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PHASE TRANSITION OF PH SENSITIVE LIPOSOMES

Master´s Programme in Chemical, Biochemical and Materials Engineering
Major in Chemistry

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Abstract of master's thesis

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Abstract

Normal tissues and blood have neutral pH whereas tumors and infected areas exhibit an acidic environment. This enables the use of drug delivery systems, for which acidification triggers the drug release. Liposomes are spherical bilayer structures that can entrap hydrophilic drug molecules. The encapsulated drug can be released e.g. by light activation, heating or upon acidification. The advantages of liposomes include low toxicity and biocompatibility.

pH sensitive liposomes are composed of lipids that can undergo phase transition from a bilayer to an inverted hexagonal phase. A protonatable amphiphile, such as cholesterol hemisuccinate (CHEMS), stabilizes the liposome at neutral pH. In an acidic environment CHEMS becomes protonated, which decreases the negative charge of the membrane and leads to liposomal destabilization. As a result, the lipids adopt an inverted hexagonal phase and the encapsulated drug is released. However, the challenge is to develop liposomes that remain stable under physiological conditions but undergo rapid destabilization in an acidic environment.

In this thesis the phase transition of pH sensitive liposomes was studied with the fluorescence probe Laurdan. pH sensitive 1,3-diolein-CHEMS liposomes were formulated and analyzed with fluorescence spectroscopy and dynamic light scattering. Fluorescence probe Laurdan that is sensitive to the polarity of the membrane, was used to detect the phase transition. pHrodo red was utilized as an additional probe to provide information about the permeability of the membrane.

The results indicated that pH sensitive liposomes became unstable below pH 6.5. Particle size increased and Laurdan emission intensity collapsed when the liposomes were prepared at low pH. Also the shape of Laurdan emission peaks changed. However, the changes were not as drastic when liposomes were prepared under neutral conditions and pH of the external solution was adjusted to lower values. pHrodo red indicated that pH sensitive formulation was more permeable towards H^+ ions than the reference liposomes.

It is proposed that aggregation of the liposomes occurs at low pH. The membrane becomes more permeable, which enables drug leakage from the liposome. In addition, the phase behavior changes: the possible changes include phase separation and a phase transition from a bilayer to an inverted hexagonal phase.

Keywords pH sensitive liposomes, phase transition, CHEMS, 1,3-diolein, Laurdan, pHrodo red

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Tiivistelmä

Normaalien kudosten ja veren pH on noin 7.4, kun taas kasvaimissa ja tulehtuneissa kudoksissa on hapan pH. Tästä johtuen esimerkiksi syövän hoidossa voidaan käyttää lääkkeenkuljettajia, jotka vapauttavat lääkeaineen kontrolloidusti happamassa pH:ssa. Liposomit ovat pallomaisia, kaksikerroksisesta lipidifilmistä koostuvia rakenteita. Niiden sisään voidaan kapseloida hydrofiilisiä lääkeaineita. Lääkeaine voidaan vapauttaa liposomista säädellysti esimerkiksi valon, lämmön tai pH muutoksen avulla. Liposomit ovat kudosten kanssa yhteensopivia ja myrkyttömiä.

pH herkät liposomit sisältävät lipidejä, jotka pystyvät muodostamaan käänteisen heksagonaalisen faasin. Protonoitu amfifiili, kuten kolesteroli-hemisukkinaatti (CHEMS), stabiloi liposomirakennetta neutraalissa pH:ssa. Neutraalissa pH:ssa lipidit järjestäytyvät kaksikerrosrakenteeksi. Happamassa ympäristössä CHEMS protonoituu, mikä vähentää lipidikalvon negatiivista varausta ja johtaa liposomien destabiloitumiseen. Samalla lipidit järjestäytyvät käänteiseen heksagonaaliseen faasiin ja myös lääkeaine vapautuu. Haasteena on kuitenkin kehittää liposomeja, jotka pysyvät stabiileina fysiologisissa olosuhteissa ja destabiloituvat happamassa pH:ssa.

Tässä työssä tutkittiin pH herkkien liposomien faasimuutosta fluoresenssimerkkiaine Laurdanin avulla. Formuloitiin pH herkkiä 1,3-dioleiinia ja kolesteroli-hemisukkinaattia sisältäviä liposomeja. Liposomeja tutkittiin fluoresenssispektroskopialla ja dynaamisella valonsironnalla. Laurdan havaitsee lipidikaksoiskerroksen polaarisuuden muutoksia, mistä johtuen sillä voidaan tutkia lipidikalvon faasimuutosta. Toisena fluoresenssimerkkiaineena käytettiin pHrodo punaista, jonka avulla voidaan mitata solunsisäinen pH ja tutkia, kuinka hyvin protonit läpäisevät lipidikalvon.

Havaittiin, että pH herkät liposomit destabiloituvat, kun pH on alle 6.5. Partikkelikoko kasvoi ja Laurdanin fluoresenssiemissiointensiteetti romahti, kun liposomit valmistettiin happamassa pH:ssa. Myös emissiopiikkien muoto muuttui. Muutokset eivät kuitenkaan olleet yhtä huomattavia, kun liposomit valmistettiin neutraalissa pH:ssa ja ulkoliuoksen pH säädettiin happamaksi. pHrodo osoitti, että pH herkkien liposomien lipidikalvo oli referenssiliposomeja läpäisevämpi H^+ ioneille.

Matalassa pH:ssa liposomit aggregoituvat ja membraanin läpäisevyys lisääntyy. Myös faasi muuttuu: mahdollisesti faasit erottuvat tai 1,3-dioleiinin faasi muuttuu kaksikerrosrakenteesta käänteiseen heksagonaaliseen faasiin.

Avainsanat pH herkät liposomit, faasimuutos, CHEMS, 1,3-dioleiini, Laurdan, pHrodo

Preface

This Master's Thesis was conducted in Physical Chemistry and Electrochemistry research group. I would like thank prof. Lasse Murtomäki for the support and opportunity to continue with the liposome research after my summer work in the physical chemistry lab. I wish to thank also my advisor Lauri Viitala for guiding me in the beginning of the master's thesis project and Maria Misukka for her excellent laboratory work. I would like to thank also prof. Jan Deska for synthesizing 1,3-diolein and prof. Mady Elbahri for letting to use the fluorescence spectrophotometer.

Last but not the least, I would like to thank my family members and friends for supporting me during my studies.

Contents

Abbreviations

BAM = Brewster angle microscopy

CHEMS = cholesteryl hemisuccinate

DAG = diacylglycerol

DiPoPc = dipalmitoleoylphosphatidylcholine

DiPoPE = dipalmitoleoylphosphatidylethanolamine

DLS = dynamic light scattering

DOPE = dioleoylphosphatidylethanolamine

DPPC = 1,2-dipalmitoyl-sn-glycero-3-phosphocholine

DSPC = 1,2-distearoyl-sn-glycero-3-phosphocholine

DSPE-PEG = 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(poly-ethyleneglycol)-2000]

EPC = egg yolk phosphatidylcholine

GP = generalised polarization

HEPES = 4-(2-hydrohyethyl)-1-piperazineethanesulfonic acid

L_α = liquid-crystalline lamellar phase

$L_{\beta'}$ = gel lamellar phase

Lyso PC = Lysophosphatidylcholine

PC = phosphatidylcholine

PE = phosphatidylethanolamine

PEG = polyethylene glycol

PSL = pH sensitive liposome

TEM = transmission electron microscopy

TRIS= Tris(hydroxymethyl)aminomethane

1 Introduction

Targeted drug delivery systems enable improved pharmacokinetics and biodistribution of the loaded drugs. Controlled release is needed for example in cancer treatment because conventional chemotherapy causes severe side effects. [1] Nanocarriers, such as liposomes accumulate in tumors after systemic administration [2]. Recently, liposomal drug delivery systems have been developed to achieve tumor specific targeting of the drug [3] [4].

Liposomes are spherical bilayer structures. Hydrophilic compounds can be loaded inside the liposomes and a phospholipid bilayer can trap hydrophobic molecules. Liposomes are biocompatible and biodegradable and therefore they do not cause allergic or toxic reactions. [5] Yet, long circulation times and stability in biological fluids are needed. Lipids with polyethylene glycol (PEG) enable reduced drug leakage from the liposomes. [6]

Drug release from the liposomes can be triggered by external or internal stimuli [7]. Light activation, local hyperthermia and ultrasound are examples of external stimuli. When combined with biosensors it is possible to obtain systems that release the drug upon physiological demand. Internal stimuli include pH and concentration of the substances. [8] [9] [10] Pathological tissues, such as tumors and inflamed areas have lower pH than healthy tissues [11]. pH sensitive liposomes destabilize in acidic environments, and therefore they can release drug content in tumors [12].

The target of this master's thesis was to study phase transition of pH sensitive liposomes. pH sensitive liposomes containing 1,3-diolein and cholesteryl hemisuccinate (CHEMS) were prepared and characterized by dynamic light scattering (DLS) and fluorescence spectroscopy. Previously, light activated liposomes were studied [13]. The hypothesis was that in acidic environments liposomes would become unstable and start to release their content, such as calcein. A change in pH would also induce phase transition, which promotes the drug release [14].

2 pH sensitive liposomes for controlled drug release

pH of blood and normal tissues is ca. 7.4 [3]. However, tumors exhibit acidic pH: due to fast cell growth, there is not enough oxygen and nutrients. Tumor cells respond by changing their metabolism into anaerobic direction and the resulting lactic acid makes the tumor tissue acidic. [15] Modification of the liposomal composition enables drug release, which is triggered by environmental conditions. pH sensitive liposomes destabilize in acidic environments and therefore they can release their drug content in tumors. [12] Moreover, inflamed or infected areas and acidic compartments of the cell are potential target sites for pH sensitive liposomes [11].

Endosomes and lysosomes are acidic parts of the cell with pH 5-6 in endosomes and pH 5 in lysosomes [16]. On the other hand, lysosomes pose a risk for drug degradation and therefore liposomal content should be released in endosomes [17] [18]. Drug release can be triggered by fusion between the liposomal and endosomal membranes. As a result aqueous content leaks into the cytoplasm. [18]

Important properties of pH sensitive liposomes include fusogenic ability, cellular internalization and stability in plasma and other biological fluids [12]. They can be divided in four different categories as follows [19]:

1. Polymorphic lipids such as phosphatidylethanolamines (PE) combined with acidic amphiphiles. The acidic amphiphiles stabilize the liposomes at neutral pH.
2. Liposomes with special chemical bonds, which can undergo acid-induced hydrolysis. This drives to membrane destabilization.
3. Liposomes for which membrane destabilization is induced by pH sensitive peptides.
4. Liposomes with pH titratable polymers.

Class 1 is the most studied. It includes widely used liposomes prepared from dioleoylphosphatidylethanolamine (DOPE) and amphiphiles such as CHEMS or oleic acid. [19] In an acidic environment, protonation of the amphiphile leads to the loss of stabilizing negative net charge and a phase transition from lamellar to inverted hexagonal takes place. [19] [20] [21] However, the disadvantage is that the

liposomes may be eliminated from the circulation since the negatively charged groups, such as carboxylates, can interact with plasma proteins [1].

In addition to PE, there are also other polymorphic lipids, which can be utilized in pH sensitive liposomes. These include diacylglycerols (DAGs) [19]. For example, 1,3-diolein and CHEMS are used in anionic liposomes [14] [17]. These are discussed further in the chapters 6.1 and 6.2.

pH sensitive liposomes enable effective intracellular drug delivery. Still, their challenges include short circulation times because of recognition and sequestration by the reticuloendothelial system's (RES) phagocytes. [12] One option to achieve longer blood circulation times is to use polyethylene glycol (PEG) or other hydrophilic polymers as steric stabilizers. Polymer chains of PEG form a repulsive barrier around the liposomes, leading to the decreased interaction with blood components. [4] [22] According to Hong *et al.* [22] DSPE-PEG improves stability of pH responsive liposomes in serum. On the other hand, PEG can reduce their pH sensitivity. Hong *et al*. observed that an addition of DSPE-PEG to pH sensitive liposomes caused a decrease in maximum leakage percentage. Their response also shifted to lower pH. DSPE-PEG reduces the pK_a of the pH sensitive moieties, which leads to the poorer pH sensitivity. [22] An option to overcome the problem is to develop a PEG-cleavable, pH sensitive liposome system. Kanamala *et al.* [4] developed liposomes, where PEG moieties were detached from CHEMS in an acidic environment, generating naked DOPE/CHEMS liposomes.

3 Membrane processes connected to liposome destabilization

Knowledge of the lipid geometry helps to predict which lipid structures are formed in given conditions [23]. Therefore, lipid geometry is covered before the general mechanisms of phase transitions and lipid membrane fusion are considered. The focus is on molecular level phenomena. This chapter provides background information for chapters, where the possible drug release mechanisms from pH sensitive liposomes are discussed in more detail.

