stabiliseringsegenskapene.

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1 Introduction

1.1 Background

Liposomes are small vesicles consisting of lipids. They can be surface modified by coating with polymers to achieve improved stability and protection (Takeuchi et al. 1998; Filipovic-Grcic et al. 2001; Mady et al. 2009; Smistad et al. 2012).

Coating of charged liposomes with charged polymers can be accomplished by electrostatic interaction between oppositely charged liposome and polymer, which will give the liposome a charged surface. This charge can cause unwanted interactions in biological fluids, e.g., saliva (Nguyen et al. 2013). Coating of charged liposomes with charged polymers is relatively much studied (Henriksen et al. 1994; Henriksen et al. 1997; Nguyen et al. 2011).

Coating of neutral liposomes with neutral polymers will not give a charged surface, which can be favorable in some cases, e.g., when the liposomal formulation is intended for oral use. This coating of neutral liposomes with neutral polymers is not extensively studied. However, there has been shown successful coating of neutral liposomes with the neutral hydrophobically modified hydroxyethyl cellulose (HEC) (Meland et al. 2014). The successful coating results with the hydrophobically modified polymer on the neutral liposomes showed that another mechanism than electrostatic interactions had to happen. The mechanism of this interaction is not yet fully understood, but it is assumed that the hydrophobic anchor of the polymer will attach to the liposomal membrane. Using hydrophobically modified HEC with different chain lengths for coating of neutral liposomes, a better understanding of the mechanism of the interaction may be obtained.

1.2 Aim of the study

The main aim of this thesis was to study the influence of the hydrophobic chain length on hydrophobically modified hydroxyethyl cellulose (HM-HEC) on the stability of HM-HEC coated liposomes. Both gel phase and fluid phase liposomes were included in the study.

The first intermediate aim was to make a survey of the influence of the hydrophobic chain length on the release of a fluorescence marker at 35 °C.

The second intermidiate aim was to investigate the influence of hydrophobic chain length on the physical stability (size, zeta potential, pH, transmittance and leakage of fluorescence marker) during storage at 4 °C.

The third intermediate aim was to reveal the mechanism of interaction between the liposome and the hydrophobically modified polymer through differential scanning calorimetry (DSC) studies.

1.3 Abbreviations

Egg PC	Egg phosphatidylcholine
Soy PC	Soy phosphatidylcholine
DOPC	Dioleoyl phosphocholine
DMPC	Dimyristoyl phosphocholine
DPPC	Dipalmitoyl phosphocholine
HEC	Hydroxyethyl cellulose
HM-Com-HEC	Hydrophobically modified hydroxyethyl cellulose, commercial quality
HM-HEC C16	Hydrophobically modified hydroxyethyl cellulose, 1 mole % palmitoyl chains
HM-HEC C8	Hydrophobically modified hydroxyethyl cellulose, 1 mole % octyl chains
MW	Molecular weight
$\mathbf{T_c}$	Transition temperature
MWCO	Molecular weight cut off
CF	Carboxyfluorescein
DLS	Dynamic light scattering
PdI	Polydispersity index
rpm	Revolutions per minute
DSC	Differential scanning calorimetry
Cp	Heat capacity
PEG	Polyethylene glycol
PNIPAAM	Poly(*N*-iso-propylacrylamide)
D (H)	Hydrodynamic diameter
D	Mutual diffusion coefficient
k	Boltzmann's constant
T	Thermodynamic temperature
η	Viscosity of the medium
U_e	Electrophoretic mobility
ε	Dielectric constant
z	Zeta potential
$f(ka)$	Henry constant

2 Theory

2.1 Liposomes

2.1.1 *General*

Liposomes consist of phospholipids in a bilayer. Phospholipids consist of two fatty acids that are hydrophobic (the tail), which are attached to a glycerol group with a phosphate group as shown in Figure 2-1. The phosphate group can be attached to different head groups.

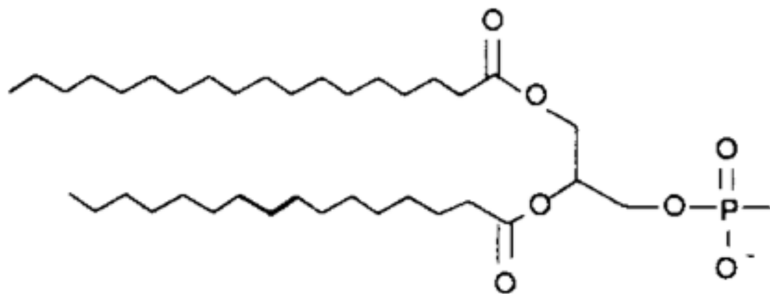

Fig. 2-1. Phosphatidyl structure

The liposomes can either be unsaturated or saturated depending on respectively the presence or absence of double bond(s) in the fatty acid chains. Known unsaturated liposomes such as egg phosphatidylcholine (egg PC), soy phosphatidylcholine (soy PC) and dioleoyl phosphocholine (DOPC) are sensitive to oxidation due to their unsaturation (Kreuter 1994). Well-known liposomes of saturated lipids such as dimyristoyl phosphocholine (DMPC) and dipalmitoyl phosphocholine (DPPC) are on the contrary less exposed to chemical degradation by oxidation due to their saturation.

The lipids are amphipathic and when they are placed in a water-consisting environment, the tail will orient away from the water, and the hydrophilic head will move towards the aqueous phase. This leads to a three-dimensional hollow sphere structure as shown in Figure 2-2.

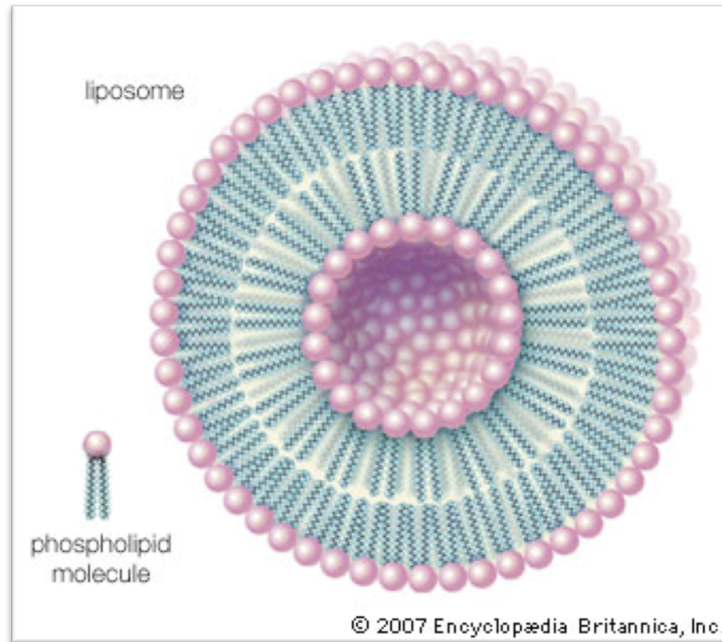

Fig. 2-2. Structure of a unilamellar liposome © 2007 Encyclopædia Britannica

The size can vary greatly from typically 20nm-1μm (Kreuter 1994). The structure will vary depending on the positions and number of lamellae, from e.g., small unilamellar vesicles (SUV) to large unilamellar vesicles (LUV), to multilamellar vesicles (MLV) and multivesicular vesicles (MVV).

The bilayer can exist in different states, depending on the temperature. Different lipids hold different phase transition temperatures (T_c). If the temperature is below T_c, the lipid will be in a "solid" gel phase, and if the temperature rises above T_c, the state will change into a liquid crystalline phase. The T_c depends on the acyl chain length and the degree of saturation (Taylor and Morris 1995).

The charge can vary from positive to negative, including neutral charge. The phosphate group bears a negative charge at neutral pH, and the head group attached to the phosphate group will determine the overall charge. Typical groups attached to the phosphate group are choline, serine, glycerol and ethanolamine (Lian and Ho 2001).

The structural properties make the liposomes suitable for drug delivery. Water-soluble drugs can be encapsulated into the aqueous core and water-insoluble drugs can be incorporated into the lipid bilayer. This can help to improve the drug solubility, protect the drug against chemical degradation in the body, and decrease the unwanted effects of toxic drugs (Gabizon 1995).

2.1.2 *Liposome stability*

The stability of liposomes can be divided into chemical stability and physical stability. The chemical stability can be divided into oxidative and hydrolytic degradation. Oxidation mainly occurs in unsaturated liposomes, but it has been observed in saturated liposomes at high temperatures. Storage at low temperature and avoidance of light and oxygen will contribute to avoid oxidation (Grit and Crommelin 1993). Hydrolysis is another type of chemical degradation that liposomes can be exposed to in solution (Grit and Crommelin 1993). When exposed to hydrolysis, the detection of hydrolysis products e.g., lyso PC, can be measured. It has been reported that liposomes that exhibit gel phase are less exposed to hydrolysis than fluid phase liposomes (Zuidam and Crommelin 1995). It has been shown that minimum hydrolysis of the ester linkage occurs at pH 6.5 (Grit et al. 1989).

Physical stability includes leakage and aggregation. When liposomes aggregate it can be observed by investigating the average size and size distribution. Liposomes that are neutral are more exposed to aggregation (and sedimentation) due to lack of electrostatic stabilization. The aggregation is brought about by Van der Waals interactions (New 1990). A charge-carrying lipid could be integrated into the lipid layer to protect the liposome from aggregation (Grit and Crommelin 1993). Aggregation could also be avoided by coating the liposomes with polymers (Nguyen et al. 2013; Zhou et al. 2014).

2.2 Polymers

2.2.1 *General*

Polymers are large macromolecules consisting of repeating subunits, known as monomers. Liposomes can be coated with various polymers for enhanced stability and protection and for increasing the circulation time in the body (Blume et al. 1993; Torchilin et al. 1994). Many drugs are intended for the use on mucosal membranes in the human body. To make the liposome more mucoadhesive and suitable for delivery to these membranes, coating with polymers that enhance the mucoadhesion can be accomplished (Khutoryanskiy 2011). Known polymers with this mucoadhesive property are, e.g., derivatives of cellulose (Salamat-Miller et al. 2005).

2.2.2 *HM-HEC*

A non-ionic hydrophilic cellulose derivative made up of a polymer backbone with hydroxyethyl groups is hydroxyethyl cellulose (HEC). The hydrophobically modified hydroxyethyl cellulose polymer (HM-HEC) can be prepared by attaching hydrophobic alkyl chains to the HEC polymer. The structures of HEC and HM-HEC are shown in Figure 2-3.

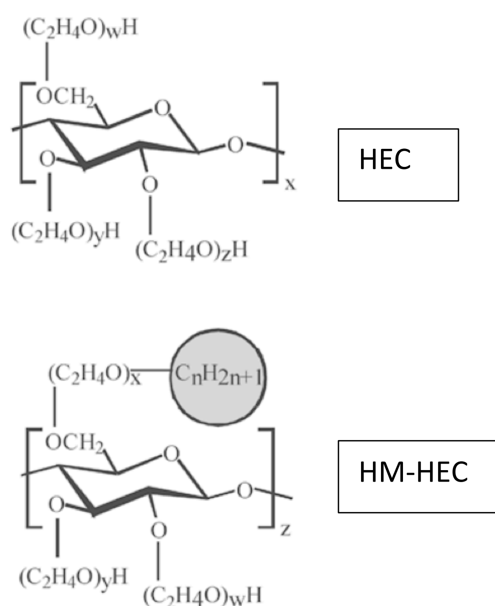

Fig. 2-3. Chemical structures of HEC and HM-HEC taken from Lashet et al. 2004

HM-HEC can be used in the production of solutions with different range of viscosity and as thickening agent in different formulations. It has been used as a drug carrier for the purpose of controlled release, and in tablets as a binder and film-coating agent (Guo et al. 1998). Another important property is the mucoadhesive effect. Surface modification of liposomes with a mucoadhesive polymer can be advantageous with a view to future use in the oral cavity (Guo et al. 1998). However, non-ionic polymers usually exhibit weaker mucoadhesive interactions than ionic polymers (Khutoryanskiy 2011).

The attachment of the hydrophobic side chain will give the polymer greater binding potential (Beheshti et al. 2006). The unmodified HEC will be able to form hydrogen bonds. The hydrophobically modified HEC will be capable of making hydrophobic interactions in addition to the hydrogen bonding.

2.3 Coating of liposomes with polymers

Surface coating of liposomes can change many properties, which can be useful when considering e.g., the stability and protection from degradation and aggregation of the liposomes (Henriksen et al. 1997). It is advantageous to know how different coating agents can change the properties.

The interaction between the neutral liposome and the neutral hydrophobically modified polymer is not fully understood. When charged liposomes are coated with charged polymers the coating is happening because of electrostatic forces, which are too week in neutral liposomes and neutral polymers. The coating of polymers onto liposomes with the opposite charge can be verified by a change in zeta potential. This will not happen when both the polymer and liposome are neutral. When neutral polymers are adsorbed on the neutral liposomal surface, small loops are formed. Further away from the surface of the liposome larger loops and tails are formed as the polymer concentration decreases (Clément and Johner 2000). It is believed that the hydrophobic chains on the hydrophobically modified polymer are interacting with the liposomal membrane (Meland et al. 2014).

The particle size can be changed by coating. Depending on the desirable target or administration route etc., different sizes can be favorable. Small-sized pegylated liposomes (about 100 nm in diameter) gave less targetability and drug delivery than large sizes (about 300 nm) in targeting tumor to endothelial cells (Kibria et al. 2013). In other cases smaller sizes can be more favorable. A study based on delivery through the skin, showed more promising results with carriers of smaller sizes. The study showed that liposomes with a diameter size of about 70 nm could penetrate deeper into the skin layers than the 300 nm sized and 600 nm sized liposomes (Verma et al. 2003). The liposome size will increase due to polymer coating. If the liposomes are not fully saturated by polymer, the size can increase because of aggregation as a result of bridging flocculation (Alund et al. 2013).

Coating can change the liposome zeta potential. Positively charged liposomes can be coated with a polymer to get the opposite charge, and vice versa (Nguyen et al. 2011). Studies have shown that the net surface charge is determined by the charge of the outer layer (Alund et al. 2013).

Many drugs are intended for the use on mucosal membranes in the human body. To make the liposome more mucoadhesive and suitable for delivery to these membranes, coating with polymers that enhance the mucoadhesion can be accomplished (Khutoryanskiy 2011). Known polymers with this mucoadhesive property are e.g., derivatives of cellulose (Salamat-Miller et al. 2005).

Liposomes can be pegylated, which will prolong the residence time of the liposome in the blood circulation (Allen et al. 1991; Parveen and Sahoo 2011). This is favorable when used as a drug delivery system to sites outside circulation due to increased probability for reaching the target (Hayes et al. 2006). The PEG protects the liposomes from being entrapped by the reticuloendothelial system and degraded (Veronese and Pasut 2005).

Liposomes can be coated with thermosensitive polymers, where poly(*N*-iso-propylacrylamide), PNIPAAM, is the most studied. This polymer undergoes phase transition at about 32°C, the lower critical solution temperature (Fujishige et al. 1989; Yang and Li 2013), from a swollen hydrated state to a shrunken dehydrated state. This property may

trigger the release and can be beneficial in the use of liposomes as drug delivery systems (Nolan et al. 2006).

2.4 Characterization of liposomes

2.4.1 *Particle size*

The particle size can be measured by dynamic light scattering. The particles in suspension move with Brownian motions. When a laser light illuminates the liposomes in a buffer solution, the liposomes will scatter the light and the intensity of the scattered light will provide the diffusion coefficient. The mutual diffusion coefficient measured by dynamic light scattering will make it possible to calculate the size using the Stoke-Einstein equation (Equation 2-1).

$$D\ (H)\ = \frac{kT}{3\pi\eta D}$$ Equation 2-1

Where D (H) is the hydrodynamic diameter, D is the mutual diffusion coefficient, k is the Boltzmann's constant, T is the thermodynamic temperature and η is the viscosity of the medium.

The polydispersity index (PdI) is a measure of the broadness of the particle size distribution. The larger the value of PdI, the broader is the size distribution (Nidhin et al. 2008).

2.4.2 *Zeta potential*

Zeta potential is the measurable net charge on a particle surface. Particles in a solution with a net charge will have ions bound to their surface. This layer is called the Stern layer. Outside this Stern layer, a second diffuse layer of ions, will occur. When the particle moves, a surface of shear will appear as a result of the strong adhesion to the particle and will follow with the particle motion, as shown in Figure 2-4.

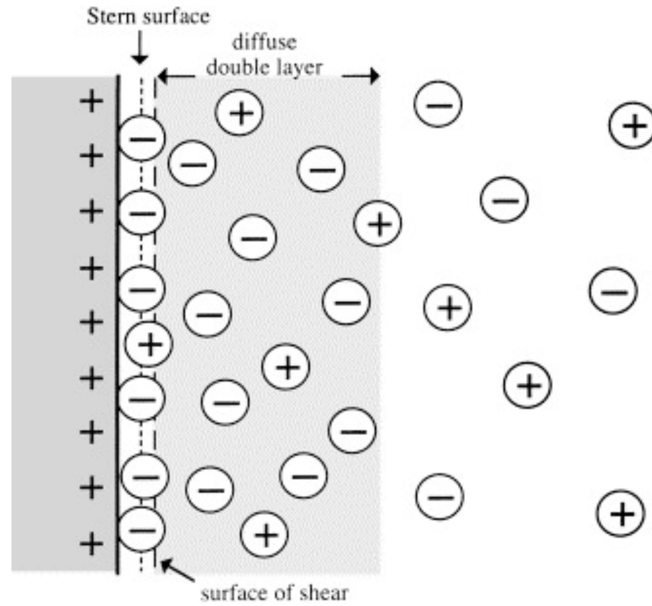

Fig. 2-4. The surface of a charged particle taken from Burns and Zydney 2000

The zeta potential is measured using laser Doppler micro-electrophoresis. It cannot be measured directly but with calculations. The motion that occurs at the surface of shear is used in the determination of zeta potential of a spherical particle using the Henry equation (Equation 2-2).

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

Where U_e is the electrophoretic mobility, ε is the dielectric constant, z is the zeta potential, η is the viscosity of the solvent/medium and $f(\text{k}a)$ is the Henry constant.

Zeta potential between -10 and + 10 mV are considered electrostatically unstable and zeta potentials around 30 mV or -30 mV provide electrostatic stabilization of the particles (Clogston and Patri 2011).

2.4.3 *Phase transition temperature*

Differential scanning calorimetry (DSC) is a thermal analysis technique that uses a material's heat capacity (Cp), how much energy a matter can hold, to find a matter's heat flow. This means that the detection of transitions, like liposomes' T_c, can be identified (Ford and Timmins 1989).

There are two different scanning methods of DSC; heat flux DSC and power-compensation DSC (Ford and Timmins 1989).

