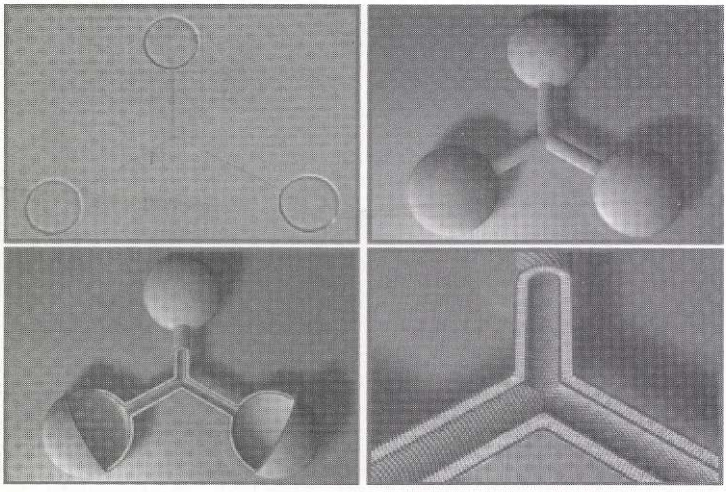

Fabrication and Dynamic Control of Surfactant-Membrane Networks with Applications in Nanofluidics and Membrane-Based Transport

ROGER KARLSSON

Department of Chemistry
Analytical and Marine Chemistry
Göteborg University

GÖTEBORG UNIVERSITY

1062

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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. 9.15 i föreläsningssal KS101, Kemigården 4, Göteborgs Universitet och Chalmers Tekniska Högskola, Göteborg.

Fakultetsopponent: Professor Staffan Nilsson, avdelningen for teknisk analytisk kemi, Lunds Universitet, Kemicentrum, Box 124, 221 00 Lund.

Analytical and Marine Chemistry
Göteborg University
2003

Abstract

Methods were developed to produce microscopic networks of vesicles (5 to 50 micrometers in diameter) conjugated by lipid nanotubes (50 - 200 nanometers in diameter). The networks are constructed from a continuous lipid bilayer membrane held together by non-covalent interactions and since it has thickness of only 5 nm, it can be viewed as a two-dimensional liquid. The membrane is very hard to stretch, but at the same time very easily bent and therefore it is possible to create complex nanoscale structures from it, for example, lipid nanotubes. Networks were produced through forced shape transformations of vesicles into nanotube-vesicle geometries, using micromanipulation-based protocols. The first technique was based on mechanical fission of vesicles, using a micromanipulator-controlled carbon fiber (5 μm in diameter). This technique was preferentially used for multilamellar vesicles with possibilities of constructing templates for solid state structures, which could be used in microfluidics or microelectronics applications. Another method for creation of networks of unilamellar vesicles and interconnecting lipid nanotubes was developed, which was based on a micropipette-assisted electroinjection technique. A microinjection pipette was inserted into a unilamellar vesicle using the electroinjection technique in which a mechanical pushing force was combined with the application of an electric field that destabilized the membrane. The membrane was allowed to reseal around the tip, whereby the pipette was pulled away to create a lipid nanotube. Buffer was injected into the nanotube (with a typical velocity of femtoliters per second) and a new vesicle was formed at the injection tip taking material from the originating vesicle. When the vesicle had reached the desired size it was allowed to adhere to the surface and the procedure could be repeated to create new vesicles. By changing the solution contained in the micropipette it was possible to differentiate the contents of the vesicle containers in a network either during or after formation. The networks have controlled connectivity, container size, nanotube-length and angle between nanotubes connected to the same vesicle.

A method to transport lipid material, fluids and particles through the nanotubes between vesicle containers was developed. The transport is based on creating a surface tension difference across nanotubes which results in a moving lipid wall having velocities ranging between 20 – 60 μm/s. Due to the small dimensions of the lipid nanotube, fluids and particles confined inside the nanotube were dragged along the lipid flow through viscous coupling. A nanofluidic system, which is capable of working with volumes down to the femtoliter (10^{-15} L) or even the attoliter range (10^{-18} L), can therefore be realized. Fluid control in nanometer-sized channels open up possibilities, for example, to perform single-molecule studies in confined spaces.

To be able to route material and fluids in larger networks, a two-point perturbation technique was developed. In this technique, the membrane tension of the vesicle from which material was to be transported was decreased, while the membrane tension of the target vesicle was simultaneously increased. Transport, therefore took place mainly through the nanotube interconnecting the two selected vesicles, without affecting the rest of the network. This way, a molecule can be shuttled throughout a complex network between selected containers, making it possible to expose it to different chemical or physical environments.

A method was developed to create and transport micrometer-to-submicrometer-sized vesicles integrated onto the interconnecting nanotubes of larger surface-adhered vesicles. The two-point perturbation technique was used to momentarily destroy the energy balance and the shape of the system, subsequently leading to formation of micrometer-sized vesicles integrated onto the nanotube. These vesicles were mobile and since they were integrated onto the lipid nanotube they could be transported to a larger target vesicle, using the principle of tension-driven lipid flows, where they could release their material.

A vesicle model system for exocytosis consisting of a pipette-attached vesicle inside another larger vesicle was constructed. The vesicles were interconnected by a lipid nanotube, making the system resemble the late stages of exocytosis, in which a synaptic vesicle ready for release of transmitter substance is fused to the outer membrane of the cell. This way, it was possible to study the last stage before release, to see how this system behaved without the controlling protein units normally present in a living cell.

Keywords: Lipid, membrane, bilayer, vesicle, biomimetics, lipid nanotube, nanotube-integrated vesicles, networks, tension-driven lipid flow, transport, nanofluidics, vesicle cell-models

ISBN 91-628-5870-X

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Printed by Chalmers Reproservice

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To Mom, Dad and Anders

Carpe diem

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List of publications

The thesis is based on the papers listed below.

