THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

METHODS DEVELOPMENT FOR BIOMEMBRANE MODEL SYSTEMS

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Abstract

The concept of a model system is fundamental to understand the role of membranes in biology. The aim of the work described herein was to develop different procedures for biomembrane model systems, using giant vesicles as a base platform. The initial focus was on investigations of lipid membrane properties with particular emphasis on nanotube systems. The outcome of this study resulted in a model system displaying transient tubulation in nanoparticle-containing surface-supported flat giant unilamellar vesicles. During this project, common problems and restrictions associated with today's conventional vesicle preparation methods were experienced. To overcome these restrictions, we have developed a set of on-demand methods for the rapid generation of giant vesicles. Our first preparation method results in the formation of individual giant unilamellar vesicles (GUVs) connected to a multilamellar vesicle, through use of localized heating. Building upon this work, we developed another protocol for the formation of giant vesicles from spin-coated lipid films. This method enables GUVs formation from different types of lipids with the ability to encapsulate molecules. We further demonstrated the applicability of this technique to another type of material, in particular non-ionic surfactants, leading to the creation of giant niosomes.

Keywords: membrane model systems, giant vesicles, liposomes, niosomes, amphiphile films, heating.

LIST OF PUBLICATIONS

I. Calcium-ion-controlled nanoparticle-induced tubulation in supported flat phospholipid vesicles
Irep Gözen, Céline Billerit, Paul Dommersnes, Aldo Jesorka and Owe Orwar
Soft Matter (2011), 7, 9706-9713.

II. Heat-induced formation of single giant unilamellar vesicles
Céline Billerit, Ilona Wegrzyn, Gavin D. M. Jeffries, Owe Orwar and Aldo Jesorka
Soft Matter (2011), 7, *9751-9757.*

III. Formation of giant unilamellar vesicles from spin-coated lipid films by localized IR heating
Céline Billerit, Gavin D. M. Jeffries, Owe Orwar and Aldo Jesorka
Soft Matter (2012), Communication.

IV. Formation of giant niosomes from spin-coated amphiphile films
Céline Billerit, Gavin D. M. Jeffries, and Aldo Jesorka
Manuscript (2013).

CONTRIBUTION REPORT

Paper I
I contributed partially, with the experimental design, performing experiments and discussion of results.

Paper II
I contributed with the experimental design, performing experiments and discussion of results. I wrote the paper.

Paper III
I contributed with the experimental design, performing experiments and discussion of results. I wrote the paper.

Paper IV
I contributed with the experimental design, performing experiments and discussion of results. I wrote the paper.

CONTENTS

1. INTRODUCTION

For all known forms of life, the cell is identified as the basic unit. Understanding the structure, functions, and the dynamics of cells at the molecular level, is generally believed to be a prerequisite for an understanding of the cells' key features, differentiating the living from the non-living[1,2]. Following the lines of thought of the American physicist Richard P. Feynman, on the relation between the understanding of something and its formulation ''what I cannot create, I do not understand'', thus, a real understanding of living cells would imply that one is able to recreate them. In other words, if one understands how living cells are built, how they function, and how the various internal components are dynamically interlinked, one should in principle be able to synthesize a cell *ab initio,* from base components. This seems to be the ultimate goal of synthetic biology[3,4].

One of the cell's key structures is the cell membrane, which is made mainly of phospholipids, cholesterol and glycolipids, arranged as a bilayer with embedded proteins. An important function of cell membranes is to define a boundary, whether between or within cells and organelles. Many cellular processes depend upon the membrane's ability to separate different regions while still allowing communication and precisely regulated transport. All cell-to-cell communications, from two-cell networks up to entire organs on whole organisms and also between organisms, are mediated by these interactions. The physical properties and many functions of the cell membrane are related to its composition[5–7]. It is becoming increasingly clear that many membrane phenomena, attributed to active control, may in fact arise passively as a result of the membranes physical properties. It is rather difficult to study membrane physics using living cells as they are complex entities, where active and passive phenomena are inextricably connected. Studying a specific physical process and quantifying it in such a crowed and dynamic environment is extremely challenging. One way around this, is to

first study the membrane in isolation, starting from a minimal single component such as a lipid vessel. The strength of such a model system is based on its simplicity, which is a prerequisite for well-controlled experimentation.

Models for membrane systems take many different forms[8], monolayers[9,10], bilayers, either planar, supported[11,12] or vesicles[13–16] of sizes ranging from tens of nanometers to several micrometers. Among these, giant unilamellar vesicles (GUVs) have sizes close to typical cells. Flexible and of finite volume and area, they are particularly suitable for the use not only as model membranes but also as cell models[17]. Challenging applications of giant vesicles include gene expressions within the vesicle, leading to the ultimate goal of constructing a dynamic artificial cell-like system[18], that is endowed with all those essential features of living cells. Although this goal still seems to be a distant hope, progress in this and other fields of giant vesicles research strongly depend on the availability of reliable methods for the reproducible preparation of giant vesicles.

In the work described in this thesis, different procedures for biomembrane model systems have been developed, using giant vesicles as a base platform. The initial focus was on investigations of lipid membrane properties with particular emphasis on nanotube systems. Detailed understanding of the properties, formation conditions, and dynamics of nanotubes is still insufficiently understood, which has placed these nanostructures in the focus of recent investigations[19,20]. The outcome of this study resulted in a model system displaying transient tubulation in nanoparticle-containing surface-supported flat giant unilamellar vesicles (Paper I).
During this project, common problems and restrictions associated with today's conventional vesicle preparation methods were experienced. This inspired a search for more efficient vesicle preparation protocols. Several methods exist for the preparation of giant vesicles, the most common one being an electroformation procedure[21]. Although various techniques exist, all of the currently known procedures are limited in one way or

another, for example, in terms of lipid composition, ionic strength conditions, or the requirement of dedicated equipment and microfabrication. To overcome these restrictions, we have developed a set of on-demand methods for the rapid generation of giant vesicles.

Our first preparation method results in the formation of individual giant unilamellar vesicles connected to a multilamellar vesicle through use of localized heating (Paper II). These vesicles are suitable for the construction of lipid nanotube-vesicle networks, which are currently the smallest and most structurally flexible nanotechnological devices known for performing controlled synthetic chemistry. A distinct advantage of this method, is the ability to create single giant vesicles, from natural and artificial lipid mixtures, but also from single lipids, at well-defined locations (*e.g.* on a microfabricated substrate).

Building upon this work, we investigated the possibility of using directed laser heating as a universal formation technique for giant vesicles from thin lipid films (Paper III). This technique enabled us to produce giant vesicles with encapsulated bioactive molecules, and allows the use of many different types of lipids, as well as various ionic strength conditions. A great advantage of this method is the convenient preparation and dry storage of lipid film surfaces for subsequent use.

The generality of these techniques has been demonstrated to be applicable also for other materials, in particular non-ionic surfactants (Paper IV). Niosomes, which are vesicles prepared from non-ionic surfactants and cholesterol, are promising structures as drug carriers as they offset the disadvantages associated with liposomes, such as chemical instability, special storage and high cost. The outcome of this study resulted in an easily applied protocol for the generation of giant unilamellar niosomes, from different types of non-ionic surfactants and with the ability to encapsulate lipophilic as well as hydrophilic compounds.

The work described herein aimed to contribute to the development of reliable methods for the preparation of giant vesicles and for expansion of accessibility of giant vesicles as membrane model system.

2. AMPHIPHILES

The earliest forms of life likely required membranes as boundaries. Phospholipids are the primary components of modern cell membranes, but it is improbable that such complex molecules were part of the prebiotic Earth[22]. Instead, simpler amphiphilic molecules probably served as precursors, evolving over time chemically into the varied and complex form of phospholipids.
Amphiphiles are molecules possessing both hydrophilic and lipophilic parts. They have a tendency to gather at interfaces, such as water-air or water-oil surfaces, and are often referred to as surface-active molecules. Amphiphilic molecules (such as lipids and other surfactants) spontaneously self-organize into a variety of structures. By means of self-assembly, the individual amphiphilic molecule adopt an ordered arrangement, with different physical properties that the individual amphiphilic molecule. However, controlling the self-assembly of amphiphiles, with the goal to form well-defined structures in a predictable and reproducible way, remains a major challenge.

2.1 Thermodynamics of self-assembly

Amphiphilic molecules tend to self-assemble in a way that allows them to expose only their hydrophilic part to water, shielding the hydrophobic part. This property of immiscibility of water and hydrophobic molecules is called the hydrophobic effect (**Figure 2.1**). This leads to the formation of microscopic aggregates (like micelles, lamellae, vesicles, etc.) which do not form a three dimensional macroscopic phase.

