Studies on the Formation and Transformation of Protein-Containing Vesicles using Cryo-Transmission Electron Microscopy

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ABBREVIATIONS

AcOH      acetic acid
BICINE    N,N-bis-(2-hydroxyethyl)glycine
bp        boiling point
CAC       critical aggregation concentration
CAPS      3-(cyclohexylamino)-1-propanesulfonic acid
CH₃Cl     chloroform
conc.     concentrated
cryo-SEM  cryo-scanning electron microscopy
cryo-TEM  cryo-transmission electron microscopy
DAP       dialkyl phosphate
DDP       didodecyl phosphate
DHP       dihexadecyl phosphate
disp.     dispersion
DLS       dynamic light scattering
DMPC      dimyristoylphosphatidylcholine
DPPC      dipalmitoylphosphatidylcholine
DSC       differential scanning calorimetry
EM        electron microscopy
Et₂O       diethyl ether
EtOAc     ethyl acetate
FA(s)      fatty acid(s)
FAT        freeze and thaw
fßEM       freeze-fracture electron microscopy
FTIR      Fourier transformed infrared
GUV       giant unilamellar vesicle
hex       hexane
hr(s)      hour(s)
K          degree Kelvin
LM         light microscopy
LUV        large unilamellar vesicle
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Me$_4$N$^+$</td>
<td>tetramethyl ammonium</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MLV</td>
<td>multilamellar vesicle</td>
</tr>
<tr>
<td>MVV</td>
<td>multivesicular vesicle</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-$sn$-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>REV</td>
<td>reverse phase evaporation</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>sec</td>
<td>second(s)</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>sol.</td>
<td>solution</td>
</tr>
<tr>
<td>susp.</td>
<td>suspension</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicle</td>
</tr>
<tr>
<td>T</td>
<td>temperature</td>
</tr>
<tr>
<td>$T_c$</td>
<td>main phase transition temperature</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofurane</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>ULV</td>
<td>unilamellar vesicle</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Vis</td>
<td>visible</td>
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A. ABSTRACT AND ZUSAMMENFASSUNG

Abstract

The present work deals with the formation and transformation of vesicles composed of either fatty acid/soap molecules, dialkyl phosphates, or phospholipids (POPC=1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine).

The first part is devoted to the development of a new autocatalytic vesicular system, similar to the oleic anhydride/oleic acid system described before. In the latter system, a hydrophobic precursor, oleic anhydride, is hydrolyzed and generates amphiphilic molecules, oleic acid and oleate, which self-assemble to form vesicles under the chosen conditions (pH 8.5). The vesicles formed during the hydrolysis -or added as preformed vesicles at the beginning of the reaction- accelerate the oleic anhydride hydrolysis and thereby the production of vesicles.

The phosphotriester didecyl p-nitrophenyl phosphate was chosen as a potential candidate for the formation of another vesicle self-reproduction system as it appeared to fulfill the requirements: (a) didecyl p-nitrophenyl phosphate is a water-insoluble compound which, (b) upon hydrolysis, generates didecyl phosphate molecules, which possess bilayer forming properties. Vesicles were indeed obtained upon hydrolysis of didecyl p-nitrophenyl phosphate under basic conditions. Didecyl phosphate vesicles were, however, endowed with catalytic properties only if the hydrophobic precursor was incorporated mechanically in the vesicle bilayer or if the hydrolysis reaction was carried out in 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer. It turned out that these dialkyl phosphate vesicles, although possessing very interesting properties, were not suitable for alternative self-reproduction systems. In order to better understand the vesicle self-reproduction process, the classical oleic anhydride/oleic acid system was therefore studied in the rest of the present work.
In the second part of this work, possible mechanisms of formation and/or transformation of vesicles upon addition of new surfactants were studied. Two systems were examined: (a) the formation and/or transformation of oleic acid vesicles upon oleic anhydride hydrolysis in the presence of preformed oleic acid vesicles (self-reproduction of oleic acid vesicles) and (b) the formation of mixed POPC/oleic acid/oleate vesicles upon addition of sodium oleate to preformed POPC liposomes. In (a) the new amphiphiles were generated by the hydrolysis of oleic anhydride whereas in (b) they were added as an aqueous micellar solution.

The addition of amphiphiles to preformed vesicles can theoretically give rise to three events: (i) the preformed vesicles grow by uptake of new surfactants, (ii) new vesicles are formed independently of the preformed ones (de novo formation), and (iii) the preformed vesicles incorporate new surfactants in their bilayer and then eventually split.

A new methodology was developed to follow the changes occurring in the vesicle suspensions caused by the addition of new amphiphiles. Ferritin, an iron storage protein containing a dense iron core, was chosen as marker and entrapped in vesicles. These ferritin-containing vesicles were used as the preformed vesicles in the different experiments (a & b).

The suspensions were frozen (vitrified) as thin aqueous layers and examined at low temperature in a transmission electron microscope (cryo-TEM). Cryo-TEM permits the determination of the true size and of the number of lamellae of a vesicle as well as the direct visualization of certain large macromolecules, such as ferritin.

A large number of cryo-TEM micrographs were analyzed and various vesicle size distributions and ferritin distributions among the vesicles were determined. Evidence regarding the formation of new vesicles and the transformation of preformed vesicles was gathered by studying the differences observed between the initial state (before amphiphile addition) and the final state (after amphiphile addition) of the vesicular suspension.
In the case of the oleic acid vesicle self-reproduction (process a), it was found that the two main mechanisms operating during this reaction were the growth of some preformed oleic acid vesicles and the de novo formation of large vesicles. The growth mechanism was evidenced by the shift of the ferritin-containing vesicles towards vesicles of larger diameters whereas the observation of large oleic acid vesicles that did not contain any ferritin molecules indicated a de novo vesicle formation. Some experimental observations suggested that some vesicles may also have been formed by a splitting process.

In the case of the formation of mixed POPC/oleic acid/oleate vesicles (process b), it was found that, upon sodium oleate addition and under the chosen conditions, some POPC liposomes incorporated oleate molecules in their bilayer to generate mixed POPC/oleic acid/oleate vesicles as evidenced by the clear shift of the ferritin-containing vesicles towards vesicles of larger diameters, and that small vesicles, smaller than the preformed POPC liposomes, were formed as well. These small vesicles, which mainly did not contain any ferritin molecules, could result either from the self-assembly of the oleic acid/oleate molecules to form vesicles (de novo vesicle formation) or from the fission of a small vesicle from a larger one.

Cryo-TEM does not yield any quantitative information about the number of vesicles present in a suspension. Therefore, a new cryo-procedure for EM specimen preparation (block-face method) was considered as a possible tool to characterize suspension. Although some technical problems are still not solved, it appeared that this method can give quantitative as well as qualitative information about suspensions that cannot be obtained using other methods.
Zusammenfassung


Der Phosphotriester Didecyl-p-nitrophenyl-phosphat wurde als möglicher Kandidat für die Entwicklung eines sich selbst-reproduzierenden Vesikelsystems ausgewählt, da er folgende Anforderungen erfüllt: (a) Didecyl-p-nitrophenyl-phosphat ist eine wasserunlösliche Verbindung, welche (b) durch Hydrolyse Didecylphosphat-Moleküle bildet, die sich zu Bilayerstrukturen organisieren können. Unter basischen Bedingungen konnten in der Tat Vesikel erzeugt werden. Didecylphosphat-Vesikel wiesen jedoch nur katalytische Eigenschaften auf, wenn entweder die hydrophoben Phosphotriester vorgängig mechanisch in die Bilayerstruktur der Didecylphosphat-Vesikel integriert wurden oder wenn die Hydrolyse in 3- (Cyclohexylamino)-propansulfonsäure (CAPS) ausgeführt wurde. Obwohl Dialkylyphosphat-Vesikel sehr interessante Eigenschaften aufwiesen, eigneten sie sich nicht als alternatives, sich selbst-reproduzierendes System. Um den Selbst-Reproduktionsprozess zu untersuchen, wurde deshalb im weiteren Verlauf dieser Arbeit das klassische Oelsäureanhydride/Oelsäure/Oleat System eingesetzt.
Im zweiten Teil werden mögliche Mechanismen der Bildung und/oder Transformation der Vesikel nach Zugabe von zusätzlichen Tensiden untersucht. Zwei Systeme standen im Vordergrund: Einerseits (System a) wurde die Bildung und/oder Transformation von Oelsäure-Vesikeln nach der Oelsäureanhydrid-Hydrolyse in Anwesenheit vorgelegter Oelsäure-Vesikel untersucht (Selbst-Reproduktion von Oelsäure-Vesikel); andererseits (System b) wurden gemischte POPC/Oelsäure/Oleat-Vesikel untersucht, die sich durch Zugabe von Natrium-Oleat zu vorgelegten POPC-Liposomen bildeten. In (a) wurden neue Amphiphile durch die Hydrolyse von Oelsäureanhydrid generiert während in (b) die Amphiphile als wässerige micellare Lösung zugefügt wurden.

Die Zugabe von Amphiphilen zu vorgebildeten Vesikeln kann theoretisch folgendens bewirken: (i) die vorgebildeten Vesikel wachsen durch Aufnahme neuer Tenside, (ii) neue Vesikel können unabhängig von den vorgebildeten Vesikeln entstehen (de novo Bildung), und (iii) die vorgelegten Vesikel können sich nach Inkorporation neuer Tenside in ihren Bilayer eventuell teilen/abschnüren ("splitting/budding process").

Eine neue Methode wurde ausgearbeitet, um die, durch die Zugabe von Amphiphilen erzeugten Veränderungen in der Vesikelsuspension zu verfolgen. Als Marker diente dabei Ferritin, ein Eisenspeicherprotein mit einem dichten Eisenkern, das in die Vesikel eingeschlossen wurde. Diese Ferritin-enthaltenden Vesikel wurden als vorgelegte Vesikel in den beiden Experimenten (a & b) benutzt.

Die Suspensionen wurden als dünne Schichten eingefroren (vitrifiziert) und bei tiefen Temperaturen im Transmissionselektronenmikroskop analysiert (Cryo-TEM). Cryo-TEM erlaubt die Bestimmung der wahren Grösse der Vesikel sowie der Anzahl Lamellen, und ermöglicht die direkte Abbildung grosser Makromoleküle, wie Ferritin.

Durch Analysieren einer grossen Anzahl von Aufnahmen wurden die verschiedenen Grössenverteilungen der Vesikel und die Verteilung der
Ferritinmoleküle pro Vesikel bestimmt. Durch Vergleich des Ausgangszustandes (vor Tensidzugabe) und Endzustandes (nach Tensidzugabe) wurde nach Hinweisen für die Neubildung von Vesikeln und/oder die Transformation der vorgebildeten Vesikel gesucht.


Im Falle der Zugabe von Oleat zu POPC-Vesikel (System b) konnte eine Zunahme der Durchmesser von Ferritin-enthaltenden Vesikeln gemessen werden was auf die Aufnahme von Oleatmolekülen in die Bilayer der vorgelegten POPC-Liposomen und demzufolge auf die Bildung von gemischten POPC/Oelsäure/Oleat-Vesikel hindeutet. Zudem wurden viele kleine Vesikel (kleiner als die vorgelegten POPC-Liposomen) gefunden. Diese kleinen Vesikel enthielten praktisch keine Ferritinmoleküle und könnten entweder aufgrund eines Selbstorganisationsprozesses der Oelsäure/Oleat-Moleküle (de novo Vesikel-Bildung) oder durch die Abschnürung aus einem grossen Vesikel entstanden sein.

Die verwendete cryo-TEM Methode hat den Nachteil, dass sie keine quantitative Aussage über die Anzahl der vorhandenen Vesikel in der Suspension geben kann. Es wurde deshalb ein neues cryo-Verfahren ("block-face method"), als mögliches Werkzeug zur Charakterisierung von Vesikelsuspensionen getestet. Trotz noch zu lösenden technischen Schwierigkeiten scheint klar, dass dieses Verfahren quantitative und qualitative Informationen über Suspensionen geben kann, die mit keiner anderen Methode erzielt werden können.
B. INTRODUCTION

1. Aim of the present work

Life, as we know it, is cellular: a physical boundary separates the living organism from the outside world. There are good reasons why a spherical, semipermeable boundary is important: it may maintain the necessary local concentration of reagent as well as pH, it may permit the traffic of selected nutrient molecules, and it may provide a micro environment in which a reaction may occur that does not necessarily occur in bulk water. One could ask if the existence of an organized supramolecular complex was the necessary requirement for the onset of the chemistry of life (Luisi, 1996).

The autocatalytic formation of oleic acid vesicles is an interesting process as it binds chemistry with reproduction, which is one of the most basic cell processes. Moreover, the oleic acid self-reproduction system is very simple as vesicles, which are of great relevance due to the importance of compartments in life, are simply formed by the hydrolysis of an hydrophobic precursor, in this case oleic anhydride. The simplicity of this system makes it also attractive in the field of the origin of life.

Up to now, self-reproduction of vesicles was only obtained upon the hydrolysis of various fatty anhydrides in an alkaline medium. The development of other vesicle self-reproduction systems, in which vesicles are endowed with similar catalytic properties, would be useful to better understand the prerequisites for such processes and to generalize this phenomenon to other chemical compounds. One aim of this work is then to find potential candidates, of possible prebiotic relevance, that are able to form a self-reproduction system.
On the other hand a deeper insight into the mechanistic aspects of the self-reproduction of oleic acid vesicles is also necessary to better understand this process. Kinetic studies have shown that the presence of vesicles accelerates oleic anhydride hydrolysis, but one does not really know what is the role of the vesicles that are already present in the suspension. What happens to these vesicles when new oleic acid/oleate molecules are released? Do the newly generated surfactants form new vesicles or are they incorporated in the vesicle bilayers that are already present in the suspension? To answer these questions a method is needed that permits the detection of the changes occurring in the vesicle suspension as a result of the release of new surfactants.

Cryo-transmission electron microscopy (cryo-TEM) permits the direct visualization of vesicles and of eventually entrapped macromolecules. Thus cryo-TEM appeared suitable to investigate the effect of new surfactants on vesicles. The idea was then to introduce a marker inside the vesicles to mark the vesicles and to follow the formation of new vesicles and the transformation of the marked vesicles by electron microscopy.

The present work is divided into two main parts:
- the investigation of potential new vesicle self-reproduction systems based on phosphate-containing amphiphiles.
- the development of a new methodology using cryo-TEM to obtain information on the formation and/or transformation of vesicles upon the addition of new surfactants. Two systems were studied: the self-reproduction of oleic acid vesicles in the presence of preformed oleic acid vesicles and the addition of oleate molecules to POPC liposomes.
2. Amphiphilic molecules and their aggregation forms

2.1 The biological membrane

All living cells are compartmented by membranes. Biological membranes are involved in almost all cellular processes, ranging from mechanical functions, such as transport, to specific biochemical processes, such as signalling and photosynthesis.

In 1972, Singer and Nicolson proposed a “fluid mosaic model” for the organization of the proteins and lipids of biological membranes (Singer & Nicolson, 1972). The essence of their model is that membranes are two-dimensional solutions of oriented globular proteins and lipids of about 5 to 10 nm in thickness. The lipids are arranged in a bilayer and serve as a matrix for the membrane proteins (Fig. 2.1). Membrane proteins are responsible for the distinctive functions of membranes. Biological membranes are dynamic structures where the lipids are free to diffuse laterally in the lipid matrix and rotate around their axis; however, they do not flip easily from one side of a membrane to the other. They are asymmetric: the composition and therefore the properties of the inner and outer leaflet of the membrane differ (Stryer, 1988).

![Fluid mosaic model](image)

**Fig. 2.1** Fluid mosaic model as proposed by Singer and Nicolson (adapted from Winter, 1990)
The lipid matrix is mainly composed of a variety of phospholipids and its principal function is to act as permeability barriers to the free diffusion of solutes.

Phospholipids belong to a special class of compounds called amphiphilic molecules. Fig. 2.2 shows the chemical structure of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), a phospholipid commonly found in biological membranes of eucaryotic cells.

![Chemical structure of POPC](image)

**Fig. 2.2** Chemical structure of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC).

### 2.2 General structure of amphiphilic molecules

Amphiphilic molecules are made of an hydrophobic (water-insoluble) and an hydrophilic (water-soluble) moiety (Fig. 2.3). These molecules are surface-active and are also called surfactants.

![Schematic representation of amphiphilic molecules](image)

**Fig. 2.3** Schematic representation of amphiphilic molecules containing (A) one and (B) two hydrophobic chains and an hydrophilic head group.
The hydrophilic part, commonly called the polar head group (or head), can be neutral or charged. Amphiphiles can be cationic, anionic, zwitterionic or non-ionic depending on the nature of their head group. The hydrophobic part, also named apolar tail, is usually made of one, two, or three hydrocarbon chains.

### 2.3 Aggregation structures

Below a certain concentration, called the critical aggregation concentration (CAC), there is -in a first approximation- no interaction between amphiphiles dispersed in an aqueous solution and they are found as monomers. Above the CAC, monomers self-assemble to form a variety of aggregates. The driving force for this behavior is known as the hydrophobic effect (Tanford, 1980), which is of entropic nature (Israelachvili, 1991; Israelachvili et al., 1976). Amphiphiles in water show a dual behavior: the hydrophilic head groups want to achieve maximal hydration while the hydrophobic carbon chains tend to avoid contact with water by spontaneously building aggregates.

The forces that hold amphiphiles together in aggregates are van der Waals, hydrophobic, hydrogen-bonding, and screened electrostatic interactions. The structures are influenced by the surfactant concentration (lyotropic polymorphism\(^1\)), insaturation of the hydrocarbon chains, pH, ionic strength, and temperature (thermotropic polymorphism). The most common structures formed by amphiphiles are micelle, hexagonal phase, cubic phase, bilayer, inverted hexagonal phase.

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1. The lyotropic phase behavior of some biological amphiphiles has been described by Seddon (1996).
Amphiphilic molecules can be approximated by simple geometrical shapes that are characterized by their effective head group area $a_0$, volume $V$, and critical chain length $l_c$. Israelachvili et al. (1976) have introduced the dimensionless "critical packing parameter" $P$ which is defined as $V/a_0 l_c$. $P$ is frequently used to predict packing properties and aggregate shape (Table 2.1). The relative size of the head group and lipid chain are partially determined by the charge state of the head group and the degree of disorder of the chains.
<table>
<thead>
<tr>
<th>Shape</th>
<th>Critical packing parameter</th>
<th>Type of surfactants</th>
<th>Structures formed</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Diagram" /></td>
<td>P &lt; 1/3</td>
<td>single-chained lipids with large head groups</td>
<td>spherical micelles</td>
</tr>
<tr>
<td><img src="image2" alt="Diagram" /></td>
<td>1/3 &lt; P &lt; 1/2</td>
<td>single-chained lipids with small head groups</td>
<td>cylindrical micelles</td>
</tr>
<tr>
<td><img src="image3" alt="Diagram" /></td>
<td>1/2 &lt; P &lt; 1</td>
<td>double-chained lipids with large head groups</td>
<td>flexible bilayers vesicles</td>
</tr>
<tr>
<td><img src="image4" alt="Diagram" /></td>
<td>P ~ 1</td>
<td>double-chained lipids with small head groups</td>
<td>planar bilayers</td>
</tr>
<tr>
<td><img src="image5" alt="Diagram" /></td>
<td>P &gt; 1</td>
<td>double-chained lipids with small head groups</td>
<td>reverse micelles</td>
</tr>
</tbody>
</table>

Table 2.1 Molecular shape, packing parameter, and preferred aggregation structure of surfactants (Israelachvili, 1991).
3. **Vesicles**

Vesicles are closed bilayers, which have in most cases a spherical shape (Fig. 3.1). The term liposome\(^2\) is also used for vesicles formed by lipids. Vesicles share many common characteristics with membranes and have therefore been widely used as model of the lipid matrix of the biological membranes. One has to keep in mind, however, that liposomes are very simple structures compared to complex biological membranes.

![Diagram of a sectioned vesicle](image)

**Fig. 3.1** Schematic representation of a sectioned vesicle with \(R_o\) the outer radius, \(R_i\) the internal radius, \(d\) the bilayer thickness, \(V\) the chain volume, \(a_0\) the head group area and \(l_c\) the critical chain length.

Due to their ability to enclose aqueous compartments, vesicles are also used as microreactors (Walde & Marzetta, 1998), and carriers for drugs and DNA (Gregoriadis, 1988; Miller, 1999).

---

2. In this work, vesicles formed by natural phospholipids will also be called liposomes.
Some physical characteristics of vesicles will be described in more detail in the particular case of dialkyl phosphates and fatty acids in Chapter 4. (page 23) and in Chapter 5. (page 34), respectively. Oleic acid, a fatty acid, and didecyl phosphate, a dialkyl phosphate, were used in this thesis. Both compounds possess amphiphilic properties and are able to self-assemble to form vesicles.

### 3.1 Types of vesicles

Vesicles can be classified according to their size and morphology. They can be composed of one, two, or even more bilayers and can enclose other vesicles (Fig. 3.2). The vesicle diameter can greatly vary, from a few tens of nm to more than hundred μm.

Multilamellar vesicles (MLVs), as shown in Fig. 3.2, consist of a sequence of concentric bilayers separated by an aqueous phase. Large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs), as their name indicates, consist of a single bilayer surrounding a central aqueous volume. The minimal radius of a SUV is determined by the maximal packing that the amphiphile head groups will tolerate in the highly curved inner leaflet of the vesicle. Multivesicular vesicles (MVVs) are the common name for vesicles entrapping other non-concentric vesicles.

Giant unilamellar vesicles (GUVs) are vesicles of a diameter of 10 μm to 100 μm and can be formed, for example, by the so-called electroformation method introduced by Angelova and Dimitrov (1986).
Fig. 3.2 Schematic and simplified drawing of various types of vesicles with their typical diameter: multilamellar vesicle (MLV), multivesicular vesicle (MVV), large unilamellar vesicles (LUV) and small unilamellar vesicle (SUV). In this scheme the bilayer is represented as a single line and an aqueous phase is present between the bilayers. The vesicles are not drawn to scale.

3.2 Manufacturing of vesicles

Vesicles are prepared according to various methods depending on the amphiphiles used, on the type of vesicles desired and on the future application of the vesicles. The chemical nature of the macromolecules to be encapsulated also plays a role in the choice of the method. Vesicles can basically be prepared by three methods (Perkins et al., 1993; Szoka & Papahadjopoulos, 1980; New, 1990): physical dispersion, two-phase dispersion and detergent solubilization. The methods used in the present work are described in this paragraph.
3.2.1 Film dispersion

MLVs were the first vesicles produced (Bangham & Horne, 1964). They were obtained by the mechanical dispersion of a lipid film in an aqueous solution. This technique consists of depositing a thin lipid film on the walls of a flask by evaporation of an organic solution containing the amphiphiles. After drying of the film under high vacuum to completely remove the organic solvent, the desired aqueous solution is added and the lipid film is hydrated at a temperature above \( T_c \). The thus produced suspension is very polydisperse and contains mostly large MLVs. Hydrophobic compounds to be incorporated in the bilayer are usually added to the organic solution that contains the lipids. Hydrophilic substrates to be entrapped in the vesicle interior are added to the aqueous solution. The major drawback of using MLVs is their relatively low internal aqueous volume and the low physical stability (aggregation).

Subsequent treatment such as sonication (see 3.2.3), or extrusion (see 3.2.4) eventually preceded by freeze and thaw treatment (see 3.2.5), are necessary to yield a more homogeneous suspension of considerably smaller vesicles.

3.2.2 Reverse phase evaporation (REV)

Large unilamellar vesicles can be obtained through the transient formation of a water-in-oil emulsion. The reverse phase evaporation method was first described by Szoka and Papahadjopoulos (Szoka & Papahadjopoulos, 1978; Szoka et al., 1980) in the late 70’s. An aqueous phase and the compounds to be encapsulated are added to an organic solution that contains the amphiphiles. The two-phase system is sonicated to yield a fine emulsion and the organic solvent is then removed with rotary evaporation. At some point a viscous gel-like intermediate phase is obtained which spontaneously transforms into a liposome dispersion. The residual traces of organic solvent are then removed under reduced pressure (Fig. 3.3).
Fig. 3.3 Schematic drawing showing the key steps in liposome formation by the REV technique as proposed by Papahadjopoulos and co-workers (Szoka & Papahadjopoulos, 1978; Szoka et al., 1980). (A) Bilayer forming amphiphiles are dissolved in a water-immiscible organic solvent to form a lipid solution. (B) The aqueous phase containing the compounds to be entrapped is added to form a two phase-system that is subsequently emulsified (C) by brief sonication to form a water-in-oil emulsion. (D) As the organic solvent is removed, the preparation becomes viscous and (E) usually forms an intermediate gel. (F) As the organic solvent is further removed, the gel disperses into a suspension of single layered vesicles (from Lasic, 1993).

The ratio lipid to water determines the type of vesicles formed. MLVs are produced when amphiphiles are in excess while LUVs are mainly obtained for a low amphiphile to water ratio (New, 1990). This method yields in any cases LUVs or MLVs (Pidgeon et al., 1986) with a high aqueous space-to-lipid ratio and the thus obtained suspension is relatively homogeneous.

The principle of using the transient formation of a water-in-oil emulsion can also be slightly modified to produce other types of vesicles, for example multivesicular vesicles (Kim et al., 1983) and cell-size vesicles (Kim & Martin, 1981). The principal disadvantage of this method is the use of organic solvents and of sonication which can denature the different compounds. Moreover, traces of organic solvents in the resulting suspension
cannot be excluded, and gel chromatography or dialysis may be necessary to remove solvent traces.
This method is also suitable for the reconstruction of proteins in bilayers (see e.g. Rigaud et al., 1983).

3.2.3 Sonication

Ultrasonic treatment (sonication) is one of the most widely used method to produce small unilamellar vesicles. MLVs are disrupted by the high energy input caused by sonication and SUVs with a typical diameter of 20-50 nm are obtained (Huang, 1969). The two main apparatus used for sonication are either a probe tip or a bath sonicator. Probe tip sonicators deliver high energy input to the lipid suspension that can be overheated and there is considerable risk of chemical degradation of the compounds. In addition, the sonication tip tends to release metal particles into the suspension which must be removed by centrifugation. Bath sonication is a milder but less efficient method.

3.2.4 Extrusion

The size and the number of lamellae of vesicles can also be reduced by extrusion (Olson et al., 1979; Szoka et al., 1980). This technique consists of forcing the suspension through polycarbonate filters with a defined pore size. Several extrusions are required to bring the average vesicle size to the approximate filter pore size. The size reduction is more efficient using double stacked membranes. The procedure has to be performed at a temperature above the gel-to-liquid crystalline transition temperature of the amphiphile (Nayar et al., 1989).
The mechanism of extrusion is however not completely understood yet and different models are proposed (Clerc & Thompson, 1994; Hunter & Frisken, 1998).
Fig. 3.4 shows a large vesicle that is deformed on entering a pore of a polycarbonate filter. The vesicle must be deformed at some energy cost to fit into the smaller pore, and it must undergo a decrease in volume, either by diffusion of water through the membrane or through rupture, to enter the pore (Hunter & Frisken, 1998). Hunter and Frisken (1998) proposed that a minimum pressure that corresponds to the lysis tension required to rupture the bilayer membrane is necessary to extrude vesicles. They found that the size and polydispersity of the extruded vesicles were a function of extrusion pressure, lipid composition and temperature.

![Figure 3.4](image)

**Fig. 3.4** Schematic drawing of a vesicle of radius $R_0$ entering a pore of radius $R_p$ of a 100 nm pore size polycarbonate membrane. The applied pressure is $P_1$ and the pressure inside the vesicle is $P_2$ (according to Hunter & Frisken, 1998).

Clerc and Thompson (1994) propounded that the liposome bilayers form cylinders in the filter pores, which then break up in smaller cylindrical structures that are unstable and seal themselves to form vesicles of indented spherical shape (also observed experimentally by Mui et al., 1993). Extrusion is often combined with freeze and thaw treatment (paragraph 3.2.5).
3.2.5 Freeze and thaw (FAT)

MLV suspensions are often treated by several freeze and thaw (FAT) cycles (Hope et al., 1985; Mayer et al., 1985; Mayer et al., 1986; MacDonald et al., 1994) prior to extrusion. FAT was found to promote solute equilibration across the various bilayers of MLVs (Mayer et al., 1985; Mayer et al., 1986; Chapman et al., 1990). This is important as it was argued that the solute distribution was not homogeneous within the MLVs obtained upon dispersion of a lipid film in an aqueous buffer (Gruner et al., 1985). FAT was also found to be associated with morphological changes. Before FAT, samples exhibited the tightly packed “onion-like” arrangements of concentric bilayers. After FAT, new structures were formed in which the interlamellar spaces were much increased, and MVVs were formed as well (Mayer et al., 1985). This method thus enhanced entrapping efficiency and the suspensions obtained after extrusion were more homogeneous than in the absence of FAT treatment. Vesicles were in some cases sonicated prior to FAT treatment (Pick, 1981; Oku et al. 1982; Oku & MacDonald, 1983). FAT induced the fusion of the small unilamellar vesicles to yield very large unilamellar vesicles that could even be detected by LM.

FAT mechanism is not completely understood. It is not clear, whether the bilayer ruptures during freezing or during thawing due to osmotic stress, or whether ice crystals are responsible for the disruption of the membrane.

3.3 Thermodynamics and kinetic aspects

The fact that preparation methods (see examples in paragraph 3.2) are necessary to yield vesicles of defined size and lamellarity indicate that vesicles are in general not thermodynamically stable states and that they do not form spontaneously (Lasic, 1999). Energy input is required (e.g. sonication, extrusion). This is the case for most conventional phospholipids.
Micelle-forming surfactants, in contrast to phospholipids, spontaneously self-assemble when dissolved in aqueous solutions to form micelles of defined size under the chosen experimental conditions. Micelles are thermodynamically stable.

Concerning the kinetic and dynamic aspects of vesicles, single-chained surfactant mixtures, e.g. cetyl trimethylammonium tosylate/sodium dodecylbenzene sulfonate, are a special class of vesicles and show a different behavior than traditional phospholipid systems (Kaler et al., 1989). It has been shown that, under certain conditions, vesiculation can occur spontaneously with formation of equilibrium systems. Fatty acid vesicles are also mixed surfactant systems (fatty acid/soap), and they can also form spontaneously in the sense that, for example, simple addition of a micellar solution to an intermediate pH buffer leads to the formation of vesicles without any input of energy (Blöchlinger et al., 1998). The thus obtained vesicles form, however, a polydisperse suspension with respect to size and lamellarity, and they can be extruded. Thereafter they display a narrower size distribution and under constant conditions are stable: they are kinetically trapped.

In all surfactant -phospholipid as well as fatty acid/soap- systems, there is an exchange between associated surfactants and monomeric (non-associated) surfactants in the bulk suspension. This exchange is very slow for conventional phospholipid liposomes (Israelachvili et al., 1977) and is expected to be considerably faster in systems formed by single-chained surfactants. In this respect, vesicles formed by single-chained surfactants are more similar to the highly dynamic micellar systems, for example, composed of sodium dodecyl sulfate (SDS).
4. Fatty acids

4.1 Introduction

Fatty acids (FAs) and their corresponding soaps are capable of self-assembling to form aggregates such as, for example, micelles or vesicles. Recently it has been found that vesicles formed by oleic acid and oleate molecules can also be obtained upon the hydrolysis of oleic anhydride and that these vesicles catalyze the anhydride hydrolysis (Walde et al., 1994). This system was described as a vesicle self-reproduction system. One part of this thesis is devoted to the study of this system (Chapter 10.) and to the effects of sodium oleate addition to POPC liposomes (Chapter 11.). Information about (i) the aggregation behavior of fatty acids, (ii) the oleic acid water system, (iii) the formation of oleic acid vesicles upon the hydrolysis of oleic anhydride, and (iv) the interaction of fatty acids with membranes are given in this chapter.

4.2 Fatty acid aggregates

The physical properties of fully protonated fatty acids and their corresponding soaps (ionized fatty acids) are quite different. For example, the solubility of long-chain protonated fatty acids in water is quite low in contrast to the very high solubility of corresponding potassium soaps above their melting-chain transition temperature. The physical behavior of fatty acids\(^3\) dispersed in aqueous solutions is rather complex. In water, fully ionized fatty acids may form micelles, liquid crystals (hexagonal type I), or crystals whereas fully protonated fatty acids separate as oil droplets or crystals, depending on the temperature. Gebicki and Hicks (1973 & 1976)

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3. For simplification, fatty acid, when used from now on without further precision, refers to the general class of compounds without specification of the ionization state of the carboxylic group.
and later Hargreaves and Deamer (1978a) showed that fatty acids could also form closed bilayers in the pH range 7 to 9. Fatty acid vesicles, in contrast to most conventional phospholipid liposomes, are made of two components, the protonated fatty acid and its soap, and are more sensitive to pH and ionic strength (Gebicki & Hicks, 1976). Thus fatty acids dispersed in water are able to form a variety of aggregates depending on fatty acid concentration, temperature (frozen or melted chain), cation (type and concentration), state of ionization (pH), and chain length (Small, 1986).