Paper I Networks of nanotubes and containers
Karlsson, A., Karlsson, R., Karlsson, M., Cans, A. S., Strömberg, A., Ryttsén, F., Orwar, O.
Nature 409:150-152 (2001)

Paper II Micropipette-assisted formation of microscopic networks of unilamellar lipid bilayer nanotubes and containers
Karlsson, M., Sott, K., Cans, A.S., Karlsson, A., Karlsson, R., Orwar, O.
Langmuir 17:6754-6758 (2001)

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

Paper IV Artificial cells: new insights into exocytosis using liposomes and lipid nanotubes
Cans, A.-S., Wittenberg, N., Karlsson, R., Sombers, L., Karlsson, M., Orwar, O., Ewing, A.
Proceedings of the National Academy of Sciences USA 100:400-404 (2003)

Paper V Nanofluidic networks based on lipid membrane technology
Karlsson, A., Karlsson, M., Karlsson, R., Sott, K., Lundqvist, A., Tokarz, M., Orwar, O.
Analytical Chemistry 75:2529-2537 (2003)

Paper VI Formation and transport of nanotube-integrated vesicles in a nanoscale bilayer network
Karlsson, R., Karlsson, A., Orwar, O.
Journal of Physical Chemistry B 107:11201-11207 (2003)

Paper VII A nanofluidic switching device
Karlsson, R., Karlsson, A., Orwar, O.
Journal of the American Chemical Society 125:8442-8443 (2003)

Papers not included in thesis

Nolkrantz, K., Farre, C., Brederlau, A., Karlsson, R., Brennan C., Eriksson, P. S., Weber, S. G., Sandberg, M., Orwar, O. "Electroporation of single cells and tissues with an electrolyte-filled capillary". Anal. Chem. 73:4469-4477 (2001).

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).

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 by performing experiments and writing the manuscript as well as literature search.

Paper II: Contributed by performing some experiments and also helped with writing the manuscript.

Paper III: Contributed by performing experiments and had main responsibility of writing the manuscript and also performed most of the literature search.

Paper IV: Contributed in designing the vesicle-model system and helped with writing parts of the manuscript.

Paper V: Contributed by performing experiments and writing the manuscript as well as literature search.

Paper VI: Contributed by performing experiments and had main responsibility of writing the manuscript.

Paper VII: Contributed by performing experiments and had main responsibility of writing the manuscript.

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Introduction

One of the greatest mysteries, which has fascinated man ever since our first conscious thoughts, is life itself. Life starts with a single cell, the embryo, which is formed when the membrane of a sperm is fused to the membrane of an egg. This single cell divides and multiplies and later these cells differentiate into various types of cells that have different functions. All these cells have to function together in order for life to be sustained; however they always retain some individuality. Just by looking at a single cell and realizing its great structural and chemical complexity, you discover that it is really a small world of its own and the more you learn about life and how it is sustained, the more awe you feel before it. Millions of processes have to be performed in the right way and even small errors in the genome, such as a simple exchange of only one base-pair in the code for a small protein might be fatal. If you also think about that it is the same cells that make it possible for you to think about their complexity, madness isn't far away…

The quest to understand life started by studies of organisms, humans, animals and plants, then alongside the technical development, the research went on to the level of organs and cells. Cell biology, genetics, biochemistry and biophysics were interwoven to form the discipline of molecular cell biology, and nowadays we have reached the sub-cellular level with studies of large biomolecules, such as DNA and protein and the smaller biomolecules, such as nucleotides, amino acids, lipids and sugars, that is, the building stones of the cells. Still, cells are very complex systems, where the whole system is always greater than the sum of its parts…

With the down-scaling from macro- to micro-scale there has been an increasing demand for techniques, methods and devices that can reach further and further down in dimensions to be able to analyze and handle smaller sample volumes, aiming at the molecular scale and the study of single molecules.

In the field of analytical and bioanalytical chemistry, for example, miniaturized fluidic separation devices have been developed. The driving force for the creation of these small scale systems is to create devices capable of sample preparation, mixing of reagents, initiation of reactions and separation of products. The breakthrough in this line of development came with the fabrication of silicon chip structures and the development of microelectromechanical systems (MEMS). In this spirit, micro-total analysis system (μ-TAS) have been created were sample preparation, mixing of reagents and separations could be performed on the same chip, a lab-on-a-chip concept (Reyes *et al.*, 2002; Auroux *et al.*, 2002). These systems have many advantages, one being that they are capable of handling very small sample volumes, thereby opening up the technique of analysing the contents of a single cell. The first devices had separation channels of millimetre sizes but today these channels have dimensions that reach towards the nanoscale (Tas *et al.*, 2002) and terms like nanofluidic systems or nanofluidics have been coined (Ramsey *et al.*, 2002; Foquet *et al.*, 2002; Haneveld *et al.*, 2003).

Reducing dimensions of analytical instrumentation presents challenges, not only in the fabrication protocols, but also how to control and transport fluids in the nanoscale channels. The shrinkage in volume will lead to an increase in the surface-to-volume ratio and an increase in the fraction of water that would be close to a surface. It has been realized both experimentally and theoretically that a fluid will behave differently if close to a surface. In macroscale systems or even microscale systems, these effects can be neglected, since the fraction of the fluid molecules associated with the surface is very small. In nanoscale systems, this might no longer be true, and the fraction of fluid which is associated and affected by the surface will have to be taken into account (Pfahler *et al.*, 1989).

The difficulties of using conventional techniques for nanoscale fabrication (especially when reaching into the third dimension) or fluid control in such ultra small scale systems

have spurred scientists to start looking for alternative solutions and materials, whereby many have been fascinated with how biology solves these problems (Ball, 2001). The most sophisticated microfabricated devices are not even close to having the same complexity, capability for parallel handling and optimizd efficiency as a single living cell. Thousands of chemical and enzymatic reactions are taking place simultaneously in specialized compartments or at specific locations. Interestingly, the cell is a highly dynamic entity, and it has recently been realized that the membranous structures in a cell, such as the endoplasmatic reticulum and the Golgi complex, are interconnected by tubular structures along which transport can take place. Due to the small dimensions of these tubular structures and the small volumes that are handled, this system can be viewed as a true nanofluidic system where controlled transport of low numbers of molecules is performed.

However, due to the high complexity and the fact that cells have a life of their own, working with living matter might not always be as reproducible and simple as one would like. Scientists have therefore begun to study and extract components from living cells, taking advantage of the specialized functions and properties that they are interested in. This has lead to the science of biomimetics, where scientists take lessons and inspiration from nature in designing molecules and materials for different purposes (Ball, 2001). It has been realized that biohybrid devices might be created in which the extracted biomolecules are used, either as they are or after modifications, in conjunction with nanostructures or nanoelectromechanical systems (NEMS) (Montemagno and Bachand, 1999). One of the most interesting molecules for this purpose is the F_1-ATP-ase, which is a molecular motor that performs a rotating motion. It has been envisioned that a biohybrid device of nanometer scale might be used as a nanoprobe inside the body, which, for example, could deliver drugs. The F_1-ATP-ase could be used by the nanoprobe as a motor for propulsion, using the cells own ATP as fuel (Montemagno and Bachand, 1999). Other types of molecular motors are the family of kinesin and myosin proteins which can be used for transport along microtubules (Dennis *et al.*, 1999). For example, using these molecular motors, DNA-molecules have been extended and stretched out along microtubules (Diez *et al.*, 2003). Another phenomenon which scientists in the field of biomimetics have wanted to study is the photosynthesis in which light is used as an energy source. The F_1F_0-ATP-synthase was inserted into liposomes containing an artificial photosynthetic membrane and by irradiation of light a proton-gradient was established across the membrane, which could drive the synthesis of ATP (Steinberg-Yfrach *et al.*, 1998). Theoretically, the produced ATP could, for example, be used by motor proteins to move the liposome along microtubules in order to transport and deliver small amounts of fluid. Not long ago, these thoughts might have seemed pure science fiction…