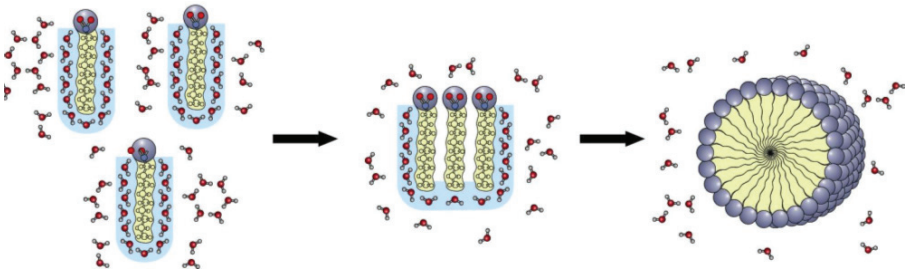

Fig. 2.1: Schematic illustration of the hydrophobic effect. Amphiphilic molecules line up in order to decrease the hydrophobic area per molecule that is exposed to water in order to decrease the free energy of the system. Above the CMC (critical micelle concentration), spherical micelles are formed where water is entirely excluded from the hydrophobic tails.

If a molecule is present in solution in aggregates of different sizes N ($\boldsymbol{N}$ representing the number of molecules in the aggregate), then the condition for thermodynamic equilibrium of the aggregates is the chemical potential of the molecule in aggregates of different size is the same[23,24]:

$$\mu(N) = \mu_N^o + k_B T/N + \ln(X_N/N) \qquad \textbf{Eq. (1)}$$

where $\boldsymbol{\mu}$ is the chemical potential of the solute molecule in the aggregate of size N, $\boldsymbol{\mu_N^o}$ is the mean interaction free energy of a molecule in an aggregate of size N, $\boldsymbol{k_B}$ the Boltzmann constant, $\boldsymbol{T}$ the temperature and $\boldsymbol{X_N}$ is the volume fraction of solute present in an aggregate of size N.

In order to study the thermodynamics of formation of these microscopic aggregates, we need to define a law which tells us how the interaction free energy, μ_N^o, varies with the size N of the aggregate. The simplest way to do this is to assume that this interaction free energy depends on the dimensionality factor $\boldsymbol{d}$ of the aggregate formed.
d is 1 for linear or one-dimensional aggregates (rods, cylinders), is 2 for planar or two-dimensional aggregates (discs, sheets) and

is 3 for three-dimensional aggregates (spheres, droplets) (**Figure 2.2**). Under this assumption, one can write[25]:

$$\mu_N^o = \mu_\infty^o + \alpha k_B T / N^{1/d} \qquad \textbf{Eq. (2)}$$

where $\boldsymbol{\mu_\infty^o}$ is the interaction energy in the bulk of the aggregate and $\boldsymbol{\alpha}$ is a constant which depends the amphiphiles-solvent interaction at the border of the aggregate.

This equation allows us to write the interaction free energy as a sum of two contributions: the bulk, which is independent from the aggregate size, and interfacial, which depends on the number interfacial amphiphiles per unit of size *N*. For example in the case of a linear aggregate (*d* is 1), the number of interfacial molecules is 2, regardless of *N*, thus the number of interfacial molecules per unit of volumes scales as $1/N$, while, in the case of disk-like aggregate (*d* is 2), the number of molecules *N* is proportional to the disk area (πR^2) and number of interfacial molecules is proportional to the perimeter ($2\pi R$) and hence the number of interfacial molecules per unit of volume scales as $1/N^{1/2}$.

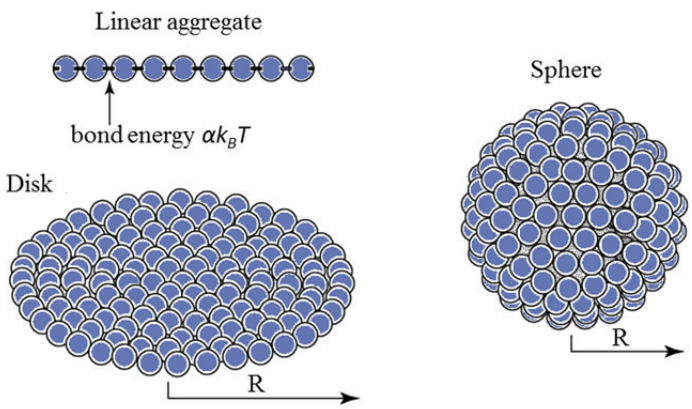

Fig. 2.2: One-, two-, and three-dimensional structures by the association of identical monomer units in solution.

The value of the interaction parameter α and dimensionality factor d in **Equation 2** are constant only for aggregates composed of fairly simple molecules that self-assemble into simple geometric shapes such spheres, discs, or rods. More complex amphiphilic molecules can have a size-dependent α and/or assemble into more complex shape as vesicles (*cf.* 2.3.2 Closed vesicles).

2.2 The concept of molecular packing parameter

The major forces that govern the self-assembly of amphiphiles into well-defined structures are derived from the delicate force balances at the interface region and at the hydrophobic core.
The idea of two opposite forces, introduced by Tanford[26], refers to (i) the attractive force caused by hydrophobic attraction of the hydrocarbon chain units in the hydrocarbon-water interface and (ii) the repulsive force between the neighboring head groups due to the hydrophilic, ionic, or steric repulsion (**Figure 2.3**).

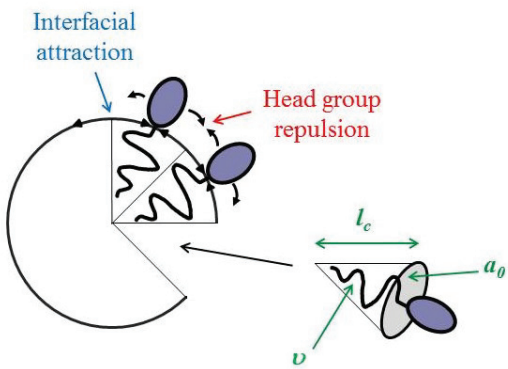

Fig. 2.3: The two opposite forces inside a micelle. Repulsive head group forces and attractive hydrophobic interfacial forces determine the optimum area $a_{o.}$ The chain volume v and chain length l_c set limits on how the fluid chains can pack together, on average, inside an aggregate.

Together, the sum of attractive and repulsive forces can be expressed as the simple relation:

$$\mu_N^o = \gamma a + K/a \qquad \textbf{Eq. (3)}$$

where $\boldsymbol{\gamma}$ is the interfacial free energy, $\boldsymbol{a}$ the head group area and $\boldsymbol{K}$ a constant.

The minimum energy can therefore be found by making, $\mathrm{d}\mu_N^o / \mathrm{d}a = 0$, which is shown in **Figure 2.4**, resulting in an equilibrium area $\boldsymbol{a_o} = \sqrt{K/\gamma}$.

By using the area a_o obtained from **Equation 3**, the packing parameter $\boldsymbol{p}$ can be estimated:

$$p = v/l_c a_o \qquad \textbf{Eq. (4)}$$

where $\boldsymbol{v}$ is the tail volume, and $\boldsymbol{l_c}$ the critical chain length referring to the effective length of the hydrocarbon chain in the liquid state.

The influence of geometrical parameters of amphiphiles on the shapes of associates is represented in **Figure 2.5**.

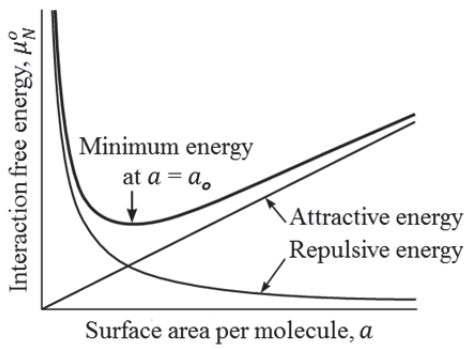

Fig. 2.4: A plot of the interaction free energy μ_N^o as function of head group surface area $\boldsymbol{a}$. The attractive and repulsive energies are combined into the total free energy with a minimum in energy located at a_o, which is the optimal head group area.

