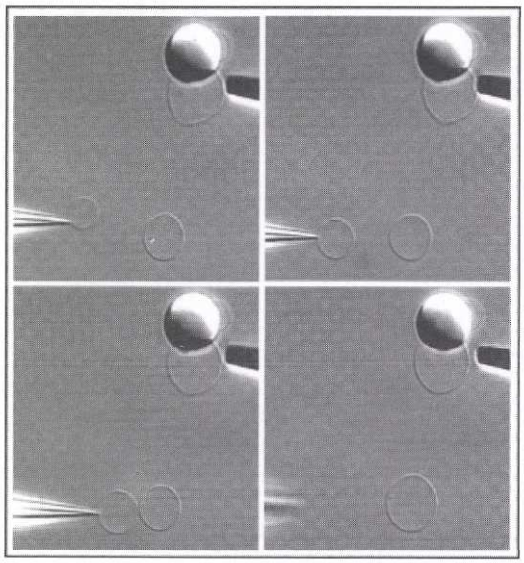

Fabrication of Surfactant-Based Nano- and Microscaled Biomimetic Compartments for Studies of Confined Chemical Reactions

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ANDERS KARLSSON

AKADEMISK AVHANDLING

för filosofie doktorsexamen i kemi (examinator Professor Daniel Jagner) som enligt kemiinstitutionens beslut offentligt kommer att försvaras fredagen den 7:e November 2003, kl. 14.15 i föreläsningssal KA, Kemigården 4, Göteborgs Universitet och Chalmers Tekniska Högskola, Göteborg.

Fakultetsopponent: Professor Francoise Brochard-Wyart, Groupe Surfaces douces, Laboratoire PhysicoChimie Curie, Institute Curie, Paris, France

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2003

Abstract

This thesis is based on the development of techniques to use liposomes and lipid nanotubes as micro- and nanoscopic reaction containers. In brief, miniaturized versions of electroporation and electrofusion techniques were developed and used to initiate chemical reactions inside single liposomes. Electroporation and electrofusion was performed by application of one or several short (typically 10-40 μs) and intense (typically 20-50 kV/cm) electric pulses, by the use of micromanipulator-controlled carbon fiber electrodes (5 μm in diameter). Chemical reactions could, for example, be induced by electrofusion of two liposomes each containing a reagent. This was demonstrated by fusion of a Ca^{2+}-containing liposome with a Fluo-3 containing liposome, which resulted in an increase of the fluorescence, upon chelation. Product formation of the enzymatic reaction between alkaline phosphatase and the substrate fluorescein diphosphate, which resulted in the product fluorescein, was measured using sensitive fluorescence microscopy and the use of a single-photon avalanche diod (SPAD) detector. Due to the small size of the liposomes, the surface-to-volume ratio is very high, making surface interactions very probable and simple computer simulations of single enzyme-single substrate encounters inside a spherical cavity revealed high collision frequency between molecules and the container wall as opposed to molecule-molecule collisions. A device consisting of sample containers connected to a common fusion chamber through microfluidic channels, consisting of ~3 mm long borosilicate glass capillaries (10-30 μm i.d., 30-100 μm o.d.) was constructed to facilitate pair-wise electrofusion of single liposomes, which could be applied to combinatorial approaches to synthesise a vast amount of different types of product liposomes with respect to membrane composition and interior contents from a small set of starting liposomes. Optical tweezers allowed selection and transport of single liposome from the sample containers into the fusion chamber.

Micromanipulator-based methods to construct two-dimensional lipid bilayer network structures of nanotubes (100-300 nm diameter) and containers (5-50 μm diameter) immobilised on surfaces were developed. The networks have controlled connectivity, container size, nanotube-length and angle between nanotube extensions emanating from a single liposome. The first method was based on simple mechanical fission of multilamellar and unilamellar liposomes using a 5 μm diameter carbon fiber as a cutting tool. A micropipette-assisted technique to create unilamellar networks of liposomes and nanotubes was also developed. A pulled microinjection pipette was inserted into a unilamellar liposome using a combination of mechanical force and an electric field. After resealing of lipid around the pipette-tip, the pipette was pulled away to create a lipid nanotube. Pressure-controlled injection of buffer (typically femtoliters per second) expanded the nanotube at the injection tip into a new liposome and when the desired size was reached, the new liposome was allowed to adhere to the surface. This technique also enables easy differentiation of interior contents in the liposomes through exchange of the fluid contained in the microinjection pipette.

Methods to control and handle fluid and material transport between the nanotube interconnected unilamellar liposomes were developed. Transport was induced by a membrane tension difference, resulting in a flow of lipids across the nanotube to diminish this difference, which dragged along the liquid column and any dispersed particles inside the lipid nanotube through viscous coupling, thus offering means of transporting and trapping of molecules in nanoscale channels. Lipid velocities usually ranged between 20-30 μm/s, however, much higher velocities, up to 60 μm/s, were occasionally observed. The nanotube segment could also function as a nanoscale flow channel, which could be combined with single-molecule-sensitive detection techniques, such as laser induced fluorescence (LIF). Also, a novel method to create, load and transport nanotube-integrated liposomes (500 nm-5 μm) between interconnected liposomes was developed. These nanotube-integrated vesicles were created by addition of excess membrane material to one of the surface-attached liposomes in a network. This was performed by merging of a nanotube-connected liposome, which resulted in a drop of membrane tension. The interconnecting lipid nanotube was subsequently destabilised and attained an asymmetric funnel-like shape, which was transformed into nanotube-integrated vesicles. As a continuation of this work, a method to route these nanotube-integrated liposomes in large networks was also developed by using a two-point perturbation technique, where the tension was increased in one liposome and at the same time decreasing the tension in another liposome that was connected to it, thus creating a larger tension-difference between these target containers, than between any other containers in the network.

Finally, network formation technology was combined with chemical reaction initiation techniques. Networks of liposomes and nanotubes were used to create two liposomes with differentiated contents with a connecting nanotube. The fluid character enables movement of nanotube attachment point across the entire network surface. Through this, two differentiated network liposomes can be created, interlinked with a nanotube. When the two compartments were brought together, *i.e.* when the nanotube length approached zero to provide a single fluid contact point, the liposome compartments spontaneously merged and their contents were mixed and the reaction could therefore be initiated. An enzymatic reaction between alkaline phosphatase and the substrate fluorescein diphosphate was used to demonstrate mixing of reagents and product formation, using this technique.

Keywords: Lipid, membrane, bilayer, liposome, biomimetic, lipid nanotube, nanotube-integrated vesicles, networks, transport, nanofluidics, electroporation, electrofusion, microinjection, reaction, initiation

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To Mom, Dad and Roger

the sun brought me
the moon caught me
the wind fought me
the rain got me

the road sent me
the years bent me
the stars overwhelmed me
time and again

Paul Waaktaar-Savoy, ***a-ha***, © 2002 Warner Chappell Music Ltd.

Abstract

This thesis is based on the development of techniques to use liposomes and lipid nanotubes as micro- and nanoscopic reaction containers. In brief, miniaturized versions of electroporation and electrofusion techniques were developed and used to initiate chemical reactions inside single liposomes. Electroporation and electrofusion was performed by application of one or several short (typically 10-40 μs) and intense (typically 20-50 kV/cm) electric pulses, by the use of micromanipulator-controlled carbon fiber electrodes (5 μm in diameter). Chemical reactions could, for example, be induced by electrofusion of two liposomes each containing a reagent. This was demonstrated by fusion of a Ca^{2+}-containing liposome with a Fluo-3 containing liposome, which resulted in an increase of the fluorescence, upon chelation. Product formation of the enzymatic reaction between alkaline phosphatase and the substrate fluorescein diphosphate, which resulted in the product fluorescein, was measured using sensitive fluorescence microscopy and the use of a single-photon avalanche diod (SPAD) detector. Due to the small size of the liposomes, the surface-to-volume ratio is very high, making surface interactions very probable and simple computer simulations of single enzyme-single substrate encounters inside a spherical cavity revealed high collision frequency between molecules and the container wall as opposed to molecule-molecule collisions. A device consisting of sample containers connected to a common fusion chamber through microfluidic channels, consisting of ~3 mm long borosilicate glass capillaries (10-30 μm i.d., 30-100 μm o.d.) was constructed to facilitate pair-wise electrofusion of single liposomes, which could be applied to combinatorial approaches to synthesise a vast amount of different types of product liposomes with respect to membrane composition and interior contents from a small set of starting liposomes. Optical tweezers allowed selection and transport of single liposome from the sample containers into the fusion chamber.

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List of publications

This thesis is based on the following publications, which are referred to in the text by their Roman numbers.

I Chiu, D.T. Wilson, C.F., Ryttsén, F., Strömberg, A., Farre, C., Karlsson, A., Nordholm, S., Gaggar, A., Modi, B.P., Moscho, A., Garza-Lopez, R.A., Orwar, O., Zare, R.N."Chemical transformations in individual ultrasmall biomimetic containers" *Science* 283, 1892-1895 (1999)

II Chiu, D.T., Wilson, C.F., Karlsson, A., Danielsson, A., Lundqvist, A., Strömberg, A., Ryttsén, F., Davidson, M., Nordholm, S., Orwar, O., Zare, R.N. "Manipulating the biochemical nanoenvironment around single molecules contained within vesicles" *Chem. Phys.* 247, 133-139 (1999)

III Strömberg, A. Karlsson, A., Ryttsén, F., Davidson, M., Chiu, D.T., Orwar, O. "Microfluidic device for combinatorial fusion of liposomes and cells" *Anal. Chem.* 73, 126-130 (2001)

IV Karlsson, A., Karlsson, R., Karlsson, M., Cans, A.S., Strömberg, A., Ryttsén, F., Orwar, O. "Molecular engineering - Networks of nanotubes and containers" *Nature* 409, 150-152 (2001)

V Karlsson, M., Sott, K., Cans, A.S., Karlsson, A., Karlsson, R., Orwar, O. "Micropipet-assisted formation of microscopic networks of unilamellar lipid bilayer nanotubes and containers" *Langmuir* 17, 6754-6758 (2001)

VI Karlsson, R., Karlsson, M., Karlsson, A., Cans, A.S., Bergenholtz, J., Åkerman, B., Ewing, A.G., Voinova, M., Orwar, O. "Moving-wall-driven flows in nanofluidic systems" *Langmuir* 18, 4186-4190 (2002)

VII Karlsson, A., Karlsson, M., Karlsson, R., Sott, K., Lundqvist, A., Tokarz, M., Orwar, O. "Nanofluidic networks based on surfactant membrane technology" *Anal. Chem.* 75, 2529-2537 (2003)

VIII Karlsson, R., Karlsson, A., Orwar, O. "Formation and transport of nanotube-integrated vesicles in a nanoscale bilayer network" *J. Phys. Chem. B* 107, 11201-11207 (2003)

IX Karlsson, R., Karlsson, A., Orwar, O. "A nanofluidic switching device" *J. Am. Chem. Soc.* 125, 8442-8443 (2003)

X Karlsson, A., Sott, K., Davidson, M., Konkoli, Z., Orwar, O. " Nanotube-mediated merging of liposomes to initiate chemical reactions" *Manuscript in preparation*

Papers not included:

Cans, A.-S., Wittenberg, N., Eves, D., Karlsson, R., Karlsson, A., Orwar, O., Ewing, A. "Amperometric detection of exocytosis in an artificial synapse" *Anal. Chem.* 75, 4168-4175 (2003)

Konkoli, Z., Karlsson, A., Orwar, O. "The pair approach applied to kinetics in restricted geometries: strengths and weaknesses of the method" In press *J. Phys. Chem. B*

Voinova, M., Karlsson, R., Karlsson, A., Karlsson, M., Lobovkina, T., Orwar, O. "Nanomechanics of conjugated lipid vesicles" Manuscript in preparation

Karlsson, M., Davidson, M., Karlsson, R., Karlsson, A., Bergenholz, J., Konkoli, Z., Jesorka, A., Lobovkina, T., Hurtig, J., Voinova, M., Orwar, O. "Biomimetic nanoscale reactors and networks" Submitted to *Annu. Rev. Phys. Chem.*

Contribution report

Paper I: Contributed with hard sphere simulation results and some complementary text writing for the manuscript. Participated in finalization of the electroporation and electrofusion experiments.

Paper II: Contributed with the hard sphere simulation results and formulation of the research project and interpretation of the results. Contributed with large parts of the manuscript text writing and literature search.

Paper III: Contributed with the development and design of the prototype microfluidic device and formulation of the research project. Performed most liposome transport and electrofusion experiments and contributed with large parts of the manuscript text writing and literature search.

Paper IV: Contributed equally with the experimental results, development of the mechanical fission technique, and the formulation of the research project. Contributed equally with interpretation of the experimental results and the manuscript text writing, as well as literature search.

Paper V: Contributed equally with the formulation of the research project and the interpretation of the experimental results. Contributed equally to the manuscript text writing, and literature search.

Paper VI: Contributed equally with the formulation of the research project and the interpretation of the experimental results as well as the manuscript text writing and literature search.

Paper VII: Contributed equally with experimental results of network formation and transport of material through nanotubes. Contributed equally with large parts of the manuscript text writing and literature search.

Paper VIII: Contributed equally with the experimental results, interpretation of the experimental results, formulation of the research project as well as large parts of the manuscript text writing and literature search.

Paper IX: Contributed equally with the experimental results, interpretation of the experimental results, formulation of the research project as well as large parts of the manuscript text writing and literature search.

Paper X: Contributed with most of the experimental results and the formulation of the research project, as well as the interpretation of the results. Contributed with most of the manuscript text writing as well as literature search.

Table of contents

Introduction

During the last decades, interdisciplinary science and technology have turned more of their focus towards reductionism and small-scale systems. When it comes to chemistry, the study of chemical reactions in confined spaces has been a subject of interest both for theorists and experimentalists (Khairutdinov and Serpone, 1996). The ambition of reductionism is motivated by several powerful driving forces that can be classified as both economic and scientific curiosity. Not only can the consumption of hazardous and/or expensive fine chemicals be drastically lowered, but the timescales of operation to complete an analysis or to perform a chemical reaction, for example, is of course also decreased substantially. This has resulted in a tremendous development of microfabrication protocols that can be implemented in chemical and biotechnological processes (McDonald *et al.*, 2000; Xia *et al.*, 1999; Haneveld *et al.*, 2003; Tas *et al.*, 2002). The outcome of this development can, for example, be demonstrated by the downscaling of chemical separation channels found in fluidic devices, where the channel size have decreased from a few millimetres to a few hundred nanometres (Woods *et al.*, 2001). Technically, a downscaling of chemical separation channels will also lead to higher separation efficiency, reduced analysis times and easier identification of separated substances. As components are downscaled much effort can instead be diverted to packing and integration of various components into functional units, which has indeed been very successful in the microelectronics industry. Nowadays, sample preparation, purification, mixing of reagents, reactions and separations can be performed on a single compact, small-sized device structure, normally referred to as a lab-on-a-chip or micro total analysis system, μ-TAS (Chován and Guttman, 2002; Reyes *et al.*, 2002; Auroux *et al.*, 2002).

A lot of effort has been devoted to the fabrication of these structures, but more and more attention is given to the performance and functionality, which also includes theoretical studies on fluid transport, mixing strategies, diffusion etcetera, to be able to optimize the system. One of the components of the μ-TAS or lab-on-a-chip is the site where chemical reactions are performed – the microreactor (Kleeman, 2001). The microreactor concept has evolved into techniques to produce ultra-small vials or reaction containers with volumes ranging from nanoliters to picoliters (Litborn and Roeraade, 2000). One of the major difficulties of using open vials is off course the rapid evaporation that can be quite dramatic for a picoliter volume of aqueous solution (Clark *et al.*, 1997). This can be avoided to some extent by including glycerol into the aqueous solution and an increased humidity in the headspace over the reaction volume or by a having a lid on top of the vials of volatile liquid (Litborn and Roeraade, 2000). Further development in surface technology and microarray technology has resulted in techniques to screen large libraries of multicomponent chemical reactions (Gosalia and Diamond, 2003)

Microreactors have many attractive features that should increase their applicability compared to traditional large-scale or bulk systems. Improved heat and mass transfer, due to high surface-to-volume ratio and the small length scale are key properties to, for example, increase the yield of a reaction. Parallelisation is very easy, thus making scale-up and re-designs of a system very accessible and fast for start-up and pilot projects. Finally, spatial control of reactants and the laminar flow properties that are inherent in these microscaled systems should enable easy characterisation and extraction of kinetic data collected from the system, thus facilitating optimisation protocols (Chován and Guttman, 2002).

Lately this type of reasoning on subjects such as miniaturization, optimization and compartmentalization has surfaced in other research fields such as biology and biochemistry. To date the best lab-on-a-chip or μ-TAS on the market are nowhere near the complexity, optimization, parallel capability, functionality and compartmentalization of the cell, which all living matter is constructed of.

It has been argued recently, that investigations of biochemical reactions are better performed inside bio-inspired or more natural environments due to unfavourable surface effects (Tsumoto *et al.*, 2001). Surface adsorption and denaturation of macromolecules on solid surfaces can be a potential problem, especially in very small-scale systems, where the surface-to-volume ratio is extremely large. Therefore much interest has been directed towards using liposomes or lipid vesicles as small-scale nano/microreactors for enzymatic reactions, for example, and other biotechnological applications (Monnard, 2003).

As a side track to this line of research, liposomes have earned much attention as prebiotic cell models. Plausibly, since all cells today are composed and constructed of lipid bilayer assemblies, early structures for compartmentalisation, necessary for maintaining integrity and separation of reactants, controlled transport of reactants and protection from outer environmental changes, this suggests an important role of lipid bilayer membranes in the early days of evolution (Monnard and Deamer, 2002).

There are different approaches to construct a common minimalistic cell model. The "bottom-up" approach involves the starting point where monomers and simple low-molecular weight molecules assemble into specific sequences and structures such as nucleic acids and proteins inside a liposome. The "top-down" approach on the other hand involves the incorporation of extant DNA, RNA and/or enzymes into liposomes by various methods to study biochemical reactions inside a closed environment (Luisi, 2002).

The last decade have witnessed a number of different biochemical reactions inside liposomes, including transcription of DNA into RNA (Tsumoto *et al.*, 2001), replication of RNA (Oberholzer *et al.*, 1995), polymerase chain reactions (Oberholzer *et al.*, 1995), growth of protein crystals (Yamashita *et al.*, 2002), and many more. The reagents can either be directly incorporated during the formation of the liposomes or by methods that penetrate the membrane barrier.

Taking the biomimickry even further, research is also aimed at literally extracting some of the cellular membranes functional properties in the form of membrane proteins (Eytan, 1982). Some of natures own building blocks are thereby taken out of its natural environment and reinserted or reconstituted into pure lipid bilayers, either to study the membrane protein in a more controlled and well-defined manner to avoid the complexity of the cell membrane or to give the lipid membrane a function. One interesting example is light-driven production of ATP, where liposome membranes were used as an artificial photosynthetic membrane to transport protons into the liposome. This creates an electrochemical potential of protons or a proton-motive force that could be used for ATP synthesis, by reconstituting F_0F_1-ATP synthase into the liposome membrane (Steinberg-Yfrach *et al.*, 1998).

In our own line of research we have tried to abstract some of the exciting mechanical properties of the fluid lipid bilayer and mimic some of the shapes and transformation events that occur in membranes. Our focus has been to develop tools to utilize lipid membrane compartments as reaction containers and ways to initiate and detect reactions inside these compartments (Chiu *et al.*, 1999a; Chiu *et al.*, 1999b). Our intention with this approach is to provide model systems that simulate or mimic the environment that prevails in the interior of cells, as opposed to standard procedures, where biochemical reaction investigations are performed in large containers (milliliters) in which, for example, surface interactions are of negligible importance (Fig. 1). Much of our attention has also been devoted to manipulate the size and structure of the reaction environment. We have developed micromanipulation protocols to mechanically and selectively induce shape changes of single liposomes and transform them into lipid membrane networks consisting of liposomes connected by lipid nanotubes (Karlsson, A. *et al.*, 2001; Karlsson, M. *et al.*, 2001). Several different fabrication strategies have been developed for this type of network structures through the years and more

are currently on the way in order to expand this field from 2-dimensional to 3-dimensional network structures. The network features container sizes in the range of a few micrometers to 50 micrometers in diameter interconnected by lipid nanotubes that in our systems range from 100 to 300 nanometers in diameter. Previous work on lipid nanotubes has demonstrated that the size of these nanotubes can be decreased even further, down to 20 nanometers in diameter (Bo and Waugh, 1989), by controlling the membrane tension in the system. The fabrications techniques are capable of controlling the sizes and position of the network liposomes with down to micrometer precision as well as the connectivity between the containers. The topology of the network can be manipulated after the first stage of network fabrication by utilizing a miniaturized electrofusion protocol, thereby expanding the possibilities of network design even further (Karlsson *et al.*, 2002).

Further on, we have studied the effect by dynamically exciting selected target containers in a network. Mechanical deformations and/or changes in the surface-to-volume ratio of target containers can give rise to membrane tension gradients or differences between two network containers connected by a nanotube. The outcome of this research has resulted in techniques to produce lipid flow from regions of low membrane tension to high tension across nanotubes interlinking network containers (Karlsson, R. *et al.*, 2002; Karlsson, A. *et al.*, 2003). Through this we have contributed to the nanofluidics research field with novel ideas and solutions to controlled fluid handling in nanometer-scaled channels. In order to route material in between selected target containers in larger networks, a two-point perturbation technique was also developed, which utilizes small (500 nm-5 μm diameter) nanotube-integrated vesicles as transport containers (Karlsson *et al.*, 2003a; Karlsson *et al.*, 2003b).

Tension-driven lipid flows and nanotube-mediated vesicle transport between membrane-enclosed compartments have also been suggested as plausible explanations for certain types of intracellular transport (Sciaky *et al.*, 1997). Thus, in addition to "pure" technological applications, such as studies of compartmentalization effects on reaction systems and implementation and studies of novel transport mechanisms in nanofluidic devices that can be combined with single-molecule detection techniques, such as confocal laser induced fluorescence (LIF), these networks of liposomes and lipid nanotubes also appear to be applicable as model systems that can be used to mimic some intracellular transport systems.

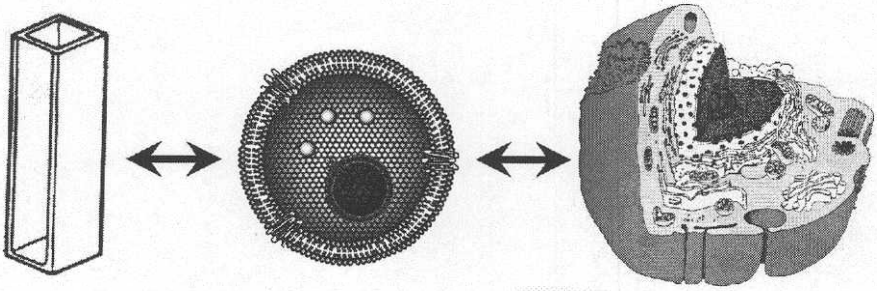

Figure 1. Schematic illustrating the use of liposomes as biomimetic reaction containers. As such, liposomes can be used for studies of chemical reactions in environments that mimic the natural situation in cellular environments, as opposed to bulk protocols, illustrated in the figure by a cuvette. The mimicking features come from the size matching and the lipid membrane material that constructs the liposome boundaries.

Structural features of the cell

A typical cell has an outer diameter (approximated as a sphere) of around 10-30 micrometers, defined by the outer plasma membrane boundary. The inner part of the cell consists of a highly complex and dynamic environment that upholds the functional internal units of the cell, which includes the nucleus containing the genome, the Golgi complex, the endoplasmic reticulum, mitochondria and a variety of small vesicular structures (Lehninger *et al.*, 1993). These structures are generally depicted as static and separated from each other in the cytoplasm, which is the fluid space in between the intracellular membrane compartments (Fig. 2). However, such structures are instead highly dynamic, are more or less in constant motion and communicate with each other through *e.g.* transport vesicles.

The endoplasmic reticulum (ER) can be described as a 3-dimensional mesh or network of membrane compartments that extends into the cytoplasm. The ER is continuous with the nuclear envelope and can be sub-divided into the rough endoplasmic reticulum (rER) and the smooth endoplasmic reticulum (sER). The rER, often depicted as flattened cisternae or flat membrane vesicles, provide sites where ribosomes synthezise proteins destined for the plasma membrane among other sites, while the sER, often depicted by a more tubular character, is the site for lipid synthesis, for example.

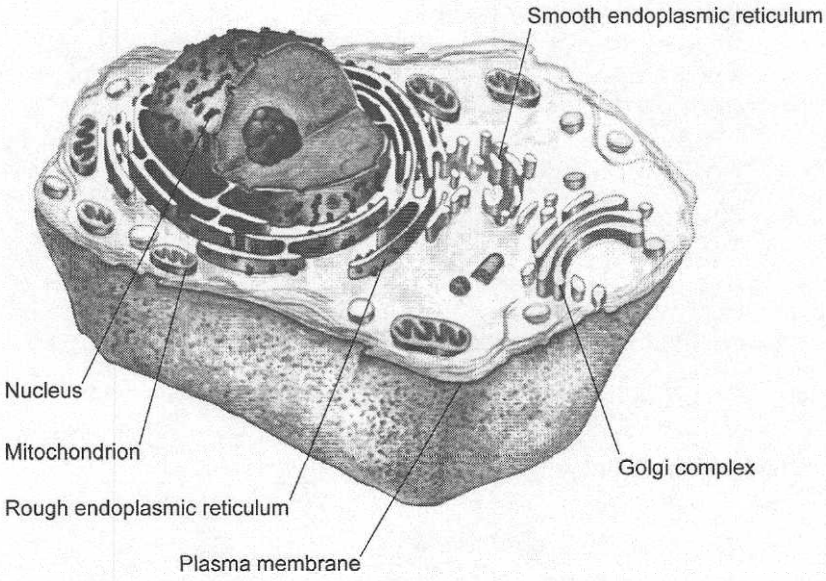

Figure 2. Schematic image of a eukaryotic animal cell. The outer boundary of the cell is defined by the plasma membrane, which protects the cytosol and the organelles in the cytoplasm. The image shows the nucelus, the rough endoplasmic reticulum, the smooth endoplasmic reticulum, the Golgi complex and the mitochondria.

The Golgi complex can be described by an assembly or cluster of cisternae. The side of the Golgi complex facing the rER is called the cis side, while the side facing the plasma membrane is called the trans side. Proteins that have been synthesized in rER are transported in vesicular carriers and merge with the cis side of the Golgi complex. Lately, is has also been suggested that some of this transport may occur through lipid nanotubes that interlink the ER and the Golgi complex, and that differences in membrane tension between these compartments give rise to a lipid flow (Sciaky *et al.*, 1997). During the passage through the Golgi complex to the trans side, proteins are modified by enzymes. One of the key functions

of this modification is to put a "name tag" of the proteins final destination, so that the modified protein ends up at the right location after leaving the trans side by another transport vesicle that is budded of the Golgi complex. Lipid nanotubes as structural elements inside cells have been identified in a number of papers (Ladinsky *et al.*, 1994; Clermont *et al.*, 1995; Sesso *et al.*, 1994; Rambourg and Clermont, 1990; Cooper *et al.*, 1990), which implies that these structures may be of great importance as, for example, material transport carriers and communication links between organelles (Sciaky *et al.*, 1997; Iglic *et al.*, 2003).