Fig. 4.1 summarizes the physical state of long-chain fatty acids at different ionization state below and above $T_c$. $T_c$ depends on the chain length, saturation, branching, and substitution of the fatty acids (Small, 1968).

$F_iq. 4.1$ Simplified summary of the aggregation states formed by long-chain fatty acids as a function of pH and temperature (adapted from Small, 1986).
At first, the stability of the lamellar phase was explained primarily through the formation of hydrogen bridges between protonated and deprotonated head groups, through the formation of acid-anion dimers (Gebicki & Hicks, 1976; Haines, 1983; Li & Haines, 1986). Cistola et al. (1986 & 1988) found, however, that bilayer aggregates of fatty acid remained stable at molar fatty acid/soap ratios that deviated significantly from 1:1. For example, oleic acid vesicles were stable up to an oleic acid/oleate ratio of 1:3. Quite generally, one can view fatty acid aggregates as extended structures stabilized by a balance of hydrocarbon chain interactions and of electrostatic interactions in the area of the head groups. Above pH 10, every surfactant bears a negative charge giving rise to a relatively large head group and strong electrostatic repulsion between the polar groups. Micelles (or hexagonal phase I) are, in that case, the most favored form of aggregation, as they can accommodate larger head groups. At pHs where fatty acids are partially ionized, the electrostatic repulsion between the polar groups is less and the mean surface area occupied by the head groups is smaller than in the case of the soap. In that case, a lamellar phase (or inverted hexagonal phase II) is built preferentially.

4.3 The oleic acid water system

Oleic acid (from olive oil) is the trivial name for \textit{cis}-9-octadecenoic acid. Oleic acid is the most abundant \textit{cis}-unsaturated fatty acid. Its chemical structure is shown in Fig. 4.2.

![Chemical structure of oleic acid](image)

\textbf{Fig. 4.2} Chemical structure of oleic acid (\textit{cis}-9-octadecenoic acid).
A titration curve of an aqueous solution of sodium oleate is shown in Fig. 4.3. Micelles, vesicles, and a separated oil phase are present in the ranges of A-C (pH > 9.7), B-E (9.7 > pH > 7.8), and D-F (pH < 7.8), respectively. The two pH plateaux observed (B-C and D-E) correspond to the ranges where two aggregate forms are coexisting (Morigaki, 1998).

![Fig. 4.3](image)

**Fig. 4.3** Equilibrium titration curve of sodium oleate. Various amounts of 1 M HCl were added to samples that contained 80 mM oleic acid and 88 mM NaOH, and the pH was measured at 25°C after equilibration (typically more than 3 days). The horizontal arrows indicate the region where micelles, vesicles (lamellar bilayers), and/or oil emulsions are present (Morigaki, 1998).

The apparent pKₐ (≥ 8.0) of oleic acid molecules in the vesicles is much higher than the pKₐ of monomeric carboxylic acids in water (pKₐ 4.8). Fatty acid vesicles have a high negative surface charge density and protons are sequestered at the bilayer surface (formation of an electrical double layer). This results in an increase of proton activity (decrease in apparent local pH) at the bilayer surface compared to the bulk solution at a given ionization state. The apparent surface pH may be as much as 3.0 units below that of the bulk pH (Haines, 1983).
The physical characteristics of oleic acid vesicles are summarized in Table 4.1. The CAC is difficult to measure and seems to depend on external parameters such as buffer used, ionic strength, and pH (Meyer, 1999). This CAC value thus slightly differs from one study to another. In any case the CAC of oleic acid is orders of magnitude higher than the CAC of conventional phospholipids (the CAC of DPPC is in the range of $10^{-10}$ M, Smith & Tanford, 1972).

<table>
<thead>
<tr>
<th></th>
<th>pKₐ</th>
<th>CAC</th>
<th>Tₑ (°C)</th>
<th>head group area</th>
<th>bilayer thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>oleic acid vesicle</td>
<td>8.0-8.5</td>
<td>21 μM</td>
<td>≈ 16°C</td>
<td>32 Å²</td>
<td>40 Å</td>
</tr>
</tbody>
</table>

Table 4.1 Some physico-chemical characteristics of oleic acid vesicles: pKₐ (Cistola et al., 1988), CAC of oleic acid (prepared in BICINE buffer 200 mM, pH 8.8) (Meyer, 1999), chain melting temperature Tₑ (Cistola et al., 1988), head group area (Jain, 1988), and bilayer thickness (Small, 1986).

Goto et al. (in press) have recently proposed a new model for the diluted oleic acid water system at intermediate pH ($10.4 < \text{pH} < 7.0$). They found experimental evidence suggesting that oleic acid vesicles always coexist with oleate micelles and propounded that the formation of giant oleic acid vesicles obtained upon extensive dilution of a oleic acid/oleate suspension (Wick et al., 1995) may involve a mechanism in which micellar aggregates and possibly non-aggregated monomers play a crucial role (Goto et al., in press). Further investigation of these basic aspects of fatty acid aggregates is certainly necessary.

4. The CAC of oleic acid at pH 7.4 is of the order of 10-20 μM (Edwards et al., 1995)
4.4 Formation of oleic acid vesicles upon hydrolysis of oleic anhydride

Fatty acid vesicles cannot be obtained only by hydration of a fatty acid film in buffered water or by titration of an alkaline micellar solution of the corresponding salt, but they can also form upon hydrolysis of the corresponding anhydride (Walde et al., 1994; Wick, 1996; Morigaki et al., 1997).

The hydrolysis of oleic anhydride was studied in detail in our group. The reaction was carried out by overlaying a drop of the water-insoluble precursor (anhydride) on top of an aqueous phase of pH 8-9. The hydrolysis of an anhydride entity yielded oleic acid and oleate, which, if present in a sufficient amount, self-assembled to form vesicles. The released oleic acid/oleate was measured by FTIR spectroscopy and the presence of vesicles was proved by LM and EM. The initial rate of anhydride hydrolysis was slow (lag phase), but after a few hours the reaction rate increased considerably (Fig. 4.4). Moreover, as depicted in Fig. 4.4, if preformed oleic acid vesicles were initially present, then there was no lag phase and the conversion of the anhydride molecules was faster. The vesicles had a catalytic effect, and it was found that the higher the vesicle concentration, the greater the effect. EM and LM investigations also suggested that the number as well as the size of the vesicles increased during the reaction (Walde et al., 1994; Wick et al., 1995; Wick, 1996).
Fig. 4.4 Hydrolysis of oleic anhydride catalyzed by spontaneously formed oleic acid vesicles at 40°C. A vesicle suspension (10 ml in 0.2 M BICINE buffer (pH 8.5)) was overlaid with 0.25 mmol of oleic anhydride and 0.025 mmol of oleic acid. The increase in concentration of oleic acid/oleate is plotted as a function of reaction time. Initial concentration of oleic acid/oleate: 0 mM (●), 20 mM (■). For an initial oleic acid/oleate concentration of 20 mM, the concentration of oleic anhydride (Δ) present in the vesicles during the reaction is also plotted (B) (from Walde et al., 1994).

The whole process was described as autocatalytic: the vesicles catalyzed the hydrolysis of anhydride and thereby their own production. In other words, the vesicles were able to self-reproduce (Fig. 4.5).
Fig. 4.5 Reaction scheme representing the hydrolysis of fatty anhydride leading to the release of fatty acids which then build vesicles that in turn catalyze the hydrolysis reaction, thus generating an autocatalytic system.

Since the anhydride molecules are water-insoluble, the hydrolysis reaction takes place either at the interface of the anhydride phase or at the surface of the vesicles. The catalytic effect of the vesicles was supposed to be due either to an increase in the nucleophilicity of $^\cdot$OH in the bilayer or to an augmentation of the surface area as a result of the presence/formation of aggregates in water (Mavelli & Luisi, 1996; Walde et al., 1994). The latter was considered the most important effect, because the surface of the vesicles is negatively charged due to the presence of carboxylates in the bilayer and thus a negative catalysis would be expected, as in the case of anionic micelles (Bunton, 1991; Bunton & Robinson, 1969).

The hydrolysis mechanism may, however, be somewhat more complex and also involve the transient formation of a (micro-) emulsion. It was also proposed that the acceleration behavior is connected to a pH drop, which has its origin in the product formed and the $pK_a$ change due to aggregate formation, such as vesicles and emulsion droplets (Blocher, 1997).
The vesicle self-reproduction system is analogous to the self-reproduction of reverse and aqueous micelles which were previously investigated in our group (Bachmann et al., 1990, 1991 a & b, 1992). Vesicles are, however, of greater biological relevance due to their bilayer structure.

4.5 Fatty acid and biological membrane (Hamilton, 1998)

The majority of the unesterified FAs (free FAs) found in blood plasma is associated with albumin or lipoproteins or bound to specific FA binding proteins. Due to their importance in various biological processes, FAs are in constant flux and need to enter and leave cells rapidly (Hamilton, 1998). The interactions of FAs with membranes have received much interest and many studies in which phospholipid liposomes were chosen as a model of the lipid matrix of cell membranes were carried out. Central questions are, for example, how FAs move across cell membranes and whether protein carriers are necessary.

Intracellular FA transport can be divided into three essential steps:
1. adsorption to one side of the membrane,
2. transmembrane movement (flip-flop), and
3. desorption from the other side of the membrane.

When FAs are in the presence of liposomes they bind rapidly and quantitatively to the bilayers. Partition coefficients ($K_p$) for long-chain FAs added to a suspension of LUV (O~100 nm) in water are of the order of $10^4$-$10^6$ in favor of the vesicles. The partition of FAs into lipid vesicles shows relatively little dependence on bilayer composition and structure, but it is dependent on the FA structure. Partition coefficient increases with increasing chain length and saturation. It seems that FA water solubility plays a key role in determining $K_p$; less soluble saturated FAs have greater $K_p$ than, for example, more soluble cis-unsaturated FAs. The cellular uptake
of FA is believed to occur without the help of proteins (Anel et al., 1993; Noy et al., 1989).

Although the studies reported in the literature and summarized in this paragraph were carried out at physiological pH (pH 7.4), about 50% of the FAs present in the phospholipid bilayer are in the protonated form. It has been proposed that the energy of activation for flip-flop must be the energy needed to create a void volume that extends across the leaflet to which FA diffuses. This energy is a property of the bilayer and should be independent of the FA chain length (Kamp et al., 1995). Flip-flop is, however, ionization dependent: protonated FAs move across the bilayer more rapidly than ionized ones (Kamp & Hamilton, 1992). The rate of transmembrane movement was found to be fast for SUV (Ø~25 nm) and LUV (Ø~100 nm), $t_{1/2} \leq 10$ msec and $t_{1/2} \leq 35$ msec, respectively (Hamilton, 1998). It indicates that FAs are capable of spontaneous flip-flop, raising the possibility that rapid transport through the lipid phase obviates the need for a transport protein (Hamilton, 1998; Kamp et al., 1995). However, studies with larger vesicles (Ø ≥ 200 nm), which theoretically more resemble the membrane cell due to their larger radius, showed that flip-flop in these larger vesicles was slow compared to that in SUVs or LUVs. Moreover, it was discussed that the rapid partition of FAs into the bilayer results in a transient asymmetry between the outer and inner hemileaflet areas, and this may compromise the integrity of vesicles. In that case flip-flop might be too slow to accommodate the flux of FA needed to support metabolic activity (Kleinfeld et al., 1997).

The rate of hydration also depends on the FA involved: the desorption rate increases with decreasing chain length and increasing unsaturation. This dependence on chain length reflects a hydrophobic effect, i.e., partitioning of FA between membrane and water. It was proposed that the transfer of FA from bilayer to albumin involves a slow, rate-determining solvation of FA bound to vesicles followed by a rapid diffusion and uptake of FA by albumin (Daniels et al., 1985). The dissociation rate was found to be
sufficiently rapid ($t_{1/2} < 1$ sec for FAs containing less than 20 carbon atoms) to suggest that complex mechanisms (e.g. protein-mediated) may not be required for their desorption from biological membranes (Daniels et al., 1985; Zhang et al., 1996; Hamilton, 1998).

From all these studies, the main trend is that passive diffusion through the lipid bilayer is the central mechanism. Hamilton (1998) summarized the transport of FAs as follows: “FA bind very rapidly to a phospholipid bilayer to establish a high concentration relative to the aqueous phase and reach an ionization equilibrium characterized by a $pK_a$ of ~7.5. They move spontaneously to the leaflet with the lower concentration of FA and desorb from the membrane at a rate that is highly dependent on the FA structure. The physical processes of adsorption to the lipid membrane, flip-flop, and desorption are mechanisms that are completely reversible and effective in ridding the cell of excessive FA, provided acceptors as albumin are available”.

The role of pH as well as the membrane perturbation caused by the uptake of FA were also investigated in the past. pH gradients were found to influence the distribution of oleic acid between albumin and phospholipid vesicles (Hope & Cullis, 1987) as well as to influence the transmembrane distribution of oleic acid in LUVs (Hamilton & Cistola, 1986). The partitioning of oleic acid to vesicles increased markedly with a small decrease in pH below 7.4, and almost all of the FA was bound to phospholipid liposomes at pH 5.5. The pH could be an important factor in regulating FA uptake and slightly acidic pH gradients could provide a driving force for removal of FA from albumin and for their uptake into a phospholipid membrane (Hamilton & Cistola, 1986). The incorporation of cis-unsaturated FAs such as oleic acid in membranes was found to induce a perturbation in the lipid acyl chain order in the bilayer. It is likely that this perturbation altered the interaction between membrane lipids and specific proteins which in turn altered the function of these proteins (Anel et al., 1993).
5. Dialkyl phosphate (DAP)

5.1 Introduction

In the previous chapter (paragraph 4.4, page 28), the oleic acid vesicle self-reproduction system was described. Up to now, only fatty acid vesicles were found to be endowed with such catalytic properties. One part of the present work deals with the development of another self-reproduction system. The phosphotriester/dialkyl phosphate system was chosen as a potential candidate (Chapter 9.). In this chapter some interesting properties of dialkyl phosphate vesicles are described.

5.2 Some properties of dialkyl phosphate vesicles

It has been known since the late 70’s that dialkyl phosphate (Fig. 5.1) is able to form vesicles (Kunitake & Okahata, 1978; Mortara et al., 1978). Vesicles formed by dialkyl phosphates and other synthetic surfactants have also been widely used as a model of the lipid matrix of biological membrane.

![Fig. 5.1 General structure of dialkyl phosphates: R_1 = C_nH_{2n+1}, R_2 = C_mH_{2m+1}.](image)

One important characteristic of vesicles is the transition temperature, \( T_c \), associated with a gel to liquid crystalline transition. This \( T_c \) can be determined by \(^{31}\text{P}-\text{NMR}, \) differential scanning calorimetry (DSC) or fluorescence depolarization, and it is characteristic of the amphiphiles
forming the vesicle and of both the concentration and nature of added solutes.

A series of vesicles formed from synthetic identical- and mixed-chain di-n-alkyl phosphate were examined. The $T_c$ was found to decrease with decreasing chain lengths, with decreasing asymmetry of the alkyl chain (Wagenaar et al., 1989 & 1992; Streefland et al., 1992; Blandamer et al., 1994), and with increasing flexibility of the hydrocarbon chain (introduction of a double bond for example) (Smits et al., 1996). The $T_c$ reflects the efficiency of alkyl chain packing in the bilayer. As electrostatic interactions between the phosphate head groups and the counterions affect the hydrocarbon chain interactions in the core of the bilayer, $T_c$ was postulated to be also dependent on the counterion. Indeed, $T_c$ decreased according to the following series: $Ca^{2+} > Na^+ > K^+ > Me_4N^+$, except for short chain amphiphiles ($R_1=R_2= C_{10}H_{21}$ or $C_{12}H_{25}$) (Table 5.1). It was suggested that the counterions bind to the phosphate head groups with decreasing effectiveness in the order $Ca^{2+} > Na^+ > K^+ > Me_4N^+$; stronger cation binding reduces head group repulsion and allows tighter alkyl chain interactions, leading to a higher $T_c$ (Wagenaar et al., 1992). The dramatic change going from $Na^+$ to $Me_4N^+$ was attributed to the modest hydrophobic character of $Me_4N^+$ cations which might penetrate into the surface of the vesicular aggregate and act as a spacer between the head groups. Compared to $Na^+$, it causes the alkyl chains of the amphiphiles to be more disordered (Fonteijn et al., 1992a; Blandamer et al., 1994). For vesicles formed from short chain amphiphiles, the head groups are further apart, more strongly hydrated, and less apt to bind to counterions, and so no effect was observed by fluorescence depolarization (Wagenaar et al., 1992). It was noticed that the stability of the vesicles increased in the same order, $Me_4N^+$DAP vesicles being more stable than $Na^+$DAP ones (Fonteijn et al., 1992a).
Table 5.1 Phase transition temperatures ($T_c$) of vesicles formed from some symmetric dialkyl phosphates ($R = R_1 = R_2$, see Fig. 5.1) (adapted from Wagenaar et al., 1992).

<table>
<thead>
<tr>
<th></th>
<th>$C_{10}H_{21}$</th>
<th>$C_{12}H_{25}$</th>
<th>$C_{14}H_{29}$</th>
<th>$C_{16}H_{33}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Na^+$</td>
<td>8°C</td>
<td>28°C</td>
<td>48°C</td>
<td>66°C</td>
</tr>
<tr>
<td>$K^+$</td>
<td>8°C</td>
<td>28°C</td>
<td>47°C</td>
<td>54°C</td>
</tr>
<tr>
<td>$Me_4N^+$</td>
<td>8°C</td>
<td>28°C</td>
<td>40°C</td>
<td>39°C</td>
</tr>
</tbody>
</table>

The effect of the pH on the formation and stability of dialkyl phosphate vesicles was also investigated, mostly using dihexadecyl phosphate (DHP) as the model compound. The apparent $pK_a$ of the phosphate in small vesicles was found to be around 5.5-6 (Carmona-Ribiero, 1990). The apparent $pK_a$ was thus about 4 $pK_a$ units higher than that of low molecular weight counterparts of DAP, as a result of the negative surface charge of the vesicles. At pH values corresponding to the $pK_a$, the number of H-bond interactions between the head groups was maximal and the bilayer was in a highly ordered state, thus resulting in a higher $T_c$ (Rupert et al., 1988; Carmona-Ribiero & Hix, 1991).

Humphry-Baker et al. (1991) pointed out that cationic or zwitterionic amine buffers, such as TRIS or imidazole, influenced the $T_c$ of dihexadecyl phosphate. The DSC spectrum indicated a strong buffer cations interaction with the anionic phosphate head groups of the vesicles. No such effect was observed for anionic buffers or at higher pH for zwitterionic ammonium sulphonate buffers such as CAPS. This might be ascribed to increased repulsive interactions between the vesicle interface and the buffer sulphonate group.
Studies on didodecyl phosphate (DDP) showed, that, above $T_c$, Ca$^{2+}$ induced the fusion of DDP vesicles and these large fused vesicles then transformed into tubular structures (Rupert et al., 1987 a & b). Similar Ca$^{2+}$-induced alterations were observed with naturally occurring amphiphilic molecules such as phosphatidic acid (Miner & Prestegard, 1984). Moreover, dialkyl phosphate vesicles are also able to engage fusion with virus membranes (Fonteijn et al., 1992b), with PS and PC liposomes as well as with erythrocyte membranes (Fonteijn et al., 1991 & 1990). This is of relevance for potential applications of DAP vesicles as drug carriers and shows once more the similarity in behavior of synthetic amphiphiles with natural lipids.

Recently it was found that cyclic double-chained phosphate amphiphiles can self-assemble to form vesicles (Buwalda et al., 1997). Single-tail phosphate containing branched (Ravoo & Engberts, 1994) or linear (Walde et al., 1997) alkyl chains are also able to form bilayers depending on the pH.
6. Ferritin

To study the mechanism of vesicle formation and transformation, such as the self-reproduction of oleic acid vesicles (Chapter 10.) and the formation of mixed vesicles (Chapter 11.), ferritin-containing vesicles were used. Some general information about the protein ferritin and its use as a vesicle marker is therefore summarized in the present chapter.

6.1 Ferritin, an iron-storage protein

The role of iron in living systems is very important. Its participation in electron transfer processes, in enzymatic reactions, and in carrying oxygen makes it essential for most organisms. Nevertheless, its presence provides a potential hazard: iron overload can cause extensive tissue damage. In aqueous solution at neutral pH, Fe(II) has the pronounced tendency to oxidize to Fe(III). Fe(III) then hydrolyzes and polymerizes to form essentially insoluble and potentially biologically inaccessible ferric hydroxides and hydrous ferric oxides such as, for example, Fe$_2$O$_3$·$n$H$_2$O. Therefore, nature has created two types of proteins with high affinity and high capacity for iron to handle this problem: the transferrins and the ferritins (Crichton & Charloteaux-Wauters, 1987; Crichton, 1981; Harrison et al., 1990).

The most important role of transferrins is the transport of iron among sites of absorption, storage, utilization, and excretion. Transferrin (Mr~80’000 Da) is a true carrier molecule which is conserved for many cycles of iron transport in its interaction with target tissues. Ferritin, on the other side, is an iron-storage protein. Ferritin is synthesized in response to iron and sequesters iron in a soluble, bioavailable, and non-toxic form. Ferritin is found in most cell types of humans and other vertebrates and in higher plants, fungi, and bacteria (Theil, 1987).
6.2 Ferritin structure and characteristics

Ferritin consists of a protein shell, called apoferritin, and a chemically rather inert inorganic ferric “iron core” (see Fig. 6.1 II for a schematic representation).

Fig. 6.1 (I) Ribbon diagram of the α-carbon backbone of an apoferritin subunit. The main body of the subunit is a bundle of four long helices A, B, C and D, with a short helix, E, lying at an acute angle to the bundle axis. The loop L joins helices B and C. The N-terminus, N, lies at the other end of the subunit to E. (II) Schematic representation of the ferritin molecule viewed down a molecular four-fold axis. Each subunit is represented by a sausage-shaped building brick with ends labelled N and E. The N-terminal region of the polypeptide chain lies close to the end labelled N and the E-helix close to that labelled E (adapted from Ford et al., 1984).

Apoferritin from horse spleen contains 24 polypeptide chains forming a hollow, roughly spherical protein shell with an average external diameter of 12 nm and internal diameter of 7.8 nm. The subunit consists of a bundle of four long α-helices and a fifth, shorter helix (Fig. 6.1 I). In the intact apoferritin, there are two types of channels, one hydrophilic and one hydrophobic, through which small molecules, ions and Fe(II) can access or leave the central cavity of the protein (Harrison et al., 1990). The large cavity inside the molecule can store up to 4500 Fe³⁺ atoms, packed in a
Microcrystalline inorganic component of approximate composition \((\text{FeOOH})_8 (\text{FeO:PO}_4\text{H}_2)\) (Crichton, 1981). The crystalline regions of the ferritin iron core have much in common with the natural mineral ferrihydrite \((5\text{Fe}_2\text{O}_3\cdot 9\text{H}_2\text{O})\) (Ford et al., 1984). The iron core of ferritin represents approximately half of the molecular weight of the full protein.

In this work, ferritin from horse spleen was used. Horse spleen apoferritin has a molecular weight of about 440,000 Da and its isoelectric point (pI) is between 4.2 and 4.6 (Urushizaki et al., 1971). The entire ferritin molecule has a molecular weight of about 900,000 Da. Ferritin can be described as a water-soluble protein containing a dense iron core. Moreover ferritin does not exhibit any type of specific affinity with lipids (Velev, 1997).

### 6.3 Ferritin as a marker

Ferritin is not only interesting because of its role in living systems, particularly in iron metabolism. Due to the very dense iron core, ferritin and conjugates of ferritin have been widely employed as markers for the localization of tissue and cell components using electron microscopy. The presence of the iron core in ferritin gives rise to scattering contrast (paragraph 7.2.2.1, page 49) which enables its visualization even in unstained electron microscopy preparations (Fig. 6.2). Ferritin is therefore well suited for its use in the bare-grid technique (paragraph 7.2.6.1, page 58) in which it appears as a black spot of about 7 nm (Massover, 1993).
Ferritin was also used in the past in several studies involving vesicles. Here are some examples from the literature:

1. As a model for protein: Velev (1997) studied the formation of ferritin shells over liposome surfaces as a model for the potential fabrication of functionalised protein and liposome/protein assemblies. These assemblies were obtained by non-specific and specific interactions:
   - ferritin arrays were formed on liposomes by electrostatic interactions
   - ferritin was attached to liposomes by specific binding, such as, for example, avidin-biotin type

The obtained liposome/ferritin structures were then investigated by EM. In this case, the negative staining procedure (paragraph 7.2.4, page 53) was chosen.
2. As ferritin is a water-soluble protein, its use as a marker within liposomes is ideal. Ferritin was employed before to find out if liposomes were indeed effective vehicles for the introduction of macromolecules into cells and to follow where exactly the entrapped molecules were transferred. This is of particular interest as liposomes are already used as drug and DNA carriers (see e.g. Miller, 1999). Other questions such as phagocytosis were also investigated using the system lipid/ferritin. In these studies, liposomes that contained ferritin in their aqueous interior were first prepared. The non-entrapped ferritin molecules were then removed from the suspension either by centrifugation or by gel permeation chromatography. In a second step the ferritin-enriched liposomes were incubated with cells or injected into tissues. Finally the migration of the liposomes or the uptake of the entrapped ferritin molecules was deduced by examining thin-sections of the biological samples with electron microscopy (see e.g. Bernon et al., 1983; Hernández-Yago et al., 1980; Petty et al., 1981; Cudd et al., 1984; Lelkes et al., 1984).

An interesting review of liposome markers used in light and electron microscopy is given by Foldvari (Foldvari et al., 1992).
7. Transmission electron microscopy: a tool to characterize vesicles

7.1 Introduction

Light microscopes and electron microscopes are instruments that allow us to get information about the microscopic world, invisible to the naked eye. For particles greater than a few micrometers in diameter, light microscopy (LM) is adequate. For smaller objects, however, LM fails because the wavelength of visible light is large compared to the object to be investigated and electron microscopy (EM) is used instead (Fig. 7.1).

![Fig. 7.1 Scale of sizes: from the macroscopic world to the atomic one.](image-url)
The eye and the different microscopes are characterized by their resolving power. Resolution is the ability to visualize two objects as distinct. The minimum distance that can be resolved can be approximated by \( d \) (Abbe Formula) (eq. 1):

\[
d = \frac{0.61\lambda}{n \cdot \sin \alpha}
\]

where \( \lambda \) is the wavelength, \( n \) is the index of refraction, \( \alpha \) is the half-aperture angle, and \( n \sin \alpha \) is the numerical aperture NA (see also Fig. 7.3).

The naked eye can resolve objects that are at least 0.1 mm apart.

### 7.1.1 Light microscopy (LM)

LM is commonly used to examine vesicles larger than 1 \( \mu \)m (giant vesicles). The interaction of the photons with the membranes is responsible for the detection of the vesicles. The bilayer has a higher refractive index than the aqueous medium, so the light wave is slowed down while going through the bilayer. Light that has crossed a vesicle bilayer has therefore a different phase than light that did not encounter any objects. LM can take advantage of this phase difference (e.g. by using phase contrast mode or differential interference contrast mode) and bilayers are then rendered visible.

Another possibility is to combine LM with fluorescence techniques. Fluorescence microscopy offers the opportunity, for example, to follow reactions occurring inside a vesicle or taking place specifically at the bilayer surface by using adequate markers (Bucher et al., 1998; Fischer et al., submitted).
7.1.2 Electron microscopy (EM)

Electrons have a dual nature: on one hand they are particles with a mass \( m \) \((m = 9.1 \times 10^{-31} \text{kg})\) and on the other hand they are waves as first proposed by Louis de Broglie. The wavelength \( \lambda \) of an electron is inversely proportional to its mass \( m \) and velocity \( v \):

\[
\lambda = \frac{h}{mv}
\]

where \( h \) is the Planck’s constant \((h = 6.626 \times 10^{-34} \text{J} \cdot \text{s})\).

If an electron is accelerated in an electric field of potential \( U \), its kinetic energy is given by \( E \):

\[
E = m \cdot v^2 = eU
\]

where \( e \) is the charge of an electron \((e = 1.6 \times 10^{-19} \text{C})\).

For an accelerating potential of 120 kV, the electrons have a wavelength \( \lambda \) of about 3.5 pm \((\lambda_{\text{green light}} \sim 545 \text{ nm})\). The resolving power of a transmission electron microscope is typically in the range of 0.2 nm.

An important difference between a light microscope and an electron microscope is that the glass lenses of a light microscope are replaced by electromagnetic lenses that are capable of deflecting electrons. In contrast to a light microscope, the spherical aberration of the electromagnetic lenses cannot be corrected. Spherical aberration means that the focal length for rays close to the optical axis of the lens is different (larger) than that for those farther away from the center. Very small numerical apertures \((10^{-2} \text{ rad})\) are used in transmission electron microscope to minimize spherical aberration. This explains why, despite the short wavelength of the electrons (in the pm range), the resolving power of a TEM is of “only” \(\sim 2 \text{ Å} \).
The most important constraint imposed by the use of an electron beam is the need of a high vacuum to avoid collisions of the beam electrons with gas molecules. Aqueous samples cannot be examined in their native state since water would immediately evaporate in the evacuated column of a microscope and therefore have to be stabilized (paragraph 7.2.3, page 52) prior to electron microscopy observations.

For the visualization of vesicles, three techniques are commonly used: the freeze-fracturing replica technique (paragraph 7.2.5, page 54), the bare-grid method (paragraph 7.2.6, page 58), and the negative staining procedure (paragraph 7.2.4, page 53). Negative staining will be only briefly described as this technique has the disadvantage of inducing artifacts in surfactant systems.

### 7.2 Transmission electron microscopy (TEM)

#### 7.2.1 Electron interactions with matter

When an electron encounters solid material, it can pass through it without any interactions or undergo elastic scattering or inelastic scattering. Fig. 7.2 summarizes the most important interactions of electrons with solid matter. If the specimen is thin, a large number of the electrons are unscattered due to the loose structure of matter. Unscattered (or undiffracted) electrons do not lose any energy and their paths remain unchanged. The probability of scattering increases with increasing atomic number and specimen thickness. Unscattered and elastically scattered electrons are used to image thin samples in TEM. Inelastic scattering events create a number of signals that provide information about the chemical composition of the sample (characteristic X-rays, Auger-electrons, transmitted electrons with specific energy loss) or allow for the description of the surface topography of the specimen (secondary electrons, backscattered electrons).
Fig. 7.2 Beam electron-specimen interactions.

### 7.2.1.1 Elastic scattering

When electrons in the beam impinge, or pass very close to, an atomic nucleus, they are deflected through relatively large angles, approximately 0.1 rad, without any loss of energy. This process is called elastic scattering. The scattering angle depends on the atomic mass (the higher the atomic mass, the larger the scattering angle) and on how close to the nucleus it passes (the closer the electron passes to the nucleus, the larger the scattering angle). Electrons scattered through a very large angle can be removed by the objective lens aperture (Fig. 7.3 & paragraph 7.2.2.1, page 49).
Inelastic scattering results from the collision of a beam electron with one of the orbital electrons. In this case, the electrons are deflected through very small angles, typically 0-1 mrad, and lose some energy. The number of inelastically scattered electrons increases with increasing specimen thickness. Inelastically scattered electrons deteriorate both the scattering absorption contrast (paragraph 7.2.2.1, page 49) and the phase contrast (paragraph 7.2.2.2, page 50) conditions. In addition, inelastically scattered electrons are polyenergetic (polychromatic) and the chromatic aberration of the objective lens becomes a problem. Chromatic aberration means that electrons of different energies (wavelength) do not have the same focal length.

Inelastically scattered electrons carry, however, information about the elementary composition of the specimen.
7.2.2 Contrast

7.2.2.1 Scattering absorption contrast

Scattering absorption represents the principal mechanism of contrast formation down to a resolution of approximately 1.5 nm. Electrons are elastically scattered according to the mass thickness variations (frequently produced e.g. by staining with metal salts or shadowing with heavy metals) in the sample. As illustrated in Fig. 7.4, electrons that are scattered into large angles are stopped by the objective lens aperture and do not reach the corresponding point in the image plane. A smaller objective lens aperture provides higher contrast but reduces resolution (eq. 1 page 45).

![Illustration of the scattering absorption contrast](image)

**Fig. 7.4** Illustration of the scattering absorption contrast. The electrons are elastically scattered through a large angle and then absorbed by the objective lens aperture. Therefore they do not contribute to the image and a kind of shadow image of the object is produced (from Kopp, 1981).

For example, the dense iron core of ferritin gives rise to scattering absorption contrast and is visualized as a black dot whereas the apoferritin remains invisible. The apoferritin can be visualized either directly by phase contrast (Massover, 1993) (paragraph 7.2.2.2, page 50) or indirectly by
negative contrast in which technique the molecules are embedded in a heavy metal layer (paragraph 7.2.4, page 53).