3.1 About lipid geometry

Lipids that have a large headgroup and a relatively small hydrocarbon chain, adopt a cone-shaped geometry. They self-assemble into micelle structures. However, lipids with an equal sized headgroup and hydrocarbon moiety have cylindrical geometry and self-assemble into bilayers. Lipids that have small headgroup exhibit inverted phases including an inverted hexagonal phase and an inverted cubic phase. It is said that the lipids with cone-shaped geometry have a positive spontaneous curvature, whereas the lipids forming inverted phases have a negative spontaneous curvature. [23] A high negative, spontaneous curvature leads to an increased repulsion between hydrocarbon chains. This again increases pressure in the lipid chains. [24] Different lipid geometries and the predicted structures are presented in the Figure 1.
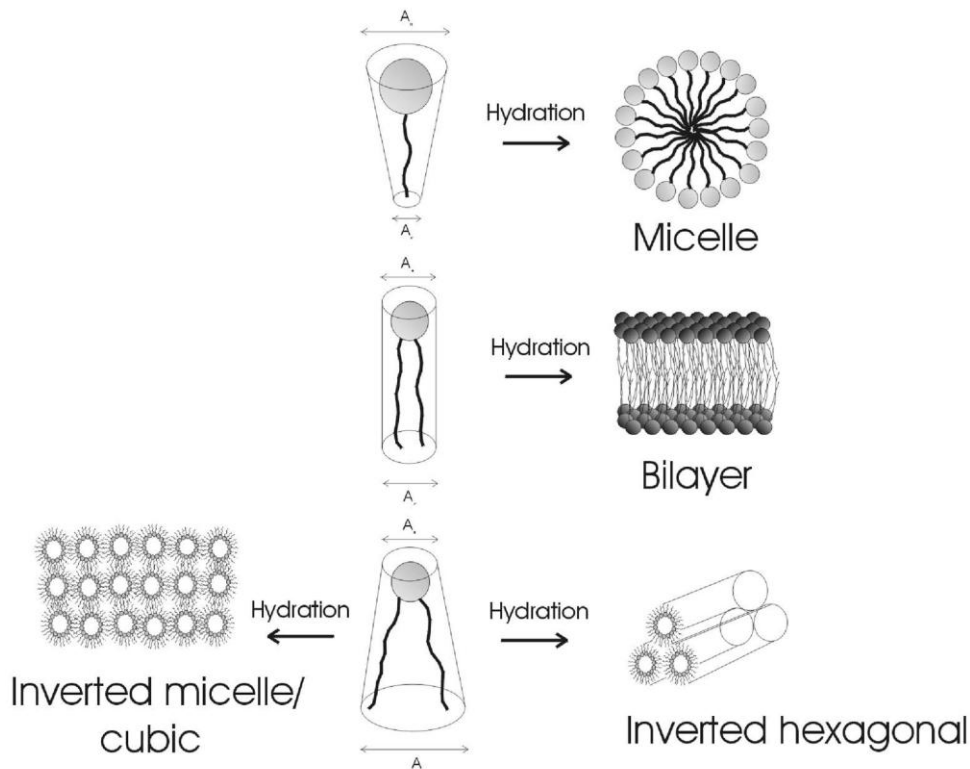
Figure 1. The effect of molecular geometry on the lipid polymorphism [23].

3.2 Phase transition

Understanding the dynamics of the lipids forms a basis for their applications in pharmaceutical and food industries [25]. The knowledge of a phospholipid main phase transition can be utilized, e.g. in drug delivery systems. In the previous study of light triggered drug delivery, drug release occurred upon the phospholipid main phase transition. Photothermal agents such as gold nanorods or Indocyanide Green molecules turned light into heat. Local heating softened the liposomal bilayer and drug release followed the phase transition. [13] [26]

Lipid bilayers form different phases that depend on the hydration level and temperature. Solid gel, fluid liquid crystalline and ripple phases are examples of the possible phases. The main transition temperature, T_m, is a temperature where the phase transition from a gel to a fluid phase occurs. Below T_m, the lipid bilayer exists in a more rigid gel phase. However, above T_m the fatty acid tails become more mobile, and the bilayer adopts a liquid crystalline phase. [25]

Apart from the well known gel to liquid crystalline phase transition, also different phase behavior is observed among the lipids. On the scope of pH sensitive liposomes, lamellar to non-lamellar phase transition is particularly interesting. During the phase transition, transformation from a bilayer to an inverted hexagonal phase takes place. Lamellar to non-lamellar phase transition can be induced, e.g. by acidic pH or by heating. [27] [21] According to Langner & Hui, [28] changes in the lipid packing caused by elevated stress within the matrix are the driving forces towards the formation of an inverted hexagonal phase. At the onset of the phase transition, bilayer properties alter [25]. These include defect formation and increased permeability towards ions [28]. Kamo *et al.* [29] studied structural changes in the molecular level even further. Before lamellar to non-lamellar phase transition, elastic deformation and hydrophobic hydration at the membrane surface occur. Elastic deformation means increased lateral pressure at the acyl chain region and it originates from forcing non-lamellar forming lipids to form planar structures. The phase transition causes a decrease in the lateral pressure.

When considering membrane fusion one interesting, but less known phase is a so called cochleate phase. Cochleates are elongated, cylindrical lipid structures. They are formed when Ca^{2+} ions rupture lipid vesicles. According to Ramani & Balasuriam [30] addition of Ca^{2+} ions into the lipid vesicle mixture resulted in the phase transition from a liquid crystalline to a rigid and dehydrated (RD) cochleate phase. They proposed that Ca^{2+} ions ruptured the lipid vesicles, which lead to the formation of flat bilayer disks. Eventually, fusion of the disks resulted in the cochleate formation.

Although cochleate formation is usually induced by Ca^{2+} ions [30] [31], similar precipitation could take place also in acidic environments. Syed *et al.* [32] prepared cochleates in acidic conditions, without multivalent cations. It is proposed that low pH stabilizes cochleate structures [33]. According to Binder [34] *et al.*, phase separation between hexagonal and cochleate phases is connected to membrane fusion.

3.3 Fusion

Membrane fusion is a part of several cellular processes, such as secretion and endocytosis. In order to a fusion to occur, there must be a close approach of the two

fusing membranes. Also, bilayers must destabilize at the fusion point. [35] Next, the fusion mechanism is considered in more detail.

Van der Waals attraction tends to link up the lipid membranes. Thus, van der Waals forces are behind membrane approach and lipid vesicle fusion. Normally a strong repulsive barrier that is composed of steric, electrostatic and hydration forces, prevents close contact between the vesicles. For example, charge loss lowers the repulsion and promotes aggregation. Similarly, high polarity and increased surface charge density prevent aggregation. [36] According to Cerv & Richardsen [36], formation of a new phase is not a prerequisite for membrane fusion, but the defects are. Accumulation of defects in the contact areas of vesicles promotes fusion. However, they consider that formation of a non-lamellar phase includes always fusion. [36]

According to Bhagat and Sofou [37] fusion is initiated by vesicle contact. After that, the interbilayer distances decrease, which finally leads to the exchange or mixing of the lipids. A mechanism of the fusion is presented in Figure 2. First, aggregation takes place. At the membrane contact areas inverted phases can appear in the form of small micelles or elongated contact structures. [36]
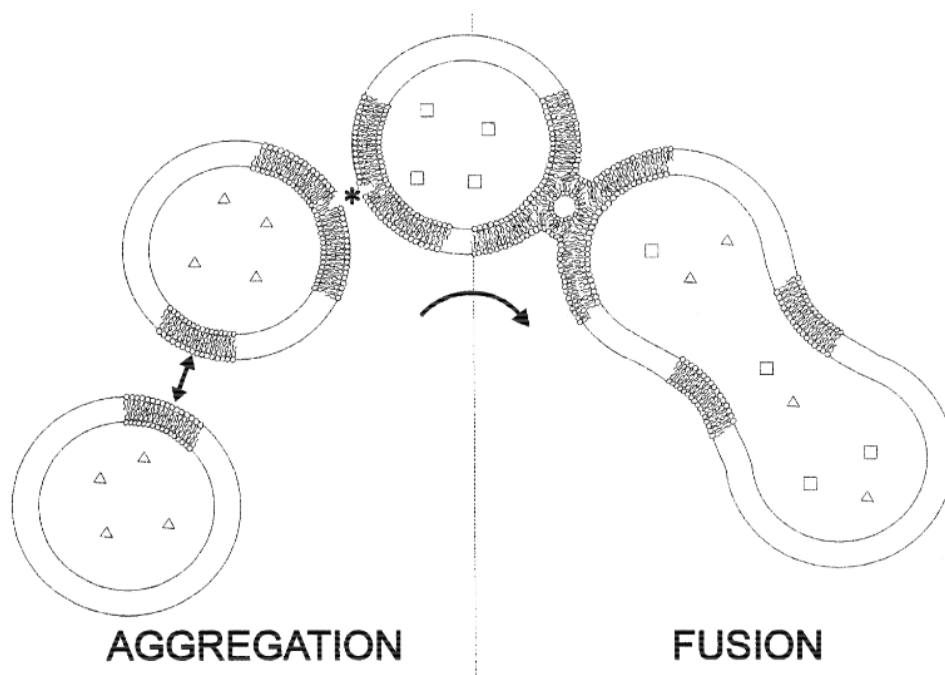

Figure 2. Mechanism of fusion (modified from Cerv & Richardson [36]).

Phosphatidylcholines and charged lipids at neutral pH act as fusion suppressing molecules. Phosphatidylethanolamines and diacylglycerols, on the other hand, are prone to fuse. [36]

To sum it up, the factors facilitating membrane fusion include formation of defects or non-bilayer structures, increased bilayer permeability, change in vesicle size or morphology and a close bilayer approach. [36]

4 Building blocks of liposomes

Next, components of the pH sensitive liposomes and the reference liposomes are discussed in more detail.

4.2 CHEMS

Cholesteryl hemisuccinate (CHEMS) (Figure 3) is a protonatable amphiphile. It is composed of succinic acid, which is esterified to the β-hydroxyl group of cholesterol. Thus, it is an acidic cholesterol ester. [38] [39]
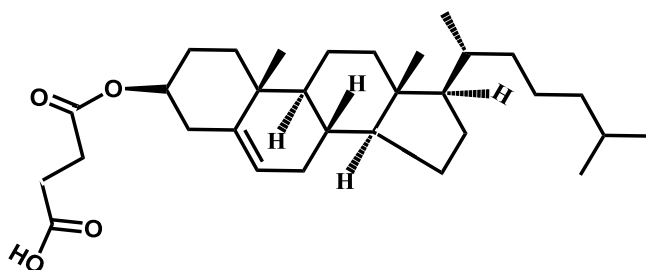
Figure 3. Structure of CHEMS.

According to Massey [40], the carboxyl group in CHEMS resides on the water interface of the lipid. Therefore, the carboxyl group increases surface charge but has little effect on the interfacial hydration behavior. CHEMS is widely used in pH sensitive liposomes containing DOPE that exhibits polymorphic phase behavior [38]. In CHEMS/DOPE systems, protonation state of CHEMS determines the phase [39]. In physiological conditions, CHEMS is negatively charged and therefore stabilizes DOPE that assumes a lamellar phase at neutral pH. Yet, in an acidic environment the stabilizing effect of CHEMS decreases and DOPE adopts an inverted hexagonal phase, promoting fusion. [20] [38] [41]. According to Hafez & Cullis [39] pK_a of CHEMS is 5.8.

Stabilization of the lamellar phase can be due to the repulsion between CHEMS's carboxylate group and the phosphate group of DOPE [38] [41]. However, according to Klasczyk *et al.* [42], membrane-bound ions have a major role in lamellar phase stabilization. They observed that bilayers containing CHEMS underwent fusion even

at neutral or alkaline conditions when ion exchange materials immobilized the counter ions.

Klasczyk *et al.* [42] also observed in their molecular dynamics simulations that counter ions, such as Na^+ or K^+, adsorb at the deprotonated carboxyl group of CHEMS and the bound ions became part of the head group volume. The increased head group size can hinder fusion and stabilize lamellar phase. When CHEMS is deprotonated, the carboxyl group binds two K^+ or Na^+ ions. [42]

4.3 1,3-diolein

1,3-diolein is a hydrophobic lipid which has a high negative curvature (Figure 4). It forms non-lamellar phases, such as an inverted hexagonal phase. [43] [29] The polar part of diolein is composed of a hydroxyl group. Therefore, it occupies only a small hydrodynamic volume. 1,3-diolein can form hydrogen bonds with adjacent diolein molecules or oxygen in a phospholipid, further reducing hydrodynamic volume. Hence, diolein promotes phase transition from a bilayer to an inverted hexagonal phase. [14] As stated above, lipids with small headgroups adopt inverted phases [23].
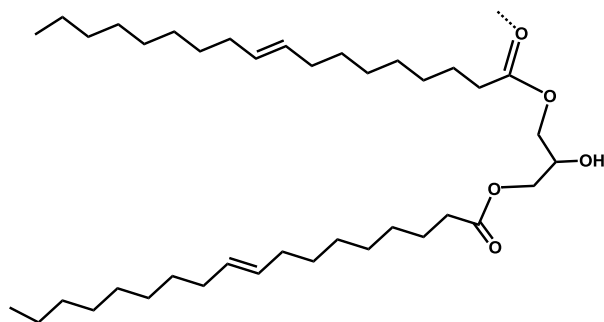

Figure 4. 1,3-diolein.

1,3-diolein belongs to the group of diacylglycerols that modify physicochemical properties of biological membranes [29]. 1,3-diolein has potential in pH sensitive liposomes because it can be combined with CHEMS to form a pH sensitive moiety in the liposomes [17] [14]. Its ability to promote membrane fusion is linked to the formation of inverted an hexagonal phase. Advantages of 1,3-diolein include an ability to remain intact in serum, while the most well-known fusogenic lipid component, DOPE, becomes inactive in the presence of serum. Serum proteins

alter bilayer composition of DOPE, which results in the loss of fusogenic properties. [14]

Kamo et al. [43] observed that increasing the fraction of diolein in egg yolk phosphatidylcholine (EPC)/diolein systems leads to a higher aggregation rate and increased size of liposomes, which can be explained by increased hydrophobicity of the lipid dispersions. A higher diolein fraction also promoted the formation of non-lamellar phases. Also, the diolein fraction determined the type of a non-lamellar liquid crystalline phase. EPC/diolein molar ratio of 8:2 resulted in a bicontinuous cubic phase, whereas higher diolein fractions lead to the formation of an inverted hexagonal phase (H_{II}). [43]

In further studies, Kamo et al. [29] investigated the effect of diolein on the membrane lateral pressure in EPC/diolein systems. During lamellar to the cubic phase transition, only the headgroup area exhibited transition to the cubic phase, but there were no changes in acyl chain packing. In the cubic phase, diolein increased headgroup exposure to water. The resulting hydrophobic hydration promoted transition to the inverted hexagonal phase. During the cubic to hexagonal phase transition, acyl chain packing loosened and headgroup packing increased further. [29]

1,3-diolein and CHEMS were chosen as pH sensitive moieties. Molar ratio of 6:4 (diolein/CHEMS) was considered to be the most suitable for pH sensitive liposomes, as suggested by Guo et al. [14] and Gosselin et al. [44] Hence, the same formulation was used also in this work, assuming that such a high diolein fraction would also promote the formation of an inverted hexagonal phase [29].