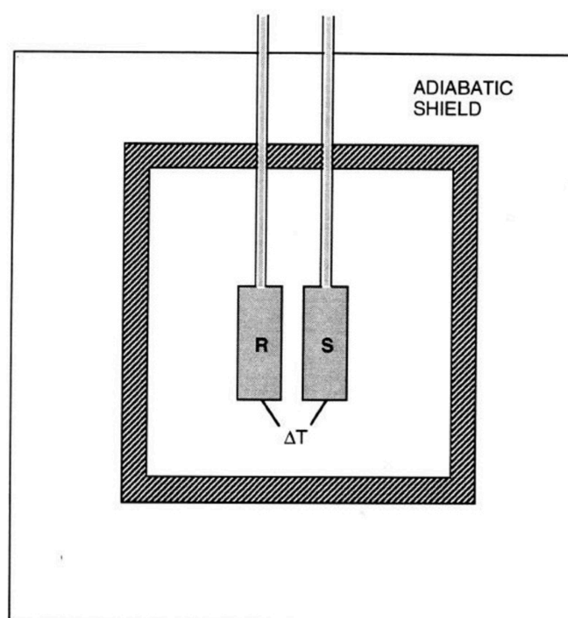

Fig. 2-5. Illustration of a heat flux DSC device taken from Freire 1995

Figure 2-5 shows a very simplified illustration of the cell compartment of a heat flux DSC. Inside the device, two identical cells are supposed to hold the sample (S) and the reference (R) during the measurement. The Cp values of the sample and the reference are different and will lead to a temperature difference (ΔT) when heated or cooled, which is used to determine the heat flow of the sample. The heat flow can be associated with transition temperatures, since the curves are plotted with temperature against time (Ford and Timmins 1989).

The DSC used in this study is heat flux DSC. The different scanning methods will give different curves. In heat flux DSC, the scanning of a material will give a curve in which the exothermic reactions will give a peak that points up. Peaks that point down are endothermic where energy is absorbed.

In the detection of liposomes' T_c, the peaks will point down, because of the energy needed to change the liposomes from a solid gel phase to a liquid-crystalline phase. Both the peak maximum and the onset temperature are of interest. Often the onset temperature will give the most accurate T_c. If the peaks are broad, however, the onset temperature can be imprecise and the peak temperature will be more accurate to define the T_c.

3 Materials and instruments

3.1 Materials

3.1.1 *Lipids*

Lipid	Abbreviation	MW (g/mol)	K-number	Manufacturer
Egg phosphatidylcholine	Egg PC	770.1	510800-04/921	Lipoid GmbH, Germany
Soy phosphatidylcholine	Soy PC	775.0	792044-01/907	Lipoid GmbH, Germany
Dioleoyl phosphocholine	DOPC	786.1	181PC-284	Avanti Lipids, USA
Dimyristoyl phosphocholine	DMPC	677.9	562191-1/20	Lipoid GmbH, Germany
Dipalmitoyl phosphocholine	DPPC	734.0	563119-01/017	Lipoid GmbH, Germany

3.1.2 *Polymers*

Polymer	Abbreviation	MW (g/mol)	K-number	Manufacturer
Hydrophobically modified hydroxyethyl cellulose, commercial quality, 1 mole % C16-chains	HM-Com-HEC	300 000	Natrosol PLUS 330 CS	Ashland, USA
Hydrophobically modified hydroxyethyl cellulose, 1 mole % C16-chains	HM-HEC C16	400 000	-	Dept. of Chemistry, UiO, Norway
Hydrophobically modified hydroxyethyl cellulose 1 mole % C8-chains	HM-HEC C8	400 000	-	Dept. of Chemistry, UiO, Norway

3.1.3 *Other chemicals*

Chemical	MW (g/mol)	K-number	Manufacturer
Sodium dihydrogen phosphate monohydrate	138.0	K25001880	Merck, Germany
Disodium hydrogen phosphate dihydrate	178.0	97352	Merck, Germany
Tris-(hydroxymethyl) aminomethane	121.1	13F130010	VWR Chemicals BDH Prolabo, Belgium
5(6)-carboxyfluorescein	376.3	10H9062, BCBJ436OV	Sigma, USA
Chloroform	-	13C260521	Merck, Germany
Ethanol 96 %	-	203031	Merck, Germany
Sodium hydroxide	40.0	70800424070C04	Merck, Germany
Hydrochloric acid 37 %	36.5	K33616217 432	Merck, Germany
Triton-X 100 (t-octylphenoxy-polyethoxyethanol)	-	10K0192	Sigma, USA
Etylene glycol	62.1	K25321821 825	Merck, Germany

3.1.4 *Solutions*

<u>Lipid stock solutions</u>

10 mg/ml lipid stock solutions were made with the different lipids. The desired amount of lipid was weighed using an analytical balance and chloroform was added to give the correct concentration.

These lipid stock solutions were stored in the freezer at ~-18 °C.

<u>1 M sodium hydroxide</u>

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).

5 mM phosphate buffer pH 6.8

690 mg sodium dihydrogen phosphate monohydrate (NaH_2PO_4 x H_2O) was weighed in a glass weighing boat on an analytical balance and transferred to a 1000 ml volumetric flask and dissolved in MilliQ-water ad 1000 ml. The procedure was repeated with 890 mg disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O) in a different 1000 ml volumetric flask. The sodium dihydrogen phosphate solution was transferred to a new 2000 ml volumetric flask and approximately 500 ml of the disodium hydrogen phosphate solution was added until the pH was 6.8. The buffer was filtered through a polycarbonate membrane filter (Nuclepore, 200 nm) and added to a 2000 ml glass bottle. The buffer was stored at 4 °C.

0.1% (w/w) HM-Com-HEC in 5 mM phosphate buffer pH 6.8

15 mg HM-Com-HEC was weight directly in a beaker on an analytical balance, and 15 g 5 mM phosphate buffer pH 6.8 was added. A small magnet was added and the solution was stirred on a magnetic stirrer (overnight) at room temperature (~20 °C) until the HM-Com-HEC was dissolved.

0.1% (w/w) HM-HEC C16 in 5 mM phosphate buffer pH 6.8

15 mg HM-HEC C16 was weight directly in a beaker on an analytical balance, and 15 g 5 mM phosphate buffer pH 6.8 was added. A small magnet was added and the solution was stirred on a magnetic stirrer (overnight) at room temperature (~20 °C) until the HM-HEC C16 was dissolved.

0.1% (w/w) HM-HEC C8 in 5 mM phosphate buffer pH 6.8

15 mg HM-HEC C8 was weight directly in a beaker on an analytical balance, and 15 g 5 mM phosphate buffer pH 6.8 was added. A small magnet was added and the solution was stirred on a magnetic stirrer (overnight) at room temperature (~20 °C) until the HM-HEC C8 was dissolved.

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

11.29 mg 5(6)-carboxyfluorescein was weighed directly in a 25 ml volumetric flask, covered with aluminum foil, added 20 ml 5 mM phosphate buffer pH 6.8 and dissolved by turning the flask. The solution was added 1 M NaOH to dissolve all the 5(6)-carboxyfluorescein. 1 M HCl was added to adjust the pH to 6.8. The solution was filtered through a polycarbonate membrane filter (Nuclepore, 200 nm) and stored at 4°C

3.1.6 *Solutions applied in release and leakage measurements*

60 mM tris buffer pH 8.0

7.2684 g of tris-(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$) was weighed in a weighing boat on an analytical balance and transferred to a 1000 ml volumetric flask and dissolved in MilliQ water ad 1000 ml. 1 M HCl was added to adjust the pH to 8.0. The buffer was filtered through a polycarbonate membrane filter (Nuclepore, 200 nm) and stored at 4°C.

60 mM tris buffer pH 8.0 with 0.05 M NaCl

292.25 mg sodium hydroxide (NaCl) was weighed in a weighing boat on an analytical balance and transferred to a 100 ml volumetric flask and dissolved in 60 mM tris buffer pH 8.0 ad 100 ml. The buffer was filtered through a polycarbonate membrane filter (Nuclepore, 200 nm) and stored at 4°C.

60 mM tris buffer pH 8.0 with 0.35 M NaCl

2.0458 g sodium hydroxide (NaCl) was weighed in a weighing boat on an analytical balance and transferred to a 100 ml volumetric flask and dissolved in 60 mM tris buffer pH 8.0 ad 100 ml. The buffer was filtered through a polycarbonate membrane filter (Nuclepore, 200 nm) and stored at 4°C.

0.1% (w/w) HM-HEC C16 in 60 mM tris buffer pH 8.0

15 mg HM-HEC C16 was weight directly in a beaker on an analytical balance, and 15 g 60 mM tris buffer pH 8.0 was added. A small magnet was added and the solution was stirred on a

magnetic stirrer (overnight) at room temperature (~20 °C) until the HM-HEC C16 was dissolved.

0.1% (w/w) HM-HEC C8 in 60 mM tris buffer pH 8.0

15 mg HM-HEC C8 was weight directly in a beaker on an analytical balance, and 15 g 60 mM tris buffer pH 8.0 was added. A small magnet was added and the solution was stirred on a magnetic stirrer (overnight) at room temperature (~20 °C) until the HM-HEC C8 was dissolved.

Triton X-100 2% (w/w) in 60 mM tris buffer pH 8.0

50 g of 60 mM tris buffer pH 8.0 was added to a glass bottle. 1 g Triton X-100 (Sigma) was added using a glass rod. Careful mixing dissolved Triton X-100. The Triton X-100 2% was stored in room temperature (~20 °C).

Stock solution of 5(6)-carboxyfluorescein 1.5 mM in 60 mM tris buffer pH 8.0

11.29 mg 5(6)-carboxyfluorescein was weighed directly in a 25 ml volumetric flask, covered with aluminum foil, added 20 ml 60 mM tris buffer pH 8.0 and dissolved by turning the flask. It was added 1 M NaOH to the solution to dissolve all the 5(6)-carboxyfluorescein and to adjust the pH to 8.0. The solution was filtered through a polycarbonate membrane filter (Nuclepore, 200 nm) and stored at 4°C.

20 mM 5(6)-carboxyfluorescein in 60 mM tris buffer pH 8.0

18.816 mg 5(6)-carboxyfluorescein was weighed directly in a glass vial covered with aluminum foil. 2.5 ml 60 mM tris buffer pH 8.0 was added. The pH was adjusted to 8.0 with 1 M NaOH. The solution was filtered through a polycarbonate membrane filter (Nuclepore, 200 nm).

100 mM 5(6)-carboxyfluorescein in 60 mM tris buffer pH 8.0

94.08 mg 5(6)-carboxyfluorescein was weighed directly in a glass vial covered with aluminum foil. 2.5 ml of 60 mM tris buffer pH 8.0 was added. The pH was adjusted to 8.0 with 1 M NaOH. The solution was filtered through a polycarbonate membrane filter (Nuclepore, 200 nm).

3.2 Instruments

3.2.1 *Preparation of liposomes*

Instrument	Model	Manufacturer
Analytical balance	AG204 DeltaRange	Mettler Toledo GmbH, Switzerland
Rotary evaporator	Vacuum pump, Mz2C, serial number 23911722	Vacuubrand 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 and 2.5 ml	Northern Lipids, Canada
Circulating refrigerating and heated water bath	MGW RC 6	Brinkman Lauda, USA
Polycarbonate membrane, 200 nm	Nucleopore Track-Etch Membrane	Whatman, UK

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
pH meter	MP 220	Mettler Toledo, Switzerland
Zeta sizer	Nano SZ	Malvern Instruments, UK
DSC	822	Mettler Toledo, Switzerland
Plate reader	Wallac Victor^3 1420	Perkin Elmer, USA
Spectrophotometer	Ultrospec II, 4052 TDS	LKB Biochrom, UK
Heating cabinet	TS8056, serial number 3-2461	Termaks, Norway
Whirlmixer	Reax Top	Heidolph, Germany

3.2.4 *Other equipment*

Equipment	Model	Manufacturer
Float-A-Lyzer, MWCO 20000 Da	G2, 1 ml	Sigma-Aldrich, USA
Desalting column	PD-10	GE Healthcare Biosciences AB, Sweden

4 Methods

4.1 Preparation of liposomes (thin film method)

The correct volume of lipid stock solution was added to a 250 ml round flask in a fume hood. If necessary, additional chloroform was added to the stock solution. The solution was evaporated to dryness in a rotary evaporator with a rotation speed of 90 rpm, and a water bath temperature of 40°C. The pressure was lowered slowly to 200 mbar. When visible dryness, the pressure was lowered to 60-65 mbar and held there for 20 minutes, while the flask was still rotating. The film was further dried in vacuum in a freeze dryer for approximately 24 hours.

The lipid film was hydrated with the desired hydration medium (5 mM phosphate buffer pH 6.8, 60 mM tris buffer pH 8.0 or 100 mM 5(6)-carboxyfluorescein in 60 mM tris buffer pH 8.0) at a temperature above the phase transition temperature (T_c) for two hours. The round flask was gently stirred occasionally.

Because of the different values of the phase transition temperatures of the different lipids, the hydration was either performed in room temperature (soy PC, egg PC and DOPC) or in a water bath holding 40°C (DMPC) or 70°C (DPPC). The hydration medium was heated up to obtain the same temperature before addition to the lipid film. The liposomal suspension was stored at 4°C for about 24 hours.

The liposomal suspension was extruded with a Lipex extruder at a temperature above T_c, using two stacked 200 nm membranes. First, the extruder/filter was rinsed with 20 ml of the hydration medium and then the liposomal suspension was extruded 10 times.

4.2 Removal of non-encapsulated carboxyfluorescein by gel filtration

4.2.1 *Column preparation*

The column, PD-10 Desalting Column, was equilibrated with 25 ml 60 mM tris buffer pH 8.0.

4.2.2 *Column saturation*

2.5 ml of the 3 mM desired liposome suspension was added. After the liposome suspension had entered the packed bed completely, 3.5 ml 60 mM tris buffer pH 8.0 was added.
The column was washed with additional 17.5 ml 60 mM tris buffer pH 8.0.

4.2.3 *Removal of non-encapsulated carboxyfluorescein before coating*

2.5 ml of the liposomes (10mM) with encapsulated fluorescence marker (100 mM) was added to the lipid-saturated column and eluted with 3.5 ml 60 mM tris buffer pH 8.0. The eluate was collected in a dark glass vial. 2.5 ml of the eluate was applied to another lipid-saturated column and eluted with 3.5 ml of 60 mM tris buffer pH 8.0. The eluate (5.1 mM liposomes) was collected in a new dark glass vial and diluted with 60 mM tris buffer 0.35 M NaCl to a 3 mM concentration of liposomes.

Immediately after the gel filtration, the liposomes were divided into three equal parts. One part was coated with HM-HEC C16 and one part was coated with HM-HEC C8 as described in Ch. 4.3. The last part remained uncoated.

4.3 Coating of the liposomes with polymer

A small glass vial was rinsed with MilliQ-water, followed by rinsing with the hydration medium used in the lipid preparation process, before adding a rinsed magnet and 4 ml of polymer solution. 1 ml liposome suspension was added to an Eppendorf tube that was rinsed with MilliQ-water and the hydration medium.

The polymer solution was put on a magnetic stirrer at medium speed. The liposomes were added in a drop-wise manner to the polymer solution, using the peristaltic pump with a speed of 20 rpm. Unsaturated liposomes were flushed with N_2 before the vial was sealed and the sample stirred on the magnetic stirrer for 5 minutes.

4.4 Fluorescence measurements

4.4.1 *Preparation of known standard concentrations*

A range of known standard concentrations was newly made every day the fluorescence was measured. The 1.5 mM carboxyfluorescein stock solution (Ch. 3.1.5) was diluted to a 0.005 mM carboxyfluorescein solution with 60 mM tris buffer pH 8.0 (Ch. 3.1.5). This solution was further diluted to different known concentrations in the range of 38.4 µM CF to 74 nM CF. The vast range of concentrations was due to the large difference in fluorescence between the leaking liposomes and the destroyed liposomes.

4.4.2 *Pipetting to plate*

50 µl of 60 mM tris buffer pH 8.0 with 0.35 M NaCl was pipetted into 27 different wells. 50 µl of Triton X-100 2% was pipetted into 27 other wells. Avoidance of air bobbles was attempted. Three wells were filled with 100 µl 60 mM tris buffer pH 8.0 0.35 M NaCl and three were filled with 100 µl Triton X-100 2%. Each standard solution was pipetted into three different wells with 100 µl in each well.

The liposomes with encapsulated carboxyfluorescein were pipetted into the wells pre-filled with either 60 mM tris buffer pH 8.0 with 0.35 M NaCl or Triton X-100 2 %. 50 µl of each liposome sample was pipetted into six different wells (three with 60 mM tris buffer pH 8.0 with 0.35 M NaCl and three with Triton X-100 2%), so each well was containing a volume of 100 µl when measurement started. The plate with the wells with different fillings is shown in Figure 4-1. The first three columns were filled with uncoated liposomes, the columns 4-6 were filled with HM-HEC C16 coated liposomes and the columns 7-9 were filled with HM-HEC C8 coated liposomes.

L+B	L+B	L+B	L+B	L+B	L+B	L+B	L+B	L+B	Std.1	Std.1	Std.1
L+B	L+B	L+B	L+B	L+B	L+B	L+B	L+B	L+B	Std.2	Std.2	Std.2
L+B	L+B	L+B	L+B	L+B	L+B	L+B	L+B	L+B	Std.3	Std.3	Std.3
L+T	L+T	L+T	L+T	L+T	L+T	L+T	L+T	L+T	Std.4	Std.4	Std.4
L+T	L+T	L+T	L+T	L+T	L+T	L+T	L+T	L+T	Std.5	Std.5	Std.5
L+T	L+T	L+T	L+T	L+T	L+T	L+T	L+T	L+T	Std.6	Std.6	Std.6
B	B	B							Std.7	Std.7	Std.7
T	T	T							Std.8	Std.8	Std.8

Fig. 4-1. The microtiter plate filled with different solutions. L = liposome suspension, B = 60 mM tris buffer pH 8.0 with 0.35 M NaCl, T = Triton X-100 2% and Std.1-8 = standard solutions with different known CF concentrations.

4.4.3 *Measurement and quantification of released carboxyfluorescein*

Immediately after the pipetting of liposomes to the microtiter plate, the plate was placed in the Wallac Victor^3 1420 Multilabel Counter plate reader, and measured with the parameters shown in Table 4-1. The measurements were performed at room temperature (~20 °C)

Table 4-1. Settings used when measuring the fluorescence with Wallac Victor^3 1420 Multilabel Counter

Technology	Prompt fluorometry
Microtiter plate	Generic, 8 x 12 size plate
Number of repeats	1
Measurement height	Default
Measurement time	0.1 second
Shaking duration	1.0 second
Shaking speed	Fast
Shaking diameter	0.10 mm
Shaking type	Linear
CW-lamp filter name	F485
CW-lamp filter slot	A5
Emission filter name	F535
Emission filter slot	A5
Emission aperture	Normal
CW-Lamp energy	500
Emission side	Above

4.5 Particle size measurements

Liposome sizes were measured using Zetasizer Nano ZS. Disposable cells were washed with 5 mM phosphate buffer pH 6.8, before 1 ml 5 mM phosphate buffer pH 6.8 was added. The cell was inspected for visible dust before adding 100 µl liposome solution to the phosphate buffer followed by careful mixing.