Following some of these inspirations, we take advantage of the interesting mechanical properties of the lipid bilayer membrane found in living cells. The lipid bilayer membrane is easily bent but hard to stretch and always strives to attain a minimum energy configuration. It is these unique material properties that make it possible to create ultra-small-scale structures, such as lipid nanotubes.

We have developed micromanipulation techniques to induce forced shape transformation of vesicles in order to create networks of vesicles and lipid bilayer membrane nanotubes (Karlsson, A. *et al.*, 2001; Karlsson, M. *et al.*, 2001; Karlsson, A. *et al.*, 2003). The vesicles usually have diameters in the range of 5 – 50 μm in diameter and the interconnecting nanotubes have diameters of 50-200 nm in diameter, but they can be made to be much smaller, in the range of 10-20 nm in diameter (Bo and Waugh, 1989). It was also possible to create closed or even fully connected networks (Karlsson, M. *et al.*, 2002), as well as to create three-way nanotube junctions, both of which added more complexity to the networks.

Not only did the unique material properties of the lipid bilayer membrane provide a means to construct nanoscale channels, the highly dynamic character also gave us the

possibility to create flows of lipid material across the nanotubes between the nanotube-interconnected vesicles. The lipid flow was created by generating a tension-difference across the nanotube through which material was to be transported. The continuous lipid bilayer membrane system responded to the generated tension-difference by moving lipids from regions of low tension towards the point of increased tension. The flow of lipid membrane across the nanotubes gave rise to a movement of the walls of the nanotube and due to the small dimensions of the nanotubes, fluids and particles were dragged along the lipid flow through viscous coupling (Karlsson, R. *et al.*, 2002).

Also since our system of nanotube-interconnected membranous compartments are very similar to the membrane system of living cells, we realized that our systems could be used for mimicking cellular events, such as exocytosis and intracellular membrane transport (Cans *et al.*, 2003).

There are several application areas and research fields where our networks of vesicles and lipid membrane nanotubes might have an impact. For example, the nanotube-vesicle networks could be used in the studies of single molecules or enzymatic reactions confined in the small volume of the nanotubes or even nanotube junctions, they could be used as nanofluidic systems, as templates for solid-state structures with impact in microfluidics and microelectronics, as sensor devices and as model systems for cells and cellular membrane-based processes.

The cell

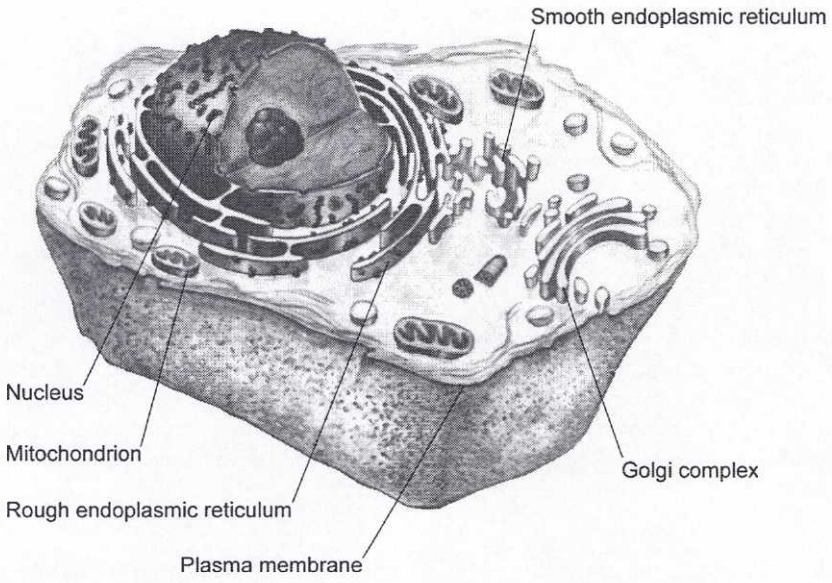


Figure 1. Schematic drawing illustrating the organization of a eukaryotic animal cell. The plasma membrane encloses the cell, protecting the cytoplasm which contains an aqueous cytosol and organelles. Shown is the nucleus, which contains the genetic material (DNA-deoxyribonucleic acid), surrounded by the rough endoplasmic reticulum, where the protein-synthesizing ribosomes are located. The smooth endoplasmic reticulum is linked to the Golgi complex, which is composed of complex vesicular structures and lipid membrane nanotubes. Also shown are the mitochondria, which produces energy in the form of adenosine triphosphate (ATP).

The size of a single animal or plant cell typically ranges between 10 – 30 μm in diameter, while bacteria are only 1 to 2 μm long. Although cells of all kinds, even in a single multicellular organism, can have different structure and function, they all share certain structural features; (1) a plasma membrane that encloses and separates the cells interior from the exterior and (2) an interior solution called the cytoplasm, which contains the aqueous cell contents, proteins and/or organelles (Fig. 1) (Lehninger *et al.*, 1993). The plasma membrane consists of a tough, flexible lipid bilayer, in which membrane proteins are incorporated. The plasma membrane provides the barrier, whereas the membrane proteins have specialized functions, for example by working as transporters, as signal transducers, mediating signals across the membrane barrier, or function as enzymes. Eukaryotic cells differ from bacteria and prokaryotic cells in that they have a nucleus, which contains genetic material, the DNA, and proteins associated with it. However, this picture is very simplified, even a single cell is a world of its own and although it has to function collectively with the other cells in the organism in which it lives, it always retains some individuality and independence. A typical single cell requires thousands of different proteins at any given moment, proteins which perform various specific functions, giving the cell its own identity. Hundreds of thousands of processes and reactions are taking place simultaneously and in parallel and everything is controlled and regulated with an unprecedented precision.