The nucleus contains the genetic information, the genome. The nuclear envelope that surrounds the nucleus is constructed by two membranes very close to each other and pinched together at certain sites called nuclear pores. The nuclear pores allow controlled transport of molecules between the nucleus and the cytoplasm by specific protein transport complexes. The deoxyribonucleic acid (DNA) in the nucleus is condensed and tightly packed into chromatin fibers, which consist of DNA and positively charged proteins, histones. This allows the entire length of DNA, which is about 2 meters, to be packed into the tiny volume of the nucleus.

The mitochondrion is the power plant organelle with a size of around 1 micrometer in diameter. The membrane structure is very complex, consisting of an outer membrane and an inner membrane, which is a highly folded and wrinkled structure. The matrix or the inner volume of the mitochondrion is therefore depicted as a concentrated aqueous solution of enzymes and intermediates in the energy producing metabolism, with the capacity to generate ATP (adenosine triphosphate). The fact that mitochondria contain their own DNA, RNA and ribosomes have lead to theories that suggest that the mitochondrion is a descendant of aerobic bacteria that had a symbiotic relationship with early eukaryotic cells.

The cytoskeleton of the cell provides stability for the cell shape, but also gives organization to the cytoplasm. The cytoskeleton is usually subdivided into actin filaments, microtubules and intermediate filaments and together creates a mesh of protein filaments throughout the entire cell. Both actin filaments and microtubules provide ways to create motion of, for example, organelles by the use of ATP and molecular motors.

The final pictorial outcome of this short exposé of cellular components is of course that the cytoplasm is a highly crowded place, with a very dynamic character and still able to perform finely orchestrated complex chemistry by tight control and ordering.

What is the Matrix? – Control.
Morpheus, The Matrix

The cytoplasmic reaction environment

How does the cell maintain order and control of chemical reaction events in the complex matrix that is defined by the inner volume of the cell? Schrödinger characterized life as the ability to establish and maintain order and many mechanisms to this ordering have been proposed through the years since then (Pagliaro, 2000). One of the key features of the cell is the high degree of compartmentalization, suggesting that this structural confinement and segregation of different micro/nanoenvironments is important. The normal view of the intracellular compartments is the membrane-bound compartments, such as the nucleus, the Golgi complex, the ER and mitochondria etcetera, that are separated from the surrounding cytoplasm. However, as opposed to these "stable" environmental compartments, other types of compartments are much more transient in character, such as protein complex assemblies, which provide functional compartments for substrates.

In a crowded and confined environment such as the interior of the cell, one also has to consider the effect of excluded volume in the system, which strongly reduces the effective volume that is present for the reactive molecules. There are also questions whether one can

really define the nature of the solvent, in this case water in such a highly crowded place. The high concentration of solvated ions together with the extremely large surface area that prevails in the intracellular environment can lead to an ordered state of the water on or near the surface of solvated ions and macromolecular assemblies. The total concentration of macromolecules in the cell, with protein being most abundant, is very high. The protein content can vary between 20 and 30% by weight, which is indeed very high, regarding the fact that some proteins have been crystallized in this concentration range (Pagliaro, 2000).

The simplest view of cytoplasmic organization and control is that compartmentalization can lead to faster catalytic throughput, when enzymes and substrates are packaged in small compartments. In addition to compartmentalization and crowding of reagents into small containers, the surface effects increases dramatically when the container shrinks, due to an increase in the surface-to-volume ratio. Surfaces of the intracellular environment are provided by membrane compartments and also macromolecular assemblies, such as proteins. It has been estimated that the total surface area of the membrane compartments can be as high as 100 000 μm^2 in a single cell of which the plasma membrane contributes with only a fraction of this number. The cytoskeleton also contributes with an extensive surface area in the interior of cells, and the total surface area inside the cell can thus reach twice the membrane surface value, that is up to 200 000 μm^2 (Luby-Phelps, 2000). Noticeably, the larger part of molecules such as proteins or enzymes is not directly involved in catalysis, but is instead coupled to protein-protein recognition, protein folding and intracellular targeting.

Many mechanisms involving surfaces as an active component in reaction pathways have been suggested. One of the earliest is the reduction of dimensionality. This type of mechanism brings molecules from 3-dimensional diffusion to instead move along surfaces, thereby reducing the dimensional freedom of motion to 2-dimensional diffusion. Examples of 1-dimensional restricted movement are also found, such as motion of DNA-binding proteins, which move along the DNA molecule. This type of reduction of dimensionality creates a functional compartment by reducing the distance to diffuse before encounter and thereby shortening diffusion times. Continuation of this type of behavior includes reversible adsorption, which involves transient localization of enzymes near a surface and also modification of the enzyme activity, where the surface attains the characteristics of an allosteric subunit, an effect which is called ambiquity. For example, it has been shown that some cytoplasmic enzymes display modified activities in the presence of cytoskeletal surfaces (Pagliaro, 2000).

Crowding of molecules also have the effect of pronouncing weak interactions and therefore provide additional driving forces for macromolecular association into assemblies that provides functional compartments that enhance catalytic rates. This process is called channeling (Ovadi and Srere, 2000). Channeling occurs in these functional compartments by increasing the probability of the reaction steps by concentrating the sequential enzymes in a reaction, which therefore makes it easier for intermediate products to be used as substrates for the next reaction step, due to the close proximity. This type of channels are usually dynamic in character, with a short life-time, but still pose interesting mechanistic solutions that might be of great importance. The complexity of the reaction environment can also be described by the number of different reactions that are performed within a single cell and by the intricate network or web of reaction pathways that can be illustrated by a metabolic pathway chart for example. The number of proteins synthesized by a typical eukaryotic cell by inspection of the mRNA (messenger ribonucleic acid) ranges between 10 and 20 000 different proteins, which indeed is a vast number considering the small size of the cell. Also, some enzymes and proteins have not only one but several targets and therefore affect several different pathways.

Biomembranes

Biomembranes or biological membranes are the walls that separate the cell from its surrounding environment and also constitute internal structures inside the cell, such as organelles (Golgi complex, endoplasmic reticulum and mitochondria, for example) and the nucleus. The functions of this wall structure include ways for the cell to regulate and control the influx and efflux of material, package and transport material inside the cell between different organelles, provide specific transport highways for certain reagents or signalling substances and of course to provide containment through formation of compartments inside the cellular volume (Lehninger *et al.*, 1993).

Many of these vital processes involve shape or morphology changes of a membrane surface, such as endocytosis (vesicle-mediated uptake of material) and exocytosis (vesicle-mediated release of material), which provide a way for the cell to communicate with their surroundings. The same shape changes also occur in internal trafficking of material, which often includes small vesicular structures or vesiculo-tubular structures (elongated vesicles or vesicles on lipid tubes) that can be budded of or fused with different organelles and function as transport carriers. On a slightly larger scale the cell must be able to grow, divide or copy itself and change its shape to adapt itself to the environment, for example, when the cell is growing on a surface. The membrane must therefore have a fluid character with a high degree of flexibility, to be able to bend easily and therefore be able to transform into shapes of various curvature, for example. The structural walls that have these critical features found in biomembranes are built by lipid molecules that form a bilayer (two opposing monolayers), due to the amphiphilic character of the lipid molecules. The two monolayers construct a wall with hydrophobic interior and hydrophilic surfaces on both sides. In a sense the biomembrane can be described as a 2-dimensional sheet or bilayer of lipid molecules with membrane-associated proteins that float around in the lipid bilayer, except those proteins interacting with the cytoskeleton, a model which is denoted as the fluid mosaic model (Singer and Nicholson, 1972) (Fig. 3).

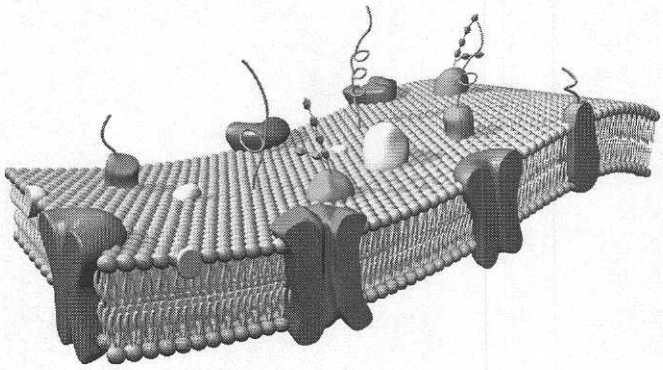

Figure 3. A schematic image of a piece of biomembrane according to the fluid mosaic model, where membrane proteins float around in a sea of lipid. Membrane proteins can span the entire membrane, so called integral proteins, or be associated to only one of the monolayers, which are then called peripheral proteins.

There is increasing evidence, however, that this "simple" description does not apply entirely in real biomembranes. For example, it has been demonstrated that lipids can segregate into domains and so called lipid rafts (Storch and Kleinfeld, 1985; Rodgers and Glaser, 1991; Glaser, 1993; Lipowsky, 2002), and that lipids can distribute laterally in the membrane in a regular pattern by formation of a superlattice (Somerharju *et al.*, 1999).

This thin sheet (~5 nm) is primarily held together by non-covalent interactions, giving the bilayer a remarkable flexibility required for the above listing of shape changes. As a consequence to the non-covalency and self-organizing properties of the bilayer, new lipids and proteins are synthesized and inserted into the bilayer without loss of the cell membrane integrity, during cell growth or division. The extreme thinness of the biomembrane can be better understood if one expands a typical cell (~10 μm diameter) into a sphere of 2 meters in diameter. The biomembrane would now still have a thickness of only 1 mm!

Passage of substances across the biomembrane barrier

The barrier to free passage of substances across the membrane lies in the properties of the lipid bilayer itself. Ions and or charged and/or polar substances have difficulties of passing the hydrophobic core of the lipid bilayer. Small molecules, such as water, can pass the membrane with ease as well as gaseous species such as oxygen; however, other vital substances must take use of transporters to be able to pass through the membrane. The membrane-associated proteins provide ways to transport nutrients and ions that are invaluable to the cell and waste products out of the cell. They also provide communication with the external environment, by signal transduction across the bilayer membrane (Fig. 4). This type of transport can be passive, but most often an active or directed transport occurs, thus requiring energy of some sort, usually provided by conversion of ATP (adenosine triphosphate) into ADP (adenosine diphosphate), a very accessible energy source in the cellular environment. This active directionality is usually needed to create and up-hold gradients of charge and/or concentration across the biomembrane.

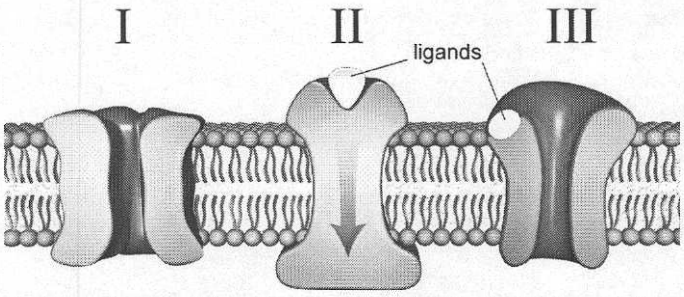

Figure 4. Schematic image showing different proteins embedded in a lipid bilayer. I. Transporter. II. Signal receptor. III. Ion channel. Transporters carry substances into and out of the cell. Signal receptors transmit a signal across the membrane upon binding of a ligand. Ion channels can be triggered to open up by binding of a ligand and allow transport of certain ions across the membrane.

Composition and organisation of biomembranes

If one examines the biomembrane composition between different cell types or even organelles within the same cell type, the variability and number of different lipid species is striking. The diversity of lipid species in biomembranes is probably coupled to the function of the membrane to some extent, for example, some proteins are only functional in the presence of a certain lipid. Also, many processes involve electrostatic control of protein adsorption, through charged lipids in the biomembrane, which itself can mediate a reaction taking place on the surface of the biomembrane.

Taking the examination of the biomembrane even further, one can also deduce an asymmetry of the distribution of the various lipid species between the two monolayers of the plasma membrane. For example, while phosphatidyl choline is mainly found in the outer monolayer of the plasma membrane, the majority of both phosphatidyl ethanolamine and phosphatidyl serine are situated in the inner monolayer (Langner and Kubica, 1999). This

most probably reflects the duality of the monolayers function, the outer monolayer providing more or less an inert barrier for the surrounding environment, while the inner surface provides sites for reactions to occur by the net charge that arises from, for example, the phosphatidyl serine component.

Lipids and lipid assemblies

Let us now focus on the building blocks of the lipid bilayer membrane. The word lipid means fat, thereby implying their insolubility in water-based solutions. This property comes from their structural features as they are amphiphiles that is they have both a hydrophilic (water-loving) and a hydrophobic (water-shedding) part. They therefore have a tendency to gather at interfaces, such as water-air or water-oil surfaces, and they are often referred to as surface-active molecules. The general structure of these amphiphiles involves a polar or charged headgroup, connected to one or two hydrocarbon tails. When placed in water based solutions, however, they can spontaneously self-assemble into structures where the hydrocarbon-water interaction is minimized (Evans and Wennerström, 1999; Lehninger *et al.*, 1993; Ben-Shaul, 1995). When a lipid molecule is exposed to water, the polar water will be reorganised from its bulk structure into a more ordered structure around the hydrocarbon chains, to be able to maximize the number of hydrogen bonds. The outcome of this water organisation is a lowering of the entropy of the system. Nature favours an increase in entropy or an increase in freedom and the water organisation around the lipid is therefore not favoured. However, if two lipid molecules exist in the water solution, some water molecules can be released into the bulk water if the two lipids associate and therefore expose less of their hydrocarbon chains. When less water is structured around the lipids, the entropy is increased and lipid association or aggregation is therefore a favoured process. This association process by increasing the entropy of the system is called the hydrophobic effect (Fig. 5).

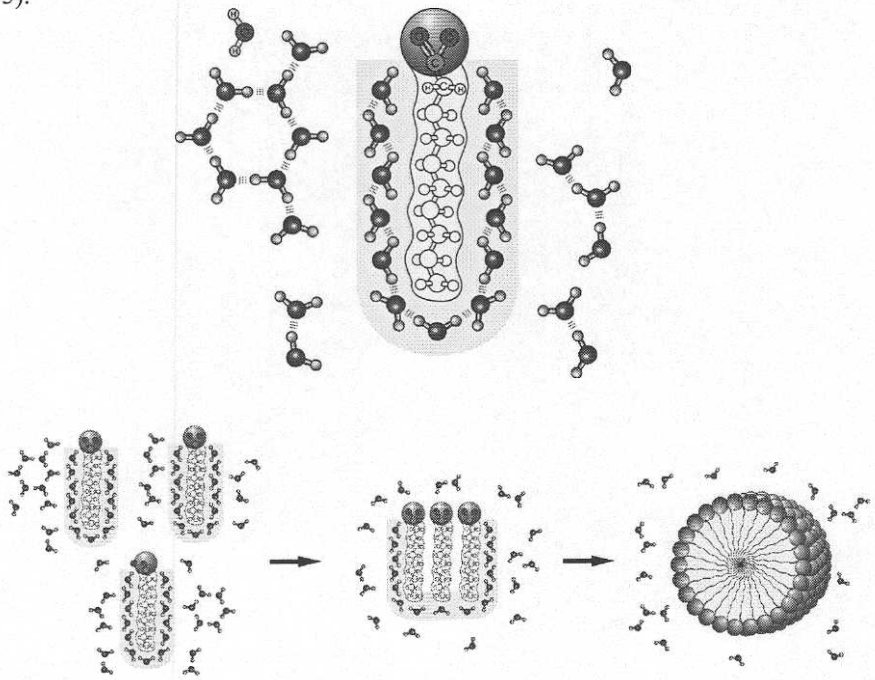

Figure 5. Schematic image of the hydrophobic effect. In bulk water, the number of hydrogen bonds is maximized, however in the prescence of long-chain fatty acids, for example, the water becomes ordered around the hydrophobic alkyl chains to be able to maximize the number of hydrogen bonds. This will lower the entropy of the system. However, through aggregation, less water is structured around the lipids and thus the entropy of the system is increased.

There are a plethora of aggregate structures that can arise and these are mainly dictated by the molecular geometry of the individual amphiphile that constructs the assembly. The individual amphiphiles can be packed into these assemblies according to delicate force balances at the interface region and inside the hydrophibic core. Looking at the interface, a number of different interactions, such as head group repulsions of purely excluded volume or electrostatic origin, acts to increase the average area per head group molecule, a. At the same time the hydrocarbon-water surface tension strives to minimize the total surface area, and thereby a decrease in head group area. The hydrophobic parts, on the other hand, experience a restricted conformational freedom, lowered entropy, since they are connected to the interface parts. Together with the tight packing of the hydrocarbon chains, this leads to inter-chain repulsion, which of course also depends on the geometry and curvature (bending) of the assembly (Ben-Shaul, 1995). The attractive force can be described as γa, where γ is the interfacial tension. The repulsive force on the other hand can be approximated by a constant, K, and an inverse proportionality of the headgroup area a. Together, the sum of attractive and repulsive forces can be expressed as the simple relation

$$\mu_N{}^0 = \gamma a + \frac{K}{a} \qquad \text{(Equation 1)}$$

in order to describe the interfacial free energy per molecule, $\mu_N{}^0$, in an aggregate. The minimum energy can therefore be found by taking $d\mu^0/da = 0$, which is shown in figure 6, which gives an equilibrium area a_0.

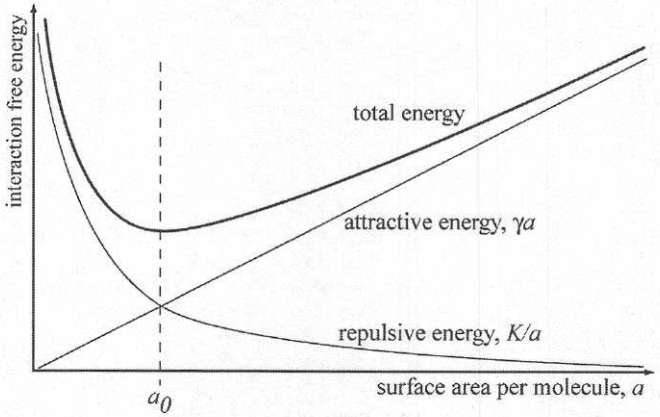

Figure 6. A plot of the interaction free energy, $\mu_N{}^0$, as a function of head-group surface area, a. The attractive and repulsive energies are combined into the total free energy with a minimum in energy located at a_0, which is the optimal head-group area.

In summary, a qualitative picture of the assembly structures can be visualized by defining a geometric shape factor, P, which depends on the optimal headgroup area , a_0, the hydrocarbon chain(s) volume, v, and hydrocarbon chain(s) length (at full extension), L (Glaser, 2001).

$$P = \frac{v}{a_0 L} \qquad \text{(Equation 2)}$$

If P is close to 1, the geometry of the amphiphile should be approximated by a small cylinder, which is a matching in the sizes of headgroup and the hydrocarbon chain(s). This type of amphiphile usually assembles into close to planar monolayer structures and to protect the hydrocarbon chains from the surrounding environment, two opposing monolayers create a bilayer structure. If the shape factor, P, is smaller than 1, this implies a structure where the headgroup is larger than the hydrocarbon tail(s), attaining the shape of a cone. The assembly structures of this type of amphiphile usually consist of a sphere, with the hydrocarbon tail(s) inside the core of the small sphere. On the other hand if the shape factor is larger than 1, the hydrocarbon chain(s) occupies larger space than the headgroup, resulting in an inverted cone structure, and therefore assemble into spherical structures with the hydrocarbons facing the outer environment and the headgroups facing the inside, thereby enclosing a small volume of polar solvent such as water, for example. These two latter structures are referred to as micelles and inverted micelles respectively. Of course one could also imagine situations where a number of amphiphiles are mixed and therefore assemble into a structure where each amphiphile contribute to the final structure. Through this, cone-shaped and inverted cone-shaped amphiphiles can be mixed with cylindrical amphiphiles to assemble into planar bilayer structures (Fig. 7). Differentiation of the molecular shapes of the lipids are also believed to enable the various shape changes that occur in a biomembrane, which require some sort of bending or curvature change (Lipowsky, 2002).

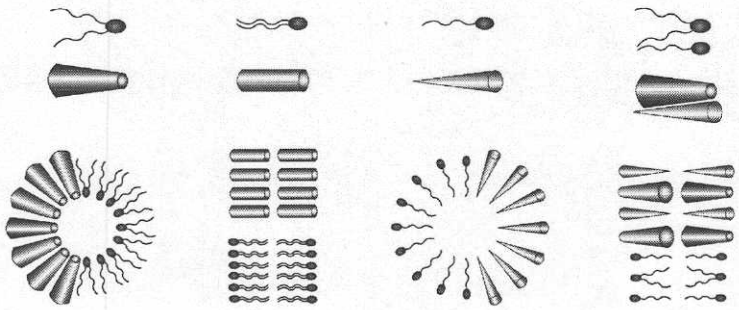

Figure 7. The figure shows different aggregate structures that can be created depending on the structure of the amphiphile. If the hydrocarbon chains occupy a larger space than the headgroup, inverted micelles are formed, as shown in the figure to the left. In the opposite situation, where the headgroup is larger than the hydrocarbon chains, micelles are formed, third from the left. If the amphiphile instead resembles the structure of a cylinder, where the headgroup and hydrocarbon chains more or less match each other in size, planar bilayers are formed, second to the left in the figure. Planar bilayers can also be created by mixing lipids that resemble cones and inverted cones, as shown to the right.

Biomembrane lipid constituents

Biomembranes contain a number of different lipids and proteins as mentioned above. The exterior plasma membrane can be visualized as an essentially flat membrane. Such flat membranes are thus most easily constructed by lipids or amphililes that have shape factors close to one, or assemblies that match up by mixing cones and inverted cone structures. There are mainly three different classes of lipids in the biomembrane, namely phospholipids, sphingolipids and sterols. The most common lipids in the biomembrane are the 1,2-dialkylphosphoglycerides or phospholipids (Gennis, 1989). These include, for example, phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl glycerol (PG). The structure of these phospholipids are summarised in figure 8.

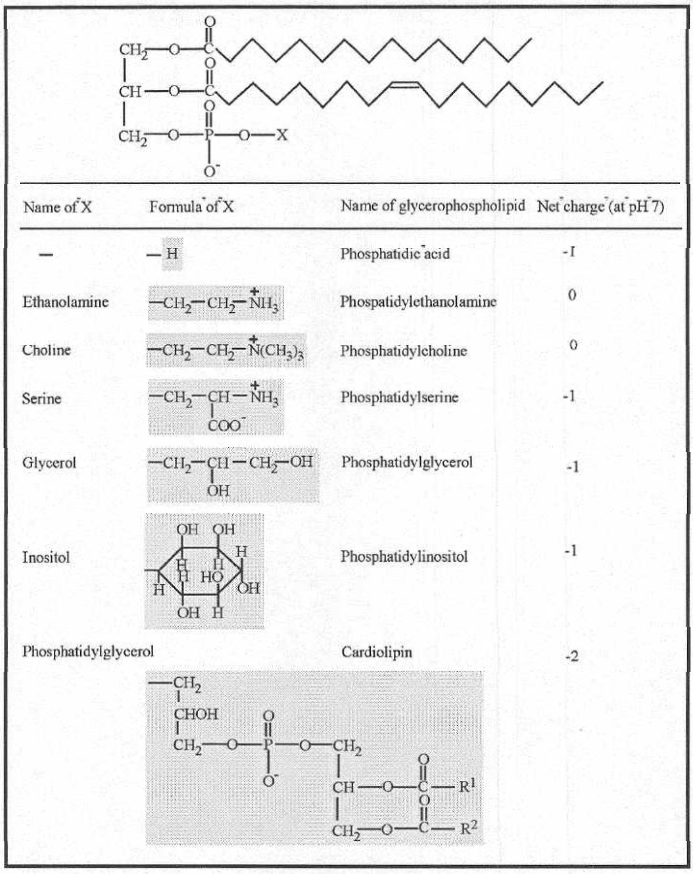

Figure 8. The structure of glycerophospholipids. The backbone of these structures is a glycerol molecule that is linked to two alkyl chains or fatty acids of various degree of saturation through ester bonds. The third link through the phosphate molecule defines the head-group of the lipids. The figure also states the net charge of the lipid at pH 7. Phosphatidyl choline is a zwitterionic lipid, which means that the lipid contains both negative and positive charges that cancel each other at neutral pH values.

The phospholipid structures consist of two alkyl chains or fatty acids that are bound to a common glycerol molecule by ester bonds. The third hydroxyl group is linked to a phosphate molecule, which is connected to the various head-groups of the lipids. The alkyl chains or hydrocarbon tails varies both in lengths (from 14-24 carbon atoms) and degree of saturation, which together dictates such fundamental properties as permeability and fluidity of the membrane, for example. The head-group on the other hand contains information of the charge of the molecule, which also affects the properties and functionality of the membrane. The most common phospholipid is phosphatidyl choline, whose head-group consists of a tertiary amine. This type of lipid is zwitterionic, which means that the structure bears a net charge of zero at neutral pH values. This occurs by balancing the charges that is located on the phosphate (negative charge) and the tertiary amine (positive charge). Phosphatidyl serine on the other hand obtains a net charge of -1 at neutral pH, since it contains both a carboxyl group (negative charge) and an amine on the head-group.

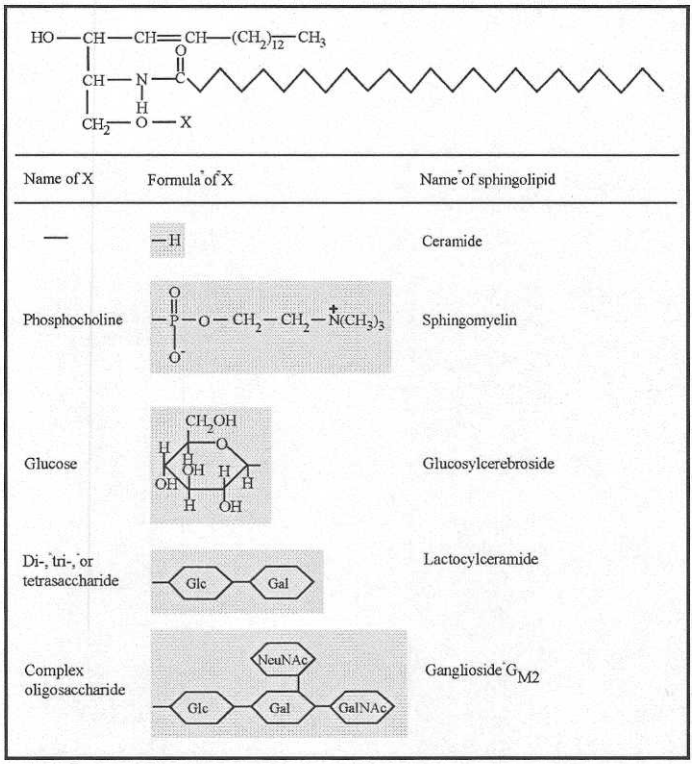

Figure 9. The structure of sphingolipids, containing a long-chain amine alcohol sphingosine as a backbone, together with a long-chain fatty acid and a polar head alcohol, which can be further linked to other polar head-groups via, for example, a phosphodiester linkage. The simplest compound in this group is the ceramide and the image also shows examples from the three different groups of the sphingolipids: sphingomyelins, glycolipids and gangliosides. The symbols for sugars used in this image are: Glc, D-glucose; Gal, D-galactose; GalNAc, N-acetyl-D-galactosamine; NeuNAc, N-acetylneuraminic acid (sialic acid).