The parameters used during the size measurements are shown in Table 4-2.

Table 4-2. Settings used when measuring the particle size with Zetasizer Nano-ZS.

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

4.6 Zeta potential measurements

After the size measurement, the same cell and sample were used to measure the zeta potential.
A dip cell, rinsed with distilled water, was added to the cell.
The parameters used during the zeta potential measurements are shown in Table 4-3.

Table 4-3. Settings used when measuring the zeta potential with Zetasizer Nano-ZS.

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

4.7 pH measurements

The pH meter, model MP220, was calibrated with two buffer solutions suitable for the pH of the sample that was to be measured (pH 4 and 7 for samples based on 5 mM phosphate buffer pH 6.8, pH 7 and 11 for samples based on 60 mM tris buffer pH 8.0). 400 µl of the sample was added to a 1 ml Eppendorf tube, and the pH was measured at room temperature (~20 °C).

4.8 Transmittance measurements

The transmittance was measured at wavelength 550 nm with an Ultrospec spectrophotometer at room temperature (~20 °C). 2 ml of sample solution was added to a disposable cuvette and the transmittance was measured. 5 mM phosphate buffer pH 6.8 was used as reference sample.

4.9 Measurement of phase transition temperatures by DSC

10 µl or about 10 mg of the sample was transferred to a pan, which was placed in the instrument (DSC 822, Mettler Toledo). The reference pan was either added the same amount of the reference sample or it was empty, depending on what was measured.
Different methods, involving temperature and scan rate, were used on the different samples.

4.10 Statistical analyses

One-way ANOVA

The statistical analyses where the differences between groups were examined were carried out 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 Preliminary tests

5.1.1 *Determination of carboxyfluorescein diffusion rate across the dialysis membrane*

The Float-A-Lyzer G2 (MWCO 20 000 Da) was prepared according to the manufacturer's instructions.
After the preparation of the dialysis device, a 100 ml beaker, covered in aluminum foil, was filled with 80 ml 5 mM phosphate buffer pH 6.8 and set on magnetic stirring for the rest of the test in room temperature (~20 °C). 1 ml 0.005 mM CF-solution in 5 mM phosphate buffer pH 6.8 was added to the Float-A-Lyzer, and 3 x 100 µl of the solution in the beaker were withdrawn at predetermined times and added to three different wells in a microtiter plate, after given times (0 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, and 24 hours.)

5.1.2 *Determination of the melting point of an ethylene glycol and phosphate buffer mixture by differential scanning calorimetry (DSC)*

Different concentrations of ethylene glycol in 5 mM phosphate buffer pH 6.8 were scanned by DSC in the temperature range -40 °C to +25 °C to find an appropriate concentration for measuring liposomes with T_c below zero. The concentrations measured were 10 %, 20 %, 25 %, 30 %, 35 %, and 100 % ethylene glycol in 5 mM phosphate buffer pH 6.8.

5.2 Release studies at 35 °C

5.2.1 *Liposomes with 20 mM encapsulated carboxyfluorescein*

The release of carboxyfluorescein from uncoated egg PC liposomes in 60 mM tris buffer pH 8.0 with 20 mM encapsulated carboxyfluorescein was investigated. The liposomes were stored at 35 °C for 24 hours. Three parallel samples of each were measured at t = 0, t = 20 minutes, t = 40 minutes, t = 1 hour, t = 2 hours, t = 4 hours, and t = 24 hours.

5.2.2 *Liposomes with 100 mM encapsulated carboxyfluorescein*

Uncoated and coated soy PC and DPPC liposomes with HM-HEC C16 and HM-HEC C8 in 60 mM tris buffer pH 8.0 were stored at 35 °C for 45 hours. Three parallels of each sample were measured at t = 0, t = 20 minutes, t = 40 minutes, t = 1 hour, t = 2 hours, t = 4 hours, t = 24 hours, and t = 45 hours. (Deviations in the time of the measurements occurred due to technical problems with the Wallac Victor^3 1420 plate reader). At each time point the fluorescence was measured according to Ch. 4.4, and the concentrations and percent release were determined using the standard curves.

5.3 Stability studies at 4 °C

5.3.1 *Size, transmittance, zeta potential and pH measurements*

Uncoated and coated soy PC and DPPC liposomes with HM-HEC C16 and HM-HEC C8 in 5 mM phosphate buffer pH 6.8 were stored at 4 °C for 12 weeks. Three parallels of each sample were measured at t = 0, t = 1 day, t = 3 days, t = 7 days, t = 14 days, t = 4 weeks, t = 8 weeks, and t = 12 weeks. At each time point the size, the transmittance, the zeta potential, and the pH were determined.

5.3.2 *Leakage measurements*

Uncoated and coated soy PC and DPPC liposomes with HM-HEC C16 and HM-HEC C8 in 60 mM tris buffer pH 8.0 were stored at 4 °C for 12 weeks. The liposomes were encapsulated with 100 mM carboxyfluorescein. Three parallels of each sample were measured at t = 0, t = 1 day, t = 3 days, t = 7 days, t = 14 days, t = 4 weeks, t = 8 weeks, and t = 12 weeks. At each time point, the fluorescence was measured according to the description in Ch. 4.4 and the concentrations were determined by using the standard curves.

5.4 Studies on interactions between liposomes and HM-HEC by DSC

The T_c was determined for the different types of liposomes by DSC. Mixtures of the liposomes and HM-HEC, and isolated HM-HEC were also scanned. The same conditions were used for the corresponding samples, e.g., egg PC alone, egg PC/HM-HEC mixture and HM-HEC alone.

6 Results and discussion

6.1 Preliminary tests

6.1.1 *Determination of carboxyfluorescein diffusion rate across the dialysis membrane*

To find a suitable method to measure the released carboxyfluorescein from the liposomes, different methods were attempted. A well-known method is to use centrifugation to separate the liposomes from the released carboxyfluorescein, and then measure the fluorescence. Earlier studies in the lab with HM-HEC coated liposomes, however, had shown that the centrifugation was very time consuming due to the relatively high viscosity of the HM-HEC solution. Liposomes coated with other polymers e.g., pectin and alginate, were more suitable for centrifugation than HM-HEC due to their viscosity, which is lower.

The intention by testing the dialysis rate with the Float-A-Lyzer was to find a method to measure the released carboxyfluorescein, which would be suitable for the HM-HEC coated liposomes. The dialysis device is designed to make small solutes, such as carboxyfluorescein, diffuse from a high concentration inside the device, to the low concentration outside the device in the buffer solution. The used membrane, a cellulose ester synthetic membrane, has small pores and this leads to a cut-off of the molecules with the largest molecular weight (MWCO 20 000 Da). In theory, this method would be an easy and efficient way to determine the release from the liposomes, since the carboxyfluorescein would diffuse across the membrane, and the liposomes would be held inside the bag. In Table 6-1 the results from the diffusion study are shown.

Table 6-1. Diffusion of CF across the dialysis membrane at room temperature (~20 °C). The fluorescence measured outside the Float-A-Lyzer, by the plate reader (Wallac Victor^3 1420 Multilabel Counter) is given. The lamp energy was 2000.

Time (hour)	Fluorescence
0	87702
0.25	94907
0.5	101261
1	104806
2	111131
4	122657
24	245860
24	4875437 (inside the dialysis device)

The results showed a very slow diffusion rate across the dialysis membrane. After 24 hours the fluorescence outside the bag was only about 5 % of the fluorescence inside the bag. It was concluded to be too slow for use in the further release studies with liposomes, since the rate determining step must be across the liposome membrane and not across the dialysis membrane in such studies.

6.1.2 *Determination of the melting point of ethylene glycol and phosphate buffer mixture by differential scanning calorimetry (DSC)*

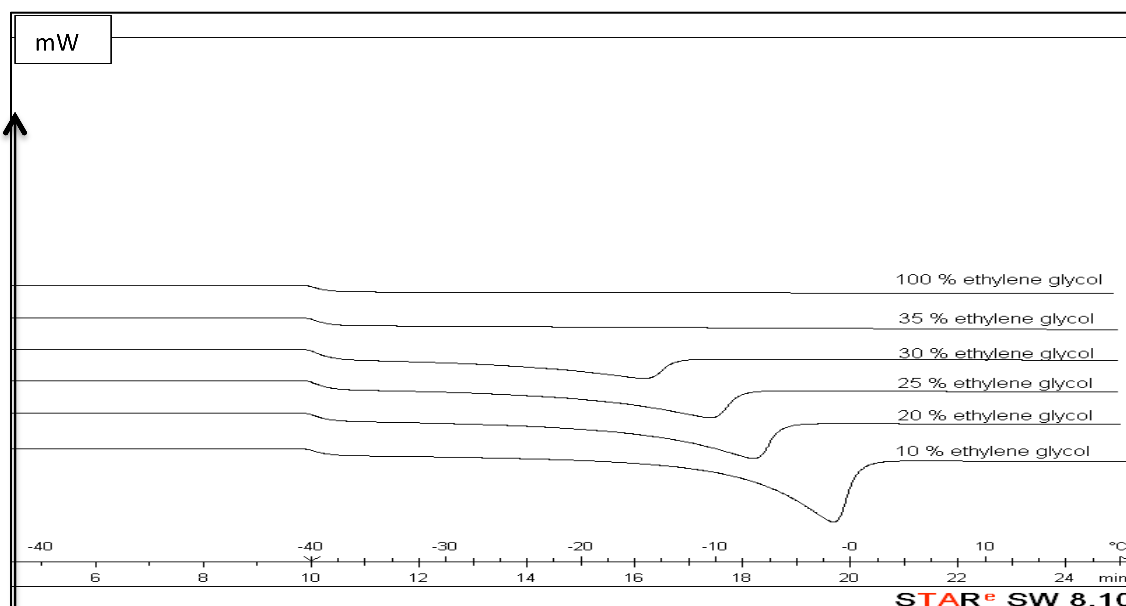

Fig. 6-1. Melting characteristics of different concentrations of ethylene glycol in 5 mM phosphate buffer pH 6.8. The temperature was held constant at -40 °C and then increased at a rate 4 °C /min until 25 °C was reached.

T_cs for the egg PC liposomes and the DOPC liposomes are below zero, around -10 °C for egg PC (Taylor and Morris 1995) and -17 °C for DOPC (Koynova and Caffrey 1998). This means that the samples have to be scanned at temperatures below 0 °C to be able to detect possible influence on the T_c by the polymer. The hydration medium for the liposomes was 5 mM phosphate buffer pH 6.8. This buffer consists mainly of water, which would give a peak around 0 °C. This water peak is not desirable because it would probably cover the peak from the T_c of the lipid and hide any possible interaction between the liposome and the polymer in the scan. Thus, the desirable medium for the liposomes would be a medium without melting point in the area where the liposomes are scanned.

A medium known for its antifreeze properties is ethylene glycol. Ethylene glycol breaks the hydrogen bonds of water and decreases the freezing point (Zimmerman et al. 1993). The freezing point of the ethylene glycol/water mixture is dependent on the concentration of ethylene glycol (Baudot and Odagescu 2004).

In Figure 6-1, the melting characteristics of different concentrations of ethylene glycol in 5 mM phosphate buffer pH 6.8 are shown. The figure shows a decreasing melting temperature with increasing ethylene glycol concentration. At 35 % ethylene glycol, no peak in the temperature range of interest was visible, which means that the freezing point was lowered to below -40 °C. The melting points in Figure 6-1 differ from literature data on melting characteristics for mixtures of ethylene glycol and water, because a phosphate buffer is used in this case. The melting points of ethylene glycol in water are higher than the melting points of the corresponding concentrations of ethylene glycol in phosphate buffer (Cordray et al. 1996). However, this is expected since the salt concentrations, or ionic strength in the ethylene glycol/buffer solution is higher than in pure water, and higher salt concentration is known to decrease the freezing point.

To summarize, the intention of these preliminary studies was to find an ethylene glycol/buffer mixture, which had the desired properties when it comes to melting characteristics, and based on Figure 6-1, 35 % ethylene glycol in 5 mM phosphate buffer of pH 6.8 was chosen as hydration medium when scanning the egg PC liposomes and the DOPC liposomes in the further studies.

6.2 Release studies at 35 °C

The dialysis method was rejected as described in Ch. 6.1.1, and another method was tried to find an appropriate way to measure the release of carboxyfluorescein. The other method relies on the fact that carboxyfluorescein is high-quenching in higher concentrations (New 1990). When carboxyfluorescein is dissolved in relatively high concentrations, phosphate buffer will not be an appropriate hydration medium because the pH is too low. Tris buffer holds a higher pH and has been found to be suitable for the encapsulation of high carboxyfluorescein concentrations (Henriksen et al. 1995).

Before the testing with carboxyfluorescein-encapsulated liposomes could be carried out, the dissolution of HM-HEC with both palmitoyl chains and octyl chains in tris buffer were examined. Both polymers were found to dissolve at 0.1 % polymer concentration in this buffer.

6.2.1 *Liposomes with 20 mM carboxyfluorescein encapsulated*

First 20 mM encapsulated carboxyfluorescein in egg PC liposomes were investigated. For the dilution of the liposomes after the gel filtration, 60 mM tris buffer pH 8.0 with 0.05 M NaCl was added to avoid osmotic shock of the liposomes (Henriksen et al. 1995). The fluorescence measurements showed that the fluorescence did not increase during the 24 hours at 35 °C, and that the method was uncertain (data not shown). The reason was possibly that 20 mM carboxyfluorescein was not enough for self-quenching (New 1990).

6.2.2 *Liposomes with 100 mM carboxyfluorescein encapsulated*

100 mM encapsulated carboxyfluorescein was also tried, which is considered high-quenching and therefore probably more accurate for the study (New 1990). For this experiment 0.35 M NaCl was added to the 60 mM tris buffer pH 8.0 used for dilution of the eluate after gel filtration to avoid osmotic shock (Henriksen et al. 1995). The experiment was carried out at 35 °C, because the intention is that the liposomes can be used in the treatment of dry mouth, and it is shown that the oral cavity often holds a temperature below the body temperature. (Sund-Levander et al. 2002) The liposomes were therefore stored in a Termaks heating cabinet at 35 °C during the study.

<u>Fluid phase liposomes (soy PC)</u>

In the preliminary experiments egg PC liposomes were investigated, but due to shortage of egg PC and easily accessible soy PC, soy PC liposomes were used in the further release and stability studies. The intention was to compare the stability and release from fluid phase liposomes and gel phase liposomes in the study, and both egg PC liposomes and soy PC liposomes are in the fluid phase at 35 °C (Taylor and Morris 1995).

The fluorescence measurements were performed by the Wallac Victor^3 1420 plate reader immediately after pipetting to plate. Usually the measurements are made after half an hour to be certain that the Triton X-100 2 % has destroyed the liposomes completely. In this case, it was necessary to measure immediately to affirm the fluorescence at the given time, because

the liposomes would be expected to continue leaking after transferring to the plate. Despite the short time between pipetting to plate and fluorescence measurements, the liposomes with Triton X-100 2 % showed nearly constant fluorescence values and the liposomes were considered fully destroyed. The mean fluorescence value for the destroyed liposomes was 2233290 at CW-lamp energy of 500 with a standard deviation of 1.6 %.

In Table 6-2 the size, PdI and pH characteristics of the soy PC liposomal samples are shown. As can be seen from the table the size increased during coating and the reproducibility of the coating process was good with small variations in the average diameter of liposomes coated with the same polymer.

Table 6-2. The size (hydrodynamic diameter) and corresponding PdI and pH of the uncoated and coated soy PC liposomes with 100 mM carboxyfluorescein encapsulated. The samples for size and PdI measurements were diluted before measurements as described in Ch 4.5.

Soy PC	Size(nm)	PdI	pH
Uncoated-1	243	0.381	8.24
Uncoated-2	238	0.376	8.25
Uncoated-3	240	0.389	8.15
Coated with HM-HEC C16-1	454	0.346	8.15
Coated with HM-HEC C16-2	456	0.364	8.20
Coated with HM-HEC C16-3	447	0.375	8.15
Coated with HM-HEC C8-1	628	0.351	8.12
Coated with HM-HEC C8-2	623	0.391	8.19
Coated with HM-HEC C8-3	632	0.363	8.16

As can be seen in Table 6-2 the uncoated liposomes were the smallest. After coating with the polymers the liposomes increase in size, which indicates successful coating. The liposomes coated with HM-HEC C8 increased more in size compared to the HM-HEC C16 coated liposomes. The sizes of the uncoated soy PC liposomes, the HM-HEC C16 coated liposomes and the HM-HEC C8 coated liposomes were significantly ($p < 0.05$) different. The pH values are nearly the same, which confirms equal conditions.

The PdI of the uncoated liposomes were unusually large (Pereira-Lachataignerais et al. 2006). The reason for this is probably due to problems during the extrusion of these liposomes. Pressure above 10 bar was needed to be able to extrude the CF encapsulated liposomes. The problems were thought to be because of the high CF concentration, and possibly because some of the CF was not dissolved. The PdI values of the uncoated soy PC liposomes, the HM-HEC C16 coated soy PC liposomes and the HM-HEC C8 coated liposomes were not significantly ($p < 0.05$) different from each other. Because of the very diluted samples during the measurements, the non-adsorbed HM-HEC was not expected to influence on the results.

The release of CF from the soy PC liposomes at 35 °C is shown in Figure 6-2. As can be seen, the release of CF increased during the period of 45 hours. The intention was to make measurements at 24 hours, however some technical problems with the Wallac Victor[3] 1420 plate reader occurred, and the measurements were made at 45 hours instead.

The release of CF was low and with small differences between the uncoated and coated liposomes.

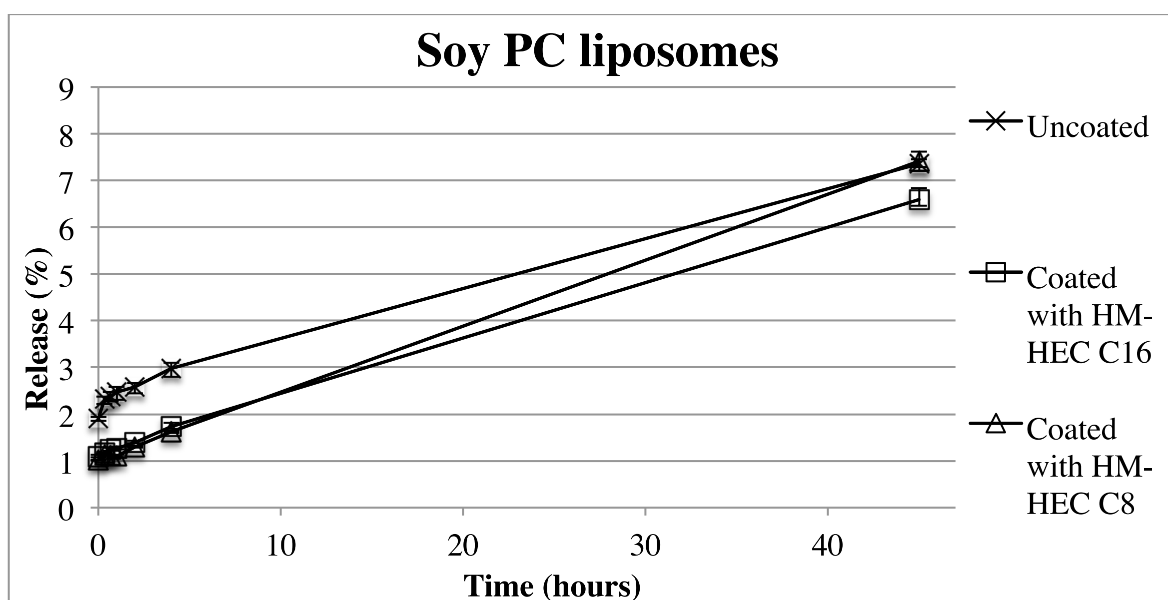

Fig. 6-2. Release of CF from uncoated and polymer coated soy PC liposomes in 60 mM tris buffer pH 8.0 with 0.35 M NaCl (polymer concentration, 0.1 %) at 35 °C during 45 hours. The error bars are equal to or smaller than the size of the symbols when not visible.