A key feature of life is the high degree of ordering and structural complexity. This not only applies to organisms, but also to a single cell. In an eukaryotic cell there are a lot of structural elements and compartments, which are separated from the bulk solution of the

cytoplasm. In fact, the cytoplasm is highly crowded and the solution inside the cell is now more thought of as a gel, rather than a simple aqueous fluid, due to the high concentration of proteins and the intermeshing of structural protein (such as cytoskeleton, actin fibers, and microtubules) and the so-called endomembrane system.

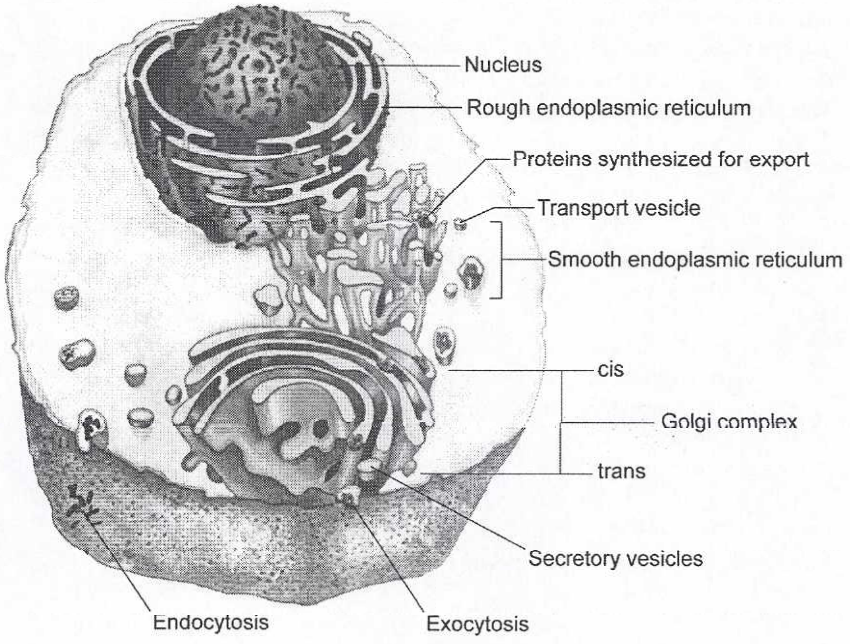


Figure 2. Schematic illustrating the endomembrane system of the cell. The endomembrane system includes the nuclear envelope, the endoplasmic reticulum, the Golgi complex and small vesicular structures, which together enclose a compartment (the lumen), which is separate from the surrounding cytosol. Contents of the lumen move from one organelle in the endomembrane system to another via transport vesicles. The vesicles are budded of one organelle and then transported to another, where they fuse and release their contents. The highly dynamic character of the endomembrane system allows newly synthesized proteins to move into the lumen of the endoplasmic reticulum and then to the smooth endoplasmatic reticulum. The proteins are moved to the Golgi complex via transport vesicles, where they are tagged with specific molecules which "addresses" them to either the cell-surface, to lysosomes or secretory vesicles. The contents of secretory vesicles are released by exocytosis. Endocytosis and phagocytosis bring extracellular materials into the cell. Fusion of endosomes (or phagosomes) with lysosomes, which are full or digestive enzymes, results in the degradation of the extracellular materials.

The endomembrane system

The endomembrane system includes the nuclear envelope, the endoplasmic reticulum, the Golgi complex, and several types of vesicles, which encloses compartments separated from the aqueous cytosol and the contents of these compartments are moved between the different parts using small transport vesicles (Fig. 2). This complex organelle system contains a lot of membrane material, in fact, when looking at a 10-µm-diameter cell and spreading out all of the membrane material in the cell, the plasma membrane only constitutes a fraction of the total area, which can as large as 0,1 - 1 mm^2, an area which can be seen by the eye without the use of a microscope. The endomembrane system is highly dynamic, transporting newly synthesized proteins through the rough endoplasmic reticulum, via the smooth endoplasmic reticulum to the Golgi with the use of transport vesicles.

The endoplasmic reticulum (ER) is a highly convoluted membrane structure that extends throughout the whole cytoplasm. It can be subdivided into two compartments, the rough and the smooth ER, which have differences in both structure and function. The rough endoplasmic reticulum is composed of flattened membrane sacks which is covered by ribosomes, thereby the name rough. Ribosomes on the ER synthesize proteins that are destined to be exported outside the cell or inserted into the nuclear or plasma membrane. The smooth endoplasmic reticulum is physically linked to the rough ER, but does not have any ribosomes attached to it, instead this is the site where lipids are synthesized.

The Golgi complex is composed of a stack of flattened membrane vesicles and this organelle can also be divided into two sub-groups, the cis and the trans side. The cis side faces the ER, while the trans side faces the plasma membrane. The Golgi modifies proteins that have been synthesized in the ER and "tags" them with certain signalling molecules which direct them to be transported to specific locations, for example, the plasma membrane.

The nucleus of eukaryotic cells contains the genome and proteins that associate with the DNA. The human DNA is composed of 46 chromosomes and the fully extended DNA can be up to 2 meters in length. However, due to clever packing using proteins, it is possible to pack all DNA into the nucleus which has a typical size of a few micrometers.

The mitochondria are the power plants of aerobic eukaryotic cells and have typical sizes of 1 μm in diameter. They are separate from the rest of the membrane system and are composed of two membranes, the outer is smooth and surrounds the inner membrane which is highly wrinkled. In the mitochondrion, organic nutrients are enzymatically oxidized and the chemical energy released is used to generate ATP (adenosine triphosphate), the major energy-carrying molecule of cells. Mitochondria contain their own DNA and they are produced only by division of previously existing mitochondria. This has led to the conclusion that mitochondria are the descendants of aerobic bacteria that lived symbiotically with early eukaryotic cells.

Intracellular transport

The mechanisms for transporting materials throughout the cell are highly important in order for the cell to maintain its complex structural differentiation and heterogenous chemical environments. It has been shown that materials are moved within the cell by diffusion or by the use of motor proteins (Bloom and Goldstein, 1998; Lane and Allan, 1998). Diffusion of small molecules, such as second messengers in the signalling pathways, is quite fast, however, it is a much slower process for macromolecules and, for example, small vesicles (Bloom and Goldstein, 1998). Diffusion is also a randomized motion and since much of the material has to be directed and controlled it cannot be used for all transport purposes. Motor protein driven transport of, for example, vesicles is faster than diffusion, having velocities of kinesin-driven microtubule translocation normally ranging between 0,4 – 0,9 micrometers/second, but values up to a few micrometers per second have been reported (Lane and Allan, 1998). This type of transport also has the advantage of being a directed motion.

