Physico-Chemical Investigations of Bilayer Discs and Related Lipid Structures Formed in Liposomal Systems Intended for Triggered Release

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Abstract

This thesis describes results from fundamental studies of liposomes intended for drug delivery and pH or temperature triggered release. In addition, the effect of lipid composition on bilayer disc formation and a potential application of the bilayer discs were investigated.

The lower pH encountered by endocytosed liposomes can be utilized to trigger drug release. The mechanisms behind cytosolic drug delivery were investigated using two different kinds of pH-sensitive liposomes. The results indicate that incorporation of non-lamellar forming lipids into the endosome membrane may allow for drug escape into the cytosol.

Temperature-sensitive liposomes containing lysolipid (LTSL) release their content almost instantly when heated to temperatures close to the gel to liquid crystalline phase transition temperature (Tc). Morphological changes of the liposomes in response to temperature cycling were studied. Temperature cycling induced liposome openings and disintegration of the liposomes into bilayer discs. Incubation of LTSL in the presence of multilamellar liposomes (MLVs) resulted in relocalisation of lysolipid into the MLVs, which affected the rapid release from LTSL. We propose that the presence of micelle-forming components, such as lysolipids and PEG-lipids, facilitates the formation of defects and membrane openings during the initial phase of membrane melting, resulting in the observed rapid release. Similar to added lysolipids, also hydrolysis generated lysolipids induce disc-formation upon heating through Tc of the lipid mixture.

Two fundamentally different micelles may form in PEG-lipid/lipid mixtures. We found that discoidal structures are preferred over cylindrical micelles when the mixture contains components that reduce the spontaneous curvature, increase the monolayer bending modulus, or reduce PEG-lipid/lipid miscibility. The large discoidal micelles found at low PEG-lipid content are better described as bilayer discs. We evaluated such discs as model membranes in drug partitioning studies, and suggest that they, in some cases, produce more accurate data than liposomes.

Keywords: liposome, triggered release, pH-sensitive liposomes, temperature-sensitive liposomes, drug partitioning, cryo-TEM, discs

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Abbreviations

PC  Phosphatidylcholine
PE  Phosphatidylethanolamine
PS  Phosphatidylserine
PG  Phosphatidylglycerol
SM  Sphingomyelin
DPPC  1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine
DSPC  1,2-distearoyl-sn-glycero-3-phosphatidylcholine
DMPC  1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine
EPC  Egg phosphatidylcholine
MSPC  1-stearoyl-2-hydroxy-sn-glycero-3-phosphatidylcholine
MPPC  1-palmitoyl-2-hydroxy-sn-glycero-3-phosphatidylcholine
POPC  1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine
DOPC  1,2-dioleoyl-sn-glycero-3-phosphatidylcholine
DSPE  1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine
DOPE  1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine
PEG  Polyethylene glycol
OA  Oleic acid
CHEMS  Cholesterylhemisuccinate
DHCho-MPEG\textsubscript{5000}  1´-(4´-dihydrocholesteryloxy-3´-buenyl)-ω-methoxy-poly(ethylene glycol 5000)ate
CF  5(6)-carboxyfluorescein
ANTS  8-aminonaphthalene-1,3,6-trisulfonic acid
DPX  p-xylene-bis-pyridium bromide
NBD  N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)
Rh  Lissamine\textsuperscript{a} rhodamine B
PHDA  1-pyrenehexadecanoic acid
LTSL  Lysolipid-containing thermosensitive liposomes
TSL  Thermosensitive liposomes containing
dDPPC/DSPE-PEG\textsubscript{2000}
NTSL  Nonthermosensitive liposomes containing
dDSPC/Chol/DSPE-PEG\textsubscript{2000}
<table>
<thead>
<tr>
<th>Acronym</th>
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<tr>
<td>SUV</td>
<td>Small unilamellar liposomes</td>
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<tr>
<td>LUV</td>
<td>Large unilamellar liposomes</td>
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<tr>
<td>MLV</td>
<td>Multilamellar liposomes</td>
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<td>RES</td>
<td>Reticuloendothelium system</td>
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<td>MPS</td>
<td>Mononuclear phagocyte system</td>
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<td>EPR</td>
<td>Enhanced permeability and retention</td>
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<td>cryo-TEM</td>
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<td>CEVS</td>
<td>Controlled environmental vitrification system</td>
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<td>DLS</td>
<td>Dynamic light scattering</td>
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<td>DSC</td>
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1 Amphiphilic molecules and self-assembly

This thesis deals with self-associated colloidal aggregates composed of amphiphilic molecules. Amphiphilic molecules are special in the way that they have a dual character. They consist of one hydrophilic, water soluble, part and one hydrophobic, oil soluble, part (Fig. 1).

![Hydrophilic part](image1)

![Hydrophobic part](image2)

Fig. 1. Schematic figure of an amphiphilic molecule.

When dissolved in either a very polar or very non-polar media, one part of the molecule interacts favorably with the surrounding solution while the other does not. Above a threshold concentration the amphiphiles may self-aggregate. In the case of a polar medium, like water, the hydrophobic moieties are embedded inside the aggregates surrounded by the hydrophilic parts. The main driving force for such assembly in water is the strong cohesion between the water molecules caused by hydrogen bonding. When hydrophobic molecules, like hydrocarbons, are embedded in water they disrupt the network of hydrogen bonds formed by the water molecules, since no hydrogen bonds can be made with the solutes. This forces the water molecules to order themselves around the aliphatic parts. Segregation of surfactants into aggregates embedding the hydrophobic parts in their interior results in a large positive entropic gain by disordering the water molecules [1]. This entropy driven association is called the hydrophobic effect.

1.1 Aggregate structure

Depending on a number of factors, such as amphiphile properties and the intermolecular forces involved, a number of more or less intricate aggregate structures may form upon self-aggregation. By considering three different geometric parameters of the surfactant, a reasonably good prediction can be made of the preferred overall aggregate structure. These geometric parame-
ters are the effective head group area, the volume and the critical length of the hydrophobic part, denoted $a_0$, $v$ and $l_c$ respectively. Relating the amphiphile shape parameters to each other gives the packing parameter, $N_s$ [2]:

$$N_s = v / (a_0 \times l_c)$$

Different aggregates are predicted depending on the magnitude of $N_s$ as illustrated in Figure 2.

Despite the crudeness of this shape method, it is very useful when predicting aggregate structure as a result of changes in solution properties such as electrolyte concentrations, pH and temperature.

![Molecular shape and packing parameter vs Preferred aggregate structure](image)

Fig. 2. The preferred aggregate structure and the geometrical shape of the surfactant.
1.2 Phase behavior
At higher surfactant concentrations, the aggregates formed can organize into liquid crystalline phases. The phase structure is dependent on the individual molecular shape and intra-aggregate interactions, and on the inter-aggregate forces that become considerable at high surfactant concentrations. A rich variety of phases often appear when, for instance, the water content is changed in a surfactant/water mixture. A hypothetical phase sequence as the amount of surfactant is increased is illustrated schematically in Figure 3. The Roman numerals I and II specify normal or inverted aggregate structures or, alternatively, aggregates with positive or negative curvature. By convention, the definition of positive curvature is when the polar interface of the aggregate is curved towards the non-polar medium and negative curvature when the polar interface is curved towards the polar medium.

\[
\begin{align*}
L_I &\rightarrow I_I \rightarrow H_I \rightarrow Q_I \rightarrow \text{L}_u \\
L_{II} &\rightarrow Q_{II} \rightarrow H_{II} \rightarrow I_{II} \rightarrow \text{L}_{II}
\end{align*}
\]

Fig. 3. A hypothetical phase sequence as a function of surfactant concentration. L denotes a micellar phase, I a micellar cubic phase, H a hexagonal phase, Q a bicontinuous phase and L_{II} the lamellar phase. I and II specify normal or inverted aggregate structures, respectively.

1.3 Phospholipids
Phospholipids are the main lipid components in most cell membranes. They consist of a polar head group containing a phosphate attached to an apolar hydrocarbon part (Fig. 4). The biologically most abundant phospholipids are glycerophospholipids. In these lipids the head group and the hydrocarbon chains are attached via ester bonds to a glycerol backbone. The head group composition may vary, but two major classes of glycerophospholipids are the phosphatidylcholines (PC) and the phosphatidylethanolamines (PE). The PC- and PE-lipids are zwitterionic over a broad pH-interval. However, at high pH the amine of the PE head group, which has a pK_a of about pH 9 [3], is deprotonated. This may result in interesting phase changes. The hydrocarbon part of the phospholipids is often comprised of two aliphatic chains that can vary in length and saturation. Lysolipids on the other hand contain only one hydrocarbon chain.
A number of both synthetically and naturally derived lipids have been used in the work presented here. A schematic figure of the lipids is shown in Figure 4.

![Figure 4](image_url)

**DMPC**: $R_1=R_2=C_{14:0}$

**DPPC**: $R_1=R_2=C_{16:0}$

**DSPC**: $R_1=R_2=C_{18:0}$

**EPC**: Average $R_1=C_{16:0}$, $R_2=C_{18:1}$

**MSPC**: $R_1=C_{16:0}$, $R_2=OH$

**DSPE**: $R_1=R_2=C_{18:0}$

**DOPE**: $R_1=R_2=C_{18:1}$

**Fig. 4.** The structure of the phospholipids used in the present investigations.

### 1.4 Phospholipid lamellar phases

Phospholipids can form a variety of aggregate structures in the presence of water. Generally the geometrical shape of phospholipids is, however, cylindrical, and hence aggregation into bilayers is preferred. Bilayer forming phospholipids tend to have a low solubility owing to their comparably long hydrocarbon chains. Phospholipid lamellar phases exhibit several thermotropic phase transitions. At low temperature the bilayers are in a lamellar crystalline phase, $L_C$ or $L'_C$, with a dense crystalline structure. The symbol ‘ indicates a phase with tilted lipids. As the temperature is increased, conformational chain excitations increase and at a certain temperature, the subtransition temperature, the bilayer transforms into a lamellar gel phase, $L_E$ or $L'_E$. In this phase the lipids are less tightly packed than in the crystalline phase, but there is still considerable chain order, with most of the chains in the all-trans configuration. Continuing to increase the temperature further increases the motion of the aliphatic chains until the order is lost. The lipids are said to melt and the bilayer transforms into the liquid crystalline phase, $L_0$, in a cooperative manner at the so-called gel to liquid crystalline phase transition temperature, $T_C$. The $L_0$ phase is characterized by rapid chain trans-gauche isomerizations and rapid lateral lipid diffusion. Membranes with a rippled bilayer surface can be found for certain classes of lipids, such as PC- and phosphatidyglycerol (PG)-lipids, between the gel and liquid crystalline phases [4].

The temperature of the phase transition depends on several factors, such as the head group structure and charge, and the length and degree of saturation of the acyl chains. Lipids with long and/or saturated chains have higher melting temperatures, owing to the high interaction energy between the chains. Consequently, unsaturated lipids will melt at lower temperatures because of the inherently lower packing order in these membranes. Further, PE-lipids display higher transition temperatures than the corresponding PC-
lipids. The PE-head groups interact more strongly, most likely via hydrogen bonding, with neighboring PE-lipids and are less hydrated than PC-lipids [3], which has the effect of raising the transition temperature.