However, at $t = 0$ and $t = 4$ hours the release from the uncoated soy PC liposomes was significantly ($p < 0.05$) higher than the coated soy PC liposomes. At $t = 45$ hours the release

from the uncoated soy PC liposomes and the HM-HEC C8 coated soy PC liposomes were not significantly different, however they were significantly higher than the release from HM-HEC C 16 coated soy PC liposomes.

Gel phase liposomes (DPPC) coated and measured three days after extrusion

In Table 6-3 the size, PdI and pH characteristics of the DPPC liposomal samples are shown. These DPPC liposomes were gel filtered, coated and measured three days after extrusion. The size decreased when coated with HM-HEC C16, however it increased with HM-HEC C8 coating. It is well known that DPPC starts aggregating immediately after extrusion (Wong and Thompson 1982).

Table 6-3. The size (hydrodynamic diameter) and corresponding PdI and pH of the uncoated and coated DPPC liposomes with 100 mM carboxyfluorescein encapsulated. The samples for size and PdI measurements were diluted before measurements as described in Ch 4.5. These DPPC liposomes were coated and measured three days after extrusion and not immediately after coating.

DPPC	Size (nm)	PdI	pH
Uncoated-1	590	0.373	8.42
Uncoated-2	618	0.333	8.46
Uncoated-3	566	0.348	8.42
Coated with HM-HEC C16-1	473	0.329	8.38
Coated with HM-HEC C16-2	457	0.350	8.31
Coated with HM-HEC C16-3	457	0.331	8.28
Coated with HM-HEC C8-1	843	0.455	8.35
Coated with HM-HEC C8-2	771	0.387	8.36
Coated with HM-HEC C8-3	794	0.442	8.38

The sizes of the uncoated liposomes in Table 6-3 indicate that the DPPC liposomes had aggregated. Before coating, the liposomes were mixed to dissolve the aggregates. It was not clear if the polymers would be able to coat the liposomes because of the aggregation, but the size values suggest that the aggregates were dissolved and that the coating most probably had been successful. The sizes of the uncoated DPPC liposomes, the HM-HEC C16 coated liposomes and the HM-HEC C8 coated liposomes were significantly ($p < 0.05$) different. The pH values are nearly the same, which confirms equal conditions.

The PdI values of the uncoated DPPC liposomes and the HM-HEC C16 coated DPPC liposomes were not significantly ($p < 0.05$) different, however they differed from the significantly higher PdI values of the HM-HEC C8 coated DPPC liposomes. It is not unusual that the HM-HEC C8 coated liposomes have higher PdI values due to their larger sizes and could be as a result of bridging flocculation.

The release of CF from the liposomes at 35 °C is shown in Figure 6.3. As can be seen in the figure the release of CF increased during the period of 45 hours. However, the release was very low. There was no significant ($p < 0.05$) difference between the uncoated DPPC liposomes and polymer coated liposomes at t = 0 and t = 4 hours. The HM-HEC C8 and HM-HEC C16 coated liposomes had nearly the same release rate during the 24 hours and the release rate was not significantly different between the coated DPPC liposomes at t = 24 hours. However, the release rate between the coated DPPC liposomes and the uncoated DPPC liposomes was significantly different at t = 24 hours.

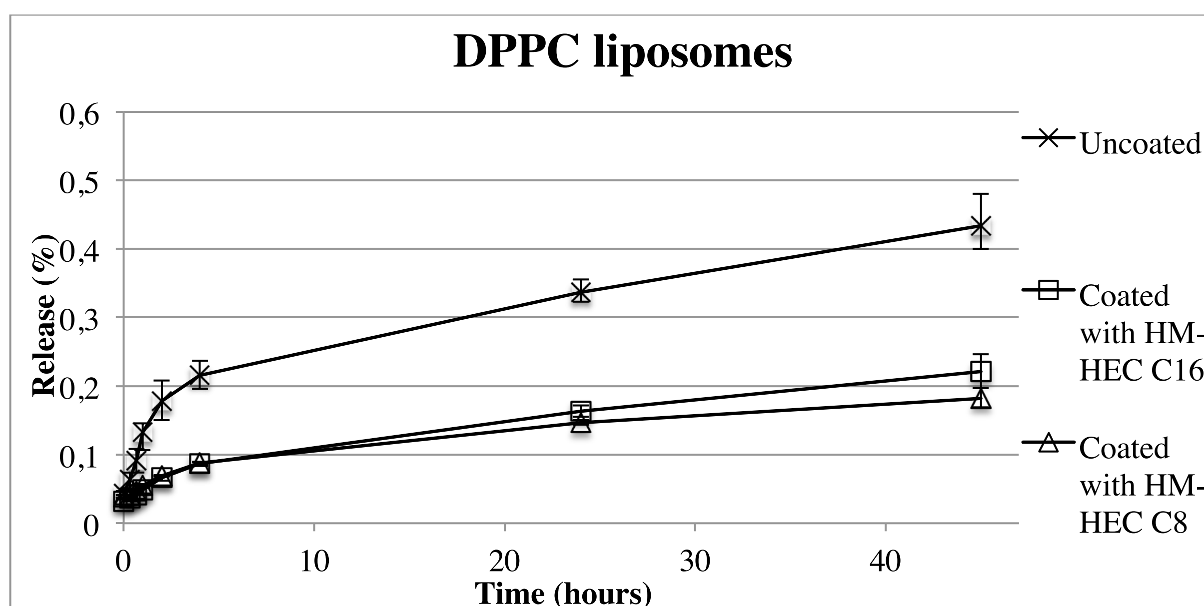

Fig. 6-3. Release of CF from uncoated and polymer coated DPPC liposomes in 60 mM tris buffer pH 8.0 with 0.35 M NaCl (polymer concentration, 0.1 %) at 35 °C during 45 hours. The DPPC liposomes were coated and measured three days after the extrusion. The error bars are equal to or smaller than the size of the symbols when not visible.

Gel phase liposomes (DPPC) coated and measured immediately after extrusion

In Table 6-4 the size, PdI and pH characteristics of another batch of DPPC liposomal samples are shown. These DPPC liposomes were gel filtrated, coated and measured immediately after extrusion. The size increased significantly ($p < 0.05$) in both the coatings with HM-HEC C16 and with HM-HEC C8 and indicated successful coating.

Table 6-4. The size (hydrodynamic diameter) and corresponding PdI and pH of the uncoated and coated DPPC liposomes with 100 mM carboxyfluorescein encapsulated. The samples for size and PdI measurements were diluted before measurements as described in Ch 4.5. These DPPC liposomes were coated and measured immediately after extrusion.

DPPC	Size (nm)	PdI	pH
Uncoated-1	220	0.269	8.74
Uncoated-2	230	0.279	8.78
Uncoated-3	236	0.267	8.83
Coated with HM-HEC C16-1	272	0.097	8.52
Coated with HM-HEC C16-2	271	0.111	8.55
Coated with HM-HEC C16-3	272	0.075	8.53
Coated with HM-HEC C8-1	406	0.229	8.46
Coated with HM-HEC C8-2	402	0.239	8.42
Coated with HM-HEC C8-3	399	0.213	8.42

The same size trend can be noted in Table 6-4, considering the trends in Table 6-2 for soy PC liposomes. The DPPC liposomes coated with HM-HEC C16 were measured to have significantly ($p < 0.05$) smaller diameters than the liposomes coated with HM-HEC C8. The uncoated DPPC liposomes were also significantly different from the coated DPPC liposomes. The pH values are nearly the same, which confirms equal conditions.

The PdI of the uncoated and HM-HEC coated DPPC liposomes in Table 6-4 are significantly ($p < 0.05$) different. The HM-HEC C16 coated liposomes have the lowest PdI values and this suggests that the coating is tightly packed around the liposomes. The PdI values of the HM-HEC C8 liposomes are higher which could suggest bridging flocculation, which can change the PdI. The PdI of the uncoated DPPC liposomes was higher than the coated DPPC

liposomes. This suggests that the uncoated liposomes had already started aggregating when the measurements started.

The release of CF from the liposomes at 35 °C is shown in Figure 6-4. As can be seen in the figure the release of CF increased during the period of 24 hours. However, the release was very low. The release was highest for the uncoated liposomes. At t = 0 and t = 4, the release from the uncoated and coated DPPC liposomes were not significantly different ($p < 0.05$). At t = 24 hours the release from the HM-HEC C16 and HM-HEC C8 coated DPPC liposomes were not significantly different, however they were significantly lower than from the uncoated DPPC liposomes.

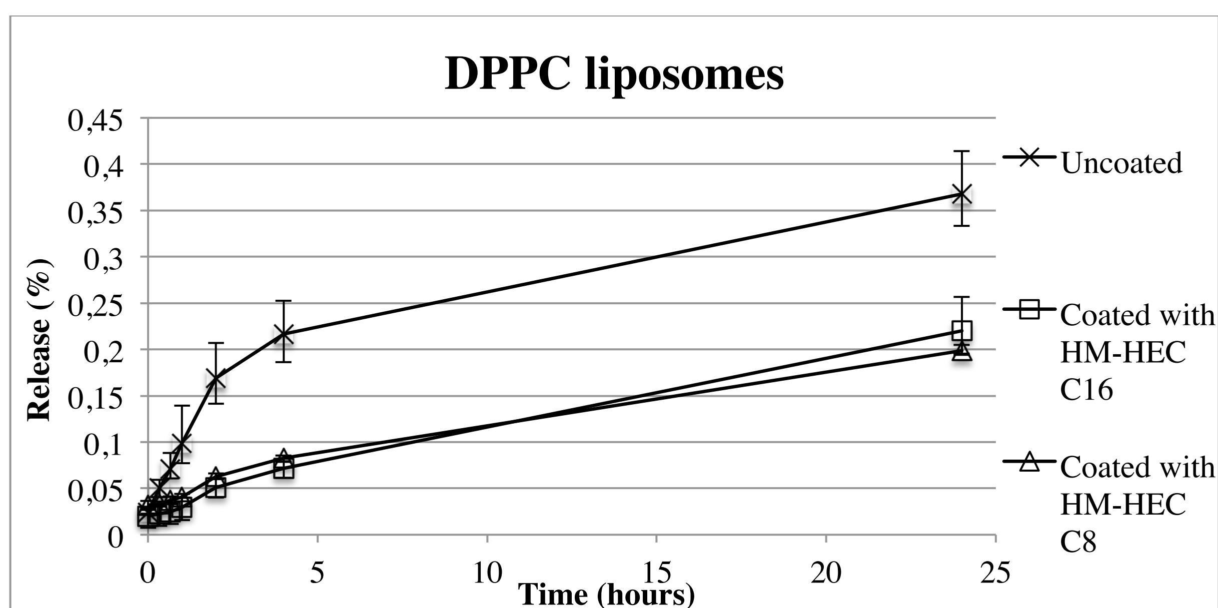

Fig. 6-4. Release of CF from uncoated and polymer coated DPPC liposomes in 60 mM tris buffer pH 8.0 with 0.35 M NaCl (polymer concentration, 0.1 %) at 35 °C during 24 hours. The DPPC liposomes were coated and measured immediately after the extrusion. The error bars are equal to or smaller than the size of the symbols when not visible.

Figure 6-4 is very similar to Figure 6-3. A comparison of the two release plots shows virtually the same tendency and percent release for all corresponding formulations. At t = 24 hours the release from the uncoated DPPC liposomes were significantly higher than from the coated DPPC liposomes in both DPPC batches. This indicated that even though the DPPC liposomes had aggregated the coating still was possible to perform. The coating aggregates were possibly dissolved during the coating process or small aggregates were coated and sufficiently protected.

Comparing the release from the soy PC liposomes in Figure 6-2 to the release from the DPPC liposomes in Figure 6-3 and Figure 6-4 shows that the release rates are significantly ($p < 0.05$) higher for both the uncoated and coated soy PC liposomes. This may be explained by the phases the soy PC liposomes and the DPPC liposomes exhibit at 35 °C. Soy PC liposomes are in the liquid phase at 35 °C due to their phase transition temperature below 0 °C, and the DPPC liposomes are in gel phase due to their phase transition temperature at 41 °C (Taylor and Morris 1995). It is known that leakage from gel phase liposomes is lower than fluid phase liposomes (New 1990). Also, the larger release from the uncoated liposomes than the polymer coated liposomes, both gel phase and liquid phase, could indicate that there is a layer coating and protecting the liposomes resulting in slower release of encapsulated CF.

Overall it seems that the HM-HEC coating protected against release at 35 °C. Other hydrophobically modified polymers, e.g., hydrophobically modified PNIPAAM has been shown to trigger the release due to its temperature sensitive property. No data has been found in the literature, which indicates that hydrophobically modified HEC is temperature sensitive.

6.3 Stability studies at 4 °C

6.3.1 *Size measurements*

Figure 6-5 shows the stability during storage of the apparent hydrodynamic diameter of uncoated and polymer coated soy PC liposomes in 5 mM phosphate buffer pH 6.8 at a storage temperature of 4 °C. In all samples, the particle size was stable during time. Since both the uncoated and the coated soy PC liposomes were stable during time, the coating did not affect the stability in this case. The uncoated liposomes had the smallest diameter, the HM-HEC C16 coated liposomes had larger diameter and the HM-HEC C8 coated liposomes had the largest diameter. This shows the same trend as in Table 6-2, but the size is much lower for all the samples. This can probably be explained by the CF encapsulation and that the buffer is different.

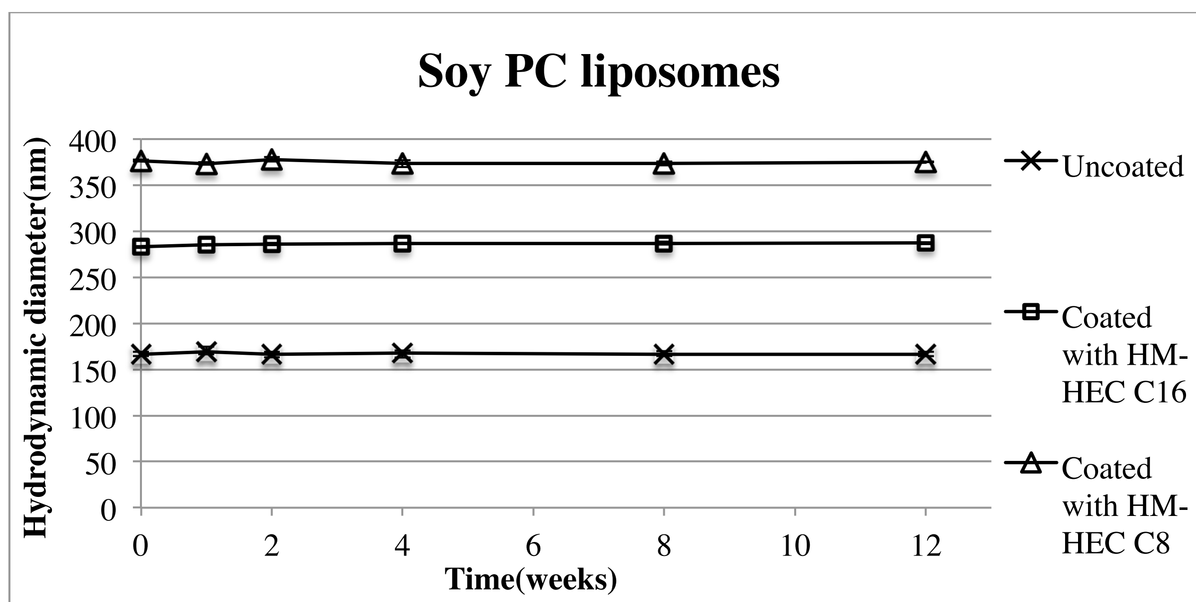

Fig. 6-5. Size (nm) of uncoated and coated soy PC liposomes in 5 mM phosphate buffer pH 6.8 with a 0.1 % polymer concentration during 12 weeks. The samples were diluted before measurements as described in Ch 4.5. The liposomes were stored at 4 °C and measured at 25 °C. The error bars are equal to or smaller than the size of the symbols.

In Figure 6-6, the effect of time on D(H) for DPPC liposomes in 5 mM phosphate buffer of pH 6.8 during 12 weeks storage 4 °C is shown. The size of the uncoated liposomes is not stable due to aggregation. The liposomes coated with HM-HEC C16 and HM-HEC C8, however, are stable during the 12 weeks. This indicates good stabilization of both liposomes coated with HM-HEC C16 and liposomes coated with HM-HEC C8.

Comparing the soy PC liposomes and the DPPC liposomes, both uncoated and coated, shows that the coatings do not affect the stability of the soy PC liposomes, however, the HM-HEC coatings clearly stabilize the DPPC liposomes.