7.2.2.2 Phase contrast

For higher resolution (1.5 – 0.3 nm), the objective lens aperture needs to be very large so that all scattered and diffracted electrons can pass and no scattering absorption contrast is produced. The same is true for samples, which consist of low molecular weight components (typically the preponderant elements in biological material), e.g. lipid vesicles embedded in vitreous ice. Such samples do no longer scatter significant numbers of electrons through large angles and the structures are rendered visible mainly by phase contrast.

The incoming electron wave, while traversing the specimen, is retarded according to the variation of the internal potential (corresponding to the refractive index in LM). This results in a small phase shift of the diffracted beam with respect to the undiffracted beam. In order to transform this small phase difference into a visible contrast, the phase of the diffracted beam has to be shifted by approximately $\pi/2$ so that it is “in phase” with the undiffracted beam. The waves can then constructively interfere to produce phase contrast.

In light microscopes (with spherical aberration corrected lenses), this $\pi/2$ phase shift can be obtained with a phase plate that shifts the phase of the undiffracted beam. In electron microscopes, however, spherical aberration of the electromagnetic lenses at present cannot be corrected. For each diffracted beam a different defocus value is therefore required to match the phase of the undiffracted beam to produce optimal contrast. At a given defocus some components (composition, object periodicity) of the specimen provide maximum contrast while others remain invisible.

Lipid vesicles or proteins embedded in vitreous ice are generally imaged at 2-3 µm in the underfocus (Dubochet et al., 1988) where adequate phase contrast is produced.
7.2.2.3 *Omega energy filter*

The energy filter is placed after the objective lens. In the energy filter of the Zeiss EM 912 OMEGA, all the electrons are spatially separated according to their energy by four magnetic prisms which are arranged so that the electron beam path forms a Ω-shape. The electrons that have lost energy in the specimen are more strongly deflected by the spectrometer and do not return on the electron-optical axis. Thus elastically (or specifically inelastically) scattered electrons can be selected for imaging and electrons with unwanted energy are trapped by the slit aperture (Fig. 7.5).

![Diagram of Omega energy filter](image)

*Fig. 7.5* Omega electron energy loss spectrometer

In the elastic imaging mode, also called the zero-loss mode, only elastically scattered and unscattered electrons, i.e. the monoenergetic electrons with an energy loss of \(-0\) eV, pass through the slit. By cutting off the inelastically scattered electrons, the contrast is clearly enhanced (see e.g. Fig. 8.6). Zero-loss imaging is advantageous for the analysis of frozen-hydrated samples, since images can be recorded closer to focus (better resolution) and with fewer electrons (Grimm et al., 1998).
7.2.3 Fixation

Fixation consists of stabilizing the structures so that they are suitable for further investigation with EM. The specimen needs to sustain the high vacuum of the electron microscope column and must be stable towards the electron beam. Therefore, the aqueous solution that constitutes the natural medium of biological samples has to be removed or transformed into another stable state. In surfactant systems, fixation is the first and most critical step in the preparation of an EM specimen as it conditions all the subsequent processes and determines how accurately the objects are reflected by the EM micrographs.

Aqueous surfactant samples are usually physically fixed by freezing (cryoimmobilized). The freezing process must be very fast to prevent the formation (nucleation) and growth of ice crystals which would destroy the sample. Moreover, fast freezing (in the μs to ms range) increases the potential of trapping native structures and dynamic events.

At ambient pressure, layers of a few μm can be adequately cryoimmobilized (in a microcrystalline state without the presence of large ice crystals). If freezing occurs fast enough, the formation of ice crystals can even be completely impeded and water can be transformed into an amorphous, vitreous state. In this case, the liquid is cooled so rapidly that the water molecules become immobilized before they have time to crystallize (Dubochet et al., 1982a). Thin films up to a thickness of 1 μm can be vitrified at ambient pressure (Dubochet & McDowall, 1981). Thicker films are frozen in a (micro-) crystalline phase.

Samples up to a thickness of ca 200 μm can, however, be adequately frozen by using high-pressure (about 2100 bar) (Moor, 1987). The pressure counteracts the tendency of water to expand thereby increasing its viscosity. The freezing point is then lowered and the rate of nucleation and of crystal growth are reduced (Bachmann & Mayer, 1987; Müller & Moor, 1983).
Liquid ethane and liquid propane are the two commonly used cryogens. Both are excellent cryogens because they can be warmed by nearly 100°C and thus absorb heat before significant evaporation takes place. The process of calefaction ("Leidenfrost Effekt"), in which a layer of gas is formed that prevents good thermal contact between the sample and the cryogen, is therefore avoided. In contrast, calefaction hinders efficient cooling with liquid nitrogen. The difference between the two cryogens is that propane (bp = -42°C) sublimates more slowly than ethane (bp = -89°C) and therefore remains longer on the sample (Dubochet et al., 1988).

Alternatively biological samples can be chemically fixed. In that case, proteins and lipids are first stabilized by chemical reactions so that they can sustain water removal.

7.2.4 Negative staining technique

Lipid-bilayers were first observed by electron microscopy using the negative staining method (Bangham & Horne, 1964) (Fig. 7.6). In this technique, vesicles are adsorbed to a carbon coated EM grid and subsequently embedded in a heavy metal salt solution that is then dried. Membranes appear as white lines on a dark background. The most frequently used staining agents are solutions of uranyl acetate, phosphotungstic acid, and ammonium molybdate. Staining with a solution of heavy metals can induce artifacts as it alters the vesicular system in two ways: first its composition changes through the addition of stain, and secondly the drying causes large changes in the total concentration of amphiphile plus stain. For example, the addition of stain can give rise to "bilayered structures" even in systems that are molecular or micellar solutions in their original state (Talmon, 1983).
Freeze-fracturing replication is a very important and widely used method to characterize cells, vesicular suspensions, and other lipid water systems. This method is relatively free of artifacts (cryofixation without chemical pretreatment) and can give a faithful representation of the surfactant system. The sample is cryoimmobilized and fractured, and a replica of the fractured face is then produced by removing the organic material. The replica is observed in a TEM at room temperature.

**7.2.5.1 Methodology**

As already mentioned, fixation, in this case freezing, is the crucial step. Freezing of the suspension should occur quickly to prevent the formation of large crystals which can lead to reorganization of the aggregates in the suspension, deformation, and even destruction of the bilayers.
One way to satisfactorily freeze a relatively small volume of bulk suspension is to use a propane jet device (Müller et al., 1980). The suspension is sandwiched between two copper platelets and subsequently frozen by the projection of an opposed pair of high velocity jets of cold liquid propane (T < 93K). The frozen bulk is then processed as described in Fig. 7.7 to produce either a coated sample that can be examined by cryo-SEM or a replica for TEM investigations.

The fracture plane passes through the hydrophobic interior of the bilayer for energetic reasons and results in the formation of holes and caps. Whether the fracture plane goes through the frozen bulk or near the copper platelets cannot be controlled. This uncertainty is a disadvantage of this method since the copper platelets might induce surface effects which lead to redistribution and alteration of the objects in the suspension.

Suspensions can also be frozen by other techniques summarized in Sitte et al. (1987). Frequently used are the spray-freezing method (Bachmann & Schmitt, 1971) and slam or impact freezing (van Harreveld & Crowell, 1964).
The vesicular suspension is frozen and then... fractured under vacuum at $p \sim 10^{-7}$ torr and $T \sim -150^\circ$ C. Fracturing occurs through the hydrophobic domains of the vesicles and thus results in the formation of caps and holes. Two complementary surfaces are obtained.

The fractured surface is shadowed with Pt/C from an angle of $45^\circ$, before being strengthened by a layer of C at $90^\circ$ --- Cryo-SEM or to yield a replica of the exposed face --- TEM.

Fig. 7.7 The different steps involved in the fabrication of a replica.

7.2.5.2 Examples of application

The direct analysis of fTEM micrographs does not give the real size distribution of the vesicles, as only a few vesicles are broken in their equatorial plane. Algorithms are, however, available to transform the size distribution of more or less homogeneous suspensions as obtained by fTEM in a true number-weighted size distribution (Egelhaaf et al., 1996). The number of lamellae of a vesicle cannot be unambiguously determined.
Large proteins incorporated in the lipid matrix can be detected, as the fracture plane goes through the bilayer; whereas macromolecules entrapped in the aqueous interior of a vesicle cannot be observed using this technique.

One of the main applications of ffEM in surfactant science is the characterization of the various phases formed by a lipid system. For example, morphological changes occurring around the pretransition temperature (ripple phase) and the main transition temperature (Meyer, 1999; Listemann, 1996; Verkleij, 1989) (see example depicted in Fig. 7.8), the fusion process of membranes (Verkleij et al., 1980; Verkleij et al., 1985), the morphology and symmetry of cubic phases (Delacroix, 1998), and carveolae structures (Meyer et al., 1998) have been investigated by ffEM.

![Fig. 7.8](image)

**Fig. 7.8** The transition from L_{β'} gel phase, through the P_{β'} phase, to the L_{α} liquid-crystalline phase of DMPC, as detected by freez-fracturing (from Verkleij, 1989).

The freeze-fracturing method is also widely used for surface analysis by scanning electron microscope (SEM). In this case the replica is not removed from the specimen and the surface is observed at low temperature (cryo-SEM) (Fig. 7.7).
7.2.6 Cryo-transmission electron microscopy (cryo-TEM)

For the last 15 years, cryo-TEM has been widely used to investigate surfactant systems. In this method the specimen is preserved in a frozen-hydrated state and is directly visualized in a transmission electron microscope at low temperature to avoid devitrification of the specimen. The bilayer of a vesicle gives rise to enough phase contrast to allow its detection without the addition of any contrasting agents. Some aspects of the use of cryo-TEM to study vesicle suspensions are given in Chapter 8.

7.2.6.1 The bare-grid method (Dubochet et al., 1988)

The specimen are either prepared on a “bare-grid” or on a grid covered with a perforated carbon film (holey carbon film) (Fig. 15.1, page 209) (Fukami & Adachi, 1965).

A drop of the suspension is first deposited on the grid and the excess liquid is then removed by blotting with filter papers, so that a thin film spanning (ideally much thinner then 500 nm) across the holes is formed (Fig. 7.9). The suspension is then vitrified by quickly plunging (≥ 3 m/s) the grid into liquid ethane. A cooling rate of 10⁶ K/sec is required to achieve vitrification (Bachmann & Mayer, 1987). The frozen-hydrated specimen is examined in a transmission electron microscope at low temperature (≈ -170°C).
The blotting time determines the thickness of the vitrified film. It is a crucial and difficult parameter to control. On one hand, a film that is too thick cannot be vitrified and electrons are hardly able to penetrate it. On the other hand, objects are excluded from a film that is too thin, and an empty aqueous layer or even no film at all is achieved. The blotting time has to be adjusted for each system. The film thickness should ideally be only slightly thicker than the investigated objects, so that they are embedded in the amorphous ice layer without deformation. They are also best examined under these conditions (optimal contrast).

Theoretically, a water layer up to 1 μm should be frozen in an amorphous state using this technique (Dubochet & McDowall, 1981). Vitrification is, however, not always successfully achieved. The state of the frozen aqueous layer can be controlled by electron diffraction. The diffraction pattern of amorphous ice is characterized by two diffuse rings at 0.224 nm and 0.370 nm and can be easily differentiated from crystalline ice.

To prevent artifacts induced by temperature changes or evaporation processes occurring during specimen preparation, the preparation has to be carried out in a controlled environment (Egelhaaf, 1996; Vinson et al., 1991; Bellare et al., 1988: paragraph 8.2, page 62).
7.2.6.2 Examples of application of the bare-grid technique

TEM of a frozen-hydrated sample provides a projection image of the object. The true vesicle size and the lamellarity of the vesicles can be directly obtained. Certain large molecules entrapped in the vesicle interior can also be visualized.

Suspensions of small biological objects such as viruses were first studied by this technique (Lepault et al., 1983; Adrian et al., 1984; for a short review see Stewart & Vigers, 1986). Lepault et al. (1985) imaged for the first time unstained vitrified DMPC liposomes. Since then cryo-TEM has found many applications in structural biological (Amos et al., 1982) and surfactant science (Vinson et al., 1991; Talmon, 1996; Almgren et al., 1996).

The transition of vesicles to micelles by the addition of detergents (such as, for example, octyl glucoside or Triton X-100) can be followed by cryo-TEM (see e.g. Vinson et al., 1989; Vinson et al., 1991; Walter et al., 1991; Edwards et al., 1989; Edwards et al., 1993; Silvander et al., 1996; Seras et al., 1996). Lamellar phase (Ponsinet & Talmon, 1997), superstructures of lipid membrane (Klösgen & Helfrich, 1997), lipid polymorphism (Frederik et al., 1991), and intermediary structures occurring during membrane fusion (Frederik et al., 1989a) were directly visualized by cryo-TEM. The interaction between cationic liposomes and DNA (Huebner et al., 1999; Battersby et al., 1998; Templeton et al., 1997; Gustafsson et al., 1995;) was also studied by cryo-TEM.

It seems that this technique is not only restricted to aqueous suspensions: Oosterwegel et al. (1995) studied the phase behavior of synthetic polymers systems in toluene.
Time-resolved cryo-TEM opens new dimensions as it permits the study of transient changes in microstructure occurring during chemical or physical transformation. These dynamic processes can be induced on the grid by a temperature change or by a change in pH, salt, or reactant concentration by rapid mixing with appropriate solutions. Such procedures are called on-the-grid processes (Talmon et al., 1990). For example, Groll et al. (1996) studied the temperature dependent intermediate structures during the main phase transition of DMPC. Siegel et al. (1989 & 1994) investigated the transition of an inverted hexagonal phase HII to a Lα phase, which is presumably an important step in the fusion process of membranes. Berriman & Unwin (1994) introduced a new method in which the transient structures are trapped by layering one reactant on the microscopic grid and spraying on a second reactant immediately before freezing. Using this technique, the effect of small molecules on macromolecules or protein-protein interactions can be studied (White et al., 1998).
C. RESULTS AND DISCUSSION

8. Cryo-transmission electron microscopy and vesicles

8.1 Introduction

Cryo-TEM, more precisely the bare-grid method, was chosen as a tool to study the formation and transformation of vesicles (oleic acid self-reproduction process, Chapter 10. and the formation of mixed vesicles upon the addition of Na-oleate, Chapter 11.). As already mentioned, this method gives information about the size and the number of lamellae of a vesicle and also permits the direct visualization of certain entrapped macromolecules, such as ferritin.

This technique, like the other electron microscopy methods, has, however, its own limitations and problems. In this chapter, some aspects of the use of cryo-TEM to investigate vesicular suspensions are discussed.

8.2 Environmental chamber

Due to their physical characteristics, vesicles are very sensitive structures: for instance, salt, temperature, pH, and concentration changes can induce morphological alterations.

Prior to vitrification, thin aqueous films have a very high surface area-to-volume ratio (see e.g. Fig. 8.4). While this enhances heat transfer, which is essential for vitrification, it also enhances water evaporation.
Fig. 8.1 The specimen were prepared in the air (no environmental chamber was used): (A) 50 nm-extruded POPC vesicles prepared in water; (B) 50 nm-extruded POPC vesicles prepared in phosphate buffer (50 mM, pH 6.5). The concentration of POPC was 5 mM. The arrows show some stomatocytes.
Fig. 8.1 shows two micrographs of frozen hydrated specimen that were prepared in the ambient atmosphere (neither the temperature nor the humidity was controlled). In both cases, the suspension contained POPC liposomes that were extruded through 50 nm pore size polycarbonate filters. Fig. 8.1 A shows liposomes that were prepared in water. They appeared as round spheres in contrast to the vesicles prepared in 50 mM phosphate buffer (Fig. 8.1 B) which frequently displayed a bilamellar-like appearance. It is likely that evaporation of water took place during specimen preparation and that the structures observed in Fig. 8.1 B resulted from an osmotic shock (Dubochet et al., 1988; Bellare et al., 1988; Vinson et al., 1989). The internal volume of the vesicles decreased while the surface area of the vesicles remained identical. A spherical shape is in this case energetically unstable. If the aqueous phase contained salt, the evaporation of water led to an increase in the bulk salt concentration. Water molecules probably permeated the bilayer to equilibrate the inner (aqueous interior of the vesicles) and outer (bulk solution) salt concentration.

Depending on the surface-to-volume ratio of the vesicle, oblate (Fig. 8.2 b) or stomatoocyte shapes (Fig. 8.2 c) are favored (Seifert et al., 1991). In the absence of salt, the evaporation of water did not affect the vesicle shape in this case.

5. The vesicle bilayer is much more permeable by water than charged ions (by a factor of ca. $10^8$; Ceve, 1993)

6. If the investigated structures are sensitive to amphiphile concentration, the evaporation of water can induce morphological or phase changes, also in the absence of salt.
Fig. 8.2 Schematic illustration representing a plausible morphological transformation of a sphere into a stomatocyte: the volume of the vesicle diminishes while its surface area remains constant. Each of these shapes represents the lowest energy configuration for the particular volume-to-area ratio (Seifert et al., 1991).

Depending on how the stomatocyte is oriented in the vitrified film, its image resembles that of a bilamellar vesicle (Fig. 8.3). An invaginated vesicle can be difficult to differentiate from a “true” bilamellar vesicle.

Fig. 8.3 Top-view projection of an invaginated vesicle.

7. Similar morphological transformations (of a sphere into a stomatocyte) have been observed by LM with DMPC and POPC giant vesicles. The changes were induced by a rise in temperature which led to an area different behavior to the inner leaflet and to the outer leaflet of the bilayer (Käs & Sackmann, 1991; Lipowski, 1991).
In order to minimize water evaporation, an environmental chamber, in which temperature and humidity are controlled, has to be used (Bellare et al., 1988; Egelhaaf, 1995). The chamber sketched in Fig. 15.2 (page 211) was used for specimen preparation in the present work.

To prevent the formation of invaginated vesicles, a relative humidity of at least 97% had to be reached inside the chamber. It was noticed that, even under controlled conditions, most of the places were free of artifacts, but there were also locations on the same grid which showed invaginated vesicles. Vinson et al. (1989) have already reported a similar behavior. They proposed that either evaporation is not uniform across the specimen or that invagination may also arise by other mechanisms. Other consequences of water evaporation are, for example, changes in surfactant concentration and pH.

All the lamellar phases investigated in this work had a gel-to-liquid crystalline transition temperature below RT; no problem was therefore encountered by preparing the specimen at RT.

Another commonly encountered problem with surface active molecules is their transfer from the bulk to the air-liquid interface as a consequence of the increase in the surface area-to-volume ratio. This effect can generate modifications in the composition of the bulk solution and also induce phase changes. This effect is potentially problematic in complex systems made of several surface active compounds in which the formed structures are susceptible to composition changes (Talmon, 1996).

The presence of surfactants can also be advantageous. It facilitates the formation of a thin film by reducing the surface tension at the air-liquid interface. Surface active compounds have been added to solution prior to investigation. The interfacial layers of surfactants can then be considered as a slide and a cover-slip (in analogy to LM preparation) enclosing a thin aqueous layer (Frederik et al., 1989b & 1989c).
8.3 Size sorting and mechanical shape deformation

Due to the biconcave shape of the film, the vesicles were often spatially arranged in the film according to their size (Fig. 8.4 and see e.g. Fig. 8.5): large ones were expelled from the center and found at the edge of the hole where the film was thicker while smaller vesicles could be found farther towards the center. In extreme cases, all the liposomes were concentrated near the rim of the hole as shown in Fig. 8.5.

Fig. 8.4 Scheme illustrating the sorting of vesicles according to their size.

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8. This phenomenon was observed relatively often and there are numerous examples throughout this work.
Fig. 8.5 Cryo-TEM micrograph of POPC liposomes prepared in water ([POPC]=5 mM, 100 nm extruded): the vesicles shown here are slightly squeezed and are only found at the edge of the hole where they are arranged according to their size. The film was presumably too thin in the middle of the hole to accommodate any vesicles. A layer of contamination which resembles fish scales covered the specimen (paragraph 8.6, page 74).

Another consequence of the finite thickness of the aqueous film is that vesicles may not find enough space in the aqueous film and are therefore deformed/flattened (see e.g. Fig. 10.26 B, page 146). Flattened vesicles may appear larger than they are. Vesicles can also be distorted and squeezed by mechanical stress, due to the fluid flows on the grid when the sample is blotted. The shear forces of this flow are sufficient to squeeze the vesicles to the wall of the carbon support and into each other (Vinson et al., 1989). Fig. 8.5 shows that vesicles have been slightly squeezed against each other at the rim of the hole.
8.4 Energy filter

As the EM 912 Omega is equipped with an energy-filter (paragraph 7.2.2.3, page 51), the frozen hydrated specimen were examined in the zero-loss mode. The inelastically scattered electrons were therefore cut off and the contrast was clearly enhanced as shown in Fig. 8.6.

The use of the zero-loss mode had the following advantages (Grimm et al., 1998):

1. The produced contrast was better and therefore the objects could be visualized closer to focus.
2. The number of multiple scattered electrons increases with increasing specimen thickness, therefore thicker specimen could be investigated as the inelastically scattered electrons were eliminated from the beam electron.
3. Due to the improved contrast, fewer electrons were needed to examine the sample and to record pictures. Thus the effects of electron irradiation could be reduced.
Fig. 8.6 Micrographs of the same specimen (ferritin-containing POPC/oleic acid/oleate vesicles) at the same location with identical electron dose (5e⁻/Å²) and defocus (A) without energy filter and (B) with energy filter. Aggregates of small ice crystals are visible on the specimen surface (see e.g. arrow in B).
8.5 Contrast

Interestingly the vesicles formed exclusively by one of the three amphiphiles studied in this work (POPC, oleic acid and didecyl phosphate) gave rise to slightly different contrast. POPC and didecyl phosphate bilayers appeared thicker and could almost be resolved⁹ at high magnification while the oleic acid bilayers always appeared as a thin line (Fig. 8.7)¹⁰. This small discrepancy presumably arose from the presence of a phosphorous atom in POPC and didecyl phosphate in addition to the carbon, oxygen, and hydrogen atoms present in oleic acid. The electron-specimen interaction is sensitive to the molecular weight of the atoms; therefore, it is reasonable to suppose that the interaction of the electron with didecyl phosphate and POPC bilayers was stronger than with pure oleic acid vesicles. Although this phenomenon could be perceived (at least by the operator), it was very difficult to quantify it. Fig. 8.7 shows (A) oleic acid, (B) POPC and (C) didecyl phosphate vesicles. All the cryo-TEM micrographs were recorded at the same magnification. Unfortunately, the defocus was different in each micrograph and the bulk solution had a different composition.

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⁹ One could almost differentiate the inner leaflet of the bilayer from the outer one.
¹⁰ The actual bilayer thickness was supposed to be similar in all cases (thickness of ca. 4 nm).
Fig. 8.7 Cryo-TEM micrographs showing (A) 100 nm extruded ferritin-containing oleic acid vesicles in BICINE buffer (200 mM, pH 8.5), (B) 100 nm extruded ferritin-containing POPC liposomes in borate buffer (100 mM, pH 8.5) and (C) didecyl phosphate vesicles in 0.25 M KOH. All the micrographs were taken at the same magnification (primary magnification 20 000X). Notice the presence of open membranes in C (arrows).

This effect may open new possibilities by allowing the distinction of vesicles made of various amphiphiles in a suspension.
8.6 Contamination

The vitrified sample kept at near liquid nitrogen temperature on a specimen cryoholder acts as a pump for water vapor. Care has to be taken to avoid the deposition of water either during specimen manipulations through the air or inside the microscope. During mounting and transfer of the sample grid, water is deposited as aggregates of small ice crystals (Fig. 8.6, arrow), while inside the microscope water vapor condenses on the specimen as a continuous layer, sometimes similar to fish scales (see e.g. Fig. 8.5).

8.7 Radiation damage

Radiation damage is a consequence of the energy transferred to the specimen by inelastic collisions of the beam electrons. It was noticed that fully amorphous ice was quite "stable" to beam damage in the sense that visible alterations were rarely observed at the electron doses used. Thicker or non-vitrified specimen were more sensitive to the electron beam; reflections emerged and poor contrast (Dubochet et al., 1988) was obtained which further hindered examination of the sample. Radiation damage could be well discerned on the carbon film, appearing as bubbles (Dubochet et al., 1982a; Talmon et al., 1986) as depicted e.g. in Fig. 10.16 B (page 132).
9. Hydrolysis of phosphotriesters

9.1 Introduction

The self-reproduction of vesicles is a very interesting system as it binds chemistry and compartmentalization with one of the most basic cell processes, namely the self-replication/self-reproduction process. The first self-reproducing system involving vesicles was studied using the hydrolysis of fatty anhydride and the corresponding long-chain fatty acid vesicles (Walde et al., 1994). Whether long-chain fatty acids are prebiotically relevant amphiphiles has been discussed (Allen & Ponnamperuma, 1967; Miller, 1998). The aim of the present project was to develop another self-reproducing vesicle system, using simple molecules of possible prebiotic relevance. Phosphate-based amphiphiles were chosen as potential candidates.

Indeed, phosphate esters and anhydrides are essential in the living world (Westheimer, 1987). The genetic material DNA and RNA are phosphodiesters. Most of the coenzymes are esters of phosphoric or pyrophosphoric acid. The principal reservoir of biochemical energy (adenyl triphosphosphate, creatin phosphate and phosphoenolpyruvate) are phosphates. Glycerophospholipids also contain phosphate. Moreover phosphate has revealed itself of prebiotic relevance. PO$_4^{3-}$ was found in meteorites and interplanetary dust particles (Oró et al., 1990) and may have arisen from volcanoes in prebiotic times. Recently, Ourisson and co-workers (Plobeck et al., 1992; Ourisson & Nakatani, 1994; Pozzi et al., 1996; Birault et al., 1996) have postulated that primitive membranes could have been more readily formed from the simplest possible terpenoids linked to an appropriate and simple polar head group such as a phosphate anion. This hypothesis is mainly based on the fact that no terpenoid-free cellular membrane is known today, and that many terpenoid-based molecular fossils have been isolated. Therefore they proposed as most primitive membrane-
building block units the polyprenyl phosphates and described the abiotic synthesis of prenyl units, a possible prebiotic phosphorylation and chain elongation of polyprenols, which could lead to the formation of primitive vesicles.

The idea in the present work was to use simple alkyl phosphates as model compounds for the elaboration of a self-reproducing vesicle system. Ideally the precursor molecule should not build structured aggregates, while the product should possess surfactant properties, such as the ability to form vesicles. One could start from a phospho-tri or -diester and hydrolyze it to the corresponding di- or mono-ester with the assumption that these hydrolysis products would be able to build vesicles under the chosen conditions. Phosphodiesters are known to be more stable than phosphotriesters and it is has long been known that dialkyl phosphates form vesicles in alkaline conditions (Kunitake & Okahata, 1978). Phosphotriesters, which bear at least two alkyl chains, appeared to fulfill the requirements: they are water-insoluble and their hydrolysis product, dialkyl phosphate, is able to form liposomes (see Chapter 5.). Therefore, they were chosen as study compounds for the development of a new self-reproduction system. Their hydrolysis had to be carried out under conditions allowing the formation of a vesicular system made of the hydrolysis product according to a scheme similar to the one already described for fatty acids (Fig. 9.1).
Fig. 9.1  Schematic illustration of the possible self-reproduction of dialkyl phosphate vesicles.

Shorter chain dialkyl phosphates form vesicular systems which have a lower $T_c$ than long chain ones and are thus easier to handle. Moreover, one could argue that, in primeval times, hydrocarbon chains must have formed by the gradual accretion of small molecules such as methane and that it is then probable that short chains initially predominated (Stephen-Sherwood & Oró, 1973; Hargreaves & Deamer, 1978b). The shortest dialkyl phosphate actually known to build vesicles is didecyl phosphate. In the present work two phosphotriesters containing at least two decyl chains were synthesized and their hydrolysis was then investigated.


9.2 Preparation of phosphotriesters

Two phosphotriesters were synthesized, a symmetric one, tridecyl phosphate (1), and an asymmetric one, didecyl 4-nitrophenyl phosphate (3) (Fig. 9.2). Didecyl phosphoric acid (2) was also obtained as by-product in the synthesis of 1.

![Chemical structure of tridecyl phosphate (1), didecyl phosphoric acid (2) and didecyl 4-nitrophenyl phosphate (3).](image)

Symmetric phosphotriesters are usually prepared by the reaction of the appropriate alcohol with phosphoryl chloride in the presence of a base, for example, triethylamine or pyridine. Asymmetric ones can be obtained by the reaction of a phosphorochloridate with sodium derivatives of phenol or alcohol (Katayshkina et al., 1956). In particular, the formation of various dialkyl p-nitrophenyl phosphates has already been studied in the 1950's. They were prepared using either p-nitrophenyl phosphorodichloridate (Cebrian, 1951) or dialkyl phosphorochloridate (De Roos & Toet, 1958) as phosphorylating agent.

The syntheses of 1 and 3 were carried out as described in the literature (Fig. 9.3). The reaction progress was followed by TLC using silica gel plates. Phosphotri-, di- and mono-esters were readily distinguishable by TLC as their different polarity resulted in differences in the migration.
1 was synthesized with a rather poor yield (38%) by the reaction of phosphoryl chloride with decanol in the presence of pyridine (Davey, 1950). The formation of 1 was accompanied by a relatively large amount (48%) of didecyl phosphoric acid (2). This may result from the presence of some traces of water, which would hydrolyze decyl phosphorodichloridate and didecyl phosphorochloridate, both intermediates of the reaction, to yield the mono- and di-ester, respectively (De Roos & Toet, 1958).

3 was prepared by reacting p-nitrophenyl phosphorodichloridate with decanol (Qiu et al., 1994) in a better yield (68%). Since p-nitrophenyl phosphorodichloridate was commercially available, it was chosen in preference to didecyl phosphorochloridate which would have had to be specially synthesized. Moreover phosphorodichloridates have been recently successfully used as phosphorylating agents, for example, in the synthesis of phosphotriesters containing nucleotides (Farrow et al., 1990) and of ether phospholipids via a phosphotriester approach (Qiu et al., 1994). The purity of the products was checked by TLC, $^1$H-NMR and $^{31}$P-NMR. Didecyl phosphoric acid (2) and phosphotriesters 1, 3 possessed a different chemical shift in $^{31}$P-NMR. Only one peak was observed for all the final compounds.
9.3 **Aggregation behavior of didecyl phosphate**

In this work, the vesicle formation properties of didecyl phosphate under selected conditions were investigated: pH and counterions were found to affect the formation and stability of the vesicles.

A titration curve of didecyl phosphate (Fig. 9.4) was determined between pH ~ 1.5 and 12.5 range using HCl and NaOH. Experimentally an apparent pKₐ of ca. 7 was obtained for the phosphate polar head group at the vesicle interface. This value was slightly higher than the pKₐ of 5.5-6 determined for dihexadecyl phosphate in small vesicles (Carmona-Ribeiro, 1990). Similar to the case of fatty acids, the pKₐ of the surfactant in the bilayer was higher than the pKₐ of lower molecular weight alkyl phosphates, which are found as monomers in aqueous solutions.

At pH 2, if the sample was heated to 50°C prior to investigation, oil droplets were observed as shown by LM (Fig. 9.5 A). However, if the sample was allowed to cool down to RT, only crystals were found (Fig. 9.5 B).

In the presence of Na⁺ as counterion, vesicles were observed from pH 5.2 to 12 (Fig. 9.5). Between pH 5 and 6 they were stable for 20 hrs at RT, but above pH 6 white shiny crystals started to precipitate after a few hours. Upon reheating of the samples, vesicles formed again. Fonteijn et al. (1992a) have already observed this behavior. They proposed that the nucleation for crystallization in the vesicle suspensions takes place at the water-air interface and that the stability of the vesicles could be enhanced by reducing the water-air interface.
Fig. 9.4 Titration curve of didecyl phosphate with HCl and NaOH ([2] = 7.1 mM). Below pH 2 mostly oil droplets were observed, above pH 6 mainly vesicles were obtained. The dashed line represents the approximate boundary between the regions where the oil phase and the lamellar phase, respectively, predominated.

A titration curve was also established with KOH to check the effect of the chemical nature of the counterion on vesicle formation. In this case, vesicles formed from pH 4.3 to 13.5. Between pH 7 and 13.2, they were stable at RT for at least 14 days. No precipitation was observed.
Fig. 9.5 LM (A, C, D) and TEM (B, E) micrographs of samples obtained from a titration curve of didecyl phosphate with HCl and NaOH. [2] = 7.1 mM: (A) oil droplets, pH 2; (B) crystal, pH 2; mainly polydisperse vesicles at (C) pH 10.3, (D) pH 11.5, and (E) pH 6.
Fig. 9.6 LM pictures of the samples obtained from a titration curve of didecyl phosphate with KOH ([2] = 7 mM). Mainly vesicles that were polydisperse in size and lamellarity were observed over all the pH range studied: (A) pH 4.3; (B) pH 7, the assembly formed was very large (O ~ 30 μm); (C) pH 11.1; (D) pH 11.7, large MVV (O ~ 20 μm); (E) pH 12.5, large MVV (O ~ 24 μm); and (F) pH 13.6, at this high pH the content of large vesicles was remarkably high.
Didecyl phosphate vesicles varied in size and lamellarity: unilamellar as well as multilamellar and multivesicular vesicles were observed by LM (Fig. 9.5 & Fig. 9.6). Interestingly, large vesicles (diameter of about 20 μm and more) were observed from pH ~ 4.3 to 13.5, particularly in the presence of K⁺ and at relatively high pH (Fig. 9.6 e.f.h.). Vesicles even formed in 0.5 M KOH, but they tended to aggregate. The vesicles were more stable in the presence of K⁺ than in the presence of Na⁺ as counterion. In more acidic conditions, only oil droplets or crystals were observed.