Interestingly, there are some intracellular transport phenomena, which seemingly cannot be explained by these mechanisms, for example the intra-Golgi (Cooper *et al.*, 1990), Golgi – ER (Sciaky *et al.*, 1997) and Golgi-to-plasma membrane transport (Polishchuk *et al.*, 2000) as well as some types of axonal transport (Kaether *et al.*, 2000).

Recently, one has found tubular elements called lipid nanotubes, connecting the various parts of the endomembrane system, especially in the Golgi complex. The tubular elements were 50-100 nm in diameter (Ladinsky *et al.*, 1994; Clermont *et al.*, 1995; Sesso *et al.*, 1994) and have been observed by electron microscopy (Sesso *et al.*, 1994; Rambourg *et al.*, 1990) as well as with fluorescence microscopy using membrane dyes (Cooper et al., 1990) and GFP-chimeras (Sciaky *et al.*, 1997). It is believed that membrane tubes are formed

and moved by using interactions with the cytoskeleton, especially the microtubule network (Allan and Schroer, 1999) The tubular structures were found to extend along the microtubule network, probably through the use of microtubule motor proteins, for example the kinesin protein (Sciaky *et al.*, 1997; Hirokawa, 1998) and it is believed that the endoplasmic reticulum and the Golgi is formed and maintained by the using the cytoskeleton network.

It has been suggested that the tubular structures found in cells are involved in some transport of material between, for example, the interconnected Golgi stacks, but also between the Golgi and the ER and most likely also between the Golgi and the plasma membrane (Sciaky *et al.*, 1997; Iglic *et al.*, 2003). A study was performed in which a drug called Brefeldin A (BFA) hindered the normal backtransport of membrane from ER to Golgi (Sciaky *et al.*, 1997). This resulted in an accelerated formation of an elaborate tubular network that extended throughout the cell, a process taking a few minutes. Suddenly, in just a few seconds, all of the Golgi membrane material was redistributed into the ER-network, a process called Golgi-blinkout. In order to investigate the transport mechanism along the tubular structures, Sciaky *et al.* (Sciaky *et al.*, 1997) considered two organelles connected by a single tubule. If diffusion was to explain the transport along such a tube, the velocity of lipid species would be approximately 0,15 micrometers per second (diffusion coefficient for lipids in a bilayer membrane $D_l = 5*10^{-12}$ m^2/s) and for a membrane protein ($D_p = 5*10^{-13}$ m^2/s) the transport would be only 0,015 micrometers per second (Almeida and Vaz, 1995). However, in the experiments studying these transport phenomena, velocities of tens of micrometers per second has been observed, which is even much faster than the motor protein driven transport phenomena. This transport process was therefore thought to take place, not by diffusion, but instead by a flow of membrane (Sciaky *et al.*, 1997). The driving force for this flow was explained by a difference in free energy and thereby a surface membrane tension gradient between the ER and the Golgi membrane compartments. However, it is not known how a difference in free energy in the different membrane compartments can be achieved. The phenomenon of membrane flows created by a difference in free energy across a membrane surface will be discussed further in later sections.

Biological membranes

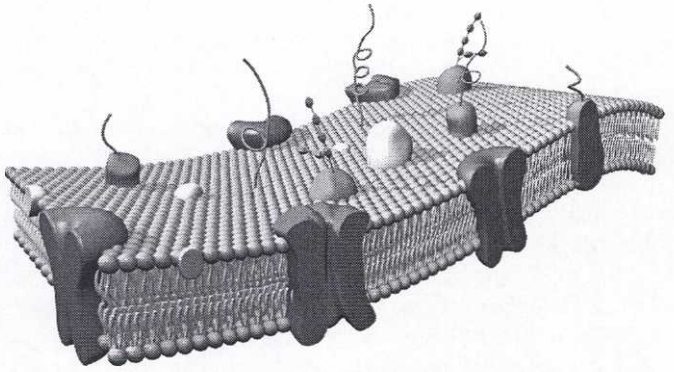


Figure 3. Schematic three-dimensional drawing illustrating a biological membrane, showing the lipid bilayer membrane and the membrane proteins. Some proteins span the whole thickness of the membrane and are called integral proteins, whereas other protein are associated to one side of the membrane, so-called peripheral proteins.

Biological membranes are constituted from a lipid bilayer membrane containing a wide range of membrane proteins. As a simple view, the lipid bilayer membrane is the barrier, separating the interior of cells and organelles from the exterior, while also providing a matrix for the membrane proteins, which works as transporters or signal conductors across the membrane barrier (Fig. 3). In biological membranes of eukaryotic cells the amount of lipid compared to the amount of protein is about 50-50. Many have studied how the lipid bilayer membrane interacts with the proteins, for example, in 1972, Singer and Nicholson (Singer and Nicholson, 1972) introduced the fluid mosaic model, where they stated that membrane proteins were free to move around in the sea of lipid, excepting those that were attached to the underlying structural cytoskeleton (Carraway and Carraway, 1989; Luna and Hitt 1982). However, it has been argued that not only can the proteins be restricted in their movement by a cytoskeletal attachment, it was also found that specific lipid domains could be formed in the biological membranes (Storch and Kleinfeld, 1985; Rodgers and Glaser, 1991), domains into which several proteins were restricted (Glaser, 1993).

Although very thin, only a few nanometers in thickness, the membrane is impermeable to ionic or charged species, only small and uncharged or polar species can migrate across the membrane, for example water, carbon dioxide and oxygen. This is due to the hydrophobic character of the internal part of the bilayer membrane. Larger molecules, for example glucose, or charged species, even protons, require some sort of transporters, for example, specialized membrane proteins. This makes it possible for the cell to be out of equilibrium with its surroundings, a vital property of life, and concentration gradients of various ions, for example protons or sodium, can be used to drive certain processes, for example, the creation of ATP by the proton-driven ATP-synthase (Nagyvary and Bechert, 1999).

Lipid bilayer membranes have extraordinary material properties making them the excellent choice for living cells as their basic building material. The membrane is constituted from two monolayers of lipid, the lipid molecules and the monolayers being held together by non-covalent interactions. The non-covalency property gives the membranes a highly dynamic character, making it possible to see them as a two-dimensional fluid. They are tough but at the same time flexible with a self-reparing feature, making it possible for the cell to

grow, migrate, and change shape. Bilayers are extremely soft with respect to bending but they are very hard to stretch and it is this unique combination of elastic properties of the lipid bilayer which allows migrating cells such as the erythrocytes to travel for several hundred km through narrow body channels without loss of material.