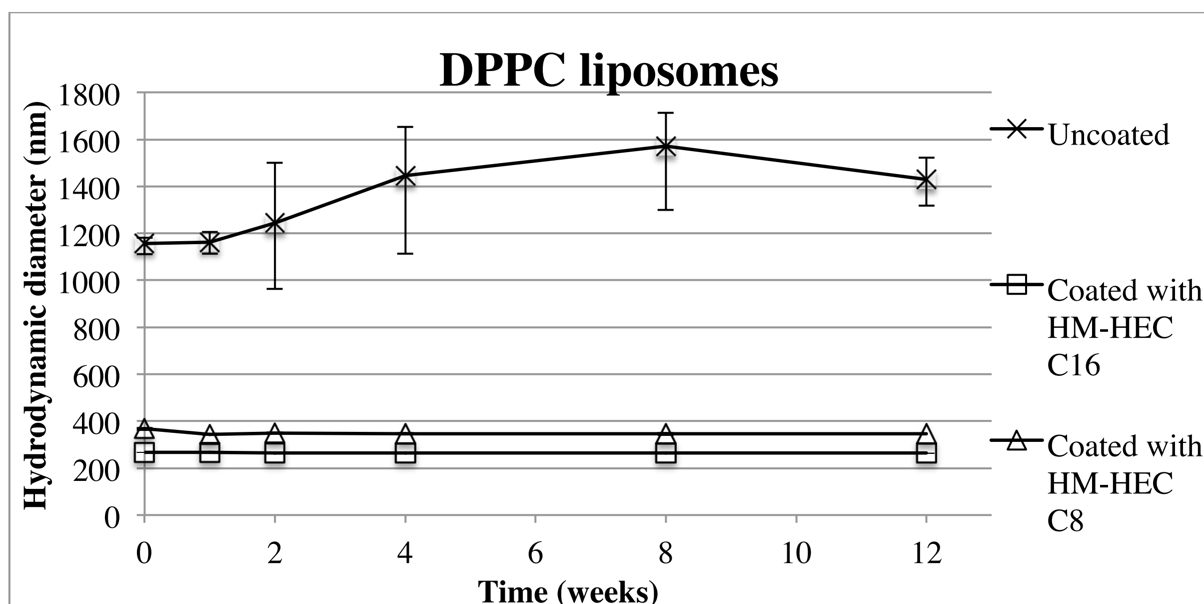

Fig. 6-6. Size (nm) of uncoated and coated DPPC liposomes in 5 mM phosphate buffer pH 6.8 with a 0.1 % polymer concentration during 8 weeks. The liposomes were stored at 4 °C and measured at 25 °C. The samples were diluted before measurements as described in Ch 4.5.The error bars are equal to or smaller than the size of the symbols when not visible.

In both the soy PC liposomes with 100 mM carboxyfluorescein encapsulated (Table 6-2) and the soy PC liposomes without carboxyfluorescein (Figure 6-5), the uncoated liposomes had the smallest diameter. By coating with HM-HEC C16 the diameter increased, and by coating with HM-HEC C8 the size increased additionally.

In the DPPC liposomes (both with and without carboxyfluorescein) shown in Table 6-3, Table 6-4 and Figure 6-6, the HM-HEC C8 coated were the biggest sized coated liposomes, and the HM-HEC C16 coated liposomes were the smallest sized. The uncoated DPPC liposomes increased in size during time, due to aggregation. The difference in size values between the HM-HEC C16 and HM-HEC C8 liposomes shows a tendency that the HM-HEC C8 made larger complexes compared to HM-HEC C16. In a way this was not expected because the HM-HEC C16 is a polymer with twice as long alkyl chain as HM-HEC C8. On the other hand this can be explained by that the HM-HEC C16 has more possible interaction sites with the liposomal membrane than the HM-HEC C8. On the basis of this the HM-HEC C16 polymer may become more tightly packed around the liposome, which lead to a smaller diameter than the HM-HEC C8, which might be more loosely packed around the liposomes.

In this study the sizes were stable during the 12-weeks test period. However, it has been observed that the size of egg PC liposomes decreased during a longer period of time (Meland et al. 2014) and DPPC/DPTAP increased during storage (Smistad et al. 2012). It was only possible to carry out this study over 12 weeks, where no change in the size of the complexes was observed. However, it is quite possible that the soy PC liposomes and the DPPC liposomes will behave in the same way as the egg PC liposomes after additional storage time.

6.3.2 *Transmittance measurements*

At time point t = 0, 2 ml of each sample was transferred to a cuvette and the transmittance was measured. The samples were stored in the cuvettes with lids at 4 °C throughout the 12 weeks test period.

During the time period, the transmittance in HM-HEC C16 and HM-HEC C8 solutions were measured and it was found that the transmittance were high (nearly 100% of the buffer reference solution), which indicated good dissolution of the HM-HEC polymers (data not shown).

The transmittance results of the soy PC liposomes during the 12 weeks are shown in Figure 6-7. The transmittance values for the different liposomes were stable during the time period. The transmittance of the uncoated soy PC liposomes was highest and the HM-HEC C8 liposomes had the lowest transmittance values. This difference in transmittance could indicate that the difference in sizes of the uncoated and coated liposomes (shown in Figure 6-5) lead to different turbidity. The larger complexes are expected to be more turbid than the smaller complexes (Klemetsrud et al. 2013).

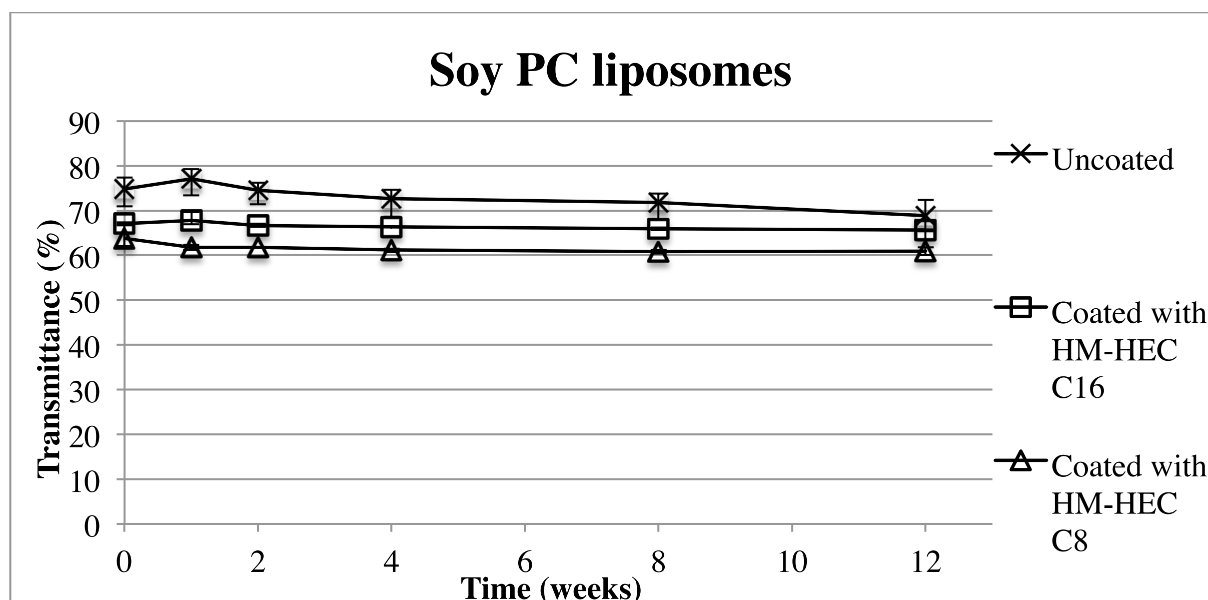

Fig. 6-7. Transmittance of uncoated and coated soy PC liposomes in 5 mM phosphate buffer pH 6.8 with a 0.1 % polymer concentration during 12 weeks. The liposomes were stored at 4 °C and measured at room temperature (about 20 °C). The error bars are equal to or smaller than the size of the symbols.

During the transmittance measurements the samples were measured twice at all time points except at t = 0 when the samples were only measured once. First, the samples were measured after motionless storage at 4 °C (called unstirred sample). Then, the samples were turned 10 times (called stirred sample) and measured again.

The differences in transmittance values between the unstirred and stirred samples are shown in Figure 6-8. The uncoated liposomes showed relatively large differences between the unstirred and stirred samples. This may indicate that there was some aggregation and sedimentation in these samples and that the soy PC liposomes were not completely stable. This is in contrast to the size measurements in Figure 6-5. However, the differences may be explained by different sample preparation procedures. The samples were diluted before the size measurements. The transmittance, however, was measured in undiluted samples. Eventual aggregates could disintegrate or become more difficult to detect when the samples were diluted, and thus not detected by the DLS. The HM-HEC C16 and HM-HEC C8 coated soy PC liposomes showed smaller differences between stirred and unstirred samples than the uncoated soy PC liposomes. The HM-HEC C8 coated liposomes had larger differences in transmittance than the HM-HEC C16 coated liposomes, which could indicate more

sedimentation in the HM-HEC C8 coated soy PC liposomes. This is reasonable due to the larger sizes of the HM-HEC C8 coated soy PC liposomes shown in Figure 6-5.

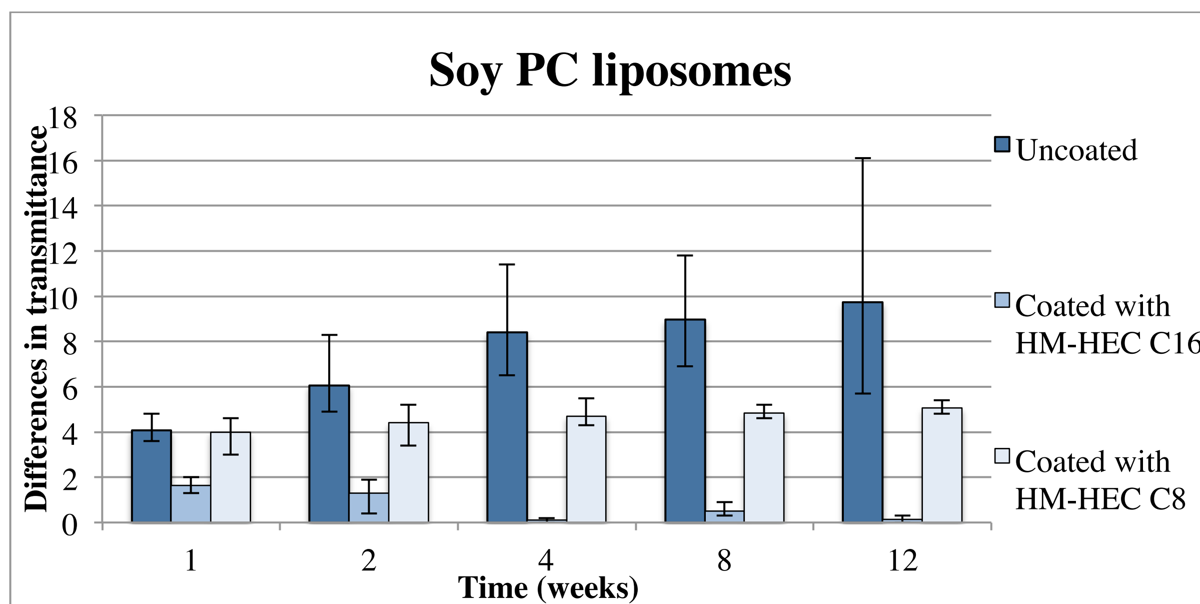

Fig. 6-8. Differences in transmittance between stirred and unstirred samples of uncoated and coated soy PC liposomes in 5 mM phosphate buffer pH 6.8 with a 0.1 % polymer concentration during 12 weeks.

Figure 6-9 shows the transmittance of uncoated and coated DPPC liposomes. The coated liposomes gave almost the same results as the coated soy PC liposomes shown in Figure 6-7. The transmittance of the uncoated DPPC liposomes, however, gave much lower values. This can be explained by the relatively large diameter values shown in Figure 6-6, and that the DPPC liposomes formed aggregates.

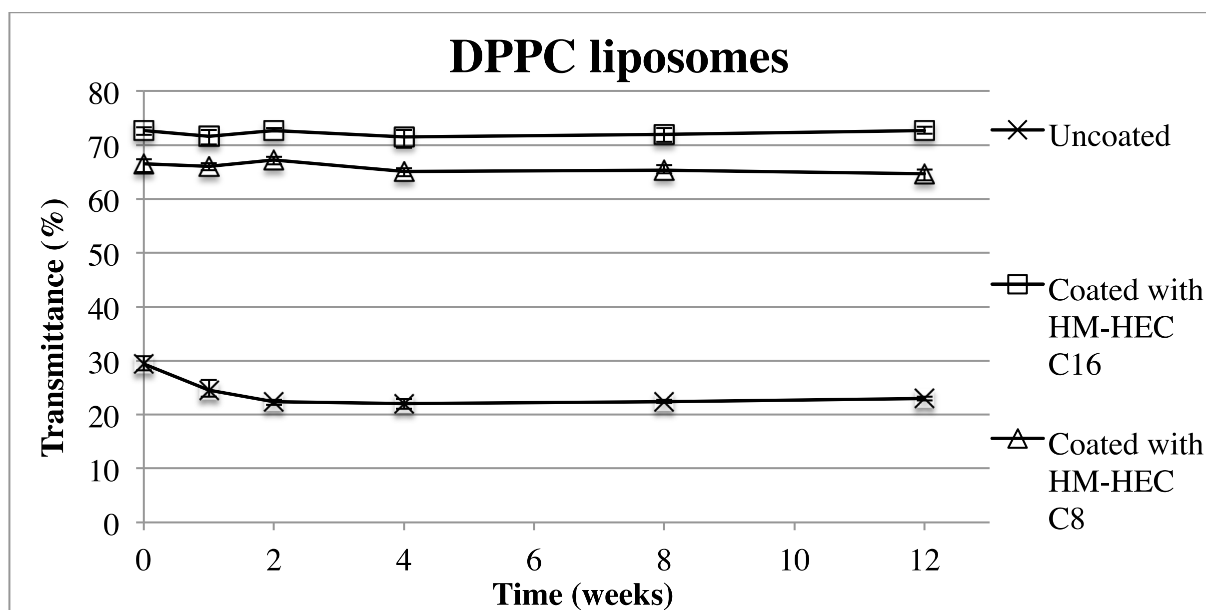

Fig. 6-9. Transmittance of uncoated and coated DPPC liposomes in 5 mM phosphate buffer pH 6.8 with a 0.1 % polymer concentration during 8 weeks. The liposomes were stored at 4 °C and measured at room temperature (about 20 °C). The error bars are equal to or smaller than the size of the symbols.

The differences in transmittance values between the unstirred and stirred samples are shown in Figure 6-10. The figure shows great differences for the uncoated liposomes. The DPPC liposomes form aggregates which sediment, and when the samples are stirred, the aggregates became evenly distributed in the sample and the differences in transmittance seem huge. The difference between the liposomes coated with HM-HEC C16 and HM-HEC C8, shows the same trend as between the coated soy PC liposomes shown in Figure 6-8. Again, the differences indicate more sedimentation in the HM-HEC C8 coated liposome samples, which is reasonable due to the larger sizes of the HM-HEC C8 liposomes compared to the HM-HEC C16 liposomes shown in Figure 6-6.

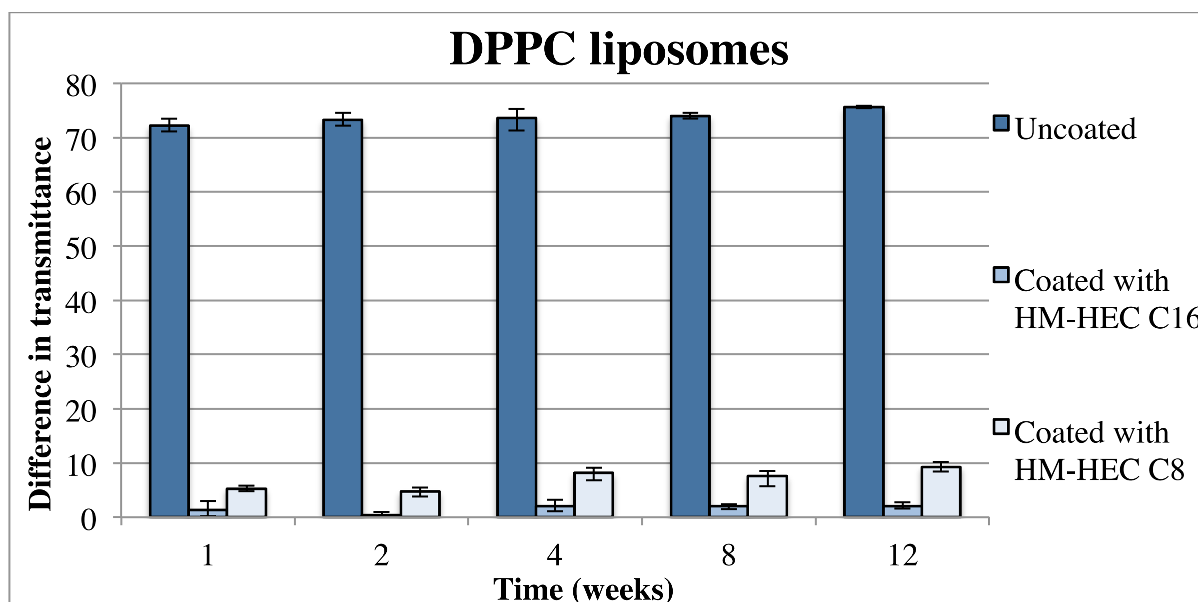

Fig. 6-10. Differences in transmittance between stirred and unstirred samples of uncoated and coated DPPC liposomes in 5 mM phosphate buffer pH 6.8 with a 0.1 % polymer concentration during 12 weeks.

6.3.3 *Zeta potential measurements*

Figures 6-11 and 6-12 show that the zeta potentials of all the formulations were slightly negative. This was expected since the liposomes were neutral and coated with neutral polymers. In an earlier study it has been observed that the zeta potential decreased during time due to degradation of the lipids, which lead to a decrease in the zeta potential (Meland et al. 2014). This was not observed in the current study.

There seem to be more fluctuations in the zeta potential measurements of the uncoated liposomes than the coated liposomes, both for the soy PC and the DPPC liposomes. This may indicate that the coating makes the liposomes more stable. However, this stabilization could not be electrostatic stabilization because the zeta potentials were only slightly negative. To be electrostatically stabilized the zeta potential of the liposomes have to be above 30 mV or below -30 mV (Clogston and Patri 2011).

The stability of the soy PC liposomes and the DPPC liposomes on the basis of the size measurements seem stable. In Figure 6-5 and Figure 6-6 the sizes are stable during the 12-week-period (except the uncoated DPPC liposomes which aggregates). According to the low zeta potential values shown in Figure 6-11 and Figure 6-12, the liposomes are not expected to

be electrostatically stabilized. The stabilization of the DPPC liposomes is probably due to the steric stabilization. The soy PC liposomes, however, were stabile without a coating of HM-HEC. This stabilization cannot be because of steric stabilization. The soy PC liposomes might be stabilized due to the hydration layer of the phospholipids (Volke et al. 1994).