Ultrasonic treatment is commonly used to produce small unilamellar vesicles (New, 1990). If 2 was sonicated in 0.25 M KOH, a completely clear solution was obtained while a turbid suspension was obtained upon dissolution of 2 in 0.25 M KOH by hand shaking or magnetic stirring. Ultrasonic treatment reduces the size of the vesicles. As scattering of the light is dependent on the concentration as well as on the size of the aggregates, sonicated suspensions can appear almost completely transparent. Kunitake and Okahata (1978) also reported the formation of a clear solution upon sonication of didecyl phosphate, which contained closed bilayers. Indeed after sonication of 2 in 0.25 M KOH for 10 min, although the aqueous phase appeared transparent, the presence of small vesicles was confirmed by cryo-TEM (Fig. 9.7). Mostly small unilamellar vesicles (diameter between 20 to 50 nm) but also few larger ones and some open bilayers (Fig. 9.7, arrow) were observed.

11. It was noticed that, not only the pH played a role in vesicle formation, but that the buffer ions also influenced the aggregation behavior of didecyl phosphate. For example, didecyl phosphate did not form vesicles in imidazole buffer (200 mM, pH 7).
Fig. 9.7 Cryo-TEM micrograph of didecyl phosphate vesicles formed in 0.25 M KOH ([2]=10 mM): the suspension was sonicated for 10 min at RT. Note the presence of some open bilayers (e.g. arrows).

If the sonicated suspension was left to stand at RT, with time it became turbid again. These sonicated vesicles were not stable and probably fused after a certain time to give larger aggregates as already proposed by Carmona-Ribiero et al. (1991). They explained this instability by the presence of bilayer fragments: due to hydrophobic attraction between the edges of these fragments, they can gather together and reform closed bilayers.
9.4 Chemically-induced formation of vesicles

9.4.1 First attempts and remarks

Although it is known that trialkyl phosphates are less labile towards hydrolysis than fatty anhydrides, it was worthwhile to investigate whether the presence of dialkyl phosphate vesicles would accelerate the hydrolysis of considerably more chemically stable phosphotriesters. The first hydrolysis reactions were carried out by using the simple symmetrical tridecyl phosphate (1) as precursor. The reactions were performed in the absence and in the presence of preformed didecyl phosphate vesicles under a variety of conditions. Tridecyl phosphate revealed itself as extremely stable, even under drastic conditions: a significant amount of 1 was still detectable after being stirred in 0.5 M KOH at 68°C for one month! 1 could be hydrolyzed quantitatively only by performing the reaction in an open flask: after 72 hrs, the water was evaporated and only a white solid residue was left at the bottom of the flask. After taking up the residue in some water, TLC revealed the presence of didecyl phosphate only, and vesicles as well as myelin structures were observed by LM. However, it would be difficult to follow the kinetics of such a reaction taking place in an open system.

Therefore, another phosphotriester, didecyl 4-nitrophenyl phosphate (3), was synthesized and its hydrolysis studied. The p-nitrophenyl group is a better leaving group than the didecyl one and, in addition, p-nitrophenol offers the advantage of bearing a chromophore which facilitates the quantitative monitoring of the hydrolysis reaction.

12. Investigations on the hydrolysis of a series of trialkyl phosphates have shown that these phosphotriesters are very difficult to hydrolyze, even in strongly alkaline solution (Conrad et al., 1986).
Preliminary experiments showed that even didecyl 4-nitrophenyl phosphate (3) was still a relatively stable molecule in aqueous solution, and extreme reaction conditions had to be used for its hydrolysis (0.25 M KOH, \( T=68^\circ C \)). The effects of preformed vesicles and of sonication on the course of the hydrolysis were investigated. Some experiments were also carried out under less drastic conditions, using 0.1 M KOH (pH 12.9) and using 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (200 mM, pH 10.5).

The kinetics of the reactions were monitored using UV spectrophotometry: the p-nitrophenolate that was released during the hydrolysis was quantified by measuring its absorbance at 391 nm. In each case, three experiments were run in parallel and the analysis of the reaction mixtures was also performed in triplicate.

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13. KOH was used instead of NaOH as it was noticed that didecyl phosphate vesicles were more stable in the presence of \( K^+ \) than in the presence of \( Na^+ \) as counterion (paragraph 9.3, page 80).
9.4.2 Hydrolysis of didecyl 4-nitrophenyl phosphate (3) in 0.25 M KOH

The preparation of the reaction mixtures as well as the macroscopic observations made during the hydrolysis of 3 in 0.25 M KOH at 68°C are summarized in Table 9.1.

<table>
<thead>
<tr>
<th>[2]</th>
<th>Preparation of the reaction mixtures</th>
<th>Macroscopic observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>a 0 mM</td>
<td>no sonication</td>
<td>→ oil drop floated on top of a clear sol. after 6-8 hrs → oil drop fell to the bottom of the flask → disp. of oil droplets as reaction proceeded, yellow color appeared</td>
</tr>
<tr>
<td>b 5 mM</td>
<td>no sonication</td>
<td>before addition of triester → turbid susp. add triester → oil drop fell directly to the bottom of the flask → disp. of oil droplets</td>
</tr>
<tr>
<td>c 30 mM</td>
<td>no sonication</td>
<td>ditto 5 mM, except that before addition of 3 the vesicular susp. was more turbid</td>
</tr>
<tr>
<td>d 10 mM</td>
<td>only 2 sonicated</td>
<td>after sonication of 2 → transparent clear sol. addition of triester → oil drop fell directly to the bottom of the flask → disp. of oil droplets</td>
</tr>
<tr>
<td>e 0 mM</td>
<td>3 sonicated</td>
<td>just after sonication → turbid/white emulsion which rapidly became yellow after few hrs → oil droplets gathered together</td>
</tr>
<tr>
<td>f 10 mM</td>
<td>2 and 3 cosonicated</td>
<td>just after sonication → turbid/white susp. which very rapidly became yellow until the end of reaction; yellow turbid susp.</td>
</tr>
</tbody>
</table>

Table 9.1 Preparation of the reaction mixtures and macroscopic observations made during the hydrolysis of 3 ([3]=10-12 mM). In (a) and (e), there were no didecyl phosphate vesicles initially present while in (b), (c), (d) and (f) vesicles formed by didecyl phosphate were present at different concentrations in the initial reaction mixture. The reactions were carried out in 0.25 M KOH (pH 13.4) at 68°C (total volume = 1ml).
If 3, which is a slightly yellow oil, is laid on top of a “pure” aqueous phase (no vesicles present), it floated on top of the solution. However, if didecyl phosphate vesicles were present in the aqueous phase, 3 dropped immediately to the bottom of the flask. After that, due to the mechanical stirring of the solution, the relatively large oil droplet split into smaller ones and a dispersion of small oil droplets in water (or emulsion) was thus achieved. In the absence of preformed vesicles, as the hydrolysis proceeded and therefore, as didecyl phosphate was released in the medium, the oil drop suddenly fell to the bottom of the flask and a dispersion of oil droplets was also obtained. A plausible explanation of this phenomenon may be that the density of the vesicular suspension was smaller than the density of pure water. To prove this hypothesis, a vesicular suspension was prepared using deuterated phosphate buffer (KD₂PO₄ 30 mM, NaCl 20 mM, pH = 7.5). Deuterated water has a higher density than “normal” water: at 25°C, the density of H₂O and of D₂O is 0.997 and 1.104 respectively, and at 70°C, 0.977 and 1.085 respectively (Handbook of Chemistry and Physics, 1976-1977). The deuterated buffer changed the properties either of the vesicular suspension or of 3. In that case, 3 remained on top of the vesicular suspension formed by didecyl phosphate at RT and at 68°C for at least one week. The conclusion seems to be that the density of the aqueous phase decreases due to the presence of didecyl phosphate vesicles.

If 3 was sonicated, a white/turbid suspension was also obtained. The turbidity was caused by the presence of a fine dispersion of oil in water. As no continuous ultrasonic treatment was applied and due to hydrophobic interactions, the initially small oil droplets gathered together with time and could again be distinctly perceived by the naked eye.
The sigmoidal curve typical of an autocatalytic reaction as obtained for the hydrolysis of oleic anhydride (Walde et al., 1994) was not observed for the hydrolysis of 3 in 0.25 M KOH. The profile of the reaction could almost be assumed to be a straight line under the conditions used (Fig. 9.8).

![Graph showing reaction profile with time on the x-axis and concentration change on the y-axis.](image)

**Fig. 9.8** Reaction profile of the hydrolysis of 3 ([3] = 10-12 mM) carried out in 0.25 M KOH at 68°C. The percentage of 3 cleaved is plotted vs. time. The data shown correspond to the mean value of three measurements and the error bars represent the standard deviation.

There was, however, no doubt that vesicles were formed upon hydrolysis of 3; some were strikingly large (up to a diameter of 65 µm) and could be characterized as giant ones (Fig. 9.9).

![LM micrographs of the suspensions obtained after the hydrolysis of 3 in 0.25 M KOH at 68°C. No preformed vesicles were present at the beginning of the reaction.](image)

**Fig. 9.9** LM micrographs of the suspensions obtained after the hydrolysis of 3 in 0.25 M KOH at 68°C. No preformed vesicles were present at the beginning of the reaction.
The initial presence of didecyl phosphate vesicles did not significantly accelerate the hydrolysis of 3 (Fig. 9.10). The minor differences noted in the hydrolysis rate (especially in the first 50 hrs) may be explained by the formation of a dispersion of oil droplets immediately after the addition of triester 3 if preformed vesicles were initially present (see Table 9.1). At least, at the beginning of the reaction, the total water/oil interface was greater in case b, c and d than in case a. The size of the vesicles did not significantly influence the course of the hydrolysis of 3 (curve d in Fig. 9.10).

![Graph showing concentration change vs. time for different cases](image)

**Fig. 9.10** Effect of didecyl phosphate vesicles upon hydrolysis of 3 ([3]=10-12 mM) in 0.25 M KOH at 68°C: (a) [2]=0 mM; (b) [2]=5 mM; (c) [2]=30 mM; and (d) the preformed vesicles were sonicated. [2]=10 mM. The percentage of 3 cleaved is plotted vs. time. The data shown correspond to the mean value of three measurements and the error bars represent the standard deviation.

To summarize, didecyl phosphate vesicles were not endowed with great catalytic activity towards the hydrolysis of 3. Neither the presence nor the size of the vesicles had a significant influence on the hydrolysis rate.
As the precursor 3 is hydrophobic, the reaction occurs at the oil/water or vesicle/water interface. If 3 is spontaneously incorporated in the bilayers, an increase in the vesicle concentration or a decrease in the vesicle size\textsuperscript{14} should enhance the rate of the hydrolysis. The previous experiments suggested that the spontaneous uptake of 3 by the vesicles did not occur efficiently under the chosen conditions and that the reaction occurred primarily at the surface of the oil drops formed by phosphotriester 3. No evidence of vesicular catalysis was found.

One way to facilitate the uptake of 3 by didecyl phosphate vesicles is to sonicate 3 in the presence of dialkyl phosphate. This method is commonly applied to mechanically incorporate water-insoluble compounds in bilayer membranes (see e.g. Kunitake & Sakamoto, 1978). In the following experiments, the effect of ultrasonic treatment on the course of the hydrolysis was investigated. Sonication was performed immediately after addition of 3 to the aqueous phase at RT for 10 min.

As a control experiment, solely 3 was sonicated. The reaction profile shows over the first 30 hrs a steep curve which then flattened out (Fig. 9.11). The thus observed profile can be explained as follows: upon sonication, the oil drop formed by 3 was splitted into many droplets, thereby increasing the surface where the alkaline hydrolysis could take place. Since this treatment was not pursued and due to evident hydrophobic reasons, the small oil droplets were brought together to reform larger droplets, thereby reducing the oil/water interface and thus leading to a decrease in the hydrolysis rate.

\textsuperscript{14}For an identical surfactant concentration, a decrease in size results in an increase in the vesicle/aqueous medium interface. In the present case smaller vesicles were obtained by sonication.
Fig. 9.11 Effect of ultrasonic treatment upon the hydrolysis of 3 ([3] = 10-12 mM) in 0.25 M KOH at 68°C: (a) no sonication, [2] = 0 mM; (e) 3 was sonicated, [2] = 0 mM; and (f) 2 and 3 were cosonicated ([2] = [3] = 10 mM). The percentage of 3 cleaved is plotted vs. time. The data shown correspond to the mean value and the error bars represent the standard deviation.

In a second set of experiments, 3 was then cosonicated with dialkyl phosphate. Small oil droplets as well as vesicles were detected by LM after ultrasonic treatment (Fig. 9.12). After one day, about 50% of the phosphotriester 3 was hydrolyzed. However, the rate diminished with time (Fig. 9.11).
Upon cosonication of 3 and 2, two events could happen:
1. Phosphotriester 3 was incorporated in the didecyl phosphate vesicle bilayers.
2. Excess of 3 (molecules that were not integrated in the bilayers) formed small oil droplets as obtained in the case of sonication of 3 (case d).

Both phenomena lead to an increase of the phosphotriester/aqueous solution interface; therefore, a larger number of precursor 3 molecules was susceptible to ‘OH attack. The triester molecules incorporated in the bilayers as well as the molecules found at the surface of the oil droplets probably hydrolyzed very quickly, which could account for the rate enhancement observed principally at the beginning of the reaction.
The hydrophobic precursor 3 was also introduced mechanically in the bilayers using the film method: a film containing both surfactant 2 and precursor 3 was prepared and then hydrated with 0.25 M KOH. After 20 hrs, more than 50% of 3 was hydrolyzed and the reaction was brought to completion in ca. 150 hrs (Fig. 9.13). The hydrolysis was somewhat faster than in the case of cosonication (compare film with f in Fig. 9.13). The most likely explanation is that the incorporation of 3 in the vesicle bilayers was more efficient using the film technique.

**Fig. 9.13** Effect of sonication, of cosonication and of film formation upon the hydrolysis of 3 ([3]=10-12 mM) in 0.25 M KOH at 68°C: (a) no sonication, [2] = 0 mM; (d) the preformed vesicles were sonicated, [2]=10 mM; (e) 3 was sonicated, [2] = 0 mM; (f) 2 and 3 were cosonicated ([2]=[3]=10 mM); and (film) a film containing 2 and 3 was formed ([2]=[3]=10 mM). The percentage of 3 cleaved is plotted vs. time. The data shown correspond to the mean value and the error bars represent the standard deviation.

15. This experiment was performed only once in order to confirm that the crucial step was indeed the uptake of 3 by the vesicle bilayers.
These last experiments suggested that
- the crucial step was not the presence of vesicles alone, but the incorporation of the hydrophobic precursor 3 in the bilayer membranes of the vesicles;
- under the conditions used, didecyl phosphate vesicles were not able to uptake the phosphotriester molecules without external input of energy.

In conclusion, only if cosonication of 2 and 3 or if a mixed film formed by 2 and 3 were used, had the vesicles a significant effect on the course of the hydrolysis as shown, for example, by comparison of curve d with curve f in Fig. 9.13.

The hydrolysis of 3 was, however, carried out under less drastic conditions to check whether the very high concentration of KOH used had an influence on the hydrolysis course.
9.4.3 Hydrolysis of didecyl 4-nitrophenyl phosphate (3) in 0.1 M KOH

Two sets of experiments were carried out under less drastic conditions, using 0.1 M KOH. The macroscopic observations made during the reactions are summarized in Table 9.2.

<table>
<thead>
<tr>
<th>[2]</th>
<th>pH\textsubscript{start}</th>
<th>pH\textsubscript{end}</th>
<th>Macroscopic observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>0 mM</td>
<td>12.9</td>
<td>12.3</td>
</tr>
<tr>
<td>b</td>
<td>10 mM</td>
<td>12.5</td>
<td>11.7</td>
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</tbody>
</table>

Table 9.2 Macroscopic observations made during the hydrolysis of 3 ([3]=10 mM) in (a) the absence and in (b) the presence of didecyl phosphate vesicles. The reactions were carried out in 0.1 M KOH at 68°C (total volume = 1 ml).

Both sets of experiments gave a reaction profile that could be approximated by a straight line (Fig. 9.14). The hydrolysis of 3 was somewhat faster in the presence of preformed bilayers. Unfortunately the scattering of the data (see the standard deviation in Fig. 9.14) made the interpretation of these results difficult. The small enhancement in the hydrolysis rate arose most likely from the formation of an oil dispersion at the beginning of the reaction if didecyl phosphate vesicles were initially present (similar to 0.25 M KOH). In any case, the vesicles did not significantly affect the hydrolysis reaction rate.
Fig. 9.14 Effect of vesicles formed by 2 upon the hydrolysis of 3 ([3]=10 mM) in 0.1 M KOH at 68°C: (a) [2]=0 mM and (b) [2]=10 mM. The percentage of 3 cleaved is plotted vs. time. The data shown correspond to the mean value and the error bars represent the standard deviation.

Compared to the reactions performed in 0.25 M KOH (Fig. 9.10), it has to be pointed out that the decrease in pH of the bulk solution did not slow down the hydrolysis: after 150 hrs, in both cases about 50% of 3 were cleaved and, afterwards, the reaction was even slightly faster in 0.1 M KOH.
9.4.4 Hydrolysis of didecyl 4-nitrophenyl phosphate (3) in 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer

As current investigations on the hydrolysis of fatty anhydrides have shown that buffer ions may play an important role in the course of the reaction (Wick, 1996), the hydrolysis of 3 in CAPS (200 mM, pH 10.5) (Fig. 9.15) was also examined.

![Chemical structure of 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS).

Fig. 9.15 Chemical structure of 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS).

The macroscopic observations made during the hydrolysis carried out in CAPS are summarized in Table 9.3.

<table>
<thead>
<tr>
<th></th>
<th>[2]</th>
<th>pH&lt;sub&gt;start&lt;/sub&gt;</th>
<th>pH&lt;sub&gt;end&lt;/sub&gt;</th>
<th>Macroscopic observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>0 mM</td>
<td>10.5</td>
<td>10.1</td>
<td>→ oil drop floated on top of a clear sol. after 20-25 hrs oil drop fell to bottom of the flask to yield. after a few hrs, a light yellow/white turbid susp. which became yelloower as the reaction proceeded.</td>
</tr>
<tr>
<td>b</td>
<td>10 mM</td>
<td>10.5</td>
<td>10.1</td>
<td>before addition of triester → turbid susp. add triester oil drop fell to bottom of the flask → disp. of oil and then yellow/white turbid susp. after 12 days clear yellow susp.</td>
</tr>
</tbody>
</table>

Table 9.3 Macroscopic observations made during the hydrolysis of 3 ([3]=10 mM) in (a) the absence and in (b) the presence of didecyl phosphate vesicles. The reactions were carried out in CAPS (200 mM, pH 10.5) at 68°C (total volume = 1ml).
In case b (Table 9.3), the mixtures always had the same aspect during the reaction progress: a yellow white/turbid suspension that became yellower with time. After 12 days, the turbidity disappeared to give rise to clear yellow solutions. However, LM and EM investigations confirmed the presence of small vesicles (difficult to see by LM due to their small size) and of a few small oil droplets (Fig. 9.16).

Fig. 9.16  LM and fEM micrographs obtained during the hydrolysis of 3 in CAPS (200 mM, pH 10.5). (A) (B) (C) LM micrographs after 6 days: big oily structure as well as MLV formation and vesicles; (D) fEM micrographs after 12 days: small vesicles.
In this case the presence of preformed didecyl phosphate vesicles affected the hydrolysis rate. However, no sigmoidal behavior was observed if no vesicles were initially present (Fig. 9.17).

**Fig. 9.17** Effect of didecyl phosphate vesicles upon the hydrolysis of 3 ([3]=10 mM) in CAPS (200 mM, pH 10.5) at 68°C: (a) [2] = 0 mM and (b) [2] = 10 mM. The percentage of 3 cleaved is plotted vs. time. The data shown correspond to the mean value and the error bars represent the standard deviation.

At pH 10.5 instead of pH 13.6, the pH of the bulk was reduced, but the phosphate head group at the surface of the vesicles were, like in 0.25 M KOH, in an ionized state (pKa ca. 7). If preformed vesicles accelerated the hydrolysis of 3 in CAPS, the reaction was overall slower than in 0.25 M KOH. A possible explanation may be that CAPS\(^{16}\) itself played an important role in the mechanism of the phosphotriester hydrolysis. The fact that the reaction could not be brought to completion also remained unexplained. CAPS may interact with phosphotriester 3 or didecyl phosphate and somehow inhibit the reaction after a certain time.

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16. It was checked that CAPS ions did not build micelles by using the dye pinacyanol chloride (Mukerjee & Mysels, 1955).
As one could argue that in 0.25 M KOH, the electrostatic repulsions between the negatively charged vesicle surface and the hydroxyl ions hindered the triester attack by \( \cdot \text{OH} \), a last experiment was attempted using BICINE buffer (200 mM, pH 8.3). At this pH, the surface of the bilayers should bear fewer negative charges. However, the presence of preformed vesicles had no influence on the rate of cleavage of the p-nitrophenyl group of 3. Moreover, the hydrolysis was extremely slow, only about 50% of 3 was hydrolyzed after 500 hrs! The decrease in charge of the vesicle surface did not favor the hydroxyl attack. A minimum concentration of hydroxide ions seemed to be necessary in order to hydrolyze 3.

### 9.5 Conclusions

One goal was reached, namely the spontaneous formation of vesicles upon hydrolysis of a water-insoluble precursor in alkaline medium. Moreover, some of the generated vesicles were remarkably large. Vesicles showed some catalytic activity if

- the precursor was incorporated in the vesicle bilayers either by input of external energy, such as that provided through ultrasonic treatment, or by the formation of a film containing the hydrophobic precursor as well as the surfactant;
- CAPS buffer was used.

The bulkiness and stability of phosphotriester 1 and 3, the negatively charged surface of didecyl phosphate vesicles under the chosen conditions, and a relatively slow monomeric-associated surfactant exchange rate presumably hindered the formation of an autocatalytic system. Once the phosphotriester was incorporated in the vesicle bilayer, vesicles revealed themselves as efficient catalysts. The effects of CAPS ions on the hydrolysis course could not be explained.
Compared to the fatty acid systems, phosphotriester 3 has three hydrophobic residues and is thereby bulkier than oleic or caprylic anhydride. In addition, fatty acid vesicles are made of single-chained surfactants and have probably a higher monomeric-associated surfactant exchange rate than vesicles made of didecyl phosphate, which possess two alkyl chains as hydrophobic rests.

The properties of the hydrophobic precursor may also play a significant role in such reactions, which take place in two phase systems. The ability of the hydrophobic precursor to form an emulsion, its behavior in the presence of amphiphiles and buffer ions, and the properties of the vesicular suspension (and surfactant itself) may be other determining factors for such reactions.

Lately it was found that the formation of didecyl phosphate vesicles could also be induced photochemically by irradiation of an emulsion of 2-methoxy-5-nitrophenyl didecyl phosphate in 0.2 M KOH with UV-light (Veronese et al., 1998). This experiment is of general interest as it indicates that membrane bilayers can indeed be formed by light-induced processes. However, phosphotriesters were in general much more stable than fatty anhydrides and dialkyl phosphate vesicles, although possessing interesting features, did not show catalytic properties similar to the ones of fatty acid vesicles. These phosphate-based amphiphiles were not suitable candidates for alternative self-reproduction vesicle systems. In order to better understand the mechanism of vesicle formation and transformation in a vesicle self-reproduction system, the previously described oleic anhydride/oleic acid system (paragraph 4.4, page 28) was chosen for further investigations and studied in more detail with the help of cryo-TEM.

10.1 Introduction

Recent studies on the self-reproduction of oleic acid vesicles have suggested that, if the oleic anhydride hydrolysis was carried out in the presence of preformed oleic acid vesicles, these vesicles also exerted a matrix effect on the formation of "new" oleic acid vesicles (Blöchliger et al., 1998). It was found that the size of the preformed vesicles influenced the size distribution of the vesicles contained in the suspension obtained after the hydrolysis of oleic anhydride. This effect was also observed for the formation of oleic acid vesicles upon injection of a surfactant stock solution (oleic acid in methanol or sodium oleate in water) to a buffer solution that already contained oleic acid vesicles. It was argued that the most likely general mechanism is one that is based on the interaction between the preformed vesicles and the newly added/generated surfactants.

The aim of the present project was to get a deeper insight into the mechanism of formation and/or transformation of oleic acid vesicles upon hydrolysis of oleic anhydride in the presence of preformed oleic acid vesicles (Fig. 10.1). If one could distinguish the preformed vesicles from the newly generated ones and if one could detect the changes in the vesicular suspension caused by oleic anhydride hydrolysis (changes between state A and state B, Fig. 10.1), one could gain information about the pathways of vesicle formation and/or transformation. For this, a water-soluble marker was entrapped in the preformed vesicles, and the suspension was examined using cryo-TEM before and after oleic anhydride hydrolysis. By analyzing a large number of cryo-TEM micrographs, the size distribution of the vesicles and the marker distribution among the vesicles were determined for state A and state B (Fig. 10.1).
Fig. 10.1 Schematic drawing of the hydrolysis of oleic anhydride in the presence of preformed oleic acid vesicles. The hydrolysis reaction was carried out in BICINE buffer (200 mM, pH 8.5) at RT. A: suspension before the hydrolysis reaction took place; B: suspension after completion of hydrolysis.

Ferritin, a water-soluble protein, was chosen as the marker. The ferritin molecule possesses a very dense iron core which gives rise to scattering contrast, facilitating its detection by cryo-TEM. Some general information about ferritin is summarized in Chapter 6. (page 38).
10.2 Possible pathways describing the hydrolysis of oleic anhydride in the presence of ferritin-containing oleic acid vesicles

The hydrolysis of oleic anhydride in the presence of preformed oleic acid vesicles releases new oleic acid/oleate molecules in the suspension. What are the effects resulting from the “addition” of new amphiphiles? Are new oleic acid vesicles formed? Do the preformed oleic acid vesicles transform? One can theoretically imagine three distinct processes resulting from the release of new oleic acid/oleate molecules:

1. The preformed oleic acid vesicles grow by uptake of the newly generated oleic acid/oleate molecules in their bilayer.
2. New oleic acid vesicles are formed independently of the preformed oleic acid vesicles (*de novo* vesicle formation).
3. The preformed oleic vesicles incorporate the newly generated surfactants in their bilayer and then eventually split.

The characteristics of each pathway is described theoretically in the subsequent sections.
10.2.1 The preformed vesicles grow (swelling of the vesicles)

![Diagram: Bicine 200 mM, pH 8.5 to oleic anhydride]

Fig. 10.2 Preformed vesicles grow.

From a theoretical point of view, if the vesicles are simply growing during the reaction (Fig. 10.2), the subsequent statements would be verified:

1. The number of vesicles would remain constant.
2. The average diameter of the vesicles would increase with the amount of oleic anhydride hydrolyzed during the reaction.
3. The vesicle size distribution would be shifted towards larger diameters: the population of large vesicles would increase and the population of small vesicles would decrease after completion of hydrolysis.
4. The ferritin distribution among the vesicles would remain identical: the number of ferritin molecules per vesicle would be the same since the number of vesicles would not vary (point 1).
10.2.2 New vesicles are formed independently of the preformed ones
(*de novo* formation)

If the preformed vesicles are inert and do not interfere with new incoming surfactants (Fig. 10.3), the following features would be observed:

1. The number of vesicles would increase.
2. The number of vesicles containing no ferritin molecules would increase drastically: all the newly formed vesicles should be empty.
3. The mean number of ferritin molecules per vesicle would decrease, as the number of vesicles increases (point 1).
4. The ferritin distribution among the ferritin-containing vesicles would remain identical.
5. The vesicle size distribution would probably broaden as it is expected that the newly generated surfactants would self-assemble in an unpredictable way.
10.2.3 The preformed vesicles grow and then eventually split

Fig. 10.4 The preformed vesicles grow and eventually split.

Vesicles incorporate newly generated surfactants in their bilayer and then split, for example, into two vesicles (Fig. 10.4). This mechanism would possess the subsequent features:

1. The number of vesicles would increase.
2. The mean number of ferritin molecules per vesicle would decrease as the number of vesicles increases (point 1).
3. The ferritin distribution among the vesicles would probably change, the ferritin molecules would possibly be distributed statistically among the vesicles.
4. The vesicle size distribution is not predictable, however, large vesicles would not be present in too high a concentration.

However, if only a few ferritin molecules are entrapped in the preformed vesicles, the probability of obtaining a vesicle that does not encapsulate any ferritin molecules by this process is relatively high. For example, if a vesicle that entraps two ferritin molecules divides into two vesicles, there is theoretically a 50% chance that one of the vesicles generated by splitting does not contain any ferritin molecules. Similarly, if a large vesicle divides into two vesicles, a large one and a small one, the probability that the larger one contains ferritin is higher than the smaller one (see path b in Fig. 10.4).
10.3 Stability of oleic acid vesicles

Before investigating the hydrolysis of oleic anhydride, the stability of 100 nm-oleic acid vesicles was checked using ffEM over a period of four days. These vesicles did not contain any ferritin molecules and were prepared in BICINE buffer (200 mM, pH 8.5). The suspension was treated with five freeze and thaw cycles prior to extrusion.

The first sample was frozen ca. 5 hrs after the last extrusion through 100 nm pore size polycarbonate filters. Other samples were frozen one, two, three, and four days after extrusion. Fig. 10.5 shows typical ffEM micrographs of the vesicle dispersion frozen at different times.

The micrographs were analyzed and a number-weighted size distribution was established for each day (Fig. 10.6). No major trend was found between freshly prepared vesicles and vesicles that were stirred at RT for up to four days, except maybe for the presence of somewhat more large vesicles. The size distributions of the suspension obtained on day 0, 1, 2, 3 and 4 are all shown to be within experimental error (Fig. 10.7). The vesicles had mean diameter of ca. 70 nm. It was smaller than the pore size of the polycarbonate filters (100 nm) used for the last extrusion cycle.

Under these experimental conditions (concentration, pH, buffer used, RT) and over this period of time, 100 nm-oleic acid vesicles were considered as forming a stable system, stable enough to study the hydrolysis of oleic anhydride in the presence of preformed oleic acid vesicles without taking into account any kind of ageing process.

The stability of ferritin-containing oleic acid vesicles in BICINE buffer (200 mM, pH 8.5) was also checked in parallel to an hydrolysis experiment (paragraph 10.5.3, page 145). These vesicles were also stable under the chosen conditions.
Fig. 10.5 Typical fFEM micrographs of the replicas obtained from the oleic acid vesicle suspension. The oleic acid vesicles were prepared by dissolving Na-oleate in BICINE buffer (200 mM, pH 8.5). The thus obtained suspension was stirred overnight and then treated by freeze and thaw cycles (5 times). The vesicles were sized down by extrusion, the last extrusion being performed using 100 nm pore size polycarbonate filters. The specimen were frozen (A) on the same day as extrusion, (B) one, (C) three, and (D) four days after extrusion through 100 nm pore size filters. The oleic acid concentration was 7.25 mM.
**Fig. 10.6** Number-weighted size distribution of oleic acid vesicles in BICINE buffer (200 mM, pH 8.5) as obtained by TEM: the specimens were frozen the same day (day 0), one (day 1), two (day 2), three (day 3), and four days (day 4) after extrusion through 100 nm pore size filters. The oleic acid concentration was 7.25 mM. The numbers in brackets in the legend indicate the number of vesicles counted. The last bars correspond to all the vesicles larger than 200 nm.

**Fig. 10.7** Average of the five number-weighted size distributions shown in Fig. 10.6. The error bars represent the standard deviation. The last bar corresponds to all the vesicles larger than 200 nm.
10.4 Preparation of oleic acid vesicles containing ferritin

10.4.1 Methods of preparation

The next step of this project consisted of preparing an homogeneous population of ferritin-containing oleic acid vesicles. Three slightly different methods were investigated:

I. Na-oleate was dissolved in the buffer containing ferritin and the suspension was stirred overnight at RT.

II. Na-oleate was dissolved in the buffer containing ferritin. The suspension was stirred overnight at RT and then treated by successive freeze and thaw (FAT) cycles (10 times).

III. A lipid film was first formed and then dissolved in the buffer containing ferritin. The dispersion was subsequently treated by FAT cycles (10 times).