The ease with which the bilayer membrane is bent also makes it possible to create very small structures. In eukaryotic cells, bilayer membranes are not only used to create the plasma membrane, separating the interior of the cell from the surroundings, they also organize the cell into various complex intracellular compartments involving structures of nanometer size, that all have different functions. This makes it possible even for a single cell to have different chemical environments separated from each other where specific reactions and processes can take place.

The dynamical properties also allow the important phenomena of membrane fission and membrane fusion to occur. Two examples of these events are endocytosis and exocytosis, both of which transport material across the plasma membrane. Endocytosis is a process in which a region of the plasma membrane invaginates forming a small membrane bud and a small volume of the extracellular volume is therefore trapped. The bud is pinched off the plasma membrane by membrane fission and is then transported deeper into the cytoplasm, where the transported material is taken care of in the intracellular components. Exocytosis is the reverse of endocytosis. A vesicle in the cytoplasm is transported to the plasma membrane and membrane fusion between the two membranous compartments makes it possible for the vesicle to release its contents to the outside of the cell. Many proteins destined for secretion into the extracellular space are released by exocytosis after being packaged into secretory vesicles. Membrane fission also occurs in the process of cell division, when a cell replicates by dividing itself into two daughter cells and another important example of membrane fusion is the fertilization process when the membrane of a sperm fuses to the membrane of an egg.

In order to understand the behaviour of the lipid bilayer membranes it is important to start at the molecular level, that is, the structural subunits constituting the membrane, and why these molecules form the supramolecular aggregate of a lipid bilayer membrane.

Lipid composition of biological bilayer membranes

The foundation for a biological membrane is a double layer of lipids which forms a barrier to large polar molecules and ions. Membrane lipids are amphiphilic, that is, they have one hydrophobic part in the form of a hydrocarbon chain, making them water insoluble, and one hydrophilic part, called the headgroup of the molecule. There are a huge amount of different lipid species occurring in nature, having different lengths in the hydrocarbon chain, having one or two hydrocarbon chains, with no or many double bonds on the hydrocarbon chain, as well as having different head groups. It has been suggested that the diversity of lipid species in biomembranes is coupled to the function of the membrane, for example, some proteins are only functional in the presence of a certain lipid. Studies of biomembranes have shown that the lipid composition varies between different cells and even different organelles within the same cell, a fact which further strengthens the idea that the composition is coupled to the function of the membrane. Although there are many different lipid species, they can be divided into groups, for example, there are three classes of structural membrane lipids: glycerophospholipids, sphingolipids and sterols.

The asymmetric distribution of lipid species does not only occur between different organelles within the same cell, interestingly, it also differs between the different monolayers within the same bilayer membrane. The distribution of the major classes of the phospholipids in the plasma membrane of human erythrocytes has been investigated and it was found that phosphatidylcholine and sphingomyelin were most often localized in the outer leaflet of the plasma membrane, while phosphatidylserine and phosphatidylethanolamine were found in the

inner leaflet (Somerharju *et al.*, 1999). This asymmetry of the lipid bilayer membrane is believed to be created by the use of lipid-translocating enzymes, called flipases, which move lipids between the two layers of the membrane (Schroit and Zwaal 1991). This is thought to reflect the duality of the monolayer function, the outer monolayer providing the inert barrier for the surrounding environment while the inner surface provides sites for reaction etcetera to occur by the net charge that arises from the surplus of negatively charged lipid species (Langner *et al.*, 1999).

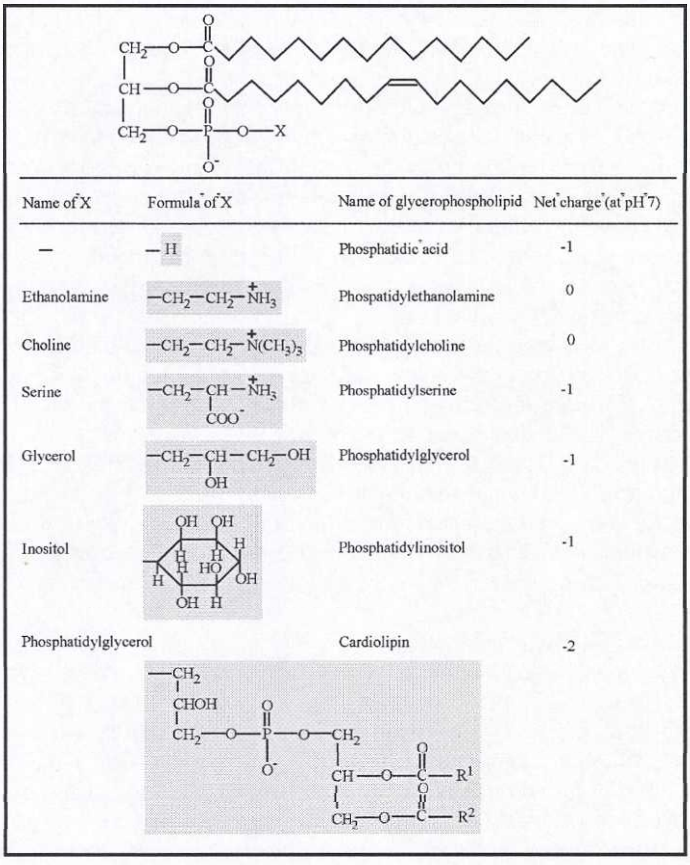


Figure 4. Schematic illustrating the class of glycerophospholipids. The backbone of these molecules is a glycerol molecule which is linked to two alkyl chains or fatty acids through ester bonds. The head-group of the lipid is linked to the third part of the glycerol molecule via a phosphate group. Shown in the figure is also 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 most common type of lipids in biomembranes is the glycerophospholipids (Gennis, 1989), which have two fatty acids joined to a glycerol molecule by ester bonds (Fig. 4). The hydrocarbon tails of the fatty acids have varying length, often between 12 and 24 carbon atoms in length, and can also have varying degree of saturation. These differences will influence parameters such as permeability and fluidity of the membrane. The third hydroxyl group is linked to a phosphate molecule which is further linked to different kinds of head groups. The head-group of the lipid will also determine the properties and the function of the

membrane, for example, if the head-group is charged or not. Phosphatidyl serine contains both a carboxylgroup and an amine besides the phosphate group and therefore, the net charge at pH 7 is negative. Phosphatidyl choline has an amine group besides the phosphate group, which means that it has a net charge of zero at neutral pH. The most common phospholipids are phosphatidylcholine (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl glycerol (PG).