4.4 Phospholipids

Phospholipids are amphiphiles that are composed of glycerol-based lipids. They have two hydrocarbon chains and a hydrophilic phosphate head group [7] [25]. Phospholipids form the matrix of the natural membranes.

At body temperature, phospholipids exist in a lamellar gel phase (L_{α}) or in a lamellar liquid crystalline phase (L_{β}). The phase depends on the acyl chain length and type of

the polar residues. Natural membranes are composed of various types of lipids. [45] The behavior of the lipid matrix can be understood by studying physical and chemical properties of artificial, self-assembled lipid entities, such as liposomes [46] [47]. Artificial systems enable the control of lipid composition and environmental factors, including temperature and pH [47]. Lipid-lipid interactions can be understood by studying phase transition. [47]

Parasassi *et al.* [48] observed that pH in the range of 4-10 did not have an effect on the Laurdan GP (eq.1) of phospholipid vesicles. In the later studies of pH sensitive liposomes, liposomes made of phosphatidylcholines (class of phospholipids) were used as a reference [5]. Consequently, DPPC liposomes were chosen as a reference system for this thesis. DPPC (Figure 5) (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) belongs to the group of phosphatidylcholines (PC). Unlike 1,3-diolein or phosphatidylethanolamines, phosphatidylcholines are bilayer forming lipids [23].
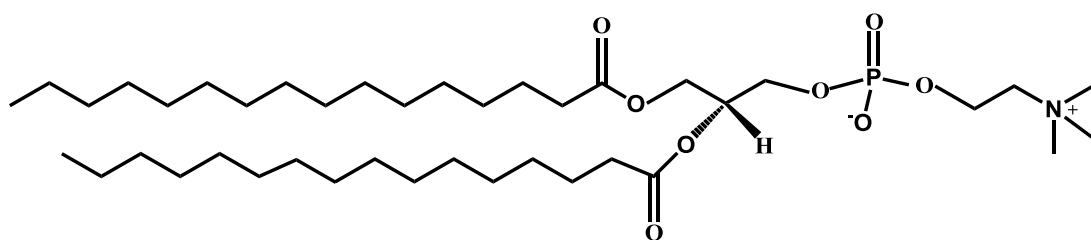
Figure 5. DPPC

5 Fluorescence probes to study lipid structures

Next, the fluorescence probes used in this work are presented. They include Laurdan that provides information about the possible phase transition, and pHrodo that can offer an insight into charge transfer across the liposomal membrane. The principles of fluorescence are covered in the chapter 7.3.

5.1 Laurdan

Laurdan (6-dodecanoyl-2-(dimethylamino)naphthalene) (Figure 7) is a fluorescence probe that is widely used to investigate phospholipid bilayers and other biological membranes. Laurdan is sensitive to polarity of the membrane and can therefore detect a phase transition in the lipid bilayer. [13] [45] [49] The mechanism can be explained by dipolar relaxation as follows.
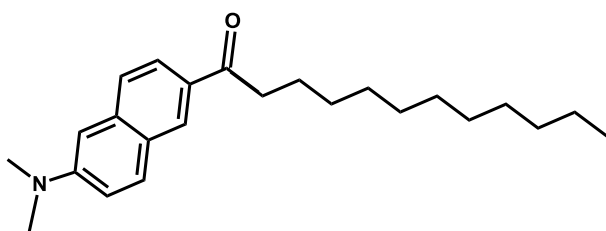

Figure 7. Fluorescence probe Laurdan [50].

The fluorescent naphthalene moiety of Laurdan has a dipole moment that increases during excitation and can result in solvent molecule reorientation. Solvent reorientation requires energy, which can be observed as a shift in the emission spectrum. The phase of the phospholipid bilayer determines the wavelength of the emission maximum: a gel phase has an emission maximum at 440 nm and a liquid-crystalline phase at 490 nm. The phase transition can thus be detected by a shift in the emission spectrum. In phospholipid bilayers, dipolar relaxation is due to the water molecules present at the glycerol backbone, where also the fluorescent moiety of Laurdan resides. [50] Figure 8 shows how Laurdan is located in the lipid bilayer. Hydrophobic interactions between the alkyl chains and Laurdan's lauric acid tail anchor the fluorescence molecule into the hydrophobic core. [51]
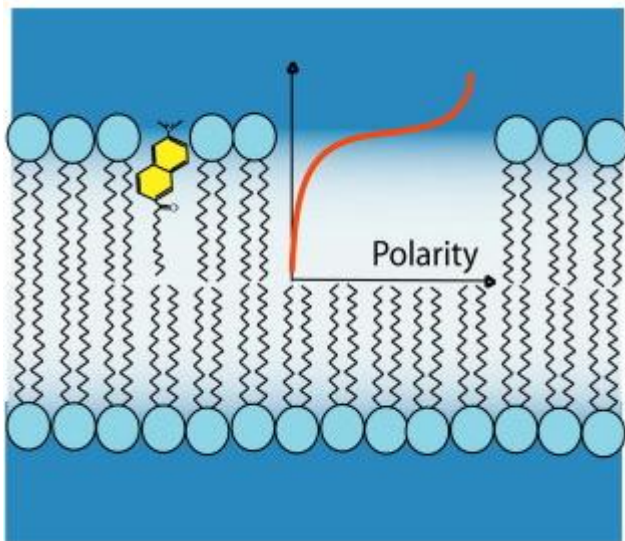
Figure 8. Laurdan anchored in the lipid bilayer. Modified from Zhang *et al.* [51].

The shift in the emission can be explained by an increased water concentration and improved mobility of the lipids [50]. When water molecules penetrate the loosely packed membrane, Laurdan emission spectrum shifts ca. 50 nm compared to the ordered membrane [52].

Generalized polarization (GP) can be used to quantify intensity data that is gathered at different emission and excitation wavelengths [45]. GP is determined as follows:

$$G = \frac{(I_{440} - I_{490})}{(I_{440} + I_{490})} \quad (1)$$

where I_{440} corresponds to Laurdan emission in a gel phase and I_{490} in a liquid crystalline phase. GP values are limited in the range between −1 and 1. [45] High GP values indicate low water penetration and high order of the lipid membrane [52]. This has been proved in several studies. In the study by Viitala *et al.* [13] the phase transition of the liposomes was monitored with Laurdan. Heating liposomes to ca. 41-43 °C resulted in a drop of the emission intensity at 440 nm and an increase at 490 nm (Figure 9). Also, Laurdan GP decreased from ca. 0.8 to −0.2, proving that the phase transition had taken place.
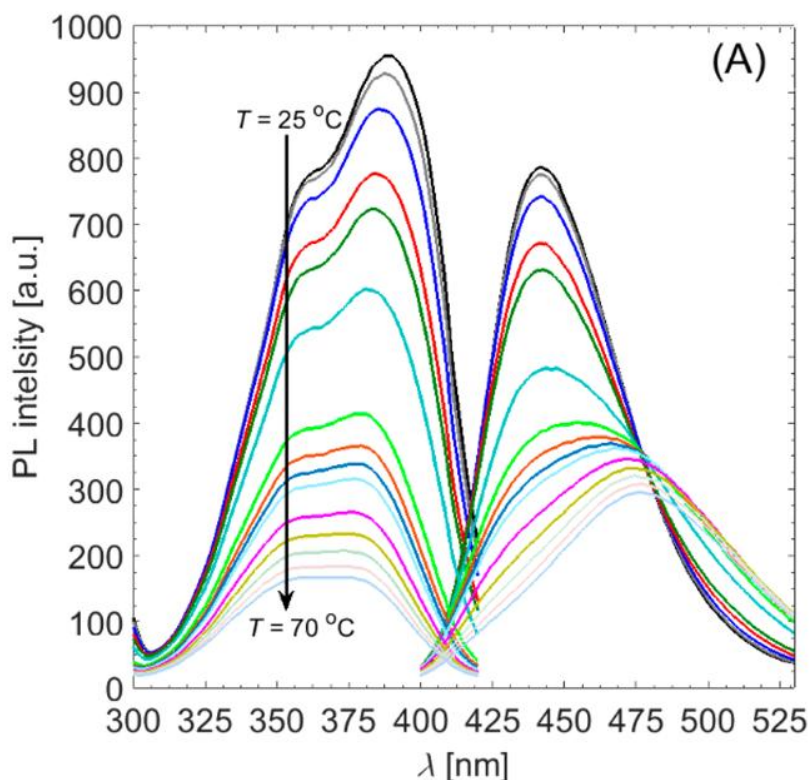
Figure 9. Emission (right) and excitation (left) spectra of DPPC-Laurdan liposomes at different temperatures [13].

In addition to Laurdan's ability to detect phase states, it can also sense mechanical strain of the bilayer. Zhang *et al.* [51] used Laurdan to study strain of DOPC vesicles that was induced by osmotic pressure. Swelling of lipid vesicles increased the membrane tension that again decreased Laurdan GP. For 100 nm vesicles the initial GP value was −0.06 and after swelling −0.12. [51] However, it is worth mentioning that differences in the GP values are of the different order of magnitude from that during a phase transition from a gel to a liquid crystalline phase.

5.1.1 Laurdan and non-lamellar forming lipids

Although Laurdan is commonly used to study phase transitions between gel and liquid-crystalline phases, it can also detect phase transitions of non-lamellar forming lipids. [27] This makes Laurdan suitable to study pH sensitive liposomes that are usually composed of non-lamellar forming lipids.

Epand & Leon [27] studied bilayer properties of dipalmitoleoyl phosphatidylcholine (DiPoPc) and dipalmitoleoyl phosphatidylethanolamine (DiPoPE) with Laurdan.

They found out that Laurdan could detect the formation of an inverted hexagonal phase of phosphatidylethanolamines, as the Laurdan GP decreased in the range of 5-28 °C. Yet, above 28 °C, GP values started to increase and the emission intensity collapsed (Figure 10). [27]
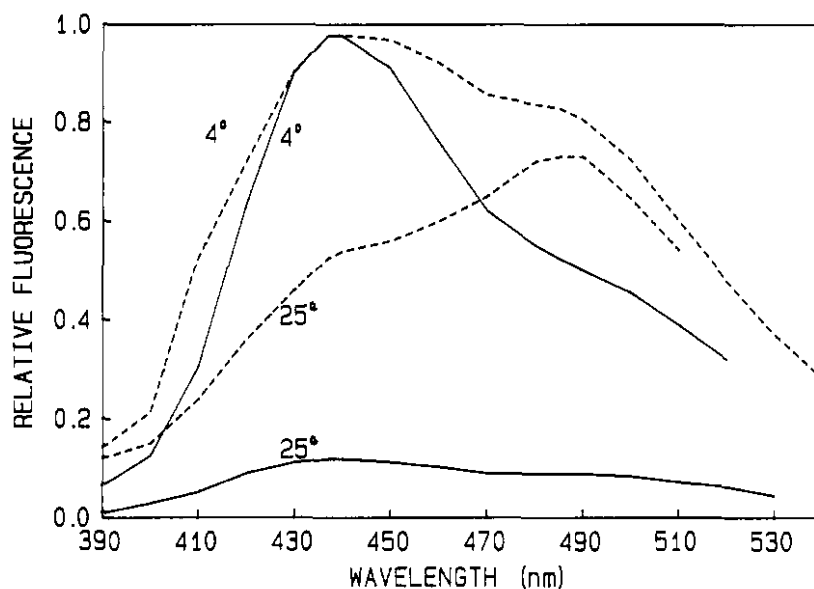

Figure 10. Fluorescence emission spectra of Laurdan-DiPoPC (dashed lines) and Laurdan-DiPoPE systems (solid lines) [27].

For DiPoPc, the phase behavior was different. As temperature increased, there was a decrease in the Laurdan GP values. Contrary to phosphatidylethanolamines, phosphatidylcholine bilayers are not prone to form hexagonal phases. [27] Although the study performed by Epand & Leon [27] did not focus on pH, it gives fundamental information about the phase transition of lipids not forming lamellae.

In this work, Laurdan was utilized to study phase behavior of pH sensitive liposomes. The target was to examine if an acidic environment can trigger a phase transition, similarly to light activation or heating.

5.2 C-Laurdan

Kim *et al.* [53] synthetized C-laurdan (6-dodecanoyl-2-[*N*-methyl-*N*-(carboxymethyl)-amino]naphthalene) for lipid raft imaging. C-laurdan is a laurdan derivative that has an additional carboxyl group (Figure 11) [49]. It reflects the cell environment (fluid

and gel phases) accurately and is more sensitive to the membrane polarity than laurdan. Advantages of C laurdan include also improved water solubility. [49] [53]
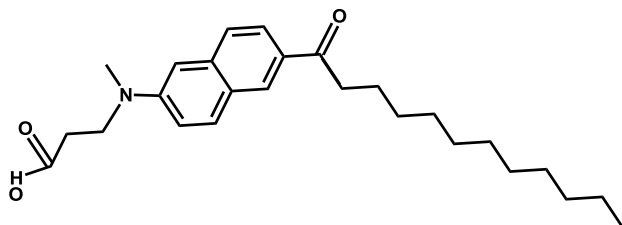

Figure 11. C-Laurdan.

An interesting property of C-Laurdan is its ability to detect a pre-phase transition from a gel to a ripple phase. Traian *et al*. [52] observed a small slope in the GP values during the pre-phase transition of DPPC liposomes, and determined the pre-phase transition temperature to be 32.8 °C. However, the transition from a gel to a ripple phase was not observed at an acidic pH 5.6. The result indicated that under acidic conditions, C-Laurdan is inserted deeper into the lipid bilayer, because the GP values were higher at pH 5.6 than at 7.4. [52]

In this work, Laurdan-C was used as an additional fluorescence probe to study the phase behavior of pH sensitive liposomes.