All these entrapment and vesicle formation procedures led to rather heterogeneous vesicle suspensions containing very large, multilamellar, as well as small vesicles. This was not ideal for the present electron microscopy study which required a more homogeneous population of not too large vesicles. Therefore, the vesicles were sized down by extrusion; the last extrusion was performed using 100 nm pore size polycarbonate filters. Finally the suspension was loaded on a Sepharose 4B column to remove the non-entrapped ferritin molecules. Fractions were collected and analyzed by UV-Vis and FTIR spectroscopy to quantify ferritin and oleic acid. A chromatogram was determined for each preparation (see e.g. Fig. 10.8). In this particular experiment (method II), fraction 18 to 22 contained ferritin as well as oleic acid, i.e., these fractions contained vesicles that encapsulated ferritin molecules.

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17. The finite thickness of the frozen hydrated specimen limits the size of the objects that can be examined by cryo-TEM without deformation.
Fig. 10.8 Separation of non-entrapped ferritin molecules from ferritin-containing oleic acid vesicles using a Sepharose 4B column (length 50 cm, Ø 1.2 cm). The oleic acid vesicles were prepared in BICINE buffer (200 mM, pH 8.5) containing ferritin (25 mg/ml) using method II. The vesicles were sized down to 100 nm by extrusion. 0.4 ml of the vesicular suspension was applied on the column and fractions of 1 ml were collected.

FAT was performed during protein entrapment, although some disruption of the protein shell of ferritin may occur as a result of freezing (Massover, 1977). In the present work, it was not crucial to have a still biologically active protein, but it was important

- to entrap as many ferritin molecules as possible in the vesicles (high entrapment efficiency), and

- to obtain an homogeneous population of unilamellar vesicles, ideally not larger than ca. 100 nm.

As mentioned in the introduction, FAT treatment is known to increase the trapped volume of the vesicles, to promote solute equilibration as well as to diminish the number of lamellae, and therefore should lead to an increase in the homogeneity of the vesicle population (Mayer et al., 1985; Mayer et al., 1986).
10.4.2 Comparison of the preparation methods using cryo-TEM

In each preparation, the fraction (collected after gel permeation) that contained the highest oleic acid concentration was examined by cryo-TEM (Fig. 10.9). The mean vesicle diameter and the vesicle size distribution, the average number of ferritin molecules per vesicle, the ferritin distribution among the vesicles, and the mean number of lamellae per vesicle were determined by analyzing the cryo-TEM micrographs.

10.4.2.1 Cryo-TEM micrographs

A ferritin molecule is visible as a black dot on a cryo-TEM micrograph. This is due to the strong electron scattering at the iron core (Massover, 1993). The bilayer of the oleic acid vesicles appeared as a thin dark line that could not be resolved under the used conditions. Fig. 10.9 A shows three unilamellar vesicles and a bilamellar vesicle that all contain ferritin molecules. The large dark particles (see e.g. arrows in Fig. 10.9 A) are ice crystals resulting from frost deposition occurring during specimen preparation or subsequent transfer into the electron microscope (paragraph 8.6, page 74). On Fig. 10.9 B, MLV and MVV vesicles are seen next to unilamellar ones. In Fig. 10.9 C some unusual “aggregates of vesicles” were observed. Some vesicles look faceted and almost touch each other, but no hemifusion was observed. The structure looked similar to a honeycomb. It was not further investigated whether these structures resulted from specimen preparation or if they were indeed present in the suspension. It is, however, likely that they arose from the mechanical stress caused by fluid flow on the grid when the excess liquid is removed to form the very thin aqueous film (Vinson et al., 1989; paragraph 8.3, page 67). At pH 8.5, which corresponds to the pKₐ of oleic acid in bilayers (Cistola et al., 1988), about half of the oleic acid molecules are ionized and the surface of the vesicles is

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18. It was assumed that this fraction also contains the largest number of oleic acid vesicles.
then negatively charged. Due to the inter-vesicular electrostatic repulsion, fusion of the vesicles was not expected and could not be detected.

Although a gel permeation separation was performed, some ferritin molecules were observed outside the vesicles (see e.g. Fig. 10.9, circles). This was unexpected as by examining the chromatograms (see e.g. Fig. 10.8), the removal of non-entrapped ferritin molecules seemed complete. At least three explanations may account for this observation:

- as oleic acid vesicles have a relatively high CAC (~ 20 μM at pH 8.5 - 9; Meyer, 1999), the dilution of the suspension occurring during gel permeation could lead to a transient partial disruption of the bilayer which in turn could lead to the release of some ferritin molecules. If this process took place near the bottom end of the column, it would then be very difficult to separate non-entrapped ferritin molecules from the vesicles.
- vesicles pulled some ferritin molecules with them, possibly sandwiched between the vesicles.
- oleic acid vesicles were not so stable and leaked during or after passage through the column.
Fig. 10.9 Cryo-TEM micrographs of oleic acid vesicles prepared in the presence of 25 mg/ml ferritin in BICINE buffer (200 mM, pH 8.5) according to method I (A & B), to method II (C) and to method III (D). The vesicles were extruded through 100 nm pore size polycarbonate filters and non-entrapped ferritin molecules were removed by gel permeation chromatography. Notice the contamination with large ice crystals (A: arrows) and free ferritin molecules (B, D: circle). See text for more details.
One has to keep in mind that a ferritin molecule is a very large macromolecule in comparison with the water-soluble markers commonly employed in liposome technology. For example, low molecular weight, water-soluble dye molecules are often used to determine the internal aqueous volume of vesicles. As the size difference between a dye molecule and a 100 nm vesicle is greater than between a ferritin molecule and a 100 nm vesicle, dye molecules are more easily separated by gel permeation chromatography than ferritin molecules. In addition, indirect methods, for example, UV spectroscopy, are then used to deduce the captured volume. On the contrary, ferritin offers the unique possibility to directly check by cryo-TEM if the separation was indeed successful. It would be interesting to entrap other directly detectable macromolecules (another protein) or colloidal gold, to use different lipid systems and then to carry out similar experiments to probe whether the presence of ferritin molecules outside the vesicles is specific to the ferritin/oleic acid system studied here or if it is a general problem which has been underestimated so far. Ferritin was also encapsulated in POPC liposomes (Chapter 11.). Some ferritin molecules could also be detected in the bulk solution after gel permeation chromatography; however, this phenomenon was observed to a lesser extent than in the case of ferritin-containing oleic acid vesicles.
10.4.2.2 Characteristics of the ferritin-containing oleic acid vesicles

In Table 10.1 the mean vesicle diameter, the mean number of lamellae per vesicle, and the mean number of ferritin molecules per vesicle determined experimentally and by making various assumptions are listed.

<table>
<thead>
<tr>
<th>Method (see 10.4.1)</th>
<th>mean diameter (± S. D.) (nm)</th>
<th>number of lamellae per vesicle</th>
<th>number of ferritin molecules per vesicle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>experimental calculated</td>
</tr>
<tr>
<td>I</td>
<td>86 ± 40 (318)</td>
<td>1.35 (316)</td>
<td>1.9 (290) 6.8 4.4 3.2</td>
</tr>
<tr>
<td>II</td>
<td>76 ± 43 (1073)</td>
<td>1.30 (1073)</td>
<td>1.8 (1053) 6.8 4.2 2.3</td>
</tr>
<tr>
<td>III</td>
<td>81 ± 23 (191)</td>
<td>1.32 (191)</td>
<td>1.9 (191) 6.8 3.5 2.3</td>
</tr>
</tbody>
</table>

a. The mean vesicle diameter and the mean number of lamellae per vesicle are based on the analysis of the cryo-TEM micrographs. The figures in brackets indicate the number of vesicles counted.

b. The number of ferritin molecules per vesicle was determined directly by counting the number of ferritin molecules entrapped in each vesicle analyzing the cryo-TEM micrographs. The figures in brackets indicate the number of vesicles counted.

c. The number of ferritin molecules per vesicle was calculated using the following assumptions: (i) the total oleic acid concentration was 80 mM (concentration of oleic acid during vesicle formation); (ii) the concentration of ferritin (25 mg/ml) inside and outside the vesicles was identical (equilibrium state) during vesicle formation; (iii) the vesicle population was monodisperse and all the vesicles had an external diameter of 100 nm (diameter of the pore size of the polycarbonate filters used for the last extrusion); and (iv) the vesicles were stable during their passage on the column and did not leak out.

d. The number of ferritin molecules per vesicle was calculated using (i) the oleic acid and the ferritin concentrations determined experimentally by spectroscopic means and (ii) assuming that all the vesicles had a diameter of 100 nm.

e. The number of ferritin molecules was calculated using (i) the experimentally determined oleic acid and ferritin concentrations (by spectroscopic means) and (ii) the mean vesicle diameter obtained by cryo-TEM analysis. Therefore this estimation took into account the fact that not all the vesicles had a diameter of 100 nm but that on average they were smaller.

Table 10.1 The mean vesicle diameter, the average number of lamellae per vesicle, and the number of ferritin molecules per vesicle were determined for the vesicular suspension obtained using the preparation method I, II and III by analyzing cryo-TEM micrographs and making various assumptions. Only the fraction containing the highest oleic acid concentration was considered.
The mean vesicle diameter, the average number of lamellae per vesicle, and the number of ferritin molecules per vesicle obtained experimentally were similar for the three methods used (Table 10.1). The vesicle diameter fluctuated between 76 and 86 nm, and the standard deviation was minimal when the vesicles were prepared using method III. In all preparations the vesicles entrapped on average two ferritin molecules. The mean number of lamellae per vesicle was always around 1.3, which statistically means that about one third of the vesicles were bilamellar while the remaining ones possessed a single bilayer. The reality was somewhat more complex: e.g. using method II, 75% of the vesicles were unilamellar while 2.6% of the vesicles consisted of more than two bilayers.

The number of ferritin molecules per vesicle was also determined by making various assumptions. It was found that not all the assumptions commonly made to determine the trapped volume of the vesicles are valid. Only the estimation calculated by using the vesicle diameter directly determined by cryo-TEM (Table 10.1 e) was in reasonably good agreement with the experimentally determined number of ferritin molecules per vesicle (Table 10.1 b). By applying methods that allow a direct visualization of the system, it was observed, for example, that not all the vesicles were unilamellar or had a diameter equal to 100 nm as assumed in a first approximation. Strictly speaking, the population should not be considered as monodisperse.

In all preparations, about 4% of the ferritin present during vesicle formation was encapsulated. This percentage was calculated using the ferritin and oleic acid concentrations determined experimentally by spectroscopic means and is independent of any size considerations.

---

19. The percentage of entrapped ferritin is expected to be dependent on the surfactant concentration. The higher the surfactant concentration, the higher the percentage of molecules entrapped.
As mean values (Table 10.1) do not give much information about the homogeneity or polydispersity of the vesicle population, the vesicle size distribution and the ferritin distribution among the vesicles were determined.

The three size distributions showed a main peak between 70 and 90 nm. Vesicles larger than 150 nm were observed with a somewhat higher frequency in the absence of FAT treatment (method I) (Fig. 10.10).

![Fig. 10.10 Number-weighted size distribution of the vesicles prepared using method I, II and III (see 10.4) as obtained by cryo-TEM. The last bars correspond to all the vesicles larger than 200 nm.](image)

However, considering the experimental error inherent in the method (statistics based on cryo-TEM micrographs), the three size distributions were in good agreement with each other (Fig. 10.11), indicating that the most important factor in determining the vesicle size was probably the pore diameter of the polycarbonate filters used in the final extrusion step (100 nm). The preparation III yielded the most homogeneous vesicle population.

In Fig. 10.11 the three data sets presented in Fig. 10.10 are combined.
as shown by the standard deviation of the mean vesicle diameter (Table 10.1) and Fig. 10.10. However, the homogeneity of the vesicle population was judged to be satisfactory for all preparations. Nevertheless these suspensions should not be described as monodisperse. In general extrusion of vesicular suspension through 100 nm pore size polycarbonate filters is not efficient enough to yield a true monodisperse population of vesicles. For example, a non-negligible percentage of the vesicles are still bilamellar. Extrusion through 50 nm filters yields more monodisperse vesicles, and most of them are unilamellar (results not shown).

![Graph showing vesicle diameter distribution](image)

**Fig. 10.11** Average of the three number-weighted size distributions shown in Fig. 10.10. The error bars represent the standard deviation. The last bar corresponds to all the vesicles larger than 200 nm.

The micrographs and the counted ferritin distribution among the vesicles (Fig. 10.12) clearly show that the ferritin molecules were not homogeneously distributed among the vesicles. It is evident from Fig. 10.12 that not all the vesicles contained two ferritin molecules like determined as mean value in Table 10.1; some did not entrap any macromolecules while others contained more than ten ferritin molecules. The percentage of
vesicles containing no ferritin molecules at all fluctuated between 34 and 37%. Using method I and II the percentage of ferritin-containing vesicles diminished logically according to the number of ferritin molecules they entrapped: more vesicles contained one ferritin molecule than two ferritin molecules and so on (Fig. 10.12). But with method III the percentage of vesicles containing one, two, and three ferritin molecules was almost identical (Fig. 10.12). The percentage of vesicles containing four ferritin molecules was again lower. It has to be noticed that only 191 vesicles were counted in this experiment (method III). Apart from these differences, the single ferritin distributions were in good agreement with each other (Fig. 10.13).

Fig. 10.12 Number-weighted ferritin distribution among the vesicles as obtained by cryo-TEM for preparation I, II and III (see 10.4).

21. For simplification, vesicles that do not contain any ferritin molecules will be referred to as empty vesicles.
In conclusion, ferritin-containing oleic acid vesicles could easily be prepared, whereby the procedure used for the preparation did not greatly influence the size and entrapment characteristics. This was somehow surprising as FAT, known to improve entrapment efficiency, did not increase the encapsulation efficiency or enhance the homogeneity of the ferritin distribution. The assumption often made that the solute to encapsulate has the same concentration in the bulk water as in the vesicle water pool (Mayer et al., 1985; Mayer et al., 1986; Perkins et al., 1993) was not corroborated, at least in our system. The previous studies were, however, performed using phospholipids and not fatty acids.

The homogeneity of the vesicle population could be increased by further extruding the vesicles through 50 nm pore size filters. This would, however, result in a decrease of the number of ferritin molecules per vesicle.
10.5 Hydrolysis of oleic anhydride in the presence of ferritin-containing oleic acid vesicles

As ferritin-containing oleic acid vesicles could easily be prepared, the hydrolysis of oleic anhydride in the presence of these ferritin-enriched vesicles was investigated. The aim of this work was to find out whether - and to what extent - the vesicles already present influenced the vesicle formation process. Ferritin was assumed to have no influence on the hydrolysis rate nor on the vesicle formation.

10.5.1 Experimental set-up

The hydrolysis was performed at RT in the presence of oleic acid vesicles as in previous studies (Walde et al., 1994; Blöchlier et al., 1998): oleic anhydride was added as a supernatant to BICINE buffer (200 mM, pH 8.5) which contained the ferritin-enriched vesicles. Due to its low density, oleic anhydride formed an oily phase (oil droplet) which floated on top of the vesicular suspension. As the hydrolysis proceeded, the oil droplet disappeared and the aqueous phase became gradually more turbid. The formation of a white gel-like mass was noticed during the reaction (already described by Wick, 1996)\(^{22}\) which disappeared upon quicker stirring. A schematic drawing of the hydrolysis experiment is shown in Fig. 10.14. For the sake of simplicity, state A will often be referred to as “before hydrolysis” and state B as “after hydrolysis”.

\(^{22}\) A white gel-like mass was also observed by Cistola et al. (1988) while studying the oleic acid/water system.
The hydrolysis experiment was performed twice. The first hydrolysis carried out will be referred to as hydrolysis I and the second one as hydrolysis II. The ferritin-containing vesicles used in hydrolysis I and in hydrolysis II were prepared in the presence of 25 mg/ml ferritin and 50 mg/ml ferritin, respectively, during vesicle formation. In both cases, non-entrapped ferritin molecules were removed by gel permeation chromatography. The fractions that contained the highest concentration of oleic acid were examined by cryo-TEM and used as preformed vesicles in the following hydrolysis. In the hydrolysis experiment I the oleic acid vesicles used as the preformed vesicles contained on average two ferritin molecules whereas in the hydrolysis experiment II they entrapped on average four ferritin molecules. The experimental data are summarized in Table 10.2.

The pH was checked before hydrolysis and after hydrolysis completion and was found to remain constant (pH=8.5).
<table>
<thead>
<tr>
<th></th>
<th>hydrolysis I</th>
<th>hydrolysis II</th>
</tr>
</thead>
<tbody>
<tr>
<td>[oleic acid]_{initial} (mM)</td>
<td>5.5</td>
<td>8.6</td>
</tr>
<tr>
<td>mean number of ferritin molecules per preformed vesicle</td>
<td>~2</td>
<td>~4</td>
</tr>
<tr>
<td>[oleic anhydride] (mM)</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>[oleic acid]_{final} (mM)</td>
<td>28</td>
<td>26.7</td>
</tr>
<tr>
<td>reaction volume (ml)</td>
<td>1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Table 10.2 Experimental data of the oleic anhydride hydrolysis experiments. Concentration of oleic acid before the hydrolysis took place (initial) and after completion of hydrolysis (final), of oleic anhydride, mean number of ferritin molecules per preformed vesicle, and reaction volume. The oleic acid concentrations were determined by FTIR spectroscopy. The mean number of ferritin molecules per preformed vesicle was obtained by cryo-TEM analysis. The first hydrolysis experiment carried out is referred to as “hydrolysis I” and the second one as “hydrolysis II”.

Oleic acid vesicles may not be stable upon dilution as suggested recently (Wick et al., 1995; Meyer, 1999; Goto et al., in press). Therefore, the prepared vesicular suspensions were never diluted. The initial oleic acid concentration (Table 10.2) was given by the concentration of the ferritin-containing vesicle fractions obtained after gel permeation chromatography. The amount of oleic anhydride was chosen so that the final oleic acid concentration -after complete anhydride hydrolysis- was still suitable for the bare-grid method without prior dilution ([oleic acid]_{final} < 30 mM).

The results of both hydrolysis experiments will be presented separately (paragraph 10.5.2 & 10.5.3), but they will be discussed together (paragraph 10.5.4, page 159).
10.5.2 Using preformed oleic acid vesicles containing on average two ferritin molecules (hydrolysis I)

The ferritin-containing vesicles obtained by procedure III (paragraph 10.4, page 114) were used as preformed vesicles in this first hydrolysis experiment. The oleic acid concentration was fivefold larger after hydrolysis completion than before hydrolysis (see Table 10.2).

10.5.2.1 Cryo-TEM micrographs

The vesicular suspension was analyzed by cryo-TEM before the hydrolysis took place (Fig. 10.9) and after hydrolysis completion (Fig. 10.15 & Fig. 10.16). Simply by examining the pictures qualitatively it was noticed that the vesicles obtained after hydrolysis were much more polydisperse in size and shape than the initial ones (compare Fig. 10.9 with Fig. 10.15 & Fig. 10.16). More large vesicles, MLV and MVV were observed. Fig. 10.15 shows two typical micrographs of the dispersion obtained after hydrolysis completion: MVV and MLV are found next to unilamellar vesicles. Fig. 10.16 shows the presence of very large unilamellar vesicles, MVV and MLV. It was not always clear if some were really MLV or represented inverted hexagonal phases that were wrapped in a bilayer (Fig. 10.16, arrows). Similar structures were already observed in the oleic acid system (Edwards et al., 1995) and in a dioleoylphosphatidylethanolamine/cetyltrimethylammoniumchloride system (Gustafsson et al., 1995). Beam damage can be observed on the carbon film (see e.g. Fig. 10.16 B).
Fig. 10.15 Typical cryo-TEM micrographs of the vesicular suspension obtained after complete hydrolysis of oleic anhydride in the presence of preformed oleic acid vesicles. The vesicles formed a polydisperse population. Notice the sorting of the vesicles according to their size (for details and experimental conditions, see text and Fig. 10.14 & Table 10.2).
Fig. 10.16 Cryo-TEM micrographs of the suspension obtained after completion of hydrolysis: they show large unilamellar vesicles, MLV or inverted hexagonal phase (arrows) as well as MVV. Beam damage (bubbling) is visible on the carbon film, especially in B (empty arrow). A layer of contamination which resembles fish scales covers both specimen (for details and experimental conditions, see text and Fig. 10.14 & Table 10.2).
Some very large structures, as shown in Fig. 10.17, were also observed after hydrolysis completion. They were not taken into account in the further established statistics as they could not be classified. Since they were not observed before hydrolysis, they were assumed to result from oleic anhydride hydrolysis. They could also represent aggregates of non-reacted oleic anhydride. Indeed it was not clear whether these aggregates were composed solely of oleic acid/oleate or also contained oleic anhydride. An oil droplet, or an oil droplet surrounded by a monolayer or a multilayer of surfactants as found in a micro-emulsion, would have the appearance of a dense (filled) black structure on a cryo-TEM micrograph. Similar structures were already observed at previous stages of the hydrolysis of oleic anhydride (Blocher, 1997). These aggregates should not be completely neglected as they consumed a large amount of oleic anhydride/oleic acid.

Fig. 10.17 Cryo-TEM micrograph showing very large and not clearly defined structures that were obtained after four days of reaction time.
A last but important point to mention is that, due to its own limitation, cryo-TEM does not give information about the absolute number of vesicles present in the suspensions (vesicle concentration). Only by counting a large number of vesicles and by establishing statistics on the vesicles counted, one can draw "semi-quantitative" conclusions from the analysis of cryo-TEM micrographs. If a marker is entrapped in the vesicles and if one assumes that the vesicles are stable, it should, however, be possible to estimate the increase in the number of vesicles in the suspension which results from the release of new surfactants.

10.5.2.2 Characteristics of the ferritin-containing oleic acid vesicles

As predicted from the qualitative examination of the cryo-TEM micrographs, the vesicles had a larger mean diameter, contained on average less ferritin molecules and possessed on average a greater number of lamellae after hydrolysis in comparison with the situation before hydrolysis. The standard deviations suggested that the suspension obtained after hydrolysis was more polydisperse in size (Table 10.3).

<table>
<thead>
<tr>
<th></th>
<th>before hydrolysis</th>
<th>after hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean vesicle diameter (nm) (± S. D.)</td>
<td>81 ± 23 (191)</td>
<td>130 ± 91 (1094)</td>
</tr>
<tr>
<td>mean number of ferritin molecules per vesicle</td>
<td>1.9 (186)</td>
<td>1.5 (999)</td>
</tr>
<tr>
<td>mean number of lamellae per vesicle</td>
<td>1.3 (191)</td>
<td>1.5 (1087)</td>
</tr>
</tbody>
</table>

Table 10.3 The mean vesicle diameter, the mean number of ferritin molecules per vesicle and the mean number of lamellae per vesicle were determined experimentally for the suspension obtained before hydrolysis and after completion of oleic anhydride hydrolysis. All the values are based on the analysis of the cryo-TEM micrographs. The figures in brackets indicate the number of vesicles counted.
Before hydrolysis the majority of the vesicles had a diameter between 50 and 100 nm with a main peak between 80-90 nm. The size distribution obtained after hydrolysis was broader and was shifted towards vesicles of larger diameters: most of the vesicles had a diameter between 90 and 120 nm. About 28% of the vesicles had a diameter larger than 150 nm after hydrolysis, while almost no vesicles were larger than 150 nm before hydrolysis (Fig. 10.18).

**Fig. 10.18** Number-weighted size distribution as obtained by cryo-TEM for the vesicular suspension analyzed before hydrolysis and after hydrolysis. The last bar correspond to all the vesicles larger than 500 nm.
A 200 nm-vesicle is composed of about four times more surfactants than a 100 nm-vesicle, therefore it was interesting to consider not only the number-weighted size distribution but also the mass-weighted size distribution (Fig. 10.19). While the mass-weighted size distribution was similar to the number-weighted size distribution before hydrolysis (73% of the fatty acid mass was contained in vesicles having a diameter between 70 to 120 nm), the mass-weighted size distribution obtained after hydrolysis (Fig. 10.19) yielded a very different picture compared to the number-weighted size distribution (Fig. 10.18). The two most striking features found were that

1. only 27% of all oleic acid molecules were dissolved in vesicles smaller than 150 nm
2. more than 24% of the oleic acid molecules were found in vesicles larger than 500 nm.

**Fig. 10.19** Mass-weighted size distributions based on cryo-TEM micrographs of the vesicular suspension examined before hydrolysis and after hydrolysis. The vesicles were assumed to be spherical and unilamellar. The last bar corresponds to all the vesicles larger than 500 nm.
As the 0.8% of vesicles larger than 500 nm consumed about 24% of the total oleic acid mass, a "restricted" mass-weighted size distribution was established in which aggregates larger than 500 nm were neglected (Fig. 10.20). Cryo-TEM is in fact not suitable for investigating such large objects. Frozen hydrated films are usually thinner than 500 nm and, therefore, vesicles larger than 500 nm may have appeared larger as a result of squeezing/flattening within the thin vitreous ice layer. This "restricted" mass-weighted size distribution agreed qualitatively much better with the number-weighted size distribution. It, however, indicated that more than 30% of the oleic acid molecules were present in vesicles larger than 250 nm. In any case the mass-weighted size distribution evidenced that even if the suspension contained only a few large vesicles, they should not be regarded as negligible as they used up a large amount of surfactants.

![Graph showing mass-weighted size distribution of vesicles](image)

**Fig. 10.20** "Restricted" mass-weighted size distribution based on cryo-TEM micrographs of the vesicles obtained after hydrolysis: vesicles larger than 500 nm were ignored.
The main difference noticed in the ferritin distributions among all the vesicles was an increase in the percentage of empty vesicles upon hydrolysis (Fig. 10.21): 37% of the vesicles were empty before hydrolysis, while 51% of the vesicles analyzed after hydrolysis did not contain any ferritin molecules.

![Graph showing ferritin distribution](image)

**Fig. 10.21** Number-weighted ferritin distribution among all the vesicles as obtained by cryo-TEM for the vesicular suspension examined before hydrolysis and after hydrolysis. The entire vesicle population (empty and filled vesicles) is considered in this figure.

The ferritin distribution among the ferritin-containing vesicles\(^\text{23}\) was also established (Fig. 10.22). In this representation (Fig. 10.22) the empty vesicles were not taken into account and the filled vesicles were assumed to form a 100% population. The percentage of vesicles containing one ferritin molecule and the percentage of vesicles containing three ferritin molecules increased and decreased, respectively, after hydrolysis. Otherwise no significant changes were observed.

\(^{23}\)Ferritin-containing vesicles will often be referred to as filled vesicles.
Fig. 10.22  Number-weighted ferritin distribution among the ferritin-containing vesicles as obtained by cryo-TEM for the vesicular suspension examined before hydrolysis and after hydrolysis. Only the filled vesicles are considered, and the sum of the filled vesicles equals 100% in this representation.

Further analyses were performed by dividing the entire vesicle population into two groups: empty vesicles and filled ones. Fig. 10.23 A & B show the same number-weighted size distribution as previously represented in Fig. 10.18, but now, filled vesicles and empty ones are represented individually. Before hydrolysis (Fig. 10.23 A) the histogram obtained matched prediction: the small vesicles (diameter < 60 nm) were predominantly empty while the larger ones (> 60 nm) were mostly filled. After hydrolysis, however, the situation changed (Fig. 10.23 B): most of the vesicles smaller than 100 nm as well as the majority of the large ones (diameter > 250 nm) were empty, whereas, most of the filled vesicles (85%) had a diameter between 100 and 250 nm.

The size distribution of the empty vesicles broadened upon hydrolysis as shown in Fig. 10.24 A. The size distribution of the filled vesicles (Fig. 10.24 B) was only slightly broader and its main peak was clearly shifted towards larger diameters. In these histograms (Fig. 10.24 A & B) each group was assumed to constitute a 100% population.
Fig. 10.23 Number-weighted size distribution as obtained by cryo-TEM for the vesicular suspension examined (A) before hydrolysis and (B) after hydrolysis. Empty and ferritin-containing vesicles are represented individually on these histograms. In (B), the x-axis scale changes after 250 nm and the last bar corresponds to all the vesicles larger than 500 nm.
Fig. 10.24 Number-weighted size distribution (A) of the empty vesicles and (B) of the filled vesicles as obtained by cryo-TEM for the vesicular suspension examined before hydrolysis and after hydrolysis. The sum of the empty vesicles as well as the sum of the filled vesicles equals 100%. The x-axis scale changes after 250 nm and the last bar in (A) corresponds to all the vesicles larger than 500 nm.
The population of the filled vesicles was further divided into different subclasses: vesicles containing exactly one (Fig. 10.25 A), exactly two (Fig. 10.25 B), exactly three (Fig. 10.25 C) or more than three ferritin molecules (Fig. 10.25 D). The main effect noticed was a shift of the size distribution of each class towards vesicles of larger diameters after hydrolysis.

Fig. 10.25 Number-weighted size distribution of different classes of vesicles before hydrolysis and after hydrolysis: vesicles containing (A) one ferritin molecule, (B) two ferritin molecules, (C) three ferritin molecules and (D) more than three ferritin molecules. The last bars correspond to all the vesicles larger than 250 nm.
The number of lamellae per vesicle was also investigated before and after completion of hydrolysis. The entire vesicle population was divided into three classes: unilamellar vesicles (ULV), bilamellar vesicles and vesicles formed by more than two bilayers \(^24\) (Table 10.4). The percentage of ULV decreased and the percentage of MLV or MVV increased after hydrolysis.

<table>
<thead>
<tr>
<th></th>
<th>1 bilayer (%)</th>
<th>2 bilayers (%)</th>
<th>&gt; 2 bilayers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>before hydrolysis</td>
<td>73</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>after hydrolysis</td>
<td>60</td>
<td>34</td>
<td>6</td>
</tr>
</tbody>
</table>

**Table 10.4** Percentage of the vesicles possessing one, two and more than two bilayers before and after completion of hydrolysis.

For each class of vesicles, the mean diameter and the average number of ferritin molecules per vesicle were determined (Table 10.5).

\(^{24}\) In these considerations the vesicles formed by a large number of lamellae (like the one in Fig. 10.16, arrows) were considered as being multilamellar vesicles. However, it was not always clear whether they really were MLV or if they represented inverted \( \Pi \Pi \) structures surrounded by a bilayer (see also description of Fig. 10.16).
Table 10.5 The vesicles were classified according to their number of lamellae, and the mean vesicle diameter and the average number of ferritin molecules per vesicle of the suspension obtained before hydrolysis and after hydrolysis were determined experimentally.

Before hydrolysis MLV were on average larger and contained less ferritin molecules than ULV, as expected. The mean vesicle diameter was found to be larger after hydrolysis, independently on the number of lamellae; the greatest increase was observed in the case MLV/MVV. A decrease in the mean number of ferritin molecules per vesicle was noticed in each population.

<table>
<thead>
<tr>
<th></th>
<th>mean diameter (nm)</th>
<th>ferritin molecules per vesicle</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>unilamellar</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before hydrolysis</td>
<td>77</td>
<td>2.1</td>
</tr>
<tr>
<td>after hydrolysis</td>
<td>113</td>
<td>1.9</td>
</tr>
<tr>
<td><strong>2 bilayers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before hydrolysis</td>
<td>90</td>
<td>1.6</td>
</tr>
<tr>
<td>after hydrolysis</td>
<td>146</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>&gt; 2 bilayers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before hydrolysis</td>
<td>111</td>
<td>1.0</td>
</tr>
<tr>
<td>after hydrolysis</td>
<td>173</td>
<td>0.8</td>
</tr>
</tbody>
</table>
10.5.3 Using preformed oleic acid vesicles containing on average four ferritin molecules (hydrolysis II)

It was hoped that additional information about the hydrolysis pathway would be obtained by using a system composed of oleic acid vesicles which entrapped more ferritin molecules. Therefore, twice as much ferritin (50 mg/ml) was used during vesicle formation (according to method III as described under 10.4, page 114). Oleic acid vesicles which contained on average four ferritin molecules were thus obtained. These ferritin-containing oleic acid vesicles were used as preformed vesicles to carry out a second anhydride hydrolysis experiment. After completion of oleic anhydride hydrolysis, the total oleic acid concentration was three times larger than before hydrolysis (Table 10.2, page 129).

FfEM was used in parallel with cryo-TEM to study the changes occurring in the suspension as a result of oleic anhydride hydrolysis. In addition, a part of the ferritin-containing oleic acid vesicles prepared was kept aside and simply stirred at RT for four days under the same conditions as the sample, by which the hydrolysis experiment was performed (same temperature, same speed of stirring, same number of days). This sample was regarded as a control to check the stability of the ferritin-containing oleic acid vesicles.