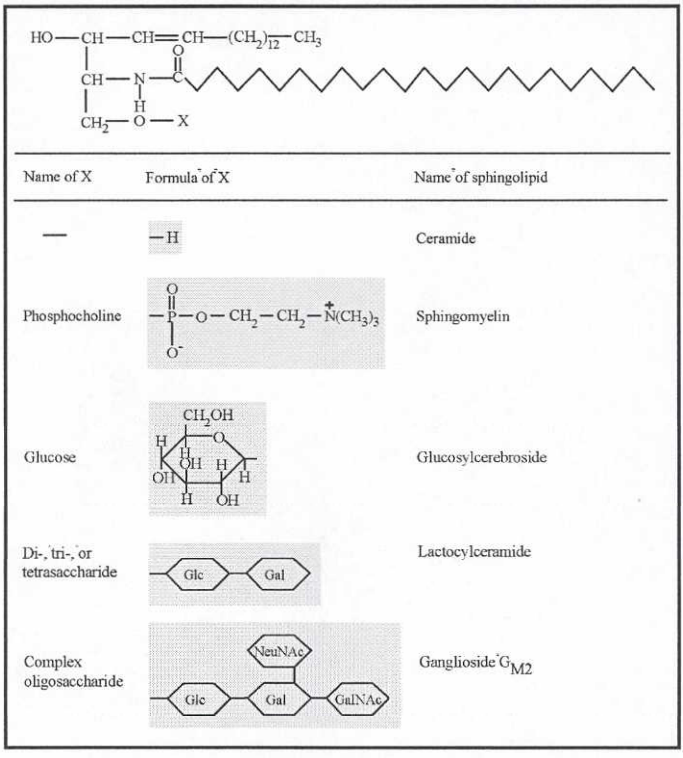


Figure 5. Schematic illustrating the class of sphingolipids. The general structure of sphingolipids containing a long-chain amine alcohol sphingosine as a backbone, together with a long-chain fatty acid and a polar head alcohol that can be further linked to other polar head-groups, for example, via phosphodiester linkage. The first three carbons at the polar end of the sphingosine is analogous to the three carbons in glycerol in the glycerophospholipids. The parent compound for this group is the ceramide and also shown are examples from the three sub-groups of the sphingolipids: sphingomyelins, glycolipids and gangliosides. Standard symbols for sugars are used in this figure: Glc, D-glucose; Gal, D-galactose; GalNAc, N-acetyl-D-galactosamine; NeuNAc, N-acetylneuraminic acid (sialic acid).

Sphingolipids are the second large class of membrane lipids and they also have a polar head and two non-polar tails, however they contain no glycerol as backbone. Sphingolipids are instead composed of one molecule of the long-chain amino alcohol sphingosine or one of its derivatives, one molecule of a long-chain fatty acid, a polar head alcohol, and sometimes a phosphodiester linkage at the polar head group (Fig. 5). The sphingolipids can be divided into three sub-groups, sphingomyelins, glycolipids and gangliosides. Sphingomyelins resembles phosphatidylcholines in properties and structure and are present in plasma membranes of animal cells. The glycolipids and gangliosides have sugar units attached to their polar head groups and are also found in the plasma membranes of animal cells, with a high presence in neural tissues, such as the brain.

Sterols are structural lipids present in the membranes of most eukaryotic cells. The sterols characteristically have a so-called steroid nucleus consisting of four fused rings, three with six carbons and one with five. The steroid nucleus is almost planar and relatively rigid because no rotation around the C-C bonds in the fused rings is allowed. Cholesterol is the major sterol in animal tissues, having a polar head-group and a non-polar hydrocarbon body that has a length about the same as a 16-carbon fatty acid in its extended form. The sterols are precursors for a variety of molecules that has specific biological functions, for example, the bile acids that acts as detergents in the intestine or some steroid hormones.

Formation and structure of amphiphilic supramolecular aggregates

The hydrophobic effect

The amphiphilic lipid molecules contain both a water-loving part and a water-shying part, which give them a dual-character, making it impossible to achieve phase-separation between a hydrophobic and a hydrophilic part of the solution. These molecules are therefore surface active, that is, they tend to gather in surface regions, for example in the air-water interface, where the polar headgroups are in the water solution, while the hydrophobic tails are in the air. In an aqueous solution, the molecules can also try to hide the hydrophobic chains from the surrounding water by forming supramolecular aggregates. The self-arranging ability arises from the dual-character structure of the membrane lipid and the properties of water, which differs greatly from the properties of the hydrocarbon chain of the lipid. This can be illustrated by the dielectric constant which is $\varepsilon = 2$ for the hydrocarbon alkyl chain and for water it is $\varepsilon = 80$. Water molecules readily form hydrogen bonds with each other, however, when a lipid is inserted into the water environment, the polar water molecules will have to adopt a more ordered structure around the hydrocarbon fatty acid chains in order to maximize the number of hydrogen bonds.

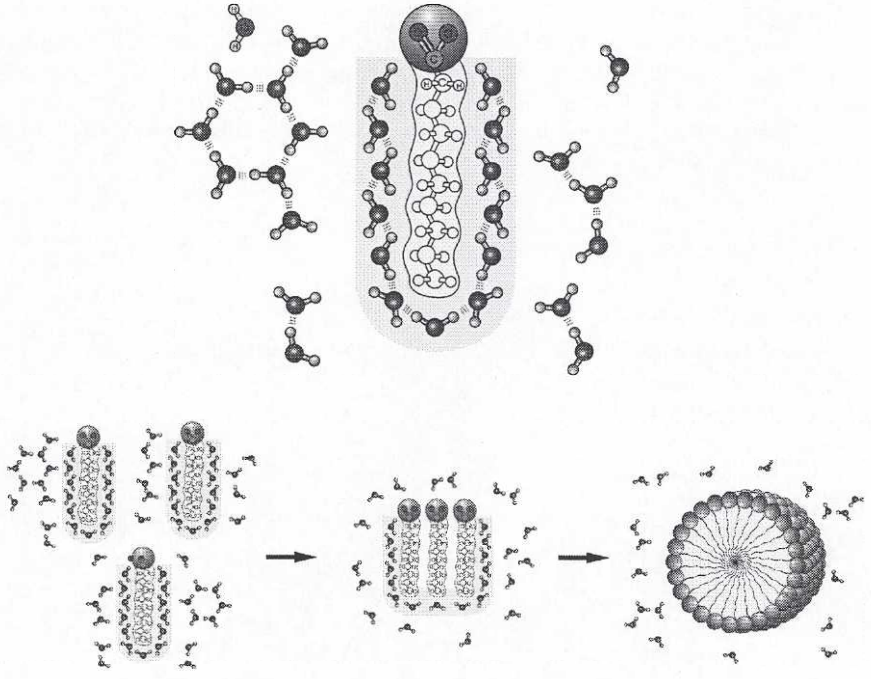


Figure 6. Schematic drawings illustrating the hydrophobic effect. In bulk water (upper left) the water molecules form "flickering clusters", where the number of hydrogen bonds is maximized and entropy is high. When a long-chain fatty acid is inserted into an aqueous environment, the water molecules will have to adopt a more ordered structure around the very hydrophobic alkyl chains. A "cage" of water molecules is formed in order to maximize the number of possible hydrogen bonds, since it cannot form these bonds with the alkyl chains. This ordering will lower the entropy of the system, thereby increasing the energy. By clustering together, for example, by forming micelles, the fatty acid molecules expose a smaller hydrophobic surface area to the water, and fewer water molecules are found in the shell of ordered water.