1.4.1 Cholesterol in phospholipid lamellar phases

Cholesterol is a major component in all plasma membranes of eukaryotic cells. Its presence in membranes has profound effects on several of their physical characteristics. The cholesterol molecule has a small hydrophilic hydroxyl head group and the hydrocarbon part contains a large, stiff ring system (Fig. 5). This molecular shape is different from that of both conformationally ordered and disordered lipid chains and will, in either case, affect the lipid packing when incorporated into a bilayer. When situated in a gel phase bilayer, the sterol will tend to break the lateral packing order and induce more disordered lipid acyl chains, while the opposite is true when incorporated into a fluid membrane [5,6]. At about 30 mol% of cholesterol the bilayer adopts a new phase, the so-called liquid ordered phase, l, [6,7]. The liquid ordered phase has mixed properties of the liquid crystalline and gel phases, with rapid lateral diffusion and at the same time a high lipid chain conformational order [6,7].

Fig. 5. The molecular structure of cholesterol.

1.5 Nanosized lamellar aggregates

1.5.1 Discs

More or less circular bilayer fragments, discs (Fig. 6), have been predicted based on theoretical arguments [8,9], and also documented in several experimental studies in various amphiphilic systems [10-13]. Normally, bilayer discs are very short lived owing to the unfavorable exposure of the hydrocarbon chains at the rim of the aggregate to the polar environment. The fragments therefore tend to fuse and grow, producing large bilayers, and/or close upon themselves forming liposomes. However, this process appears to be
prevented or retarded in the presence of certain surfactants. The surfactants act as edge-actants and accumulate at the rims of the discs creating a protective hemispherical cap around the disc. Hence, the formation of discs is a consequence of component segregation. Obviously, this is associated with a significant free energy penalty in the form of reduced mixing entropy. The reduced mixing entropy is counterbalanced by the relieved curvature constraints experienced by the segregated membrane components. At the edge, the surfactant may adopt a preferred micellar-like conformation while bilayer-forming components constitute the flat part of the disc.

Fig. 6. A schematic figure of a disc. Dark grey amphiphiles correspond to edge-actants with a preferred micellar-like conformation. © Göran Karlsson.

1.5.2 Liposomes
Liposomes are artificial spherical self-closed colloidal particles composed of lipid bilayers with a solvent filled interior (Fig. 7) [14]. The size of the liposome and the lamellarity, number of bilayers surrounding the aqueous interior, can vary substantially. Depending on these factors the liposomes are referred to by various names such as multilamellar vesicles, MLV, large unilamellar vesicles, LUV, and small unilamellar vesicles, SUV. The ability of liposomes to encapsulate polar solutes in their interior or incorporate hydrophobic substances in the membrane, and their similarity to cell membranes, have made them interesting for applications in several areas from medicine and cosmetics to food technology and ecology.
Generally, liposomes are not thermodynamically stable structures and hence some energy must be added to the system for them to form. However, the existence of thermodynamically stable liposomes has been suggested [15-18]. The mechanism of liposome formation has been discussed and can be generalized as follows [19-21]. The formation of vesicles is a result of closure of bilayer fragments as dictated by the energy balance between edge tension, due to the exposure of hydrophobic bilayer edges, and curvature energy, due to the cost of bending the bilayer. The edge tension is expressed as:

\[ E_b = \gamma 2\pi r (1 - (r^2 / 4R^2))^{1/2} \]

where \( \gamma \) is the effective edge tension, \( r \) the circular bilayer fragment radius and \( R \) the radius of curvature (Fig. 8).

The fragment strives to minimize edge area and will do so by bending the bilayer inducing a curvature energy contribution. The elastic curvature energy is expressed as:
where \( k_c \) is the bending modulus. The total excess energy of the closed vesicle \((r = 2R)\) may then be written as:

\[
E_e = 2k_c \pi r^2 / R^2
\]

The excess energy of the vesicle is independent of size, and hence large liposomes are preferred energetically whereas many small ones are entropically favored. The value of \( k_c \) for membranes containing the most common PC lipids is in the order of \(10 – 35 \text{ kT} \) [22] from which it follows that the vesicles are thermodynamically metastable aggregates. However, they are stable for some extended time (days to even years) due to the large cost of creating a pore in the bilayer and initialize vesicle destabilization and subsequently the formation of lamellar phase.

**Liposomes for drug delivery**

When a conventional drug is added intravenously it spreads out in the system with no specific targeting and sometimes with toxic side effects. To achieve therapeutic levels of a drug at a target site while at the same time avoiding toxic side effects can be a problem. One solution to this problem may be to encapsulate the drug in a biocompatible carrier that can deliver a large amount of a drug specifically to the site of disease. Liposomes have proved promising as such delivery vehicles.

Various types of drugs can be transported with liposomes. Hydrophobic drugs can be included in the lipid bilayer and hydrophilic drugs can be encapsulated in the liposome water compartment. In the latter case efficient drug loading methods have been developed [23,24]. Often an ion concentration gradient across the membrane is being exploited, such as a pH gradient, inducing transport of the drug into the liposome.

When administered in the blood compartment the liposomes must stay intact and not release the drug instantaneously. Interactions with lipoproteins or phospholipases present in the plasma may be detrimental in this aspect. Depending on liposome distribution and drug pharmacodynamics, different drug release profiles are wanted. In some cases the drug is required to leak out slowly from the liposomes leading to a more or less constant level of free drug. However, often the drug is needed to be retained within the liposomes while in circulation and then rapidly be released at a target site, such as a tumor. To reach the target site the circulation time must be sufficiently long. One critical problem, encountered early in the development of liposomes as
drug delivery vehicles, is the fast elimination of liposomes from the blood circulation as they are recognized by the host defense system as foreign particles. One important aspect of liposome clearance from the blood stream appears to be the adsorption of certain proteins, opsonins, onto the liposome surface. These mark the liposomes for destruction by the mononuclear phagocyte system (MPS), or reticuloendothelial system (RES). The circulation time may however be significantly prolonged by including polymer-grafted lipids, such as polyethylene glycol (PEG)-lipids, in the liposome bilayer [25-27]. This effect has been explained by a reduction of protein interactions with the liposome surface and consequently macrophage resistance [28]. However, there seems to be a more complicated picture behind the increased circulation time observed for stERICally stabilized liposomes [29]. Steric stabilization is further discussed below.

With increased circulation time the probability of the liposomes accumulating in tissue with leaky vasculature increases. Inflamed tissues and solid tumors have defective blood vessels with large gaps between the endothelial cells, allowing for relatively large particles to extravasate into the interstitial space [30,31]. Further, tumors have defective lymphatic drainage [32] and vessels that end in loops or dead ends and hence particles such as liposomes concentrate in these areas. This effect is called the enhanced permeability and retention effect (EPR) and is the cause of so-called passive targeting of drug carriers.

Passive accumulation of liposomes in diseased tissues does not necessarily mean that the liposomes are internalized by the cells, nor is the treatment of separate cells efficient. Developments to further improve target specific drug delivery has utilized the attachment of ligands or antigens, directed towards specific or overexpressed cell surface receptors, onto the liposome surface [33]. This is referred to as active targeting. Directing the liposomes to cell surface receptors may result in internalization via endocytosis, which may be a significant advantage.

Triggered release
When drug release during circulation is unwanted it is important that the liposomes stay intact until they reach the target tissue. A fast and controlled release resulting in high local drug levels is often preferable since the substance is normally inactive inside the carrier. Different approaches to achieve site specific release have been proposed [34]. The use of external triggers in the form of light or heat sources to destabilize the liposomes have been utilized in light-sensitive liposomes [35,36] and temperature-sensitive liposomes [37-41] respectively. The use of an external trigger may however, be rather limited to spatially well defined targets and it would sometimes be more preferable to exploit naturally occurring changes which could be used as a trigger. Such triggers could be tissue specific enzymes [42,43] or pH-changes [44-46].
Stability
Liposome shelf life is limited since physical and chemical degradation occurs with time. Examples of physical degradations are liposome fusion, aggregation or leakage of encapsulated molecules. Chemical degradation can occur through lipid oxidation or hydrolysis, which may affect the physical integrity of the liposomes.

Oxidation
Lipid oxidation occurs via a free radical chain mechanism, catalyzed by e.g. metal ions, radiation or sonication. Lipid oxidation may result in chain scissions or the formation of cyclic peroxides. All lipids are susceptible to oxidation but lipids with unsaturated fatty acyl chains are particularly sensitive, and the more unsaturated the higher the oxidation rate [47]. In order to minimize oxidation samples should be stored in the dark at low temperature and protected from oxygen. The use of highly purified and saturated lipids further decreases the risk of oxidation. Additionally, oxidation rates may be significantly decreased by the addition of antioxidants [47].

Hydrolysis
In aqueous environments lipids in liposome dispersions can also be subjected to degradation through hydrolysis. The most common lipids in pharmaceutical liposome formulations are glycerophospholipids and the discussion of hydrolysis will thus focus on these lipids. All four ester bonds present in phospholipid molecules are susceptible to hydrolysis. However, the carboxy esters at sn-1 and sn-2 position are by far more prone to hydrolysis than the phosphate esters [48]. Hydrolysis products after cleavage at the sn-1 or sn-2 positions are initially 2-acyl and 1-acyl lysophospholipids respectively and the corresponding fatty acid (Fig. 9). Further hydrolysis of the lysolipid generates another fatty acid and a glycerophospho compound. The last step is hydrolysis of the phosphate head group. Under pharmaceutical relevant conditions glycerophosphoric acid is the end product.
Fig. 9. Hydrolysis scheme of PC-lipids.

The hydrolysis follows pseudo first-order kinetics and depends on temperature according to the Arrhenius equation and is thus significantly reduced at lower temperatures [49,50]. Further, hydrolysis is highly pH dependent being catalyzed in both acidic and basic environment with a minimum around pH 6.5 [49,50].

Apart from being spontaneously hydrolyzed, the ester bonds in phospholipids may also be cleaved by specific enzymes, phospholipases [51]. This may be utilized when designing liposomes for drug delivery applications. It has for example been shown that phospholipase A₂, PLA₂, is over expressed at certain sites of disease [52,53] and may be used as a natural trigger for site specific liposomal drug release [42,54].

Steric stabilization

The interaction between liposomes is a sum of several individual interaction parameters. There is an attractive van der Waals force, counterbalanced by various repulsive forces such as electrostatic, undulation and hydration interactions. The contribution from each of these forces depends on the liposome composition and environment conditions. Liposomes with charged mem-
brane components do not easily aggregate due to electrostatic repulsion while membranes containing uncharged lipids and cholesterol readily aggregate as both electrostatic and undulation repulsions are minimized.

Enhanced colloidal stability can be achieved through steric repulsion if a polymer is either grafted or adsorbed on the liposome surface [55,56]. Liposomes are often sterically stabilized by inclusion of PEG grafted lipids in the membrane (Fig. 10) [25-27]. The repulsive force arises when the polymer layers of two liposomes begin to overlap. The chemical potential of water will be lower in the interaction region resulting in a repulsive osmotic pressure. Further, the conformational freedom of the polymer segments will be decreased giving rise to a repulsive contribution of entropic origin. Similarly, when a large protein approaches a PEG-ylated liposome entropic and osmotic repulsion will impede protein adsorption.

Fig. 10. Schematic figures of a sterically stabilized liposome and a PEG-modified lipid. © Göran Karlsson.
2 Present investigations

2.1 Aims

The aim of paper I was to increase the fundamental knowledge about the release from DOPE-based pH-sensitive liposomes and also evaluate a new cleavable PEG-lipid as membrane stabilizer. Further, despite a vast amount of studies in the area of pH-sensitive liposomes detailed knowledge about the actual mechanisms of cytosolic drug delivery is still lacking. Hence, studies of interactions between pH-sensitive liposomes and endosome-like membranes are of importance for the design of potent drug carriers.