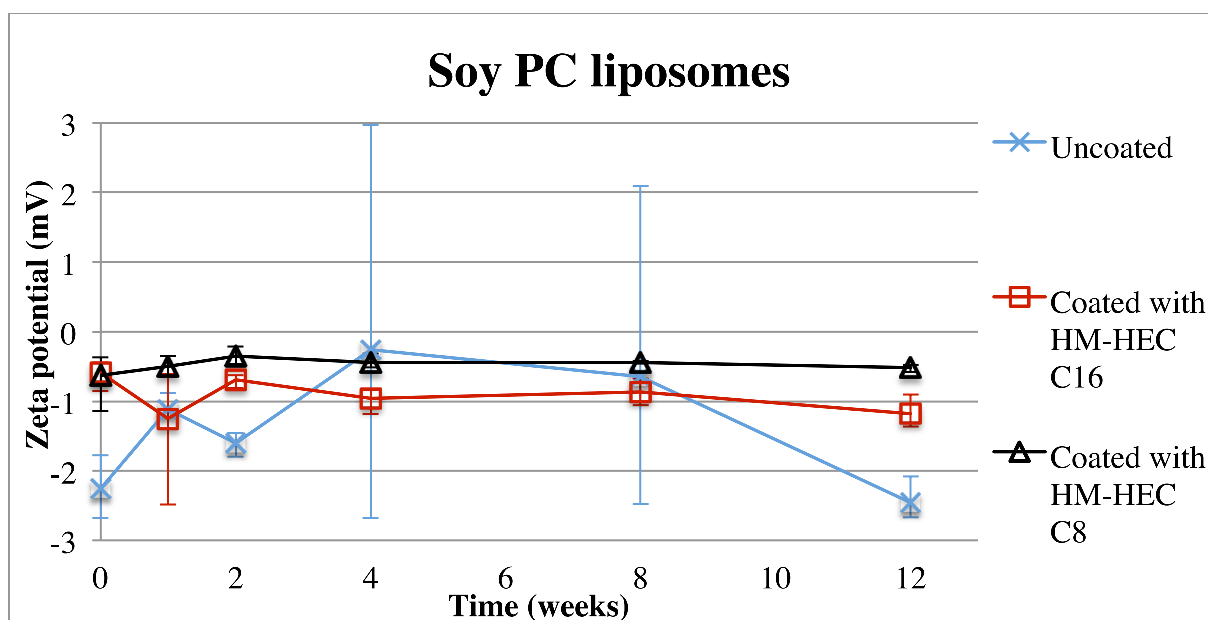

Fig. 6-11. Zeta potential of uncoated and coated soy PC liposomes in 5 mM phosphate buffer pH 6.8 with a 0.1 % polymer concentration during 12 weeks. The samples were diluted before measurements as described in Ch 4.5. The liposomes were stored at 4 °C and measured at 25 °C.

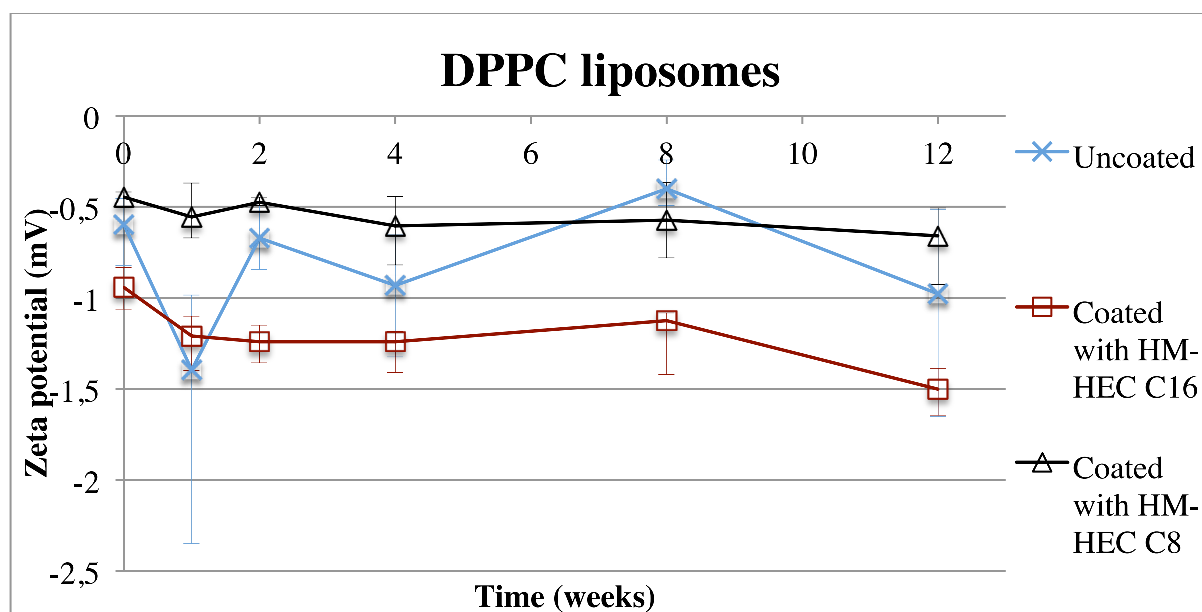

Fig. 6-12. Zeta potential of uncoated and coated DPPC liposomes in 5 mM phosphate buffer pH 6.8 with a 0.1 % polymer concentration during 12 weeks. The samples were diluted before measurements as described in Ch 4.5. The liposomes were stored at 4 °C and measured at 25 °C.

6.3.4 *pH measurements*

In the Figures 6-13 and 6-14, the pH values of the soy PC and DPPC liposomes during 12 weeks are shown. The pH values were relatively constant during the study, however, there seems to be a trend against lower pH in the first four weeks for both the soy PC liposomes and the DPPC liposomes. It is not easy to give a good explanation for this tendency. The variations are, however, very small.

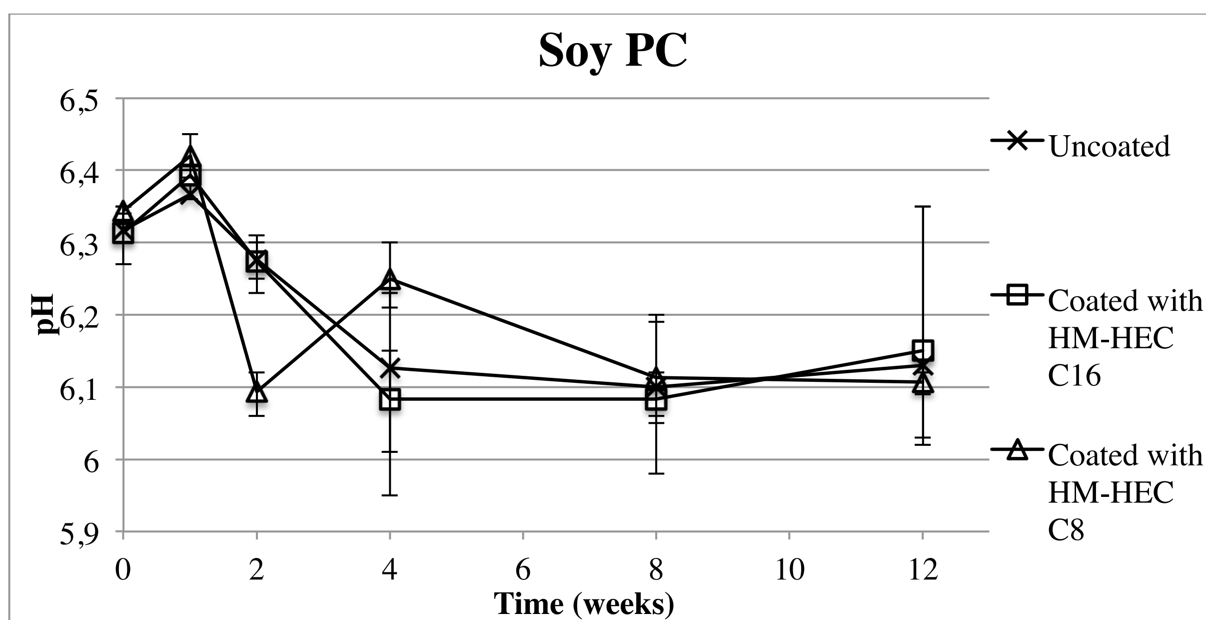

Fig. 6-13. pH of uncoated and coated soy PC liposomes in 5 mM phosphate buffer pH 6.8 with a 0.1 % polymer concentration during 12 weeks. The liposomes were stored at 4 °C and measured at room temperature (about 20 °C).

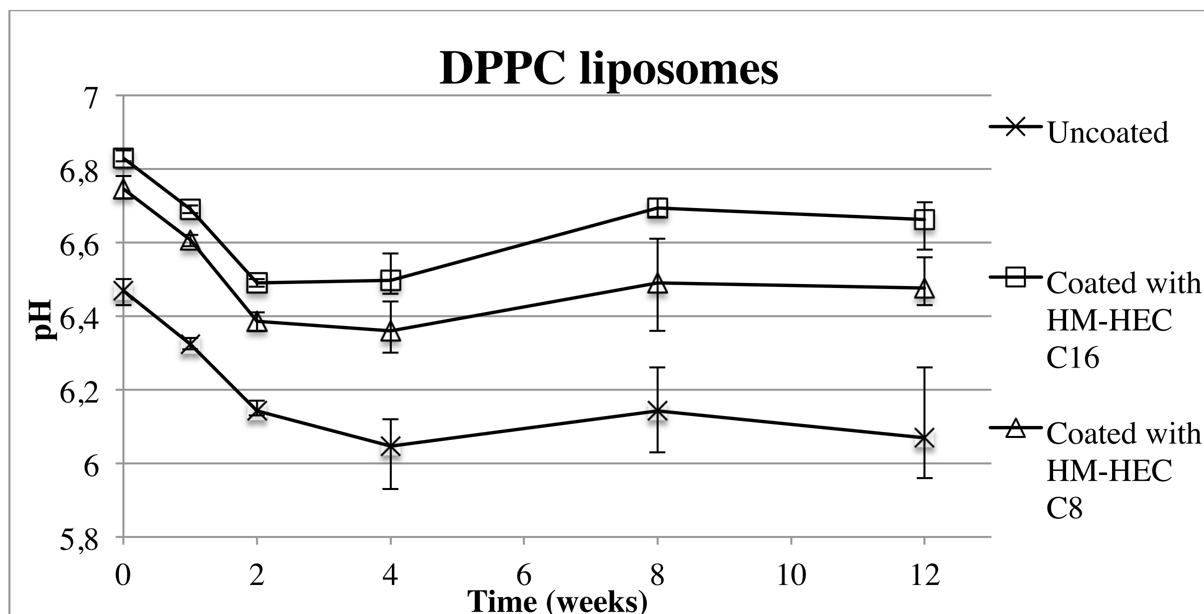

Fig. 6-14. pH of uncoated and coated DPPC liposomes in 5 mM phosphate buffer pH 6.8 with a 0.1 % polymer concentration during 12 weeks. The liposomes were stored at 4 °C and measured at room temperature (about 20 °C).

6.3.5 *Leakage measurements*

The leakage from the liposomes during storage at 4 °C for 12 weeks is shown in Figures 6-15, 6-16 and 6-17. Comparing the leakage from the samples based on soy PC and DPPC at 4 °C, show that the uncoated soy PC liposomes leak the most, but the uncoated DPPC liposomes, however, leak the least. The gel phase liposomes leak less than the fluid phase liposomes due to their more organized structure. Also, upon transition from gel to fluid phase, the bilayer thickness decreases, which will make the liposome structure less protective against leakage (New 1990).

Comparing the leakage from the soy PC liposomes at 4 °C (Figure 6-14) to the release from the soy PC liposomes at 35 °C (Figure 6-2), the release rate at 35 °C is higher even though the measurements were made during a shorter period. This has been observed before with uncoated and pectin coated liposomes (Smistad et al. 2012). This indicates that the storage temperature of 4 °C makes the liposomes more stable and less leaky. Comparing the release from the DPPC liposomes in Figures 6-3 and 6-4 to the leakage in Figures 6-16 and 6-17 show that there is a difference between the release at 35 °C and the leakage at 4 °C. For the

DPPC liposomes at 35 °C, the uncoated liposomes had the highest release rate, however for the DPPC liposomes at 4 °C, the uncoated liposomes had the lowest leakage rate and there was a change between the uncoated and coated liposomes during the 12 weeks. The phase transition temperature of DPPC is as mentioned earlier 41 °C. The phase transition temperature is closer at 35 °C than at 4 °C, which could lead to a less arranged membrane, and thereby increased leakage. At 4 °C the diffusion is low and the membrane is far from the phase transition.

The reason for the higher release rate for the coated DPPC liposomes could possibly be that the hydrophobic chain on HM-HEC probes the membrane and makes disorder, which could lead to increased leakage.

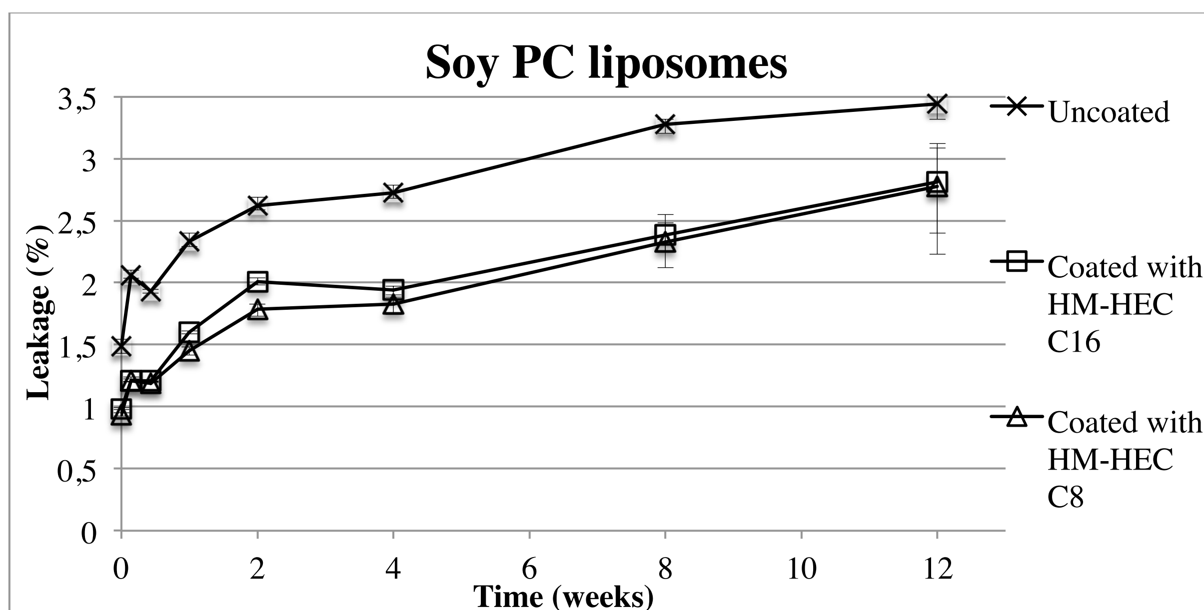

Fig. 6-15. Leakage from uncoated and coated soy PC liposomes in 60 mM tris buffer pH 8.0 with 0.35 M NaCl with a 0.1 % polymer concentration during 12 weeks. The liposomes were stored at 4 °C and measured at room temperature (about 20 °C).

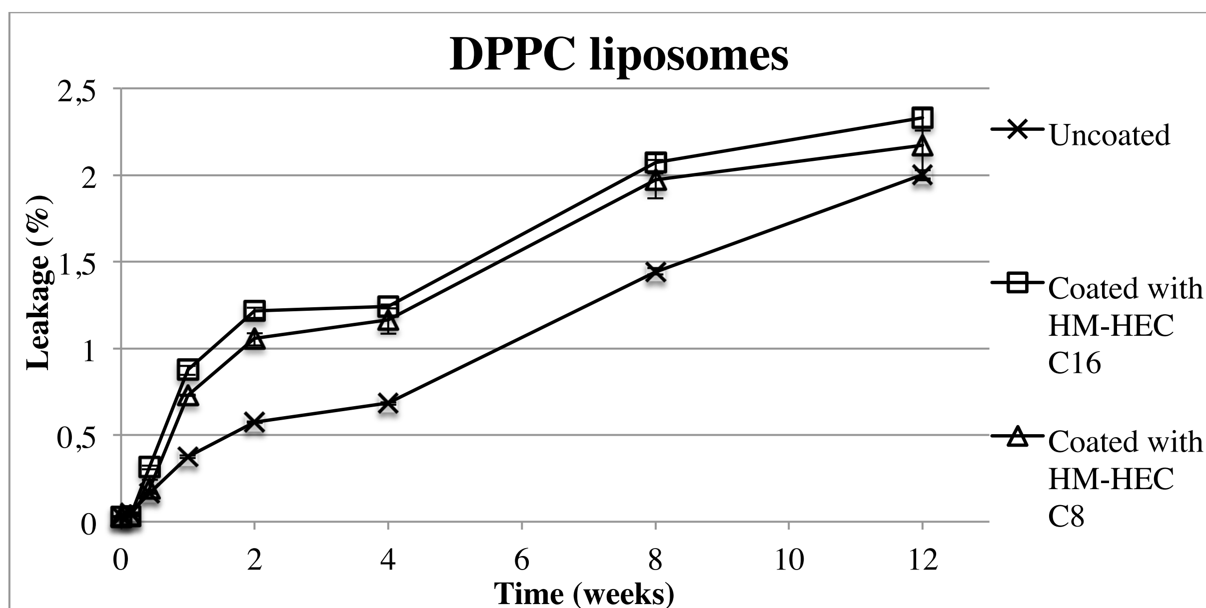

Fig. 6-16. Leakage from uncoated and coated DPPC liposomes in 60 mM tris buffer pH 8.0 with 0.35 M NaCl with a 0.1 % polymer concentration during 12 weeks. (The DPPC liposomes were not coated and measured immediately after extrusion). The liposomes were stored at 4 °C and measured at room temperature (about 20 °C). The error bars are equal to or smaller than the size of the symbols.

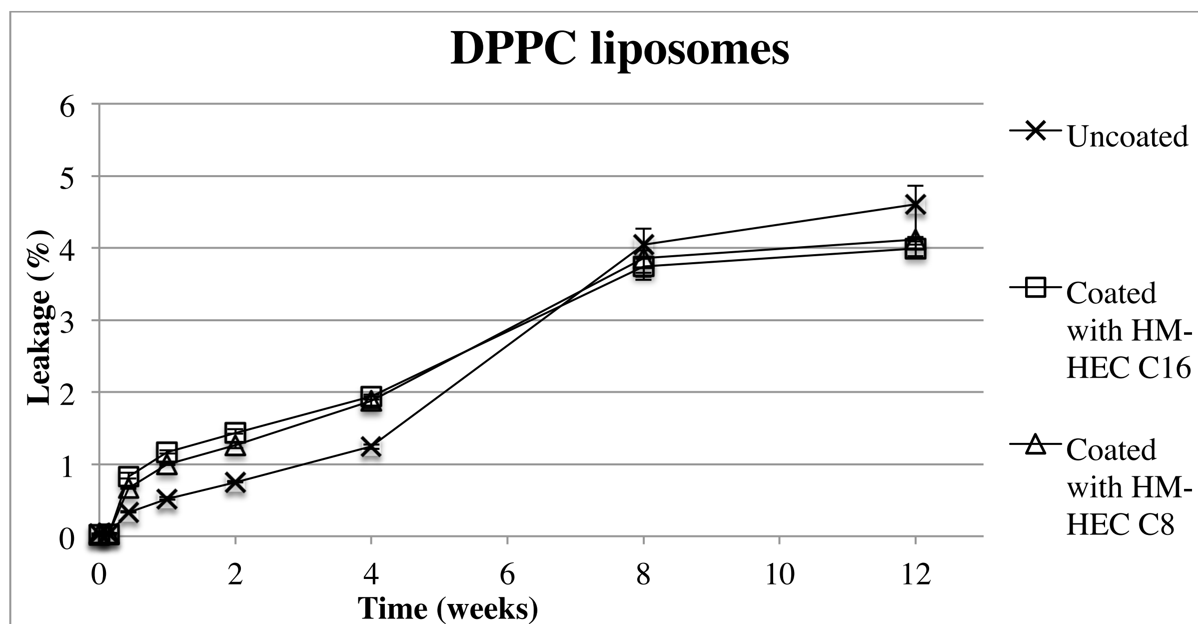

Fig. 6-17. Leakage from uncoated and coated DPPC liposomes in 60 mM tris buffer pH 8.0 with 0.35 M NaCl with a 0.1 % polymer concentration at 4 °C during 12 weeks. (The DPPC liposomes were coated and measured immediately after extrusion). The liposomes were stored at 4 °C and measured at room temperature (about 20 °C).