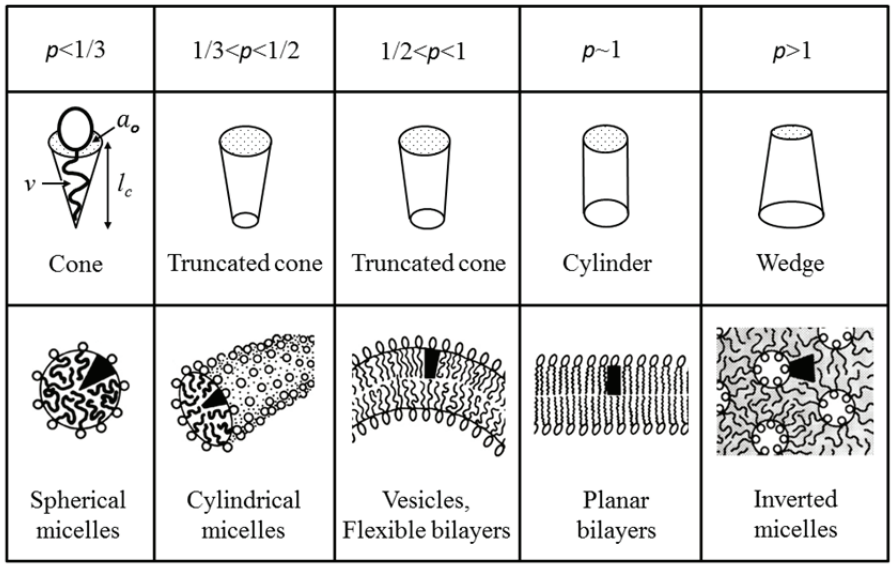

Fig. 2.5: Packing parameters and the related shapes of associated aggregates.

2.3 Bilayers and closed vesicles

Bilayers formed by amphiphilic molecules occupy a central position in the study of self-assembly for two reasons. Firstly, they represent the midpoint between normal and inverted structures, and under suitable conditions, the majority of amphiphiles aggregate into bilayers. Second, the bilayer is the basic building unit of the biological membrane. A multitude of processes in the living cell rely on the unique and versatile properties of the membranes.

Amphiphiles that form bilayers, are those that cannot pack into micellar structures due to their small head group area a_o or because their hydrocarbon chains are too bulky to fit into small aggregates, while maintaining the surface area at its optimal value. Different amphiphiles can form bilayers (*e.g.* surfactants, block copolymers), the most common being lipids. In order to gain more understanding of the behavior of biological

membranes in cells and model membranes it is necessary to acquire some basic understanding of lipid bilayer mechanics.

2.3.1 Lipid bilayer mechanics

Several theoretical approaches to the mechanics of lipid or surfactant bilayers have been described, where the bilayer is usually depicted or approximated by a thin elastic shell[27,28]. Upon application of a force, this thin film undergoes shape changes that affect the elastic energy of the system. Stretching, shearing and bending are the three main types of deformations that can be applied to an elastic shell[29].

Stretching

The first type of deformation is an isotropic stretch. If the area element of an amphiphile bilayer is dilated by in-plane stretching, the bilayer will expand elastically (**Figure 2.6**). For such stretching deformation, the cost of surface free energy per unit area is:

$$e_{stretch} = (k_s/2A_0)(A - A_0)^2 \qquad \textbf{Eq. (5)}$$

where k_s is the elastic stretching modulus, A_0 is the surface area before deformation, A is the area after deformation.

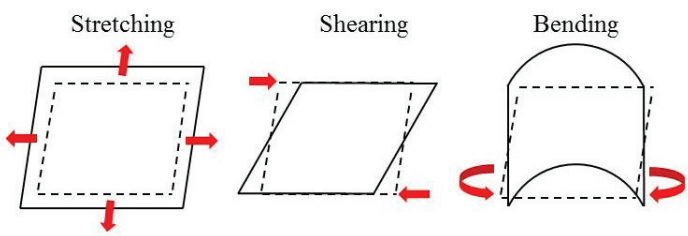

Fig. 2.6: Schematic illustration of stretching, shearing and bending.

Shearing

The second type of deformation is the in-plane extension of a bilayer at constant surface area (**Figure 2.6**). This type of deformation occurs only in bilayers that are frozen. In the fluid

state, amphiphile bilayers have no resistance to shearing due to the freedom of lateral motion within the monolayers.
The surface shear energy can be described by:

$$e_{shear} = \mu_s \beta \qquad \textbf{Eq. (6)}$$

where $\boldsymbol{\mu_s}$ is the shear modulus of the bilayer and $\boldsymbol{\beta}$ the degree of deformation.

Bending
The third elastic relationship represents the curvature elasticity of the bilayer (**Figure 2.6**). Due to the extreme thinness of the bilayer, bending rigidity offers little opposition to deformation for vesicles that are cell-sized. In the case of nanoscopic membrane structures, the situation is fundamentally different due to high curvature and therefore the bending can represent the dominating contribution to the surface free energy. The curvature of a surface in space can always be described by the principal curvatures $\boldsymbol{C_1}$ and $\boldsymbol{C_2}$ (**Figure 2.7**) and $\boldsymbol{C_0}$, the spontaneous curvature.

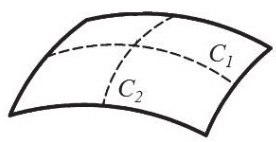

Fig. 2.7: Schematic illustration of the principal curvatures C_1 and C_2.

The widely used approach of Helfrich[28] is to describe the curvature energy per unit area, $\boldsymbol{e_{bend}}$ of a thin elastic shell as quadratic function of the principal curvatures:

$$e_{bend} = \left[\kappa/2 \ (C_1+C_2-C_0)^2\right] + \overline{\kappa} C_1 C_2 \qquad \textbf{Eq. (7)}$$

where $\boldsymbol{\kappa}$ is the bending modulus and $\overline{\boldsymbol{\kappa}}$ the Gaussian modulus.

2.3.2 Closed vesicles

By using the molecular packing parameter described in the previous section, for ½< p <1, vesicles and lamellae are expected to form. While this concept is convenient to explain an observed amphiphile aggregation behavior, it has little predictive power. For example, the equilibrium area a_o is not a simple geometric area, but an equilibrium parameter derived from thermodynamic considerations. Consequently, a_o can assume widely different values depending on the environmental conditions such the temperature, salt concentrations, etc[30]. Hence, it becomes a rather frustrating exercise to predict the packing behavior of an amphiphile in a solution containing salts and additives at a certain temperature and concentration, if the relative weight of these parameters is unknown. In addition, the influence of the amphiphile tail on the packing behavior is neglected in the original concept of molecular packing parameter[30]. A steric hindrance interaction parameter also needs to be included, making a prediction of the packing behavior even more ambiguous from a practical point of view[31].

Another approach to gain better understanding of amphiphile self-assembly, takes into account the instability of flat membranes and the energy required to form closed vesicles[32]. In this case, the formation of vesicles can be viewed as a two-step self-assembly process, in which amphiphiles first form a bilayer, which then, in a second step, closes to form a vesicle.

Imagine that we have a bilayer disk. Amphiphilic molecules of this disk will arrange themselves in a way such that the hydrophobic parts of the amphiphiles will avoid water and form an arrangement to curve at the edge (**Figure 2.8**). This membrane edge is characterized by an edge tension energy E_{edge}:

$$E_{edge} = 2\pi\gamma r \qquad \textbf{Eq. (8)}$$

where r is the radius of the open bilayer disk and γ the edge tension. The bending energy of any materials is:

$$E_{bend} = 4\pi(2\kappa + \overline{\kappa}) \quad \textbf{Eq. (9)}$$

where κ is the bending modulus and $\overline{\kappa}$ the Gaussian modulus. These two moduli have the dimension of energy per unit volume and are typically on the same order of magnitude to k_BT. This explains why amphiphile membranes in solution are not rigid but subjected to thermal fluctuations[33].

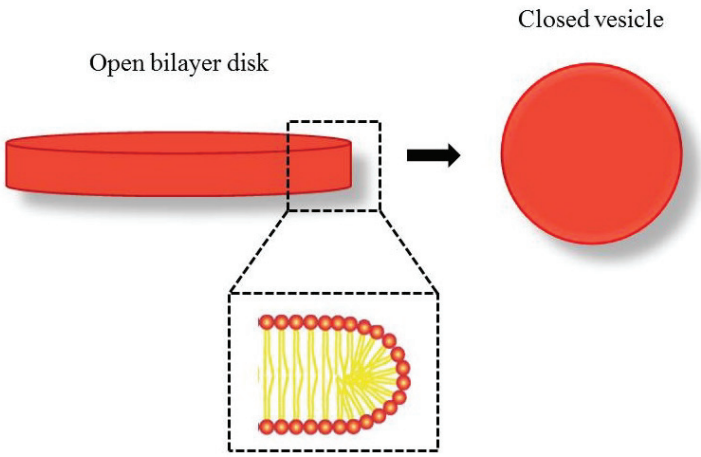

Fig. 2.8: Formation of a closed vesicle from an open bilayer disk. Left: schematic drawings of an open bilayer disk with an enlargement showing amphiphiles curve at the edge. Right: Schematic drawing of a closed vesicle.