A new type of temperature-sensitive liposomes with lysolipids in the membrane exhibits very promising characteristics from a drug delivery perspective. They release their drug cargo very fast at temperatures easily attainable clinically. Further, they have a significant effect on tumor growth inhibition. The mechanisms behind the rapid release, which are interesting also from a fundamental point of view, have not been thoroughly investigated. The focus of papers II and III was to elucidate possible drug release mechanisms from lysolipid containing temperature-sensitive liposomes, LTSL. Morphological changes of the liposome structure with respect to temperature changes were studied in paper II. Further, paper III focused on membrane component segregation and retention in these liposomes.

The results of paper II lead us to believe that also hydrolysis generated membrane components can affect the morphology of gel phase liposomes. This may have serious implications on the evaluation and performance of liposomes intended for various applications. We found in paper II that PEG-lipids promote disc formation. Further, most liposomes intended for drug delivery are PEG-ylated. Hence, the morphological effect of hydrolysis was investigated in PEG-stabilized gel phase liposomes as presented in paper IV.

In papers II and IV, bilayer discs were observed as a result of temperature cycling and hydrolysis. This intrigued us to explore more specifically under which conditions discs may form in PEG-lipid/lipid mixtures. This is the focus of paper V. Furthermore, the discs are interesting for various biotechnical applications. Paper VI addresses one of these applications, i.e., the use of the discs as membrane models for studies of drug/membrane interactions.
2.2 Experimental techniques

2.2.1 Cryo-TEM

Cryo-TEM is a powerful technique which allows for direct imaging of the aggregates formed by amphiphilic molecules in aqueous solutions [57]. Sample perturbing preparation steps, such as drying and staining, are avoided. Instead, a thin sample film, 10-500 nm, is rapidly cooled in liquid ethane producing a vitrified water matrix rather than crystalline ice formed during slow cooling. In the course of rapid cooling it is also unlikely that any important reorganization of the large aggregates occur. The thin amorphous matrix allows for electrons to pass readily through it. Visualization of the amphiphilic aggregates is then possible due to the difference in electron density between water and the atoms making up the aggregates. Figure 11 shows how to translate the obtained 2D images into 3D objects [58].

![Diagram showing cryo-TEM process]

Fig. 11. The two uppermost figures are schematic representations of the vitrified sample film and the corresponding 2D image, respectively. The bottom figure is a cryo-TEM picture displaying discs from various angles from flat on to edge on, and liposomes. © Göran Karlsson.
The lower resolution limit is around 4 nm. Hence, surfactant micelles are only seen as dots. Under specific conditions, however, the two leaflets of liposome bilayers may be resolved. An upper limit is set at around 500 nm due to the film thickness. A thicker film may be difficult to vitrify properly and also electron scattering from water becomes more pronounced. Further, in a thick film aggregates residing at different depths enhance electron scattering and blur the contrast. A high sample concentration will similarly cause crowding and contrast blurring and, in addition, a too high sample viscosity makes it difficult to produce the thin sample films. Hence, cryo-TEM is limited to dilute aqueous solutions, >95 wt% water.

When producing a sample film special care is needed to control the environmental conditions. Hence, sample preparation is made in a controlled environmental vitrification system (CEVS) (Fig. 12). A small copper grid covered with a holey polymer film is used as support for the sample film (Fig. 12). A small sample droplet is placed onto the grid and excess solution is blotted away with a filter paper leaving a thin, 10-500 nm, layer. This procedure is conducted in a chamber with controlled temperature and humidity. The humidity is kept high to avoid water evaporation from the film. The grid is then plunged into liquid ethane for vitrification. Finally, the grid is mounted into a sample holder and transferred into the microscope. All handling of the vitrified sample is performed at a temperature below 108 K to prevent ice crystal formation and sample perturbation.

Fig. 12. A schematic representation of the sample preparation chamber and a polymer covered grid. © Göran Karlsson.
Artefacts in cryo-TEM

In order to interpret cryo-TEM images correctly, knowledge about possible structural artifacts is required. One common artifact is invaginated liposomes (Fig. 13A). This has been explained by an osmotic stress of the liposomes caused by water evaporation during the film preparation [57]. Evaporation results in a modified salt concentration in the thin film and as result water from within the liposomes leaks out causing liposome collapse. However, far from all salt containing liposome samples appear invaginated. Liposomes with rigid membranes may resist collapse and stay spherical. Such examples are gel phase liposomes and liposomes containing relatively large amounts of cholesterol (Fig. 13B).

Fig. 13. A) Cryo-TEM image of invaginated EPC liposomes in HEPES buffer containing 150 mM NaCl, B) Cryo-TEM image of liposomes composed of palmitoyl-oleoylPC (POPC):cholesterol (60:40 mol%) in phosphate buffer containing 150 mM NaCl.

A second artifact is size sorting. The sample film supported by the holey polymer network is not uniformly thick. In the middle the film is thinnest and grows in size towards the support. This will cause a non-uniform aggregate size distribution in the film. The larger aggregates are found in the thick part of the film and only small or flat objects may be found in the middle, thin, part. It is important to remember that very large aggregates will be excluded from the cryo-TEM sample. Based on these facts, care should be taken when estimating the size distribution from cryo-TEM micrographs. However, it has been found that for samples containing relatively small objects the size distribution found by cryo-TEM and dynamic light scattering agree [59].

2.2.2 Light scattering

When light is shone onto a colloidal solution some of the light will be scattered by the particles in the sample. This fact may be used to explore proper-
ties of the particles such as size distribution, molecular weight and particle interactions. The amount of scattered light is dependent on factors such as the size, number and shape of the scattering centers. This may be utilized when investigating a structural change in a sample. For example, when liposomes are solubilized into micelles the light scattered will be reduced. Such relative structural changes can be monitored by apparent absorbance, turbidity, measurements. A high turbidity indicates large particles in the solution, provided that the sample is non-absorbing. Relative aggregate sizes may also be measured using static light scattering at a constant angle. A high intensity results from a large portion of scattered light and hence, large particles reside in the solution. Turbidity measurements have been used in Paper I and static light scattering have been used in Paper I and IV.

Information on particle sizes may also be collected by measuring the time dependent intensity-fluctuations of the scattering from the sample, i.e. the dynamic light scattering (DLS). The variation of the intensity contains information about the random motion of the particles and may be used to determine the diffusion coefficient, D, of the particles. The measured diffusion coefficient may then be used to determine the hydrodynamic radii, \( R_h \), of the particles \textit{via} the Stokes-Einstein equation. Assuming spherical particles:

\[
D = \frac{k_B T}{6\pi \eta R_h}
\]

where \( k_B \) is the Boltzmann constant, \( T \) the temperature and \( \eta \) the viscosity of the solvent.

DLS has been used in Paper IV.

2.2.3 Fluorescence measurements

\textbf{Leakage measurements}

Investigations of liposome leakage were performed by encapsulating fluorescent probes in the liposome interior. Two different leakage assays based on fluorescence quenching were used in this thesis.

In Paper I leakage from pH-sensitive liposomes was studied by co-encapsulating 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and \( p \)-xylene-bis-pyridium bromide (DPX) in the liposomes [60]. This is achieved by preparing the liposomes in a buffer containing the probes in comparatively high concentrations. Unencapsulated probes are then removed by gel filtration. ANTS fluorescence is efficiently quenched by DPX when the two probes are in close proximity. As the liposomes start to leak and the probes are diluted into the external buffer a relief of the fluorescence quenching is seen. The fluorescence from ANTS is relatively insensitive to pH [60] which is a prerequisite when studying leakage at different pH values.
However, when studying leakage at pH 7.4 as in Paper III 5(6)-carboxyfluorescein (CF) was used as a probe. CF is not suitable for leakage at lower pH since the fluorescence intensity then is significantly lower [61]. CF is more than 95% self-quenched at a concentration of 100 mM. As the liposomes start to leak CF is diluted into the external buffer and the fluorescence intensity is increased. Liposomes encapsulating concentrated CF-solution were prepared as described above for ANTS/DPX.

**Lipid mixing**

Liposome fusion was monitored by studying the energy transfer between the two lipid coupled fluorophores N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD) and lissaminea rhodamine B (Rh) [62]. The emission band of NBD overlaps with the excitation band of Rh and if these molecules are spatially close together an effective energy transfer between them can occur. If one population of liposomes containing both of the probes is allowed to fuse with another population devoid of NBD- and Rh-lipids, the efficiency of energy transfer will be reduced. This is a consequence of lateral diffusion of the energy acceptor and donor in the bilayer of the newly formed liposome, which increases the spacing between the probes. Hence, monitoring the emission from NBD and Rh will reveal to what extent the lipids in the sample mix.

This assay has been used in Paper I.

**PHDA accumulation**

The pyrene labeled hexadecanoic acid, PHDA, was used as a marker to study distributional changes of membrane components. PHDA forms excited state dimers, excimers, when an excited and a ground state PHDA are in close proximity to each other [63]. When the membrane PHDA content is low enough the formation of excimers will mainly be possible due to PHDA membrane accumulation or segregation. The emission maximum of the excimers is different from that of the monomer [63]. Monitoring the ratio between the excimer and monomer (E/M ratio) gives information about the membrane distribution of PHDA.

This assay was used in Paper III.

2.2.4 Calorimetry

**Differential Scanning Calorimetry**

In a differential scanning calorimetry (DSC) experiment the temperature of the sample is continuously changed and the response of the sample relative to a reference is monitored. In the case of a reaction or physical reorganization of the sample heat will be evolved or consumed. This is measured as a difference between the sample and reference in heat needed to increase or
decrease the temperature according to the programmed temperature scan. An example is the gel to liquid crystalline phase transition of phospholipids bilayers which is an endothermic, heat-consuming, event and hence more heat is needed compared to the reference to increase the temperature to the same extent. The result of a DSC scan is a heating and cooling curve. Peaks appearing correspond to physical changes or reactions and may be used to determine for instance the enthalpy of the change and at which temperature it occurs. In the field of liposome science, DSC is most commonly used to determine phase transitions and so in this thesis.

DSC was employed in Paper III and IV.

**Isothermal Titration Calorimetry**

Isothermal titration calorimetry (ITC) is commonly used to study binding reactions, such as the interaction of small solutes with macromolecules or membranes. It may also be employed in liposome solubilization studies or to determine critical micelle concentration, cmc, values. In this thesis it was used in Paper VI to study the partitioning of drugs into lipid membranes. Commonly, in ITC experiments exact amounts of solutes are titrated into the sample cell containing the lipid aggregate causing a heat change due to solute/membrane interaction. Solute dilution will also contribute to the observed heat change. The difference in temperature between the sample cell and a reference cell is monitored and corrected for to maintain an equal temperature. The resultant plot contains peaks corresponding to each injection and may be used to calculate partition coefficients. In Paper VI, a slightly modified assay was used called the solvent null method [64]. The drugs were allowed to equilibrate between buffer and lipid membranes in the syringe and then titrated into the sample cell containing various amount of a drug. As the amount of the free drug in the syringe matched the concentration of drug in the sample cell no heat change occurred due to drug partitioning or desorption from the membrane and the concentration of unbound drug, [drug]_{free}, could be determined. The partition coefficient, K, could then be calculated according to:

\[
K = \frac{[\text{drug}]_{\text{bound}}}{[\text{lipid}][\text{drug}]_{\text{free}}}
\]

where [lipid] is the lipid concentration in the syringe and [drug]_{bound} is the amount of drug associated with the lipid.