Sphingolipids are also common in biomembranes and consist of one molecule of the long-chain amino alcohol sphingosine or one of its derivatives, one molecule of a long-chain fatty acid and a polar head alcohol, which sometimes have a phosphodiester linkage. The sphingolipids can also be sub-divided into three groups, sphingomyelins, glycolipids and gangliosides (Fig. 9). Sphingomyelins have similarities with phosphatidylcholines in properties and structure and are present in plasma membranes of animal cells. The glycolipids and gangliosides are also found in animal cell plasma membranes, with a high presence in neural tissues, such as the brain, and have sugar units attached to their polar head groups.

The sterols of which cholesterol is the most common in animal tissues have a polar head-group and a non-polar hydrocarbon body with a length about the same as a 16-carbon fatty acid in its extended form. Sterols are often precursors for molecules with specific biological functions, such as the bile acids that act as detergents in the intestine or steroid hormones.

Pure lipid bilayer membranes

Pure phospholipids membranes can have features that are attributed to both liquids and solids and are therefore sometimes referred to as liquid crystals. However, the liquid and solid

properties exist in a very thin sheet and therefore the lipid membrane can be viewed as a 2-dimensional liquid (Fig. 10).

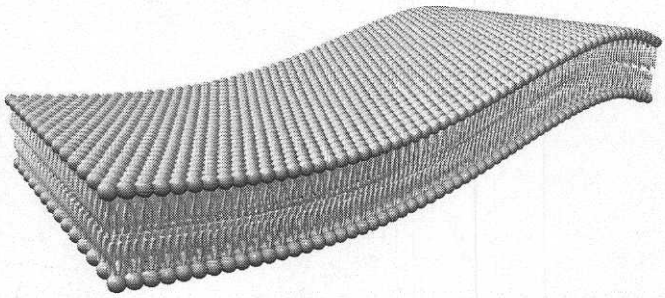

Figure 10. Schematic image showing a fluid lipid bilayer membrane, represented as a two-dimensional liquid sheet, which is constructed of two opposing monolayers, where the polar headgroups face the surrounding aqueous solution.

The properties of this assembly can change with temperature and attain different phases and is therefore a thermo-tropic material. At ambient temperatures, such as body temperature and room temperature, most lipids display a liquid phase, with high lateral freedom of motion within the opposing monolayers and undergo lateral Brownian motion, with diffusion coefficients ranging between 10^{-12} – 10^{-11} m^2/s (Almeida and Vaz, 1995). The crossing or exchange of lipid constituents between the two different monolayers is fundamentally very improbable, due to the transport of bulky polar and even charged head-groups through the inner hydrophobic core of the membrane. This transport is often referred to as "flip-flop" of the lipid molecules and usually occurs on time-scales of days or even longer (Wimley and Thomson, 1991; Svetina *et al.*, 1998). However, under substantial mechanical stress of the bilayer, for example during tether formation, this type of intermonolayer exchange can be more pronounced (Svetina *et al.*, 1998). Exchange rates between the membrane and the solution is also very low for bilayer-forming lipids, which depends on the solubility limit or CMC, critical micellar concentration, for the amphiphile. The CMC for micelle-forming amphiphiles ranges from 10^{-2} to 10^{-4} M, while the CMC, or better, critical association concentration, for bilayer forming lipids ranges between 10^{-7} and 10^{-12} M (Israelachvili, 1992; Sackmann, 1995). This affects the residence times, τ_r, for the amphiphiles in the membrane. For bilayer lipids this time is approximately 10^4 seconds, while for micelle amphiphiles this time is approximately 10^{-4} seconds. As mentioned above the temperature can affect the membrane, for example, lower temperatures can give rise to more structural ordering of the membrane and at some point solidify or freeze the membrane, thus producing an ordered solid crystalline phase. This type of behaviour typically occurs in lipid membranes with one pure component and it is therefore possible to ascribe a certain type of lipid with a critical transition temperature, T_c, which describes the temperature where the liquid and solid states are equally probable (Evans and Needham, 1987). The transition between the fluid and the solid crystalline phase, sometimes display intermediate crystalline phases, referred to as gel-phases, due to a higher degree of disorder. It has also been demonstrated that two different phases can coexist in a single bilayer membrane, by fluorescent staining of the two phases and the use of confocal fluorescence microscopy (Korlach *et al.*, 1999).

As a two-dimensional fluid, the lipids in the bilayer have a high degree of lateral and rotational freedom. Interactions between lipids in the monolayers still persist to some extent and thereby give rise to a shear surface viscosity of the bilayer. This surface viscosity, η_s, is a two-dimensional parameter, but can be converted into a three-dimensional viscosity, η_3, by

division of the surface viscosity with the thickness of the bilayer, h (Chizmadzhev *et al.*, 1999).

$$\eta_3 = \eta_s/h \qquad \text{(Equation 3)}$$

Measurements of η_s have given values from 10^{-3} to 10^{-7} g/s from cell membranes, lipid bilayer membranes and lipid monolayers (Meiselman *et al.*, 1978). The highest value comes from cells, where additional forces arise from cytoskeleton-membrane interactions. The lowest value can be compared to the viscosity of olive oil, η_3 = 1 Poise = 1 g/s cm. Several different techniques have been developed to study and measure the shear surface viscosity of a membrane. Lateral diffusion of fluorescent probes situated in the membrane can give information of the mobility and the surface viscosity of the membrane. Values for the surface viscosity using this technique usually range between 10^{-6} to 10^{-5} g/s (Meiselman *et al.*, 1978; Tocanne *et al.*, 1994; Galla *et al.*, 1979). Giant unilamellar liposomes or vesicles (GUVs) have the advantage of being observable and manipulated under a microscope, which has lead to different techniques to estimate the surface viscosity of GUVs. Tether pulling, falling ball viscosimetry and optical dynamometry are all techniques that can be used to extract information regarding the shear surface viscosity (Dimova *et al.*, 2002).

Liposomes

One of the more well-known structures that can assemble in water solutions is the liposome, which consists of a self-closed lipid bilayer. The appearance of liposomes or lipid vesicles as they are also called in the literature dates back to as early as the middle of the 19th century. Back then scientific research was performed on the colloidal behaviour of lecithin and other phospholipids. Lecithin had earlier been isolated from brain tissue by using hot ethanol. Much later, more than 100 years later actually, characterisation of liposomes started to appear in the literature. Alec Bangham used electron microscopy, osmotic and permeability studies to confirm that the liposomes were actually separated from the aqueous phase and also entrapped part of the solvent in which they were formed, as shown in figure 11 (Lasic, 2000).

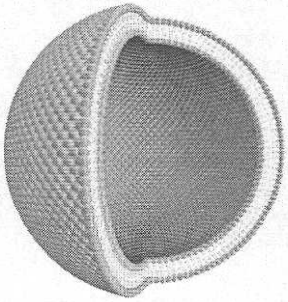

Figure 11. A schematic image showing a unilamellar liposome consisting of a single bilayer that encloses a volume of fluid, which is separated from the external medium. The liposome shown in the image has been cut open in order to show the bilayer structure.

Liposomes can be classified according to their morphological appearance (Lasic, 1993). Based on their size, the liposomes can be referred to as small (20-50 nm diameter), large (50-1000 nm diameter) and giant (> 1 μm) liposomes. The liposomes are then further classified according to the number of bilayers or lamellae they contain. Therefore names such as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), giant unilamellar vesicles (GUV) and multilamellar vesicles (MLV) and many other descriptions of liposomes appear in the literature (Fig. 12).

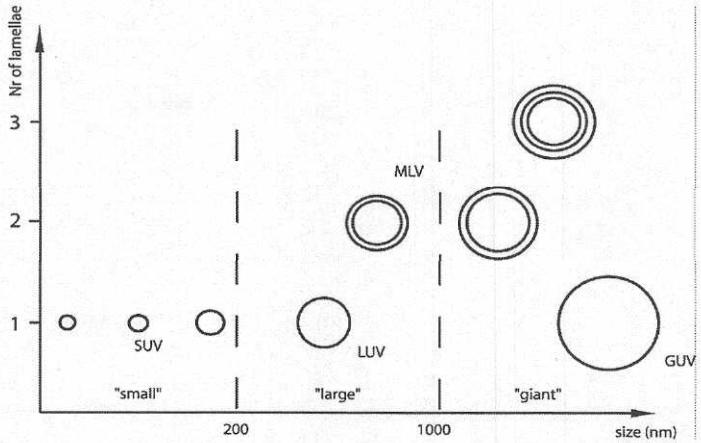

Figure 12. The liposomes can be classified according to their size and number of lamellae or lamellarity. Small unilamellar liposomes or vesicles (SUVs) range between a few tens of nanometers up to 200 nm. Large unilamellar vesicles (LUVs) range between 200 nm and 1 micrometer, which is followed by giant unilamellar vesicles that range between 1 μm up to hundreds of micrometers. If the liposome contains more than one bilayer it is usually classified as a multilamellar liposome.

Formation of liposomes

The formation of liposomes can be achieved by many existing protocols. When it comes to the formation of giant unilamellar vesicles, the type which has mainly been used in this thesis, the thin film hydration technique is by far the most commonly used. This technique was first described by Reese and Dowben and involves the deposition of a thin lipid film with subsequent careful hydration in a moist nitrogen atmosphere (Reese and Dowben, 1969). The lipid is then swelled in aqueous solution above the critical temperature T_c in order to maintain the fluid character of the bilayer. Vesicles are created by self-closure of the bilayer in a process called budding, where the connections to the original lipid sheet, or tethers as they are also called, are severed through convection or gentle agitation.

There are several parameters that affect the yield of formation of giant vesicles. One of the most important is the interbilayer separation, when swelling the lipid. If the bilayer separation is small, the formation of giant vesicles is less probable. One way to increase this separation before and during the swelling is to increase the temperature. An increased temperature leads to a higher degree of undulations or vibrations of the bilayers, which subsequently promotes the bilayer separation by sterically occupying a larger space. Besides sterically increased separation, an electrostatic repulsion can also increase the bilayer separation. Through this, lipids of the same charge repel each other when situated in opposing bilayers in close contact. In accordance to this, the ionic composition of the rehydrating media should also affect the swelling procedure. The formation of giant vesicles under physiological salt conditions is very hard and normally much lower ionic strengths must be utilised. Divalent cations, such as magnesium, Mg^{2+} and calcium, Ca^{2+}, can also function as bridging ligands, thereby attracting negatively charged bilayers and decreasing the bilayer separation. In contrast a number of articles have surfaced lately, describing a positive effect of divalent cations on the formation of giant unilamellar vesicles (Akashi *et al.*, 1996; Akashi *et al.*, 1998). This is mainly thought to be an effect of reversing the charge of the bilayer to net positive thereby increasing the electrostatic repulsion again, and hence promote vesicle formation.

Another preparation technique that has attracted much interest lately is the electroformation technique (Angelova and Dimitrov, 1986; Angelova, 2000). Briefly, lipids dissolved in an organic solvent or mixtures of organic solvents are deposited and dried onto

two platinum electrodes. An electric field is applied and the chamber containing the two electrodes is filled with water or aqueous solution. A number or various field strengths have been tried for various lipids and lipid mixtures (Fischer *et al.*, 2000). Normally, the starting voltage is around 0,2 Volts with a frequency of 10 Hertz, when the filling of the chamber starts. The voltage is then raised to between 1 and 7 Volts for a period of time, before the frequency is lowered in the end of the procedure, normally to around 0,5 Hertz. This is believed to promote the separation of the vesicles from the electrodes and attaining a spherical structure. This is followed by a gradual decrease of the voltage back to 0,2 Volts. The electroformation method produces giant vesicles in the size range of 5-200 μm in diameter with usually a slight excess of membrane area, resulting in a flaccid fluctuating vesicle. The tension of the vesicle seems to be dependent on the kinetics of the formation of the vesicle. The underlying mechanism for electroformation is thought to be mainly the electroosmotic periodic movement of the water medium, which creates vibrations perpendicular to the electrode surface and therefore pulling lipid lamellae apart and inducing vesicle formation. Several vesicles can also merge together at the electrode surface, thereby increasing the size, before detachment occurs.

The multilamellar vesicle (MLV) is easiest to produce by rehydrating dry lipid followed by vortexing or vigorous shaking. This type of vesicle suspension or lipid dispersion contains a large size distribution of vesicles with different lamellarity and 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 dipersion. The MLVs undergoes defragmentation into smaller vesicles and with decreasing number of lamellae. Both of these types of liposome suspensions can then be dehydrated as described above to increase the size of the vesicles and thus produce large and giant vesicles. Controlled reduction in the size and the lamellarity can also be done by extrusion protocols, where the multilamellar vesicles are extruded through polycarbonate filters of well-defined pore size. Alternative preparation methods include reversed phase evaporation and ethanol injection. Reversed phase evaporation includes the removal of organic solvents, in which the lipids are dissolved, from an emulsion. The ethanol injection protocol is based on the injection of lipids dissolved in ethanol into a larger volume of aqueous phase, where the ethanol is diluted and the lipids assemble into lamellae that self-close to form vesicles.

Trapping efficiency

One of the most important features of vesicles, which is very dependent on the method of preparation, size of vesicles, lamellarity etc, is the so called entrapment or trapping efficiency. The entrapment efficiency is defined as the amount of solute entrapped within the vesicle as opposed to the total amount of solute in the solution during preparation (Imai *et al.*, 2000). Multilamellar vesicles usually have low trapping efficiency due to the internal complex structuring. Values normally range between 5 and 15%, however, higher values have been reported in the literature. Small unilamellar vesicles have an even lower trapping efficiency, usually because of their small size, and values of less than 10% are normally reported. Vesicles that form upon dehydration-rehydration procedures are more efficient and values between 15 and 45% are found in the literature and are therefore a good choice when high trapping efficiency is a requirement (Walde and Ichikawa, 2001).

Giant unilamellar liposomes

In this thesis giant unilamellar or thin-walled liposomes have been used. A dehydration/rehydration technique described by Criado and Keller (Criado and Keller, 1987) was used with modifications to produce the giant unilamellar liposomes or vesicles, GUVs,

(Karlsson *et al.*, 2000). One of the advantages of this technique is the possibilty to produce vesicles using high ionic strengths in the buffer. Entrapment of buffer salts occur in the dehydration step which will lead to to osmotic pressure differences when the lipid is rehydrated and result in an influx of water to swell the lipid film.

The main advantage of this type of vesicle is of course the ability to visualise them under an optical microscope. Through this, GUVs provide unique opportunities to study interactions of near 2-dimensional surfaces in three-dimensional space with broad research areas in, for example, physical chemistry and statistical physics (Döbereiner, 2000). Due to their size, shape changes and thermal fluctuations of the membrane that exist above the transition temperature are easily observable. By application of forces or by changing the chemistry in the environment, one can achieve variations in the vesicle configurations. The shape of the vesicle is determined by several parameters, including the enclosed volume and membrane area, but also on the elastic energy of the membrane that arises due to bending deformations. Area and volume are usually fixed (at constant temperature and osmotic conditions). If there are any osmotic imbalances, this difference will be eliminated by flux of water across the membrane, since the lipid bilayer is a semi-permeable membrane with quite high water permeability. The total lipid area can be considered fixed due to the extremely low exchange rate of lipids between the membrane and the bulk solution as described above.

To be able to understand what types of shapes are possible for a lipid assembly, such as the liposome, a closer look at the mechanics of lipid membranes is needed.

Mechanics of lipid membranes

Many theoretical approaches to the mechanics of lipid bilayers have been described, where the lipid bilayer is usually depicted or approximated by a thin elastic shell (Canham, 1970; Helfrich, 1973; Evans, 1974). Upon force application, this thin sheet can undergo shape changes that affect the elastic energy of the system and there are three main types of deformation that can be applied to the elastic shell (Evans and Needham, 1987).

Stretching

The first is an isotropic stretching (Fig. 13) or in other words an increase or dilation of the membrane area from the starting equilibrium area.

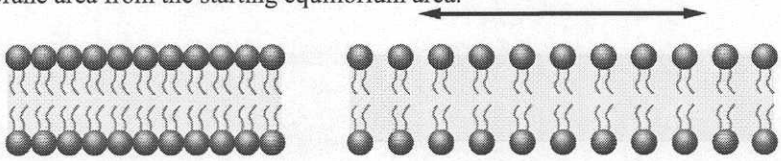

Figure 13. Schematic image illustrating in plane stretching of a lipid membrane. (**left**) An unstretched lipid membrane. (**right**) A stretched lipid membrane.

Conversely it can also describe the condensation or compression of the area, although lipid membranes are often referred to as being incompressible. The energy of this type of deformation (isotropic stretching or compression) can be described by a Hookean relation:

$$E_{stretch} = \frac{K_a}{2A_0}(A - A_0)^2 \qquad \text{(Equation 4)}$$

The equilibrium area is denoted by A_0 and the stretched membrane area is A. The elastic stretching or compressibility modulus is described by K_a, which is dependent on the lipid composition of the bilayer.

Shearing

The second type of deformation is the in-plane extension of a membrane at constant surface area. This type of deformation occurs only in bilayers that are frozen or in other words subsides in an environment where the temperature is kept below the transition temperature. Lipid membranes that are in the liquid phase have no resistance to shearing due to the freedom of lateral motion within the monolayers. The surface shear energy can be described by

$$E_{shear} = \mu_s \beta \qquad \text{(Equation 5)}$$

The shear energy is thus dependent on a shear modulus for the membrane, μ_s, and the degree of deformation, β. This term is often neglected, since most experiments are performed at temperatures above the transition temperature and the lipid exists in the fluid phase.

Bending

Since the number of molecules in the membrane is essentially fixed, an equilibrium area of the unperturbed membrane exists. The bilayer is only 4 nm across as mentioned previously, while the giant vesicles are in the size range of 5-50 μm in diameter usually. This separation of length scales enables the description of the surface as a 2-dimensional surface in 3-dimensional space (Seifert, 2000). Any such surface can be described by two principal

curvatures, c_1 and c_2, which are the reciprocal of the orthogonal radii, r_1 and r_2 respectively (Fig. 14, 15).

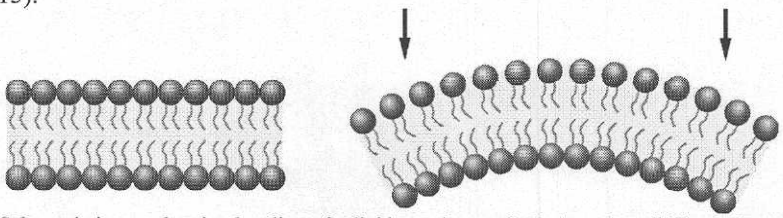

Figure 14. Schematic image showing bending of a lipid membrane. (**left**) An unbent lipid membrane. (**right**) A bent lipid membrane.

The two principal curvatures can be used to obtain the mean curvature, H, and the Gaussian curvature, K, of the membrane. The mean curvature is therefore described by

$$H = \frac{(c_1 + c_2)}{2} = \frac{\left(\frac{1}{r_1} + \frac{1}{r_2}\right)}{2} \qquad \text{(Equation 6)}$$

The Gaussian curvature on the other hand describes the product of the two curvatures

$$K = c_1 c_2 = \frac{1}{r_1 r_2} \qquad \text{(Equation 7)}$$

This means that for cylindrical geometries, where one of the curvatures is zero, the Gaussian curvature is zero and the other curvature is the reciprocal of the cylinder radius. For spheres, $c_1 = c_2 = 1/r$, where r is the radius of the sphere and for planar surfaces of course both $c_1 = c_2 = 0$. At so called saddle points, where $c_1 = -c_2$, the mean curvature is zero. Figure 15 show some of the deformations for a membrane surface element.

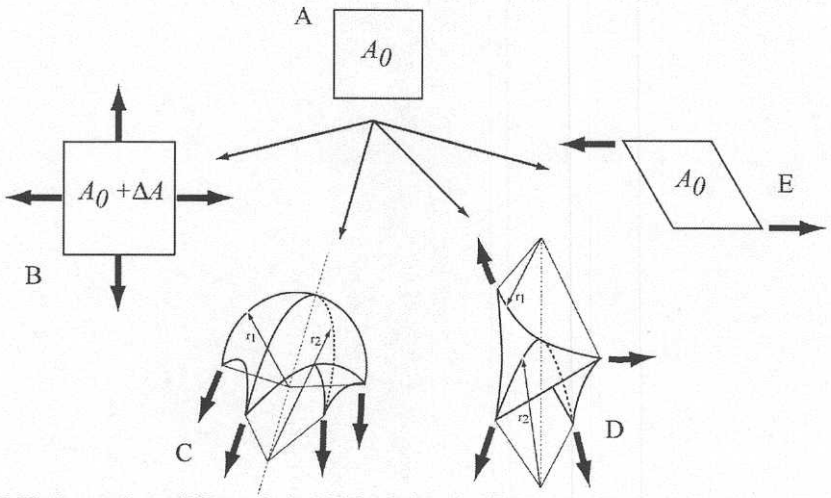

Figure 15. The image shows different kinds of deformation of a lipid membrane. (**A**) shows the starting situation, with an equilibrium area A_0 for the lipid membrane. (**B**) shows an isotropic stretching or dilation of the membrane, which results in an increased area by ΔA. (**C**) and (**D**) both illustrate two types of bending of the membrane, creating different curvatures for the surface. (**E**) shows an in-plane shear deformation of the surface at constant surface area, A_0.

The local energy cost for bending this type of membrane is therefore the sum of these two terms multiplied by the local bending, k_c, and Gaussian bending, k_G, moduli respectively

$$E = \frac{k_c}{2}(2H)^2 + k_G K = \frac{k_c}{2}(c_1 + c_2)^2 + k_G c_1 c_2 \qquad \text{(Equation 8)}$$

A vesicle is a topological sphere, having a genus of zero. In practice the topology of the vesicles does not change, since this would imply the formation of edges and holes, which cost considerable amounts of energy. Therefore, when integrating over the entire surface, the second term describing the Gaussian bending can be neglected since it will be constant no matter the shape of the vesicle (Seifert, 2000).

Spontaneous curvature

The lipid membrane has so far been described as a symmetric bilayer, which implies that the chemical composition and environment is the same (Seifert, 2000). This type of discrepancy can be corrected by introducing a preferred or spontaneous curvature, c_0, which a membrane would have in reality (Döbereiner, 2000). Therefore the equation now becomes

$$E = \frac{k_c}{2}(2H - c_0)^2 = \frac{k_c}{2}(c_1 + c_2 - c_0)^2 \qquad \text{(Equation 9)}$$

The bending energy of the vesicle, E_{ves}, therefore becomes

$$E_{ves} = \frac{k_c}{2}\int (c_1 + c_2 - c_0)^2 \, dA \qquad \text{(Equation 10)}$$

By summing up both stretching and bending energy contributions one obtains

$$E_{vestot} = \frac{K_a}{2A_0}(A - A_0)^2 + \frac{k_c}{2}\int (c_1 + c_2 - c_0)^2 \, dA \qquad \text{(Equation 11)}$$

What energy parameter contributes most? One can make a gedanken experiment, where the bending and stretching contributions can be compared to some extent. Consider a perfect spherical liposome with no spontaneous curvature, i.e. $c_0 = 0$, $c_1 = c_2 = 1/R$, where R is the radius of the sphere. The curvature energy of a sphere is therefore

$$E_{curv} = \frac{k_c}{2}\int (c_1 + c_2)^2 dA = \frac{k_c}{2}\left(\frac{2}{R}\right)^2 4\pi R^2 = 8\pi k_c \qquad \text{(Equation 12)}$$

The bending energy of a sphere is therefore independent of the size of the sphere! This reasoning can be viewed as the energy that is needed to bend a flat membrane into a sphere. The bending rigidity or modulus, k_c, usually have values near 10^{-19} Joules for lipid bilayer membranes (Rawizc *et al.*, 2000). The bending energy is thus $2{,}5*10^{-18}$ Joules for a perfect spherical liposome. If instead this liposome is expanded in size by area stretching instead the energy can be calculated from the stretching energy above. By considering an area increase of only 1% of a vesicle having a radius, R, of 10 μm and an area expansion modulus, K_a, of 200 mN/m, the stretching energy equals

$$E_{stretch} = \frac{K_a}{2A_0}(1{,}01A_0\text{-}A_0)^2 = \frac{10^{-4} K_a 4\pi R^2}{2} \approx 1{,}25*10^{-14}\, Joules \qquad \text{(Equation 13)}$$

The stretching of the bilayer followed by an area increase of only 1% is thus 20000 more costly than the bending of a flat membrane into a sphere!

Area difference elasticity

Up to now the theory describes a thin elastic sheet or shell with no concerns on the bilayer architecture. The fact that the bilayer consists of two opposing monolayers is missing so far. The monolayers are coupled together; however they have more or less unrestricted freedom (especially flat surfaces) to move laterally over each other to relieve stress that is imposed on the membrane (Seifert, 2000; Döbereiner, 2000; Svetina *et al.*, 1998; Miao *et al.*, 1994). Also, the exchange of lipid between the monolayers is very slow, thus conserving the number of lipid molecules that constitute the two monolayers. The difference in the lipid number gives rise to a preferred area difference ΔA_0 as opposed to the actual area difference, ΔA, that is described by

$$\Delta A = h \oint (c_1 + c_2) dA \qquad \text{(Equation 14)}$$

where h is the distance between the neutral surfaces of the bilayer (approximately half the bilayer thickness). The difference between the two states also cost energy to achieve and this gives rise area difference elasticity term

$$E_{ADE} = \frac{k_r}{2A_0 h^2}(\Delta A - \Delta A_0)^2 \qquad \text{(Equation 15)}$$

where the coefficient k_r describes the non-local bending modulus. This energy also contributes to the total energy, which thus becomes

$$E_{vestot} = \frac{K_a}{2A_0}(A - A_0)^2 + \frac{k_c}{2}\int (c_1 + c_2 - c_0)^2 dA + \frac{k_r}{2A_0 h^2}(\Delta A - \Delta A_0)^2 \qquad \text{(Equation 16)}$$

Equilibrium shapes of vesicles have been obtained by minimizing the energy from curvature or from the sum of curvature energy and area difference. In the latter the shape is thus determined by the volume-to-area ratio and the area difference or preferred curvature of the vesicle. Through this it is possible to construct so called phase diagrams of vesicle shapes, where the area difference is plotted as a function of the volume-to-area ratio. Shapes such as prolates, oblates, pears, starfish and stomatocytes appear in a phase diagram (Döbereiner, 2000; Seifert, 1997).

Shape changes can be triggered by different external stimuli. One well-known example is the temperature-induced budding (Berndl *et al.*, 1990; Seifert and Lipowsky, 1995). An increase in temperature can result in a thermal expansion of the bilayer membrane, which results in a transformation of the shape of the liposome from its near spherical form into a prolate liposome and further on into a pear shaped liposome (Fig. 16). The final step involves formation of a small bud that is expelled from the liposome, however still connected to the "mother" liposome by a narrow neck, and increased temperature can lead to the formation of several buds interconnected by narrow necks. More exotic shape changes, such as inside-out topological inversion have also been reported. This type of shape transformation was induced by addition of surfactants to the outside solution of the liposomes. The effect of

this addition was interpreted as an exclusion of lipid molecules from the outer leaflet of the lipid bilayer, by penetration of surfactant molecules into the bilayer. This results in a relative change in the number of lipid molecules in the two monolayers, which would ultimately lead to a different preferential curvature of the membrane and in some cases inversion of the liposome (Nomura *et al.*, 2001).