A closed vesicle forms when the bilayer disk is so large, that the energy loss due to the edge tension is greater than the bending energy cost of curving the disk bilayer to a vesicle (**Figure 2.8**). The ratio E_{bend}/E_{edge} will determine the critical vesicle radius r_c:

$$r_c = (4\kappa + 2\overline{\kappa})/\gamma \quad \textbf{Eq. (10)}$$

For fluid lipid bilayers, typical experimental values are; 10pN for γ[34,35], 10-20k_BT for κ[36] and - 0.9κ for $\overline{\kappa}$[37,38]. That implies that r_c

must be larger than $20k_BT/10$pN (~nm). This means that practically all free membranes exist in the form of vesicles.

2.3.3 Vesicle adhesion

In many applications and in this work, vesicles are brought into contact with a rigid substrate. Vesicle adhesion can occur when the vesicle experiences various forces, such as electrostatic, Van der Waals and hydration forces, from the surface. Usually these forces are collected into a contact potential, W, for the surface. When the vesicle adheres to the surface, it will have a contact area, A^*, with the surface thus gaining the adhesion energy:

$$E_{ad} = -WA^* \qquad \textbf{Eq. (11)}$$

For weak adhesion situations, the gain in adhesion energy is balanced by the cost in curvature energy.

3. LIPOSOMES

Lipids are amphiphilic molecules which in water may self-assemble into various kinds of aggregates such as micelles or vesicles. Vesicles made of lipid bilayers are called liposomes. They consist of one or more lipid bilayers surrounding an "aqueous" compartment, where the hydrophilic head groups are oriented towards the interior and exterior aqueous phases. Liposomes are used as model membranes, but also as cell models. They are well recognized as promising candidates for drug delivery systems and in this regard more tailor-made lipid formulations are being investigated for pharmaceutical and cosmetic applications.

3.1 Lipids and lipid bilayers

The most common of the biological membrane lipids are phospholipids. Phospholipids consist of a hydrophilic head group, which is linked to two alkyl chains though a phosphate group. The alkyl chains, or hydrocarbon tails, vary in both length and degree of saturation, which together dictates the fundamental properties of the membrane, such as permeability and fluidity[39]. The head group holds the charge of the molecule which also affects the properties and functionality of the membrane. The dominant phospholipids in the plasma membrane of mammalian cells are: phosphatidylcholine, phosphatidylserine, phosphatidylinositol phosphatidylethanolamine (**Figure 3.1**) and sphingomyelin. Due to their cylindrical shape and amphiphilic nature, phospholipids spontaneously form bilayers. Phospholipids residing in a bilayer membrane can move freely in the lateral direction, and therefore membranes are often referred to as 2-dimensional fluids. A lipid molecule can also move from one monolayer to the other (flip-flop), but this occurs on a very slow timescale, since the polar head group has to transverse through the hydrophobic core of the bilayer. The degree of membrane fluidity is dependent on both the temperature as well as the molecular structure of the

hydrocarbon tails. For example, within each bilayer, the lipid molecules can exhibit a fluid phase at high temperature, in which the nonpolar chains of the lipids are disordered, and one or several gel-like phases at low temperature, in which the chains are rigid. The main phase transition temperature T_m describes the temperature at which lipids undergo a transition from the gel to liquid phase. All pure lipids have a characteristic T_m. This aspect of lipid bilayers will be further discussed in section 3.3.

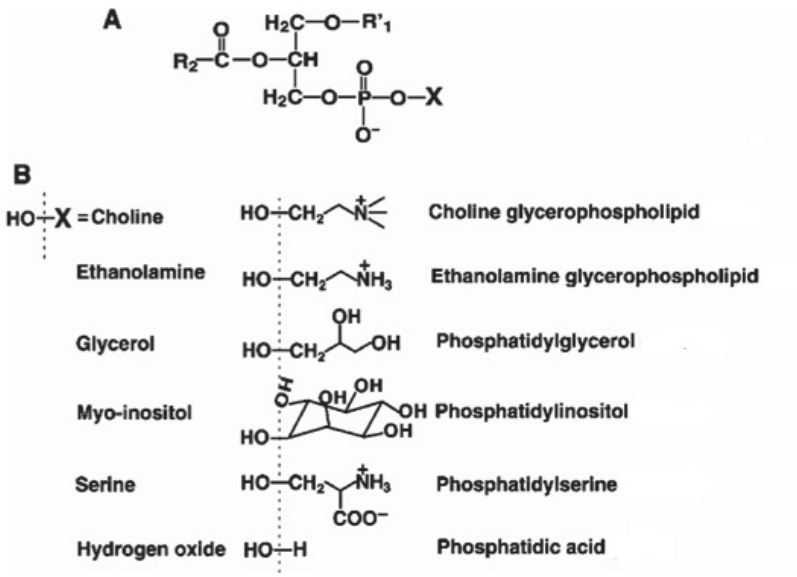

Fig. 3.1: Structure of some phosphoglycerides.

3.2 Classification of liposomes

Liposomes are, from a morphological perspective, frequently classified by their size and number of membrane bilayers (lamellae) (**Figure 3.2**).

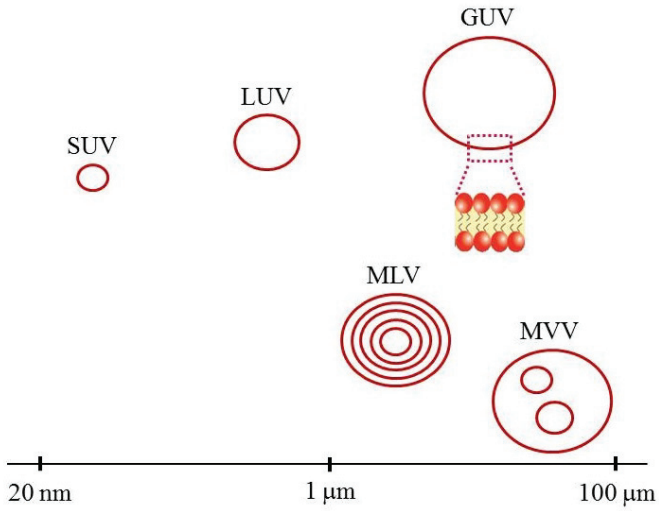

Fig. 3.2: Different types of liposomes according to their size and number of membranes bilayers. SUV = Small Unilamellar Vesicle, LUV = Large Unilamellar Vesicle, GUV = Giant Unilamellar Vesicle, MLV = Multilamellar Vesicle (MLV) and MVV = Multivesicular Vesicle.

Unilamellar vesicles are of special interest in research, mostly due to their well-characterized membranes properties. They are divided into three size catagories: small, large and giant. Giant unilamellar vesicles (GUVs) are useful models for the study of biological cell membranes, owing to their similar size and composition, which can be directly observed and micromanipulated using light microscopy. GUVs allow real time monitoring of morphological changes of individual vesicles and also monitoring of surface reaction and interactions, an area of importance in our present studies.

Liposomes containing several bilayers are denoted as multilamellar vesicles (MLVs). Few details are currently known about the morphology of MLVs, but it seems that internal layers are connected to each other by nanochannels, which can be perceived as handle-like pores, semi-pores or micelle like defects[40,41]. Multilamellar vesicles often show physical characteristics and behavior that are very different from unilamellar species, and are commonly used for industrial

applications such as drug delivery. In this thesis, MLVs have been used to form double-bilayer-patches (Paper I), and to create individual GUVs (Paper II). They have been also employed as lipid reservoirs in the process of nanotube-vesicle network fabrication (Paper II and section 3.6 Formation of linear nanotube-vesicle networks).

3.3 Preparation of giant vesicles

To obtain liposome preparations, crude or purified plant or animal lipid extracts are often used. Plant and cyanobacteria lipid extracts contain mostly phosphoglycerides, whereas extracts from erythrocytes or liver cells are mainly composed of phosphoglycerides, sphingolipids and sterols. Unpurified extracts, contain other hydrophobic molecules, such as membrane protein material as contaminants and are therefore of limited use[42,43].