6.4 Studies on interactions between liposomes and HM-HEC by differential scanning calorimetry (DSC)

The stability studies described in Ch. 6.3 suggest that both the liquid crystalline phase soy PC liposomes and the gel phase DPPC liposomes were surface coated with HM-HEC C16 and HM-HEC C8. It is not entirely clear, however, what mechanism that is responsible for the formation of these liposomes-polymer complexes.

An earlier study has shown that the liposome size did not increase by mixing HEC with egg PC liposomes, but by mixing hydrophobically modified HEC with the fluid phase egg PC liposomes and the gel phase DPPC, the size increased (Meland et al. 2014). The difference between the HEC and the HM-HEC is the hydrobobic alkyl chains attached to the HEC backbone, therefore it is natural to assume that these chains will fasten to the liposomes in a way.

Usually charged liposomes are coated with a polymer with the opposite charge. Coating of neutral polymers onto neutral liposomes has not been studied to a great extent, but some data have been reported. The hydrophobically modified poly(*N*-isopropylacrylamides) was found to interact with the neutral liposomal membrane (Polozova and Winnik 1997).

Only HM-HEC was included in this study. Both the soy PC liposomes and the DPPC liposomes, which appear in respectively liquid phase and gel phase at room temperature, acquired increased sizes due to mixing with HM-HEC. This increase in size may be an indication of that there is some sort of binding between the liposome and polymer, and it is believed that this appears by anchoring of the hydrophobic alkyl chains of HM-HEC into the liposome bilayer.

In the DSC measurements, DMPC was used instead of the DPPC liposomes, which were used as the gel phase liposomes in the release and stability studies. Both DMPC and DPPC have phase transitions above zero. The idea was to investigate liposomes with phase transition below and above zero in the studies by DSC. DMPC has C14 chains and DPPC has C16 chains. It was believed that a possible interaction between the HM-HEC C16 polymer and the

DPPC liposomes would be difficult to detect due to their equally long chains. Therefore, DMPC with the C14 chains was chosen for these interaction studies.

The DSC, an instrument that can give information about a material's thermodynamic properties, was thought to be a useful tool in the study on interactions between liposomes and HM-HEC.

6.4.1 *DMPC*

First, the DMPC liposomes with a phase transition in the positive temperature range were investigated to find the appropriate concentration and scanning parameters for detection of the T_c. DMPC liposomes have a T_c of 24 °C (Needham and Evans 1988). Different concentrations of DMPC in 5 mM phosphate buffer pH 6.8 were scanned in the temperature range of about 5 °C to 40 °C, which would cover the area of T_c. Different DSC scans of the DMPC concentrations are shown in Figure 6-18. The 5 mM phosphate buffer pH 6.8 was used in the reference pan.

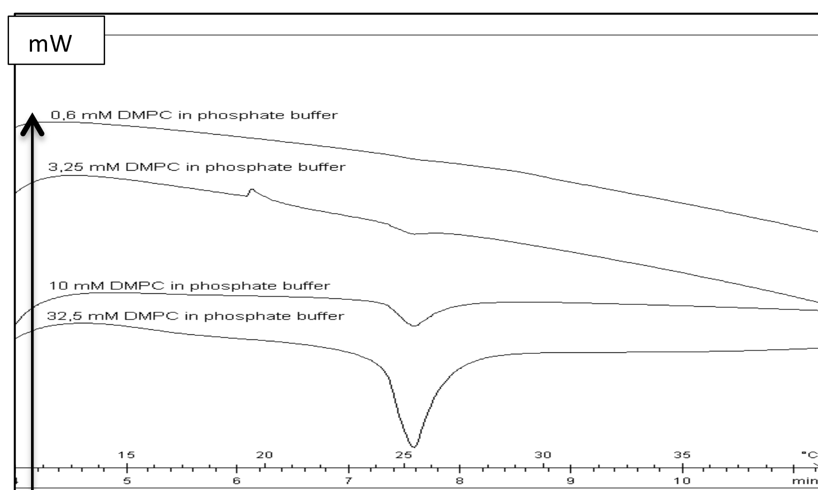

Fig. 6-18. Phase transition characteristics of different concentrations of DMPC in 5 mM phosphate buffer pH 6.8. The temperature was held constant at 10 °C and then increased at a rate of 4 °C/min until 40 °C was reached.

The peak of the phase transition of DMPC is easy to spot at about 25 °C. The onset temperature can be seen at about 24 °C both for 10 mM and 32.5 mM DMPC in 5 mM

phosphate buffer pH 6.8. The T_c of 0.6 mM DMPC is not visible and the T_c peak of the 3.25 mM DMPC was concluded to be too small for further studies.

It is more difficult to obtain successful polymer coating of liposomes at high liposomes concentrations. Therefore, 10 mM DMPC in 5 mM phosphate buffer pH 6.8 was chosen instead of 32.5 mM DMPC for the further study.

Figure 6-19 shows DSC scans of a sample of 10 mM DMPC in 5 mM phosphate buffer pH 6.8 and a mixture of 10 mM DMPC and HM-HEC C16 0.1 %. Phosphate buffer 5 mM pH 6.8 was used as the reference sample.

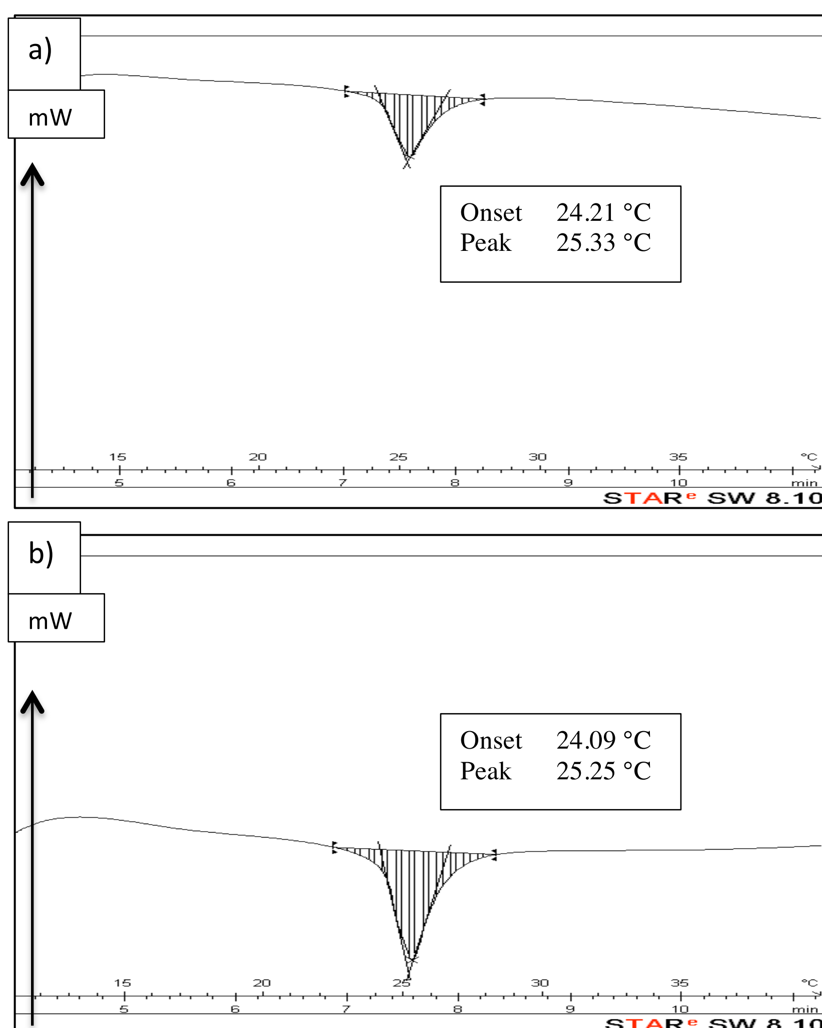

Fig. 6-19. Phase transition characteristic of a) 10 mM DMPC in 5 mM phosphate buffer pH 6.8 and b) 10 mM DMPC in 5 mM phosphate buffer pH 6.8 with 0.1 % HM-HEC C16. The temperature was held constant at 10 °C and then increased at a rate of 4 °C/min until 40 °C was reached.

Figure 6-19(a) shows a phase transition peak with an onset temperature of 24.21 °C. The T_c of the DMPC was measured several times with the different samples to find out if the method was reliable. The results were reproducible with measured onset values between 24.21 and 24.89 °C.

In Figure 6.19(b) the scan of 10 mM DMPC mixed with 0.1 % HM-HEC C16 in 5 mM phosphate buffer pH 6.8 is shown. The components were mixed by using a whirlmixer.

The hypothesis was that it should be a change in the onset curve because of the interactions between the liposome and the polymer. However, no significant ($p < 0.05$) differences between the onset values of the two samples could be detected.

The same mixtures of 10 mM DMPC and HM-HEC were stored at both 4 °C and 45 °C overnight to try to improve the interactions, and new scans were conducted the next day. These scans gave the same results as in Figure 6-19(b) with no significant differences in the onset values compared to the DMPC alone (data not shown). One possible explanation could be that 0.1 % polymer concentration was too low for an interaction to be detected. Also, it has to be remembered that of the 0.1 % concentration the hydrophobic moiety constituted only one mole %. Another factor is that the difference between the C14 in DMPC and C16 in the HM-HEC polymer is very small and an eventual interaction could be difficult to detect. The same scanning was conducted with 10 mM DMPC with 0.1 % HM-HEC C8, with the same result (data not shown). It was believed that it could be easier to detect an interaction between the DMPC and the HM-HEC C8 because the chain lengths were more different than between the DMPC and the HM-HEC C16. However, the same results with not detectable interaction occurred.

6.4.2 *Egg PC*

The egg PC liposomes exhibit phase transition below 0 °C and were scanned in a mixture of 35 % ethylene glycol, to avoid the melting peak of water (Ch.6.1.2).

The melting temperature for the DMPC liposomes was determined using the onset temperature. However, when peaks are broad, like in the naturally occurring egg PC due to the mixture of the fatty acids in the molecule, the onset temperature will be more accurate to use in the determination of the transition temperature.

In the Figure 6-20(a) the phase transition of 32.5 mM egg PC liposomes is shown with a peak at -10.40 °C. This seems reasonable when the literature have shown a T_c in the range -10 to -15 °C. The characteristic peak, which occurs when the temperature is increased, indicates a phase transition from gel to liquid crystalline phase. The same peak can also be observed as an exothermic peak when the temperature decreases before the constant temperature at 20 °C. This is due to the phase transition from the liquid state to the gel state.

The T_cs of the egg PC liposomes were measured several times with different samples to find out if the method was reliable. The results were reproducible with measured peak values between -10.23 and -10.47 °C.

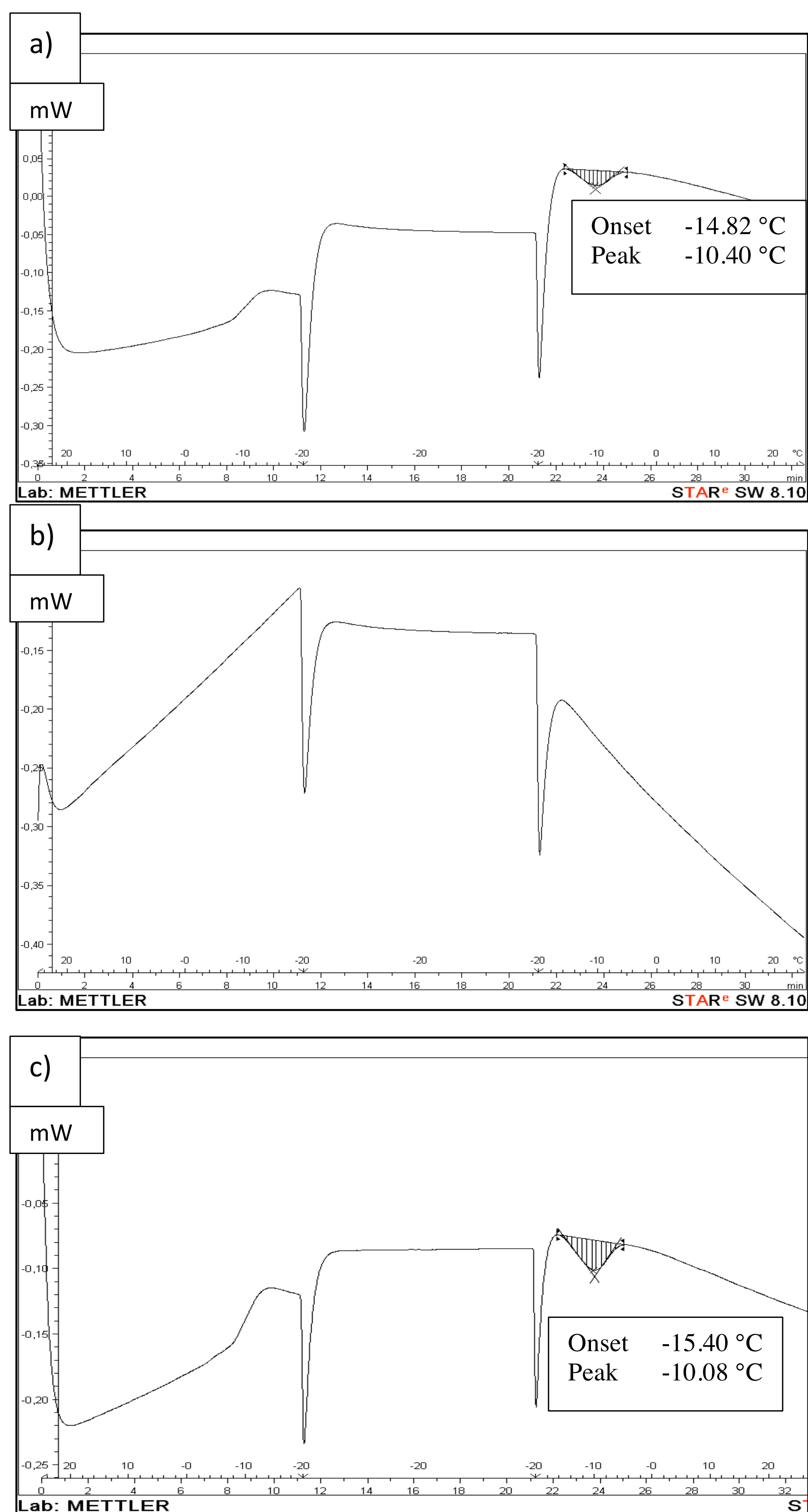

Fig. 6-20. Phase transition characteristic of a) 32.5 mM egg PC in 35 % ethylene glycol in 5 mM phosphate buffer pH 6.8, b) 35 % ethylene glycol in 5 mM phosphate buffer pH 6.8 and c) 32,5 mM egg PC in 35 % ethylene glycol in 5 mM phosphate buffer pH 6.8 with 0.1 % HM-Com-HEC. The temperature was decreased until it reached -20 °C and held constant at -20 °C for 10 minutes. Then the temperature was increased at a rate of 4 °C/min until 25 °C was reached.

In Figure 6-20(b), the melting characteristics of 35 % ethylene glycol in 5 mM phosphate buffer pH 6.8 are shown with the same temperature parameters as used in Figure 6-20(a). The Figure shows no peak at around -10 °C, neither before nor after the constant temperature at -20 °C. This supports what is seen in Figure 6-19(a), that the peak occurs because of the presence of egg PC liposomes.

In Figure 6-20(c), the phase characteristic of 32.5 mM egg PC liposomes with 0.1 % HM-Com-HEC is shown. The mean peak value was -10.08 °C and was not significantly ($p < 0.05$) different from the egg PC alone.

Thus, no interactions could be detected by DSC. Again, this could be explained by the low HM-HEC concentration. Also, egg PC is a natural product containing fatty acid chains of different lengths including C16. This results in a broad peak and this will also make it difficult to detect any interaction.

Even though the scanning of the egg PC liposomes with the polymer (Figure 6-20(c)) gave no sign of an interaction, the egg PC liposomes' T_c has been detected. It is difficult to find exact values in the literature on the T_c of the egg PC liposomes. This method did, however, seem to be precise and reliable due to the reproducible data.

6.4.3 *DOPC*

As mentioned above, the egg PC liposomes gave a very broad peak, which would make eventual interactions harder to detect. DOPC is a synthetic lipid and contains only C18 chains with one double bond. This will give a narrower peak and interactions with saturated C16 chains would probably be easier to detect.

Figure 6-21 shows melting characteristics of 10 mM DOPC in 35 % ethylene glycol in phosphate buffer. It was believed that the same method by mixing the egg PC liposomal suspension with the ethylene glycol would be transferrable to the DOPC liposomes. However, Figure 6-21 shows no T_c peak. The method was tested with several samples, but still no peak occurred.

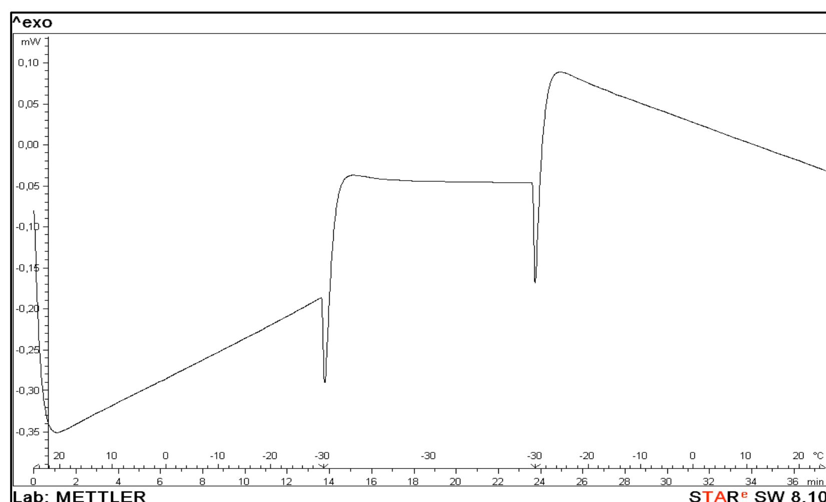

Fig. 6-21. Melting characteristic of 10 mM DOPC in 35 % ethylene glycol in 5 mM phosphate buffer pH 6.8. The temperature was decreased until it reached -30 °C and held constant at -30 °C for 10 minutes. Then the temperature was increased at a rate of 4 °C/min until 25 °C was reached.