**2.2.5 Drug Partitioning Chromatography**

Drug partitioning chromatography (DPC) is a method to investigate the interactions of drugs with immobilized membranes [65,66]. One way to im-
mobilize the lipid membranes is by rehydrating gel beads with the lipid suspension. This procedure traps the lipid aggregates inside the bead pores and the beads are then packed in a column. The drugs are applied to the column and their elution volume monitored spectroscopically. The drug partitioning into the immobilized membranes is then evaluated from the retention volume giving a normalized capacity factor, $K_S$, according to:

$$K_S = \frac{(V_E - V_0)}{A}$$

where $V_E$ is the elution volume of the drug, $V_0$ the elution volume of an analyte that presumably does not interact with the lipids in the column, in our case we used $\text{Cr}_2\text{O}_7^{2-}$, and $A$ is the amount of immobilized lipid. There is also a very small interaction between the drugs and the gel beads that can be taken into account but was neglected in this thesis.

DPC was used in paper VI.

Experimental studies

2.3 pH-sensitive liposomes

One major route for cells to internalize extracellular material is through the endocytic pathway [51]. During endocytosis a part of the cell membrane invaginates, enclosing the material to be transported, and buds off into a vesicle or vacuole. The vesicle produced following endocytosis, the endosome, eventually matures and fuses with other intracellular compartments such as lysosomes. During the endosome maturation process the pH gradually decreases [67,68]. The pH decrease experienced by liposomes internalized via endocytosis constitutes one potential intrinsic stimulus for drug release.

Liposomes sensitive to pH can be constructed in different ways. Inclusion of various molecules into the liposomal membrane such as pH-sensitive peptides, fusion proteins or titratable polymers are some examples [44,45]. Another common approach is to combine polymorphic lipids, like unsaturated phosphatidylethanolamines (PE), with mildly acidic amphiphiles that stabilize the liposome at neutral pH. Dioleoylphosphatidylcholine (DOPE) constitutes the main component in many of the formulations used to construct pH-sensitive liposomes. At physiological pH and salt conditions DOPE has a relatively small headgroup area compared to the hydrocarbon tails and hence form inverted hexagonal structures under these conditions [69]. By combin-
ing DOPE with e.g. oleic acid (OA) or cholesterylhemisuccinate (CHEMS), a stabilization of the lamellar structure, and hence the liposomes, at physiological conditions can be obtained [60,70,71]. When the pH is lowered, and the stabilizing molecule gets protonated, an L_{\alpha} to H_{II} transition occurs during which the liposome cargo is released.

Another approach is to use membrane stabilizers that are hydrolyzed at lower pH and thereby lose their stabilizing properties. Cleavable PEG-lipids constitute one interesting alternative [72-75]. Inclusion of PEG-lipids in DOPE mixtures make liposome formation possible due to the molecular shape of the PEG-lipid [76,77], and at the same time a compound offering long circulation time is introduced in the bilayer. If the linkage between the PEG-chain and the bilayer anchor is made pH-sensitive, the PEG-chain may be cleaved off in acid environment inducing a transition from lamellar into hexagonal structures resulting in drug release (Fig. 14).

Fig. 14. Schematic figure of pH triggered structural reorganization due to the cleavage of lamellar phase stabilizing polymer. © Göran Karlsson.

In paper I an aim was to increase the understanding of pH-induced drug release from different pH-sensitive liposome formulations. Further, we investigated a novel cleavable PEG-molecule, dihydrocholesteryl-butenylmethoxyPEG (DHCho-MPEG_{5000}) (Fig. 15).

Fig. 15. Acid-catalyzed hydrolysis reaction of DHCho-MPEG_{5000}. The number of PEG units, n = 112.
A methoxy-PEG, MPEG, moiety is attached to a membrane anchor, consisting of dehydrogenated cholesterol, via an acid labile vinyl ether bond. At acidic conditions DHCho-MPEG_{5000} is hydrolyzed into dihydrocholesterol (DHCho) and an MPEG derivative (Fig. 15).

Inclusion of 5 mol% DHCho-MPEG_{5000} effectively induces and stabilizes liposomes composed of DOPE as is seen in Figure 16A, where all liposomes appear normal and are nicely separated. This is in contrast to the pure DOPE sample that, despite the high pH, contains aggregates with morphologies representing intermediates between lamellar and inverted phases and aggregated liposomes (Fig. 16B). Lowering the DHCho-MPEG_{5000} content to 1 mol% lowered the stabilizing effect significantly resulting in increased aggregation and abundance of alternative aggregates (Fig. 16C).

![Fig. 16. Cryo-TEM micrographs of DOPE and DHCho-MPEG/DOPE liposomes at 37°C, pH 9.5, 3mM total lipid concentration. A) 5:95 DHCho-MPEG_{5000}:DOPE, B) pure DOPE liposomes, and C) 1:99 DHCho-MPEG_{5000}:DOPE. Bar = 100 nm.](image)

The acid sensitivity of the vinyl ether bond of DHCho-MPEG_{5000} is low, as determined from TLC analysis, at pH 4.5. Hydrolysis is not even completed after 5 days at this pH. This is mirrored in the slow release rate and fusion, measured as lipid mixing, observed for DOPE/DHCho-MPEG_{5000} liposomes containing as little as 1 mol% DHCho-MPEG_{5000} (Fig. 17). The presence of a lag phase in the lipid mixing curve indicates that a certain amount of PEG must be cleaved off before the, for fusion, necessary membrane contact can occur. Even though a certain amount of leakage occurs prior to membrane contact and hence prior to the structural reorganization into hexagonal phase the slow destabilization process hinders a rapid content release. The low acid sensitivity of DHCho-MPEG_{5000} makes these molecules non-ideal as components in rapid release DOPE liposomes intended for *in vivo* use.
2.3.1 Cytosolic drug delivery

The low pH encountered by the liposomes upon endocytosis can trigger a drug release into the endosomal compartment. This is, however, not always sufficient. In many cases the drug needs to be delivered into the cytosol to achieve a therapeutic effect. Further, compounds that cannot escape the endosome and end up in the lysosome are exposed to the risk of destruction by degrading enzymes. A number of studies utilizing pH-sensitive liposomes have demonstrated the delivery of hydrophilic substances and macromolecules into the cytosol of target cells [78-81]. However, the mechanisms behind cytosolic delivery are complex and far from fully understood.

Depending on the character of the drug substance, a simple diffusion across the endosomal membrane into the cytosol could occur. Relocation via specific membrane transporters is another possible mechanism. These options are not applicable for many substances however. Another possibility is a direct fusion between the liposome and endosome membranes resulting in a microinjection of the liposomal content into the cytosol (Fig. 18). It is well documented that various pH-sensitive liposome systems become fusogenic when acidified and fuse with self-similar liposomes [71,75,82,83]. However, few studies have been carried out investigating the fusion properties between pH-sensitive liposomes and endosome-like membranes [84]. Evidence to support a mechanism where the pH-sensitive liposomes fuse directly with the endosome membrane is thus largely lacking. An alternative route for cytosolic drug delivery could be achieved from a series of events. Liposome destabilization and drug release in the endosome can be followed by incorporation of the liposomal components into the endosome membrane. This may cause a destabilization or even structural reorganization of the en-
dosome membrane allowing for the drug molecules to escape into the cytosol (Fig. 18).

![Fig. 18. Schematic representation of two possible cytosolic drug delivery mechanisms.]

To be able to distinguish between various mechanisms, systematic studies of the relevant lipid mixtures are required. We investigated the interactions between pH-sensitive liposomes and endosome-like membranes. The composition of early endosomes is generally believed to be similar to the plasma membrane and thus varies between different cell types [85]. Further, the membrane composition varies with endosome maturation stage [85,86]. However, the endosomes normally includes PC, PE, sphingomyeline (SM), cholesterol (Cho) and phosphatidylserine (PS) as major components [86,87]. To avoid any possible complications due to the presence of charge, PS was excluded from our endosome liposomes that were comprised of EPC:DOPE:SM:Cho (40:20:6:34 mol%).

We started the interaction study by investigating the lipid mixing properties between OA:DOPE (40:60 mol%) or DOPE:DHCho (97:3 mol%) liposomes and endosome liposomes. Note that the DOPE/DHCho liposomes correspond to the composition found in pH-sensitive liposomes of DOPE/DHCho-MPEG5000 after complete cleavage of the DHCho-MPEG5000 conjugate. We found that no significant lipid mixing of the different liposome populations took place even after 2 days at 37 °C and pH 5.5 (Fig. 19). Hence, a spontaneous fusion between the pH-sensitive liposome and endosome membranes seems unlikely. This is however a very simplified sys-
tem and it cannot be excluded that a fusion mediated by endosome specific proteins may take place \textit{in vivo}.

We also wanted to investigate the possibility of endosome membrane destabilization upon incorporation of lipids from the acid destabilized liposomes leading to endosomal drug escape. In order to do so we compared leakage from liposomes with compositions that might result after lipid exchange between OA:DOPE (40:60 mol%), DOPE:DHCho (99:1 mol%), DOPE:DHCho-MPEG₅₀₀₀ (97:3 mol%), or DOPE:distearoylPE-PEG₅₀₀₀₀ (DSPE-PEG₅₀₀₀₀) (97:3 mol%) and endosome liposomes (Table 1). The initial size of early endosomes has been reported as in the range of 100 nm [88]. Samples 1-4 (Table 1) have compositions corresponding to a 1:1 ratio of liposome and endosome membranes and would hence represent models for events taking place very early in the endocytotic process. During the endosome maturation the sizes of the endosomes increases significantly [88,89]. To investigate the effect of endosome to liposome size we included one sample with a composition representing a membrane obtained after incorporating DOPE:DHCho (97:3 mol%) liposomal lipids into a four times as big endosome (sample 5). The leakage from the various liposomes at 37 °C and pH 5.5 is displayed in Figure 20. Leakage from samples representing liposome/early endosome interactions is significantly hampered in the presence of PEG-lipids (samples 3 and 4) as compared to a comparably rapid release from samples 1 and 2. The slow leakage rate from liposomes containing DHCho-MPEG₅₀₀₀₀ is not surprising with previous knowledge of the slow hydrolysis of this molecule. Increasing the endosome to liposome size ratio representing a later stage of endocytosis decreased the leakage rate (sample 5). These results indicate the importance of H₂ phase promoters, such as DOPE and OA, as membrane destabilizers inducing leakage. This effect is
efficiently counterbalanced by very small amounts of PEG-lipids that stabilize the lamellar phase.

Table 1

Lipid composition\(^a\) and size ratio of endosome:liposome mixtures.

<table>
<thead>
<tr>
<th>Sample</th>
<th>EPC</th>
<th>DOPE</th>
<th>SM</th>
<th>Cho</th>
<th>OA</th>
<th>DHCho</th>
<th>DHCho</th>
<th>DSPE</th>
<th>SR(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.9</td>
<td>38.1</td>
<td>3.3</td>
<td>18.6</td>
<td>18.1</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>22.8</td>
<td>53.1</td>
<td>3.4</td>
<td>19.4</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>21.1</td>
<td>57.2</td>
<td>3.2</td>
<td>18.0</td>
<td></td>
<td>0.5</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>23.2</td>
<td>52.3</td>
<td>3.5</td>
<td>19.8</td>
<td></td>
<td></td>
<td>1.2</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>31.7</td>
<td>35.9</td>
<td>4.8</td>
<td>27.0</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
<td>3.5</td>
</tr>
<tr>
<td>6</td>
<td>40</td>
<td>20</td>
<td>6</td>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n/a</td>
</tr>
</tbody>
</table>

\(^a\) mol\%; \(^b\) SR is the size ratio, i.e. the size of the endosome/size of the liposome

Fig. 20 Leakage as a function of time for ◆ sample 1, ◇ sample 2, ● sample 5, ○ sample 3, ■ sample 6 (endosome liposomes), □ sample 4. Numbers 1-6 refer to the sample numbers used in Table 1.