The formation of giant vesicles can be achieved by many existing protocols. Generally for multilamellar vesicles, the film hydration technique is used[44]. Lipid solution is initially dried, either by using an evaporator or by spray drying/lyophilization for larger scale preparations[45]. Alternatively, this step can be performed at very low pressure for a few hours in the presence of neutral desiccant. For subsequent multilamellar vesicle formation, the sample is mechanically agitated in the presence of a hydration medium such as buffer. MLVs in the micrometer-size range are spontaneously formed when the film is exposed to an excess volume of aqueous buffer. This type of multilamellar lipid suspension is usually the starting material for vesicles with more well-defined characteristics, such as number of lamellae and size. For example, small unilamellar vesicles are normally produced by sonicating a multilamellar lipid suspension.
In this thesis, a method known as dehydration/rehydration, originally developed by Criado and Keller[46] was used, with some modifications. First, as described previously, a suspension of small unilamellar vesicles is produced by sonication of a multilamellar lipid suspension. Then, 5 μl of this suspension is

placed on glass cover slip and dehydrated in a vacuum desiccator for about 10 minutes, until a lipid film is formed. During the dehydration step, buffer salt is trapped between the bilayers of lipid film. After the sample is dry, it is first rehydrated with 5-10 μl of buffer. After 5 minutes, it is further diluted by carefully transferring the sample to a chamber (typically a frame composed of PDMS on a treated glass coverslip) and filled with 2 mL of buffer. Due to the presence of buffer salts within the dehydrated film, an osmotic pressure gradient forces water between the individual bilayers, and the lamellae separate to form liposomes. This procedure is used to form GUVs, which are connected to a MLV. The latter vesicles provide lipid material that is needed for building nanotube-vesicle networks (see 3.6 Fabrication of linear nanotube-vesicle networks). A critical factor that affects the formation yield is the degree of bilayer separation, which is influenced by; temperature, lipid composition, and the ionic strength of the surrounding medium. Inclusion of negatively charged lipids is known to enhance separation of the lamellae. A relatively low-ionic-strength buffer (10-50 mM) and the absence of multivalent ions that interlink charged lipids are essential. The dehydration/rehydration technique is still the only method available to obtain GUVs connected to MLVs. The process is relatively fast and giant vesicles are produced with a high yield. But so far this method has been optimized only on soybean polar extract (SPE) and not on pure single lipids. SPE is an unpurified extract that contains 6.9 % of unknown and has therefore limited use. To overcome this limitation, a novel method to form GUVs from MLV by means of directed laser heating has been developed (Paper II).

Formation of single GUVs can be approached by two widely used techniques; one is based on spontaneous swelling of lipids (called gentle hydration), and the other on the effect of electric fields (called electroformation). A number of modified procedures for preparing GUVs has been reported[47], but a detailed review is outside our scope. Instead, we give a short summary and some typical features of the two protocols.

Traditionally, GUVs were made by simple hydration of lipids also called the gentle hydration technique. This technique was originally developed by Reeve and Dowben[48] in the late 1960's and involves the deposition of a thin lipid film with subsequent careful hydration in a moist nitrogen atmosphere. Vesicles are created after few days by self-closure of the bilayer in a process called budding, where the connections to the original lipid sheet, or tethers as they are called, are severed through convection or gentle agitation. It is important to note that the hydration has to be carried out in the liquid-disordered state of the bilayers, that is, at a temperature above the T_m of the lipid film. A recent atomic force microscopy (AFM) study reported on a correlation between physical state of lipid layers and ability to generate giant vesicles: hydration of smooth, liquid-crystalline state of the lipid film resulted in the successful formation of giant vesicles, while hydration of rough, gel state of lipid film did not yield giant vesicles[49,50]. This method is very simple but requires a few percent of negatively charged lipids in the mixture[51] and the yield of GUVs is variable.
Another preparation technique that has attracted much interest is the electroformation technique, whereby the lipid film is swelled in an electric alternative current (AC) field (**Figure 3.3**).

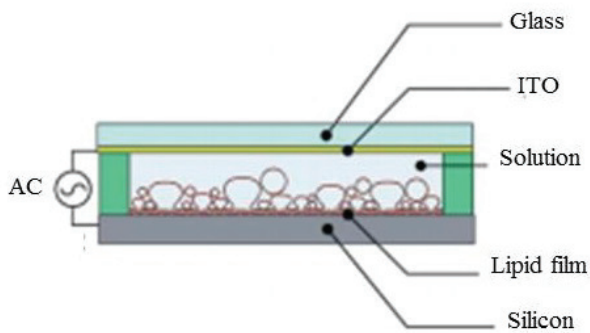

Fig. 3.3: Experimental set-up of electroformation technique of giant phospholipid vesicles on a silicon substrate[52]. Adapted with permission from ref. 52. Copyright 2008, American Chemical Society.

This protocol was originally developed by Angelova and Dimitriov[21] in 1986 and is based on the idea that the applied electrical field will induce fluctuated movements in the bilayer, thus generating an increased separation of the bilayers. The underlying mechanism for electroformation is thought to be mainly the electro-osmotic periodic movement of the water medium, which creates vibrations perpendicular to the electrode surface and pulls the lipid lamellae apart and induces after a few hours vesicle formation. In contrast to gentle hydration, the standard electroformation method has high reproducibility but does not work well with charged in the lipids mixture[53] and requires an aqueous phase of low ionic strength.

With some reported exceptions[54,55], the gentle hydration and the electroformation methods generally do not allow efficient encapsulation of large water-soluble molecules or charged compounds during the formation process, as the molecules to be encapsulated have to somehow move below the outermost layer of the deposited film; this is difficult due to slow trans-bilayer movements of large or charged molecules.

Two alternative approaches that simultaneously create GUVs of a well-defined size and load them with high encapsulation efficiencies are the water/oil (w/o) emulsion method and microfluidic techniques. If a (stable) w/o emulsion can be prepared from an oil and a bilayer-forming lipid, this w/o emulsion can be used as a starting system for the preparation of giant vesicles[56–58]. This method has a high encapsulation efficiency and high yield of vesicles, nonetheless the possible presence of oil in the vesicle membrane might significantly affect the physical properties of the lipids in the bilayer, or properties of reconstituted membrane proteins. In the case of microfluidic techniques[59], giant vesicles can be formed from an initially planar bilayer. The planar bilayer is first formed between two aqueous solutions in a double-well chamber. Vesicle formation is then induced by detaching fragments of the planar bilayer by jet-blowing an aqueous solution onto the bilayer[60]. Giant vesicle formation depends on the precise way in which the aqueous solution is blown onto the bilayer. Recently, the general concept of the method was applied and elegantly extended for the high-

throughput production of hundreds of giant vesicles per minute by using a microfluidic T-junction[61]. Microfluidic techniques have a clear potential for high encapsulation efficiencies, control of vesicle size, and high yield of vesicles but require special equipment and expertise.

All of the currently known procedures are limited in one or another way, for example in terms of lipid composition, ionic strength conditions, or they require dedicated equipment and microfabrication. This inspired us to search for more efficient vesicle preparation protocols. By following the work reported in Paper II, we have investigated the possibility of using direct laser heating as an alternative method to electroformation. Through this, a method for the generation of giant vesicles from thin lipid films has been developed (Paper III).

3.4 Formation of flat giant unilamellar vesicles

Model membranes can be assembled by self-organization processes from individual phospholipids to form either three-dimensional fully enclosed aqueous compartments (liposomes), or two-dimensional planar lipid films (supported membranes). The most commonly studied type of supported membranes, well suited as mimics for the cell's plasma membrane, are supported lipid bilayers. Supported lipid bilayers can be formed in a variety of ways; the two most common techniques being Langmuir-Blodgett deposition[62,63], and vesicle adsorption with subsequent rupture[64,65]. Lipid bilayers can be assembled on many types of high energy surfaces such as metals, metal oxides, semi-conductor oxides and nitrides. The high surface energy is a consequence of the strong cohesion within these materials.
An alternative approach to membrane assembly on high energy surfaces is by spreading a rolling double bilayer (**Figure 3.4**). First, silicon dioxide is deposited onto solid substrates in thin transparent layers. These surfaces are hydrophilic and have somewhat negative zeta potential. An important factor

contributing to the adhesion between the lipid membrane and the solid support is the presence of multivalent ions[66,67].