5.3 pHrodo red

pHrodo red is a hydrophilic, rhodamine based fluorescence dye. Its advantages include good photostability and improved brightness. [54] In an acidic environment, fluorescence emission of pHrodo red increases remarkably. Thus, pHrodo red can be used to sense and image pH in cellular compartments, such as endosomes and lysosomes. Its pK_a value is ca. 6.5. [55]

When embedded in lipid vesicles, pHrodo red shows pH response from 4 to 7. However, the challenge is to load sufficient amounts of the fluorophore into the liposomes. An alternative is to link pHrodo red to a membrane forming phospholipid such as DOPE [54]. In an even more developed system only the inner leaflet of the

liposomal membrane is labeled, which minimizes background fluorescence and enables more reliable observations about ion transport [56].

In this work pHrodo red was used to study charge transfer through liposomal membrane.

5.4 Calcein

Calcein is a hydrophilic and self-quenching fluorescence dye, which is widely used to study drug release from liposomes. Calcein encapsulates in lipid vesicles at high concentrations (55 mM and higher [57]) where self-quenching takes place. Self-quenching can be observed as a low fluorescence intensity. When calcein leaks out of the liposomes the intensity of the fluorescence signal increases notably. [5]

Calcein release from the liposomes can be calculated from the following equation [17]:

$$R = \frac{F - F_0}{F_{100} - F_0} * 100 \ \% \quad (2)$$

where R is the release percentage (%), F_0 is background fluorescence (reference sample), F_{100} is fluorescence after Triton-X addition and F is fluorescence of the sample.

6 Possible mechanisms of drug release from pH sensitive liposomes

In a large number of studies, liposome aggregation and model drug release from pH sensitive liposomes have been observed [3] [14] [58] [59]. Therefore, liposome aggregation and the drug release in acidic environments are discussed before considering the possible mechanisms of liposome destabilization in more detail.

6.1 About liposome aggregation and drug release

Different lipid combinations can be utilized to obtain liposomal drug release in acidic environments. Fan *et al.* [3] used oleic acid, CHEMS and linoleic acid as pH sensitive molecules. Liposomes containing PE, cholesterol and pH sensitive molecules were analyzed at pH 5, 6 and 7.4. For PE-CHEMS liposomes, the initial diameter was less than 150 nm at pH 7.4, but at pH 5 the diameter increased up to ca. 250 nm. At low pH, CHEMS's carboxyl group became protonated, resulting in decreased negative membrane charge, which again led to the liposome aggregation. Zeta potential measurements supported the particle analyses results: at physiological conditions, zeta potential of PE-CHEMS liposomes was below −30 mV, which indicated that negatively charged particles repelled each other. However, at pH 5 a slight increase in zeta potential (−25 mV) was obtained. As was seen from the particle size analyses, lowered negative membrane charge leads to the liposome coagulation. [3]

Furthermore, Fan *et al*. [3] observed that the release of a model drug, docetaxel, was more rapid in an acidic environment than at neutral pH. CHEMS was the most suitable pH sensitive molecule for the effective drug release. During 24 hours ca. 88% of docetaxel was released from CHEMS liposomes at pH 5, whereas liposomes containing linoleic acid released only 64.6%.

Guo *et al.* [14] studied calcein release from pH sensitive DOPE/CHEMS and diolein/CHEMS liposomes formed with different diolein/CHEMS ratios. Liposomes with 6/4 diolein/CHEMS ratio were stable at physiological pH, but in an acidic environment (pH 5), 40% calcein was released after 30 minutes incubation at 37 °C.

The mean diameter of the liposomes increased from 150 nm to 1500 nm after 60 min incubation at pH 4.8 (RT). However, at pH 5.35 and 7.4 no significant changes were observed. [14] Particle size analyses would be needed also at pH 5 to thoroughly compare drug release and aggregation.

Although the combination DOPE and CHEMS in pH sensitive liposomes has been widely investigated there are only few studies concerning diolein. In addition to the studies carried out by Guo *et al*. [14] and Gosselin *et al*. [44], Lajunen *et al*. [17] utilized 1,3-diolein and CHEMS in thermo- and pH sensitive liposomes. The temperature gradient experiments showed that pH had an effect on liposomal calcein release. At pH 4.5 the most optimal formulation DPPC/DSPC/Lyso PC/diolein/CHEMS (10:90:15:15:10) released over 80% calcein at 45 °C. At pH 7.4, the release was less than 10% at the same temperature. [17]

Therefore, it is evident that pH sensitive liposomes with varying lipid compositions can release their cargo in acidic environments. Size increase also indicates that lipid aggregation takes place. However, the mechanism behind the drug release remains unclear. When considering liposome destabilization and the consequent drug release under acidic conditions, two possible mechanisms have been proposed. At low pH, carboxyl group of amphiphile or head group of PE becomes protonated, which can lead to the charge loss and aggregation. [3] [58] [20] The protonation also promotes a phase transition from a lamellar to an inverted hexagonal phase, which leads to the liposome destabilization [20]. Possible mechanisms and their connection are discussed next in more detail.

6.2 Charge loss and phase transition

When considering the possible drug release mechanisms, it is important to note that drug release from liposomes can be significant also below the maximum permeability. This means that drug release begins below the main phase transition temperature. Heating causes premelting at the grain boundaries, which can improve permeability. [9]

In an acidic environment, protonation of the amphiphile leads to liposome destabilization. Phase transition occurs since the bilayer converts into an inverted

hexagonal phase. [20] This indicates that the charge loss and the phase transition resulting from destabilization are connected. The lipid structures that are formed during the phase transition, are presented in Figure 12. Liposomal bilayer collapses during the phase transition, and the resulting lipid structures are composed of a monolayer. As presented in the Figure 12, hydrophobic chains are pointing outwards from the monolayer surface.
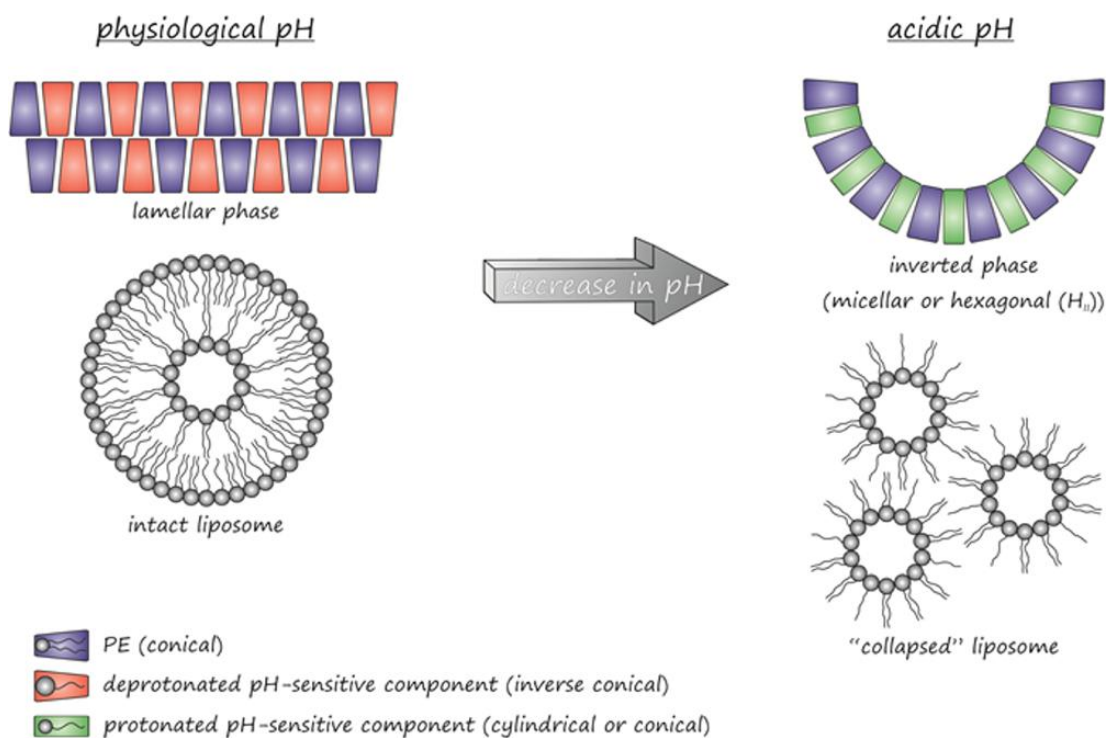

Figure 12. Scheme of phase transition of pH sensitive liposomes containing DOPE [60].

The fusogenic properties of the lipids can be connected to the phase transitions that are facilitated by high unsaturation, trans-configuration and a short lipid chain length. [18] Interestingly, non-bilayer structures can be important intermediates in membrane fusion [19] [61]. During lamellar to inverted phase transition, bilayer membranes transform into hexagonally packed tubes that have monolayer walls. The first step of the phase transition includes small connections between bilayer interphases. After that, the initial connections aggregate and rearrange into a structure with hexagonal symmetry. The same mechanism is proposed for membrane fusion. [61] The similarity between the mechanisms makes it probable

that both the membrane fusion and the phase transition take place during liposomal destabilization and drug release.

Bambeke *et al.* investigated alterations in lipid organization upon acidification. They proposed that structural changes could be the first factor in liposomal destabilization. Their biophysical studies showed that leakage occurs at higher pH than fusion. Membrane permeabilization increased below pH 6.5 whereas the mean diameter of liposomes started to increase below pH 6. Lipid reorganization, resulting to an inverted hexagonal phase, progressed from pH 8 to 5.

Although the phase transition is considered as the primary reason for drug release, only few studies have focused on the phase transition mechanism. Lajunen *et al.* [17] examined the phase behavior of lipid systems containing 1,3-diolein and CHEMS. Brewster angle microscopy (BAM) images showed that diolein/CHEMS moieties gave rise to the lipid domains, which caused a phase separation at pH 5 between gel and liquid crystalline phases. The phase separation was absent at neutral pH and the lipids formed a homogenous, gel phase film. 1,3-diolein/CHEMS Langmuir films did not either have a clear collapse pressure. These results imply that in an acidic environment diolein/CHEMS components induced partial collapse of the monolayer or formed multilayers or soluble aggregates that were separated from the monolayer. [17] Also Karve *et al.* [57] studied phosphatidylcholine (PC) and phosphatidic acid containing lipid membranes at varying pH and observed phase separation. Lipid heterogeneities were formed under acidic conditions, but membrane permeability was improved only for lipid pairs with different acyl chain lengths.

Also Fan *et al.* [3] considered the mechanism of phase transition in more detail. They examined the morphology of pH sensitive liposomes with transmission electron microscopy (TEM). In an acidic environment, the liposomes became more irregular and looser than in neutral conditions. At pH 5 TEM images of CHEMS containing liposomes showed flower-like structures, resembling the packing of hexagonal tubes. They proposed that a possible reason for a bilayer to hexagonal phase transition could be the loss of stabilizing effect of CHEMS, which was expected also in other studies, such as by Simoes *et al.* [20]. However, also head

group dehydration of CHEMS occurred. Dehydration changed its shape from conical to cylindrical, promoting phase transition. [3]

Overall, it is probable that the loss of the negative charge of the liposome membrane promotes a phase transition from a bilayer to an inverted hexagonal phase. Yet, it is good to bear in mind that the most of research has focused on PE, especially on DOPE, and other lipids may have different mechanism. In the studies by Lajunen *et al*. [17] and Fan *et al.* [3] phase separation and phase transition were confirmed with BAM and TEM images. Fluorescence spectroscopy, however, has not been used to investigate the phase transition of pH sensitive liposomes containing 1,3-diolein or CHEMS. Therefore, Laurdan, Laurdan-C and pHrodo red were utilized in this work to understand the mechanism of the phase transition in more detail.

7 Characterization methods

7.1 Zeta potential

Zeta potential measurement is a common analysis method in nanoparticle surface characterization. Zeta potential corresponds to the potential at the slipping plane (Figure 13). The slipping plane determines the boundary where the particle and the attached ions move as one unit. Potential decreases with the distance from the particle surface. The highest value, surface potential, is obtained at the direct particle surface. Negatively charged liposome attracts positive ions that form a Stern layer on the surface. Beyond that, there is a more loosely packed layer, slipping plane, where zeta potential is measured. [62]
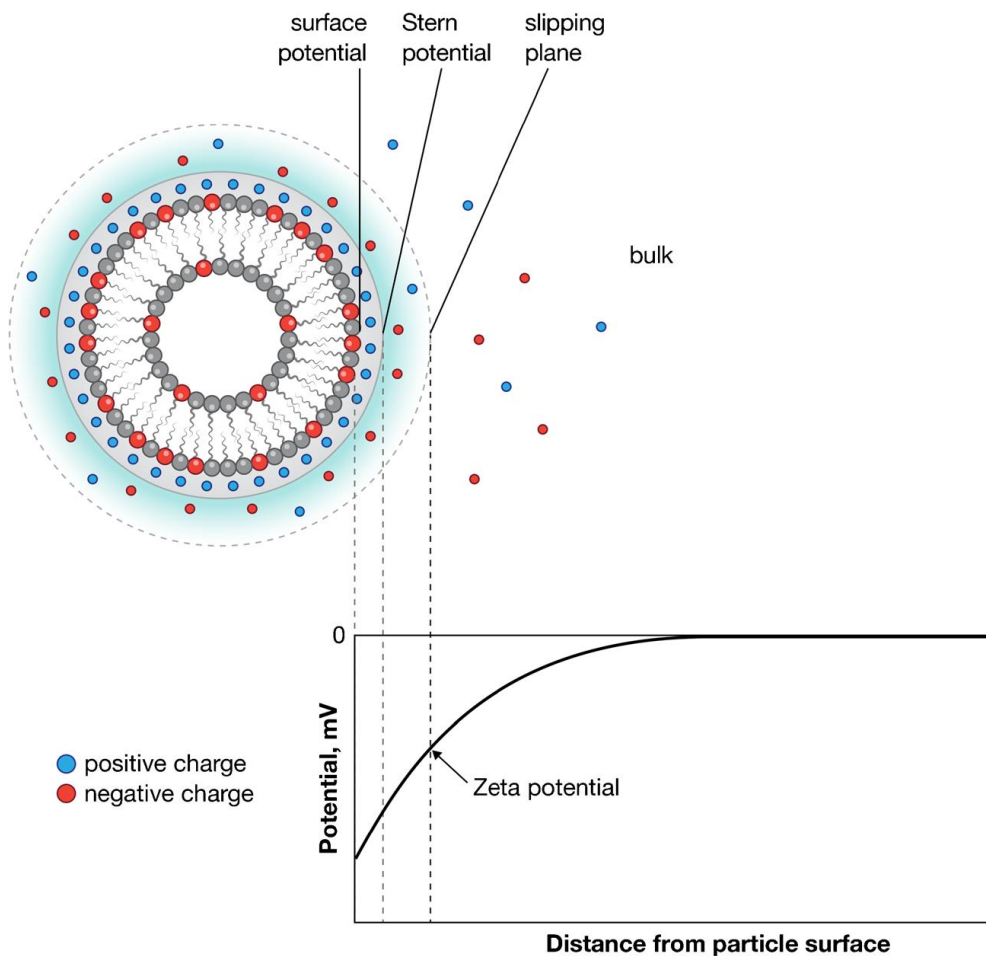

Figure 13. Zeta potential of negatively charged liposome [62].