A cryo-TEM analysis was carried out in order to investigate any potential correlation between leakage rate and changes in aggregate structure (Fig. 21). The endosome liposomes displayed a polydisperse collection of liposomes at 37 °C and pH 5.5, however all aggregates were lamellar (Fig. 21A). Including DOPE/OA or DOPE/DHCho in the preparations resulted in samples with structures representing intermediates between lamellar and inverted hexagonal phases correlating well with the observed rapid leakage rate from these preparations (Fig. 21B). Figure 21C shows how even a small amount of PEG-lipids efficiently stabilize the lamellar arrangement of the liposomes, even after acidification, corroborating the leakage result from this formulation.
The investigation of possible cytosolic drug delivery mechanisms in our simplified systems lead us to conclude that the incorporation of material from destabilized DOPE containing pH-sensitive liposomes into the endosomal membrane aid in endosomal drug escape. However, it is also possible that cytosolic delivery cannot be explained solely by lipid interactions but that specific proteins facilitate the transport across the endosomal membrane.

2.4 Temperature-sensitive liposomes

As mentioned earlier, another method to trigger liposomal drug release is the use of temperature-sensitive liposomes in combination with local tumor/tissue hyperthermia. Hyperthermia means, in this context, heating a part of the body to slightly elevated temperatures using an external heating source utilizing e.g. microwaves, ultrasound or radio frequencies [90]. Combining hyperthermia with temperature-sensitive drug-containing liposomes may result in increased therapeutic efficacy. Apart from inducing drug release from the liposomes, hyperthermia also induce increased tumor blood flow and microvasculature pore size [91] leading to increased accumulation of liposomes in the target area [92].

One approach of forming temperature-sensitive liposomes is based on the increased membrane permeability observed at the formulation specific gel to liquid crystalline phase transition temperature (T_c) [93]. The increased permeability around T_c is due to mismatches in molecular packing in interfacial regions between solid and fluid domains. The area of these interfacial regions is greatest at T_c correlating well with observed release rates [94].
Several studies have demonstrated an increased amount of drugs at the target site when using temperature-sensitive liposomes in combination with hyperthermia [41,92,95-97]. However, this does not always lead to an effect on tumor growth [98]. Despite accumulation of liposomes in the tumor area, drug still encapsulated in the carriers is inactive. Thus, there is a need to further improve the amount or rate of drug released at the target site. Recently, a new liposome formulation containing the commercially available anti-cancer drug doxorubicin was developed, which releases its content almost instantaneously when heated to temperatures close to its \( T_C \) (41.5°C). Further, it exhibits superior efficacy in tumor growth delays [91,99-101]. The liposomes were composed of dipalmitoylPC (DPPC) and DSPE-PEG\(_{2000}\), and contained furthermore 10 mol% of either monopalmitoylPC (MPPC) or monostearoylPC (MSPC). In paper II and III we investigated the mechanisms behind the rapid temperature induced release from such lysolipid containing temperature-sensitive liposomes (LTSL).

2.4.1 Drug release mechanisms from lysolipid containing liposomes

**Morphological changes upon phase transition**

In paper II we focused on examining morphological changes of LTSL and related liposome compositions before and after cycling the liposomes through their \( T_C \). We found that LTSL liposomes prepared above \( T_C \) and then lowered to room temperature displayed a faceted morphology, typical of gel phase liposomes (Fig. 22A). In addition few bilayer discs could be found. When increasing the temperature again to above \( T_C \) the sample contained open liposomes and some bilayer discs (Fig. 22B). Further cycling of the sample through \( T_C \) increased the amount of open liposomes and discs (Fig. 22C).

![Cryo-TEM images of freshly prepared LTSL before and after cycling through \( T_C \) after extrusion. A) 22°C, B) 50°C, C) after cycling liposomes five times through their \( T_C \). White arrow indicates open liposome, black arrows indicate bilayer discs. Bar = 100 nm.](image)
Two other liposome formulations were investigated in order to study the contribution of the lysolipids and PEG-lipids to the observed effect of temperature cycling. LTSL devoid of PEG-lipids displayed no open structures even after five cycles through $T_C$ (Fig. 23A,B). Liposome formulations containing DPPC and PEG-lipids, thermosensitive liposomes (TSL), however, did respond to the temperature cycling by forming discs but to a lesser extent than when both lysolipids and PEG-lipids were present in the membrane (Fig. 23C,D).

In order to understand the mechanism behind the temperature-induced liposome fragmentation and opening we must first describe the mechanisms of phase transitions and the formation of grain boundaries.

The polygonal appearance of gel phase liposomes can be explained by the presence of highly ordered, rigid membrane domains intersected by domain boundaries, or grain boundaries, formed as the membrane is transformed from the liquid crystalline phase to the gel phase. When the lipid membrane is cooled from the liquid crystalline phase through $T_C$ multiple gel phase nuclei with highly ordered lipids form in the bilayer. These nuclei grow in size, forming domains, until the entire membrane is in the gel phase. The grain originating from a particular nucleus meet neighboring grains at so called grain boundaries. The individual plates do not necessarily have the same lattice orientation and hence, the membrane disorder is highest in these grain-separating domains [102]. The smaller the liposome the higher is the bending stress and thus the curvature in these boundary regions.

Certain incorporated membrane additives are excluded from the flat gel phase patches during gel phase formation, and segregate into distinct membrane regions [103] such as disordered grain boundaries. The lysolipids and PEG-lipids likely represent such additives. The melting temperature at the boundaries is lower than that of the membrane domains [104]. Thus, when
increasing the temperature through $T_C$ the transition from gel into liquid crystalline phase will be initiated at these regions.

Both lysolipids and PEG-lipids are micelle-forming components. Based on the assumption that they accumulate in the grain boundaries, they may adapt their favored micellar shape within the bilayer when the grain boundaries melt prior to the rest of the membrane. This would stabilize the rim of open liposome structures. Depending on the amount of lysolipid and PEG-lipid segregation this can lead to the formation of pores or promote the dispatch of membrane discs (Fig. 24).

![Fig. 24. Schematic representation of proposed pore- and disc-formation in LTSL. A, C) Accumulation of micelle-forming lipids at grain boundaries. B) Pore formation during phase transition. D) Disc formation during phase transition. PEG-lipids are not included in the drawing.](image)

It appears as if the PEG-lipid is necessary to sterically stabilize the open liposomes and discs and prevent them from closure or fusion (Fig. 23). Further, both lysolipids and PEG-lipids are necessary to induce a significant amount of membrane discs upon temperature cycling (Fig. 22 and 23).

The rapid drug release from LTSL may, however, not be solely explained by the cycling induced liposome opening and fragmentation. The mere presence of lysolipids and PEG-lipids in disordered regions enhance the formation of structural defects upon melting. This mechanism probably constitutes an important contribution to the observed rapid release.

**Membrane component segregation and lysolipid retention**

Our proposed drug release mechanisms require that a certain amount of component segregation into grain boundaries occur. This has not previously been proven in LTSL membranes. Hence, in paper III we investigated the membrane distribution of additives using the fluorescent probe PHDA.

Evidence of an inhomogeneous distribution of membrane components in the gel phase liposomes LTSL and TSL via the accumulation and dimerization of the fluorescent probe PHDA is shown in Figure 25. In contrast, no accumulation of PHDA could be observed in liposomes containing 45 mol%
cholesterol, non-thermosensitive liposomes (NTSL). Several other membrane markers have previously been shown to segregate into distinct membrane regions in gel phase liposomes [105-107]. This may be explained by component segregation during the transformation of the liposome membrane from liquid crystalline phase into gel phase, see discussion above. As described in chapter 1, above a threshold concentration cholesterol abolishes the phase transition of phospholipid membranes and induces the formation of the liquid ordered phase over a wide range of temperatures, thus explaining the observed absence of component segregation in cholesterol containing liposomes.

Additionally, the E/M ratios for the TSL and LTSL samples increase with time (Fig. 25), which could be due to several factors. Incubation at pH 4 and 38°C is likely to cause some lipid hydrolysis producing fatty acids and lysolipids. This may affect the mobility and packing density of the PHDA molecules in the TSL and LTSL membranes. An alternative explanation could be a slow accumulation of PHDA molecules retained in the bulk gel phase membrane over time. However, considering the reported diffusion rates in gel phase membranes [3] and possible domain sizes it seems likely that the accumulation occurred prior to the first measurement.

Due to the large polymeric head group, PEG-lipids are likely to be expelled from domains of well-packed gel phase lipids. Therefore it is plausible to assume that also PEG-lipids accumulate in defects in gel phase membranes, as discussed in paper V. Further, even though the molecular structure of PHDA is different from that of the lysolipid they may both be considered as membrane impurities. Therefore we suggest that trends observed for PHDA are also applicable to lysolipids and thus lysolipids are not homogeneously distributed in the LTSL membrane.

Fig. 25. PHDA excimer/monomer (E/M) ratios of ○ LTSL, □ TSL, and △ NTSL, containing 5 mol% PHDA. (Mean ± S.D.; n=4 for TSL and n=3 for LTSL and NTSL). Liposomes were stored at 38°C and analyzed for 7 days.
An alternative release mechanism has been put forward based on the observation of lysolipids leaving or partitioning into liquid crystalline phase membranes when added or removed from the buffer [108-110]. It has been proposed that the membrane permeability increases significantly when the lysolipids leave the LTSL membrane during phase transition [39,99]. Independent of release mechanism it is important that the lysolipids do not leave the membrane prior to $T_C$. In paper III we therefore also investigated the lysolipid membrane retention in the presence of a membrane pool consisting of egg-PC (EPC) multilamellar liposomes (MLVs). By tracing the lysolipid distribution via radiolabelled MPPC we found that lysolipids leave the LTSL membrane well below $T_C$ of the formulation and partition into the EPC membrane pool (Fig. 26). Approximately half the amount of lysolipid included in the LTSL membrane transferred within 10 minutes without any further exchange at 30 minutes (Fig. 26). Assuming a more or less equal distribution of lysolipids between the inner and outer membrane leaflet and a slow lipid flip flop [111] this result indicates that all lysolipid in the outer membrane layer have left LTSL.

![Graph showing the distribution of 14C-MPPC in LTSL and EPC MLVs after co-incubation at 37°C (mean ± S.D.; n=6).](image)

We also investigated the effect of the lysolipid transfer on the release of CF from LTSL. Importantly, we found that the lysolipid transfer did not lead to significant leakage from gel phase LTSL. When increasing the temperature to values around $T_C$ of the formulation, the amount of CF released from liposomes preincubated in the presence of EPC MLVs was on average 1.4 times lower than for liposomes incubated in the absence of a membrane pool (Fig. 27A). LTSL lacking lysolipids in the outer membrane leaflet did, however, still release around 5.4 times more CF than liposomes completely devoid of lysolipids, TSL (Fig. 27A). The CF release curve shows two distinct phases, one rapid initial phase followed by a second slower phase. It seems as if an initially lower membrane lysolipid content results in a lower percentage of CF released during the rapid release phase while the second phase
is not affected by the lysolipid content. The first release period, likely represents the state of the liposome membrane during melting and reorganization of the grain boundaries. Our results thus indicate that the extent of packing defects and membrane openings formed in grain boundaries is directly correlated with the amount of lysolipids present in these membrane regions. The second phase probably represents leakage from liposomes after the lysolipids have left the membrane or redistributed in the liquid crystalline areas and would then, as expected, not be dependent on lysolipid content.

Fig. 27. A) Percentages of CF released over time from ○ TSL and LTSL at 41.5°C. LTSL were co-incubated without △ or with □ a 100-fold excess amount of EPC liposomes for 10 min at 37°C. (Mean ± S.D.; n=6 for TSL, n=4 for LTSL). B) Percentages of CF released over time from LTSL at 41.5°C. Concentrated stock solution of LTSL was added to pre-heated buffer containing ○ 0, □ 0.5, and △ 1 μM MSPC. (Mean ± S.D.; n=3).