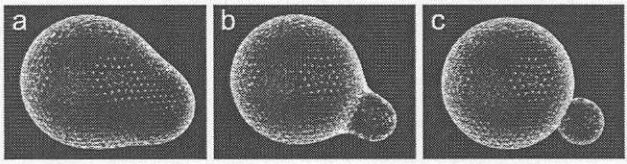

Figure 16. Schematic three-dimensional drawing of temperature induced budding of vesicles. Increased temperature leads to shape changes from near spherical shapes into pear structures (**a**, **b**) and into a budding transition (**c**).

Adhesion of liposomes to surfaces

So far vesicles that are free in solution have been described. However, the shape of the vesicle can also be perturbed by interactions with a surface (Seifert and Lipowsky, 1990, Seifert and Lipowsky, 1991). Vesicle adhesion can occur when the vesicle experience 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

$$E_{ad} = -WA^* \qquad \text{(Equation 17)}$$

The energy equation including interactions with surfaces is thus described by

$$E_{vestot} = \frac{K_a}{2A_0}(A\text{-}A_0)^2 + \frac{k_c}{2}\int(c_1 + c_2\text{-}c_0)^2\,dA + \frac{k_r}{2A_0h^2}(\Delta A - \Delta A_0)^2 - WA^* \qquad \text{(Equation 18)}$$

The contact potential for surfaces ranges roughly between 10^{-4} mJ/m^2 for a weak adhesion to 1 mJ/m^2 for strong adhesion (Seifert and Lipowsky, 1995). Figure 17 shows different adhesion situations for a liposome ranging from weak to strong adhesion.

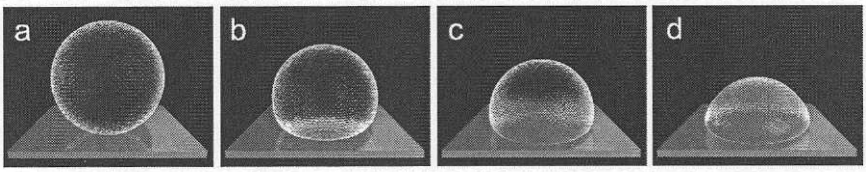

Figure 17. Schematic three-dimensional image showing different adhesion situations. (**a**) A liposome with very small or no adhesion to the surface, thus retaining its spherical shape. (**b**) The liposome show weak adhesion by a slight deformation of the spherical shape. (**c**) A liposome with an increased adhesion strength compared to b and the liposome has a spherical cap appearance. (**d**) The situation of strong adhesion, where the liposome resembles a hemisphere.

For weak adhesion situations the gain in adhesion energy is balanced by the cost in curvature energy. In the strong adhesion regime the shape of the vesicle approaches that of a spherical cap, except for the parts that are near the line of contact and an effective contact angle can be estimated as in figure 18.

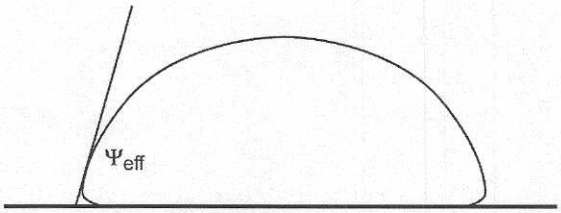

Figure 18. At strong adhesion potential, an effective contact angle, Ψ_{eff}, can be defined.

In this case the energy balance is not between the adhesion energy and the curvature energy but rather on the balance between the adhesion energy and the elastic stretching energy of the membrane. For very strong adhesion it is possible that the membrane will rupture and spread onto the surface (Sandre *et al.*, 1999). Typically, this occurs at the so called lysis tension for the lipid membrane, with values ranging from 4-10 mN/m (Olbrich *et al.*, 2000). Experimental studies of adhesion of vesicles have been performed using for example micropipette aspiration techniques (Evans, 1980) and reflection interference microscopy (Zilker *et al.*, 1987; Rädler and Sackmann, 1993).

Lipid tethers – cylindrical tubes of lipid membrane

Other shapes that can arise include tethers or cylindrical lipid tubes that form more or less spontaneously when swelling dry lipids (Bar-Ziv *et al.*, 1998), or by using lipids that self-assemble spontaneously into cylindrical structures (Schnur, 1993; Schnur and Shashidar, 1994). These self-assembled lipid cylinders have been demonstrated to be utilized as templates for metallization and also for protein crystallisation (Schnur, 1993; Wilson-Kubalek *et al.*, 1998). In contrast to these self-assembled structures, there are other ways to produce these cylindrical nanostructures under much more controlled conditions. Today, several techniques exist to induce forced shape transitions of bilayer membranes, which includes pulling of lipid nanotubes or tethers using pipets (Evans and Yeung, 1994), optical tweezers (Dai and Sheetz, 1999), pulling of lipid tubes under hydrodynamic flows (Rossier *et al.*, 2003), pulling of tubes by the action of polymers (Fygenson *et al.*, 1997) or molecular motors (Roux *et al.*, 2002).

As mentioned previously, it is easy to bend a membrane but hard to stretch it. By applying a point load to a lipid membrane it will deform and buckle from the near planar starting situation and a lipid membrane tether will start to form (Heinrich *et al.*, 1999; Derényi *et al.*, 2002). This type of structures have developed into a research area of its own and the formation of tethers under more controlled conditions dates back to the mid 70´s, when Hochmuth *et al.* studied how red blood cells that were attached to a surface behaved when subjected to a flow (Hochmuth *et al.*, 1973). The red blood cells seemed to detach from the surface but suddenly stopped and it appeared anchored to the surface where it originally rested by an invisible link, which turned out to be a tether or lipid tube, to small to visualize at the time. Earlier it was implied that the membrane stretching contributed more to the overall elastic energy of the system, than the bending term. However, this is true for larger vesicles and not for highly curved membranes, such as in membrane tethers or nanotubes, where the bending contribution will be more prominent. The bending or curvature energy of a tether can be found from the curvature energy equation above. For a membrane tube or cylinder, the principal curvatures $c_1 = 1/R_t$, where R_t and $c_2 = 0$. The curvature energy, assuming no spontaneous curvature, thus becomes

$$E_{tubecurv} = \frac{k_c}{2}\int (c_1 + c_2)^2 dA = \frac{k_c}{2}\frac{1}{R_t^2} 2\pi R_t L_t = \pi k_c \frac{L_t}{R_t} \qquad \text{(Equation 19)}$$

Consequently, the bending energy of the tube segment will therefore be linearly dependent on the tube length, L_t, and inversely proportional to the radius of the tube, R_t. By now many different methods have been developed to produce and study tethers and tether formation. One of the most powerful techniques is the micropipette aspiration controlled technique (Hochmuth and Evans, 1982; Hochmuth *et al.*, 1982). This technique is based on the aspiration of a liposome into a micropipette under controlled suction pressure. The applied suction pressure, the geometry of the pipette and the vesicle size governs the resulting membrane tension of the aspirated vesicle. Membrane tether formation can therefore be performed under very controlled conditions, where membrane tension can be set and held constant. A plot of the membrane tension and the corresponding area stretching or expansion of the aspirated vesicle revealed two different regimes (Rawizc *et al.*, 2000). At low suction pressure an exponential rise of the tension versus area expansion can be found. This is believed to be an effect of diminishing thermal fluctuation or undulations, which exists in the membrane. These undulations can therefore be considered as an extra membrane reservoir for the vesicle. This regime is often referred to as the low tension regime. The corresponding high tension regime dominates at tensions approximately above 0,5 mN/m. This regime features a direct stretch response and therefore has a linear dependence of membrane tension, σ, versus area expansion, *i.e.*

$$\sigma = K_a \frac{(A - A_0)}{A_0} \qquad \text{(Equation 20)}$$

The energy of the tube thus can be written as the energy arising from both membrane bending and tension and also the pulling force, f, acting on the membrane (Derényi *et al.*, 2002).

$$E_{tube} = \left[\frac{k_c}{2R_t^{\,2}} + \sigma \right] 2\pi R_t L_t - f L_t \qquad \text{(Equation 21)}$$

The equilibrium radius, R_{t0}, can be found by $\delta E_{tube}/\delta R_t = 0$, which gives the relation

$$R_{t0} = \sqrt{\frac{k_c}{2\sigma}} \qquad \text{(Equation 22)}$$

The bending modulus will therefore favour a larger tube radius, while the surface tension instead wants to decrease the tube radius. Tube radius estimations have been reported down to around 10 nm using micropipette techniques (Bo and Waugh, 1989).

Correspondingly, an equilibrium force f_0, to pull such a tube can be found as

$$f_0 = 2\pi\sqrt{2\sigma k_c} = \frac{2\pi k_c}{R_t} \qquad \text{(Equation 23)}$$

Lately, the formation of tubes has been described in detail and the result was that the formation of tubes is a non-trivial process involving first-order shape transitions. It was found that in the process of tube formation, the required force has to overcome a barrier, in order to create the tubes. This may have implications in biological processes, where motor proteins must be able to surmount a force about 13% larger than the force to pull tubes, during the creation of the tubes (Derényi *et al.*, 2002).

The methods described above can be used to probe the physical and chemical properties of the bilayer membrane, which has resulted in accurate measurements of local bending modulus, non-local bending modulus (Waugh *et al.*, 1992) and also the intermonolayer friction that can arise when lipid tubes are pulled from liposomes (Evans and Yeung, 1994). A rapid dynamic transformation of the shape of the liposome, for example by extraction of a tether from a liposome, pronounces the interaction between the two monolayers, which results in a viscous resistance or drag to lipid flow out onto the tether. This effect was called the "hidden dynamic coupling" by Evans *et al.* and describes the frictional interactions between the two monolayers, which have been proposed to even dominate the extraction force when pulling tethers from liposomes. This interlayer drag comes from the fact that an enormous relative motion between the monolayers arises due to a 1000-fold curvature increase going from the liposome to the tether (Fig. 19). It has been estimated that molecules in the opposing monolayers pass each other at rates close to 10^5/s, which indeed is an impressive number. Values of this frictional drag have been measured to be between $4*10^{-7}$ and $1{,}8*10^{-6}$ g/s up to $2*10^{-4}$ g/s when pulling tethers out of neuronal growth cones (Evans and Yeung, 1994; Raphael and Waugh, 1996; Dai and Sheetz, 1995).

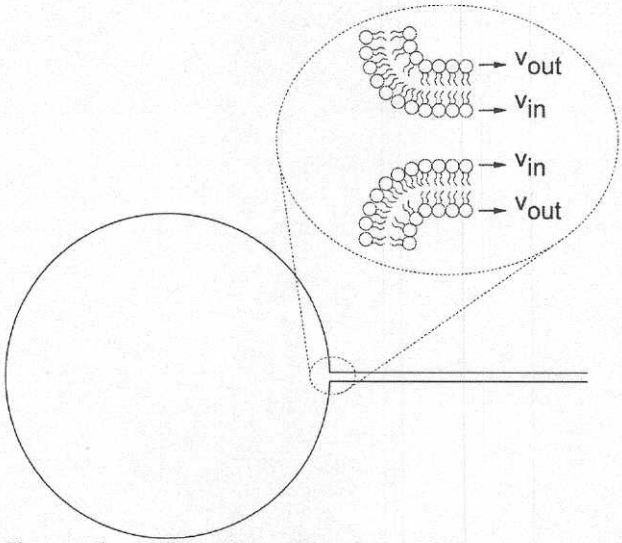

Figure 19. Tether pulling experiments illustrating a difference in velocity between the two monolayers. Lipids situated in the giant liposome membrane experience the surface to be essentially flat, especially when compared to the situation that exists in the nanotube, where the membrane is highly curved. The monolayers in the nanotube will have different areas and since the outer monolayer will have a larger area, the velocity of lipids in the outer monolayer will be higher than the inner monolayer. This gives rise to an intermonolayer friction.

Recently, it was demonstrated that extrusion of relatively long tubes at high velocities can lead to a non-uniform radius of the tube, due to the fact that the contribution of the friction force of the surrounding water will become more important. This will produce a gradient of surface tension along the tube and since tension is related to the radius of the pulled tube, a non-uniform tube radius will be obtained (Rossier et al., 2003).

Networks of liposomes and lipid nanotubes

As mentioned above, lipid membranes and liposomes can undergo a multitude of different shape changes and transitions by various types of external stimuli. As a continuation of this line of research our own contribution in this field of research has been the development of tools to induce forced shape changes of liposomes, using a more direct and hands-on approach. We have developed several micromanipulation-based techniques to distort liposomes from their original structure into networks of liposomes that are interconnected by lipid nanotubes (Fig. 20). There is a short background story to the network formation techniques. As a side project, we had discovered that multilamellar liposomes sometimes disrupted onto surfaces of high contact potential and seemed to peel of lipid layers that spread onto the surface. By scraping with a micromanipulator controlled carbon fibre across the lipid film, the film could sometimes be removed from the surface. The film could also be divided into two films and it seemed that these films could then merge together again at a single point and repair the scraped surface. My supervising professor and a professor colleague from USA joined the lab session, because the procedure looked peculiar indeed.

- Can you do that with a liposome, the American professor said?
- Sure, why not said the PhD-student at the micromanipulator controls.

Sure enough, the liposome could easily be divided if it was attached to the surface and under the microscope it seemed as if one liposome had been converted into two liposomes. However, the liposomes started to drift towards each other by some invisible pulling force. When they reached each other, the liposomes merged together into the original liposome. Spontaneously. This intrigued us to the point that we wanted to follow this merging in fluorescence, by staining the lipid membrane with a fluorescent dye. The procedure was repeated and a thin tether was found that connected the liposomes after division. A lipid nanotube had been formed between the liposomes. This technique was called the mechanical fission technique.

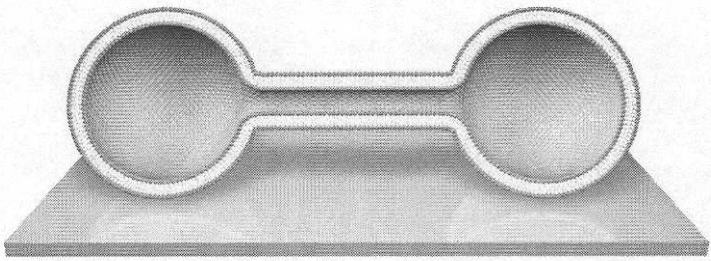

Figure 20. A network of two surface-attached liposomes interconnected by a lipid nanotube.

Mechanical fission technique

This technique, which is described in papers IV and VII, is based on a mechanical division or fission procedure, although the term fission is not entirely correct since it implies that the liposomes are fully disconnected from each other. The technique works best with multilamellar liposomes, due to the mechanical strain that is imposed during the division stages. However, flaccid unilamellar or thin-walled liposomes or liposomes connected to a multilamellar liposome seems to cope with this treatment due to excess of membrane material and access to extra lipid material donated from the multilamellar part (Karlsson *et al.*, 2000; Karlsson, A. *et al.*, 2003). When the liposomes have adhered to the coverslip surface, a micromanipulator controlled carbon fibre (5 μm in diameter) is brought down into the solution and carefully positioned above the liposome under the microscope (Fig. 21a). The carbon fibre now acts as a cutting tool and is positioned near horizontally with the surface or

at a very shallow angle. By bringing the carbon fibre down towards the surface, it cuts through the liposome and leaves a thin lipid tube in between (Fig. 21b, c). The sizes of the resulting pair of liposomes are set by the division site on the liposome. If the division site is located on the equator, the liposome is divided into two equally sized liposomes. If the cutting tool is placed at any other site it results in two differently sized liposomes. After division, the carbon fibre is translated in the plane of the surface and therefore separates the two liposomes and elongates the connecting tube (Fig. 21d). When a target site is reached the translated liposome is allowed to adhere to the surface and a network structure is obtained (Karlsson, A. *et al.*, 2001; Karlsson, A. *et al.*, 2003). The main drawback of the technique is that the starting material (one liposome) is limited. The liposome gets smaller and smaller, until the offspring or daughter liposomes that are created are too small to divide further (limited by the size of the cutting tool). This can be solved by electrofusion of a large liposome into the network, as described later. Another drawback is the surface attachment. The surface must be able to attract the liposomes, but still allow translation of the liposome along the surface, without leaving a trace of lipid debris. This can be hard to control, however by patterning the surface with repelling and attracting surfaces, this should be able to make the division or fission technique more controllable.

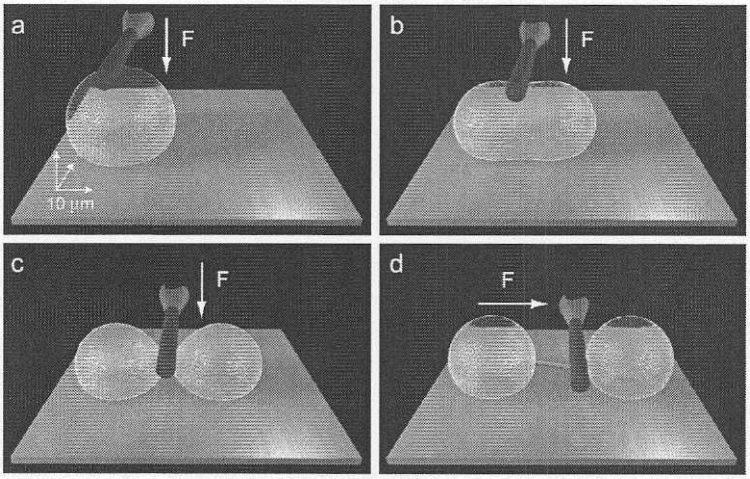

Figure 21. Schematic of the mechanical fission technique. (**a**) A carbon fibre is placed above a liposome attached to the substrate surface. (**b**, **c**) As the carbon fibre is brought down towards the substrate surface it deforms the liposome into two spheres connected by a thin tube. (**d**) The interconnecting tube could be elongated by horizontal movement of the carbon fibre, until the liposome had reached its target site on the surface. The white arrow represents the direction of the force, F, in each step.

Micropipette-assisted network formation

The second technique of network formation is based on a microelectroinjection technique, which will be described later. It is the primarily used technique when using unilamellar or thin-walled liposomes. This type of liposomes can be formed by the dehydration/rehydration technique, which can produce unilamellar/multilamellar twinned or paired liposomes, which are very useful for microinjection studies (Karlsson *et al.*, 2000). In this technique, the unilamellar protrusion is first penetrated by microelectroinjection (Fig 22a). The lipid is then allowed to re-seal around the injection tip, which is followed by a retraction of the injection tip away from the liposome (Fig 22b). This result in a lipid tube connected between the injection tip and the original liposome (Fig 22c). By slowly injecting buffer (tens of femtoliters per second), the nanotube expands at the injection tip, thereby

taking the extra lipid material for growth from the multilamellar part, which flows across the nanotube and the original unilamellar protrusion (Fig 22d). This flow of lipid is stipulated by the stress imposed on the bilayer membrane, when the membrane is expanded and stretched at the injection tip and the membrane tension is increased. It has been demonstrated both theoretically and experimentally that a gradient or difference in membrane tension across a lipid membrane surface, drives the lipids to flow from regions of low tension to regions of high tensions, in order to eliminate the tension difference (Chizmadzhev *et al.*, 1999). Therefore the technique relies on the lipid reservoir in the form of a multilamellar liposome attached to the unilamellar protrusion during growth and formation of the network. When the expanding liposome growing on the injection tip has reached the desired size it is placed on the surface and allowed to adhere and the pipette is removed (Fig 22e, f). This procedure is repeated until the final structure is obtained (Karlsson, M. *et al.*, 2001).

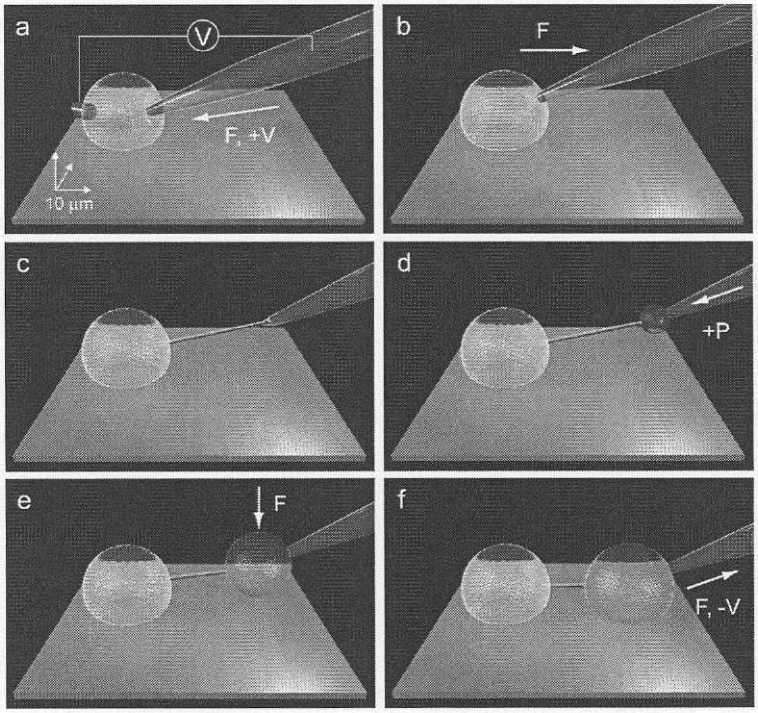

Figure 22. Schematic of the micropipette-assisted network formation technique. (**a**) By a combination of mechanical deformation and electric pulses across the liposome a microinjection needle is inserted into a unilamellar liposome connected to a multilamellar protrusion (not shown). (**b**) After the lipid has adhered to the injection needle, the micropipette is pulled away from the liposome. (**c**) A lipid nanotube is created between the tip of the micropipette and the liposome. (**d**) By applying a low pressure, P, to the microinjection needle, the nanotube is expanded into a liposome at the microinjection tip, by taking lipid material from the multilamellar liposome (not shown). (**e**) After the liposome has reached a desired size it is allowed to adhere to the surface. (**f**) The micropipette is removed by applying electric pulses of reversed polarity and simultaneously pulling it out of the liposome. The white arrow show the direction of the applied force, F, and V represents the electric field.

The main advantage of this technique is the easy handling of unilamellar liposomes, which is a much more well-defined system than the multilamellar system. Also, the differentiation of the contents in the different network containers or liposomes is very easy, since the liposomes are filled with their content when they are formed. The contents are

therefore set by the solution in the microinjection needle that constructs the network. By exchanging the solution in the pipette (that is change to another injection needle filled with another solution), the new network liposomes contains the new solution. This technique is more flexible when it comes to surface adhesion effects, compared to the mechanical fission technique, however, as mentioned above strong adhesion can lead to deformation of the liposomes, increased membrane tension and rupture or lysis of the membrane, which can compromise the structural integrity of the network.

Micropipette writing technique

The third network formation technique developed so far is called a writing technique, which is a very good name for it. The technique involves a reversible micropipette aspiration technique capable of collecting lipid material into the micropipette by applying a negative pressure to the micropipette followed by careful expulsion of the lipid out of the micropipette (Fig. 23 a, b). The technique can therefore be described as writing a pattern or a network on a surface (Sott *et al.*, 2003). When lipid material has been collected, usually by suction of a large liposome into the micropipette, the pressure is reversed and the liposome starts to eject from the tip (Fig. 23b). The tip is brought to the surface and the lipid is allowed to adhere to the surface. When enough lipid material has been ejected the injection pressure is stopped and the micropipette is retracted from the surface (Fig. 23c).

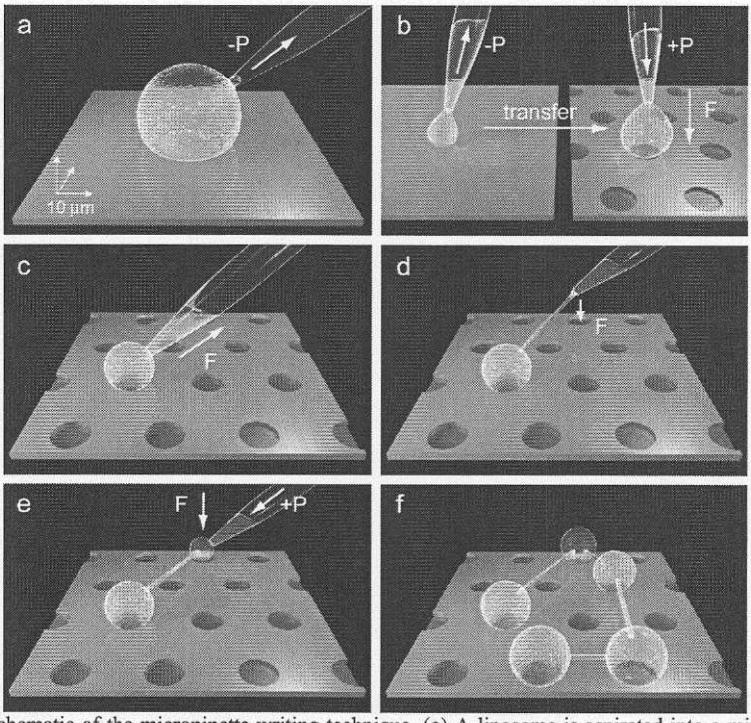

Figure 23. Schematic of the micropipette writing technique. (**a**) A liposome is aspirated into a micropipette by applying a suction pressure, P. (**b**) The micropipette is transferred to a new site or a new surface, where the pressure, P, is reversed and lipid material starts to eject from the pipettetip. (**c**, **d**) When the new liposome has reached the desired size, the pipette is again pulled away from the liposome attached to the surface and a tether is formed. (**e**, **f**) The procedure is repeated to create the final network structure. In this case the image illustrates the use of a patterned surface in order to achieve better control over the liposome adhesion sites. The white arrow shows the direction of the applied force, F.

The attached liposome is now separated from the lipid reservoir in the micropipette, but is still connected by a tether as before (Fig. 23d). The micropipette is moved to a new target site and a positive pressure is applied. A new liposome is formed at the tip and placed on the surface as before and this liposome is connected to the previously formed liposome with the tether created in the first stage and this process is repeated to create a network (Fig. 23e, f). The technique is therefore very similar to the mechanical fission technique in some aspects since a large lipid reservoir is divided or partitioned into several smaller liposomes that are attached to the surface. It has the same drawbacks, except for the adhesion problem, since no scraping along the surface occurs, but instead liposomes are placed at specific sites and the micropipette is removed away from the surface during each division.

All of the above described techniques have the capability of creating 2-dimensional lipid networks with control over daughter liposome size, connectivity, angle between two liposomes connected to a common liposome, nanotube length etcetera. After formation, these types of networks can be further processed and developed into more complex structures by utilising a miniaturised electrofusion protocol which will be described later. In brief electrofusion enables fusion of network containers by application of electric fields (Chiu *et al.*, 1999a). This will of course expand the design possibilities of the network architecture to include structures such as closed networks and fully connected networks, where all network containers is connected to every container in the network, with subsequent changes in the topology of the system (Fig. 24) (Karlsson, M. *et al.*, 2002).