In pH sensitive liposome characterization, zeta potential provides information of the surface charge, which is connected, e.g. to liposome aggregation [58] [3]. Negatively charged liposomes can repel each other, which prevents aggregation [3].

7.2 Dynamic Light Scattering

Dynamic Light Scattering (DLS) or photon correlation spectroscopy (PCS) are common analysis methods in nanoparticle characterization. DLS measures Brownian motion of suspended nanoparticles. It detects particle sizes between 1-1000 nm. It is also suitable for a broad concentration range. Because the large particles can mask signal of the smaller ones, the technique has limitations on the samples with high polydispersity.[63]

7.3 Fluorescence spectroscopy

Fluorescence can simply be understood as a reverse process of absorption [64]. Principles of fluorescence are presented in the Jablonski diagram in Figure 14. First, light absorption of a molecule induces electron transfer from the ground state to an excited state. Relaxation to the ground states includes two steps. First, an internal conversion takes place, i.e. the molecule releases some energy into the surrounding medium and returns to the first excited state. From the first excited state, it returns to the ground state by emitting a photon. Apart from fluorescence, there are also other competing processes such heat dissipation, phosphorescence and energy transfer to nearby molecules. [65]
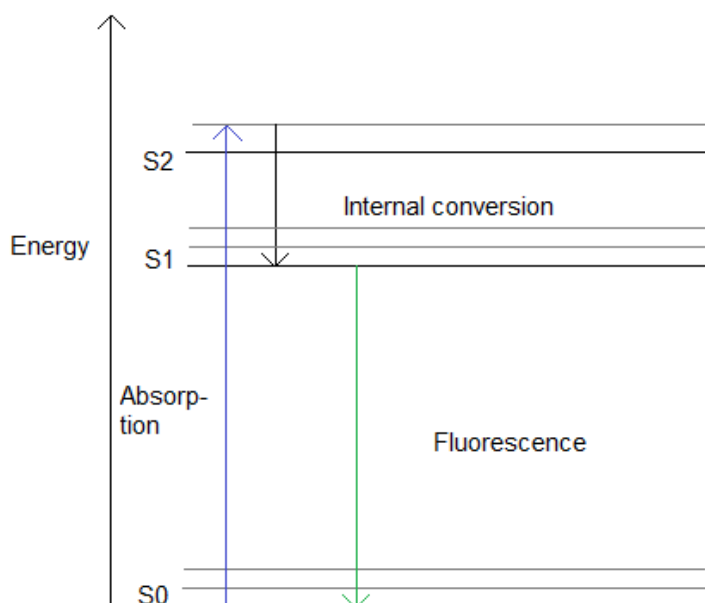
Figure 14. Principle of fluorescence (Jablonski diagram).

Since the total absorbed energy is released into the surroundings via several routes, the emission energy is always lower than the absorption energy [65]. Compared with the excitation spectrum, the emission spectrum shifts to the longer wavelengths, which is known as a Stoke's shift. [64]

The time scale of fluorescence depends on the electronic structure of the molecule. For the most fluorophores fluorescence occurs in the range of 1-100 ns. Important fluorescence properties include quantum yield, lifetime and intensity. Quantum yield describes the efficiency of a fluorophore, that is, how efficiently incoming photons are converted to emitted light. A fluorescence lifetime corresponds to the average time a fluorophore remains on the excited state. The lifetime depends on the emission and non-radiative relaxation that return the system to the ground state. Fluorescence intensity is determined by the amount of excited molecules that are generated upon excitation. [64] Fluorescence parameters also respond to the physical properties of the surroundings, and fluorescence is widely used to study lipid membranes. When considering the lipid behavior, such as phase coexistence and dynamics, fluorescence spectroscopy has a few advantages, because its time scale corresponds to that of membrane processes. [48]

Fluorescence spectroscopy is a highly sensitive and non-destructive technique. Fluorometer instrumentation is composed of an excitation source, cuvette holder,

emission and excitation monochromators/filters, detector and recorder. Lamps or lasers can be used as an excitation source. [65] There are several fluorescence probes, which can be utilized in membrane labelling, such as Laurdan and Prodan [50] [13]. In this work, Laurdan and pHrodo red were used to examine membrane behavior of the liposomes.

8 Experimental

1,3-diolein/CHEMS liposomes were prepared for the study of pH triggered drug delivery and plain DPPC liposomes were used as the reference in fluorescence measurements. In DLS and zeta potential measurements, DPPC-DSPE-PEG(2000) liposomes were used as the reference.

DPPC (25mg/ml in chloroform), DSPE-PEG(2000) and CHEMS were purchased from Avanti polar lipids. Laurdan and calcein were purchased from Sigma Aldrich. Etax Aa was the product of Altia. pHrodo ™ red dextran 10,000 MW was purchased from Thermo Fischer Scientific. Prof. Jan Deska kindly synthesized 1,3-diolein and Laurdan-C.

Fluorescence measurements were carried out with Perkin Elmer LS55 fluorescence spectrophotometer. Particle analysis and zeta potential measurements were performed with Malvern Zetasizer Nano ZS.

8.1 Liposome preparation

Liposome samples were prepared according to ref. Viitala *et al*. [13] [66]. First, lipids (see Table 1 and Table 2) and 0.55-1 ml chloroform were added to a round flask. Chloroform was evaporated under nitrogen flow, and the flask was heated in a water path. When the temperature of 60.5 °C was reached, heating was continued for 30-45 minutes. The dry lipid film was next hydrated with a suitable buffer (see Tables 1-3). To ensure proper dispersion, the flask was kept in an ultrasonicator for ca. 30-45 minutes after the lipid film was dissolved. Finally, a liposome solution was extruded 11 times through a polycarbonate membrane with the pore size of 200 nm or 400 nm. Extrusion temperature was ca. 61 °C. In the case of 1,3-diolein/CHEMS liposomes, the sample solution was first extruded through a 800 nm membrane to facilitate the extrusion process.
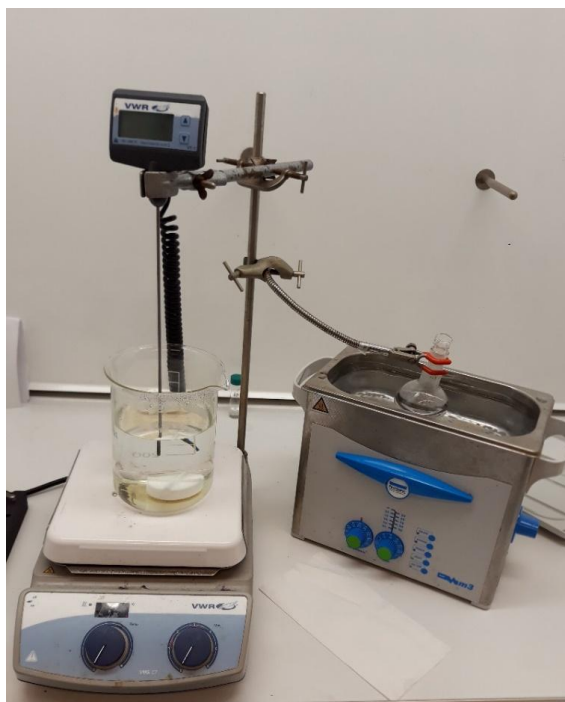
Figure 15. Water bath on the left and ultrasonicator on the right.

Figure 16. Extrusion apparatus.

When preparing liposomes for fluorescence measurements, 10 µl Laurdan/Laurdan-C (0.2 mg/ml in ethanol) was added to the mixture (750 µl) in the first step. For calcein containing liposomes calcein solution (calcein 60mM, NaCl 280 mOsm pH 7.4) was used instead of a buffer. Laurdan/Laurdan-C was not used in the calcein embedded liposomes. For samples containing pHrodo red, 10 µl pHrodo red (1mg/ml in MQ water) was added to 1ml PBS hydration buffer; they contained also Laurdan to make the sample visible in gel filtration.

More dilute buffers (2mM Hepes 15 mM NaCl) were used in zeta potential measurements to prevent electrode degradation. TRIS buffer was used for Laurdan/Laurdan-C containing DPPC liposomes instead of HEPES because pure DPPC is more stable in TRIS buffer.

Table 1. Details of the liposomes prepared for DLS measurements.

Liposome composition	Lipid molar ratios	Lipid conc.	Buffer
1,3-diolein-CHEMS	60:40	3mM	20 mM Hepes 150 mM NaCl (pH 7.4, 6.5, 6, 5.5 or 5)
DPPC-DSPE-PEG2000(carboxylated)	97:3	6 mM	20 mM Hepes 150 mM NaCl (pH 7.4, 6, or 5)

Table 2. Details of the liposomes prepared for zeta potential measurements.

Liposome composition	Lipid molar ratios	Lipid conc.	Buffer
1,3-diolein-CHEMS	60:40	3 mM	2 mM Hepes 15 mM NaCl (pH 7.4, 6.5, 5, 5.5 or 5)
DPPC-DSPE-PEG2000(carboxylated)	97:3	3 mM	2 mM Hepes 15 mM NaCl (pH 7.4, 6.5, 5, 5.5 or 5)

Table 3. Details of the liposomes prepared for fluorescence measurements.

Liposome composition	Lipid molar ratios	Lipid conc.	Buffer
1,3-diolein-CHEMS-Laurdan/Laurdan-C	60:40	2 mM	20 mM HEPES 150 mM NaCl (pH 7.4, 6.5, 6, 5.5 or 5)
DPPC-Laurdan/Laurdan-C	100	2 mM	Tris NaCl (pH 7.4, 6 or 5)
DPPC-1,3-diolein-CHEMS-Laurdan	50:30:20	2 mM	20 mM HEPES 150 mM NaCl (pH 7.4 or 5)
1,3-diolein-CHEMS-Laurdan-pHrodo red	60:40	2 mM	PBS
DPPC-Laurdan-pHrodo red	100	2 mM	PBS
1,3-diolein-CHEMS-calcein	60:40	2 mM	PBS

8.2 Preparation of buffers and other solutions

When preparing TRIS and HEPES buffers, HEPES or TRIS and NaCl were first weighed and transferred into a volumetric flask. Then the volumetric flask was filled with MQ water to ca. 95 % of the full volume. pH was adjusted to pH 7.4, 6.5, 6, 5.5 or 5 with HCl (1M) or with NaOH (0.5 M) and the volumetric flask was filled to the full volume with MQ water.

PBS buffer was prepared by dissolving five tablets to 1 l of MQ water.

Calcein solution was prepared by dissolving calcein and NaCl into MQ water. pH was adjusted to 7.4. Heating and sonication were used to dissolve calcein.

Laurdan and Laurdan-C were first dissolved in ethanol (Etax Aa) to obtain fluorophore concentration 0.2 mg/ml. Lipids in powder form, (DSPE-PEG(2000) and CHEMS), were diluted in chloroform. pHrodo red was dissolved in 500 μl MQ water to obtain concentration of 1 mg/ml.

8.3 Liposome characterization

8.3.1 Fluorescence

Prior to fluorescence experiments, the samples were gel filtrated using Sephadex G-50 column to remove an excess of a fluorophore. In practice 200-700 μl of the extruded and cooled liposome sample was injected into the column. UV-light lamp was used to distinguish Laurdan/Laurdan-C containing liposomes. The UV-visible fraction was gathered into five aliquots of ca. 500 μl. Sample number 4 or 5 was used in the fluorescence experiments. However, because of acid-induced destabilization, 1,3-diolein-CHEMS liposomes prepared at pH 6.5, 6, 5.5 and 5 were not visible in UV-light. For them fluorescence was measured from non-filtrated samples. To obtain comparable results, emission and excitation slits were adjusted to 10 nm for 1,3-diolein-CHEMS liposomes at pH 6, 5.5 and 5. Fluorescence signal of liposomes prepared at pH 6.5 and 7.4 was so strong that the slits were narrowed to 5.4 and 5.6 nm. Because of instability of the liposomes prepared below pH 6, fluorescence was measured from two independent liposome batches and the averages were used for the figures and calculation of the GP values.

Due to the calcein fluorescence, calcein loaded liposomes were not visible to the naked eye. Yet, the liposomes were sedimenting in the column faster than free calcein. Thus, the liposome sample was gathered before bright orange, dilute calcein solution reached the bottom of the Sephadex column (Figure 17).

Figure 17. Gel filtration of calcein-loaded liposomes.

To study release in an acidic environment, pH of the gel filtrated solution was adjusted with HCl (250 mM and 100 mM) to pH 5. The effect of pH on calcein fluorescence was minimized according to ref. [57]. After one hour incubation at T= 37 °C, the samples were diluted with PBS buffer (pH 7.4). In practice, 20-40 µl of the sample (pH 5) was added to 1960-1980 µl of PBS buffer (pH 7.4). The same procedure was conducted also for pH 7 sample to compare release under neutral and acidic conditions.