To further test the hypothesis that the liposomes leak as a result of lysolipids leaving the membrane, we studied liposomal leakage profiles in the presence of extravesicular lysolipid. During the leakage measurements the liposomes were diluted approximately 500-fold in lysolipid-free warm buffer. This allows for the lysolipids still left in the membrane to leave, directly from the outer membrane or through defects or transient pores from the inside leaflet. Including MSPC in the extravesicular buffer is expected to decrease the propensity of membrane bound MSPC to desorb. We found that the leakage increased with increasing concentration of MSPC in the buffer (Fig 27B), which could be interpreted as the lower probability of MSPC to desorb. This means that the lysolipids are required in the membrane for the rapid release to occur and is not due to them leaving the membrane.

It can be concluded from this study that lysolipids most likely desorb from LTSL upon dilution or in the presence of other membrane pools, which may compromise their in vivo thermosensitivity.
2.4.2 Effect of hydrolysis

Accumulation of hydrolysis products in the liposome membrane may alter the integrity of the bilayer inducing leakage, fusion and transformation into alternative aggregate structures [112-114]. This may seriously affect the performance of liposomes intended for various pharmaceutical or biotechnical applications. It is thus important to accumulate knowledge about the effects of hydrolysis on the liposome formulations. This way it becomes easier to determine for instance shelf lives or find explanations for changes in liposome performance. Further, the results of paper II lead us to believe that hydrolysis of gel phase liposomes can significantly affect liposome integrity. In paper IV we investigated the effect of lipid hydrolysis on aggregate structure in sterically stabilized DPPC liposomes after gel to liquid crystalline phase transition.

The DPPC/DSPE-PEG<sub>2000</sub> liposomes were stored at pH 2, 4 or 6.5 at a temperature of 4 or 22°C. The hydrolysis rates were, as expected, found to be faster at lower pH and higher temperature (Table 2).

Table 2

<table>
<thead>
<tr>
<th>Storage temperature</th>
<th>pH 2</th>
<th>pH 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>22°C</td>
<td>4.95x10&lt;sup&gt;-7&lt;/sup&gt; s⁻¹</td>
<td>0.59x10&lt;sup&gt;-7&lt;/sup&gt; s⁻¹</td>
</tr>
<tr>
<td>4°C</td>
<td>0.85x10&lt;sup&gt;-7&lt;/sup&gt; s⁻¹</td>
<td>0.18x10&lt;sup&gt;-7&lt;/sup&gt; s⁻¹</td>
</tr>
</tbody>
</table>

The morphology of the stored liposome samples before and after heating the liposomes through T<sub>C</sub>, one T<sub>C</sub>-cycle, were analyzed using cryo-TEM. Samples stored at pH 6.5 contained mainly polygonal intact liposomes and did not change morphology after temperature cycling even after storage for 33 weeks at a temperature of 22°C (Fig. 28).

Fig. 28. Cryo-TEM images of DPPC:DSPE-PEG<sub>2000</sub> (molar ratio: 90:4) liposomes stored at 22°C and a pH of 6.5 for 33 weeks after one T<sub>C</sub>-cycle. Bar = 100 nm.

When storage conditions were changed to pH 4 the liposomes disintegrated into discs upon T<sub>C</sub>-cycling after prolonged storage. When stored at 22°C for 10 days the liposomes appeared intact before the T<sub>C</sub>-cycle (Fig. 29A) but disintegrated completely into discs when cycled once (Fig. 29B).
However, the disintegration did not occur at a specific threshold value of hydrolysis. Samples stored under the same conditions for 7 days did not disintegrate completely but still contained a significant amount of discs after the T<sub>c</sub>-cycle. The same trend was observed for liposomes stored at pH 4 and 4°C although the disintegration occurred at later time points. A 35 days old sample displayed a significant amount of discs after the T<sub>c</sub>-cycle and disintegrated completely into discs after 67 days (Fig. 29C and D).

![Fig. 29. Cryo-TEM images of DPPC:DSPE-PEG<sub>2000</sub> (molar ratio: 90:4) liposomes stored at pH 4. A) Liposomes stored for 10 days at 22°C before the T<sub>c</sub>-cycle. B) The same sample as in A after one T<sub>c</sub>-cycle. C) Liposomes stored for 35 days at 4°C after one T<sub>c</sub>-cycle. D) Liposomes stored for 67 days after one T<sub>c</sub>-cycle. Arrowheads indicate discs positioned face-on and arrows indicate discs positioned edge-on. Bar = 100 nm.](image)

When samples were stored at the extreme pH 2, morphological changes induced by T<sub>c</sub>-cycling occurred much earlier than when stored at higher pH. As a considerable amount of hydrolysis occurs already during sample preparation, even freshly prepared samples contained a significant amount of discs (Fig. 30A). After one day at 22°C temperature cycling induced total disintegration into small discs (Fig. 30B). Upon further storage at pH 2 and 22°C for 3 weeks the sample appearance started to change even before the T<sub>c</sub>-cycle. Liposomes appeared to be more round in shape and bilayer flakes were present. When such a sample was cycled large bilayer sheets and large discs were found (Fig. 30C). Eventually, bilayers, large liposomes and large clusters of partially fused liposomes could be observed before the T<sub>c</sub>-cycle (Fig. 30D).
These results present a strong correlation between the degree of phospholipid hydrolysis and the amount of disc formation induced by one T\textsubscript{C}-cycle. Based on the hydrolysis rate constants (Table 2) the degree of hydrolysis at complete liposome disintegration into discs can be calculated. Liposomes stored at pH 4 and 22 or 4\textdegree{}C disintegrated completely when DPPC concentrations were decreased by 5\% or 9.9\% respectively. The corresponding values for samples stored at pH 2 were 4.2\% and 3.6\%. However, it is important to notice that the onset of disc formation occurred at even lower levels of hydrolysis. In previous studies the onset of disc formation in pure DPPC liposome samples did not occur until DPPC concentrations had decreased by approximately 10\% through hydrolysis [112,115,116]. It can thus be concluded that the presence of PEG-lipids in the liposome membrane considerably increases the disc formation propensity in hydrolyzed samples.

The results presented in Paper IV correlate well with the findings of paper II where we present evidence for disc formation in lysolipid containing DPPC/DSPE-PEG\textsubscript{2000} liposomes after T\textsubscript{C}-cycling. Hence, hydrolysis-generated accumulation of lysolipids in the membrane of e.g. DPPC liposomes also increases the propensity for transformation of liposomes into discs.

So far only full T\textsubscript{C}-cycles (increasing the temperature to above T\textsubscript{C} and cooling back to temperatures below T\textsubscript{C}) have been employed to investigate disc formation in partially hydrolysed liposome samples stored in their gel phase. In order to find out whether a full T\textsubscript{C}-cycle is required for liposomes to disintegrate or if a transition from gel to liquid crystalline phase would be
enough static light scattering experiments were performed. Light scattering from a fresh DPPC/DSPE-PEG$_{2000}$ liposome sample was compared to light scattered from an identical sample stored at pH 4 and 22°C for 10 days. Storage under these conditions will lead to disc formation after $T_C$-cycling (Fig. 29) while the corresponding fresh sample stays intact. Results strongly indicate that disintegration of the liposomes occurs already when heated through $T_C$, i.e. no $T_C$-cycling was needed (Fig. 31).

![Fig. 31. Light scattering intensities of DPPC:DSPE-PEG$_{2000}$ (molar ratio: 90:4) liposomes at the day of preparation and after storage for 10 days at 22°C and a pH of 4. Light scattering intensities were determined in the sample that was never cooled below 48°C (black bar, n=4), cooled to 25°C (white bars, n=4), heated again to 48°C (grey bars, n=2), and after cooling back to 25 °C (hatched bars, n=2).]

Phospholipid hydrolysis also produces fatty acids. Apparent pK$_a$ values of fatty acids under physiological salt conditions range in liposomes from 7.2 to 8 [117]. This means that fatty acids in the present study are predominantly protonated and prefer structures with a negative curvature [118,119]. However, the presence of the fatty acids appears to promote rather than inhibit the formation of discs. Although MSPC concentrations of 9 mol% are not sufficient to induce complete disc formation in DPPC/DSPE-PEG$_{2000}$ liposomes after one $T_C$-cycle (compare with Fig. 22), complete disc formation occurred in partially hydrolysed liposomes where DPPC concentrations had decreased by 10% or less (Fig. 29). A possible explanation for this effect is that fatty acids in grain boundaries weakens their cohesive strength and thus promotes liposome fragmentation.

After prolonged storage, the lysolipid/fatty acid ratio will decrease as the lysolipid is further hydrolysed and produces another fatty acid. This influences the structural transformations in the sample. Large bilayer sheets appear and it seems as if small discs fuse into larger aggregates when the amount of lysolipid that can stabilize the rim of the discs is decreased. This process is likely facilitated by the hydrolysis of PEG-lipids, which otherwise would prevent aggregate interactions. At extensive hydrolysis the amount of fatty acids in the sample is high enough to allow the formation of more com-
plex aggregates (Fig. 30D) similar to those found as intermediates during lamellar to inverted aggregate structures [120,121].

It can be concluded that storage conditions and thermal history of liposome samples should be carefully considered as these may significantly affect the intended performance of the liposomes. The formation of the metastable liposomes presented here may be highly unwanted in certain applications but can be exploited for others such as liposomes intended for temperature triggered drug release.

2.5 Discs in PEG-lipid systems

2.5.1 Factors promoting disc formation

PEG-lipids with sufficiently long polymer chains are micelle-forming molecules, and will transform lipid membranes into mixed lipid/PEG-lipid spherical micelles if the PEG-lipid concentration is high enough. The transition structures formed during vesicle to micelle conversion may however vary depending on the lipid composition, with the transition commonly occurring via the formation of either of the two fundamentally different micelles, cylindrical or disc micelles (Fig. 32).

![Fig. 32. Schematic figure of A) PEG-lipid/lipid disc and B) PEG-lipid/lipid cylindrical micelle.](image)

Irrespective of the micellar structure formed, the PEG-lipids experience less of a curvature constraint, when compared to PEG-lipids situated in a flat bilayer, and the polymer-lipid may adopt its preferred conformation. The curvature constraint experienced by the bulk lipids is, however, significantly different between the two aggregates. Further, as discussed in chapter 1.5.1, disc formation is associated with an entropy loss due to component segregation.

It appears logical that sample components, or environmental conditions, that reduce lipid/PEG-lipid miscibility should increase the tendency for disc
formation. Further, properties of the lipid mixture such as the monolayer bending modulus and spontaneous curvature should have a decisive influence on whether thread-like or discoidal mixed micelles form in the system. In paper V we investigated how these properties affected the structure in lipid/PEG-lipid systems with a fixed amount of 25 mol% DSPE-PEG2000.

EPC:DSPE-PEG2000 (75:25 mol%) samples displayed a variety of aggregate structures, such as liposomes, bilayer flakes with cylindrical extensions and cylindrical micelles (Fig. 33A). Inclusion of cholesterol drastically affected the structural outcome. Cholesterol induced the formation of bilayer flakes and discs, to the cost of a decreased amount of cylindrical micelles and liposomes. The amount of cylindrical micelles was almost negligible at 10 mol% cholesterol and the liposome population largely diminished (Fig. 33B). Further, as the amount of cholesterol increased from 0 to 40 mol% the flakes and discs adopted a more circular shape (Fig. 33C).