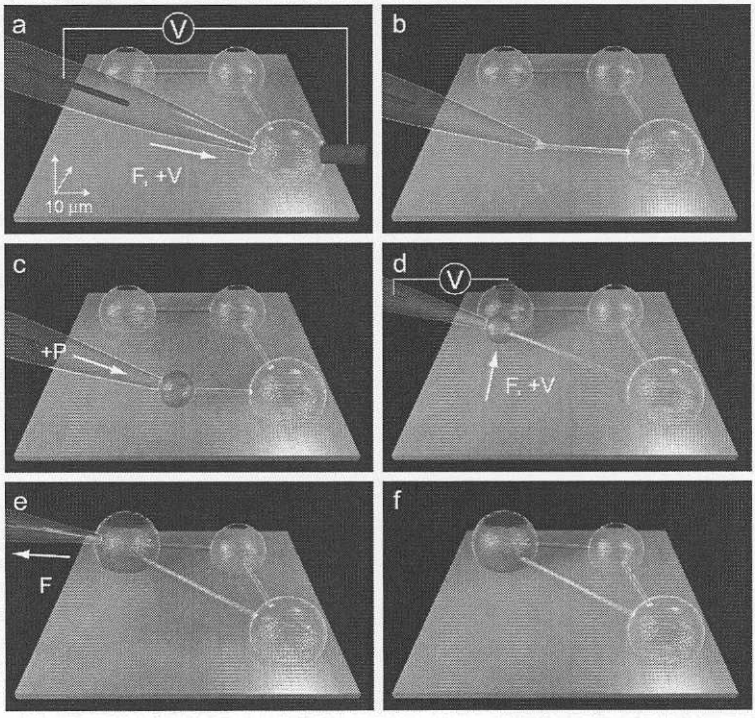

Figure 24. Formation of closed networks. (**a-c**) By the use of electroinjection a micropipette is inserted into a network container and a new liposome is created at the tip of the micropipette. (**d-f**) The newly formed liposome can then be electrofused into another network liposome in order to create a closed network system. The white arrow shows the direction of the applied force, F, whereas V represents the electric field and P the applied pressure.

Network design can also be based on the fluidity, self-organising and energy minimisation characteristics of the bilayer membrane. For example, it is possible to create nanotube junctions, which is networks of nanotubes connected to each other. These nanotube networks follow a pathway energy minimisation and therefore adopt three-way junction structures with 120° angles at the nanotube junctions (Karlsson, A. *et al.*, 2001; Karlsson, M. *et al.*, 2002; Evans *et al.*, 1996). Liposome-nanotube junctions that emanate from the same liposome can coalesce into one nanotube if they are in close proximity of each other (Derenyi *et al.*, 2002). After coalescing the nanotubes will spontaneously rearrange into the minimum energy configuration with a 120° angle at the junction site (Fig. 25). This has been utilised and studied by producing highly interconnected networks followed by a triggered merging of nanotubes to induce a dynamic structural transformation in the network (Karlsson, M. *et al.*, 2002).

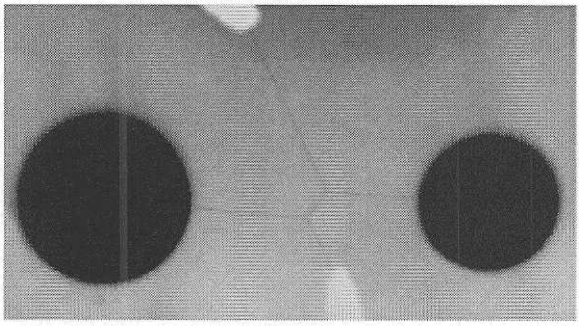

Figure 25. Negative fluorescence image of nanotube junctions. The membrane was stained with a membrane fluorescent dye in order to visualize the nanotubes. Two micromanipulator controlled carbon fibers (white) was attached to the lipid nanotube interconnecting the two liposomes (black spheres) and positionally manipulated to produce the two nanotube junctions, which follow a minimum pathway.

Initiation of reactions

Solitary liposomes and also discrete network components such as liposomes, lipid nanotubes and lipid nanotube junctions, as well as complete networks described above may provide nano/microcompartments with biomimetic features to be used as model systems and studies of confined chemical reactions. In the following, a summary of techniques to initiate chemical reactions inside these types of compartments is given, including some micromanipulation protocols developed in this thesis.

Direct incorporation methods

Direct incorporation means that the entire reaction system is enclosed upon formation of the liposome and the reaction is initiated as soon as the reagents are mixed (Fig. 26a). As mentioned above, the trapping efficiency is dependent on many parameters including the type of lipid(s) used, the number of lamellae (lamellarity) in the liposome, size and also preparation method etcetera (Monnard, 2003). For example, multilamellar liposomes, having many lamellae, tend to exclude solutes from their interior, thus giving them a low trapping efficiency, usually between 5 and 15%. Liposomes formed by dehydration/rehydration protocols on the other hand often display much higher trapping efficiencies, ranging from approximately 15 to 45% (Walde and Ichikawa, 2001). Consequently, reactions requiring many components will be difficult to incorporate at a high yield or efficiency, using direct incorporation techniques.

In some cases it is possible to evade the start of the reaction by the use of a photoactivable or "caged" reactant (Pelliccioli and Wirz, 2002). The "cage" protects the substance, for example, a reactant, and hinders the on-set of the reaction when mixing the caged substance with the other reactants during the preparation. Flash photolysis, by the use of a laser-light source for example, of the photoactivable probe will destroy the "cage" that protects the reactant and release it, making it accessible to the other reactants whereby a reaction can take place. This release is highly controlled, both spacially and temporally, and provides a good starting point for the reaction, since mixing of the reactants by diffusion or convection is unnecessary. In the early experiments using this technique, the release of the caged compounds was somewhat uncontrolled and it was hard to know the precise amount that was released. Nowadays, however, by fine tuning of the properties of the "cages" and by knowing the laser light power and the exposure time of light to the protected compound, one can give a more or less precise answer to how much is released. In cell research areas, these photoactivable probes have made a large impact, giving cell researchers means to deliver bioactive materials such as neurotransmitters, ATP or simply calcium ions rapidly to small target sites.

Transport across membranes - membrane penetrating techniques

Many techniques have been developed to penetrate the membrane barrier to supply the interior volume of liposomes with reagents (Fig. 26b)

Reagent permeability

The permeability property of the bilayer is highly dependent on the lipids that constitute the membrane. For example, adenosine diphosphate, ADP, had some permeability across phosphatidylcholine, PC, bilayers with hydrocarbon chains of 12 carbons, while PC bilayers with 16 and 18 carbons were almost impermeable to ADP (Chakrabarti *et al.*, 1994). The permeability is also very dependent on the transition temperature between gel and liquid crystalline phases. It is believed that solutes that normally display low permeability can increase their flux across the membrane barrier through transient defects in the bilayer membrane, which can occur at boundaries between gel and liquid phase domains of the

membrane (Monnard, 2003). This type of enhanced passive diffusion can thus be promoted by controlling the temperature near the transition temperature. The bilayer can also be rendered more permeable through addition of for example detergents at sublytic concentrations and therefore increase the flux of substrates across the membrane barrier. Cholate was used to make POPC (palmitoyl oleoyl phosphatidyl choline) permeable to glucose-1-phosphate, which was transformed into glycogen on the inside of the liposomes by phosphorylase A (Oberholzer and Luisi, 2002). Based on the fact that giant liposomes display other physico-chemical properties than small and large unilamellar liposomes it has been questioned whether permeability assays on small liposomes can be extended to giant unilamellar liposomes. The major differences are the larger membrane curvature of small and large liposomes compared to giant liposomes and increased fluidity and undulating properties of the membrane surface found in giant liposomes. It has been shown, for example, that low molecular weight molecules, such as YO-PRO-1 and fluorescein diphosphate, as well as proteins such as DNases and RNases, not belonging to the class of membrane associated proteins, demonstrated passive transport across a lipid membrane boundary of giant liposomes. This type passive transport was, however, not found in small and large unilamellar liposomes (Fischer *et al.*, 2000).

Membrane proteins as transporters

Another way to produce more persistent holes in the membrane is of course to abstract structural and functional units and mechanistic solutions from biomembranes, where transport across membranes is mainly regulated by membrane proteins. These membrane proteins can be extracted from cell membranes and their functionally can be inserted or reconstituted into the lipid membranes of liposomes, to provide a way for ions and reagents to pass the membrane (Fig. 26c) (Racker, 1972; Eytan, 1982). A number of different reconstitution protocols have been developed in order to increase the efficiency of incorporation and functionality of the membrane proteins. One example is detergent solubilisation, where membrane proteins and lipids are co-solubilized, thus forming mixed micelles of lipid-detergent or lipid-protein-detergent. The detergent used for this purpose is often cholate since it can easily be removed by dialysis and after removal liposomes with reconstituted proteins or proteoliposomes are formed. Proteoliposomes can also be created by mechanical means such as freeze-thawing and sonication of mixed suspensions of lipids and proteins or by direct incorporation techniques, where the proteins are added to preformed liposomes. The transport across membranes mediated by proteins can also be subdivided according to the transport mechanism. Facilitated diffusion involves incorporation of a protein with a simple channel function, through which either non-specific or specific transport can occur. Secondary active transport involves for example formation of an electrochemical gradient to drive the transport of a substance across the membrane through the protein. Finally, active transport is described by ATP-dependent transport, where ATP is hydrolysed to facilitate transport of a substance across the membrane. Apart from reconstitution of proteins into smaller liposomes, much effort has been directed into reconstitution of membrane proteins into giant unilamellar liposomes (Kahya *et al.*, 2001). Also, reconstitution of membrane proteins could also be combined with liposome networks. Plasma membrane proteins from erythrocytes were prepared with eosin-5-maleimide (EMA) labelled anion exchanger AE1. One of the results from this study was that the membrane proteins displayed free lateral movement in the bilayer across the interlinking nanotubes of the network (Davidson *et al.*, 2003).

Lately, peptides have earned some attention as membrane translocating carriers of substances. It was observed that peptides had the ability to deliver polypeptides and oligonucleotides into the cytoplasm and nucleus of living cells. One of the most well-studied

membrane translocating peptide, penetratin, was shown to translocate across lipid bilayers of liposomes (Thorén et al., 2000).

Both uptakes through passive permeability and through membrane proteins, however, lacks the control over a well-defined time of reaction initiation, since reactants are replenished continuously and therefore more direct approaches to membrane penetration have been developed in order to gain more control over the reaction events.

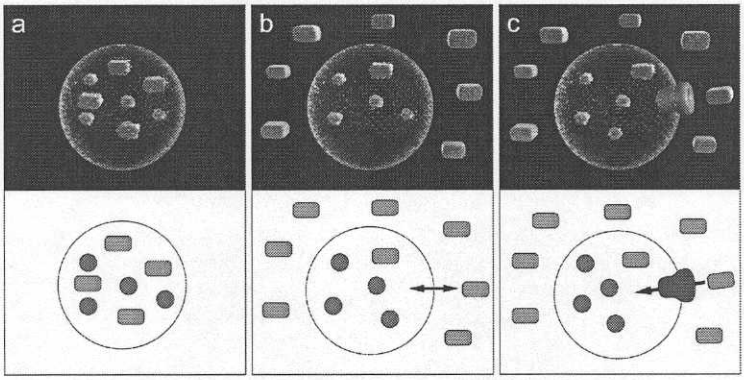

Figure 26. Schematic images of different techniques to initiate chemical reactions inside liposomes. (**a**) Direct incorporation. The reaction is initiated upon mixing of the reagents (depicted as spheres and rectangular boxes) and the reaction is contained in the liposome upon formation. (**b**) Reagent permeability. One of the reagents (rectangular boxes) is permeable across the membrane boundary and can therefore initiate a reaction by crossing the lipid bilayer. (**c**) Membrane protein mediated transport. Reagents (rectangular boxes) can be transported through the lipid bilayer membrane mediated by membrane proteins.

Electroporation

Of course the permeability would increase if one could controllably produce transient holes or pores in the membrane. This can be done by the use of electric fields applied across the bilayer membrane through a process called electroporation (Weaver and Chizmadzhev, 1996). Electroporation has been a known phenomenon for half a century by now and has been applied to cells and pure lipid membranes to study the effects of the electric field. Early studies of this phenomenon quickly deduced the fact that the membrane could be rendered permeable through dielectric breakdown, when the induced potential difference reached a critical value. In an electric field, the membrane will act as a capacitor, where oppositely charged species will be attracted to either side of the membrane to produce a charge separation across the membrane. The capacitance of the membrane can be written as

$$C = \frac{\varepsilon_r \varepsilon_0 A}{h_e} \qquad \text{(Equation 24)}$$

, where A is the membrane area, ε_0 is the permittivity in vacuum and h_e is the dielectric thickness of the membrane. ε_r is the relative dielectric constant, which describes the membrane polarisability and the response of the membrane dipoles in an electric field. When this charge separation is large enough, the critical dielectric breakdown potential is reached, which leads to a structural breakdown and increased conductance across the membrane.

The mechanism of this structural breakdown has not yet been fully concluded, however plausible explanations have been given. One of the most widely accepted mechanisms involves the formation of pores through the membrane (Fig. 27). The size of the final pore

structure usually ranges from a few nanometers up to 10 nanometers. The energy of formation of such a hydrophilic pore can be written as

$$E_{H\_philic} = 2\pi\gamma r_{pore} - \pi\sigma_{membrane} r_{pore}^{\ 2} \qquad \text{(Equation 25)}$$

, where r_{pore} is the pore radius at the narrowest part of the pore structure, γ is the line tension along the pore edge (one dimensional tension) and the $\sigma_{membrane}$ is the surface membrane tension (Glaser *et al.*, 1988). This means that energy is created along the edge of the pore due to bending and packing constraints of lipids in this region, according to the first term in the equation and that energy is lost according to the second term, since the pore area is removed from the total membrane surface. If the pore radius is very small, below the radius that is defined by γ/σ, the edge energy of the pore dominates and hence the pore will try to shrink and re-seal the membrane. If, however, the surface tension dominates instead, the pore radius will increase and this can sometimes lead to irreversible membrane breakdown (Wilhelm *et al.*, 1993).

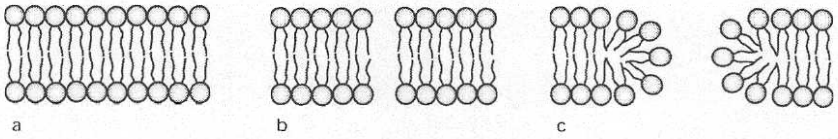

Figure 27. Schematic of the hydrophilic pore formation. (**a**) The starting situation of an intact membrane. (**b**) The hydrophobic pore where the hydrocarbon chains are exposed to the water contained inside the pore. (**c**) The final hydrophilic pore structure where the lipids in the pore regions organize so that the polar headgroups face the inside of the pore.

The formation of pores can be very fast (μs-regime) and is governed by the magnitude of the critical potential difference across the membrane. The pores will re-seal again when the field is turned of and this process is much slower, usually taking milliseconds to seconds to complete, however much longer re-seal times have been reported. The formation of pores can be highly promoted when the membrane is under mechanical stress, such as increased tension. This was realised as the magnitude of the applied electrical field to perform membrane permeabilisation seemed to decrease when a mechanical force was applied to the bilayer membrane. According to this interpretation, the electric field across the membrane creates an electro-compressive mechanical stress, $\sigma_{elec.mech}$ that acts normal to the plane of the membrane (Needham and Hochmuth, 1989). This will lead to a slight decrease in membrane thickness. This mechanical stress is related to the voltage drop, V_m, across the membrane and can be written as

$$\sigma_{elec.mech} = \frac{1}{2}\varepsilon\varepsilon_0\left(\frac{V_m}{h_e}\right)^2 \qquad \text{(Equation 26)}$$

where ε is the relative dielectric constant, ε_0 is the permittivity in vacuum and h_e is the dielectric thickness of the bilayer membrane. The sum of the electro-compressive stress and the isotropic membrane tension is called the differential mechanical work that is done on the lipid membrane and can thus be described by

$$dW = \left[\sigma_{membrane} + \frac{1}{2} \varepsilon \varepsilon_0 \left(\frac{V_m}{h_e} \right)^2 h \right] dA \qquad \text{(Equation 27)}$$

where $\sigma_{membrane}$ is the isotropic membrane tension, h is the overall thickness of the bilayer and A is the membrane area.

The membrane pores have been visualized using various approaches. For example, rapid freezing followed by freeze fracture electron microscopy was used to investigate the structural changes in a cell membrane after electroporation (Chang and Reese, 1990). The membrane pores were visualised as "volcano-shaped" openings in the membrane. Larger pores could be stabilised and visualised by applying tension in the membrane, using the micropipette aspiration technique. By loading the liposomes with media having a different refractive index than the outside solution, the pores could be visualised as the small "jet" of fluid passing through the pore out of the liposome (Zhelev and Needham, 1993). The fast dynamics of the pore opening and closing could be slowed down dramatically by the use of a viscous solvent. In a low viscosity solution, such as water, the leak out is fast and the pores close fast if the tension is relaxed. However, when the solvent is more viscid the leak out is slow and the pores can grow up to micrometers in size (Sandre *et al.*, 1999). As a continuation of this technique it was possible to study the effects of cholesterol or detergents on the line tension of pores in the membrane. Cholesterol was shown to increase the line tension, thus decreasing the lifetime of the pores. Detergents on the other hand decreased the line tension with a subsequent increase in pore lifetime (Karatekin *et al.*, 2003).

During the states of pore opening and re-sealing, molecules can be transported across the membrane. The dominant mechanism of this transport is believed to be diffusion of substances down their concentration gradients according to Fick's first law. However, this type of diffusion must be considered as somewhat hindered compared to free diffusion, since size, charge and shape of the transported molecule are likely to influence the flux through the pore (Weaver, 1993). In our own research in this field we have developed miniturised versions of this technique, to be able to address the chemical composition and contents of single cells and liposomes (Fig. 28) (Lundqvist *et al.*, 1998; Ryttsén *et al.*, 2000; Åberg *et al.*, 2001).

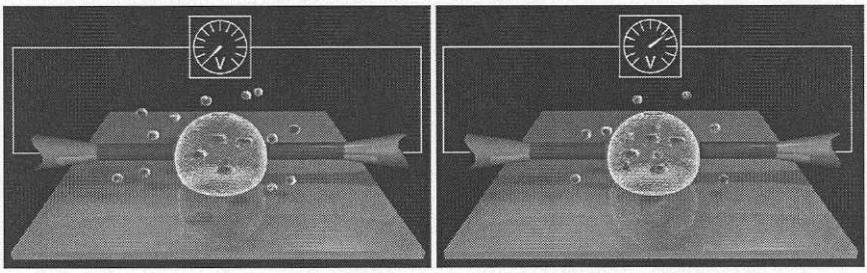

Figure 28. Schematic illustration of microelectroporation. (**left**) Two micromanipulator controlled carbon fibres are positioned close to the membrane of a surface-attached liposome. (**right**) Upon application of an electric field across the two carbon fibres, the membrane is destabilised which creates membrane pores. Reagents that are in the outer solution (spheres) can diffuse through the pores and into the liposome and mix with reagents contained in the liposome (rectangular boxes) and a reaction can be initiated.

This miniaturised version also enables selective penetration of the membrane barrier of a specific network liposome as opposed to the other techniques described so far. It is therefore possible to initiate reactions in a single network container and follow the evolution of the

reaction and also possible spreading of the reaction throughout the network structure. The main drawback of the electroporation technique is the lack of control of the amount of substances that pass through the membrane.

Microinjection

Penetration of the membrane can also be performed by more mechanical means, such as microinjection, which utilizes a pulled glass micropipette with a very fine tip (usually submicrometer) to deliver reagents into the lumen of the liposome (Bucher *et al.*, 1998). Microinjection into giant vesicles has been described in many papers and there are some important issues to address. First of all, the vesicle must endure one or preferably several punctuations or injections to be able to function as a microreactor (Oberholzer and Fischer, 2000). However, due to the mechanics of the bilayer it is very hard to stretch, as discussed above, and therefore cannot accommodate more than a few percent of area increase before reaching the critical lysis tension, where the membrane is disrupted. It is therefore vital that the liposome either has a surplus of membrane material from the start or has access to some lipid reservoir. Liposomes that are formed by the electroformation technique, which are still attached to the platinum electrode have proven to fulfill these requirements. Another type of liposome that has similar capabilities is the giant unilamellar liposomes having a multilamellar liposome attachment, formed by the dehydration/rehydration technique. This type of unilamellar/multilamellar pairs has been shown to be very compatible with microinjection techniques since the unilamellar liposome can accommodate extreme volumetric increase. This is due to the donation of extra lipid material from the multilamellar liposome functioning as a local lipid reservoir (Karlsson *et al.*, 2000).

Microelectroinjection

The usual procedure when performing microinjection into liposomes, is to stab the liposome with the micropipette in order to punch through the membrane. This is often a difficult task if the liposome is not immobilised and also because the bilayer is soft and easily bent and can therefore change shape to evade the injection tip. Our own contribution to this field of research has been the development of a technique that combines conventional microinjection with electroporation or electropermeabilisation of the membrane (Karlsson *et al.*, 2000). Penetration of the injection tip turned out to be much easier by a simultaneous combination of mechanical stress and electric pulses across the liposome. The mechanical stress was imposed by pressing the liposome with the microinjection tip and a microelectrode carbon fibre on the other side (Fig. 29).

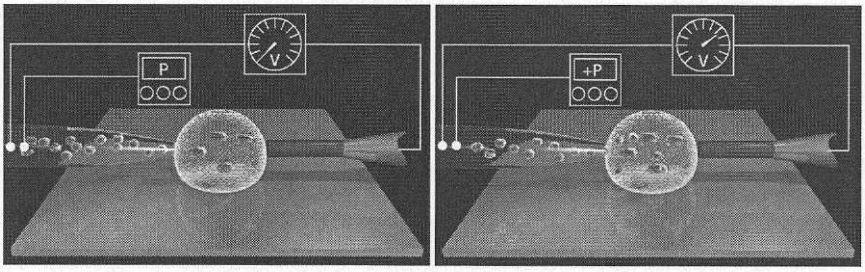

Figure 29. Schematic of the microelectroinjection technique. (**left**) Reagents can be supplied to a liposome by penetrating the membrane with a micropipette, using electroinjection techniques. (**right**) By application of a mechnical pushing force and an electric field across the liposome, the microinjection needle penetrates the membrane. By applying an injection pressure to the micropipette, reagents (spheres) flow into the liposome and mix with the other reagent (rectangular boxes) and initiate the reaction.

The carbon fibre on one side and the use of a modified microinjection needle, with a platinum wire electrode in contact with the inner solution of the needle, created the electric field pulses.

An additional benefit using this technique is that much larger injection tips can be used and it is therefore very useful, when injecting larger molecules or particles into liposomes. As before with the miniaturised electroporation method, microinjection enables differentiation of the interior contents of individual network containers after formation of the network, in order to initiate reactions.

Penetration of two membranes – fusion of two separated compartments

Up to now, techniques to initiate reactions and mix reagents by penetration of the bilayer membrane have been described. Another useful method to initiate reactions is to mix the contents of chemically differentiated liposomes in a procedure called fusion. Fusion of membrane compartments is a well-known and vital process in the cellular environment, where fusion of small transport or carrier vesicles merges with internal structures (organelles etcetera) or the plasma membrane. Through the years, many techniques have been developed to study the fusion of membranes by the use of liposomes as model membranes and this type of fusion can be stimulated by a variety of methods such as electrical, chemical and optical means for example. The first requirement of membrane fusion is a close contact of the membranes. At very small separation distances, ~ 2,5 nanometers, hydration forces repel the membranes and prevent close contact. It has been estimated that very large mechanical forces, such as an external pressure of more than 100 atmospheres, is needed to overcome this energy barrier and induce membrane fusion (Chernomordic *et al.*, 1987; Chernomordic, 1996). There are predominantly two theories for membrane fusion, the stalk mechanism and the pore formation mechanism. In the stalk mechanism membrane defects in the two opposing membranes in close contact, join and create a cylindrical micelle-like lipidic tether or stalk that connects the two membranes (Markin and Albanesi, 2002). This result in an intermediate hemifused structure, where only the outer monolayers of the two fusing membranes mix. Membrane stress, such as membrane tension imposed on the intermediate structures is believed to promote and complete the membrane fusion.

Electric-field induced fusion

Electrical stimulation to promote membrane fusion is commonly called electrofusion. One mechanism to this technique is very similar to electroporation and structural breakdown of the bilayer and is therefore called the pore formation mechanism. As before an electric field is placed across the chemically different liposomes that are destined to fuse. The electric field will lead to defects or pores in the membrane as described above and if two liposomes are in close contact with each other, these defects or pores can coalesce and subsequently span two membranes instead of one. Eventually, this will result in a pore connecting the two compartments, which provide a fluid contact point between the two liposomes (Zimmermann, 1982). This structure is referred to as the fusion pore. After this stage, the pore expands and completes the fusion of the liposomes, which result in mixing of the contents of the two liposomes. If several pores coalesce at the same time, inverted or inside-out configured liposomes can form between the fusing membranes (Fig. 30).

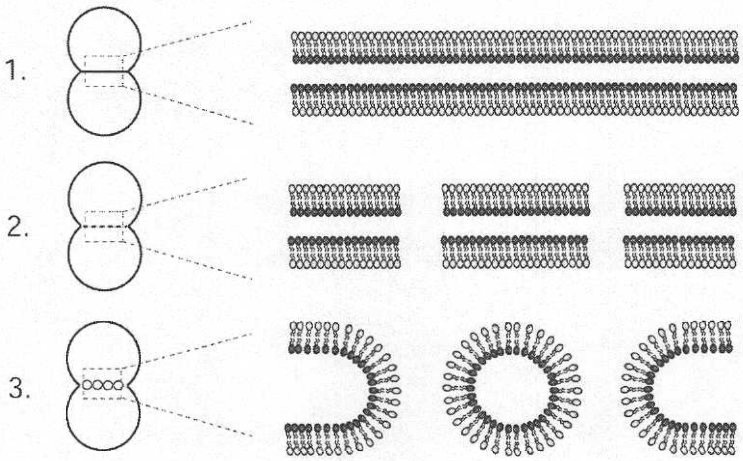

Figure 30. Schematic of the pore formation mechanism for electric field assisted membrane fusion. When two membranes are brought into close contact (step 1) and an electric field is applied, membrane pores are created (step 2). If the pores in the two membranes overlap, membrane fusion can occur. In some cases, small liposomes have been observed to form after during the membrane fusion and according to the proposed fusion mechanisms these liposomes have an inside-out configuration (step 3).

Traditional electrofusion and electroporation protocols are normally performed in bulk experiments (Stoichova and Hui, 1994), where two different liposome suspensions are mixed and the whole mixture is subjected to the electric field. It is therefore difficult to determine which fusion partners and the number of fusion partners in the final product liposome. We have developed a miniaturised electrofusion technique, where individual cells or liposomes can be selected and fused, by the use of micromanipulator controlled carbon fibre electrodes (Fig. 31) (Strömberg *et al.*, 2000, Chiu *et al.*, 1999a). The advantage is that individual liposomes can be studied one at a time since the electric field that is applied is locally produced and does not affect surrounding liposomes. Of course it is easy to choose various fusion partners and the number of fusion partners in the final liposome using this technique, which also is of great importance when performing controlled reactions inside liposomes.

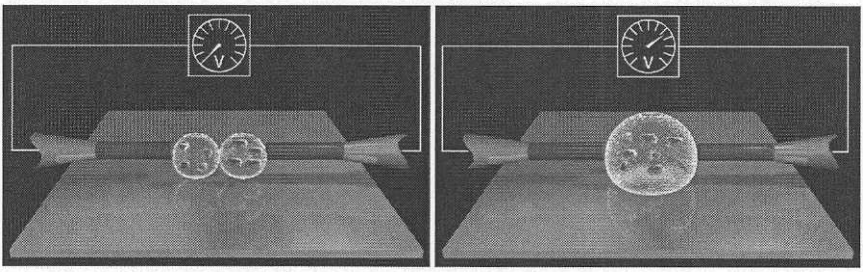

Figure 31. Schematic illustration of the microelectrofusion technique. (**left**) If two liposomes are differentiated with respect to their interior contents with different reagents, for example, illustrated in the figure by spheres and rectangular boxes, a reaction can be initiated by electrofusion. (**right**) When an electric field is applied across the two liposomes, pores are created in the liposome membranes and if the two liposome membranes are in close contact, two pores can coalesce into a fusion pore, which expands and completes the liposome fusion. After fusion, the contents of the two liposomes are mixed and the reaction is initiated.