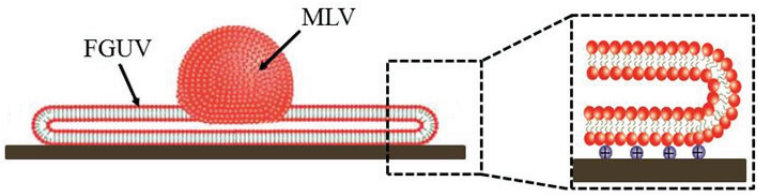

Fig. 3.4: Formation of a flat giant unilamellar vesicle. FGUV = Flat Giant Unilamellar Vesicle, MLV = Multilamellar Vesicle (MLV). Schematic drawing illustrating a double bilayer spreading from a MLV. The inset shows the advanced edge of the FGUV. The lower bilayer is fixed on the substrate while the upper bilayer slides over the lower bilayer and rolls onto the substrate.

On such negatively charge surfaces, divalent ions such as Ca^{2+} often bind to available functional groups (*e.g.* $Si\text{-}O^-$) and reduce the surface charge density. Then, when multilamellar vesicles in a buffer droplet are allowed to settle onto silicon dioxide surface substrates, they spread in a circular manner as a rolling double bilayer, wetting the solid surface and forming a flat giant unilamellar vesicle in the process. The spreading of lipids from a multilamellar vesicle can be described by tension driven flow, which is often referred to as Marangoni flow. The driving force for such a flow would be a free energy gain in the system. This energy gain emanates from the difference in surface tension at two different interfaces: the substrate wetted with the aqueous buffer and the substrate wetted with the lipid film. For lipid spreading to be favorable the latter should be lower. Flat giant unilamellar vesicles (FGUVs) have previously been established as effective model architectures, *e.g.* for biomimicking applications[68,67]. In this work, FGUVs have been used as model system for calcium-ion dependent tubulation in the presence of nanoparticles (Paper I).

3.5 Lipid nanotubes

Lipid nanotubes are cylindrical hollow structures with a diameter of about 50-200 nm. They were discovered when red blood cells were subjected to shear stress by hydrodynamic flow[69]. Also, they have been observed within cells in the Golgi apparatus and the endoplasmic reticulum as well as between cells[70,71]. These biological nanotubes, often called tunneling nanotubes enable transport of cell components, signal transduction, but also virus particles and bacteria are implied in transmission[72–74]. Several *in vitro* experimental techniques to pull nanotubes from lipid vesicles and cells have been developed, in order to gain better understanding in the underlying physics and elastic properties of the lipid membranes[75,19,76]. But many *in vivo* functions are still insufficiently understood, which has placed these nanostructures of current interest. In this thesis, nanoparticle-induced formation of lipid nanotubes from flat vesicles has been studied (Paper I), and lipid nanotubes have been pulled from giant vesicles (Paper II).

3.6 Fabrication of linear nanotube-vesicle networks

Phospholipid nanotube-vesicle networks (NVNs) represent some of the smallest and structurally most flexible devices known to date for performing controlled chemistry[77]. We have developed NVNs as a means for transport of reactive material between containers, for initiation and control of chemical reactions in ultra-small volumes and as analytical devices with a resolution down to the single-molecule level[78]. A recent article by Jesorka *et al.*[79] describes in details the generation of these systems.

The method used in the present work is based on a combination of micromanipulation, electroporation, and microelectroinjection techniques (**Figure 3.5**). The liposomes are prepared in such a way that a GUV is attached to a MLV (see 3.3 Formation of giant vesicles). The MLV is required as a source of lipid material for building the network. During the course of the experiment, constant adhesion of the vesicles to the surface is assumed.

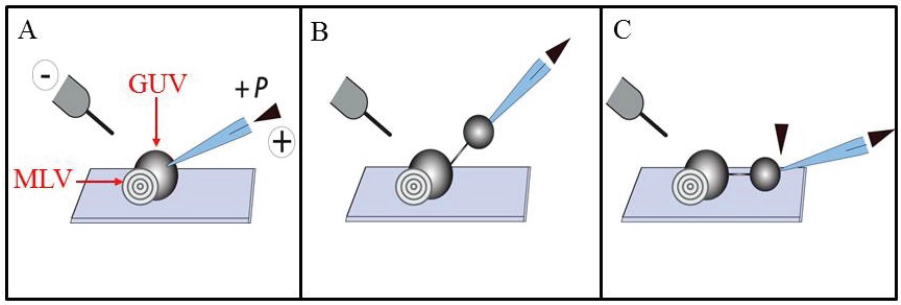

Fig. 3.5: Formation of a linear nanotube-vesicle network. (A) Electroporation and microinjection. (B) Nanotube extraction and daughter vesicle creation. (C) Daughter vesicle deposition/adhesion.

The experiment begins with placing a microelectrode and, a micropipette filled with buffer solution and containing a counter electrode, close to the surface of the GUV. By applying an electric pulse and piercing the GUV with the micropipette, it is possible to penetrate the membrane, that the pipette tip enters the vesicle. The membrane then seals around the micropipette tip. By pulling the micropipette away from the GUV, a lipid nanotube is created, which connects the pipette tip and the vesicle. Next, a positive pressure is applied through the micropipette. This leads to injection of a buffer solution, and a small daughter vesicle is formed at the pipette tip. The size of the daughter vesicle is controlled by the amount of injected liquid. This new vesicle is then positioned on the surface, and the pipette can be detached from the daughter vesicle by pulling the pipette away from the vesicle with applying electric pulses. Linear nanotube-vesicle networks have been fabricated in Paper II to verify the stability of heat-induced GUVs.

4. NIOSOMES

Non-ionic surfactant based vesicles, also called niosomes, are formed from the self-assembly of non-ionic amphiphiles and cholesterol, in aqueous media, resulting in closed bilayer structures. They consist of one or more bilayers surrounding aqueous compartments, where the hydrophilic head groups are oriented towards the interior and exterior aqueous phases. Niosomes were first introduced as a formulation tool by the cosmetic industry. In recent years niosomes have been extensively studied for their potential to serve as carriers for the delivery of drugs[80], antigens[81], and hormones[82]. Analogous to liposomes, niosomes show great advantages in stability, cost and safety and in this regard an increasing number of tailor-made formulations are investigated for pharmaceutical applications.

4.1 Non-ionic surfactants and bilayers

Non-ionic surfactants are the most common type of surface agent used in preparing vesicles, due to the superior benefits they impart with respect to stability, compatibility and toxicity compared to their anionic, amphoteric or cationic counterparts[83,84]. They have many functions including acting as solubilizers, wetting agents, emulsifiers and permeability enhancers. They are also strong P-glycoproteins inhibitors, a property useful for enhancing drug absorption and for targeting to specific tissues[85].

Sorbitan fatty acid esters are the most commonly used non-ionic surfactants, a subgroup being Span 20, 40, 60, and 80. All span types have the same head group and different alkyl chain length (**Figure 4.1**). Another class of non-ionic surfactants is polyoxyethylene alkyl ethers also called Brij, which have the ability to form bilayer vesicles when mixed with cholesterol. In the last decade, the use of Tweens as non-ionic surfactants in

niosomes has been reported. Tweens sorbitan esterified with fatty acids are polysorbates derived from PEGylation.

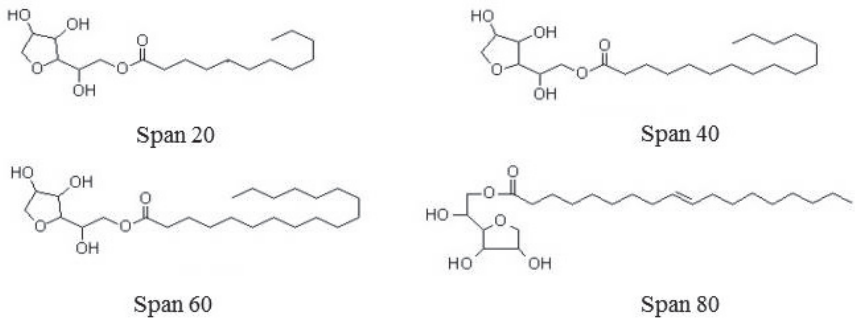

Fig. 4.1: Structure of Span 20, 40, 60 and 80.

The formation of bilayer vesicles instead of micelles is dependent on the critical packing parameter (CPP) but also on the chemical structure and on the hydrophilic-lipophilic balance (HLB) of the surfactant. The HLB value represents a relative proportion of the hydrophobic and hydrophilic groups comprising the molecule and provides a guide for evaluating potential vesicle formation. Generally, surfactants with alkyl chain length from C_{12}-C_{18} and Span series having HLB numbers of between 4 and 8, are suitable for niosome preparations[86]. However, with an optimum level of cholesterol, non-ionic surfactants with varying HLB values have been used to prepare vesicles[87,88]. Both cholesterol and the HLB of the surfactant tends to affect important vesicular properties, such as vesicle stability, and drug entrapment efficiency. For HLB>6, cholesterol must be added to the surfactant in order to form vesicles and for lower HLB values, cholesterol enhances stability of vesicles[89]. It is also shown that the addition of cholesterol suppresses the tendency of the surfactant to form aggregates, and the leakiness of the membranes[90].