However, when scanning solid DOPC (~10 mg) hydrated with a small amount of buffer (~2.2 µl) a large peak appeared as shown in Figure 6-21(a). This small amount of buffer was expected to fully hydrate the phospholipids (Ulrich et al. 1994).

Figure 6-22(a) shows the T_c of the DOPC at -18.15 °C.

In Figure 6-22(b) a thermogram scan of a 50/50 mixture of DOPC and HM-HEC C16 hydrated with a small amount of phosphate buffer is shown. The components were first mixed in excess of phosphate buffer. This was tried to see if an interaction between the liposome and the polymer could be detected just by mixing the materials together. The sample was left to dry at room temperature for several days. The 11th day the sample was added ~2.2 µl 5 mM phosphate buffer pH 6.8 and scanned (Figure 6-22(b). Figure 6-22(b) shows a peak with melting onset at -17.49 °C. This is a bit higher but not significantly different from the T_c of DOPC alone. The large peak around 2 °C was supposed to be the phosphate buffer peak.

Figure 6-22(c) shows the same as Figure 6-22(b), but with the HM-HEC C8 polymer instead of HM-HEC C16. A peak with a melting point onset of -17.75 °C is seen. This onset temperature was not significantly different from the peak onset of DOPC alone.

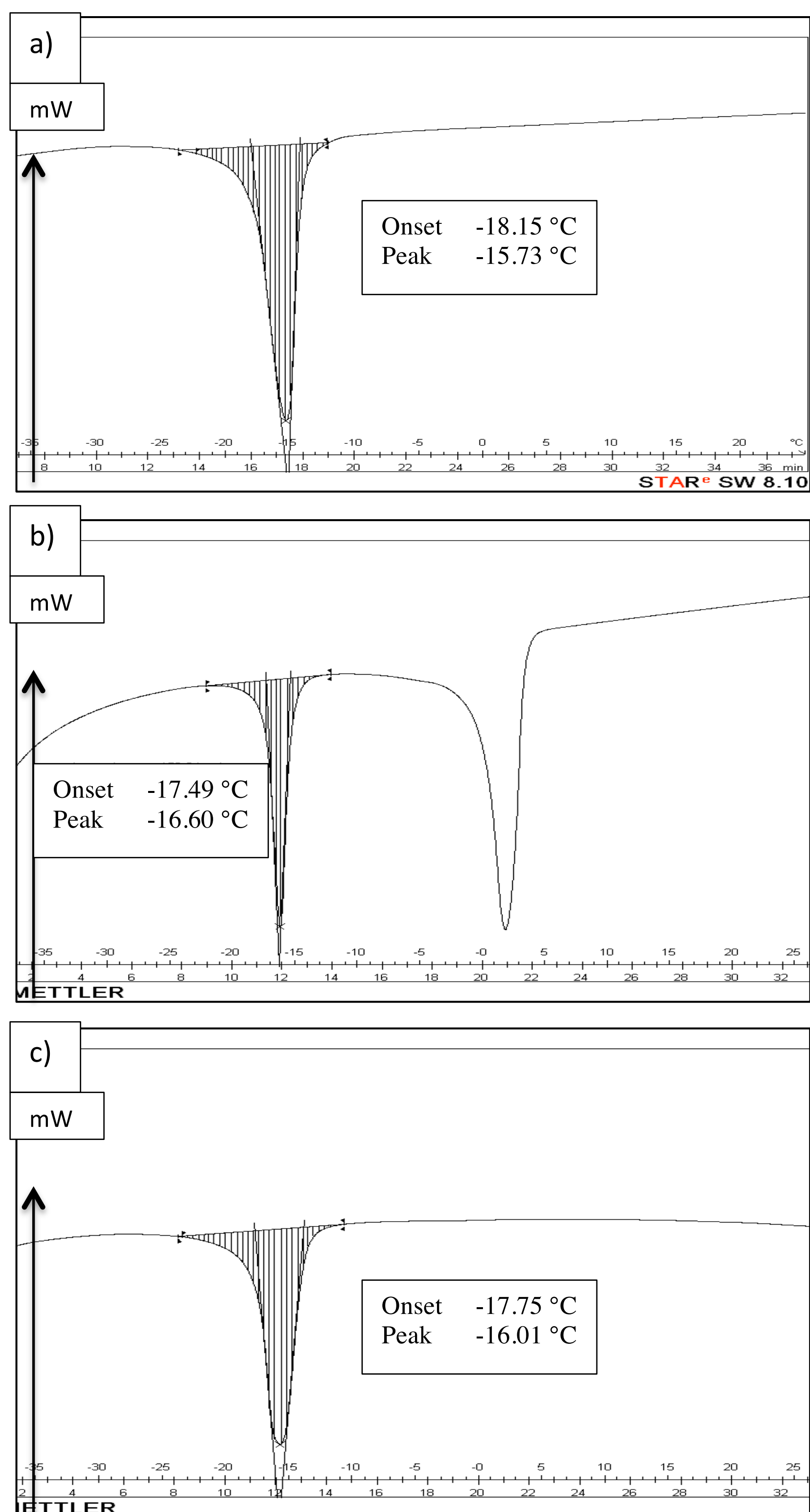

Fig. 6-22. Phase transition characteristic of a) DOPC, b) DOPC with HM-HEC C16 and c) DOPC with HM-HEC C8. The temperature was decreased until it reached -35 °C and increased at a rate of 2 °C/min until 25 °C was reached.

To summarize this section, the phase transition temperatures for all the liposomes were detected by DSC. The intention was to detect the hydrophobic interaction between the HM-HEC and the liposomal membrane, however, using this method gave no significant difference between the T_c of the liposome alone compared to the samples with liposome-polymer complex. This does not necessary mean that a hydrophobic interaction does not take place. A possible explanation could be that the method is not sensitive enough, because the amount of hydrophobic alkyl chain on HM-HEC is very small.

7 Conclusion

In this study the influence of the hydrophobic chain length of HM-HEC on the stability of polymer-coated liposomes has been studied.

The release of carboxyfluorescein at 35 °C from both fluid phase and gel phase liposomes was low. Overall it seemed that the HM-HEC coating protected against release at 35 °C, for both the gel phase and liquid crystalline phase liposomes, however, with no significant difference between the HM-HEC C16 coating and HM-HEC C8 coating. The liquid phase soy PC liposomes had higher release rate than the gel phase DPPC liposomes.

Both the uncoated and the polymer coated soy PC liposomes were size stable during 12 weeks of storage at 4 °C. This suggests that the polymer coating did not influence on the size stability of the soy PC liposomes. The uncoated DPPC liposomes, however, were not stable due to aggregation, but became stable after coating with both the HM-HEC C16 and HM-HEC C8 polymer. The stabilization of the liposomes was concluded to be steric and not electrostatic due to the low zeta potential of the samples. There were no differences between the zeta potential of the HM-HEC C8 and HM-HEC C16 coated liposomes. Slightly lower transmittance values for the HM-HEC C8 coated liposomes than the HM-HEC C16 coated liposomes were found. The pH values were relatively constant and with no great differences between the HM-HEC C8 and HM-HEC C16 coated liposomes. There were no differences between the leakage of the HM-HEC C8 and HM-HEC C16 polymer coated liposomes during storage at 4 °C.

The phase transition temperatures were sucessfully detected in all the liposomes investigated. The DSC studies showed, however, no detectable interaction between the liposomal membrane and the hydrophobically modified polymers.

Although no interactions of HM-HEC alkyl chains with the liposome membrane could be verified by DSC, this study has shown that the liposomes in fact were coated and that the polymer coating stabilized the liposomes. No differences between the stabilization properties of C8 and C16 chain length of the hydrophobic alkyl chain of HM-HEC could be detected.

8 List of references

Allen, T. M., C. Hansen, F. Martin, C. Redemann and A. Yau-Young (1991). "Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo." Biochim Biophys Acta **1066**(1): 29-36.

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

Baudot, A. and V. Odagescu (2004). "Thermal properties of ethylene glycol aqueous solutions." Cryobiology **48**(3): 283-294.

Beheshti, N., H. Bu, K. Zhu, A. L. Kjoniksen, K. D. Knudsen, R. Pamies, J. G. Hernandez Cifre, J. Garcia de la Torre and B. Nystrom (2006). "Characterization of interactions in aqueous solutions of hydroxyethylcellulose and its hydrophobically modified analogue in the presence of a cyclodextrin derivative." J Phys Chem B **110**(13): 6601-6608.

Blume, G., G. Cevc, M. D. J. A. Crommelin, I. A. J. M. Bakker-Woudenberg, C. Kluft and G. Storm (1993). "Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times." Biochimica et Biophysica Acta (BBA) - Biomembranes **1149**(1): 180-184.

Burns, D. B. and A. L. Zydney (2000). "Buffer effects on the zeta potential of ultrafiltration membranes." Journal of Membrane Science **172**(1–2): 39-48.

Clément, F. and A. Johner (2000). "Adsorption of neutral polymers: loops and tails." Comptes Rendus de l'Académie des Sciences - Series IV - Physics **1**(9): 1135-1142.

Clogston, J. D. and A. K. Patri (2011). Characterization of Nanoparticles Intended for Drug Delivery

Cordray, D. R., L. R. Kaplan, P. M. Woyciesjes and T. F. Kozak (1996). "Solid - liquid phase diagram for ethylene glycol + water." Fluid Phase Equilibria **117**(1–2): 146-152.
Filipovic-Grcic, J., N. Skalko-Basnet and I. Jalsenjak (2001). "Mucoadhesive chitosan-coated liposomes: characteristics and stability." J Microencapsul **18**(1): 3-12.

Ford, J. L. and P. Timmins (1989). Pharmaceutical thermal analysis. Ellis Horwood Limited, Chichester.

Freire, E. (1995). Protein Stability and Folding, Humana Press.

Fujishige, S., K. Kubota and I. Ando (1989). "Phase transition of aqueous solutions of poly(N-isopropylacrylamide) and poly(N-isopropylmethacrylamide)." The Journal of Physical Chemistry **93**(8): 3311-3313.

Gabizon, A. A. (1995). "Liposome circulation time and tumor targeting: implications for cancer chemotherapy." Advanced Drug Delivery Reviews **16**(2–3): 285-294.

Grit, M. and D. J. A. Crommelin (1993). "Chemical stability of liposomes: implications for their physical stability." Chemistry and Physics of Lipids **64**(1–3): 3-18.

Grit, M., J. H. Desmidt, A. Struijke and D. J. A. Crommelin (1989). "Hydrolysis of phosphatidylcholine in aqueous liposome dispersions." International Journal of Pharmaceutics **50**(1): 1-6.

Guo, J.-H., G. W. Skinner, W. W. Harcum and P. E. Barnum (1998). "Pharmaceutical applications of naturally occurring water-soluble polymers." Pharmaceutical Science & Technology Today **1**(6): 254-261.

Hayes, M. E., D. C. Drummond, K. Hong, W. W. Zheng, V. A. Khorosheva, J. A. Cohen, C. O. N. th, J. W. Park, J. D. Marks, C. C. Benz and D. B. Kirpotin (2006). "Increased target specificity of anti-HER2 genospheres by modification of surface charge and degree of PEGylation." Mol Pharm **3**(6): 726-736.

Henriksen, I., S. A. Sande, G. Smistad, T. Ågren and J. Karlsen (1995). "In vitro evaluation of drug release kinetics from liposomes by fractional dialysis." International Journal of Pharmaceutics **119**(2): 231-238.

Henriksen, I., G. Smistad and J. Karlsen (1994). "Interactions between liposomes and chitosan." International Journal of Pharmaceutics **101**(3): 227-236.

Henriksen, I., S. R. Våagen, S. A. Sande, G. Smistad and J. Karlsen (1997). "Interactions between liposomes and chitosan II: Effect of selected parameters on aggregation and leakage." International Journal of Pharmaceutics **146**(2): 193-203.

Khutoryanskiy, V. V. (2011). "Advances in mucoadhesion and mucoadhesive polymers." Macromol Biosci **11**(6): 748-764.

Kibria, G., H. Hatakeyama, N. Ohga, K. Hida and H. Harashima (2013). "The effect of liposomal size on the targeted delivery of doxorubicin to Integrin alphavbeta3-expressing tumor endothelial cells." Biomaterials **34**(22): 5617-5627.

Klemetsrud, T., H. Jonassen, M. Hiorth, A.-L. Kjøniksen and G. Smistad (2013). "Studies on pectin-coated liposomes and their interaction with mucin." Colloids and Surfaces B: Biointerfaces **103**(0): 158-165.

Koynova, R. and M. Caffrey (1998). "Phases and phase transitions of the phosphatidylcholines." Biochim Biophys Acta **1376**(1): 91-145.

Kreuter, J. (1994). Colloidal Drug Delivery Systems, Taylor & Francis.

Lashet, M., J. P. Plog, C. Clasen and W.-M. Kulicke (2004). "Examination of the flow behaviour of HEC and hmHEC solutions using structure-property relationships and rheo-optical methods." Colloid and Polymer Science **282**(4): 373-380.

Lian, T. and R. J. Ho (2001). "Trends and developments in liposome drug delivery systems." J Pharm Sci **90**(6): 667-680.

Mady, M. M., M. M. Darwish, S. Khalil and W. M. Khalil (2009). "Biophysical studies on chitosan-coated liposomes." Eur Biophys J **38**(8): 1127-1133.

Meland, H.-G., A. Røv-Johnsen, G. Smistad and M. Hiorth (2014). "Studies on surface coating of phospholipid vesicles with a non-ionic polymer." Colloids and Surfaces B: Biointerfaces **114**(0): 45-52.

Needham, D. and E. Evans (1988). "Structure and mechanical properties of giant lipid (DMPC) vesicle bilayers from 20.degree.C below to 10.degree.C above the liquid crystal-crystalline phase transition at 24.degree.C." Biochemistry **27**(21): 8261-8269.

New, R. R. C. (1990). Liposomes: a practical approach. Oxford, IRL Press.

Nguyen, S., S. J. Alund, M. Hiorth, A.-L. Kjøniksen and G. Smistad (2011). "Studies on pectin coating of liposomes for drug delivery." Colloids and Surfaces B: Biointerfaces **88**(2): 664-673.

Nguyen, S., M. Hiorth, M. Rykke and G. Smistad (2013). "Polymer coated liposomes for dental drug delivery – Interactions with parotid saliva and dental enamel." European Journal of Pharmaceutical Sciences **50**(1): 78-85.

Nidhin, M., R. Indumathy, K. J. Sreeram and U. N. Balachandran (2008). "Synthesis of iron oxide nanoparticles of narrow size distribution on polysaccharide templates." Bulletin of Material Science **31**(1): 93-96.

Nolan, C. M., L. T. Gelbaum and L. A. Lyon (2006). "1H NMR investigation of thermally triggered insulin release from poly(N-isopropylacrylamide) microgels." Biomacromolecules **7**(10): 2918-2922.

Parveen, S. and S. K. Sahoo (2011). "Long circulating chitosan/PEG blended PLGA nanoparticle for tumor drug delivery." Eur J Pharmacol **670**(2-3): 372-383.

Pereira-Lachataignerais, J., R. Pons, P. Panizza, L. Courbin, J. Rouch and O. López (2006). "Study and formation of vesicle systems with low polydispersity index by ultrasound method." Chemistry and Physics of Lipids **140**(1–2): 88-97.

Polozova, A. and F. M. Winnik (1997). "Mechanism of the interaction of hydrophobically-modified poly-(N-isopropylacrylamides) with liposomes." Biochimica et Biophysica Acta (BBA) - Biomembranes **1326**(2): 213-224.

Salamat-Miller, N., M. Chittchang and T. P. Johnston (2005). "The use of mucoadhesive polymers in buccal drug delivery." Advanced Drug Delivery Reviews **57**(11): 1666-1691.

Smistad, G., S. Bøyum, S. J. Alund, A. B. C. Samuelsen and M. Hiorth (2012). "The potential of pectin as a stabilizer for liposomal drug delivery systems." Carbohydrate Polymers **90**(3): 1337-1344.

Sund-Levander, M., C. Forsberg and L. K. Wahren (2002). "Normal oral, rectal, tympanic and axillary body temperature in adult men and women: a systematic literature review." Scand J Caring Sci **16**(2): 122-128.

Takeuchi, H., H. Yamamoto, T. Toyoda, H. Toyobuku, T. Hino and Y. Kawashima (1998). "Physical stability of size controlled small unilameller liposomes coated with a modified polyvinyl alcohol." International Journal of Pharmaceutics **164**(1–2): 103-111.

Taylor, K. M. G. and R. M. Morris (1995). "Thermal analysis of phase transition behaviour in liposomes." Thermochimica Acta **248**(0): 289-301.

Torchilin, V. P., M. I. Shtilman, V. S. Trubetskoy, K. Whiteman and A. M. Milstein (1994). "Amphiphilic vinyl polymers effectively prolong liposome circulation time in vivo." Biochimica et Biophysica Acta (BBA) - Biomembranes **1195**(1): 181-184.

Ulrich, A. S., M. Sami and A. Watts (1994). "Hydration of DOPC bilayers by differential scanning calorimetry." Biochim Biophys Acta **1191**(1): 225-230.

Verma, D. D., S. Verma, G. Blume and A. Fahr (2003). "Particle size of liposomes influences dermal delivery of substances into skin." International Journal of Pharmaceutics **258**(1–2): 141-151.

Veronese, F. M. and G. Pasut (2005). "PEGylation, successful approach to drug delivery." Drug Discov Today **10**(21): 1451-1458.

Volke, F., S. Eisenblätter, J. Galle and G. Klose (1994). "Dynamic properties of water at phosphatidylcholine lipid-bilayer surfaces as seen by deuterium and pulsed field gradient proton NMR." Chemistry and Physics of Lipids **70**(2): 121-131.

Wong, M. and T. E. Thompson (1982). "Aggregation of dipalmitoylphosphatidylcholine vesicles." Biochemistry **21**(17): 4133-4139.

Yang, Y. and J. Li (2013). "Lipid, protein and poly(NIPAM) coated mesoporous silica nanoparticles for biomedical applications." Adv Colloid Interface Sci.

Zhou, W., W. Liu, L. Zou, W. Liu, C. Liu, R. Liang and J. Chen (2014). "Storage stability and skin permeation of vitamin C liposomes improved by pectin coating." Colloids Surf B Biointerfaces **117**: 330-337.

Zimmerman, R. J., H. Chao, G. D. Fullerton and I. L. Cameron (1993). "Solute/solvent interaction corrections account for non-ideal freezing point depression." Journal of Biochemical and Biophysical Methods **26**(1): 61-70.

Zuidam, N. J. and D. J. A. Crommelin (1995). "Chemical hydrolysis of phospholipids " Journal of Pharmaceutical Sciences **84**(9): 1113-1119.