10.5.3.1 Cryo-TEM micrographs and ffEM micrographs

Fig. 10.26 show typical features of the suspension obtained (A) before hydrolysis and (B) after hydrolysis. Notice in Fig. 10.26 A the presence of a slightly elongated vesicle enclosing another distorted vesicle (arrow). In Fig. 10.26 B large and empty vesicles are observed near the edge of the carbon film, while smaller ones which mainly contained ferritin molecules, are found more towards the center of the vitrified film. The large vesicles were deformed (flattened) as they did not have enough space in the thin aqueous film (Fig. 10.26 B, arrows).
Fig. 10.26 Cryo-TEM micrographs of the suspension obtained (A) before hydrolysis and (B) after hydrolysis of oleic anhydride. The hydrolysis was carried out in the presence of preformed ferritin-containing oleic acid vesicles (for experimental details see Table 10.2 and Fig. 10.14). Note the presence of a slightly elongated vesicle enclosing another distorted vesicle in A (arrow). The large vesicles were flattened during specimen preparation in B (arrows).
Fig. 10.27 FfEM micrographs of the vesicular suspension obtained (A) before hydrolysis and (B) after hydrolysis of oleic anhydride in the presence of preformed oleic acid vesicles. The surface of some vesicles (see e.g. arrow in B) is typical for a Lα phase near the main phase transition temperature.
Fig. 10.27 shows typical fEM micrographs of the suspension examined (A) before hydrolysis and (B) after completion of the hydrolysis. The only striking difference found between the two states was the presence of some larger vesicles after hydrolysis.

The surface of some fractured vesicles (Fig. 10.27, arrow) was typical for a Lα phase near the main phase transition temperature. The suspensions were frozen at RT, only some degrees above the phase transition temperature of oleic acid vesicles (Tc ~ 16°C).

### 10.5.3.2 Characteristics of the ferritin-containing oleic acid vesicles

The suspension was examined by cryo-TEM after extrusion and gel permeation chromatography (before hydrolysis), after stirring at RT for four days (for simplification this control will be referred to as “after stirring”) and after completion of hydrolysis, i.e. four days after the addition of oleic anhydride to the ferritin-containing oleic acid vesicle suspension (Table 10.6).

<table>
<thead>
<tr>
<th></th>
<th>before hydrolysis</th>
<th>after stirring</th>
<th>after hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mean diameter (nm)</strong></td>
<td>78 ± 31 (1417)</td>
<td>80 ± 33 (1572)</td>
<td>100 ± 122</td>
</tr>
<tr>
<td>(± S. D.)</td>
<td></td>
<td></td>
<td>(2343)</td>
</tr>
<tr>
<td><strong>mean number of</strong></td>
<td>3.9 (1195)</td>
<td>3.8 (1400)</td>
<td>3.8 (2115)</td>
</tr>
<tr>
<td>ferritin molecules</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>per vesicle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>mean number of</strong></td>
<td>1.27 (1261)</td>
<td>1.31 (1516)</td>
<td>1.39 (2185)</td>
</tr>
<tr>
<td>lamellae per vesicle</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 10.6**  The mean vesicle diameter, the mean number of ferritin molecules per vesicle and the mean number of lamellae per vesicle were determined experimentally for the suspension obtained before, after stirring at RT for four days, and after completion of oleic anhydride hydrolysis. All the values are based on the analysis of the cryo-TEM micrographs. The figures in brackets indicate the number of vesicles counted.
Looking at the mean values characterizing the oleic acid vesicle suspension before and after stirring at RT for four days, the preformed ferritin-containing oleic acid vesicles used in this experiment seemed to be stable. In comparison with the almost identical experiment described above (paragraph 10.5.2), the mean values determined for the present system were different: the average number of ferritin molecules as well as the mean number of lamellae per vesicle hardly changed during hydrolysis. The only tangible difference found was the increase in the mean vesicle diameter from 78 to 100 nm upon hydrolysis. The polydispersity of the suspension probably increased since the standard deviation obtained after hydrolysis was greater than before hydrolysis (Table 10.6).

It was interesting to see whether the size distribution yielded a picture comparable to the one obtained after simply looking at the mean values. Firstly, the stability of the vesicles upon stirring at RT for four days could be confirmed: apart from the decrease in the percentage of vesicles having a diameter between 50 and 60 nm, the two size distributions were almost identical (Fig. 10.28).

![Number-weighted size distribution as obtained by cryo-TEM for the vesicular suspension examined before hydrolysis and after stirring at RT for four days.](image-url)
The size distribution of the vesicles present in the suspension obtained after stirring at RT for four days and after oleic anhydride hydrolysis occurring during the same period of time were compared. The differences noticed between these two distributions were thus considered as resulting exclusively from the hydrolysis process. Vesicles larger than 200 nm were observed with a higher frequency after oleic anhydride hydrolysis than after simply stirring the oleic acid vesicles at RT for four days (Fig. 10.29): only 0.5% of the vesicles were larger than 200 nm after stirring at RT, while 4.2% belonged to this class after hydrolysis. The two size distributions were otherwise much alike.

![Number-weighted size distribution as obtained by cryo-TEM for the suspension examined after stirring at RT for four days and after completion of hydrolysis. The last bar corresponds to all the vesicles larger 300 nm.](image)

If the number-weighted size distribution of the suspension obtained after hydrolysis is converted into a mass-weighted size distribution, it is found that 62% of the total lipid mass was consumed by the 1.6% (in number) aggregates larger than 500 nm!
As this experiment was followed by ffEM as well, it was interesting first to probe whether both methods, cryo-TEM and ffEM, yielded similar results (Fig. 10.30) and secondly to compare the size distributions of the three systems investigated (before hydrolysis, after stirring and after hydrolysis) as obtained by ffEM analysis (Fig. 10.31).

The size distributions determined by the two electron microscopic methods were in general in good agreement (Fig. 10.30). The population of vesicles smaller than 50 nm and the one of vesicles larger than 300 nm seemed to be somewhat overestimated in ffEM analysis compared to cryo-TEM analysis. This was not surprising, since during replica preparation (more precisely, the fracturing step), most of the vesicles are not fractured through their equatorial plane; therefore, they appear with this technique smaller than they actually are\(^{25}\). On the other hand, large vesicles tend to be excluded from the thin frozen hydrated film, which leads to an underestimation of this class using the bare-grid method.

\(^{25}\)It has to be mentioned that no correction of the ffEM data was performed to obtain the true number-weighted distribution (Egelhaaf et al., 1996).
Fig. 10.30  Comparison of the number-weighted size distribution as obtained by cryo-TEM and by ffEM of the suspension obtained (A) before hydrolysis, (B) after stirring at RT for four days, and (C) after completion of oleic anhydride hydrolysis. The last bars correspond to all the vesicles larger than 300 nm.
Fig. 10.31 Number-weighted size distribution as obtained by ffEM for the suspension analyzed before hydrolysis, after stirring at RT for four days, and after hydrolysis. The last bars correspond to all the vesicles larger than 300 nm.

Despite these differences, similar trends to the ones noticed using the cryo-TEM technique were observed using the ffEM method: for example, 2.8% of the vesicles were found to be larger than 200 nm after stirring, while 10.4% were larger than 200 nm after hydrolysis (Fig. 10.31).
The ferritin distribution among the vesicles was also examined. Note that some vesicles entrapped a great number of ferritin molecules (more than twenty molecules) whereas ~24% of the vesicles did not contain any ferritin molecules. The small increase in the percentage of empty vesicles was the only noteworthy change resulting from oleic anhydride hydrolysis (Table 10.7 & Fig. 10.32). No significant differences were detected in the ferritin distribution among the ferritin-containing vesicles (Fig. 10.33).

<table>
<thead>
<tr>
<th></th>
<th>empty vesicles (%)</th>
<th>filled vesicles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>before hydrolysis</td>
<td>24</td>
<td>76</td>
</tr>
<tr>
<td>after stirring</td>
<td>27</td>
<td>73</td>
</tr>
<tr>
<td>after hydrolysis</td>
<td>32</td>
<td>68</td>
</tr>
</tbody>
</table>

Table 10.7 Percentage of empty and ferritin-containing (filled) vesicles present in the suspension obtained before hydrolysis, after stirring at RT for four days, and after oleic anhydride hydrolysis.
Fig. 10.32 Number-weighted ferritin distribution among all the vesicles as obtained by cryo-TEM for the vesicular suspension examined before hydrolysis, after stirring at RT for four days and after hydrolysis. The entire vesicle population (empty and filled vesicles) is considered in this figure. The x-axis scale changes after 20.

Fig. 10.33 Number-weighted ferritin distribution among the ferritin-containing vesicles as obtained by cryo-TEM for the suspension examined before hydrolysis, after stirring at RT for four days and after hydrolysis. Only the filled vesicles are considered, and the sum of the filled vesicles equals 100% in this representation. The x-axis scale changes after 20.
As previously done in the analysis of the first hydrolysis experiment (paragraph 10.4.2.2), the entire vesicle population was divided into two classes: empty vesicles and filled ones.

Fig. 10.34 A, B, and C show the same number-weighted size distributions as formerly depicted in Fig. 10.28 and Fig. 10.29, but this time filled and empty vesicles are represented individually. Before hydrolysis, the size distributions obtained for the empty and for the filled vesicles were basically the same, except that the percentage of empty and filled vesicles differed for each size category. Apart from the vesicles smaller than 30 nm, which were mainly empty, most of the larger vesicles contained ferritin molecules. After hydrolysis the majority of the vesicles with diameters between 10 and 50 nm as well as most of the vesicles larger than 200 nm were empty. The vesicles of intermediate size (50 nm < diameter < 200 nm) were predominantly filled.

By examining the different size distributions obtained for the filled vesicles, apart from a minor shift towards vesicles of larger diameters, no significant changes were detected during hydrolysis (Fig. 10.35 B). On the contrary, by comparing the size distributions obtained for the empty vesicles population, the increase in vesicles larger than 200 nm during hydrolysis was striking (Fig. 10.35 A).
Fig. 10.34 Number-weighted size distribution of the vesicles (A) before hydrolysis, (B) after stirring at RT for four days, and (C) after anhydride hydrolysis. Empty and ferritin-containing vesicles are represented individually on these histograms. The last bars correspond to all the vesicles larger than 200 nm.
Fig. 10.35 Number-weighted size distribution (A) of the empty vesicles and (B) of the filled vesicles as obtained by cryo-TEM for the suspension investigated before hydrolysis, after stirring at RT for four days, and after hydrolysis. The last bars correspond to all the vesicles larger than 200 nm.
10.5.4 Discussion

The stability of oleic acid vesicles was checked by ffEM (paragraph 10.3, page 111) and once by cryo-TEM in a control experiment carried out in parallel with the hydrolysis of oleic anhydride (paragraph 10.5.3, page 145). The vesicles were stable during storage at RT for four days; the only changes observed were the slightly more frequent appearance of aggregated vesicles and the presence of somewhat more large vesicles. In any case, these changes were negligible if compared to the changes observed after complete hydrolysis of oleic anhydride. These stability tests allowed us to regard the oleic acid vesicles as forming a rather stable system under the conditions used and to consider the changes observed between state A and B (Fig. 10.14) as resulting mainly from the hydrolysis of oleic anhydride and not from some sort of ageing processes.

The various size and ferritin distributions gave information about the effects of the release of oleic acid/oleate upon oleic anhydride hydrolysis on a suspension that already contained ferritin-enriched vesicles. Ferritin-containing vesicles found after hydrolysis were assumed to be related to the originally present oleic acid vesicles, whereas on the other hand empty vesicles were regarded in a first approximation as newly formed vesicles.

The clear shift of the size distribution of the ferritin-containing vesicles towards larger diameters observed in the hydrolysis experiment I (Fig. 10.24 B & Fig. 10.25) evidences that some vesicle grew upon hydrolysis. In the hydrolysis experiment I the entire vesicle population (filled and empty vesicles) was actually biased towards larger vesicles after complete anhydride hydrolysis (Fig. 10.18 & Fig. 10.24). In contrast, in the hydrolysis experiment II, no real shift of the size distribution of the ferritin-containing vesicles was detected after hydrolysis completion (Fig. 10.35 B). Only large vesicles were observed (Fig. 10.29 C & Fig. 10.31).
If growth takes place, the mean vesicle size would increase upon oleic anhydride hydrolysis. The mean vesicle diameter as obtained by cryo-TEM for the suspensions examined before and after hydrolysis, as well as a theoretical value of the expected mean vesicle diameter after complete hydrolysis postulating that the preformed vesicles only grew\textsuperscript{26}, are listed in Table 10.8.

<table>
<thead>
<tr>
<th></th>
<th>mean vesicle diameter (nm)</th>
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<tbody>
<tr>
<td></td>
<td>before hydrolysis\textsuperscript{a}</td>
</tr>
<tr>
<td>hydrolysis I</td>
<td>81</td>
</tr>
<tr>
<td>hydrolysis II</td>
<td>78</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The mean diameters were determined experimentally by analyzing cryo-TEM micrographs.

\textsuperscript{b} The theoretical mean diameter (after hydrolysis) was calculated postulating that only swelling of the preformed vesicles occurred. It was assumed that the number of vesicles remained constant during the hydrolysis and that the vesicles were unilamellar.

Table 10.8 The experimental mean vesicle diameter obtained for the suspension before hydrolysis and after hydrolysis of oleic anhydride, and a theoretical mean vesicle diameter (after hydrolysis) were determined.

The theoretical mean vesicle diameter was in both cases larger than the experimentally determined one (Table 10.8). In addition, the percentage of empty vesicles increased significantly in both hydrolysis experiments (I & II) (Fig. 10.21 & Fig. 10.32). These observations indicate that some vesicle growth occurred, but this process was certainly not the only mechanism taking place.

\textsuperscript{26} Knowing the oleic acid concentration as well as the mean vesicle diameter, the number of vesicles initially present in the suspension can be calculated under the assumptions of monodispersity and unilamellarity, and assuming that the monomer concentration can be neglected. A mean head group area of 32Å\textsuperscript{2} and a bilayer thickness of 40Å were used in this calculation.
The presence of large vesicles which are not related to the preformed vesicles would also result in an increase of the mean vesicle diameter. Indeed, large vesicles that predominantly did not contain any ferritin molecules were observed in a much higher frequency after hydrolysis completion in both experiments (I & II) (Fig. 10.24 A & Fig. 10.34 C & Fig. 10.35 A). This suggests that some vesicles were formed \textit{de novo}. Even if the absolute number of large vesicles may not be so large, these vesicles consumed most of the surfactants as shown by the mass-weighted distributions (Fig. 10.19 and comments on page 150). The increase in the percentage of empty vesicles (of all sizes) noticed after hydrolysis is another hint in favor of a \textit{de novo} vesicle formation.

In the hydrolysis experiment I, the mean number of ferritin molecules per vesicle decreased. Before hydrolysis, the vesicles contained on average 1.9 ferritin molecules whereas they entrapped on average 1.6 ferritin molecules after hydrolysis (Table 10.3). This indicates that the number of vesicles raised by a factor of ca. 1.3 upon hydrolysis, assuming that the absolute number of ferritin molecules did not change during hydrolysis. The increase in the number of vesicles resulted either from a \textit{de novo} vesicle formation or a splitting/fission process. This increase was actually not so large considering that the oleic acid concentration was fivefold larger after hydrolysis. This suggests that most of the newly generated surfactants were used to form new large vesicles (probably by a \textit{de novo} vesicle formation process) or were incorporated in the bilayer of the preformed vesicles.

In the hydrolysis experiment II, the mean number of ferritin molecules per vesicle hardly changed. It is, however, not reasonable to get at the same time an increase in the percentage of empty vesicles and an almost identical mean number of ferritin molecules per vesicle (Table 10.6 & Table 10.7). An increase in the percentage of empty vesicles implies an increase in the total number of vesicles whereas the same mean number of ferritin molecules per vesicle means that the number of vesicle remained constant. This observation enlightens the limits of the method.
To find out whether the vesicles split, optimally all the preformed vesicles should contain exactly the same number of ferritin molecules (ideally more than two ferritin molecules). Such a vesicular suspension is, however, not trivial at all to prepare! A splitting process would result in a redistribution of the ferritin molecules among the ferritin-containing vesicles.

Since the preformed oleic acid vesicles used in the hydrolysis experiment I contained on average only two ferritin molecules, it was difficult to follow the changes occurring in the ferritin distribution. For example, as outlined in paragraph 10.2.3, if a vesicle that entraps two ferritin molecules divides into two vesicles, the probability to get one filled vesicle (with two ferritin molecules) and another empty one theoretically equals 0.5.

However, it was found that the percentage of vesicles containing one ferritin molecule increased whereas the percentage of vesicles containing two, three, five and six ferritin molecules decreased (Fig. 10.22, ferritin distribution among filled vesicles). This observation could suggest that some vesicles also split. The initial suspension showed, however, a rather strange behavior since the percentage of vesicles containing one, two and three ferritin molecules was almost identical (Fig. 10.12) and possibly the number of vesicles containing one ferritin molecule was underestimated. The size distribution of the vesicles containing one ferritin molecule was shifted towards larger diameters (Fig. 10.25 A), which rather indicates a growth process.

If a vesicle divides into two vesicles of different size, it is likely that the larger one has a higher probability to entrap ferritin. The splitting mechanism would, in this case, also lead to an increase in the empty population, above all to an increase in the number of small empty vesicles. Splitting is therefore difficult to differentiate from the second pathway (de novo vesicle formation).

It appeared that in the hydrolysis experiment I, the three processes may have played a role. In this experiment, the number of MLV and MVV also increased after completion of the hydrolysis. These vesicles may have been
formed by the self-assembly of newly generated oleic acid/oleate molecules or new bilayers may have been formed around a preformed vesicle.

No redistribution of ferritin among the filled vesicles was observed in the hydrolysis experiment II (Fig. 10.33). In that case, the only noteworthy change was the large increase in the percentage of large empty vesicles (Fig. 10.34 C). This suggests that the *de novo* formation of new large oleic acid vesicle was the main effect of the release of oleic acid/oleate. In this hydrolysis experiment, the suspensions were also examined by ffEM which gave information about the size of the vesicles. The size distributions obtained by cryo-TEM and ffEM were in good agreement with each other.

To summarize, slightly different results were obtained for the first and for the second hydrolysis experiment. While in the hydrolysis experiment I swelling of the preformed vesicles appeared to be the main consequence of the release of new oleic acid/oleate molecules, in the hydrolysis experiment II the formation of presumably new large empty vesicles clearly predominated. The discrepancies observed between the two hydrolysis experiments can arise from the different concentrations of oleic acid vesicles and oleic anhydride used. In the hydrolysis experiment I, the total oleic acid concentration was fivefold larger at the end of the reaction than at the beginning, whereas in the second hydrolysis experiment the final suspension contained “only” three times more oleic acid than the initial one. Besides this, the reproducibility of this type of experiments, which take place in a biphasic system, is difficult to control. Many external parameters, such as the speed of stirring, the concentrations used, the temperature, play a crucial role. It was found that the reproducibility of oleic anhydride hydrolysis could be enhanced by working at 50°C (Blocher, 1997). At this temperature, the formation of the undefined white gel-like mass (probably a dense inverted phase) does not form. Since the previous experiments regarding the matrix effect of oleic acid vesicles were performed at RT the experiments in the present work were done under the same conditions in order to allow a better comparison. The dynamics of oleic acid bilayers is probably different at 50°C than at RT.
10.6 Control Experiments

To check whether the ferritin molecules that were not completely removed by gel permeation chromatography did neither interfere with the oleic acid vesicles nor with the hydrolysis reaction, the subsequent control experiments were performed.

10.6.1 Empty oleic acid vesicles were stirred with ferritin

![Diagram of oleic acid vesicle stirring with ferritin](image)

**Fig. 10.36** Schematic drawing representing the stirring of oleic acid vesicles in the presence of externally added ferritin. Oleic acid vesicles were prepared in BICINE buffer (200 mM, pH 8.5) and were then sized down to 100 nm. Ferritin was added externally to the suspension and the thus obtained suspension was stirred at RT for 5.5 days (total volume ~ 0.25 ml).

100 nm-oleic acid vesicles that did not contain any ferritin molecules were prepared and subsequently stirred at RT in the presence of externally added ferritin (Fig. 10.36). Ferritin was removed by gel permeation chromatography using a spin column. Cryo-TEM analysis showed that most of the vesicles did not entrap any ferritin molecules (Fig. 10.37).
Fig. 10.37 Cryo-TEM micrograph of the oleic acid vesicle suspension obtained after stirring of the oleic acid vesicles in the presence of externally added ferritin at RT for 5.5 days (for experimental conditions, see also Fig. 10.36). Ferritin was then removed by gel permeation.
10.6.2 Hydrolysis of oleic anhydride in the presence of empty oleic acid vesicles and externally added ferritin

100 nm-oleic acid vesicles that did not contain any ferritin molecules were prepared. Ferritin in a large excess followed by oleic anhydride were added to these vesicles and the hydrolysis reaction was carried out as usual (Fig. 10.38).

**Fig. 10.38** Schematic drawing of the hydrolysis experiment: Oleic acid vesicles were prepared in BICINE buffer (200 mM, pH 8.5) and were then sized down to 100 nm. Ferritin followed by oleic anhydride were added to the vesicular suspension. The hydrolysis reaction was carried out at RT for 5.5 days (total volume = 0.25 ml).

After completion of the hydrolysis, a gel filtration was carried out using a spin column to remove non-entrapped ferritin molecules. Unfortunately non-entrapped ferritin molecules were not completely separated from the vesicles by this short column. However, almost no ferritin molecules were detected inside the vesicles (Fig. 10.39). Solely large vesicles contained sometimes ferritin. This type of aggregates (large vesicles) was found to be in general empty in the hydrolysis experiments I and II. Therefore, it seems that the few ferritin molecules that were not entrapped in the preformed vesicles in the former experiments (hydrolysis I & II) did not bias the obtained results and can be indeed neglected.
Fig. 10.39 Cryo-TEM micrograph of the oleic acid vesicle suspension obtained after the hydrolysis of oleic anhydride in the presence of empty oleic acid vesicles and externally added ferritin. The hydrolysis reaction was carried out at RT for 5.5 days (for experimental conditions, see also Fig. 10.38). Ferritin was then removed by gel permeation prior to cryo-TEM examination.
10.6.3 Hydrolysis of oleic anhydride in the presence of free ferritin

Finally, a last hydrolysis experiment was carried out in the absence of preformed vesicles but in the presence of free ferritin (2 mg/ml) dissolved in the buffer (Fig. 10.40). After complete hydrolysis of oleic anhydride, the vesicles were separated from the free ferritin molecules using a Sepharose 4B column.

![Diagram](image)

Fig. 10.40 Schematic drawing of oleic anhydride hydrolysis in the presence of free ferritin dissolved in BICINE buffer (200 mM, pH 8.5), but in the absence of preformed oleic acid vesicles. The reaction was carried out at RT for 4.5 days (total volume = 1 ml).

Three fractions that were collected after gel permeation chromatography were examined by cryo-TEM. The following observations were made. Many more large vesicles were observed than in the same type of experiment, but carried out in the presence of preformed vesicles. These large vesicles often entrapped ferritin molecules in their aqueous interior (Fig. 10.41). On the other hand, most of the small vesicles formed did not contain any ferritin molecules (Fig. 10.42). Many vesicles showing a protuberance caused by the enclosure of another much distorted vesicles were also observed (Fig. 10.41, arrows).
Fig. 10.41 Cryo-TEM micrographs of the suspension obtained in a control experiment in which oleic anhydride was hydrolyzed in the presence of ferritin, but in the absence of preformed vesicles. The hydrolysis reaction was carried out at RT for 4.5 days and free ferritin was removed by gel permeation chromatography (for experimental conditions, see also Fig. 10.40). Some of the large vesicles contained ferritin within their aqueous pool (A) and some showed a protuberance that was caused by the enclosure of another vesicle (B, arrows).
Fig. 10.42 Cryo-TEM micrograph of small oleic acid vesicles obtained after hydrolysis of oleic anhydride in the experiment depicted in Fig. 10.40. They were mostly empty.

As mostly very large vesicles contained ferritin, an hypothesis about the formation of oleic acid vesicles upon hydrolysis of oleic anhydride in the absence of preformed vesicles can now be formulated. First of all, it is unlikely that these large vesicles were formed by the growth of small vesicles, since the probability that a ferritin molecule, which has a diameter of 12 nm, is entrapped in a small vesicle is low. Instead the following mechanism is proposed: First a bilayer or myelin structure grows from the surface of the oleic anhydride droplet, surrounded or not by a mono- or multi-layer of oleate. This process was already observed by LM (Fischer, personal communication). After a certain time the bilayer detaches itself from the droplet and, due to unfavorable hydrophobic interactions with water, seals to form a closed bilayer. During this process the bilayer is at some point probably not optimally closed and ferritin molecules can be encapsulated. It is plausible that the hydrolysis itself (attack of HO⁻) does not only take place at the surface of the vesicles (Walde et al., 1994; Wick,
1996; Mavelli & Luisi, 1996) but also occurs at the surface of the oleic anhydride droplet (and possibly via the transient formation of a (micro-) emulsion) as proposed by Blocher (1997).

10.7 Conclusions

The use of preformed ferritin-containing oleic acid vesicles and of cryo-TEM as study tool showed that the release of oleic acid/oleate molecules upon oleic anhydride hydrolysis had at least two effects on the suspension: some preformed vesicles grew and some new oleic acid vesicles formed.

The clear shift of the ferritin-containing oleic acid vesicles towards larger diameters observed in the hydrolysis experiment I shows that some vesicles swelled by incorporation of new surfactants in their bilayer. On the other hand the increase in the percentage of empty vesicles suggests a de novo vesicle formation or/and the formation of empty vesicles by a splitting process. These two mechanisms were difficult to differentiate from each other under the studied experimental conditions. Should all the vesicles formed by fission of a ferritin-containing vesicle still entrapped ferritin? The answer to this question is not trivial. Whether the ferritin molecules are distributed statistically among the “split” vesicles depends on the splitting mechanism. The size difference of the vesicles produced by such a process, the relatively small number of ferritin molecules entrapped in the preformed vesicles (in the studied systems) render the answer to this question even more difficult.

The fact that the percentage of large empty vesicles increased upon hydrolysis in both experiments (hydrolysis I & II) is very much in favor of a de novo vesicle formation. In hydrolysis I, however, the percentage of vesicles that contained one ferritin molecule also increased. This observation may be a hint that splitting also took place.
One can conclude that, overall, probably more than one single mechanism is operating. By changing the experimental conditions (buffer, ionic strength, pH, temperature, speed of stirring, concentrations...) one or the other pathway could probably be favored. The factors influencing the hydrolysis also need to be clarified in more detail in order to enhance the reproducibility of the reaction. One way could be to carry out the reaction at 50°C.

Finally, the hydrolysis of oleic anhydride in the absence of preformed vesicles suggests that at least part of the hydrolysis did not take place at the surface of the vesicles but also at the surface of the oleic anhydride droplet.
11. Formation of mixed POPC/oleic acid/oleate vesicles: A cryo-TEM study

11.1 Introduction

In the previous chapter, the formation of oleic acid vesicles upon hydrolysis of oleic anhydride was studied. In the past our group has also focused attention on the formation of oleic acid vesicles obtained by injecting of a micellar solution of sodium oleate in aqueous solution. The latter studies showed that the size distribution of oleic acid vesicles formed simply in buffer (pH 8.8) by injecting an aqueous micellar solution of oleate was much broader than the size distribution of oleic acid vesicles generated in buffer containing preformed and relatively monodisperse oleic acid vesicles. The size of these preformed oleic acid vesicles seemed to influence the size distribution of the final vesicular system, thus suggesting a "matrix effect". Moreover, the vesicle formation was faster when the buffer already contained preformed oleic acid vesicles (Blöchliger et al., 1998).

Recently similar experiments have been carried out, in which sodium oleate in water was added to a relatively monodisperse suspension of POPC liposomes\textsuperscript{27} in borate buffer (100 mM, pH 8.5). Turbidity as well as dynamic light scattering measurements (DLS) suggested that the lipid vesicles also influenced the size of the mixed POPC/fatty acid vesicles thus formed. The formation rate of the mixed vesicles was also higher when preformed POPC liposomes were initially present. This matrix effect was observed for preformed POPC liposomes extruded both through 50 and 100 nm pore size polycarbonate membranes; however, the effect seemed to be weaker for the larger (100 nm extruded) POPC liposomes (Lonchin et al., 1999).

\textsuperscript{27} The term "liposome" will be exclusively used for phospholipid vesicle. The term "vesicle" will be used in all the other cases.
The methodology previously applied to study the hydrolysis of oleic anhydride (Chapter 10) appeared appropriate to find out which pathways were followed in the formation of mixed POPC/oleic acid/oleate vesicles obtained upon addition of sodium oleate to POPC liposomes. In a first step, ferritin was entrapped in POPC liposomes and, in a second step, a micellar solution of sodium oleate was added to these preformed ferritin-containing POPC liposomes (Fig. 11.1).

**Fig. 11.1** Schematic drawing illustrating the addition of Na-oleate dissolved in water to ferritin-containing POPC liposomes. The initial POPC concentration was 0.25 mM and the mixed POPC/oleic acid/oleate vesicles obtained after addition of Na-oleate had the following composition: \([\text{POPC}] = 0.2 \text{ mM}, [\text{oleic acid}] = 5 \text{ mM}\).

Evidence regarding the formation of mixed POPC/oleic acid/oleate vesicles should be gathered by studying the differences observed between the initial state (prior to sodium oleate addition) and the final state (after sodium oleate addition) of the vesicular suspension. The initial and the final state of the vesicular system was therefore examined by cryo-TEM and various size and ferritin distributions were determined.
Moreover, the uptake of oleic acid by POPC liposomes is of biological relevance due to the importance of fatty acids transport in body fluids and of the interaction of fatty acids with biological membranes. From the literature it is known that PC liposomes can incorporate oleic acid molecules in their bilayer (paragraph 4.5, page 31).

From a theoretical point of view addition of Na-oleate to POPC liposomes can give rise to three events. These events are analogous to the ones described for the formation of oleic acid vesicles upon oleic anhydride hydrolysis in the presence of preformed oleic acid vesicles (paragraph 10.2, page 107).

1. Swollen mixed vesicles are formed by the incorporation of oleic acid molecules in the POPC bilayers (simple swelling of the initially present POPC liposomes, Fig. 11.2 case 1).
2. New vesicles are formed independently of the preformed POPC liposomes (de novo formation) (Fig. 11.2 case 2).
3. POPC liposomes incorporate oleic acid molecules in their bilayer and then eventually split (Fig. 11.2 case 3).

The three cases are again schematically depicted in Fig. 11.2. The theoretical considerations presented in paragraph 10.2 are also valid for the formation of mixed vesicles upon addition of Na-oleate to POPC liposomes.
Fig. 11.2 Schematic illustration of the various events that may occur upon Na-oleate addition to POPC liposomes in borate buffer (100 mM, pH 8.5): (1) the preformed liposomes simply grow by uptake of oleic acid molecules, (2) "new" oleic acid vesicles are formed independently of the preformed ones (de novo formation), and (3) the liposomes incorporate new surfactants in their bilayer and then eventually split (see also text and paragraph 10.2, page 107 for theoretical considerations).
11.2 Preparation of POPC liposomes containing ferritin

Large POPC liposomes containing ferritin were prepared in borate buffer (100 mM, pH 8.5) according to the reverse phase evaporation method (REV) first described by Szoka and Papahadjopoulos (1978)\(^{28}\). The lipid concentration in the aqueous phase (33 mM) was chosen so that unilamellar liposomes should be preferentially formed (New, 1990). The liposomes were sized down to 100 nm by extrusion and non-entrapped ferritin molecules were removed by gel permeation chromatography using a Sepharose 4B column as previously performed for the preparation of ferritin-containing oleic acid vesicles. The separation of free ferritin from ferritin-containing POPC liposomes was more efficient than the separation of free ferritin from ferritin-containing oleic acid vesicles. POPC liposomes probably formed a more stable system than oleic acid vesicles. However, some free ferritin molecules were sometimes observed in the bulk suspension (Fig. 11.4 B, circle). The POPC and ferritin concentrations of the collected fractions were quantified using the Steward assay and spectrophotometry, respectively. The fraction containing the highest concentration of POPC was examined by cryo-TEM and DLS, and further used to study the formation of mixed vesicles (paragraph 11.3).

In former experiments (Lonchin et al., 1999) it was found that the “matrix effect” was more pronounced using 50 nm-POPC liposomes. In our case, the liposome suspension could be extruded very easily through 100 nm pore size filters but could not be extruded through 50 nm pore size membranes.

\(^{28}\)Ferritin-containing POPC liposomes were also prepared using the film method followed by FAT treatment. The entrapment efficiency was, however, poorer than when the ferritin-containing vesicles were prepared according to the REV method.
Fig. 11.3 shows the suspension obtained after extrusion through 100 nm pore size polycarbonate filters. Large structures formed either by the aggregation of vesicles and ferritin molecules or by the clustering of ferritin molecules only were not observed. The presence of such structures would have explained why the suspension could not be further extruded. Therefore, the most likely explanation is that the filled liposomes were not "flexible" enough to enter or to go through the cylindrical pores and then simply clogged the polycarbonate filters. Indeed, some liposomes entrapped so many ferritin molecules in their aqueous interior (Fig. 11.4) that it is conceivable to regard them as too stiff to undergo further deformation.