When it comes to applications of this technique to networks, it is possible to fuse end-liposomes of a linear branched network into a closed network structure, using this technique. Also, electrofusion can be used to connect individual networks, by fusion of two liposomes belonging to two different networks, thereby increasing the size and complexity of the network architecture. If the two networks display different chemical identity, network reactions can also be initiated.

Chemically induced fusion

Chemical stimulation to fusion can also be performed in a variety of ways. Perhaps the best known is the effect of divalent cations, for example calcium ions, that can induce fusion of negatively charged lipid membranes. The underlying mechanism is believed to be an effect of adsorption of calcium ions onto the negative surface of the lipid membrane (Menger and Gabrielson, 1995; Hui *et al.*, 1999). The calcium then acts as a bridging ligand between two membranes and therefore provides a very close contact, which is normally not conceivable, due to thermal fluctuations etcetera. This results in a surface dehydration by water exclusion effects, which then triggers the fusion of the lipid membranes.

Optically induced fusion

Optical induced fusion or photoactivated liposome fusion can be promoted by incorporating a photochromic lipid that can polymerise under the influence of light of a certain wavelength (photopolymerisation) or undergo structural changes (Bennet and O´Brien, 1995, Morgan *et al.*, 1995). For example, by incorporation of a sorbyl lipid, the fusion process was enhanced by UV-light, resulting in photopolymerisation of the membrane. The structural changes in the membrane and domain formation were believed to be the mechanism of the membrane fusion in this system (Bennet and Morgan, 1995). Another elegant approach utilise optical trapping of liposomes in order position the liposomes into close contact, by the use of near infrared laser and fusion was initiated by a high energy ultraviolet laser pulse at the contact point between the liposomes (Kulin *et al.*, 2003). Since optical trapping of unilamellar liposomes is difficult, multilamellar liposomes was used in this work.

Nanotube-mediated merging of liposomes

The unique mechanical properties of the lipid bilayer membrane allow the formation of spherical structures (liposomes) inter-connected by ultra-small cylindrical nanotubes, as described above. We have utilized the network formation technology to create liposomes of well-defined size, which are differentiated with respect to interior contents and interconnected by nanotubes. The differentiation is performed on-line during the formation of the network, by exchanging the solution in the micropipette used for network fabrication. Thus, we are able to load containers in a network with specific concentrations or in other words specific numbers of reagent molecules (set by the size of the container and the reagent concentration). We used these features to develop a new method to initiate chemical reactions inside liposomes, described in paper X.

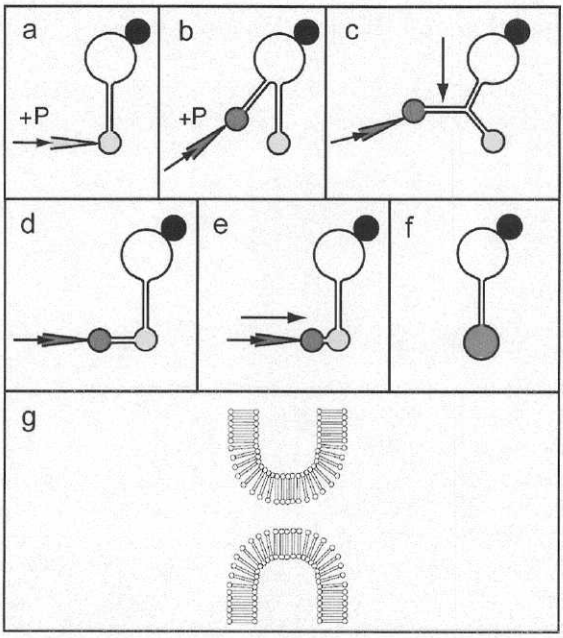

Figure 32. Schematic of the nanotube-mediated merging of differentiated liposomes. (**a**, **b**) By utilizing the micropipette assisted network formation technique two network liposomes with differentiated contents are created. (**c**) Due to the fluid character of the lipid bilayer, the nanotube attachment point is freely movable over the entire bilayer membrane network, thus creating an intermediate nanotube junction. (**d**) The nanotube attachment point has been moved to the liposome created in the first step. (**e**) By bringing the two liposomes containing different reagents into close contact, they spontaneously merge into one. (**f**) After merging, the reagents are mixed and product starts to form. (**g**) Schematic picture of the "fusion pore" structure created when the two liposomes are brought into close contact. The liposomes can more or less be considered as planar membranes, when compared to small size of the fluid contact point.

The technique involves formation and translation of nanotube attachment points across the fluid bilayer membrane system of liposomes and nanotubes (Fig 32a-c). The purpose is to create two liposomes that are differentiated with respect to interior contents, interconnected by a single nanotube (Fig 32d). The interconnecting nanotube provides fluid contact between the two compartments and at minimal separation length, *i.e.* when the nanotube length approaches zero (Fig 32e), the fluid contact point resembles a "fusion pore" between the liposomes, similar to the structure found when two solitary liposomes are fused using electrofusion, as described above (Fig 32g). When the two liposomes are pushed together, the interconnected liposomes merge and form a product liposome and chemical reactions can thereby be initiated, without any external forces, such as electric fields (Fig 32f).

Communication and transport between network containers

The use of solitary liposomes and liposome containers in a network as microreactors has so far been described. One can also envision the use of network structures as miniaturised chemical laboratories in accordance with the spirit of μ-TAS and lab-on-a-chip devices. This means that network containers attain different functions, such as reagent containers, mixing containers, product containers and detection containers etcetera. One key feature in such a miniaturised chemical laboratory of vital importance is missing so far, which is the transport of material between liposome containers in a network.

Tension-driven lipid flow

The dynamical character and fluid features of the bilayer membrane network enables lipid transport across the nanotubes. Such flows are believed to be the result of membrane tension differences across the nanotube. The general name for such flows is Marangoni flows, which, for example, describes spreading of films across an air-water interface (Probstein, 1994). Mechanical equilibrium requires that the interfacial tension of an interface surface must be constant throughout the system. By chemical or mechanical perturbation from the equilibrium situation, tension gradients or differences can be created across the system, which leads to a flow of material to diminish these differences. This type of membrane tension-driven lipid flow has been theoretically investigated in previous work (Chizmadzhev *et al.*, 1999). Lipid flow was studied through fusion pore models, where two membranes with differing membrane tensions fuse. The lipid flow was modelled by a balance between the work done by tension forces and the viscous energy dissipation in these systems. Lipid velocity and area flux was estimated through the fusion pore structure as a function of the tension difference and geometry of the fusion pore. The lipid velocities usually ranged between a few micrometers per second to 50 micrometers per second depending on pore geometry and tension difference.

This kind of lipid flow has also earned some attention as transport mechanisms between the ER and the Golgi complex in the cell. Tension differences were thought to be the driving force to produce convective flow of lipid rather than diffusive movement of lipid, mediated by lipid nanotubes interlinking the two compartments. Lipid velocities of around 10 micrometers per second were estimated from redistribution experiments. These inspiring results suggest that lipid flow can be of great importance to describe some types of material transport between organelles and also the plasma membrane (Sciaky *et al.*, 1997).

In lipid bilayer networks, any difference in membrane tension between nanotube connected network containers will thus lead to a lipid flow between the containers across the lipid nanotube from regions of low tension to regions of high tension. We make use of this important result first of all when networks are created using the micropipette-assisted network formation technique described above, since lipid material flows across the network system from the lipid reservoir in the form of a multilamellar liposome. The second field of application makes use of the lipid transport phenomena as a key mechanism to create communication between network containers. In our systems of bilayer networks we mechanically perturb a target container to induce a tension difference, which can be done, for example, by micropipette aspiration techniques. We used two carbon fibres to pinch one of the surface attached liposomes in the network in order to change the surface-to-volume ratio and increase the membrane tension as described in paper VI. This created a tension difference between the two liposomes and lipid flowed towards the perturbed liposome to diminish this difference. Estimated lipid velocities ranged between 20-30 μm/s, however much higher velocities, >60 μm/s were also observed (Karlsson, R. *et al.*, 2002).

Fluid and material transport in nanoscale channels

Due to the small scale dimension of the nanotube in our systems and the viscosity of the fluid contained inside the nanotube, this fluid will be dragged along the lipid flow. Creation of nanometer scaled fluidic systems and control and handling of fluid in such small-scale systems may be difficult using more traditional techniques. However, there is an increasing development of fabrication methods that reaches down to the nanometer scale (Haneveld *et al.*, 2003; Tas *et al.*, 2002) and solutions to fluid handling in small channels (Desmet and Baron, 2000), including principles of hydrodynamic focusing of fluids to produce nanoscaled submerged fluid jets (Knight *et al.*, 1998). Following this sprit we have developed new fabrication protocols using soft materials and creative principles of fluid movement to overcome problems with handling and controlling fluid transport in nanometer scaled systems, through the development of membrane tension driven lipid flow, as demonstrated in papers VI and VII (Fig. 33).

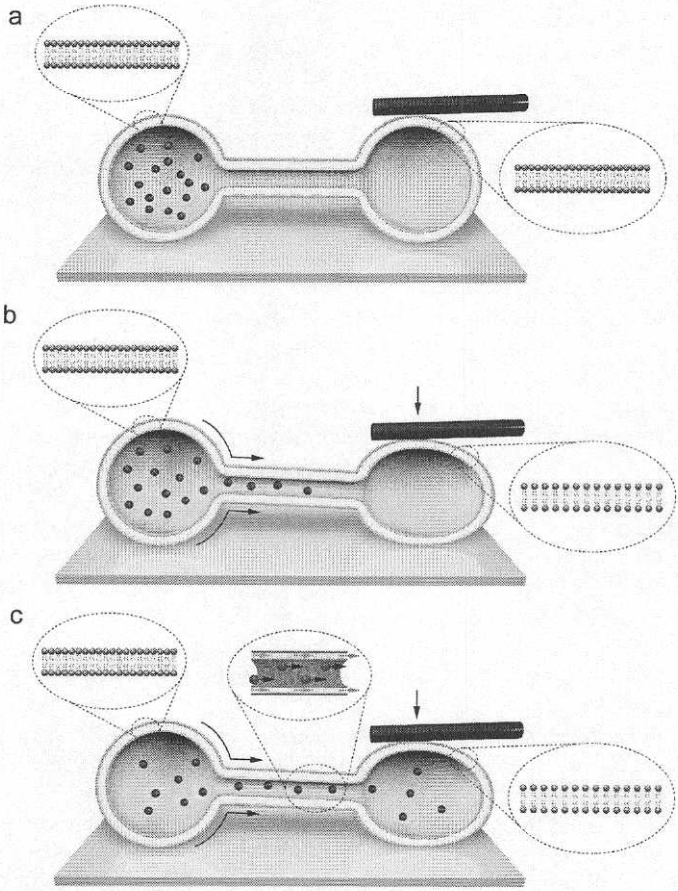

Figure 33. Schematic illustrating the tension-driven lipid flow principle. (**a**) A cut-through view of a two surface-immobilised liposomes interconnected by a lipid nanotube. In the starting situation there is no active transport of lipid across the nanotube. (**b**) By shape deformation, for example, by pressing the liposome with a micromanipulator carbon fiber, a tension difference is created, which results in a flow of lipid material from regions of low tension to regions of high tension. (**c**) Fluid and material trapped inside the lipid nanotube will be dragged along the lipid flow with a plug-like flow profile.

In our system we assume that the flow profile should be essentially flat and plug-like, which means that particles and substances dissolved in the fluid matrix will have approximately the same velocity as the fluid and the lipid flow (Goveas *et al.*, 1997). In such small scale channels as demonstrated by the lipid nanotubes presented here, the so-called Reynolds number, Re, is very low, thus indicating that the flow is in the laminar flow regime, which is demonstrated by Re << 1. The Reynolds number is defined as

$$\mathrm{Re} = \frac{\rho v l}{\eta} \qquad \text{(Equation 28)}$$

, where ρ is the density of the fluid, v is the velocity, l is the systems length scale and η is the viscosity of the fluid. Considering transport of water (ρ = 1000 kg/m^3, η = 0,001 Pa s) in a lipid nanotube with diameter 100 nanometers with a velocity of 50 µm/s, the Reynolds number is $5*10^{-6}$.

Fluid transport was vizualised by tracking trapped particles inside the nanotubes. The system demonstrated very rapid response to mechanical perturbations, which was illustrated by the fact that particles could be transported and trapped at any point along the nanotube by controlling the extent of deformation of the target vesicle. It has been estimated that steady-state fluxes of lipid can be obtained very fast in these systems, usually in the nanosecond regime (Chizmadzhev *et al.*, 1999). By relieving the imposed stress, the system reverted back to its original starting situation, thus making it possible to create reversible flows in these lipid nanochannel systems (Karlsson, R. *et al.*, 2002).

Formation and transport of nanotube-integrated vesicles

A continuation of this work was done by developing techniques to produce nanotube-integrated mobile vesicles as transport containers. This type of nanotube-vesicle assembly has been described in several other papers and is commonly called pearl-chain vesicles. However, the pearling states arise from other starting situations than described in our systems and the appearance of pearl-chain vesicles are triggered by other excitations, such as the use of optical tweezers (Bar-Ziv *et al.*, 1998), gradual disruption of actin cytoskeleton (Bar-Ziv *et al.*, 1999) or by anchoring polymers to the bilayer membrane (Tsafrir *et al.*, 2001). The use of optical tweezers induces a shape transformation of long cylindrical lipid tubes into a string of pearls. This type of instability was believed to be a result of a competition between the induced membrane tension, created by the optical tweezers, and the bending elasticity of the membrane (Bar-Ziv and Moses, 1994; Bar-Ziv *et al.*, 1998). Anchoring of polymers on the other hand gives rise to another type of pearling, which could be explained by both area difference elasticity models and spontaneous curvature models (Tsafrir *et al.*, 2001). Noticeably, pearl-chain vesicles have also been found in cells, thereby motivating the study of formation and dynamics of these structures (Kosawada *et al.*, 1999). Recently, it has also been suggested that lipid nanotubes may have an important role in directed transport of vesicles inside cells (Iglic *et al.*, 2003).

The starting situation in our system was again two liposomes connected by a lipid nanotube, which can be described by the delicate balance between tension, bending and adhesion forces as described above (Fig 34a). By perturbing the system, a dynamical response is obtained, which can lead to shape changes of the system. For example, nanotube-integrated vesicles were created by rapidly adding excess membrane to one network container by merging this target container with another nanotube-connected vesicle (Fig 34b). This results in a momentary reduction in membrane tension. The dynamical response was an assymetrical shape change of the nanotube into a funnel-like structure, by increasing the nanotube diameter severalfold (Fig 34c). After the dynamical shape change the system tried to minimize the

energy by assuming the starting situation with a nanotube connecting the surface attached vesicles. Excess membrane flowed into the unperturbed surface attached vesicle, thus trapping some of the fluid that had entered the nanotube during the shape change. Undulations are thereby transformed into nanotube-integrated vesicles, which thus contain the same fluid as the perturbed vesicle, since this vesicle donates both the membrane and the fluid to create these integrated vesicles (Fig 34d).

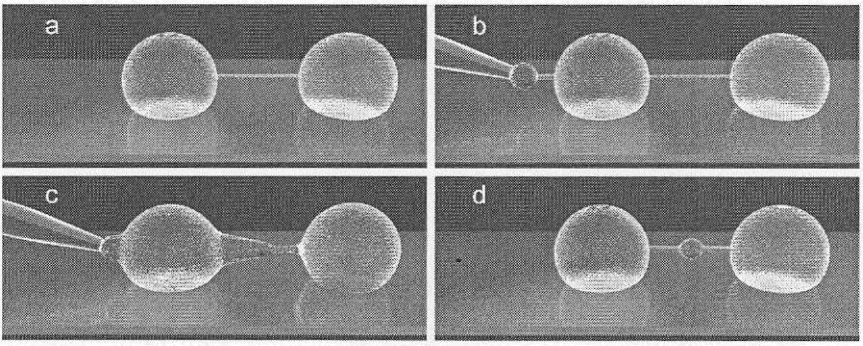

Figure 34. Schematic three-dimensional image showing the formation of nanotube-integrated vesicles on nanotubes. (**a**) Starting system of two surface-attached nanotube-connected unilamellar liposomes (a multilamellar liposome is usually attached to one of the unilamellar liposomes, not shown in the image). (**b**) By using the micropipette-assisted technique of network formation, a new liposome is created at the microinjection tip. (**c**) By merging of the micropipette attached liposome with one of the surface attached liposomes, the system is perturbed and the nanotube is opened up assymetrically. (**d**) The system tries to create the starting system with two nanotube-connected liposomes, however, excess fluid is trapped within the nanotube system, thereby forming nanotube-integrated vesicles.

These nanotube-integrated vesicles could be shuffled back and forth between the surface-attached vesicles by subsequently changing the membrane tension of target containers, since these vesicles follow the direction of the lipid flow in the system (Goveas *et al.*, 1997). It was also possible for the nanotube-integrated vesicles to merge and release their content into one of the surface-attached vesicles, thereby illustrating controlled material transport between nanotube connected vesicles. This can be used for controlled delivery of small volumes of reagents into a network liposome to start reactions or to titrate a chemical substance in order to find the concentration of this substance in a network liposome (Karlsson *et al.*, 2003b).

Routing of nanotube-integrated vesicles in large networks

For routing of materials and reagents in larger networks we developed a two-point perturbation technique in order to direct the material transport between two specific network containers. Normally, if only one network container is perturbed by, for example, mechanical shape deformation to increase the membrane tension, this result in a symmetric flow of lipid from the surrounding connected containers towards the perturbed liposome container. The two-point perturbation technique decreases the membrane tension in one container by adding excess membrane as described above and at the same time increase the membrane tension at another nanotube connected liposome container by mechanical shape deformation, for example. A much larger difference in membrane tension is therefore created across one specific nanotube, which results in a more directed flow. As before, the drop in membrane tension results in nanotube-integrated vesicles, which can function as transport containers. A fluidic switch could therefore be constructed through selection of the directionality of material

transport between target containers. To demonstrate the switching function in these networks, we created a network of three liposome containers interconnected by two nanotubes. Upon perturbation of the middle liposome container by adding surplus membrane through merging of a nanotube-connected liposome, the two nanotubes were deformed as described above and transformed into nanotube-integrated vesicles. By mechanical deformation of one of the outer liposomes in the network, using a carbon fibre, these nanotube-integrated vesicles could be transported to and merge with the target vesicle either to the right or to the left (Fig. 35) (Karlsson *et al.*, 2003a)

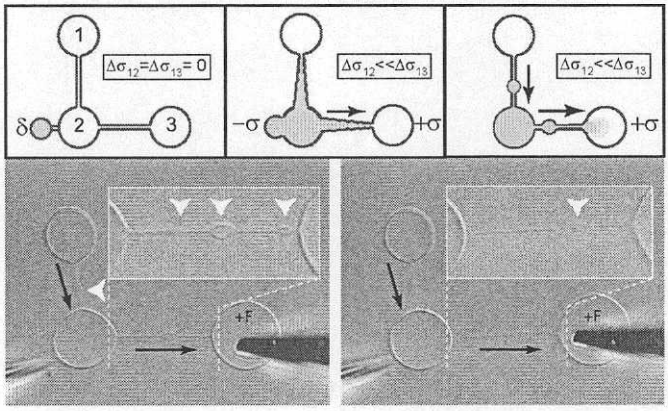

Figure 35. Schematic images and corresponding experimental images of routing of nanotube-integrated vesicle in a network. By using a two-point perturbation technique, i.e. increasing the tension, σ, in one container by mechanical shape deformation, for example, and at the same time lowering the tension in another network container by adding excess membrane, a directed flow of nanotube-integrated vesicles can be achieved. The addition of excess membrane to one of the surface-immobilized liposomes was performed by merging with a nanotube-integrated liposome (denoted by δ). Black arrows show the lipid flow direction when force, F, was applied to induce shape deformation to one of the surface-immobilized liposomes.

Lipid nanotubes and nanotube junctions as reaction containers

The unexplored area so far is the use of other parts of the network besides the liposomes as reaction containers, namely the lipid nanotubes and the nanotube junctions. So far, solitary liposomes and network liposomes have been used as reaction containers, however these kinds of structures still belong to the microscopic size range between a few micrometers to a several tens of micrometers in diameter. To be able to shrink the reactor volumes further and still have the ability to manually manipulate the system and maintain control over reaction events inside these confined volumes (which can be difficult using solitary liposomes of submicrometer size), lipid nanotubes and nanotube junctions appears to be good candidates. These structures range between a few tens of nanometers up to a few hundred nanometers in diameter, thereby displaying structural features of a truly nanoscaled reaction container. Reagents that are dissolved in the fluid medium contained within the bilayer membrane boundary can thus be transported by several mechanisms through the nanotubes, which includes diffusion, moving-wall driven lipid flow mediated transport and transport in the form of nanotube-integrated vesicles. The diffusional transport through confined structures, such as nanotubes have been shown to be significantly reduced compared to the bulk value. This can be attributed to hindered diffusion (Deen, 1987), which has several contributions, such as surface interactions, and also the anomalous behaviour of fluids confined in nanometerscaled systems (Di Leo and Marañón, 2003; Phaler *et al.*, 1989).

Moving-wall driven lipid flows as described above can be used to transport the liquid column within the lipid nanotube. It should therefore possible to create a nanofluidic flow reactor by constructing a three-way junction and letting the lipid flow drag along reagents from two different reagent reservoirs and mix the reagents in the nanotube junction, where the nanotubes meet. For example, figure 36 describes a hypothetic enzyme catalyzed reaction, where substrate is converted into products inside a third nanotube.

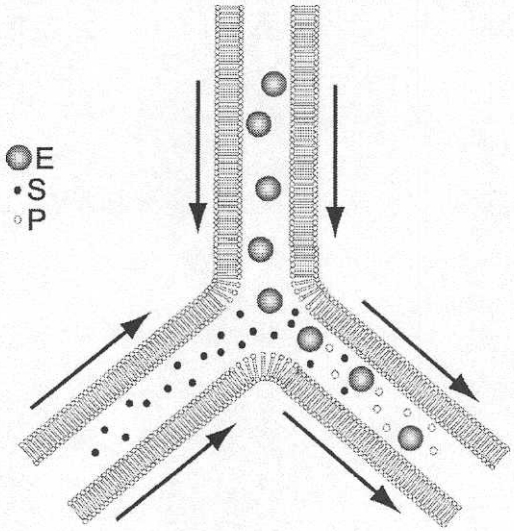

Figure 36. Schematic of a hypothetical continuous flow reactor system of lipid nanotubes. By inducing a lipid flow in the system, the lipid will drag along the fluid and particles inside the nanotubes. The black arrows show the direction of the lipid flow in this system. If substrate and enzyme are contained in two different nanotubes, this type of flow can direct the substrate and enzyme into a nanotube junction, where they mix and start to produce products in the third nanotube channel.

The same kind of experiment could also be performed by using the nanotube-integrated vesicles as transport containers. Such vesicles tend to be stabilized in nanotube junctions, thus making it possible to create and transport nanotube-integrated vesicles into a junction point, where they stay. Reactants can thus be supplied into the junction point in the form of two vesicles on two separate nanotubes. These vesicles can be moved into the shared nanotube junction as before, using tension-driven lipid flow, from two reservoirs into a nanotube junction, where the contents of the transported nanotube-integrated vesicles mix. However, in this case the majority of the reaction instead takes place inside a small vesicular structure at the junction point, rather than a cylindrical geometry (Fig. 37).

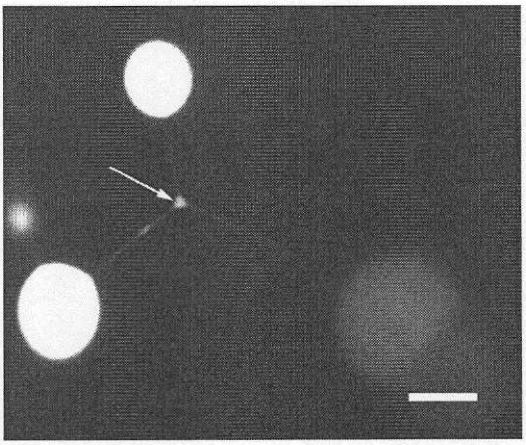

Figure 37. Image showing formation and transport of nanotube-integrated vesicles into a nanotube junction. The two "white" network liposomes was filled with fluorescent dextrane (molecular weight 10 kDa) and nanotube-integrated vesicles were formed by merging one of these network liposomes with a micropipette-attached nanotube-connected liposome. The nanotube-integrated vesicles moved into the nanotube junction, where they accumulated and the white arrow show the thus formed small vesicular structure trapped in the junction region. Scale bar 10 μm.

Chemical reactions – implications in confined compartments

One of the aims of this thesis was to develop tools to be able to manipulate liposomes and lipid assemblies in order to use them as biomimetic reaction containers. This has resulted in miniaturized techniques of electroporation, electrofusion and modified microinjection protocols to be able to pass the semi-permeable membrane and add reagents to the interior of liposomes. It has also lead to the development of techniques to create networks of lipid nanotubes and liposomes in order to decrease the size of the reaction environment down to the nanometer scale and to study reactions in more complex geometries and in networks. The network formation technique has also resulted in novel reaction initiation techniques, such as the nanotube-mediated merging of liposomes presented in paper X. Much effort has also been devoted to transport and route material and fluid in between these network containers.

Although the focus has been to develop these reaction environments and transport mechanisms, the goal is to study chemical reactions in these confined systems and therefore some words have to be said about reactions and some implications that may arise in confined systems.

Historically, reaction kinetics and reaction dynamics have been more or less treated separately, however kinetics (reaction rate measurements) and dynamics (molecular reaction mechanism) both try to elucidate what factors that drives reactants into products (Laidler and Meiser, 1995). For example, experimentally it has been known for a long time that rates and rate coefficients of many reactants depend on temperature. A well-known relationship between the temperature, T, and the rate coefficient, k, is called the Arrhenius equation

$$k = Ae^{-E/RT} \qquad \text{(Equation 29)}$$

This type of dependence arises because the reactants have to overcome an energy barrier to be rearranged and produce products. A is called the pre-exponential factor, E is the activation energy and R is the gas constant. Estimates of the pre-exponential factor have, for example, been made according to the hard-sphere collision theory. (A modified version of this hard-sphere collision model was used in paper II). In this theory, collision between gaseous species treated as hard spheres were used as a model to predict the magnitude of the pre-exponential factor. Collision theory provides a simple picture of how bimolecular reactions can occur, however large discrepancies between theory and experimental results have been noted. Often a steric factor must be included to be able to match and correct theory with experiments due to the fact that molecules must orient themselves in a specific way in order to react, that is, just a simple collision is usually not enough. Much attention has therefore instead been directed to developing other theories, such as the activated complex theory or the transition-state theory. In this theory, the reactants are considered to move along a reaction coordinate and the energy profile of the reaction is followed. This usually results in potential energy surfaces, which maps the reaction system as bonds are broken and as well as the creation of a transition state, which is depicted as a saddle point on the potential energy surface. Calculation of potential energy surfaces have been done for a few very simple systems, however, more complicated reactions are very difficult to describe in detail (Laidler and Meiser, 1995).