4.2 Classification and preparation of niosomes

Niosomes like liposomes, are classified according to their size and number of membrane bilayers. They can be divided into three groups:
- Multilamellar Vesicles or MLV (1 μm-20 μm of diameter),
- Large Unilamellar Vesicles or LUV (50-1000 nm of diameter),
- Small Unilamellar Vesicles or SUV (20-50 nm of diameter).

The preparation of niosomes can be achieved by many existing protocols and should be chosen according to the use of niosomes, since the preparation methods influence the number of bilayers and the size. For instance, MLVs are generally used for the encapsulation of lipophilic drugs and their micron size scale is suitable for ocular drug delivery, while LUVs are employed more for their ability to entrap hydrophilic compounds and their sub-micron sizes are suitable for intravenous administration and transdermal delivery.

When it comes to the formation of multilamellar niosomes, the film hydration technique is the simplest method[91]. In this method, surfactants and cholesterol are dissolved in a volatile organic solvent in a round bottom flask. Then the solvent is removed at room temperature using a rotary evaporator leaving behind a thin layer of solid mixture deposited on the wall of the flask. The dried surfactant film can be hydrated by an aqueous buffer above the main transition temperature of the surfactant. Shaking yields a dispersion of MLVs.
Concerning the preparation of LUVs, a reverse phase evaporation method is commonly used[92]. First, the surfactants and cholesterol are dissolved in an organic solvent. Then an aqueous phase is added to the organic phase and the mixture is sonicated in order to form an emulsion, following by slow removal of the organic phase, yielding large unilamellar niosomes.
The size range of niosomes has a major impact on their *in vitro in vivo* fate. Generally, nanoformulations increase the dissolution of the drug compound and improve bioavailability. Hence, size

reduction of niosomes is essential. MLV dispersions prepared by the film hydration technique are converted into SUVs generally by sonication[86].
Niosomes seems to be a promising vehicle for drug delivery and understanding the molecular mechanisms that underlie the interaction of the niosomes with the cell, their uptake properties and retention will be beneficial for the successful development of these systems. This inspired us to search for the generation of giant unilamellar niosomes as a membrane model system to obtain information about the background mechanism. By following the work reported in Paper III, we have developed a method for the generation of giant unilamellar niosomes (Paper IV).

4.3 Niosomes versus liposomes

Niosomes and liposomes have similar application in drug delivery, but have different chemical structures. Niosomes are constituted from non-ionic surfactants whereas liposomes comprise of phospholipids. They are functionally the same, have the same physical properties and act as amphiphilic vesicles. Both can be used in targeted and sustained drug delivery system. The property of both depends upon the composition of the bilayer and methods of their preparation[93]. In spite of these comparable characteristics, niosomes offer several advantages over liposomes such as intrinsic skin penetration enhancing properties[94], higher chemical stability and lower costs[95]. Both of the last features make the niosome more attractive for industrial manufacturing[96]. Also, niosomes do not require special conditions such as low temperature or inert atmosphere during preparation and storage[93]. Both niosomes and liposomes have a risk of aggregation, drug leakage, or hydrolysis of entrapped drugs during storage.

5. METHODOLOGY

5.1 Surfaces preparation

Methods of micro- and nanofabrication are numerous but they are all based on deposition, or selectively removal of layers of various materials. In this work, sputtering and photo-polymerization have been used to form layers of certain materials.

5.1.1 Sputtering

Direct Current (DC) sputtering is a method for the deposition of various materials, electrically conducting or insulating. The sputtering process takes place in an evacuated chamber, where a disc-shaped target material, which serves as a cathode, is bombarded with a flow of argon ions (**Figure 5.1**).

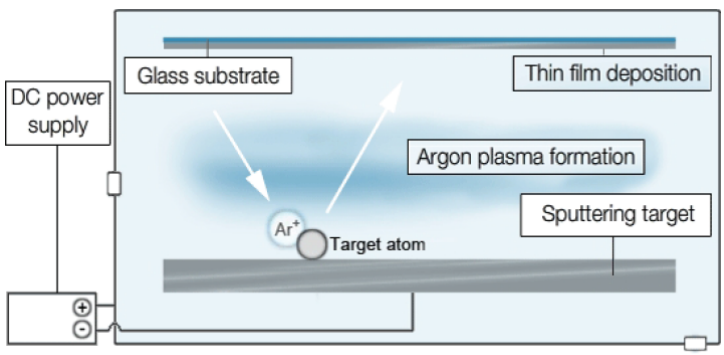

Fig. 5.1: Schematic drawing of DC sputtering deposition.

The argon ions are created in a plasma. They interact differently with the target material depending on their energy of impact they receive from the plasma. If this energy is between 10 to 5000 eV,

surface atoms are removed from the target, which is known as the sputtering process. A stream of ejected surface atoms away from the target is created, and these atoms condense at the opposing substrate which is placed above an anode. The condensation process at the substrate is called deposition. In this thesis, DC magnetron has been used for the fabrication of gold surfaces, by deposition respectively of a titanium adhesion layer and a gold layer, for the adhesion of vesicles (Paper II).

In Radio Frequency (RF) sputtering, there is no direct current between anode and cathode, but the current alternates at high radio frequencies. It is mostly used to sputter insulators that require high bias for the sputtering process to occur. By using an alternating current, a build-up of charge at the target and the substrate is prevented. In this work, the RF sputtered material is silicon which in the presence of oxygen in the sputter chamber, forms films of SiO_2. In this process called reactive sputter deposition, atoms of the ambient gas in the chamber react with surface atoms and are built into the film. The SiO_2 surfaces have been used for the formation of flat giant unilamellar vesicles (Paper I) and for the formation of spin-coated lipid films (Paper IV).

5.1.2 Photo-polymerization

A photoresist is a light-sensitive material used in several industrial processes, such as photolithography and photoengraving to form a patterned coating on a surface.
SU-8 is a commonly used epoxy-based negative photoresist, originally developed for the microelectronics industry. It is now mainly used in the fabrication of microfluidics and parts of micro-electromechanical systems. The key components of the resist are epoxy resin, a photo catalyst and solvent. In SU-8, triarylsulfonium hexafluoroantimonate (SbF_6-) serves as a photo-catalyst and cyclopentanone as a solvent. The procedure of photo-polymerization includes four steps (**Figure 5.2**). First, a glass coverslip is covered with photoresist. This is typically

performed by spin-coating, where photoresist is dispensed onto the coverslip, and then thinned by spinning.

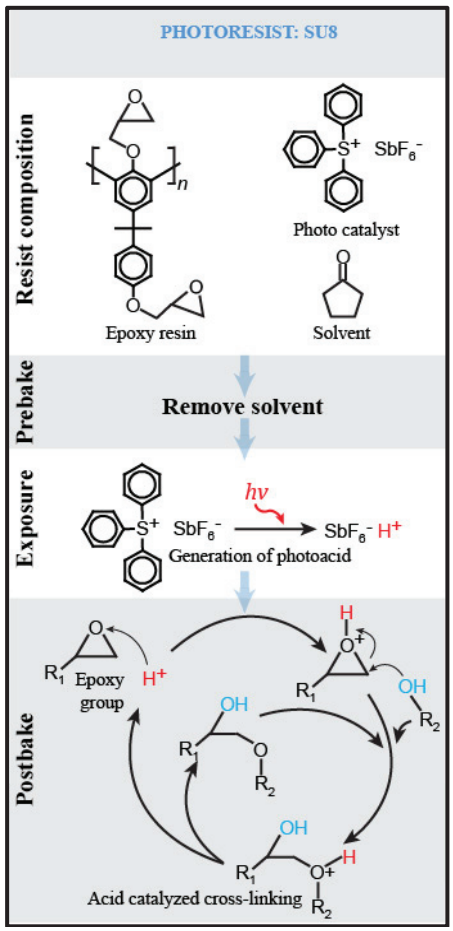

Fig. 5.2: Chemistry of photo-polymerization of SU-8.

The final thickness depends on viscosity, spin speed and time, and the rate of evaporation. After this coating step, the substrate is heated to evaporate the solvent (also called soft-baking), forming a flat polymer layer. Then the substrate is exposed to UV light. During this step, the photo-catalyst is turned into a Lewis acid. After this exposure, a post-baking step catalyzes the epoxy

group opening and cross-linking reactions causing the solidification of the material. The SU-8 surfaces have been used in this work for the adhesion of vesicles in Paper II and for the formation of spin-coated surfactants films in Paper IV.