Fig. 11.3 Cryo-TEM micrograph of ferritin-containing POPC liposomes obtained by REV and after extrusion through 100 nm pore size filters, but before removal of the external ferritin molecules by gel permeation chromatography.
11.3 Addition of sodium oleate to preformed ferritin-containing POPC liposomes

Sodium oleate was first dissolved in H₂O and then added to the ferritin-containing POPC liposomes. The experimental procedure (addition of Na-oleate to POPC liposomes) was done as described by Lonchin et al. (1999), except that, in the present case, the preformed POPC liposomes entrapped ferritin molecules. The formation of the mixed vesicles was followed by turbidity, and the initial and final suspension was investigated by DLS and by cryo-TEM. The mol ratio of POPC with respect to oleic acid was 1:25.

11.3.1 Results from dynamic light scattering (DLS)

According to DLS data (Table 11.1) the thus formed mixed system contained vesicles that were much larger and more polydisperse in size than the preformed ferritin-containing POPC liposomes.

<table>
<thead>
<tr>
<th>Vesicles</th>
<th>Rₜ (60°) (nm)</th>
<th>Rₜ (90°) (nm)</th>
<th>Rₜ (120°) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>59.0 ± 0.3</td>
<td>58.0 ± 0.4</td>
<td>57.4 ± 0.3</td>
</tr>
<tr>
<td>POPC/oleic acid/oleate</td>
<td>109.1 ± 1.4</td>
<td>89.8 ± 0.5</td>
<td>75.0 ± 0.5</td>
</tr>
</tbody>
</table>

Table 11.1 Mean hydrodynamic radius of the ferritin-containing POPC liposomes and of the mixed POPC/oleic acid/oleate vesicles formed upon addition of Na-oleate to ferritin-containing POPC liposomes at three different observation angles as obtained by DLS measurements.

29 For simplification the term “mixed vesicles” will also be used instead of mixed POPC/oleic acid/oleate vesicles.
11.3.2 Results from cryo-TEM

The mean vesicle diameter, the average number of ferritin molecules per vesicle, and various size distributions and ferritin distributions among the vesicles were established on the basis of cryo-TEM micrographs (Fig. 11.4 & Fig. 11.5).

11.3.2.1 Cryo-TEM micrographs of the POPC liposomes and of the mixed POPC/oleic acid/oleate vesicles

The comparison of Fig. 11.3 with Fig. 11.4 illustrates well the efficiency of gel permeation chromatography to remove non-entrapped ferritin molecules from the bulk: after the passage of the suspension through the column, almost no free ferritin molecules were left in the bulk.

The bilayer of the mixed vesicles appeared thinner than the bilayer of the POPC liposomes. Theoretically it should therefore be possible to distinguish liposomes built only by POPC molecules from vesicles formed by a mixture of POPC and oleic acid molecules or from vesicles made from solely oleic acid entities (paragraph 8.5, page 71). No clear difference was, however, noticed on the micrographs obtained after oleate addition to POPC liposomes.

At least two explanations can account for this observation:
1. All the vesicles had a similar composition and contained both POPC and oleic acid molecules and therefore gave rise to the same image contrast.
2. Some technical problems (insufficient knowledge about the image contrast transfer function, suboptimal specimen preparation) may have impeded the differentiation of the various types of vesicle bilayers.
By looking qualitatively at the cryo-TEM micrographs (Fig. 11.4 & Fig. 11.5), it was observed that:

- POPC liposomes formed a rather homogeneous suspension and mostly entrapped ferritin molecules (Fig. 11.4).

- The mixed system obtained after oleate addition contained more polydisperse vesicles. It looks as if the large vesicles still contained ferritin, while the small ones were mainly empty (Fig. 11.5).
Fig. 11.4 Cryo-TEM micrographs of ferritin-containing POPC liposomes obtained by REV and sized down by extrusion. Free ferritin was removed by gel permeation chromatography. A large number of ferritin molecules were entrapped in some liposomes. Almost no free ferritin molecules were observed (B, circle). The POPC concentration was 1.9 mM.
Fig. 11.5 Cryo-TEM micrographs of the mixed vesicles formed upon oleate addition to preformed ferritin-containing POPC liposomes; the large vesicles mainly contain ferritin molecules while the small ones are mostly empty ([POPC] = 0.2 mM, [oleic acid] = 5 mM). In (B), the vesicles are arranged according to their size in the vitrified film.
11.3.2.2 Characteristics of the POPCliposomes and of the mixed POPC/oleic acid/oleate vesicles

While the number of ferritin molecules per vesicle diminished, surprisingly the mean vesicle diameter did hardly increase after oleate addition. The standard deviation was, however, much larger after oleate addition and suggested an increase in the polydispersity of the suspension. About 90% of the POPC liposomes were unilamellar, and the mean number of lamellae per vesicle did not change upon oleate addition (Table 11.2).

<table>
<thead>
<tr>
<th></th>
<th>POPC liposomes</th>
<th>mixed vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean diameter (± S. D.) (nm)</td>
<td>97 ± 33 (811)</td>
<td>101 ± 63 (2167)</td>
</tr>
<tr>
<td>mean number of ferritin molecules per vesicle</td>
<td>11 (597)</td>
<td>2.4 (1587)</td>
</tr>
<tr>
<td>mean number of lamellae per vesicle</td>
<td>1.1 (787)</td>
<td>1.1 (2074)</td>
</tr>
</tbody>
</table>

Table 11.2 The mean vesicle diameter, the average number of ferritin molecules per vesicle and the mean number of lamellae per vesicle were determined experimentally for the ferritin-containing POPC liposomes and for the mixed POPC/oleic acid/oleate vesicles obtained upon oleate addition to ferritin-containing POPC liposomes. All the above listed values are based on the analysis of cryo-TEM micrographs. The figures in bracket indicate the number of vesicles counted.

Assuming that all the POPC liposomes have a diameter of 100 nm and using the experimentally determined ferritin and POPC concentrations, the POPC liposomes (prior to oleate addition) should entrap on average twelve ferritin molecules. This value is in good agreement with the experimental value determined by analyzing the cryo-TEM micrographs (Table 11.2).
The vesicle size distribution (Fig. 11.6) shows that POPC liposomes formed a narrow distribution around the main peak at 90-100 nm. After oleate addition the obtained size distribution was broader and the main peak was shifted towards smaller diameters (50-60 nm). Mixed vesicles having a diameter larger than 150 nm represented ~19% of the entire population, whereas only 2% of the POPC liposomes were larger than 150 nm before oleate addition.

Fig. 11.6 Number-weighted size distributions as obtained by cryo-TEM for the POPC liposomes ([POPC] = 1.9 mM) and for the mixed POPC/oleic acid/oleate vesicles ([POPC] = 0.2 mM, [oleic acid] = 5 mM) formed upon addition of Na-oleate to preformed POPC liposomes at RT.
The number-weighted size distributions presented in Fig. 11.6 were converted into mass-weighted size distributions (Fig. 11.7). The mass-weighted distribution of the POPC liposomes was quite similar to its number-weighted size distribution. Most of the POPC molecules were dissolved in vesicles having a diameter between 90 and 130 nm. The mass-weighted distribution of the mixed vesicles yielded a picture different from its number-weighted size distribution: most of the lipids was found to be present in vesicles larger than 200 nm. The 8.5% vesicles larger than 200 nm consumed 40% of the total lipid mass.

Fig. 11.7 Mass-weighted size distribution based on cryo-TEM micrographs of the POPC liposomes ([POPC] = 1.9 mM) and of the mixed POPC/oleic acid/oleate vesicles ([POPC] = 0.2 mM, [oleic acid] = 5 mM) formed upon addition of Na-oleate to preformed POPC liposomes at RT. The vesicles were assumed to be spherical and unilamellar. The last bars correspond to all the vesicles larger than 200 nm.
The most striking feature noticed in the ferritin distributions among the vesicles (Fig. 11.8) was the great increase in the percentage of empty vesicles upon oleate addition: whereas ~ 15% of the POPC liposomes did not contain any ferritin molecules, the empty vesicles represented ~ 77% of the entire population after addition of oleate.

![Ferritin distribution among all the vesicles as obtained by cryo-TEM](image)

**Fig. 11.8** Ferritin distribution among all the vesicles as obtained by cryo-TEM for the POPC liposomes ([POPC] = 1.9 mM) and for the mixed POPC/oleic acid/oleate vesicles ([POPC] = 0.2 mM, [oleic acid] = 5 mM) formed upon addition of Na-oleate to preformed POPC liposomes. The entire vesicle population (empty and filled vesicles) is considered in this figure. The x-axis scale changes after 10.

The ferritin distribution among the filled vesicles (Fig. 11.9) shows that, upon oleate addition, the percentage of vesicles containing one or two ferritin molecules increased leading to some redistribution of the ferritin molecules among the filled vesicles. In Fig. 11.9 the empty POPC liposomes and the empty mixed vesicles are not taken into account. The filled POPC liposomes and the filled mixed vesicles constitute a 100% population.
Fig. 11.9 Ferritin distribution among the filled vesicles as obtained by cryo-TEM for the POPC liposomes ([POPC] = 1.9 mM) and for the mixed POPC/oleic acid/oleate vesicles ([POPC] = 0.2 mM, [oleic acid] = 5 mM) formed upon addition of oleate to preformed POPC liposomes. Only the filled vesicles are considered, and the sum of the filled vesicles equals 100% in this representation. The x-axis scale changes after 10.

The entire population of vesicles was further divided into two groups: empty vesicles and filled ones.

Fig. 11.10 A shows the size distribution of the POPC liposomes in which empty and filled liposomes are represented individually: as expected only the vesicles smaller than 40 nm were generally empty, and the majority of the other ones entrapped at least one ferritin molecule. The size distribution obtained for the mixed vesicles (Fig. 11.10 B) was quite different: the vesicles smaller than 160 nm were mainly empty while the ones larger than 160 nm were mostly filled.

Fig. 11.11 A shows that, upon oleate addition, the size distribution of the empty vesicles broadened and that the main peak was shifted towards smaller sizes. About 70% of the empty mixed vesicles still had a diameter between 30 and 100 nm. Fig. 11.11 B indicates that while the filled POPC liposomes were more or less narrowly distributed around 90-100 nm, the filled mixed vesicles constituted a broad, flat distribution.
Fig. 11.10 Number-weighted size distributions as obtained by cryo-TEM (A) for the POPC liposomes ([POPC] = 1.9 mM) and (B) for the mixed POPC/oleic acid/oleate vesicles ([POPC] = 0.2 mM, [oleic acid] = 5 mM) formed upon Na-oleate addition to preformed POPC liposomes. Empty and ferritin-containing liposomes are represented individually on the histogram.
Fig. 11.11 Number-weighted size distributions as obtained by cryo-TEM (A) for the empty POPC liposomes and the empty mixed POPC/oleic acid/oleate vesicles and (B) for the ferritin-containing POPC liposomes and the ferritin-containing POPC/oleic acid/oleate vesicles. Empty and ferritin-containing liposomes/mixed vesicles were each assumed to represent a 100% population.
11.3.3 Discussion

Most of the results concerning the ferritin-containing POPC liposomes were predictable. Once more the ferritin molecules were not distributed equally among the liposomes: some liposomes contained more than fifty ferritin molecules while ~15% were still empty (Fig. 11.8).

The number of lamellae per vesicle determined for both the POPC liposomes and the mixed vesicles indicates that vesicles were mostly unilamellar. Under the studied conditions, the addition of oleate did not result in an increase in the number of lamellae of the vesicles.

The changes observed in the size and in the ferritin distributions between the initial and the final suspension suggests that the formation of mixed vesicles followed at least two pathways: a growth process and a process leading to the formation of empty vesicles.

The broadening of the overall size distribution (Fig. 11.6) and above all the broadening of the size distribution of the filled vesicles upon oleate addition (Fig. 11.11 B) demonstrates that the POPC liposomes did indeed grow to form mixed POPC/oleic acid/oleate vesicles. The fact that most of the lipid mass was present in large vesicles is an indication that the vesicle growth was an important effect of the addition of Na-oleate.

The mixed vesicles contained on average 2.4 ferritin molecules whereas the POPC liposomes encapsulated on average 11 ferritin molecules. This suggests that the vesicle population increased by a factor of ca. 4.5 assuming that the absolute number of ferritin molecules did not change upon oleate addition. This increase was the result of either a de novo vesicle formation or/and a splitting process.

The large increase in percentage of the empty population noticed after oleate addition (Fig. 11.8) suggests that some “new” vesicles were formed de novo.
If the preformed POPC liposomes swelled or if the added oleate molecules self-assembled to form new oleic acid vesicles, the number of filled vesicles would not change and therefore, the ferritin distribution among the filled vesicles would remain identical. The percentage of vesicles containing one and two ferritin molecules was, however, found to increase after oleate addition (Fig. 11.9). A plausible explanation for this observation could be that some vesicles containing ferritin did indeed split thus leading to a ferritin redistribution among the filled vesicles. Splitting could happen as a result of a perturbation of the bilayer caused, for example, by a sudden excess of oleate molecules in the outer leaflet of the vesicle membrane. It is known that flip-flop of charged molecules is relatively slow (paragraph 4.5, page 31). An excess of negatively charged oleate molecules in the outer leaflet of the vesicles or an increase in the surface area of the outer monolayer could possibly induce the budding off a vesicle. Slower changes and in particular asymmetric membrane changes can result in budding off, either externally after growth of protrusions or internally after invagination (Winterhalter & Lasic, 1993). If a small vesicle is generated by budding off a large one as described in Fig. 11.2 (case 3.b), the probability that the small vesicle does not contain any ferritin molecules is relatively high. Overall, one would also observe an increase in the empty population, and more specifically an increase in the percentage of small empty vesicles. This phenomenon is, therefore, difficult to differentiate from a de novo vesicle formation. It has to be mentioned that whether the ferritin molecules are distributed statistically among the vesicles depend on the budding/splitting mechanism.

The slight shift of the size distribution of the empty population towards smaller diameters (Fig. 11.11 A) and the fact that the vesicles smaller than 160 nm were mostly empty (Fig. 11.10 B) could support both mechanisms, a de novo formation and/or a budding/splitting process.
The last remark concerns the discrepancy observed between DLS measurements and cryo-TEM analysis. While for POPC liposomes the mean radius obtained by each method were in good agreement (DLS $<R_h> \sim 58$ nm; cryo-TEM $<R> \sim 48$ nm), the mixed POPC/oleic acid/oleate vesicles yielded very different results. The mean radius found by DLS was almost twice as large as the one obtained by cryo-TEM. In the case of an heterogeneous population of vesicles, $<R_h>$ determined by DLS is always expected to be larger than $<R>$ obtained from cryo-TEM.

The “limits” of both techniques were presumably reached. On one hand, DLS has the propensity to overestimate large aggregates, especially in polydisperse suspension. The size distribution obtained in a DLS experiment is weighted by the ability of the particles to scatter light (intensity-weighted). Small particles are only very weakly weighted in DLS experiments. In contrast, large ones are easily detected in DLS. Even a very small number of large particles leads to a considerable change in the obtained size distribution and will have a tremendous effect on the resulting average sizes (Egelhaaf, 1996).

On the other hand, due to the need of a thin frozen hydrated sample, large aggregates tend to be excluded from the water film during sample preparation for cryo-TEM study. Cryo-TEM has therefore the tendency to underestimate large vesicles. This problem is not so consequential while investigating homogeneous population of relatively small objects, such as 100 nm-POPC liposomes, but is more dramatic if polydisperse suspensions are studied.
11.4 Conclusions

The addition of Na-oleate to preformed POPC liposomes under the chosen conditions (borate buffer, 0.1 M, pH 8.5; ratio POPC:oleic acid, 1: 25) led to:

1. the formation of mixed POPC/oleic acid/oleate vesicles obtained by incorporation of oleate molecules in the POPC bilayer as evidenced by the clear shift of the ferritin-containing vesicles towards larger diameters;
2. the formation of small empty vesicles which could have resulted either from a de novo vesicle formation or a budding/splitting mechanism.

In this experiment, it was not possible to quantify the importance of each pathway and to distinguish between the budding/splitting and the de novo vesicle formation mechanism.

The addition of Na-oleate to borate buffer (0.1 M, pH 8.5) (in the absence of preformed liposomes) was studied with DLS (Lonchin et al., 1999). It would be interesting to examine the vesicle suspension obtained after oleate addition to borate buffer with cryo-TEM and to compare the vesicle size distribution obtained in the absence of preformed vesicles with the size distribution obtained in the presence of preformed vesicles.

It could also be worthwhile to investigate the formation of mixed POPC/oleic acid/oleate vesicles under other conditions, for example, at another pH. As the properties of oleic acid and oleate are different from each other, it is reasonable to suppose that the pH of the solution can have an influence on the formation of mixed vesicles. The ratio oleate to POPC could also influence the final state of the suspension. For example, one could imagine that POPC liposomes can only incorporate a certain amount of oleate molecules in their bilayer.
12. Use of microscopy as a quantitative tool

12.1 Introduction

The use of protein-containing vesicles and of the bare-grid method gave some hints on the mechanism of oleic acid vesicle self-reproduction and of mixed vesicle formation upon addition of Na-oleate to POPC liposomes. Although it was possible to determine an approximate ratio between the number of vesicles present before and after surfactant addition, it was not possible to determine the absolute number of vesicles contained in the investigated suspensions (vesicle concentration). Moreover large vesicles tend to be underestimated using this method. A new cryo-procedure for EM specimen preparation was therefore examined as a possible tool to quantitatively characterize the bulk of vesicle suspensions (number, size distribution, number of lamellae).

In the first part of this chapter, some problems associated with specimen preparation in the case of the bare-grid method and of the ffEM technique using the “sandwich” method are summarized, and the block-face method is described.

In the second part, preliminary studies on vesicle and latex suspension using the block-face method are reported.

12.2 The block-face method

In this work the bare-grid method (cryo-TEM) was primarily used to examine ferritin-containing vesicles as it permitted the direct visualization of ferritin molecules entrapped in the vesicles. Unfortunately this technique does not yield any information about the absolute number of vesicles present in a dispersion and in addition is not ideal for the observation of large objects (> 500 nm). During the blotting step the excess suspension is removed by filter papers. What is left on the grid does not necessarily reflect the true situation of the bulk suspension. Moreover, due to the finite
thickness of the film, this method tends to overestimate smaller particles. The bare-grid method is also not suited to examine highly concentrated suspensions as, with such suspensions, thin aqueous films are more difficult to achieve. In addition, overlapping of vesicles may happen which renders the interpretation of the micrographs even more delicate.

The other widely employed method for the visualization of colloidal suspensions is the freeze fracturing technique. Although this approach may give an approximate idea of the number of vesicles contained in a suspension, it is limited as well. If propane-jet freezing (Müller et al., 1980) is used to cryofix thin aqueous layers, the suspension sandwiched between the copper platelets has a thickness of about 10 μm. Surface effects due to the presence of copper platelets cannot be ruled out. This as well as the formation of ice crystals may lead to a redistribution of the vesicles. In the fracturing step, it is not known where the fracture plane proceeds, e.g. through the bulk suspension or near the copper platelets.

For the quantification of particles an alternative method, in which the above uncertainties are eliminated was needed. A recently developed technique which uses gold tubes of “large” diameter (inner Φ 200 μm) as sample holder was investigated (Shimoni & Müller, 1998). The suspension is sucked into a gold tube and high-pressure frozen as described by Shimoni and Müller (1998). The tube is then either fractured or cut with a cryo-ultramicrotome to expose a large surface, called the block-face, which can then be visualized by cryo-SEM (Walther & Müller, 1999). High-pressure freezing is used because, at ambient pressure, vitrification (or at least satisfactory freezing) can only be achieved for thin objects, typically thinner than a few μm (paragraph 7.2.3, page 52).

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30. This method is mainly used in our laboratory to achieve satisfactory freezing of relatively small volume of aqueous specimen at ambient pressure.
The freezing of a large bulk volume results in an increase of the ratio volume of the suspension to surface of the metal and therefore, the eventual alterations caused by surface effects are less significant. In contrast to the "sandwich method" described above, the sample is not fractured in a total random manner in the block-face procedure, but a cut through the bulk of the suspension is achieved.

12.3 Block-face of suspensions containing oleic acid vesicles

An oleic acid vesicle suspension was therefore prepared, high-pressure frozen in gold tube and cryo-sectioned using a cryo-ultramicrotome (Michel et al., 1992) (for experimental details, see paragraph 15.3, page 214). The tube was cut until a smooth surface was obtained. The surface then required substantial sublimation of water in order to reveal its topography.

Cutting through the bulk actually represents the only method to get a true representation of the suspension. The block-face does not show the compression artifacts caused by cutting which are visible on thin cryo-sections obtained simultaneously by this procedure (Michel et al., 1992). Cut and intact vesicles were easily differentiated on SEM micrographs. The two main difficulties were:

1. Freezing damage: large segregation patterns were often observed (Fig. 12.1 C).
2. It was difficult to control the sublimation of water. If water removal was insufficient, no vesicles could be observed (flat surface). If too much water was sublimated, the cut vesicles collapsed and only intact spheres were visible "deeper inside the sample" (well below the surface) (Fig. 12.1 C).
When freezing and water sublimation of the specimen were nearly optimal, the vesicles could, however, be nicely observed (Fig. 12.1 A & B). This method yielded information about the morphology of the vesicles, such as the number of lamellae. The oleic acid bilayer membrane appeared approximative 10 nm thick, i.e. much thicker than the theoretical value of 4 nm. This could partially arise from the deposition of Pt (~3nm) on both sides of the cut membranes. Another possible explanation is, that some water may still be bound to the membranes, because it was held by the hydrophilic head groups of the amphiphiles and therefore, was not removed during sublimation at ~ -110°C. In contrast to cryo-TEM the true size of the vesicles cannot be directly measured. The vesicle size distribution can, however, be obtained by morphometric techniques (Howard & Reed, 1998).

Fig. 12.1 Typical SEM micrographs of the block-face of high-pressure frozen oleic acid vesicles ([oleic acid] = 20 mM). (A) and (B) “ideal” freezing and water sublimation: unilamellar as well as vesicles composed of more than one bilayer are visible. The elongated vesicle entrapping a smaller one in A (arrow) looks very similar to some typical oleic acid vesicles previously observed using cryo-TEM; (C) excessive water sublimation: round intact vesicles are found dispersed in a network of salt (buffer component). The block-face was rotary-shadowed with Pt/C.
12.4 Experiment with latex spheres

In order to test the validity of the method to quantify particles in a suspension, a more defined suspension than the polydisperse oleic acid vesicle suspension was needed. Latex spheres have the advantage of being supplied as monodisperse suspensions. Two suspensions containing a different concentration of latex spheres (Ø 110 nm) were high-pressure frozen. The first latex suspension (suspension I) contained 59.5 particles/µm³ whereas the second one (suspension II) contained 23.8 particles/µm³. Block-faces of the bulk suspension were obtained by cryo-sectioning as well as by cryo-fracturing of the gold tube at 163 K in a freeze etching unit (paragraph 15.3, page 214).

12.4.1 Cryo-SEM micrographs

Looking at the two overviews (Fig. 12.2, insets) the difference between cryo-sectioning and cryo-fracturing is well perceivable. The surface of the block-face is much smoother in the case of cryo-sectioning. Contamination (deposition of large hexagonal ice crystals) was more significant in that case, although great care was taken during the transfer of the cut sample to the freeze-etching unit.

The latex particles had a strange appearance when they were fractured (see Fig. 12.2 B): it looks as if their inner content was spitted out (plastic deformation). In the case of cryo-sectioning, the removal of water was also very difficult to control. Excessive sublimation of water caused partial eviction of the particles from the bulk: some were found to lay loosely on top of the surface, their cut edge perpendicular to the cut plane. Some particles may also have been lost. In addition, intact (uncleaved) spheres newly appeared at the surface (Fig. 12.2 A). These particles probably emerged with water sublimation. It was sometimes difficult to differentiate these intact spheres from the cut ones.
Fig. 12.2 Typical cryo-SEM micrographs showing (A) fractured and (B) cut latex spheres at a primary magnification of 50,000 X. Overviews of the cryo-sectioned and of the cryo-fractured tube are shown in the inset in (A) and (B), respectively. The blockface was shadowed with Pt/C from an angle of 45°.
12.4.2 Quantification of the latex particles

When the tube was cryo-fractured, the fractured spheres as well as the holes, which were assumed to be left by latex particles that were pulled out of the bulk during the fracturing step, were counted. Only the cut spheres were counted in the case of cryo-sectioning. The experimental number of latex per \( \mu \text{m}^3 \) was obtained by dividing the number of spheres counted by the total volume considered. The volume was estimated by multiplying the area of the micrograph (length by width) with the diameter of a sphere. Indeed, all the spheres which have their center of mass within 110 nm (2 x 55 nm) should be theoretically cut (Fig. 12.3).

**Fig. 12.3** Schematic drawing representing the bulk suspension containing latex spheres having a diameter of 110 nm. The plain line represents the cutting plane. The dash lines are drawn 55 nm (radius of a latex sphere) away from the cutting plane.
Table 12.1 Number of latex particles per μm$^3$ as obtained theoretically and experimentally using a height of 110 nm (Fig. 12.3), and experimental error in% for the latex suspension I and II. When the sample was cryo-fractured, fractured spheres and holes were counted. When the sample was cryo-sectioned, only the cut spheres were counted.

<table>
<thead>
<tr>
<th></th>
<th>suspension</th>
<th>theoretical (particles/μm$^3$)</th>
<th>experimental (particles/μm$^3$)</th>
<th>error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cut in the microtome</td>
<td>I</td>
<td>23.8</td>
<td>15.4</td>
<td>35.1</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>59.5</td>
<td>39.9</td>
<td>32.9</td>
</tr>
<tr>
<td>fractured in the BAF</td>
<td>I</td>
<td>23.8</td>
<td>16.8</td>
<td>29.3</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>59.5</td>
<td>46.5</td>
<td>21.9</td>
</tr>
</tbody>
</table>

The experimental values deviated substantially from the theoretical ones (35 to 20% error). Such a discrepancy could originate from the way the total volume was calculated. Taking a height of 110 nm implies that even the spheres that were just touching the cutting plane were cut and still detectable after sample preparation. This is arguable and taking a height of 100 nm is more realistic. The error was then reduced (15 to 30%). As mentioned above, when the sample was cryo-sectioned, it was difficult to discern undoubtedly the cut particles from the uncleaved ones on the micrographs and some cut spheres may have been lost. The number of cut ones was possibly underestimated.
However, the experimental ratio of the number of particles present in the two investigated suspensions correlated in both cases well with the theoretical value (Table 12.2). Error in the range of 4-11% can be regarded as very satisfactory considering the different steps involved in these experiments. It seems that this method allows us to compare suspensions of different concentration and therefore to determine a concentration ratio between two suspensions.

<table>
<thead>
<tr>
<th></th>
<th>theoretical ratio</th>
<th>experimental ratio</th>
<th>error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cut in the microtome</td>
<td>2.5</td>
<td>2.59</td>
<td>3.9</td>
</tr>
<tr>
<td>fractured in the BAF</td>
<td>2.5</td>
<td>2.76</td>
<td>10.4</td>
</tr>
</tbody>
</table>

Table 12.2 Theoretical and experimental ratio of the number of particles contained in suspension I and II, and experimental error in%.

The same technical problems were encountered with latex spheres and with oleic acid vesicles (freezing, water sublimation). Moreover, it was difficult to differentiate cut latex spheres from uncleaved ones. For a further evaluation of the method, it may be advantageous to prepare a more monodisperse suspension of phospholipid vesicles, for example, by using the detergent method preparation (Zumbühl & Weder, 1981).
12.5 Conclusion

The current limitation of these experiments was, that several preparation steps, such as freezing, condensation and sublimation of water, could not be controlled well enough, and were therefore, not sufficiently reproducible. Contamination of the sample surface by ice crystals or hoar frost and water condensation occurred mainly during the transfer from the cryo-sectioning device to the freeze-etching unit and from there to the microscope. The sublimation of water was the major problem: too much water removal destroyed the bilayers and rendered them difficult to observe whereas insufficient water sublimation yielded a flat surface with no morphological details. As the rate of water sublimation depends on the surface temperature of the sample, which cannot be exactly measured, the sublimation step is very difficult to control with the instrumentation currently available.

In the case of vesicular suspensions, adequate freezing of the sample was very difficult to achieve as well. In contrast to most biological samples investigated with this technique until now, vesicular suspensions contain no natural cryo-protectants which could help to improve freezing quality. For this, one will have to resort to thinner aqueous layers. It is concluded, that the technical equipment must be significantly improved in order to routinely conduct block-face studies. A vacuum transfer device, for example, from the cryo-ultramicrotome to the freeze-etching machine and from there to the SEM would greatly facilitate the procedure and eliminate the water contamination problem.

However, this technique is potentially very powerful as the block-face represents the true situation of a dispersion:

1. It can allow the quantification of the objects in a dispersion, such as vesicle concentration.
2. The size distribution of the objects can also be obtained by morphometric techniques, provided enough micrographs are taken (Howard & Reed, 1998).
13. General conclusions and outlook

The use of protein-containing vesicles and of cryo-TEM as study tool gave information about the mechanism of vesicle formation and transformation upon addition of new amphiphilic molecules to a suspension that already contained vesicles.

In both systems studied, the self-reproduction of oleic acid vesicles and the formation of mixed vesicles upon Na-oleate addition to POPC liposomes, experimental observations clearly evidenced that some vesicles grew. More empty vesicles were also observed after the addition of surfactants in both cases. This suggested a de novo vesicle formation. The de novo vesicle formation mechanism was, however, very difficult to differentiate from the splitting/budding process, in which, under the given experimental conditions, empty vesicles could also be produced. A small vesicle obtained by budding off a large ferritin-containing vesicle does not necessary contain ferritin and has a lower probability to entrap ferritin molecules than a larger one. These experiments did not permit a quantitative validation of each pathway. Cryo-TEM of ferritin-containing vesicles gave a hint about the increase in the number of particles, however this information should be taken with care as frozen-hydrated sample do not necessarily represent the actual situation in the bulk suspension.

It may be also possible to obtain the number of vesicles present in a suspension by using light scattering techniques. A methodology was recently developed which permits the measurement of concentrated suspensions and the quantification of particles present in a dispersion (Urban, 1999; Urban & Schurtenberger, 1998). In that case no marker is needed and this method may be more straight forward. Further studies in this field should attempt to use light scattering in addition to electron microscopy.

The use of electron microscopy in a quantitative way seems to be another possibility as outlined in Chapter 12. (page 195). For this, a larger volume of the bulk solution has to be frozen instead of a thin aqueous layer as in the
bare-grid method. The frozen bulk sample can then be processed by cryo-
sectioning to yield a flat surface of the bulk (block-face). A cut through the
bulk gives a true representation of the suspension and one should be able to
quantify the number of particles present in the suspension. Morphometric
analysis can then be performed on high resolution SEM micrographs of the
block-face in order to obtain the vesicle size distribution.
Further investigations should be performed in order to answer the following
question: How can one distinguish the splitting/budding process from the \textit{de novo} vesicle formation?

It is difficult to design experiments that permit the differentiation between
the splitting/budding process and the \textit{de novo} vesicle formation. One should
be able to mark the membrane of the small preformed vesicles. In this way,
the distribution of the initially present amphiphiles in the vesicle bilayer
could be theoretically followed. If this is conceivable in theory, it is
experimentally not trivial to achieve. The small amphiphilic molecules
should be detectable using electron microscopy and at the same time their
physical properties should not change so that they are still able to form
vesicles. Recently, undecagold cluster have been covalently attached to
lipids. These gold-labelled lipids were able to form vesicles (Hainfeld et al.,
1999). Such lipids could eventually serve as markers.
D. MATERIAL & METHODS

14. Chemicals

Sodium oleate (>99%), N,N-bis-(2-hydroxyethyl)glycine (BICINE) (>99.5%), boric acid (>99.5%), 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), 4-nitrophenyl phosphorodichloridate, phosphorus oxychloride, decanol, iso-octane (spectroscopic grade) were purchased from Fluka, Switzerland. Oleic anhydride (99%), ferritin (from horse spleen) were purchased from Sigma. Standard 1 M solutions of HCl (Titrisol) and NaOH (Titrisol) were purchased from Merck. Didecyl phosphoric acid was from TCI (Japan). POPC was from Avanti Polar Lipids, Inc. Sepharose 4B was from Pharmacia (Sweden) and Bio-Gel A-15m was from Bio-Rad Lab (Richmond, USA).

15. Methods and instruments

15.1 Cryo-transmission electron microscopy of frozen hydrated samples (cryo-TEM)

15.1.1 Preparation of the copper grids coated with a holey carbon film

Copper grids (300 mesh, Athene) covered with a holey carbon film were used for the preparation of frozen hydrated samples. They were prepared according to a modified procedure described by Fukami (Fukami & Adachi, 1965; Egelhaaf, 1995).