Of course, the situation turns even more complex when reactions take place in solution. The environment is much denser, making movement of molecules much more hindered and complex in character. For example, nitrogen gas at room temperature and normal pressure (1 atmosphere) will only occupy 0,2% of the total volume, while liquids usually occupy more than 50 %. Also, the solvent molecules will contribute with effects that can alter the reaction kinetics, for example, by stabilization of ionic molecules. Motion through the solution or diffusion is often envisioned as a random walk, which can be

described as molecules moving randomly in new directions all the time, uncorrelated to the original direction.

The investigation of diffusion for individual solute molecules was initiated in 1828 by the Scottish botanist Robert Brown. He noted that when pollen grains were observed under a microscope they seemed to be in constant and irregular motion. He therefore thought that this behaviour was a property of living matter. However, the same motion was also observed for dye particles and Robert Brown had no explanation for this. The explanation was given in 1888 when Louis Georges Gouy made a thorough investigation of the motion. The motion had come to be called Brownian movement or Brownian motion. By eliminating other possibilities Gouy concluded that the explanation lies in the thermal movements of the liquid, which cause solute molecules to move by colliding with the solvent molecules. In a liquid, a Brownian particle will collide with the solvent molecules about 10^{20} times per second depending on the size of the particle (Chandrasekhar, 1943). These collisions are so frequent that separate collisions cannot be treated explicitly. Each collision is believed to induce a change in the path of the molecule and therefore it is impossible to follow the path in detail. In the beginning of the century, Albert Einstein and Marian von Smoluchowski both treated the problem from different points of view with the same results. One important result that was obtained by Einstein was the probability distribution for the distance travelled, in any direction, in a certain time t. The mean square distance travelled, x^2, can thus be evaluated and this gives

$$x^2 = 2Dt \qquad \text{(Equation 30)}$$

, where D is the diffusion coefficient and t is the time the particle has travelled. This is called the random-walk equation in one dimension.

An approximation that can make the problem easier to solve is to treat the collisions not individually, but rather as ensemble-averaged collisions. In this way, the path of a molecule is changed only after many collisions with the solvent molecules. The molecules will now experience a frictional force in the path and also a random change in their direction. This frictional force was shown to be related to the diffusion coefficient and lead to the formulation of the well-known Stokes-Einstein equation

$$D = \frac{k_B T}{6\pi\eta r} \qquad \text{(Equation 31)}$$

, where r is the spherical radius of the particle, η is the solvent viscosity, k_B is the Boltzmann constant and T is the absolute temperature. If the viscosity of the solvent is known, a measurement of the diffusion coefficient gives an estimate of the particle radius. Conversely, measurement of the rate of diffusion-controlled reactions of known species can give information of the physical parameters of the system, for example the microviscosity within micelles (Rothenberger and Grätzel, 1989; Gösele *et al.*, 1979).

The difference between gas and solution phase reactions can further be depicted by the collision approach above. When two reagent molecules meet in solution by diffusion or Brownian motion they are sometimes trapped in a dynamical "cage" of solvent molecules, which forces the reagent molecules to collide a number of times before they separate again. A group of collisions is called an encounter. This type of encounter effects were also found from simulations made in paper II. The result is that although the average collision frequency can be almost similar in gas and in liquid, the distribution of collisions is very different if the collisions are performed in a dense media such as a liquid.

Reactions in solution can be classified into diffusion-controlled reactions and activation-controlled reaction (Pilling and Seakings, 1995). Diffusion-controlled reactions occur when the reaction has small activation energy or if the separation of reactants is difficult, for example, in a viscous solvent or a crowded environment. The rate-determining step is thus the approach of reactants and once they reach each other the reaction is fast (Calef and Deutch, 1983). Activation-controlled reactions are instead limited by large activation energy and the reaction rate is determined by the equilibrium concentration of the encounter pairs and the passage rate of the energy barrier. The crowded, complex and compartmentalized interior of cells give rise to hindered diffusion of molecules, thereby slowing down transport. Intracellular reactions are therefore often considered to be of a diffusion-controlled reaction type (Luby-Phelps, 2000).

Another special feature of cellular reaction systems is the use of biological catalysts, enzymes. A catalyst is by definition a substance that influences the rate of reaction without itself being used up. Enzymes provide alternative reaction pathways by drastically lowering the activation energy of a reaction of substrate into product (Cornish-Bowden, 1995). The catalytic activity depends on the amino acid sequence and especially the functional groups present in the enzyme´s active or catalytic site. This active site can be described as a complex three-dimensional structure, where the functional groups of the amino acids have access to the substrate. The precise shape and size of the active site of enzymes also provides specificity for the substrates. Enzymes can both be activated or inhibited by a number of mechanisms in order to follow the demand and availability situation of the involved reaction and enzymes are therefore in some sense considered as molecular machines. The simplest case involves conversion of a single substrate into product. Usually, the rates of such reactions display different limiting kinetics with regards to the substrate concentration. At low substrate concentration first-order kinetics is displayed, while at high substrate concentration zero-order kinetics is found. These types of behaviour lead to the development of the Michaelis-Menten mechanism, where enzyme and substrate reversibly form an enzyme-substrate complex, which can be transformed into enzymes and product. Steady-state treatment leads to the well-known Michaelis-Menten equation

$$v = \frac{V[S]}{K_m + [S]} \qquad \text{(Equation 32)}$$

, where v is the rate of the reaction, V is referred to as the limiting rate, K_m is the Michaelis constant and [S] is the substrate concentration.
This is under the assumption that the enzyme concentration is much smaller than the substrate concentration. However, in reality it has been argued that the opposite situation can exist in cellular environments at various compartmentalised sites, where local concentrations of enzymes can be very high. In that situation, the availability of substrate may instead be the rate limiting step, thus strengthening the argument of the phenomenon of channelling substrates through a complex of enzymes to increase the overall efficiency (Pagliaro, 2000; Ovadi and Srere, 2000).

The effects of "pure" compartmentalisation or simple containment of a reagent system has also been considered. It has been argued that under certain conditions, biochemical reaction systems can be subjected to a synchronous operation mode by development of so called molecular networks (Mikhailov and Hess, 1996; Stange *et al.*, 1999). These molecular networks can be viewed as a population of reactive molecules that interact and communicate with each other, with the result that individual reaction cycles coalesce into a single population cycle. The outcome of this self-organising synchronous behaviour is a correlated product release from an enzyme population, which gives rise to product spikes. It has been

shown that the emergence of this type of behaviour can occur in an allosterically regulated enzyme population by variation of the timescales involved in physical and chemical processes taking place in a small spatial volume.

The time it takes for a regulatory molecule to be found anywhere in the confined volume, with the same probability after release somewhere in the volume, is called the mixing time. This can be estimated by

$$t_{mix} = \frac{L^2}{D} \qquad \text{(Equation 33)}$$

The mixing time, t_{mix}, is thus dependent on the linear size of the reaction volume, L, and the diffusion coefficient, D, of the molecule. The time it takes for this regulatory molecule to find its target site on the enzyme is called the traffic time, $t_{traffic}$, which can be estimated by

$$t_{traffic} = \frac{L^3}{DR} \qquad \text{(Equation 34)}$$

, where R is the target site radius on the enzyme molecule. If there are several enzyme targets (N equal targets) the transit time, $t_{transit}$, which is the time needed for a regulatory molecule to find one of the targets, can be defined as

$$t_{transit} = \frac{L^3}{NDR} \qquad \text{(Equation 35)}$$

In small compartments, strong diffusional mixing occurs, which can lead to a much smaller mixing time compared to the transit time. When this occurs, the regulatory molecule can find its target anywhere in the volume with equal probability. These diffusion and transport times can then be compared to the time for an enzyme molecule to perform a catalytic cycle, denoted by τ_0. The catalytic cycle of an enzyme includes binding of substrate, creation of substrate-enzyme complex, formation of product, release of product and finally resetting to the first stage to be able to accept a new substrate molecule again. Slow enzymes could have cycle times between 10 and 100 ms and in small compartments this can lead to the following situation

$$\tau_0 >> t_{transit} >> t_{mix} \qquad \text{(Equation 36)}$$

For enzymatic reactions that occur in highly compartmentalized volumes, with structural features between sub-micrometers to a few micrometers, the characteristic molecular cycle time may thus be greater than both the transit time and the mixing times in the system. When regulatory molecules are produced by the enzyme population, interactions can start to occur and when the transit time is larger than the mixing time the regulatory molecule can influence the enzyme molecules with equal probability. If the allosteric regulation is strong, the individual cycles may start to affect each other so much that the dynamics will be coherent, resulting in synchronous product release. This demonstrates that only by shrinking the reaction volume, effects that are usually not observed can start to evolve when reactants are confined in a crowded confined space, suggesting that such effects may be of importance in environments of high compartmentalization, such as in the cell (Stange *et al.*, 1999).

In cellular environments it has been noted that the finite volume sometimes results in very low copy number of species in the intracellular environment, even if the molar

concentration is appreciable, and that number fluctuations of a certain substrate can thereby play an important role and limit the availability at a certain location and time in the cellular environment (Pagliaro, 2000). The usual approach of classical chemical kinetics does not involve fluctuations of the concentration of reacting species; however in restricted systems fluctuations are inherent (Khairutdinov and Serpone, 1996). It is therefore not possible to apply classical chemical kinetics to describe reactions taking place in confined volumes, instead stochastic approaches are often used in order to analyse statistical fluctuations of the reacting species.

There are several excellent reviews on the implications of chemical reactions in confined structures, such as micelles, reverse micelles, microemulsions and small vesicles (Kalyanasundaram and Grätzel, 1995; Rothenberger and Infelta, 1995; Khairutdinov and Serpone, 1996). In some aspects the theoretical advancement has by far exceeded the experimental part in this field of research. A number of different models exist to describe the distribution of solutes in micellar media and diffusion of reactants in order to explain intramicellar reactions in these microheterogenous environments (Tachiya, 1987; Bug *et al.*, 1992; Gösele *et al.*, 1979). This also includes diffusion models for diffusion-controlled reactions inside micelles and analytical expressions for the so-called survival probability of reactants exist for situations where one of the reactants is fixed at the center of the reaction volume. Other cases include the motion of both reactants inside the reaction volume, which is of course more similar to experimental situations and here random walk methods and simulations must be used instead, since analytical expressions are hard to formulate. In either case the survival probability can usually be described by a single exponential with a first-order rate coefficient, which in general has been demonstrated to be a good approximation. The result from these approaches is that confinement of reactant molecules in spherical volumes, like the interior of micelles, results in faster reactions at large times compared to a similar reaction in infinite space. The micellar compartment features extreme confinement due to its small size (typically a few nanometers in diameter), thus giving a lifetime for a reactant pair somewhere between 50-100 ns (Rothenberger and Infelta, 1995). Excellent agreement has been found for some quenching and triplet-triplet annihilation using appropriate analytical equations and results from simulations. When it comes to reactions inside vesicles, there have been some reports; however, vesicles are more complex and more difficult to study, thus presenting more experimental challenges, which perhaps explain the large imbalance between the numbers of studies performed on micellar reactions compared to vesicular reactions (Khairutdinov and Serpone, 1996).

Of course, theoretical models have also been developed for other geometries than spherical structures, which include two-dimensional and one-dimensional structures, to describe reactions that take place on surfaces and in confined cylindrical environments. All diffusion-limited reactions have been shown to be affected by the spatial dimension of the reaction volume, and below some critical dimension the reaction turns out to be non-classical with a time-dependent rate coefficient, which results from the formation and growth of depletion zones of reactants. Experimental studies of reactions inside capillaries have displayed this type of anomalous kinetics (Lin *et al.*, 1997).

Studies of reaction kinetics in restricted geometries not only will enrich our understanding of reactions taking place inside nanostructures, such as the intracellular compartments, but will also lead to a development of more efficient and better designed nanodevices (Khairutdinov and Serpone, 1996). Together with the vast biochemical complexity that underlies the function of cells and life itself, a formidable task is established for the scientist to unravel. Technical advancement has lead to techniques to investigate the contents and building blocks of cells, through single-cell cytoplasmic analysis and single-organelle content etcetera (Olefirowicz ans Ewing, 1990; Chiu *et al.*, 1998). However, cells

belong to that peculiar type of objects, where the whole is always greater than the sum of all its parts. Even so, efforts have been made to create *in-silico* cell models, where a set of software tools can be used to build cell models of interactions between some specific genes, proteins and nutrients for example (Normile, 1999). Approaches to describe some parts of the vast complexity that prevails inside cellular environments have been made recently through models and increased understanding of biological signaling systems, which possess attributes such as dynamic assembly, translocation, degradation and channeling of chemical reactions (Weng *et al.*, 1999). Still, when it comes to interpreting the system as a whole, in terms of the individual components functionality, the outcome is that increased knowledge so far only expands the complexity and increases the level of difficulty.

Detection

Due to the small-scale of the systems used in this thesis, the number of molecules that are to be detected in a chemical reaction for example is very small, usually a few thousand. To be able to detect low numbers of molecules, sensitive detection techniques must be utilized. Luckily, both optical and electrochemical methods have been developed to probe and detect molecules even down to the single-molecule limit (Xie and Trautman, 1998; Fan and Bard, 1995). In this thesis, we have used fluorescence, or specifically laser induced fluorescence (LIF), as detection technique for fluorescent products in a chemical reaction as described in papers I, II, X or for membrane associated dyes for visualisation purposes of the lipid membrane.

When a molecule is exposed to electromagnetic radiation, *i.e.* light, it can absorb this energy if the amount is close to the energy gap between two electronic, vibrational or rotational energy levels. An electronically excited molecule can relax through several different processes, including both radiative (emission of light) and non-radiative (heat production, photoionisation and photodestruction) processes and usually have a life-time of a few nanoseconds. Radiative processes include fluorescence and phosphorescence. Fluorescence is the more common of the two and occurs between two singlet electronic states S_1 and S_0, where S_1 is the electronically excited state and S_0 is the ground state. A singlet state means that the electrons have different spin states (up and down).

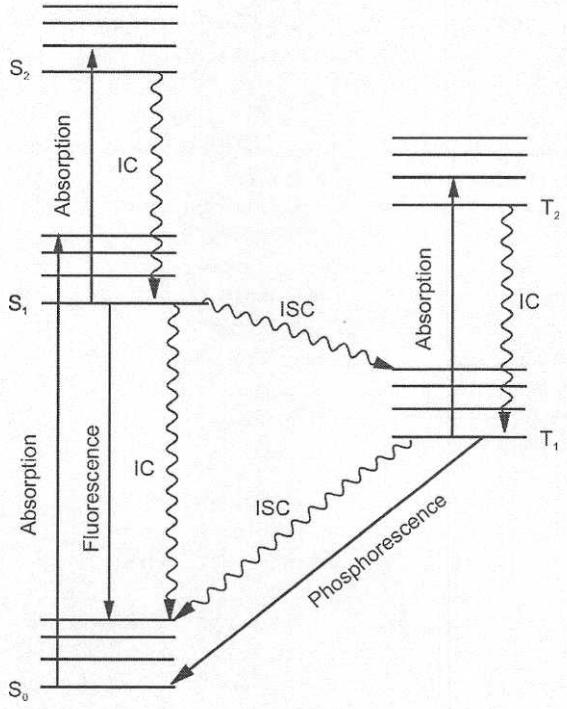

Figure 38. Jablonski diagram of a photoluminescent system. Singlet states are denoted by S and triplet states by T. S_1, S_2, T_1, T_2 are all excited states, while S_0 is the singlet ground state. Non-radiative processes are shown by internal conversion (IC) and intersystem crossing (ISC) in the figure. Each of the electronic states (S_0, S_1, S_2, T_1, T_2) also have vibrational states superimposed on the electronic states, and after absorption fast relaxation occurs to the lowest vibrational state in the electronic level.

Phosphorescence occurs when intersystem crossing (ISC) occurs, where the singlet excited state is converted into a triplet excited state. A triplet state means that the excited electron has the same spin state as the electron in the ground state. Relaxation between a triplet excited state and the singlet ground state is spin-forbidden, since two electrons of the same spin state cannot occupy the same energy level. An illustration of these transitions can be summarised in a Jablonski diagram (Fig. 38). Internal conversion (IC) describes a non-radiative process, where energy is passed over to a lower electronic state without emission of radiation. One major advantage of using fluorescence as a detection method is a high sensitivity, due to the fact that the signal is measured against a dark background as opposed to for example absorbance where a small decrease in the total light intensity is measured.

A linear relationship exists at low absorbance and irradiance, between the resulting fluorescence intensity and the incident light intensity and therefore lasers have been used as excitation sources, in order to improve the fluorescence detection sensitivity. This technique is therefore denoted as laser induced fluorescence (LIF). Laser light display a high intensity beam of light that is highly coherent, have very narrow spectral bandwidth and small beam divergence, making it possible to focus the beam to a very small diffraction-limited spot, which is an excellent feature when detection is applied to small detection windows of chemical separation techniques, such as capillary electrophoresis (CE) (Lundqvist *et al.*, 2003).

Confocal fluorescence microscopy

Normal fluorescence microscopy will excite the fluorescent molecules of the entire object under study and much of the fluorescence thus comes from regions both below and above the focal plane. Confocal fluorescence microscopy on the other hand introduces two pinholes or apertures, one at the excitation side and one at the emission or detection side, which eliminates out-of-focus fluorescence signals. The signal loss of introducing these pinholes can be severe, up to 90-95% for small pinholes, however this can be compensated to some extents by the use of lasers as excitations source, as discussed above. Also, very sensitive detectors, such as single photon avalanche diode detectors (SPAD), as described in papers I and VII, can be used to collect weak fluorescence signals. The result of this confocal setup is that an extremely small detection or probe volume is created, down to femtoliter volumes (Hill and de Mello, 2000), thus making this setup very compatible as a detection technique to monitor reactions inside single liposomes (Chiu *et al.*, 1999a) and also to detect fluorescent flow markers and reaction products inside lipid nanotubes (Karlsson, A. *et al.*, 2003). The sensitivity of this detection technique has reached to the ultimate limit by the detection of single molecules (Xie and Trautman, 1998).

Methods used in the thesis not described so far

Optical trapping

Vesicle transport and manipulation through channels and topographically complex structures, for example, through capillaries was performed by the use of an optical tweezers as described in paper III (Strömberg *et al.*, 2001). Optical tweezers are sometimes compared with "tractor beams", which are encountered in science-fiction films or television shows. They work almost the same, using light or some kind of invisible radiation to take hold of the object in question and move it over some distance without touching or causing damage to it. The only difference is the size of the object. Where the "tractor beam" can move macroscopic spacecraft the optical tweezers can operate on cells, organelles, bacteria and also vesicles (Block, 1990).

The development of optical tweezers was performed by Arthur Ashkin and colleagues at AT&T Bell Laboratories and among the first applications was to cool, stop and trap atoms

and molecules with the help of lasers (Ashkin, 1978). Laser-based atom traps are today used to cool atom beams down to micro-Kelvin temperatures. This eliminates Doppler line-broadening and hence permits a very high resolution for spectroscopic measurements. Larger particles of micrometer sizes in diameter were also trapped in an "optical bottle" or optical potential well. In these experiments two weakly focused laser beams were used to create the optical potential well at the intersection of the two beams.

Lasers that are used to create these optical traps operate in the TEM 00 mode, which means that the laser beam has a Gaussian intensity distribution normal to the direction of light. The trapping of particles using a single laser beam was discovered by the introduction of the gradient force optical trap. The gradient force creates a trap, which is derived from the spatial gradient in the light intensity. The particles will now experience a pulling force towards a point where the light intensity is the highest, which is near the focus. As before, when the laser operates in the TEM 00 mode, the highest light intensity is found in the middle of the beam because of the Gaussian distribution. In a single beam trap, the gradient force is optimised to be as large as possible and also to point towards a single stable trapping zone.

Optically trapped particles must fulfill some general requirements, which include transparency, a higher refractive index than the surrounding solution and a spherical appearance. Multilamellar liposomes can be approximated as nearly transparent and refracting objects having a higher refractive index than the outside solution, and are therefore easily trapped and manipulated using optical tweezers. When the liposome is placed in a beam of light the rays are bent by refraction at the interfaces where they enter and leave the sphere. Before the rays hit the sphere their momentum lie in the z-direction, but after exiting the spherical liposome the rays have gained momentum in other directions. To compensate for this and therefore conserve the momentum in any direction, the liposome gains a momentum that is equally large but has opposite sign to the momentum change of the rays. This means that when the beam has a light gradient, the sphere is pulled towards the point where the light is brightest to compensate for the changes in momentum (Fig. 39).

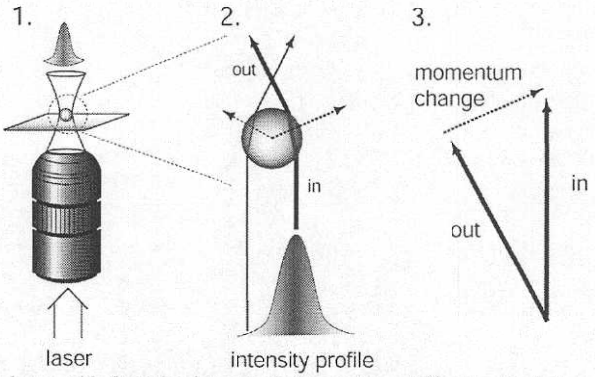

Figure 39. 1. The laser beam with Gaussian intensity is focused to a diffraction-limited spot, using a microscope objective with high magnification and high numerical aperture. 2, 3. The light rays are refracted at the boundaries of the spherical object, both into and out of the sphere. There is a change in the momentum from the incoming light rays compared to the outgoing light rays, which leads to a force in the direction of the dashed arrows. To conserve the momentum of the light, this results in movement of trapped particles toward the beam axis, where the intensity of the light is highest.

Conclusions

We see an increasing interest in studying and performing chemistry in confined compartments. The motivation to this interest comes from both a technological side, where a strong development of micro- and nanofabrication exist and also from a biological/biochemical side, where the interest lies in the study of intracellular reactions, which are performed within nanometer scaled compartments, in environments that clearly differ from bulk situations (Khairutdinov and Serpone, 1996; Pagliaro, 2000). Thus, there is a demand for an increased knowledge of how chemical reaction kinetics and dynamics are affected by confinement from a physico-chemical perspective and also how to apply this knowledge to biological systems. Furthermore, this would lead to an understanding of how to increase the functionality and efficiency of nanoscopic chemistry devices that can operate with only a few numbers of molecules.

Theoretical advancement in this line of research has revealed several compelling results that confinement can lead to anomalous kinetics as a function of size and dimensionality and examples include coherent dynamics of enzyme populations, which clearly motivates the need for development of experimental model systems (Stange *et al.*, 1999). Technologically, the production of nanoscaled chemical reactors with capability of initiating and monitoring reaction events down to the single molecule level is very difficult and therefore also very challenging.

An interesting approach to meet these demands and challenges comes from biomimetics, where information to mechanistic solutions is extracted from biology in order to solve a technological problem (Ball, 2001). The cell displays fascinating features such as extreme compartmentalisation, high degree of control over reaction events, transport of material between compartments etcetera and thus constitutes a major source of inspiration to build nanoscopic devices using biocompatible and biomimetic building blocks.

The use of liposomes as small-scale nano/microreactors for biotechnological applications has been suggested lately (Monnard, 2003), and several reports of liposomes as hosts for biochemical reactions can be found in the literature. As a continuation of this insight we have developed a number of techniques and tools to initiate and monitor chemical reactions inside lipid membrane assemblies, such as solitary liposomes, but also inside networks of liposomes and lipid nanotubes, through development of several micromanipulation protocols to produce networks of liposomes and nanotubes. Finally, in order to fully appreciate the ability of these networks to function as micro/nanoreaction systems, we have also developed means to transport material between containers in a network mediated by the interconnecting nanotubes.

The different techniques to initiate reactions inside liposomes presented in this thesis therefore provide this field of research with a set of tools, which can be applied to studies of confined chemical reactions in solitary liposomes or in network liposomes. This will have impact in the development of model systems for intracellular biochemical reactions inside biomimetic environments, since the liposomes can be tailored with different surface characteristics, for example, by altering the lipid composition of the bilayer, and involve functionality by reconstitution of membrane proteins. The development of network-based technologies also extends and broadens the size range and geometry obtainable for the reaction containers by utilizing lipid nanotubes and nanotube-nanotube junctions. Thus, the idea of performing controlled chemical reactions inside these nanoscaled structures seems very promising.

As mentioned above, the biomimetic capabilities of the lipid bilayer material make it compatible with reconstitution procedures, thereby expanding the applicational areas of these structures as chemical sensors. Insertion of transporters and receptors could give the networks the same functionality as these proteins have in their natural environment. Thus it would be

interesting to study how networks with differentiated contents and reconstituted membrane proteins respond to external stimuli, for example. The biocompatibility properties may also have impact in bionanotechnology, since the network structures developed in this thesis can be directly coupled to biological cells through lipid nanotubes. This establishes a communication link between the liposome network and the cell, which should provide possibilities to donate material into or extract material from the cell, mediated by the lipid nanotubes (Davidson *et al.*, 2003).

The transport capabilities of these systems demonstrated in some of the papers included in the thesis have several application areas. Nanotechnological applications involves the creation of nanofluidic systems capable of transporting and handling low copy numbers of molecules, which can be difficult using traditional fabrication protocols and materials as noted above. These liposome network systems provide fluidic channels down to a few tens of nanometers in diameter and demonstrate novel solutions for liquid transport through a moving-wall driven transport mechanism, induced by application of membrane tension differences. The small-scale features of these systems make them very compatible with single molecule detection technique as demonstrated in paper VII, and can thus provide novel solutions for construction of nanoscale devices operating with single molecules.

Noticeably, this type of tension driven lipid transport mediated by lipid nanotubes have been suggested as a transport mechanisms between organelles inside cells (Sciaky *et al.*, 1997). Together with the development of nanotube-integrated vesicles, these systems can therefore be used as model systems for some membrane-based processes in biological cells and membrane biophysics. This also includes models for membrane fusion and models based on lipid membrane technology have already been applied to the study the final stages of exocytosis events (Cans *et al.*, 2003).

In some context it might be of value to use these lipid networks as template structures, thereby presenting new ways to create solid state devices based on network architecture and design (Evans *et al.*, 1996). Lipid tubules have been demonstrated to function both as templates for metallisation procedures in order to fabricate small scale metall structures (Schnur, 1993; Schnur and Shashidar, 1994), and also as sites for protein crystallisation (Wilson-Kubalek *et al.*, 1998). As mentioned above the micromanipulation protocols can create network structures of great diversity and there is an ongoing development of techniques to extend network formation to include three-dimensional structures, by the use of topographically structures substrates (Hurtig *et al.* Manuscript in preparation).

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Finally, I would like to thank the people that have made this journey possible.

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Past and present members of the Orwar research group. I have great difficulties in formulating the right sentence to describe you. You are: *crème de la crème*, best of the best, top of the line, intelligent, encouraging, stimulating, bla bla bla.
To sum it up, I am deeply honored to have worked with you all.

Many thanks to Daniel Fagerlund for help with the illustrations. You are truly an artist.