5.2 Infrared Laser Heating

Temperature is an important parameter for the thermodynamic and kinetic studies of many processes notably in the self-assembly of amphiphiles. Localized temperature control in microscopy experiments offers exquisite control of local environment.[97,98] One convenient approach to heat locally, is to use focused laser light in an existing microscope set-up. Due to the low absorption of biological samples in the near infrared (IR-A) range and below, this approach is not considered to be efficient. Since in the biological matter adsorption increases significantly above 1450 nm, a better alternative is to use the long wavelength infrared (IR-B). From 750 nm, the absorption of water increases over 6 orders of magnitude with an absorption maximal at 2940nm.
For the purpose of localized heating of lipids (Paper II & III), an IR-B semiconductor diode laser connected to 8A power supply has been used as an optical heat source (**Figure 5.3**). The use of a 1470 nm semi-conductor diode laser, originally developed for optical communication purposes, in conjunction with a narrow optical fiber, provides a cheap and portable scheme for localized heating without the need for extraneous optical components or a specially equipped microscope.

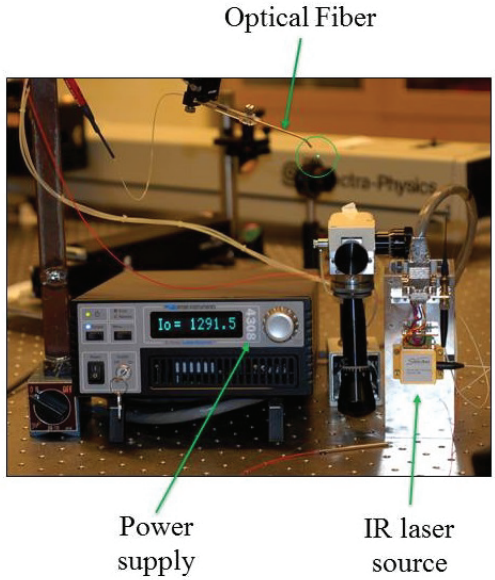

Fig. 5.3: Infrared laser heating system.

5.3 Imaging techniques

Different techniques exist to obtain a highly resolved image of a sample by means of microscopy. Common bright field microscopy with Differential Interference Contrast (DIC) and Laser Scanning Confocal Microscopy have been used in this work.

5.3.1 Microscopy

The capability of a microscope to resolve small objects is dependent on the numerical aperture (***NA***) of the objective used, which describes both the focusing power and the light collection ability and can be expressed as:

$$NA = n_{\lambda} \sin(\theta) \qquad \textbf{Eq. (12)}$$

where $\boldsymbol{n_\lambda}$ is the refractive index of the medium and $\boldsymbol{\theta}$ the maximum angle relative to the objective axis from which light can be collected. A higher numerical aperture provides better resolution of the object.
The resolution of the objective, $\boldsymbol{r_\lambda}$, depends on the wavelength $\boldsymbol{\lambda}$ and describes the distance in which two objects can be distinguished as separate entities. There are several equations that have been derived to express the relationship between NA, λ and r_λ. In the ideal case, when the objective is aberration-free and provides a uniform circular aperture, r_λ can be expressed:

$$r_\lambda = 0.61\ \lambda/NA \qquad \textbf{Eq. (13)}$$

Equation 13 is based upon a number of factors (including a variety of theoretical calculations).

5.3.2 Differential Interference Contrast

Differential Interference Contrast (DIC) is a method to obtain bright field transmission images of transparent samples with a three dimensional appearance. In this mode, the light beam is polarized and subsequently split. Coherent beams travel through the sample, interference between them occurs and in this way information about the optical density is collected, giving a 3D effect as improved contrast on the visible image.
Even in DIC microscopy mode, it is possible to observe but not to resolve very thin lipid nanotubes or supported bilayer membranes. For this reason fluorescence microscopy has been employed as well in this thesis.

5.3.3 Fluorescence microscopy

Fluorescence microscopy requires staining of the specimen with fluorescent molecules, or dyes. Fluorescent molecules absorb light in one wavelength band (excitation) and emit light at longer wavelengths (emission). When fluorescent molecules absorb light with excitation wavelength, they are excited to a higher electronic

state. This state has a short lifetime and the molecules will release the energy in the form of light and heat. Since some energy is dissipated as heat, the emitted light contains less energy and therefore has a longer wavelength than the absorbed excitation light. This emission of light is referred to as fluorescence. In the present work, a fluorescence technique known as laser scanning confocal microcopy is used.

5.3.4 Laser Scanning Confocal Microscopy

In conventional microscopy, all light coming from the sample is detected, often rendering the image blurry. The confocal concept evolved as an alternative to wide-field microscopy (**Figure 5.4**). The principal advantage of confocal microscopy is the introduction of a pinhole in the light path in front of the detector, which eliminates light that is originating from out-of-focus planes. This results in elimination or reduction of background information that leads to image degradation. Confocal illumination occurs only at a resolution-limited point, which allows optical sectioning and 3D reconstruction of images.

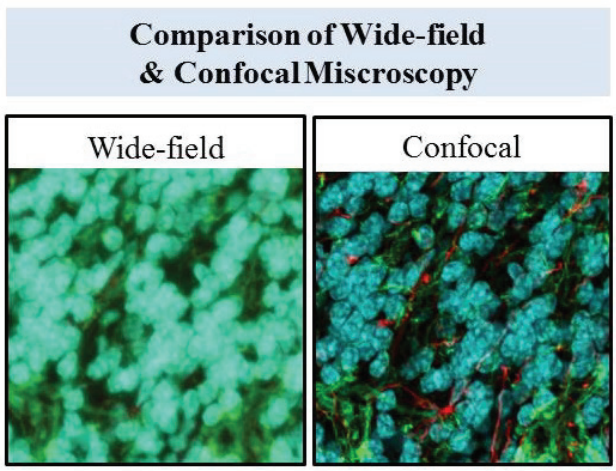

Fig. 5.4: Wide-field *versus* confocal microscopy. On the left, wide-field fluorescence micrograph of a brain tissue and on the right confocal micrograph of the same brain tissue.

6. SUMMARY OF PAPERS & REMARKS

Paper I. Nanoparticles of virus particle-like morphology were inserted into flat giant unilamellar vesicles. The nanoparticles induced the spontaneous formation of lipid nanotubes, emerging from the flat vesicles and elongating gradually into the ambient solution. The tube formation could be dynamically controlled by changing the Ca^{2+} concentration in the ambient buffer.

Paper II. An on-demand method for the generation of giant unilamellar vesicles from single multilamellar liposomes by means of localized heating was reported. This technique enables formation of individual giant unilamellar vesicles from natural and artificial lipid mixtures but also from single neutral lipids at selected locations.

Paper III. An on-demand method for the generation of giant unilamellar vesicles from spin-coated lipid films by means of localized heating was presented. This technique enables giant unilamellar vesicles generation from charged and neutral single lipid species, as well as from a complex mixture, in different ionic conditions and with the ability to encapsulate molecules.

Paper IV. A method for the preparation of giant unilamellar niosomes from spin-coated amphiphile films was reported. This technique enables generation of giant niosomes from different non-ionic surfactants with the ability to encapsulate lipophilic as well as hydrophilic compounds.

The main purpose of this thesis has been to develop reliable methods for the preparation of giant vesicles. More specifically, simple and rapid methods without the need of specialized chambers have been reported. These protocols enable reproducible formation of mainly giant unilamellar vesicles from a number of amphiphiles or mixtures of amphiphiles. The possibility to form giant vesicles at selected locations by means of

localized heating can be advantageous for surface-based single vesicle experiments *e.g.* experiments on microfabricated substrates. Moreover, the ability to form vesicles from different types of amphiphiles may greatly expand the accessibility of giant vesicles as a model system for the investigation of membrane physical properties but also for the study of physiological processes such as membrane-binding, artificial cell synthesis or drug delivery mechanism.

It is likely that supplementary studies on the amphiphile morphological changes with high temperature would provide interesting information about the mechanism of giant vesicles formation. Likewise about entrapment ability of vesicles formed, further exploratory work has to be done to characterize encapsulation.

However challenging these obstacles, it is expected that our simple and rapid methods for giant vesicles formation, will be developed further, leading most likely to additional applications of giant vesicles as cell-like systems and as drug delivery vehicles.

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