A cleaned microscope slide was immersed in a 0.1% aqueous Triton-X 100 solution for 5 min and then allowed to dry at RT. This treatment rendered the slide surfaces hydrophobic. Thereafter the slide was cooled down in a freezer at -24°C for 3 min, where it was laid on top of an aluminium block. It was then transferred quickly (ca 5 sec in the air) to a water saturated chamber and left there for 1 min (RT). Upon warming in the humid
chamber, dew minute droplets formed on the slide surface. The slide, which was covered with water droplets, was then plunged briefly into a precooled (4°C) triafol solution (0.4% triafol in EtOAc), and allowed to dry at RT overnight. Thus a plastic film with holes was obtained. The quality of the holey film was checked by LM. The film was then floated off on top of water, covered with copper electron microscopy grids (300 mesh, Ø 3 mm) and finally lifted with a piece of newspaper. Once dried, carbon was evaporated onto the grids (ca 20 nm). The triafol film was dissolved by placing the coated grids over a pile of filter papers soaked with EtOAc in a glass container for 2 days. Depending on the suspension to be investigated, the grids were glow discharged shortly before sample preparation (Dubochet et al., 1982b). Glow discharge renders the film more hydrophilic which leads to a better wetting with aqueous samples. This results in a more homogeneous coverage of the grid. Fig. 15.1 shows a holey carbon film. Ideally holes should have a diameter between 1 and 5 μm.

Fig. 15.1 TEM micrograph of a grid covered with a holey carbon film.
15.1.2 Preparation and observation of the frozen hydrated samples

The thin frozen hydrated films were prepared under controlled temperature (25°C) and humidity conditions within a custom-built environmental chamber (Egelhaaf, 1995).

The vitrification system (Fig. 15.2 A) consisted of two major parts: the environmental chamber, in which the specimen were prepared, and the cryogen reservoir, into which they were plunged.

The controlled environmental chamber is briefly described below (for more details, see Egelhaaf, 1995). Two concentric plexiglas tubes surrounded the environmental chamber. While the top was fixed, the bottom with the blotting system could easily be removed. The blotting system (Fig. 15.2 B) consisted of filter papers stretched over two plexiglas blocks that were covered with foamed plastics. Through the space between the two plexiglas tubes water of the desired temperature could be circulated. This allowed one to thermally isolate the chamber from the surrounding and to control the temperature inside the chamber. The desired humidity inside the environmental chamber was obtained by gentle flow of water-saturated nitrogen.

A polystyrene box isolated the cryogen reservoir. The temperature of the atmosphere inside the cryo compartment was controlled through a continuous flow of cold and dry nitrogen. It resulted from the evaporation of liquid nitrogen caused by the heat produced by a resistor. The ethane, kept in a small vessel, had the same temperature as the surrounding atmosphere (~ 108 K).
Fig. 15.2 Scheme of the vitrification and of the blotting system used in the present work (Egelhaaf, 1995).
Once a relative humidity of 97-99% was reached inside the chamber, the grid and new filter papers were mounted. They were allowed to equilibrate for 10-15 min and then 10 µl of the suspension was put on the perforated carbon film using the side opening. After ca 10 sec, the tweezers that hold the grid was conducted between the filter papers (Fig. 15.2 B) and the excess liquid was blotted from both sides by the filter papers for 2 to 6 sec. Symmetrical blotting was found to reduce demixing and evaporation effects (Cyrklaff et al., 1990 & 1994). The obtained thin film was very rapidly plunged into liquid ethane ($T_{ethane} \sim 108K$). A plunge velocity of $\sim 3$ m/s was used. The vitrified sample was kept under liquid nitrogen for storage or for transfer to a cryoholder (Gatan, Inc., Pleasanten, CA, USA). The cryoholder was inserted into a Zeiss EM 912 OMEGA microscope, equipped with an energy filter. The specimen chamber was pumped for ca 15 min to remove water vapors that entered the specimen chamber during transfer and the shutter above the specimen was then opened. The frozen hydrated samples were examined at 120 kV and at a specimen temperature of $\sim 105K$. Electron micrographs were digitally recorded using a cooled 1024 x 1024 pixel CCD camera (Proscan). Images were taken with an electron dose of $2-10$ e/Å². Gratings with 2160 lines/mm were used to calibrate the magnification.

15.1.3 Analysis of the cryo-TEM micrographs

The cryo-TEM micrographs shown in the present work were processed with a high pass function (Photoshop software) to reduce background noise. The quality of the printed pictures was improved and the analysis was thus facilitated. Only vesicles were taken into account to establish the various distributions. For example, undefined aggregates of lipids and ferritin were not considered. The vesicle diameter was measured and the number of ferritin molecules entrapped in each vesicle was counted. Number-weighted and mass-weighted size distributions were determined on the basis of the cryo-TEM micrographs. Number-weighted distributions were obtained by
plotting the relative number of vesicles (given in percentage of the total number of vesicles) against the vesicle diameter (nm). The mass-weighted distribution was obtained by calculating the frequency of each size weighted by the corresponding surface area. The vesicles were assumed to be spherical and unilamellar. In this case the relative mass (given in percentage of the total lipid mass) was plotted against the vesicle diameter (nm).

15.2 Freeze fracture electron microscopy (ffEM)

15.2.1 Preparation and observation of the replicas

Freezing and freeze-fracturing of the samples were carried out according to the procedure described by Müller et al. (1980) and outlined in paragraph 7.2.5 (page 54). A gold grid (Ø 2.5 mm, 300 mesh) was dipped into the suspension and sandwiched between two copper platelets that were previously washed with cone. sulfuric acid followed by water and acetone. The sandwich was then rapidly frozen in a propane-jet at -180° C, fractured at -150° C and 2 x 10⁻⁷ mbar in a Balzer BAF 300 freeze fracturing apparatus (BALTEC Inc., Balzers, Liechtenstein) and the fractured faces were immediately shadowed with 2 nm Pt/C at an angle of 45° followed by 10 nm C at 90°. The replicas were floated on distilled water and examined without further cleaning in a Hitachi 600 EM (Hitachi, Tokyo, Japan). Electron micrographs were digitally recorded using a cooled 1024 x 1024 CCD camera (Gatan). Gratings with 2160 lines/mm were used to calibrate the magnification.

15.2.2 Analysis of the ffEM micrographs

The apparent vesicle diameter was determined by measuring the radius of the profiles on the pictures perpendicular to the shadowing direction,
thereby minimizing the apparent enlargement by the deposited platinum. The number-weighted size distributions were determined in the same way as in the case of the cryo-TEM analysis (see 15.1.3).

15.3 Cryo-scanning electron microscopy

15.3.1 Sample preparation

Gold tube with an inner diameter of 200 μm and a wall thickness of 50 μm (Goodfellow Cambridge Limited, Cambridge, U.K.) were used. The gold tube was inserted in a plastic pipette tip and the plastic was slightly melt with a soldier lamp around the gold tube to produce a tight tip. This “customized” tip was mounted on a pipette man and 50 μl of the suspension was withdrawn. The filled tube was sealed by compressing and flattening the ends with a tweezers while pushing out some suspension in order to avoid the presence of air bubbles inside the tube (Fig. 15.3).

![Fig. 15.3 Experimental procedure describing the preparation of a suspension sample for high-pressure freezing (Shimoni, 1998).](image)

The sealed tube was then mounted in the HPF-holder according to the method described by Shimoni and Müller (1998). The tube was then frozen in the high-pressure freezing machine (HPM 010, BAL-TEC, Balzers, Liechtenstein). After freezing the tube was stored in liquid N₂.
15.3.1.1 Cryo-fracturing in BAF

The tube was cut in the middle with a scalpel. One half was clamped in a holder (Bastacky et al., 1995) and cryo-fractured in a freeze etching unit (BAF 300, Balzers, Liechtenstein) (Fig. 15.4). The sample was first shadowed with 3 nm of Pt/C from an angle of 45° followed by 5 nm of C evaporated perpendicularly (at 90°) (Walther & Müller, 1997; Walther et al., 1995).

Fig. 15.4 Processing of the frozen gold tube (Shimoni, 1998).
15.3.1.2 Cryo-sectioning in an ultramicrotome

Half of a gold tube was clamped in a holder and the holder was mounted in a cryo-ultramicrotome (Reichert Sc 4 cryo-ultramicrotome). Semi-thin (0.5 μm, 0.6 mm/s) and thin (50 nm, 0.05 mm/s) sections were then cut using a glass knife and a cryo-diamond knife (Diatome AG, Biel, Switzerland), respectively (Fig. 15.4). The holder with the cut tube (block-face) was cryo-transferred to the freeze-etching unit. Water was then evaporated at -109°C for 2 min and the sample was either shadowed from an angle of 45° or rotary shadowed with 3 nm of Pt/C and the Pt layer was strengthened by 5 nm of C evaporated perpendicularly (Walther et al., 1995; Walther & Müller, 1997).

The coated tube was mounted on a precooled cryoholder (Gatan, Inc., Pleasanton, CA, USA) and cryo-transferred into the SEM (Hitachi S-900, in-lens field emission SEM, Hitachi, Tokyo, Japan). The specimen chamber was pumped for ca 10 min to remove water vapor that entered the specimen chamber during transfer and the shutter above the specimen was then opened. Samples were examined at a temperature of ca 143 K and a primary accelerating voltage \( V_0 \) of 10 kV. Micrographs using the BSE signal were recorded with a Gatan DigiScan 688 (Digital Micrograph 2.0, Gatan, Inc., Okearabteb, CA, USA). The contrast is thereby formed by the scattering events taking place at the Pt layer. As the Pt layer is in direct contact with the investigated sample, the true surface of the specimen is revealed. Pictures were typically taken at a primary magnification of 50 000 x.

15.4 Light microscopy

Light microscopy micrographs were obtained by using an Axioplan microscope from Zeiss. All micrographs were taken using the DIC (Differential Interference Contrast) mode.
15.5 Extrusion

The extrusion procedure was performed using

- a Liposofast device manufactured by Avestin Inc. (Canada) for volumes between 0.2 to 1 ml. The suspensions were first forced 21 times through two stacks of polycarbonate filters with pore sizes of 400 nm in diameter. The same procedure was then repeated with 200 nm and finally with 100 nm pore size filters.

- an extruder from Lipex Biomembranes Inc. (Canada) for larger volumes. The suspensions were first passed 10 times through two 400 nm Nucleopore polycarbonate membrane filters. The same procedure was then repeated with 200 nm and finally with 100 nm pore size filters.

The polycarbonate membranes were purchased from Nucleopore (USA).

15.6 Gel permeation chromatography

Gel permeation chromatography was carried out using either

- a column (length 50 cm, Ø 1.2 cm) that was filled with Sepharose 4B and then equilibrated with the desired buffer prior to use. The suspension (0.3 to 1 ml) was loaded on the column and fractions of approximately 1 ml were collected.

- “spin columns” (length 7 cm, Ø 0.5 cm) that were filled with Bio-Gel A-15m. The suspension (0.2 ml) was loaded on the spin column, which was previously equilibrated with BICINE buffer (200 mm, pH 8.5), and centrifuged at 165 g for 2 min as described elsewhere (Chonn et al., 1991). Fractions of approximately 50 µl were collected.
15.7 **UV-Vis spectroscopy**

The UV-Vis absorption spectra were recorded either on a Cary 1E UV-Visible spectrophotometer from Varian (Australia), or on a DU-68 spectrophotometer from Beckman (USA). Quartz cells of a pathlength of 1 or 0.5 cm from Hellma were used.

15.8 **FTIR spectroscopy**

FTIR spectrum were recorded with a Nicolet 5SXC using a CaF$_2$ cell with a 0.2 mm optical pathlength.

15.9 **Dynamic light scattering (DLS)**

Vesicle sample were analyzed with a fiber-optics based spectrometer consisting of an argon laser (Coherent, Innova 200-10, $\lambda_0 = 488$ nm), a digital autocorrelator (ALV 5000), and a computer controlled rotational stage (Newport, Model 496 and Controller PMC 400). A photomultiplier ALV/PM-15 was used as detector.

15.10 **Dialysis**

Dialysis was performed using 0.5 - 3 ml Slide-A-Lyzer™ cassettes from Pierce.

15.11 **Sonication**

Bath sonications were performed in a Brandlin Sonorex RK 100 H instrument at RT.
16. **Synthesis of the phosphate esters**

All the reactions were carried out under Ar atmosphere. The reaction progress was followed by TLC. Phosphodi- and mono-ester were revealed at RT by spraying the TLC plate with a modified Dittmer reagent (Ruy & MacCoss, 1979). To detect phosphotriesters, the TLC plate had to be heated. In the synthesis of 3, all the compounds bearing a nitrophenyl part could be easily visualized on the TLC plate under UV-light.

16.1 **Tri-n-decyl phosphate (1) and didecyl phosphoric acid (2)**

Redistilled benzene over Na (40 ml), redistilled decanol (6 ml, 31.42 mmol) and pyridine kept under Ar (2.9 ml, 36.03 mmol) were mixed in a 250 ml 3-necked round bottom flask and stirred at RT for 0.5 hr. Phosphoryl chloride (0.92 ml, 10.05 mmol) was added dropwise over a period of 15 min. The thus obtained suspension was stirred a further 5 min at 0°C, 10 ml of benzene were added and the reaction mixture was then heated to reflux for 8 hrs. The mixture was then allowed to cool down to RT, the white pyridinium salt was filtered off and the benzene was taken off using a rotary evaporator. The remaining pyridine was evaporated using HV and a yellow oil was obtained. The crude product was purified by silica gel column chromatography using hex:EtOAc (2:1) to yield tridecyl phosphate (1) (1.9963 g, 38.5%) as an oil and then using CHCl₃:MeOH:AcOH (70:10:2) to give didecyl phosphoric acid (2) (1.629 g, 43.1%) as a white powder.

(1) **Rₚ(hex:EtOAc, 1:2) 0.5; **¹H-NMR (CDCl₃): δ = 4.02 (dt, J=6.7 Hz, 6H, -O-CH₂-CH₂-), 1.63 (m, 6H, -O-CH₂-CH₂-), 1.26 (m, 42H, alkyl chain), 0.87 (t, J=6.7 Hz, 9H, CH₃); ¹³C-NMR (CDCl₃): δ = 67.6 (d, J= 5.8 Hz, -O-CH₂-CH₂-), 31.8 (CH₂), 30.2 (d, J= 6.8, -O-CH₂-CH₂-), 29.47 (2C, CH₂), 29.24 (CH₂), 29.1 (CH₂), 25.42 (CH₂), 22.6 (CH₂), 14.01 (CH₃); ³¹P-NMR

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31. The polarity of the solvent mixture was increased gradually to avoid "washing" of the column and thus collection of mixed fractions containing simultaneously the di- and mono-ester of phosphate.
(CDCl₃): δ = -0.03; MS m/z (%): 519 (M⁺1, 100%), 1037 (2M⁺1, 33%), 379 (M-C₁₀H₂₁, 12%), 179 (H₄P₂O₇+1, 19%).

(2) Rₓ (CDCl₃): (MeOH:AcOH:CDCl₃; 70:10:2) 0.5; H-NMR (CDCl₃): δ = 11.16 (s, 1H, -OH), 4.01 (dt, J=6.7 Hz, 4H, -O-CH₂-CH₂-), 1.67 (m, 4H, -O-CH₂-CH₂-), 1.26 (m, 28H, alkyl chain), 0.88 (t, J=6.6 Hz, 6H, CH₃); ³¹P-NMR (CDCl₃): δ = -2.03; MS m/z (%): 401 (M⁺Na, 100%), 379 (M⁺1, 68%), 757 (2M⁺1, 18%).

16.2 Didecyl 4-nitrophenyl phosphate (3)

Redistilled decanol (4.2 ml, 22 mmol) was put in a 50 ml 2-necked flask containing freshly distilled pyridine (4 ml, 49.7 mmol). THF (5 ml) was then added and the solution was stirred at RT for 15 min. 4-nitrophenyl phosphorodichloridate (2.562 g, 10 mmol) in THF (5 ml) was added dropwise to the decanol mixture cooled to 0°C over a period of 35 min. The formation of a white solid was observed after the addition of the first drop. The reaction mixture was stirred at room temperature for 5 hrs and then H₂O (100 µl) was added to quench the reaction. After 30 min of further stirring, the white solid was filtered off and THF was evaporated using a rotary evaporator. The remaining pyridine was taken off using HV to give a yellow oil which was then purified by silica gel column chromatography using EtOAc:hex (1:2) to yield didecyl 4-nitrophenyl phosphate (3) (3.42 g, 68%) as a slightly yellow oil.

Rₓ (EtOAc:hex, 1:2) 0.5; H-NMR (CDCl₃): δ = 8.24 (m, 2H, Hₐrom) 7.37 (m, 2H, Hₐrom), 4.15 (m, 4H, O-CH₂), 1.7 (m, 4H, O-CH₂-CH₂) 1.3 (m, 28H, alkyl chain), 0.88 (t, 6H, J=6.1 Hz, CH₃); C-NMR (CDCl₃): δ = 14.09 (CH₃), 22.68 (CH₂), 25.38 (CH₂), 29.07 (CH₂), 29.29 (CH₂), 29.5 (CH₂), 30.18 (CH₂), 30.23 (CH₂), 31.8 (CH₂), 69.15 (O-CH₂), 120.52 (Cₐrom), 125.66 (Cₐrom), 144.6 (Cₐrom), 155.7 (Cₐrom); P-NMR (CDCl₃): δ = -6.25; MS (m/z) (%): 500 (M⁺1, 44%), 220 (100%); UV (MeOH), λmax = 267.
17. Characterization of didecyl phosphate

17.1 Titration of didecyl phosphate

The stock solutions were prepared by dissolving $2^{32}$ in an aqueous phase (10-12 ml) and stirring at RT overnight. The aqueous solution contained an equimolar ratio of NaOH or KOH to 2 to ease experimental work. $2$ in pure bidistilled water gave rise to a two phase system, either oil in water or solid in water depending on the temperature, while in the presence of an equimolar amount of base an homogeneous suspension was obtained.

17.1.1 Using NaOH and HCl

When the base used was NaOH, some crystals were still visible in the suspension after stirring at RT. The stock solution was therefore heated for 2 min at 56°C to give a turbid suspension. Samples of 1 ml were taken with a prewarmed glass pipette and further diluted with $\text{H}_2\text{O}$, HCl or NaOH. The samples were warmed again to 56°C and then allowed to cool down at RT for 0.5 hr before measuring the pH and a titration curve was established.

17.1.2 Using KOH

When the base used was KOH, the stock solution was stirred at 37°C for a further 0.5 hr to ensure complete dissolution of $2$. The turbid suspension was allowed to cool down to RT and samples of 1 ml were removed with a glass pipette. The samples were diluted with $\text{H}_2\text{O}$, HCl or KOH. The pH of the samples was measured at RT. A titration curve was established.

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32. Didecyl phosphoric (2) purchased from the firm TCI (Japan) was used.
17.2 General procedure for the preparation of vesicular suspensions

All the vesicular suspensions were prepared by adding the desired amount of aqueous solution to 2 and subsequently stirring the suspension at RT overnight if not otherwise stated.

18. Hydrolysis of didecyl 4-nitrophenyl phosphate (3)

18.1 Material and general procedure

The hydrolysis reaction of didecyl 4-nitrophenyl phosphate (3) was carried out in a closed 3 ml flat-bottom flasks (Ø 13 mm) containing a magnetic stirrer (10 x 5 mm) in a Reacti-Therm heating/stirring module 18971X from Pierce (USA) under slow magnetic stirring (position 1.5) at 68°C. The total reaction volume was 1 ml and the initial concentration of 3 fluctuated between 10 and 12 mM. Each experiment was made in triplicate.

18.2 Analysis of the hydrolysis reaction

The p-nitrophenolate released during the reaction was quantified spectrophotometrically at 391 nm (maximum of its absorbance). From time to time 10 µl of the aqueous phase was removed with a 10 µl Hamilton syringe and diluted with 10 µl of CAPS buffer (400 mM, pH=10.5). The sample was further diluted with 1 ml of MeOH and a UV-Vis spectrum was then immediately recorded. Each measurement was made in triplicate.

A calibration curve of p-nitrophenolate was established under these experimental conditions to determine its molar extinction coefficient ε at 391 nm (Fig. 18.1).
18.3 Hydrolysis of didecyl 4-nitrophenyl phosphate (3)

18.3.1 Without the use of a sonication bath

a) In pure aqueous solution (no vesicles initially present)

3 (~ 5 µl) was laid on top of the aqueous phase (1 ml) and the hydrolysis reaction was carried out at 68°C.

b) In the presence of didecyl phosphate (2) vesicles

2 was weighted in the reaction flask and 1 ml of aqueous solution was added. The sample was then stirred at RT overnight and a turbid suspension was obtained. 3 (~5 µl) was then laid on top of the vesicular suspension and the hydrolysis reaction was carried out at 68°C.
18.3.2 With the use of a sonication bath

a) In pure aqueous solution

3 was weighted in the reaction flask, 1 ml of aqueous solution was added and the mixture was sonicated at 20°C for 10 min. A fine dispersion of oil droplets in water was obtained. The hydrolysis reaction was then carried out at 68°C.

b) In the presence of didecyl phosphate (2) vesicles

(i) only 2 was sonicated

2 was weighted in the reaction flask and 1 ml of aqueous solution was added. The sample was sonicated at 20°C for 10 min and a clear solution was obtained. 3 (~5 μl) was then laid on top of the sonicated vesicular suspension and the hydrolysis reaction was carried out at 68°C.

(ii) 2 and 3 were cosonicated

2 and 3 were weighted in the reaction flask and 1 ml of aqueous solution was added. The sample was sonicated at 20°C for 10 min and a turbid suspension was obtained. The reaction flask was transferred at 68°C.

18.3.3 Using the film method

2 (2.14 mg, 5.65 x 10^{-3} mmol) and 3 (2.84 mg, 5.68 x 10^{-3} mmol) were dissolved in CHCl₃ (3 ml) and MeOH (1.5 ml). The solvent mixture was then slowly evaporated using a rotary evaporator under reduced pressure. The thus obtained film was dried under HV overnight, dissolved in 0.25 M KOH (1 ml) and vortexed for 5 min at RT. A turbid suspension was obtained. The reaction flask was then transferred at 68°C.
19. Preparation of ferritin-containing oleic acid vesicles

19.1 Preparation of the ferritin solutions

The purchased stock ferritin solution had the following characteristics: ferritin 102 mg/ml, NaCl 150 mM.

   a) 25 mg/ml ferritin solution

A 25 mg/ml ferritin solution was obtained by dilution of the 102 mg/ml ferritin solution with BICINE buffer (200 mM, pH 8.5).

   b) 50 mg/ml ferritin solution

The 102 mg/ml ferritin solution was first dialyzed (three times) against BICINE buffer (200 mM, pH 8.5), and then diluted with BICINE buffer (200 mM, pH 8.5) in order to obtain a 50 mg/ml ferritin solution.

19.2 Formation of ferritin-containing vesicles

Oleic acid vesicles were formed in the presence of either 25 mg/ml or 50 mg/ml ferritin in BICINE buffer (200 mM, pH 8.5). 0.5 to 0.6 ml vesicle suspension were prepared. Three slightly different methods were used.

Method I: stirring overnight

Sodium oleate was transferred into a 1.4 ml eppendorf tube and the ferritin solution was added. The mixture was then stirred at RT overnight to give a turbid suspension.

Method II: stirring overnight followed by freeze and thaw treatment

Sodium oleate was transferred into a 1.4 ml eppendorf tube and the ferritin solution was added. The mixture was stirred at RT overnight to give a turbid suspension. The suspension was then frozen in liquid nitrogen (3 min) and subsequently thawed in lukewarm water (30°C). Between each FAT cycle
the suspension was briefly vortexed. Ten FAT cycles were performed (if not otherwise stated).

**Method III: film formation followed by freeze and thaw treatment**

Sodium oleate was transferred into a 10 ml graduated reagent flask and MeOH (2 ml) was added. MeOH was removed with a rotary evaporator under reduced pressure ($T_{water\ bath} < 40°C$). The thus obtained lipid film was further dried under high vacuum overnight. The ferritin solution was then added to the flask and the dispersion was vortexed until complete dissolution of the film. The vesicular suspension was then treated by FAT as described above in *Method II*.

The oleic acid concentration of the vesicular suspension prior to extrusion was 80 mM unless otherwise stated. Vesicles were sized down using a liposofast device (see 15.5). Ca. 1 to 2 hrs after the last extrusion through 100 nm pore size filters, the vesicular suspension (0.38 - 0.6 ml) was loaded on a Sepharose 4B column (see 15.6) previously equilibrated with BICINE buffer (200 mM, pH 8.5) to remove non-entrapped ferritin molecules.

**19.3 Analysis of the fractions**

The concentration of oleic acid and of ferritin was determined by FTIR and by UV-Vis spectroscopy, respectively.

**19.3.1 Oleic acid/oleate determination**

The oleic acid concentration was determined through the intensity of the C-O (st) band at 1715 cm$^{-1}$ by FTIR spectroscopy. A calibration curve of oleic acid in isooctane was first established (Fig. 19.1).
The samples were prepared as follows: 50 μl of the aqueous suspension was taken, then HCl 1 M (100 μl) and isooctane (200 μl) were added. The mixtures were subsequently vortexed for 1 min and stored at RT for at least 5 hrs. The isooctane phase containing the extracted oleic acid was then analyzed by FTIR. The extraction of oleic acid according to this procedure is quantitative (99%) (Wick, 1996).

![Graph](attachment:image.png)

**Fig. 19.1** Calibration curve of oleic acid in isooctane at 1715 cm⁻¹ using FTIR.
19.3.2 Ferritin determination

The concentration of ferritin was determined by measuring its absorbance either at 280 nm or at 440 nm by UV-Vis spectrophotometry.

![Graph showing calibration curves for ferritin at 280 nm and 440 nm.](image)

**Fig. 19.2** Examples of two calibration curves of ferritin at 280 nm (borate buffer (0.1 M, pH 8.5): cholate (80 mM), 1:1) and at 440 nm (BICINE buffer (200 mM): cholate (80 mM), 1:1).

The ferritin concentration of the ferritin-containing POPC liposomes was determined in the same way. After destroying the vesicles with 40 mM cholate, the fractions obtained from the gel permeation chromatography were analyzed. A calibration curve of ferritin was established at 280 nm and 440 nm under the same experimental conditions (Fig. 19.2).
20. Stability of oleic acid vesicles checked by ffEM

A suspension (1 ml) of oleic acid vesicles was prepared according to method II (see 19.2, except that no ferritin was used during vesicle formation and that the oleic acid concentration was 7.25 mM) in BICINE buffer (200 mM, pH 8.5). The suspension was treated by FAT five times and the vesicles were then sized down to 100 nm using a liposofast device (see 15.5). The thus obtained suspension was stirred at RT for four days. Samples were withdrawn and frozen for ffEM examinations (see 15.2). One sample was frozen 5 hrs (day 0), another one 24 hrs (day 1), 49 hrs (day 2), 72 hrs (day 3) and 96 hrs (day 4) after the last extrusion cycle.

21. Hydrolysis of oleic anhydride

21.1 In the presence of ferritin-containing oleic acid vesicles

The ferritin-containing oleic acid vesicles were prepared according to method III described in 19.2. The ferritin-containing vesicles used in the hydrolysis experiment I were prepared in the presence of 25 mg/ml ferritin whereas the vesicles used in the hydrolysis experiment II were prepared in the presence of 50 mg/ml ferritin. Free ferritin molecules were removed from the suspension by gel permeation chromatography (see 15.6). The fractions that contained the highest concentration of oleic acid were used in the hydrolysis of oleic anhydride.

The ferritin-containing oleic acid vesicle suspension was transferred to a flat-bottom flask (Ø 13 mm) containing a magnetic stirrer (10 x 5 mm). Oleic anhydride was laid on top of the vesicular suspension and the reaction mixture was slowly stirred at RT for 4 days. The oleic acid concentration of the initial and of the final suspension was determined by FTIR as described in 19.3.1.
21.2 In the absence of oleic acid vesicles

Oleic anhydride (24 μl, 0.04 mmol) was laid on top of 1 ml of BICINE buffer (200 mM, pH 8.5) containing ferritin (2 mg/ml). The hydrolysis reaction mixture was stirred at RT for 4.5 days. Non-entrapped ferritin molecules were removed by gel permeation chromatography using a Sepharose 4B column (see 15.6). Fractions 20 to 22 were examined by cryo-TEM.

21.3 In the presence of empty oleic acid vesicles and externally added ferritin

2.5 μl of ferritin stock solution (102 mg/ml) followed by oleic anhydride (1.8 μl, 0.003 mmol) were added to a suspension of 100 nm-oleic acid vesicles ([oleic acid] = 6 mM, 0.25 ml) in BICINE buffer (200 mM, pH 8.5). The suspension was stirred at RT for 5.5 days. Non-entrapped ferritin molecules were removed using a “spin column” (see 15.6).

21.4 Stirring of empty oleic acid vesicles in the presence of free ferritin

2.5 μl of ferritin stock solution (102 mg/ml) was added to a suspension of 100 nm-oleic acid vesicles ([oleic acid] = 6 mM, 0.25 ml) in BICINE buffer (200 mM, pH 8.5). The mixture was then stirred at RT for 5.5 days. Non-entrapped ferritin molecules were removed using a “spin column” (see 15.6).

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33. The oleic acid vesicles were prepared according to method II except that no ferritin was used during vesicle formation and that the oleic acid concentration was 6 mM.
22. Formation of mixed POPC/oleic acid/oleate vesicles

22.1 Preparation of POPC ferritin-containing liposomes (New, 1990; Szoka & Papahadjopoulos, 1978)

A 50 mg/ml ferritin solution was prepared as described in paragraph 19.2, except that borate buffer (0.1 M, pH 8.5) was used instead of BICINE buffer. POPC (23 mg) was dissolved in Et₂O (5 ml) in a 50 ml round-bottom flask. 1 ml of the 50 mg/ml ferritin solution in borate buffer (0.1 M, pH 8.5) was added to the POPC solution with the help of a syringe. The mixture was sonicated for 5 min at 20°C using a sonication bath to yield an homogeneous red suspension. Et₂O was removed by evaporation using a rotary evaporator (p = 400 mbar, T=25°C) until a viscous (gel-like) suspension was obtained. The gel-like mixture was then vortexed until a liquid-like suspension was obtained. An extra 1 ml of borate buffer was added and the suspension was evaporated again using a rotary evaporator at 100 mbar for 10 min and finally at 50 mbar for a further 5 min. The thus obtained liposomes were sized down to 100 nm using an extrusion device from Lipex Biomembranes (see 15.5).

Non-entrapped ferritin molecules were removed by gel permeation chromatography using a Sepharose 4B column equilibrated with borate buffer (see 15.6). Ferritin concentration was quantified spectrophotometrically (see 19.3.2) while the Stewart assay was used to determine POPC concentration (see 22.2) (Fig. 22.1).
Fig. 22.1 Separation of non-entrapped ferritin from ferritin-containing POPC liposomes using a Sepharose 4B column (length 50 cm, Ø 1.2 cm). For liposome preparation and fraction analysis, see text.

Fraction 19 was examined by cryo-TEM and by DLS and subsequently used to study the effects of oleate addition to POPC liposomes (see 22.3).

22.2 POPC determination (Stewart, 1980)

The Stewart assay can be used to determine phospholipid concentrations in the range of 0-50μg lipid/ml. Ammonium ferrothiocyanate (Stewart solution) was prepared by dissolving ferric chloride hexahydrate (27.03 g) and ammonium thiocyanate (30.4 mg) in water (1000 ml). Samples were prepared as follows: 2 ml CHCl₃ and 2 ml Stewart solution were added to the aqueous sample containing POPC. The biphasic system was vortexed for 1 min and let to stand at RT for 2 hrs. A clear phase separation was obtained. The lower CHCl₃ phase was then removed with a Pasteur pipette and the OD was recorded at λ = 488 nm using a quartz cell of 1 cm path length. A calibration curve was established in parallel to each
determination and further used for the quantification of POPC in the samples.

22.3 Addition of sodium oleate to preformed POPC liposomes (Lonchin et al., 1999)

The procedure described by Lonchin et al. (1999) was followed. An aqueous micellar solution of oleate (25 mM) was prepared by dissolving Na-oleate in H$_2$O. This oleate solution (0.2 ml) was then added to the ferritin-containing POPC liposomes (0.8 ml, [POPC] = 0.25 mM prepared by dilution of fraction 19 with borate buffer). The reaction progress was followed by turbidity measurement ($\lambda = 400$ nm) (Fig. 22.2) over the first 4 hrs. The POPC liposomes and mixed POPC/oleic acid/oleate vesicles were then examined by cryo-TEM and DLS (ca. 5 hrs after Na-oleate addition).

![Graph showing change in turbidity](image)

**Fig. 22.2** Change in turbidity of the ferritin-containing POPC liposome suspension upon Na-oleate addition over the first 4 hrs ($\lambda = 400$ nm).
23. Cryo-SEM investigations

Oleic acid vesicle suspension
Oleic acid vesicles were prepared according to Method II (see 19.2) except that no ferritin was used during vesicle formation. The oleic acid concentration was 20 mM.

Latex particles
The stock solution of latex particles had a concentration of 8.3% by volume and contained spheres having a diameter of 110 ± 10 nm. Two suspensions were prepared by dilution of the stock solution 1 to 1 and 1 to 4 (v:v) with bidistilled water.

Both suspensions were prepared for cryo-SEM analysis as described under 15.3.
E. REFERENCES


Curriculum Vitae

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