Collaborators and co-workers in this research field who have enriched my knowledge through the years: Professor Andrew Ewing, Professor Daniel Chiu, Professor Evan Evans, Doctor Wolkmar Heinrich, Professor David Needham, Associate professor Johan Bergenholtz, Professor Sture Nordholm, and Professor Marina Voinova.

Past and present co-workers and colleagues at the former department of analytical and marine chemistry. Although my own research have been aimed slightly in other directions than pure analytical and marine chemistry I am glad to say that you all have broadened my knowledge in many different forums of which the corridor science rules.

To my family, relatives and friends. Thank you for support through these long years of studying. Looks like I am finished more or less now (only took about twenty years to come this far)

Finally, to Mom, Dad and Roger. Thank you for endless support and loads of fun through the years. I am grateful to you, especially for letting me be a bit lazy these last few years, when I needed it. I couldn't have done this without you.

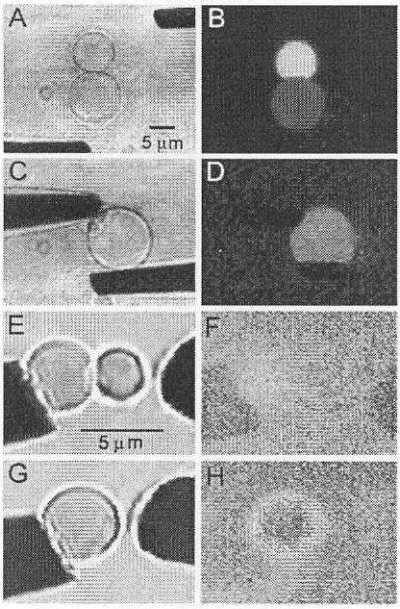
Anders Karlsson

Summary of papers

In paper I-III we developed tools, such as electrofusion, electroporation etc, to initiate and study chemical reactions inside liposomes. In order to shrink the reaction volume described by the bilayer membrane, we developed tools to create lipid nanotubes and networks of nanotubes and liposomes, which are described in paper IV-V. As an extension to these papers we developed various techniques to transport fluid and material between different liposomes mediated by the lipid nanotubes, which is described in paper VI-IX. In the final paper, paper X, we combined the techniques of studying chemical reactions inside liposomes with techniques to produce networks of nanotubes and liposomes, taking advantage of the benefits of both approaches.

Chemical reactions in liposomes, paper I-III

Paper I Chemical transformations in individual ultrasmall biomimetic containers



In this paper we present techniques to initiate and monitor chemical reactions inside liposomes. Due to their small size, in this work 1-5 µm in diameter, rapid diffusional mixing occurs and the surface-to-volume ratio is high. Molecules contained inside the liposomes will thus experience wall-collisions at high frequency according to Brownian motion hard sphere simulation. This implies that surface interactions can be pronounced in cellular environments and that a simple model system that can be used to evaluate and study such effects would be very valuable.

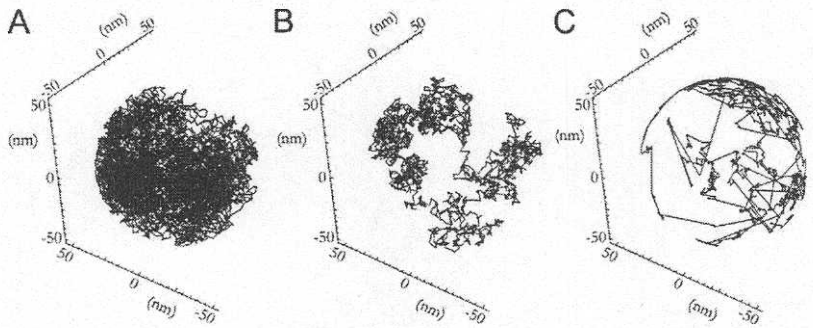
Three different approaches were made to initiate chemical reactions inside the liposome. The first included the entire reaction system, in this case alkaline phosphatase catalysing the transformation of a substrate fluorescein diphosphate into fluorescein, inside the liposome at the time of formation. The liposome was optically trapped and product formation was monitored by sensitive laser induced fluorescence (LIF) in combination with an avalanche photodiode detector. The fluorescence intensity, related to the number of products formed, was measured at 60 second intervals with subsequent product

photobleaching in between the measurements. This way, the reaction was set to zero after the products formed had been photobleached and the reaction was re-started again. This gives information of the product formation, dP/dt, which is the number of products formed during 60 seconds typically. It was estimated that less than 100 product molecules were formed between the time of bleaching and detection. The product formation was plotted as a function of time, which normally resulted in an exponential decay. This type of experiment is easy to perform and requires little micromanipulation but the time of initiation of the reaction is not determined and the number of reactants is difficult to estimate. Also, the reactant number is limited in the small volume element, which leads to rapid depletion even during the preparation and formation of the liposome.

The second approach involved one reagent inside the liposome and the other on the outside in the surrounding medium. The lipid bilayer now separates the two reagents and the only way to initiate the reaction is to permeabilise or transiently destroy the lipid barrier to be able to transport reagents inside the liposome. This was performed by a miniaturised electroporation method, by using 5 μm diameter carbon fibre electrodes. Pore formation in lipid bilayers can be performed by applying pulses of an electric field of well-defined strength and pulse duration across the liposome. When the nanometersized pores are open, reagents are transported through the membrane by diffusion, driven by concentration gradients across the lipid membrane. The pores formed in this process typically reseals in microsecond, although sometimes longer, after the electric field is turned off. Fluorescein was trapped inside liposomes in 140 mM NaCl of pH 7.2 and the surrounding medium was constituted by a citrate buffer of pH 4.3. During the electoporation, influx of H^+ ions occurred, which protonated the fluorescein and resulted in a quenching of the fluorescence. This approach enables a fast and precise way to determine the time of reaction initiation, but still has very little control over the amount of reagents that is delivered into the liposome during electroporation.

The final approach involved having the two reagents inside two different liposomes. To initiate the reaction, the two liposomes must mix their contents without much loss of material to the outside solution. This was performed by a miniaturised electrofusion method using the same carbon fibres as in the electroporation method. By applying an electric field across two liposomes in close contact, the pores created in the separate lipid bilayers can coalesce between the liposomes and create a fusion pore structure. The liposomes can then undergo complete fusion and mix their contents and initiate the reaction. The mixing was demonstrated by enclosing green fluorescent carboxyrhodamine-6G inside one of the liposomes and red fluorescent TOTO-3 intercalating 15-mer DNA during preparation. When mixed, both constituents could be detected inside the final liposome, which resulted in an orange colour when the two colours were superimposed. To demonstrate initiation of chemical reactions, 10 μM Fluo-3 and 10 μM Ca^{2+} were loaded into different liposomes. Upon fusion, Fluo-3 binds to calcium ions and responds by an increase in the fluorescence emission.

Paper II Manipulating the biochemical nanoenvironment around single molecules contained within vesicles



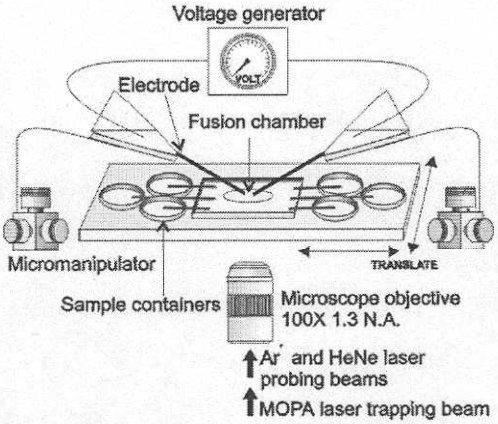
This paper is devoted to the study of chemical reactions in ultra-small volumes provided by the liposome. A Brownian motion Monte Carlo study was presented where a deeper understanding of the reaction environment in small volumes could be envisioned. Single enzymes and single substrates was treated as hard spheres performing random (Brownian) motion contained in a small spherical cavity with hard walls. Simple analytical treatments provided equations for substrate-enzyme, substrate-wall and enzyme-wall collisions to be compared with results from simulations.

The outcome of this study was a confirmation of the vision that when the reaction container shrinks, the surface-to-volume ratio is increased and wall effects can be more pronounced. Indeed, the number of molecule-wall collisions is extremely high in these systems, for example the substrate-wall collision is roughly 200 MHz in a 170 nm sphere! Effects that are believed to contribute to alter reaction kinetics and/or dynamics include electrostatic effects such as attraction/repulsion or simply reduction of dimensionality from 3D motion to 2D etc.

Off course reactants of differing sizes (such as enzymes and substrates) sample the reaction volume in differing time. It was also shown that the collisions often took place in clusters, which agrees with the intuitive picture that molecules close to each other can collide with each other several times before departing again, sometimes referred in the literature as a solvent caging effect.

Experimentally, reactions inside liposomes were performed with the reaction alkaline phosphatase catalyzing the substrate fluorescein diphosphate into fluorescein. As in paper 1, the product formation was followed as a function of time. Normally, by plotting the peak intensities (number of fluorescein molecules formed during one minute) against time, gives an exponential decay, reflecting the exponential decay of the number of substrate molecules. Occasionally, however, an erratic pattern was obtained that showed oscillatory behavior of fast and slow product formation. If this was inherent to the small size (~1 μm diameter) of the liposome or other effects such as multilamellar structure of the liposome has not yet been determined.

Paper III Microfluidic device for combinatorial fusion of liposomes and cells



This paper describes a prototype microfluidic device for sorting, selection and fusion of various types of liposomes. The device was constructed on an ordinary cover slip glass with pieces of pulled glass capillaries (~3 mm long, 10-30 μm i.d. and 30-100 μm o.d.) working as fluid conduits and transparent glue as separating wall structures.

Liposomes with differing properties were sorted in the sample containers that were connected to a common fusion container. Individual liposomes were then trapped by optical tweezers and transported into the fusion container through the capillaries by careful movement of the microscope stage. The selected liposomes were then allowed to adhere to the surface in the fusion container and fused using a miniaturized electrofusion protocol developed in earlier work.

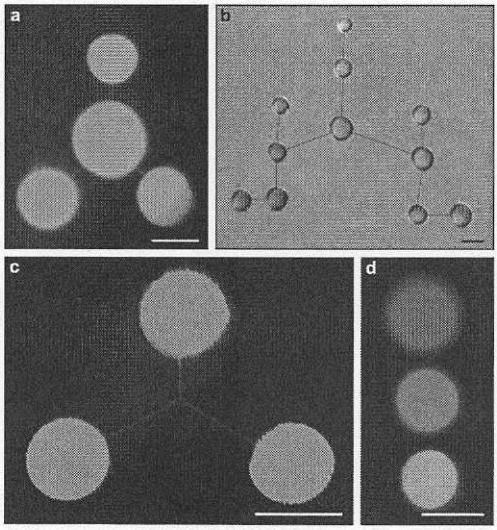
The partitioning of the liposome suspensions serves mainly two purposes. The first is storage of the liposome preparations without mixing, which gives the advantage of control over the identity of the fusion partners. This is normally a difficult task in bright-field microscopy if different liposome preparations are mixed. The second is purification and isolation of the selected liposome from the extra-liposomal solution, since the same components that are to be trapped inside the liposome are usually a component of the surrounding medium during preparation.

The device can be applied to combinatorial fusion schemes were pair-wise electrofusion of selected liposomes can lead to a vast number of different "product" liposomes with regards to membrane composition and internal composition. This was demonstrated by a sequential pair-wise fusion of a liposome doped with DiO (green fluorescent) with a plain (unlabelled) liposome. This hybrid liposome was then fused with a liposome doped with DiI (red fluorescent) to produce a liposome with both red and green fluorescence, giving rise to an orange color when superimposed.

Finally, the device structure could also be used for transport and subsequent electrofusion of single cells, which was demonstrated by fusion of red blood cells.

Formation of networks of lipid nanotubes and containers, paper IV-V

Paper IV Networks of nanotubes and containers



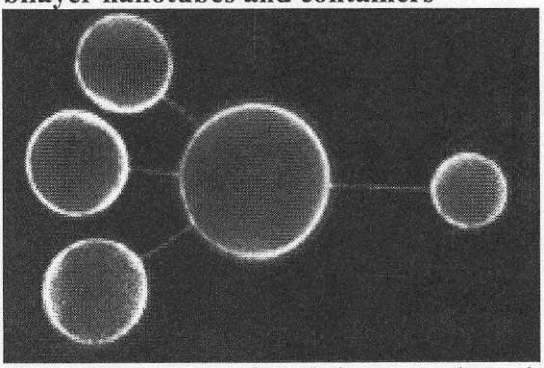
A novel method to produce 2D networks of liposomes of micrometer size connected by lipid nanotubes (typically ~300 nm in diameter) was presented in this work. These network structures were constructed by mechanical fission of the liposomes attached to the coverslip surface by using a micromanipulation protocol with a 5 μm diameter carbon fiber as a cutting tool. Repetitive fission of a single liposome could yield networks containing more than 10 "new" liposomes all connected by nanotubes.

With this method it is possible to control parameters such as container size, connectivity, nanotube length and angle between nanotube extensions emanating from a single common liposome. It was also possible to produce nanotube three-way junctions, with minimal pathway self-organizing properties always producing 120° angles.

The chemical identity could also be post-modified by various methods, which was demonstrated by selective photobleaching of the fluorescent compounds in different network containers to produce a micrometersized stop-light.

Finally, preliminary results from unilamellar networks showed that materials could be transported between network containers mediated by the interconnecting nanotubes. The transport was initiated by applying a difference in the membrane tension between two connected liposomes.

Paper V Micropipete-assisted formation of microscopic networks of unilamellar lipid bilayer nanotubes and containers



The paper was devoted to the development of a technique to produce microscopic networks of unilamellar lipid bilayer nanotubes and containers. The technique was based on a micromanipulation protocol and controlled microinjection. Briefly, the technique involves insertion of a micropipette into a giant unilamellar liposome connected to a multilamellar liposome. A lipid nanotube was pulled out of the unilamellar liposome, after the lipid had resealed around the tip. By careful injection into the nanotube at the micropipette orifice, a new liposome started to form and grow in size. This liposome was then allowed to adhere to the underlying surface, which concluded the procedure or could be repeated to involve more containers in the network.

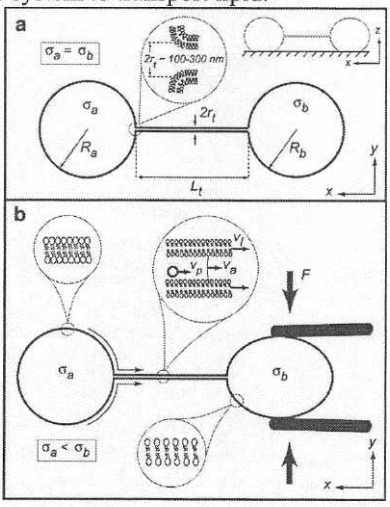
The use of a pair of unilamellar and multilamellar liposomes as starting material is vital when constructing large networks. Due to the mechanics of the lipid bilayer, it is very hard to stretch and therefore the excess membrane that can be used to create network structures is almost non-existing. However, the multilamellar part coupled to the unilamellar liposome can donate some of its lipid material into the unilamellar part to relive stress implied when the network is created. Compared to the previous technique of mechanical fission of liposomes presented in paper IV, the use of unilamellar liposomes is greatly facilitated, the degree of control is enhanced and the differentiation of internal composition of network liposomes is much easier.

Transport of fluid and material between liposomes mediated by lipid nanotubes, paper VI-IX

Paper VI Moving-wall driven flows in nanofluidic systems

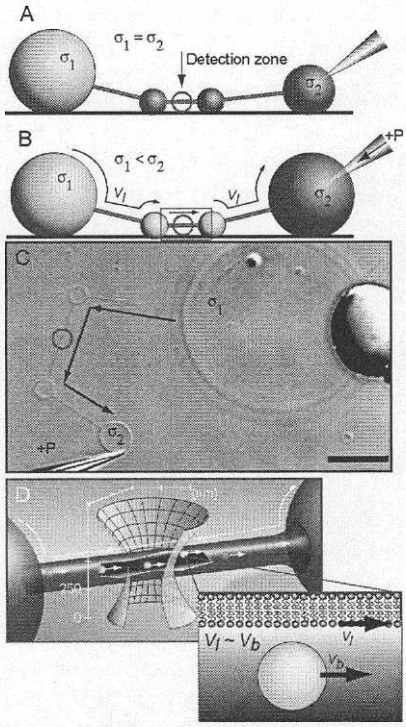
Controlled transport of the fluid contained inside lipid nanotubes is described. The transport was induced by applying a force to one of the vesicles connected to the nanotube, thereby deforming the shape of the vesicle and hence the surface-to-volume ratio. Through this the membrane tension in the target vesicle, where the force application occured, was increased. The lipids that are the building blocks of the bilayer network system tried to minimize the stress in the system by rapid translocation of lipids from regions of lower tension to regions of higher tension, which resulted in a directional transport of lipids across the nanotube. This can be viewed as a cylinder of lipids that flow through the water between the vesicles. The water that is contained inside the lipid cylinder couples to the lipid membrane and due to the small size of the lipid nanotube (radius 50-150 nm), the water was transported in the same direction as the lipid flow, thus resulting in a situation where the flow of water was driven by the moving wall.

In this work we used two carbon fibers as mechanical tweezers to perturb the equilibrium situation by pinching one of the vesicles and thereby change its shape. The extremely rapid dynamics of the membrane made it possible to trap the system in different excited states along the nanotube. This was proved by translocation of a particle along the nanotube by applying varying degress of shape deformations to a target vesicle. The degree of deformation was directly coupled to the distance traveled by the particle and when the deformation was removed the system returned to its original equilibrium situation. Transport velocities normally ranged between 20-30 μm/s, however much higher velocities could be achieved. The transport velocity turned out to be dependent on a number of parameters, such as the transient membrane tension difference between the vesicles and the viscous resistance of the nanotube-vesicle system to transport lipid.



Paper VII Nanofluidic networks based on surfactant membrane technology

Figure 5.



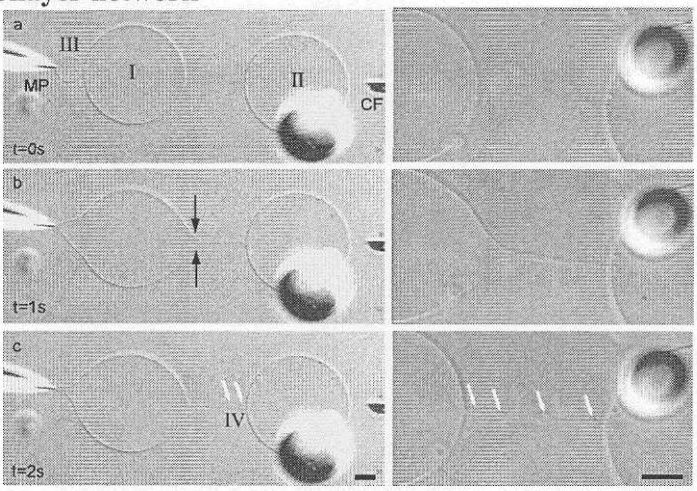
In order to explore the possibilities of utilizing these network structures and especially the nanotubes as flow channels for ultra-small analytical platforms we devised a system of vesicles and nanotubes that could be coupled to single-molecule detection setups. A continuous injection into a target vesicle in the network created a lipid flow over the system and fluorescent nanoparticles was detected by sensitive laser induced fluorescence (LIF) detection as they flowed through the nanotubes.

The detection setup required that the detection window, described by the lipid nanotube in this case, was placed near the substrate surface to facilitate the detection alignment. The nanotube-vesicle system therefore consisted by a large vesicle loaded with fluorescent beads (~30 nm diameter) connected to a multilamellar vesicle functioning as a lipid reservoir. From this vesicle two small vesicles of equal size (~4 μm in diameter) and another vesicle connected to an injection micropipette was created using the micropipette assisted protocol described in paper V. The detection site was placed between the two small vesicles, where the interconnecting nanotube was residing less than 2 μm from the substrate surface. Injection into the vesicle attached to the pipette-tip created a lipid flow that dragged along the nanoparticles trapped inside the nanotube-vesicle system. Fluorescence bursts was recorded when the fluorescent beads at various concentrations passed through the detector volume.

In this work we also described how the nanotube-substrate surface separation distance could be controlled to some extent by changing either the size of the vesicles to which the nanotube was connected or the strength of adsorption to the substrate surface. Strong adhesion leads to a shape deformation of the vesicles leading to a more hemispherical appearance and a

subsequent reduction of the separation distance between the nanotube and the surface. Also, the self-organizational quality that these types of lipid membrane possess was disrupted by strong adhesion, which resulted in network structures that could not be attained in systems of weak adhesion.

Paper VIII Formation and transport of nanotube-integrated vesicles in a nanoscale bilayer network

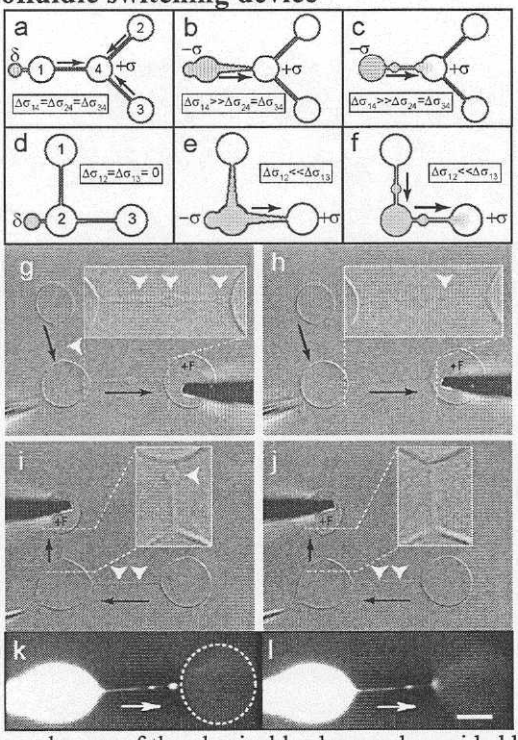


A method to create, load and transport small vesicular structures integrated into the nanotube bilayer is presented. These nanotube-integrated vesicles (~500 nm-5 μm in diameter) could be transported between the surface-adhered vesicles to which the nanotube was connected, by applying membrane tension gradients as described in paper IV-VII, and release the cargo into a target vesicle. The formation of these mobile nanotube-integrated vesicles was initiated by rapidly adding excess membrane material to one of the surface-adhered vesicles by merging it with another nanotube connected vesicle. This type of perturbation leads to a reduction of the membrane tension of the "product" vesicle formed after the merging and a subsequent increase of the diameter of the connecting nanotube. The nanotube was first distorted into a funnel like geometry, which was then rapidly transformed into a nanotube with one or several nanotube-integrated vesicles by energy minimisation of the bilayer system.

The contents of these small nanotube-integrated vesicles is exclusively determined by the content of the surface adhered vesicle with the reduced membrane tension after merging, since both the lipid and the fluid that form the nanotube-integrated vesicles had been demonstrated to come from this vesicle.

In this work we explore the possibility of using this type of setup to move small packets of volumes controllably between two vesicles that have different contents from the start. We successfully loaded nanotube-integrated vesicles with fluorescent dextrane and moved these to a neighbouring vesicle with no fluorescent dextrane, by using a two-point perturbation technique, i.e. lower the membrane tension in one container while at the same time increasing it at another vesicle. The nanotube-integrated vesicles were made to inject their contents by merging into the vesicle with no fluorescent dextrane, and after repetitive formation, transport and injection an increase in the fluorescence in the target vesicle could be measured. Through this one could create systems capable of transport of femtoliter volumes of reagents into a vesicle or to titrate a substance contained inside the vesicle.

Paper IX A nanofluidic switching device

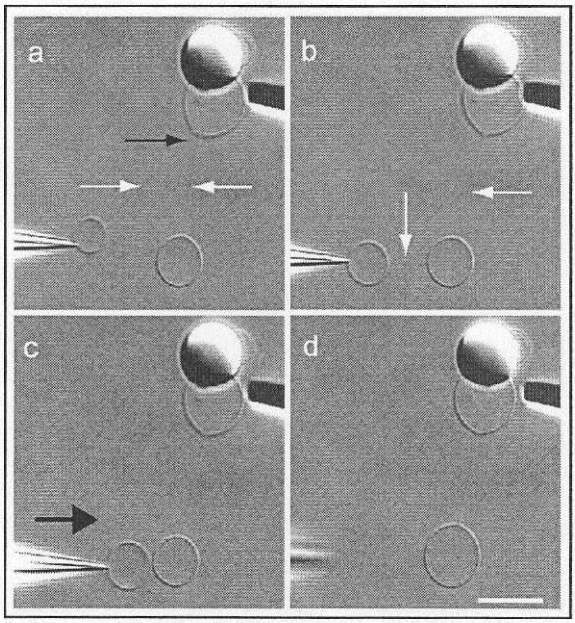


In this work we make use of the physical background provided by paper VIII to create nanotube-integrated vesicles in a network of vesicles and nanotubes. The two-point perturbation technique, where the membrane tension is decreased in one surface adhered vesicle while increasing it at another vesicle was utilized to create a nanofluidic switch. Through this a membrane tension difference was created between the target vesicles that was much larger than between the rest of the nanotube connected vesicles, which leads to a directed flow of lipids and also nanotube-integrated vesicles.

In this paper we show how to direct the transport of nanotube-integrated vesicles that have been created on multiple nanotubes in large network system. A linear system of three vesicles was created and the middle vesicle was subjected to a decrease in membrane tension by merging it with a nanotube-connected vesicle attached to a microinjection pipette. The middle vesicle was deformed and nanotube-integrated vesicles were created on both nanotubes emanating from the middle vesicle as described in paper VIII. By applying the two-point perturbation technique to this system, the nanotube-integrated vesicle could be made to move and inject their material into one of the outer vesicles in the linear system of vesicles, by applying a force and hence increase in tension in the target vesicle. A switch function in these types of lipid bilayer networks was therefore demonstrated where the fluid packets could alternately be directed in two ways.

Combination of lipid network technology and initiation of chemical reactions, paper X

Paper X Nanotube-mediated merging of liposomes to initiate chemical reactions



A novel technique to initiate chemical reactions inside liposomes is presented. The procedure abstracts the advantages of the micropipette-assisted technique of creating networks of lipid nanotubes and liposomes, described in paper V. This technique displays features such as control of size of liposomes in the network and control of interior chemical composition of the individual containers as mentioned above. Through this, chemical reagents can be filled into separate containers in a network structure, simply by exchanging the solution in the micropipette that is used to construct the network. In this work this was demonstrated by formation of two network containers filled with substrate, fluorescein diphosphate, and enzyme, alkaline phosphatase, respectively. One of the key features of this technique is the control of the size of the reagent liposomes and the concentration of the reagents that are contained in the liposomes. Through this one can vary the number of reagents that are to react in the final product liposome with good control and flexibility.

Since the lipid bilayer membrane displays a fluid character, this enables movement of nanotube attachment points over the entire membrane surface after formation of the network. Through this, nanotube-connected liposomes can also be translated across the membrane surface in order to change the way the network liposomes are connected. The goal of this procedure is to create two liposomes interconnected by a nanotube and differentiated with respect to their interior contents. The two different reagents were now contained in two liposomes that had fluid contact through the connecting nanotube. These liposomes were then brought into close contact, where the nanotube length approached zero. In this instance, the structure with a small fluid contact point in between two liposomes resembles the fusion pore structure found in electrofusion experiments as described above. The liposomes were the pushed together and spontaneously merged into a product liposome, where the two reagents mixed and product was formed, in this work fluorescein with fluorescein monophosphate as an intermediate product.

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