The reference sample was not incubated at T=37 °C but the dilution to PBS was similar.

The sample volume for all fluorescence measurements was 450 µl. Four-window fluorescence cuvette (total volume 700 µl, Thorlabs) was used. For Laurdan/Laurdan-C containing liposomes excitation wavelength was set to 365 nm and the emission spectrum was recorded. For calcein loaded liposomes excitation wavelength was 480 nm whereas for pHrodo red containing samples excitation wavelength was set to 580 nm. Temperature was adjusted to 37 °C using external water circulation.

In the case of homogenous sample series, 1,3-diolein-CHEMS-Laurdan/Laurdan-C and DPPC-Laurdan/Laurdan-C, fluorescence was measured after ca. 15 minutes incubation at T = 37 °C. Measurement was repeated three times to calculate an average of the results.

To examine how an acidic environment affects liposomal membrane, pH of external solution was adjusted to the lower values. In practice, 2.5-3 ml of gel filtrated liposome solution was mixed with 7.5-8 ml neutral buffer (see Table 1) to enable pH adjustment with HCl (1M, 250mM, 100mM or 25 mM). First, the neutral sample was measured. After that, pH was adjusted to the lower values (6.5 ,6, 5.5, 5 and 4.5). Samples were incubated 15 minutes at T= 37 °C before measuring fluorescence.

8.3.2 DLS

Particle size was analyzed with Malvern Zetasizer Nano Zs apparatus. Approximately 400 µl of undiluted liposome sample was pipetted into a disposable plastic cuvette. Size of the lipososomes was analyzed 2 x 3 times. Temperature was set to 25 °C. The samples were stored in refrigerator (4 °C) and they were measured again one and two weeks after preparation to examine stability.

8.3.3 Zeta potential

Zeta potential was analyzed with Malvern Zetasizer Nano ZS apparatus. Various dilutions and buffer salt concentrations were tested before obtaining a good response. In addition, disposable zeta potential cuvette was replaced with Malvern Zetasizer Dip Cell cuvette (ZEN1002). The final samples contained 100 µl liposomes and 900 µl buffer. More dilute buffers (2mM HEPES and 15 mM NaCl) were used in both liposome preparation and dilution to avoid electrode degradation. Temperature was set to 25 °C. Depending on the sample, measurement was repeated 1-2 x 3-5 times. 1 x 5 times was more suitable because the applied voltage damaged the liposomes.

9 Results and discussion

9.1 Particle size analysis

Significant changes in the particle sizes were obtained for 1,3-diolein-CHEMS liposomes (Figure 18). At neutral pH 7.4, the particle size was quite similar to the DPPC-DSPE-PEG reference samples (Figure 18) with an average diameter of 211 nm. However, below pH 6 the particle size increased remarkably. At the day of preparation the average diameter of 1,3-diolein-CHEMS liposomes was 918 nm at pH 5. This indicated rapid aggregation or coagulation of the sample. 1,3-diolein-CHEMS liposomes at pH 5.5 were relatively stable during the day of preparation with an average diameter of 390 nm. Yet, after a week the diameter was increased up to 900 nm. At pH 6, no remarkable changes were detected during the week.

Standard deviations increased in accordance with the size enlargement, proving instability and heterogeneity of the sample. For nearly neutral 1,3-diolein-CHEMS samples (pH 7.4 and 6.5) and for DPPC-DSPE-PEG the standard deviations were very small, between 0.2 and 4.6, and therefore the error bars in the figures are very narrow.
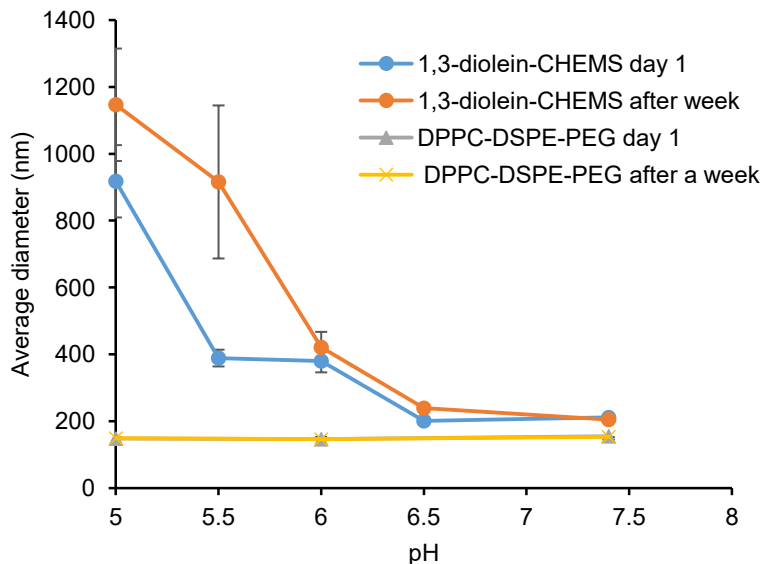

Figure 18. Average diameters ± standard deviations of 1,3-diolein-CHEMS and DPPC-DSPE-PEG liposomes at different pH.

Intensity distributions of 1,3-diolein-CHEMS liposomes at pH 7.4 and pH 6.5 were overlapping in a great extent, but under more acidic conditions the distributions were shifted to higher wavelengths (Figure 19). Intensity distributions of the reference liposomes DPPC-DSPE-PEG were overlapping at all pHs (Figure 20).
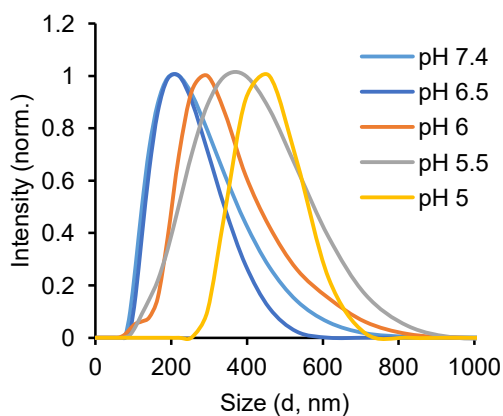

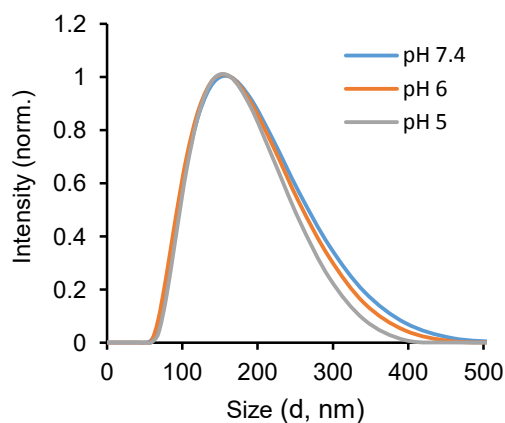Figure 19. Intensity distributions of 1,3-diolein-CHEMS liposomes at different pH.

Figure 20. Intensity distributions of DPPC-DSPE-PEG liposomes at different pH.

After two weeks the particle sizes of DPPC-DSPE-PEG liposomes remained unaltered but 1,3-diolein-CHEMS liposomes showed great degradation under acidic conditions (Table 4). In addition to the very large particles, also small structures were detected. This could have been due to the destabilization and precipitate formation. However, at pH 7.4 and 6.5 no significant changes took place.

Considering the stability of the neutral 1,3-diolein-CHEMS liposomes, it is remarkable that the particle size remained unaltered for two weeks. In this respect, it could be possible to obtain a stable formulation at physiological conditions without PEG. However, it should be noted that the liposome samples were stored at
T = 4 °C, which does not correspond physiological conditions.

Table 4. Average diameters of the liposomes two weeks after preparation.

Liposomes	pH	Average diameter
DPPC-DSPE-PEG	7.4	155
DPPC-DSPE-PEG	6	147
DPPC-DSPE-PEG	5	149
1,3-diolein-CHEMS	7.4	206
1,3-diolein-CHEMS	6.5	240
1,3-diolein-CHEMS	6	324
1,3-diolein-CHEMS	5.5	8000

In conclusion, the average diameters of 1,3-diolein-CHEMS liposomes were strongly dependent on pH. The results were in line with the other studies, where liposome enlargement in acidic environment was observed [14] [3].

9.2 Zeta potentials

Zeta potentials of homogenous 1,3-diolein-CHEMS and DPPC-DSPE-PEG liposome samples are shown in Figure 21. Zeta potential of 1,3-diolein-CHEMS liposomes was clearly negative with ca. −70 mV at pH 7.4 and −59 mV at pH 5. Considering the pH scale between pH 7.4 and 5, the zeta potential was constantly increasing but the changes were not very big. This could be due to the protonation behavior of CHEMS: with pK_a of 5.8 [39] more acidic environment would be needed to cause a more drastic increase in zeta potential. It is also possible that the ion content of the buffer affected the results. When pH of external solution was adjusted to the pH 4.36, remarkable increase of zeta potential was detected (Figure 22). For the experiment, the liposomes were prepared at pH 7.4 and pH of the external solution was lowered to the desired values.

In a case of DPPC-DSPE-PEG, the slightly negative zeta potentials, −22 mV at pH 7.4 and −16 mV at pH 5 were probably due to the carboxyl group of PEG.
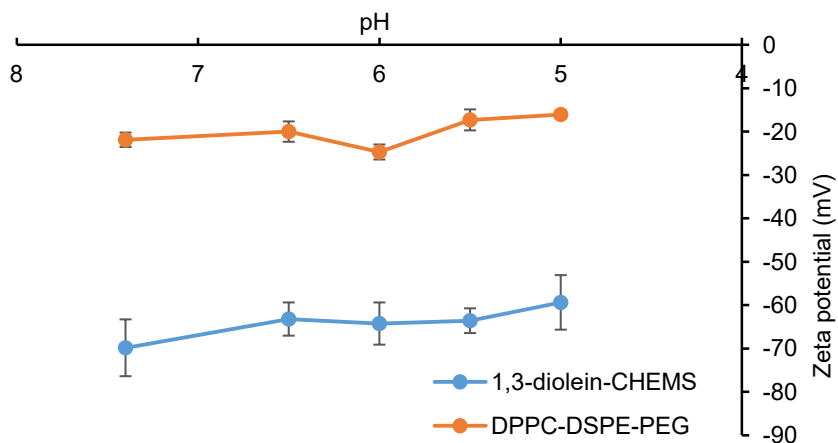
Figure 21. Zeta potentials and standard deviations of 1,3-diolein-CHEMS and DPPC- DSPE-PEG.
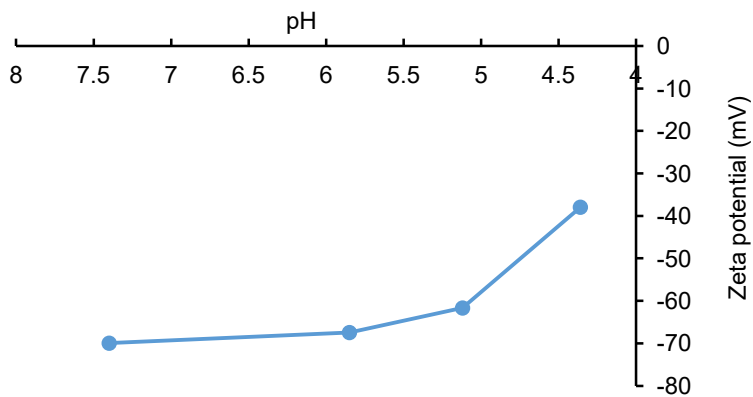

Figure 22. Zeta potentials of 1,3-diolein-CHEMS liposomes when pH of external solution was adjusted to lower values.

The zeta potential results supported the hypothesis that the negative charge of the liposomal membrane decreases upon acidification. The results were in accordance with Fan *et al.* [3], who also observed a decrease in the absolute value of zeta potential. For CHEMS containing liposomes, zeta potential was a bit below −30 mV at pH 7.4 and −25 mV at pH 5 [3]. However, they used PE instead of 1,3-diolein, which can explain the higher values.

9.3 Fluorescence experiments

9.3.1 Homogenous series

Fluorescence spectra of Laurdan-containing, homogenous 1,3-diolein-CHEMS liposomes are shown in Figure 23. Emission spectra of Laurdan and DPPC containing reference liposomes were overlapping at pH 5, 6 and 7.4 (Figure 24). Therefore, Laurdan is well suitable to study pH induced changes in the membrane behavior in the pH range of 5-7.4.
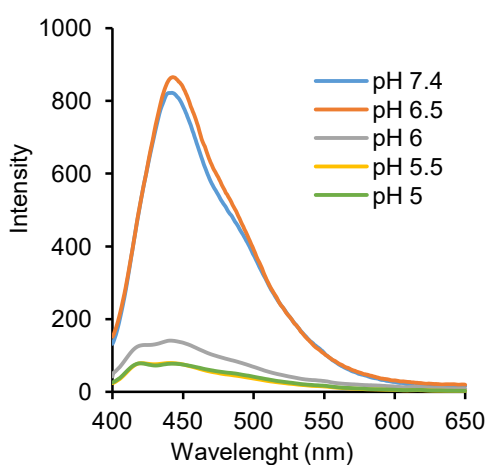

Figure 23. Emission spectra of 1,3-diolein-CHEMS-Laurdan liposomes at different pH at T = 37 °C.
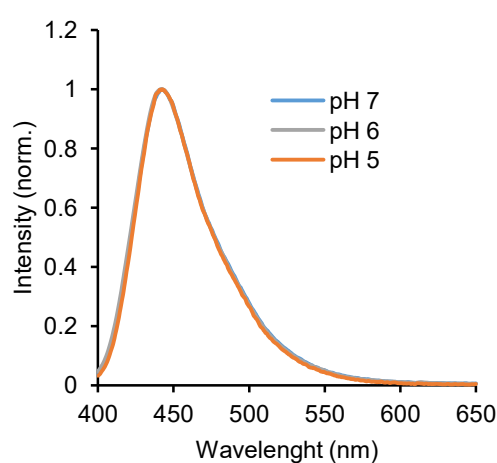
Figure 24. Emission spectra of DPPC-Laurdan liposomes at different pH at T = 37 °C .