As discussed in section 1.4.1 cholesterol has the ability to increase chain order in liquid crystalline membranes and at concentrations of around 25-30 mol% to induce the formation of the liquid-ordered phase, l0. It could be speculated that PEG-lipids are less soluble in the l0-domains than in the liquid disordered domains thus leading to PEG-lipid segregation. Cholesterol/lipid interactions mainly take part in the acyl chain region and possibly involve hydrogen bonding in the vicinity of the hydrocarbon-water interface. Thus the PEG-chain should not influence this interaction. However, a reduced PEG-lipid/lipid miscibility in the liquid ordered phase is possible in the case of very different hydrocarbon chains of the PEG-lipid and PC-lipid. Results obtained from studies of DOPC/DOPE-PEG2000 mixtures show that cholesterol can inhibit the formation of cylindrical micelles as well for this system (Fig. 34). In this case it is not expected that cholesterol will have any significant influence on the PEG-lipid/lipid miscibility, and therefore re-
duced miscibility cannot explain why cholesterol induces component segregation and discs formation when added to PEG-lipid/lipid mixtures.

It is more probable that the preference of cholesterol to be situated in discs rather than cylindrical micelles is due to its molecular shape. The small head group and comparatively large and stiff hydrocarbon region indicate that cholesterol is not well suited to be situated in aggregates with high positive curvature. In line with this, it has been found that the inclusion of cholesterol into phospholipid membranes increases the membrane bending modulus [122] and decrease the spontaneous curvature of the mixture [123]. Further, the same arguments may also be applied to explain the decreased irregularity of the discs as the cholesterol content is increased. With a higher amount of cholesterol, less material suitable for the rim is available, resulting in the formation of perfectly circular discs as this minimizes the circumference of the aggregates.

Lanosterol is a related sterol with three additional methyl-groups on the hydrocarbon body (Fig. 35).

The chain ordering effect of lanosterol is markedly smaller than that of cholesterol [124-127]. This is directly correlated with the impact of the two sterols on the bending modulus of POPC membranes. We found, however, that lanosterol induced disc formation to at least the same extent as cholesterol.
These results indicate that the bending modulus does not play a significant role in determining which aggregate structure is formed. It thus appears that the change in lipid spontaneous curvature induced by the sterols is the main determinant for disc formation.

In order to find out if curvature reducing components other than sterols could prevent the formation of cylindrical micelles in EPC/DSPE-PEG\textsubscript{2000} mixtures and possibly induce the formation of discs, we included DSPE in the samples. DSPE has a similar structural effect as cholesterol, though not as pronounced. At 40 mol\% DSPE there were still irregularly shaped bilayer flakes and patches present (Fig. 37A). Exchanging all EPC for DSPE, however, resulted in the formation of exclusively circular discs of varying size (Fig. 37B). DSPE forms lamellar gel phase at the experimental temperature used. In the pure DSPE/DSPE-PEG\textsubscript{2000} sample this fact likely contributes to the observed sample appearance of only circular discs, as will be discussed below.

Fig. 36. Cryo-TEM images of samples containing A) EPC:lanosterol:DSPE-PEG\textsubscript{2000} (64:11:25 mol\%) and B) EPC:lanosterol:DSPE-PEG\textsubscript{2000} (35:40:25 mol\%). Bar = 100 nm.

Fig. 37. Cryo-TEM images of dispersions of A) EPC:DSPE:DSPE-PEG\textsubscript{2000} (35:40:25 mol\%), B) DSPE:DSPE-PEG\textsubscript{2000} (75:25 mol\%), C) EPC:DSPE:DSPE-PEG\textsubscript{2000}:MSPC (40.5:26.8:22.7:10 mol\%). Bar = 100 nm.
The potential of DSPE to promote discs in EPC/DSPE-PEG$_{2000}$ mixtures may be explained by considering the geometrical shape of DSPE. DSPE has a small polar head group and thus tends to reduce the spontaneous curvature of the mixture. Inclusion of 10 mol% of the lysolipid MSPC into an EPC:DSPE:DSPE-PEG$_{2000}$ (45:30:25 mol%) mixture induced the formation of thread-like micelles (Fig. 37C). MSPC has a high positive curvature and obviously counteracts the curvature reducing effect of DSPE and makes the formation of cylindrical micelles favourable. These results again indicate the importance of spontaneous curvature on aggregate shape.

The inclusion of DSPC into EPC/DSPE-PEG$_{2000}$ mixtures did not induce disc formation (Fig. 38A). The hydrocarbon chains of DSPC and DSPE are identical, and the disc promoting effect of the latter thus stems from the PE head group rather than the saturated nature of the hydrocarbon chains. However, in pure DSPC/DSPE-PEG$_{2000}$ samples discs were the only structures formed (Fig. 38B).

Fig. 38. Cryo-TEM images of A) EPC:DSPC:DSPE-PEG$_{2000}$ (45:30:25 mol%), and B) DSPC:DSPE-PEG$_{2000}$ (75:25 mol%). Bar = 100 nm.

Importantly, the discoidal aggregates in DSPC/DSPE-PEG$_{2000}$ mixtures were observed at a temperature well below the $T_C$ of DSPC. The promotion of discs below $T_C$ of the bulk lipid may be understood by considering that the bending modulus is roughly ten times larger for membranes in the gel phase compared to the liquid crystalline phase [128]. Further, the PEG-lipid/lipid miscibility is likely reduced in the gel phase, as discussed above, thus promoting component segregation. The structures formed in DMPC/DMPE-PEG$_{2000}$ above and below $T_C$ corroborate this reasoning. In the liquid crystalline phase, long thread-like micelles were observed (Fig. 39A). However, lowering the temperature below $T_C$ resulted in the formation of small disc shaped micelles (Fig. 39B).
In conclusion, discs are favoured over thread like micelles upon inclusion of components that reduce the spontaneous curvature of the mixture and increase the monolayer bending modulus. A reduced PEG-lipid/lipid miscibility, as might be expected in gel phase membranes, also promote disc formation.

2.5.2 Discs as model membranes

Since most drugs are required to pass through membranes in order to reach its site of action, knowledge about drug membrane interactions are of great importance. Often, a simple octanol-water system is used to model partitioning of drugs into biological membranes but octanol is a crude membrane model. Artificial phospholipid bilayers, such as liposomes, constitute better models due to their structural similarity with naturally occurring membranes. Accordingly, several liposome-based methods have been developed for drug membrane interaction studies [65,129-131]. Although liposomes appear to be good models in this context, a number of problems have been identified. Liposome preparation of common lipid compositions normally results in a fraction of bi- or multilamellar vesicles. Depending on the properties of the drug, and the analytical technique used, this fact may give rise to significant difficulties concerning the interpretation and quantification of the results. If the drug is not allowed to equilibrate between the various layers in the multilamellar structures a proportion of the lipids will in effect be hidden from interaction. Further, in the case of the unilamellar liposomes, the fact that initially only the outer membrane leaflet is accessible for interaction may be a problem for certain drugs. Another obstacle facing the use of liposomes is their thermodynamical instability. Conventional liposomes thus exhibit poor long-term stability. In paper VI we developed and evaluated the use of membrane discs as alternative model membranes in drug/membrane partitioning studies using isothermal titration calorimetry (ITC) and drug partitioning chromatography (DPC).

With increasing amount of DSPE-PEG_{5000} in DSPC/cholesterol mixtures, with a fixed amount of 40 mol% cholesterol, bilayer discs become more
abundant. At 5 mol% DSPE-PEG5000 predominantly unilamellar liposomes form (Fig. 40A). Increasing the PEG-lipid concentration to 12 mol% resulted in samples containing almost exclusively bilayer discs which are relatively large (Fig. 40B). Further increasing the PEG-lipid content resulted in a continuous decrease in disc size (Fig. 40C). We anticipated that large discs with a small edge to body surface area would constitute the most suitable model membrane and hence, the preparation with 12 mol% DSPE-PEG5000 was chosen in the drug partitioning studies.

Drug partitioning into multilamellar liposomes, unilamellar liposomes and discs, all composed of DSPC, 40 mol% cholesterol, and 0, 5 or 12 mol% DSPE-PEG5000 respectively, were compared. ITC was used to investigate partitioning of three different drugs into either discs or liposomes and it was found that the partitioning was similar for both aggregate structures indicating the potential of the disc as a model membrane (Table 3).

Table 3

<table>
<thead>
<tr>
<th>Drugs and solutes</th>
<th>Charge at pH 7.4</th>
<th>Unilamellar liposomes</th>
<th>Discs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Log K</td>
<td></td>
</tr>
<tr>
<td>Alprenolol</td>
<td>+</td>
<td>1.22 ± 0.06</td>
<td>1.21 ± 0.04</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>+</td>
<td>1.18 ± 0.12</td>
<td>1.10 ± 0.10</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>-</td>
<td>0.85</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Average log K values ± S.E.M. for positive drugs of two determinations and log K value of a negative drug.

The ITC results support the fact that the solvent-null method (see Experimental techniques) used in these experiments allows for the analyte to equilibrate between lipid and water phases prior to the measurement, thus eliminating the potential problem of hidden lipid layers in the liposome case.
Further, the various amounts of PEG-lipid in the two different lipid aggregates do not seem to affect the equilibrium of drug distribution in these cases. Unfortunately, the ITC method is limited to analytes having relatively high water solubility and is therefore not suitable for many drugs. Additionally, multilamellar liposome samples sedimented in the injection syringe and for this reason could not be investigated using this technique.

We further evaluated the discs as model membranes in partition studies using the chromatographic technique DPC, giving the capacity factor log $K_s$ (see Experimental techniques). A high log $K_s$ value indicates a high membrane partitioning. DPC is a relatively rapid and easy method allowing for a vast amount of various drugs to be investigated. Further, all three different lipid preparations can be immobilized in the gel beads. We found that generally the drugs had higher log $K_s$ values for discs compared to liposomes, and higher for unilamellar than multilamellar liposomes (Fig. 41A). This trend coincides with an increase in PEG-lipid content in the lipid aggregates. However, inherent drug/PEG-lipid interactions may be ruled out based on the ITC results. Further, comparison of drug partitioning between extruded EPC liposomes with or without PEG-lipid obtained from DPC measurements showed no significant difference. We did, however, observe an electrostatic effect due to the negative charge of DSPE-PEG5000. Comparisons of the drug partitioning into MLVs and discs, where this effect is most prominent, indicated a disproportionally high interaction for the positively charged drugs with discs and the opposite for negatively charged drugs (Fig. 41B). However, it is important to remember that the general trend, irrespective of charge, is a higher log $K_s$ value for discs compared to liposomes. Later studies with uncharged discs have shown the same trend [132].

![Fig. 41. A) Drug partitioning for neutral (white), positively charged (grey), and negatively charged (black) drugs into multilamellar liposomes (MLV), unilamellar liposomes (ULV), and discs expressed as average log $K_s$ values. B) Log $K_s$ for drug partitioning into discs against into multilamellar liposomes (MLV) for (▲) negatively charged, (○) neutral and (■) positively charged drugs.]

The observed trend of higher drug partitioning into discs than liposomes, may be explained by the above-mentioned problem of a hidden lipid frac-
tion. The capacity factor is obtained by dividing the retention volume with the amount of immobilized lipid (see Experimental techniques). Hence, if only a portion of the lipid is accessible for interaction the obtained capacity factors are underestimated. In the case with multilamellar liposomes the amount of inaccessible lipid may be very large. To what extent this effect influences the capacity factors depends on the properties of the drug. A hydrophilic drug has a low propensity to partition into the membrane and will therefore equilibrate slowly between the lamellas. A hydrophobic drug will preferably interact with the membrane and therefore equilibrate slowly across the inner aqueous compartments in a multilamellar liposome. Hence, in both cases, long equilibration times are needed in order to obtain true capacity factors. The use of discs may overcome this problem and allow for the rapid and convenient screening of drugs using fast chromatography- or electrophoresis-based techniques.