Higher intensity of 1,3-diolein-CHEMS pH 6.5 sample is due to the gel filtration of the sample prepared at pH 7.4. Below pH 6.5, the emission intensity collapsed and shape of the emission peaks changed. This shows that membrane properties of 1,3-diolein-CHEMS liposomes alter in acidic environments. The intensity drop at 440 nm indicates that the gel phase disappears at low pH values. Yet, the phase behavior deviates from that of liposomes that exhibit a phase transition from a gel to a liquid crystalline phase. During heating, when a phase transition of DPPC containing liposomes occurred, emission intensity at ca. 490 nm increased while intensity at 440 nm collapsed [13]. In the case of 1,3-diolein-CHEMS liposomes also intensity at 490 nm dropped in acidic environments, indicating that a phase transition from a gel to a liquid crystalline phase does not take place. However, Epand *et al.* [27], who studied non-lamellar forming lipids, observed similar changes in the emission

spectrum when the system was approaching the hexagonal phase transition temperature. Emission intensity collapsed and the spectrum resembled that of Laurdan in a buffer. Therefore, it is suggested that 1,3-diolein-CHEMS lipid system adopts an inverted hexagonal phase in acidic environments.

In addition to the peaks corresponding gel and liquid crystalline phases, also a third emission peak at n. 420 nm was observed below pH 6.5. The third peak becomes even more evident as pH drops. One possibility is that it is due to precipitate formation. Ramani & Balasuriam [30] observed also third peak at 425 nm in a study where they examined cochleate structures with Laurdan. In the emission spectrum, the phase transition from a liquid crystalline to a rigid and dehydrated cochleate phase was detected as a shift from 490 nm to 425 nm. Therefore, it is possible that similar precipitates are formed also in acidic conditions. In addition, phase separation between an inverted hexagonal and a rigid, dehydrated phase can explain the shape of the emission peaks.

In addition to the drop of emission intensity, slight decrease in the Laurdan GP values was observed (Figure 25). This deviates from the results obtained by Epand *et al*. [27] who observed a slight increase in GP values during a lamellar to hexagonal phase transition. However, as Epand *et al*. [27] denoted, low fluorescence intensity makes the GP values less accurate. Different lipid compositions were also used in this work.
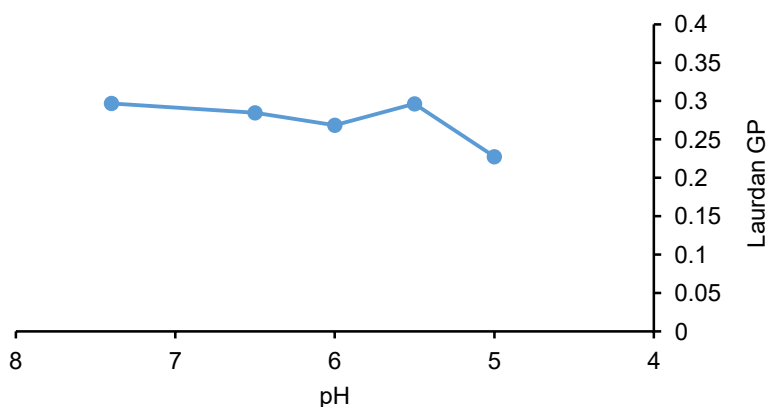

Figure 25. GP-values for 1,3-diolein-CHEMS-Laurdan liposomes at different pH.

Considering the GP values and the shape of the emission peaks, there are significant differences between 1,3-diolein-CHEMS and DPPC liposomes. For diolein-CHEMS liposomes, the peak is boarder already at neutral pH, indicating a less ordered membrane. This is confirmed with the GP values: for 1,3-diolein-CHEMS GP is 0.29 at pH 7.4 whereas GP value of DPPC liposomes at the same conditions is 0.45.

Emission spectra of Laurdan-C containing 1,3-diolein-CHEMS liposomes are shown in Figure 26. The results are in line with those obtained with Laurdan. Yet, Laurdan-C labeled liposomes showed slightly greater decrease in the GP values (Figure 28). This could be due to the improved ability of Laurdan-C to detect pre-phase transition [52]. However, DPPC-Laurdan-C reference samples showed also pH dependency, although it was different to that of pH sensitive formulation. Under acidic conditions, the emission peaks of DPPC-Laurdan-C liposomes narrowed and shifted to slightly shorter wavelengths (Figure 27). The emission intensity remained unaltered. The changes were probably due to the protonation of carboxyl group of Laurdan-C.
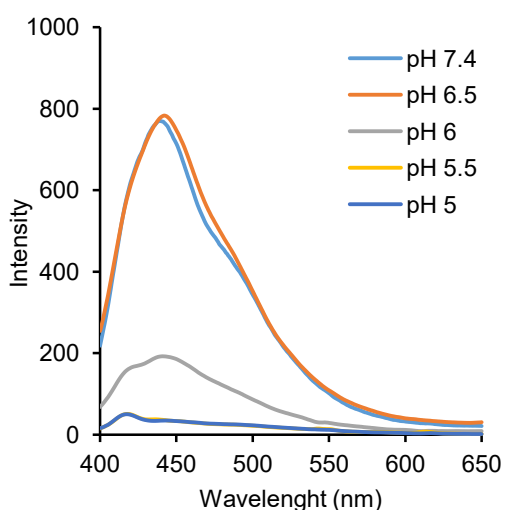

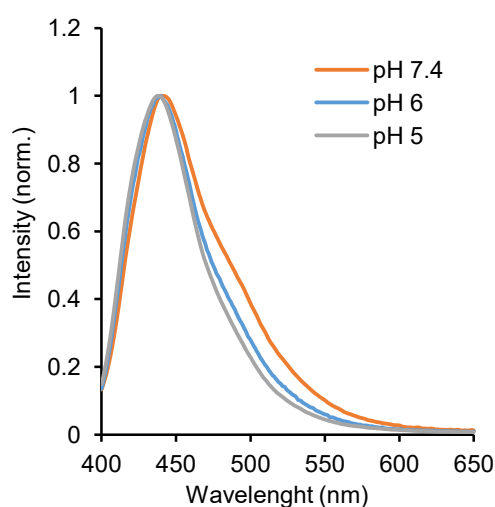Figure 26. Emission spectra of diolein-CHEMS-Laurdan-C liposomes at different pH.

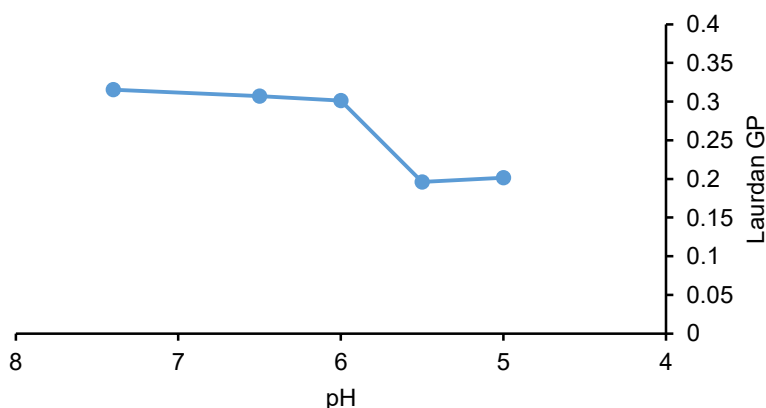Figure 27. DPPC Laurdan-C liposomes at different pH.

Figure 28. GP-values for 1,3-diolein-CHEMS-Laurdan-C liposomes at different pH.

When 1,3-diolein and CHEMS moieties were combined with DPPC, the formulation showed no remarkable pH dependency. As seen from the Figure 29, the emission peak of the liposomes prepared at pH 5 was slightly narrowed. Intensity collapse was absent, which indicates that 50 % DPPC suppressed pH sensitivity of the system. At pH 7.4 GP value was 0.35 whereas at pH 5 it was 0.43.
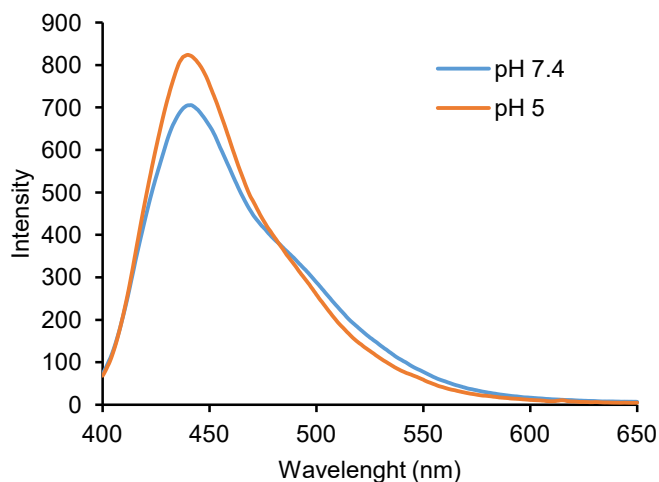

Figure 29. Laurdan containing DPPC-diolein-CHEMS (50:30:20) liposomes prepared at pH 7.4 and pH 5.

In the particle size analyses, there was only a small increase in the average particle size, with 183 nm at pH 7.4 and 230 nm at pH 5. Possibly 1,3-diolein and DPPC formed a bilayer structure at neutral pH, causing a broader emission peak than that of pure DPPC. However, when the protonation behavior of CHEMS changed, it did not cause a phase transition of the lipid system. 1,3-diolein-CHEMS moieties could have been separated from the DPPC bilayer structure that remained unaltered in an acidic environment.

Although the goal of the work was to study how pH affects liposomal membrane, heating experiments provided additional information of the phase behavior. Figure 30 shows that 1,3-diolein-CHEMS liposomes respond to heating differently from DPPC vesicles. For both Laurdan and Laurdan-C containing DPPC liposomes, collapse of GP values is observed at ca. T = 42-43 °C where the phase transition takes place [13]. However, for Laurdan containing 1,3-diolein-CHEMS liposomes the GP value decrease slowly from 0.34 at T = 25 °C to 0.16 at T = 70 °C. This indicates that the phase transition is not as sharp as for DPPC liposomes.
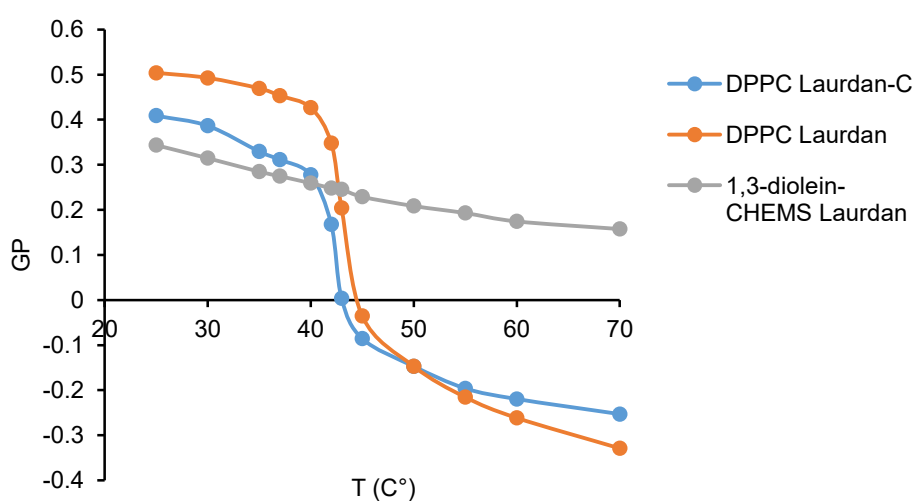

Figure 30. GP values of Laurdan/Laurdan-C containing reference liposomes and 1,3 diolein-CHEMS liposomes during heating.

9.3.2 Liposomal response for lowered pH of external solution

To obtain a homogenous series, liposomes were prepared in acidic conditions that are not suitable for drug delivery. Therefore, in addition to the homogenous series, phase behavior of Laurdan containing 1,3-diolein-CHEMS liposomes was examined upon lowering the pH of an external solution. For the experiments, the liposomes were prepared at pH 7.4. The results are presented in the Figures 31-33. Although the emission intensity dropped at an acidic pH, the changes are not as remarkable as those obtained by the homogenous series. After two weeks storage at RT the shape of the peaks at pH 5.5 and 5 were quite similar to the homogenous series. White precipitates were observed by naked eye. It is interesting that after one week at RT, the emission of pH 5.1 sample is lower than that of pH 4.5 sample. According

to Lee & Thompson [67] the ability of diacylglycerols to promote bilayer destabilization, including phase transition to inverted hexagonal phase, and vesicle fusion is maximized at pH 5. Therefore, the pH sensitive point of the formulation could be around 5.
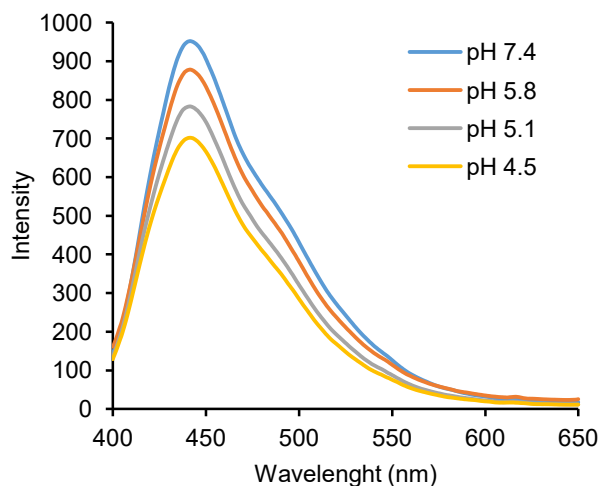

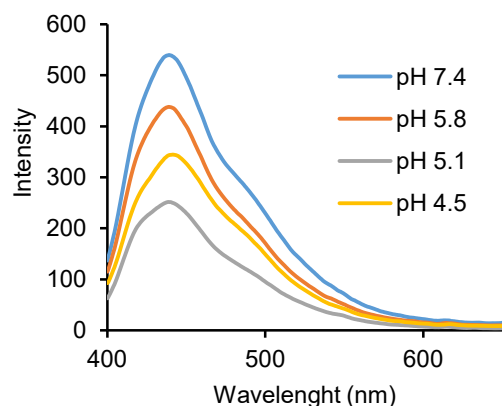Figure 31. Emission spectra of 1,3-diolein-CHEMS-Laurdan sample at different pH.