The discs used in this study can be improved, and hence the development of uncharged discs with a lipid composition mimicking that found in biological membranes has recently been performed [132].
3 Conclusions

Paper I

DOPE-based pH-sensitive liposomes do not interact or fuse with endosome resembling membranes containing EPC, DOPE, SM and cholesterol. Furthermore, the data collected for the pure OA/DOPE and DHCho-MPEG5000/DOPE systems indicate that the liposomes leak before fusion upon acidification. Liposomes up taken \textit{via} the endocytic pathway are thus believed to discharge their contents into the endosomal compartment upon pH reduction. Subsequent transport of the liposomal cargo across the endosomal membrane may then take place \textit{via} a number of different mechanisms. The present study shows that the permeability of the endosomal membranes may increase significantly upon incorporation of extra DOPE. Cytosolic delivery of the liposomal contents may, therefore, be aided by the transfer and incorporation of DOPE molecules from either pH-sensitive liposomes or from precipitated particles of inverted hexagonal phase, formed as a consequence of the endosomal acidification.

These results should be considered in the design of future effective pH-sensitive liposome formulations. Our findings suggest, for instance, that the time needed to achieve complete cleavage of the PEG moiety is a crucial parameter for any formulation based on acid labile PEG-lipids. Slow cleavage kinetics will delay liposome destabilization and hence drug release. This will allow the endosomes to mature into larger, less DOPE-sensitive endosomes. In case of very slow cleavage kinetics the endosomes may even have time to mature into lysosomes and the drugs are exposed to the risk of being digested by lysosomal enzymes.

Paper II and III

Cycling PEG-stabilized DPPC liposomes containing 10 mol% lysolipid, LTSL, through their Tc results in the formation of open liposomes and membrane discs. Open structures are also found in liposome formulations devoid of the lysolipid, but to a lesser extent compared to when both lysolipids and PEG-lipids are present in the membrane. We found that PHDA
molecules accumulate in grain boundaries of LTSL and DPPC/PEG-lipid liposomes. Based on this we draw the conclusion that also lysolipids and PEG-lipids have a tendency to accumulate in these regions. The presence of these components in the liposome membrane facilitates the formation of defects and membrane openings during the initial phase of the gel to liquid crystalline phase transition. Further, the PEG-lipids and lysolipids may adapt a micellar conformation enabling bilayer discs to form and dissociate from the liposomes, or alternatively stabilize the rim of open liposomes. Taken together, these temperature-induced effects explain the instant release of encapsulated material observed from LTSL at temperatures slightly below their $T_c$.

However, upon dilution or in the presence of acceptor liposomes, lysolipids desorbs rapidly from LTSL. This has implications for the release of encapsulated CF from the liposomes. The initial amount of CF released is significantly decreased in lysolipid poor LTSL. These results imply that in the in vivo situation transfer of lysolipids into biological membrane pools and loss of lysolipid upon dilution in the blood stream may compromise the thermosensitivity of the LTSL.

**Paper IV**

Prolonged storage of gel phase DPPC/DSPE-PEG$_{2000}$ liposomes result in the membrane accumulation of hydrolysis generated lysolipids and fatty acids. Drastic structural reorganizations occur in partially hydrolyzed liposome samples upon increasing the temperature through $T_c$. The most prominent effect is an increased tendency of the liposomes to disintegrate into discs with increasing degree of hydrolysis. Complete disintegration occurs when the amount of DPPC has decreased by, in some cases, as little as 3.6%. However, even if considerable amounts of hydrolysis products accumulate in the membrane the liposomes stay intact below $T_c$. Hence, the compositional changes may remain unnoticed. These results stress the importance of careful consideration of storage conditions and thermal history when evaluating liposome formulations. The formation of metastable liposomes with the propensity of disintegrating into discs may be utilized for certain applications, i.e. in the case of a temperature-induced release of a liposomal cargo.

**Paper V**

Discoidal structures are preferred over cylindrical micelles in PEG-lipid/lipid mixtures when the mixture contains components that reduce the spontaneous curvature and increase the monolayer bending modulus. Cholesterol, lanosterol and DSPE are components that induce disc formation.
Discoidal structures are, furthermore, preferred at temperatures below the $T_C$ of the lipid mixture. In this case, disc formation is likely promoted by a combination of high bending modulus and a reduced lipid/PEG-lipid miscibility.

Paper VI

Stable bilayer discs can be formed by dispersion of DSPC/cholesterol/DSPE-PEG$_{5000}$. By varying the PEG-lipid content, the size of the discs can be altered. The discs exhibit long-term stability and their size and structure remain unchanged in the temperature range of 25°C to 37°C. The discs function well as model membranes for drug partitioning studies. For certain classes of drugs, the discs may even produce more accurate partitioning data when compared to ULV or MLV.
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…Katarina för att du antog mig som doktorand och för att du är en så bra handledare. Jag har verkligen lärt mig jättemycket!

…Mats för biträdande handledarskap och för att du alltid tar dig tid när man kommer med frågor.

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…Emma, PEr, Anna och Nill för att ni är så himla bra helt enkelt!!!

…Kajsa för hjälpen med svenska sammanfattningen och för en himla massa annat förstås!

…min familj för glada tillrop och stöd!

…världens bästa Niclas och Alfred!
När man häller diskmedel i smutsigt diskvatten ser man hur diskmedlet sprider sig på ytan och pressar undan fetten. Denna egenskap beror på de amphifila molekylerna som finns i diskmedlet. Amphifila molekyler är speciella på grund av sina dubbla egenskaper. De består av en hydrofil, vattenlöslig del, och en hydrofob, fettlöslig, del (Fig. 42). När amphifila molekyler blandas med vatten kommer de, över en kritisk koncentration, att självassociera och bilda aggregat med de fettlösliga delarna vända mot varandra omgivna av de vattenlösliga delarna (Fig. 42). Ett flertal olika typer av aggregatstrukturer kan bildas beroende på faktorer såsom amphifilens form och den omgivande miljön.

Fig. 42. Schematisk bild på en amphifil molekyl och ett aggregat, en så kallad micell.

Amphifila molekyler utgör en viktig klass av molekyler. De finns runt omkring oss i vårt dagliga liv i t.ex. tvättmedel och tvål. En annan sort amphifila molekyler, så kallade lipider, utgör de huvudsakliga komponenterna i biologiska membran, såsom i membranet som omger en cell. Lipider är intressanta för läkemedelsdistribution i kroppen, då användandet av biologiskt kompatibla molekyler är ett krav i designen av läkemedelstransportörer. När ett läkemedel administreras intravenöst sprider det sig i kroppen utan något specifikt mål, vilket kan leda till toxiska biverkningar. Att uppnå effektiv behandling, vilket ofta innebär höga halter av läkemedel, och samtidigt undvika bieffekter kan därför vara svårt. En lösning på problemet kan vara att kapsla in läkemedlet i en transportör som levererar den aktiva substansen till målet.

Liposomer har visat sig lovande som sådana läkemedelsbärare. Liposomer är stärrika ihåliga partiklar i nanometerstorlek bestående av ett bilagermembran uppfylt av lipider (Fig. 43). Den speciella strukturen möjliggör transport av såväl hydrofoba som hydrofila läkemedel. Fettlösliga substanser
kan inkorporeras i membranet, medan vattenlösning kan inkapslas i det vattenfyllda inre hålrummet. För att nå sitt mål måste liposomerna cirkulera relativt länge i kroppen utan att elimineras av immunsystemet. Detta kan uppnås genom att inkorporera en polymer-lipid, så kallad PEG-lipid, i membranet. En förlängd cirkulationstid ökar sannolikheten att liposomerna anslas i vävnad med defekta blodkärl, såsom i tumörer och inflammaderad vävnad. Alternativt kan specifika målsökare, såsom små proteiner eller antikroppar, som söker sig till och fäster vid receptorer på de sjuka cellernas ytor, fästs på liposomens yta. Oftast strävar man efter liposomer som är intakta under cirkulationen och frisläpper sin last vid den sjuka vävnaden. En snabb och kontrollerad läkemedelsfrisläppning som resulterar i höga lokala halter är att föredra.


Fig. 43. Schematisk bild på en liposom.

Delarbeten I fokuserar på pH-känsliga liposomer. En naturlig pH-förändring som kan utnyttjas för att destabilisera pH-känsliga liposomer sker vid så kallad endocytos (Fig. 44). Endocytos är ett vanligt sätt för celler att ta upp extracellulärt material. Vid endocytos omsluter en del av cellmembranet...
materialet och knopas sedan av som en vesikel, en så kallad endosom (Fig. 44). Endosomen har ett lägre pH än blodet och denna pH-förändring kan användas för att stimulera en frisläppning av läkemedel från liposomerna. Ofta krävs dessutom att läkemedlet tar sig ut ur endosomen och in i cytosolen för att verka effektivt. Sådan delokalisering har observerats i tidigare studier.


Fig. 44. Schematisk bild på liposom fylld med läkemedel som 1) binder till en receptorn på cellens yta, 2) tas upp via endocytos och 3) hamnar i en endosom. 4) pH-förändringen kan resultera i destabilisering av liposomen och frisläppning av läkemedel som kan diffundera in i cytosolen.

I delarbeten II och III har vi studerat mekanismen bakom temperaturinducerad läkemedelsfrisläppning från en viss typ av temperaturkänsliga liposomer. Denna typ av liposomer är baserade på den ökade membranpermeabiliteten vid formuleringens småtttemperaturen, d.v.s. den temperatur då membranet övergår från gelfas till flytande kristallin fas. Studie II och III fokuserar på lysolipid-innehållande temperaturkänsliga liposomer (LTSL) som har uppfattats lovande egenskaper i tidigare studier. Man har funnit att innehållet i LTSL läcker ut väldigt snabbt vid temperaturer strax under den så kallade småtttemperaturen. Dessutom har liposomerna, fyllda med läkemedel mot
cancer, visat signifikant hämningseffekt på tumörtillväxt i djurstudier. Mekanismen bakom den snabba frisläppningen har inte studerats i detalj tidigare. Delarbete II fokuserade på morfologiska förändringar vid temperature Cyrkling och betydelsen av olika membrankomponenter. Delarbete III fokuserade på komponentsegregering och lysolipidretention i membranet och effekten av dessa på frisläppningsprofilen.

Resultaten från studierna av LTSL indikerade att även produkterna av lipiderhydrolyser kan påverka morfologin hos liposomer i gelfas. Sådana förändringar kan ha stor effekt på utvärderingen av resultat och liposomernas beteende i olika applicationer. I delarbete IV presenteras en studie av hydrolysekseffekterna på aggregatstrukturerna hos liposomer i gelfas och inverkan av temperaturförändringar.

I studierna presenterade i delarbete II och IV fann vi att liposomerna disintegrade till bilagerdiskar. Diskar har även observerats i andra sammanhang. Vi blev intresserade av under vilka omständigheter dessa membran-diskar kan bildas. I delarbete V har vi systematiskt undersökt hur olika membrankomponenter påverkar vilken typ av aggregat som bildas i PEG-lipid/lipid system.

Diskarna har potential att utnyttjas i flera olika biotekniska applicationer. I delarbete VI utvecklades en viss typ av diskar och utvärderades sedan som modellmembran för studier av interaktioner mellan läkemedel och lipidmembran.

Förhoppningsvis kan resultaten som presenteras i denna avhandling hjälpa till i den framtida designen av effektiva liposomer för läkemedels-transport. Vidare är den grundläggande förståelsen för under vilka omständigheter diskar bildas viktig för utveckling av diskar till olika biotekniska applicationer.
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