Figure 32. Emission spectra of 1,3-diolein-CHEMS-Laurdan samples after one week at RT.
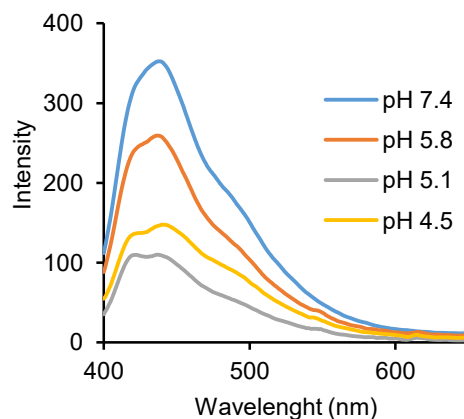
Figure 33. Emission spectra of 1,3-diolein-CHEMS-Laurdan samples after two weeks at RT.

The GP values of the samples increased slightly upon acidification as shown in Figure 34. However, below pH 5 the values started to decrease after ca. 24 hours of preparation. Also, at pH 5 the GP value decreased slightly after two weeks at RT. Interestingly, this deviates from the results obtained from the homogenous series, where a slight decrease in the GP values was observed. However, when the shape

of the peaks become similar to that of the homogenous series, also the GP values start to decrease. One possible reason could be that first the lower pH leads to tighter packing of the lipid membrane. After that, the sample starts to degrade and undergoes a phase transition.
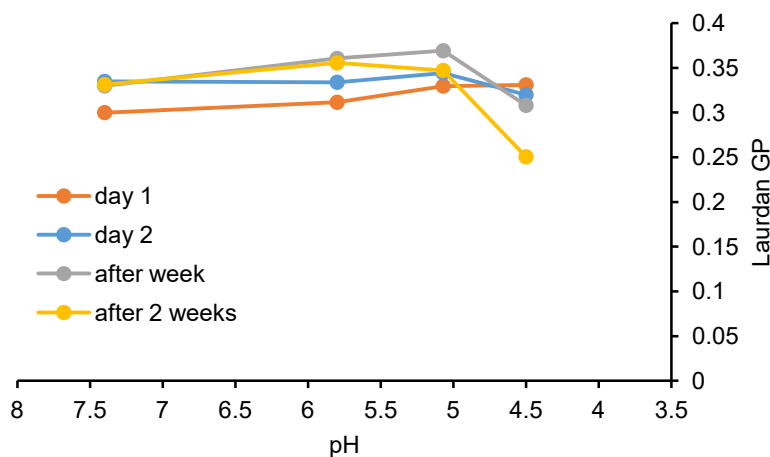

Figure 34. Laurdan GP values of 1,3-diolein-CHEMS systems.

pHrodo red was used to study proton transfer across the liposomal membrane. Figures 35-36 show that particularly at low pH there were differences between 1,3-diolein-CHEMS and DPPC liposomes. In the case of DPPC liposomes, the changes in emission intensity are smaller, which is probably due to a higher pH inside the liposomes. The membrane of 1,3-diolein-CHEMS liposomes is more permeable towards H^+ ions and therefore the intracellular pH becomes more acidic as pH of the external solution drops. The sensitivity of pHrodo red declines towards pH 4, as shown in the Figure 37. Thus, the emission intensities overlap for DPPC-pHrodo red system at pH 5, 5.5 and 4.5. Because of a lower intracellular pH, the emission intensities of pHrodo red in 1,3-diolein-CHEMS liposomes were distinguishable also at low pH.

Overall, the emission spectra of pHrodo red containing 1,3-diolein-CHEMS liposomes are more close to pHrodo red in buffer than that of DPPC liposomes. This supports the suggestion that the membrane of 1,3-diolein-CHEMS liposomes is more permeable towards H^+ ions than the DPPC membrane. Because of relatively small differences in the intensities, the measurements were repeated with fresh liposome batches to confirm the results. The average diameter of pHrodo red

containing 1,3-diolein-CHEMS liposomes increased upon acidification. The initial diameter was 206 nm at pH 7.2 whereas at pH 4.9 the diameter was 486 nm.
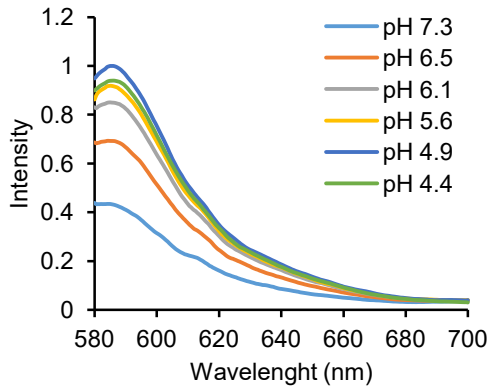

Figure 35. pHrodo red containing 1,3-diolein-CHEMS liposomes at different pH.
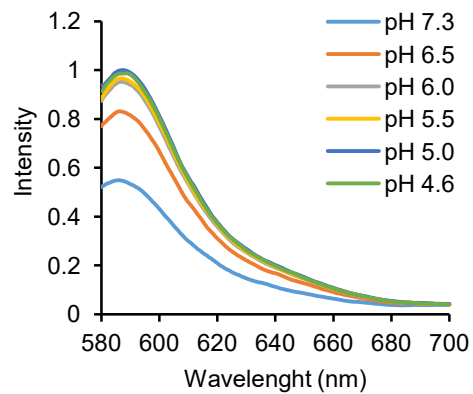
Figure 36. pHrodo red containing DPPC liposomes at different pH.
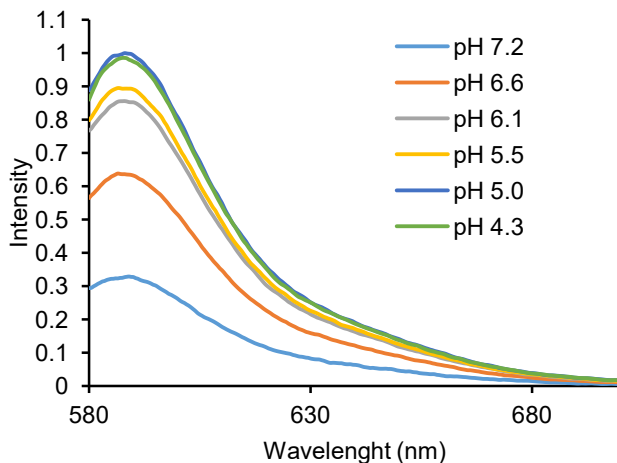
Figure 37. pHrodo red in PBS buffer.

It is worth noting that the phase transition occurs more slowly than charge transfer across the membrane. The change in the phase behavior is not close to the homogenous sample series until after two weeks at room temperature. Therefore, it is possible that release occurs already at the pre-phase transition. Another option is that the liposomes encapsulate the drug but the release remains inefficient. In cancer treatment, the goal of pH sensitive liposomes is to release their content already in endosomes, before possible degradation in lysosomes [68] [41]. For this,

the phase transition is too slow, at least if the drug release is not sufficient already during pre-phase transition.

To obtain a more profound understanding about the drug release and its connection to lipid membrane behavior, calcein release experiments were performed. Calcein fluorescence showed pH dependency and therefore different sample preparation procedures were tested. With an adequate dilution, the effect of pH on calcein fluorescence was minimized.

Calcein fluorescence assay showed that calcein was encapsulated efficiently in the 1,3-diolein-CHEMS liposomes (Figure 38). At pH 7.25, before addition of Triton-X, fluorescence intensity of the sample was small. Triton-X ruptured the lipid membrane, which resulted in calcein release from the liposomes. Calcein release was observed as an increased fluorescence intensity. The capability to encapsulate calcein also indicates that 1,3-diolein-CHEMS lipid systems form vesicle structures in a neutral environment.

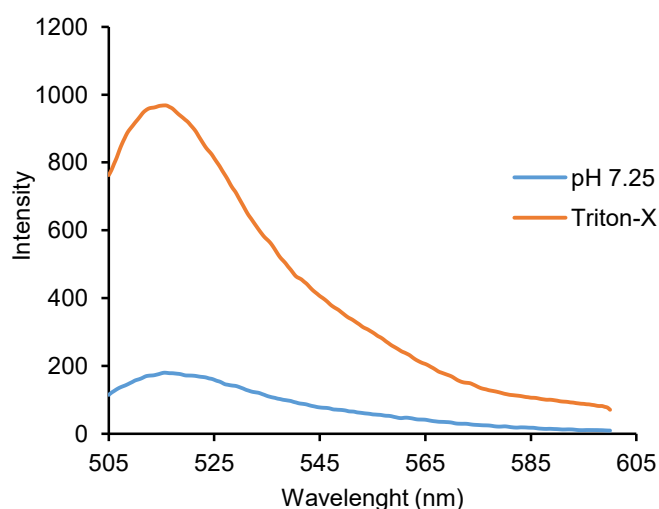Figure 38. Calcein release from 1,3-diolein CHEMS liposomes after addition of Triton-X.

After one hour incubation at T = 37 °C 10.1 % of encapsulated calcein was released at pH 4.8. At pH 7.3, the release was 4.1 %. After one day storage at RT the release percentage was 37.8 % at pH 4.8 and 19.4 % at pH 7.3. All the results are presented in Table 5. The release percentages were calculated from eq. 2. The

release was well below the release percentages obtained by Guo *et a*l. [14] who used the same lipid combination in pH sensitive liposomes. It is possible that the effect of pH on calcein fluorescence was not completely eliminated. The lower release percentage could also have been due to different liposome preparation method or dilution of the sample solution upon HCl addition. In addition, the fluorescence following Triton-X addition was varying upon time. Therefore, the release percentages presented in the table are only approximate.

Table 5. Calcein release from 1,3-diolein-CHEMS liposomes

Time	pH	Release %
Day 1	4.8	10.1
Day 1	7.3	4.1
Day 2	4.8	37.8
Day 2	7.3	19.4
Day 1	5	8.5
Day 1	7.3	2.3

To obtain more reliable results, some calibration method could be used to remove the effect of pH on calcein fluorescence. Also, the formulation of the liposomes could be improved to minimize drug leakage at neutral pH and improve the release under acidic conditions.

10 Conclusions

As a summary, DLS and fluorescence experiments indicate that 1,3-diolein-CHEMS liposomes become unstable below pH 6.5. Size enlargement begins at pH 6 and becomes even more remarkable as pH drops. In a similar fashion emission spectra of homogenous 1,3-diolein-CHEMS fluorescence samples show little variation between pH 6.5 and 7.4. Yet, at pH 6, the intensity drops significantly and shape of the peaks change. Fluorescence experiments carried out with pHrodo showed that the membrane of 1,3-diolein-CHEMS liposomes is more permeable towards H^+ ions than the DPPC membrane. Thus, in acidic environments liposomes aggregate and their phase behavior changes. The results also proved that Laurdan is a suitable fluorescence probe to study pH induced changes in liposomal membrane.

The phase transition of 1,3-diolein-CHEMS liposomes deviates from that of light or heat activated liposomes [13]. The differences are due to the different lipids used in the formulations. Unlike phospholipids, the lipids adopting inverted phase structures can undergo acid induced destabilization. Instead of a gel to a liquid crystalline phase transition, 1,3-diolein can transform from a bilayer to an inverted hexagonal phase [14]. The changes in the phase behavior are observed as collapsed Laurdan emission intensity.

For further research, a microscope technique such as Cryo-transmission electron microscopy (Cryo-TEM) could be utilized to acquire more information about the lipid structures and to confirm formation of inverted hexagonal phase structures. Cryo-TEM technique is suitable for aqueous, dilute solution [35]. In addition, it does not rupture the lipid vesicles [66].

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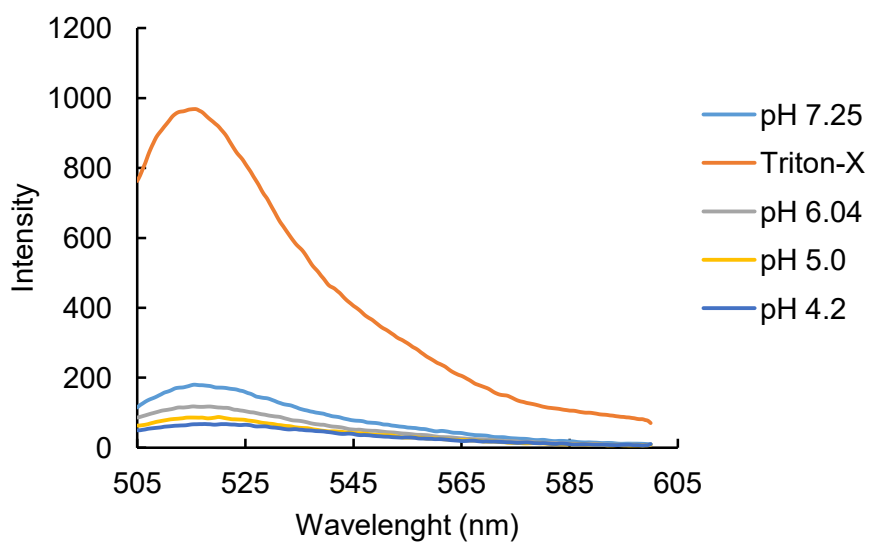
Figure. Fluorescence spectra of 1,3-diolein-CHEMS calcein samples at different pHs, when the effect of pH on calcein fluorescence was not minimized. Complete calcein release was achieved with Triton-X.