This ordering of the water molecules will lower the so-called entropy, that is, the degree of freedom of the system, which will lead to a higher energy. If one considers two such lipid molecules with their hydrocarbon chains fully exposed to water, and then let these two associate with the chains together, much of the water molecules are released from the ordered structure and the entropy is increased. This is the so-called hydrophobic effect (Fig. 6) (Evans and Wennerström, 1999; Ben-Shaul, 1995, Lehninger *et al.*, 1993).

Formation of supramolecular aggregates

When the aggregate is formed, there are two opposing forces that will work on the lipid molecules, one attractive and one repulsive force. The attractive force, which will dominate the repulsive forces, comes mainly from the interfacial tension between the aqueous solution and the lipid hydrocarbon interior (~35 mN/m), see discussion on the hydrophobic effect above, and also to a small degree from very short range van der Waals attractions between the lipid tails. The repulsive forces can be divided into two groups, one arising at the headgroups and one at the hydrophobic tails. A "surface pressure" (~21 mN/m) may be said to exist in the head groups due to electrostatic and steric repulsions. Similarly, a "chain pressure" (~14 mN/m) arises because of thermal motions of the chains and their steric repulsions. (Ben-Shaul, 1995; Oster *et al.*, 1989)

The attractive forces tends to decrease the interfacial area, a, per molecule (headgroup area) exposed to the aqueous phase, and is proportional to γa, where γ is the interfacial tension. The repulsive force of the headgroups on the other hand tends to increase the headgroup area. This latter force is approximately inversely proportional to the surface area occupied per headgroup. The total interfacial free energy per molecule in an aggregate can now be expressed, to first order, as

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

where K is a constant (Ben-Shaul, 1995).

The minimum energy of the molecule is therefore when $d\mu^0/da = 0$ as illustrated by figure 7 (assuming that the attractive and repulsive forces act in the same plane at the hydrophilic-hydrophobic interface).

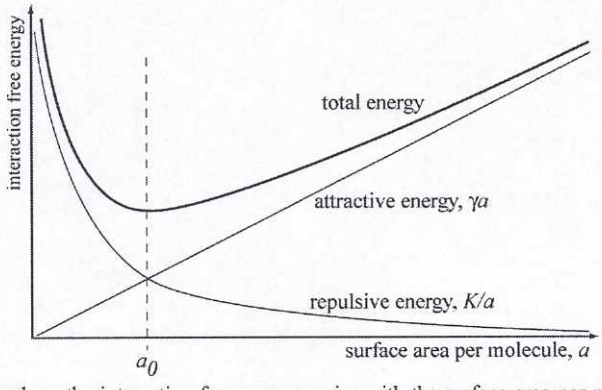


Figure 7. Plot showing how the interaction free energy varies with the surface area per molecule according to equation 1. The attractive energy will increase with the surface area per molecule, a, as γa, while the repulsice energy will decrease as K/a. When the opposing forces of head group repulsion and interfacial attractions are balanced, an optimal head group area, a_0, can be found where $d\mu_0/da=0$.

The aggregate system therefore wants to minimize the total interfacial energy and will form an aggregate of size N where μ^0 is at global minimum. If the number of molecules are to few in the aggregate, they have to much space and the system will strive to insert more molecules and if the number of molecules are to many, they feel crowded and the system instead wants to get rid of some of the molecules.

Aggregate structures

As discussed, the size of the aggregate is determined by the total interfacial energy of an amphiphile in an aggregate. However, the geometry, or shape, of the supramolecular assembly will critically depend on the individual geometry of the molecules constituting the aggregate (Israelachvili, 1992). Lipid molecules can be characterized by a dimensionless "shape parameter", P, which can be described as a function of the optimal headgroup area, a_0, the volume of the hydrocarbon chain(s), v, and the maximum effective length of the hydrophobic tails of the molecule, l_c (Glaser, 2001). This length is of the same order as the fully extended molecular length of the hydrocarbon chains.

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

From this shape parameter, lipids can be classified as having the shape of a cylinder, cone or inverted cone. Note that this classification depends on the environment in which the lipid finds itself. It is then also possible to predict the shape of the aggregate that these molecules will form (Fig. 8).

- If P is less than one, it will be classified as a cone and the corresponding aggregate will be in the form of a sphere, which is called a micelle.
- If P is larger than one, the molecule will have the shape of an inverted cone, or frustum, and the corresponding aggregate will have the form of an inverted micelle.
- If P is close to 1, the molecular shape of the amphiphile is a cylinder and the corresponding supramolecular aggregate will be a planar membrane. However, a planar monolayer of amphiphiles would mean that all of the hydrophobic tails are exposed to the surrounding water. Therefore, bilayer membranes are instead formed, where the hydrophobic tails of the two monolayers are facing each other, completely protected from the water.

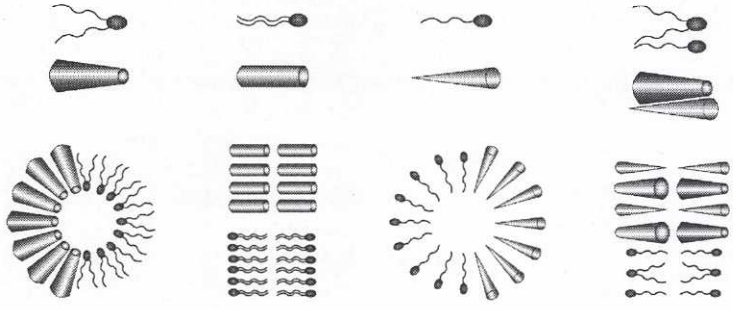


Figure 8. Schematic showing the correlation between monomer shape factors and aggregate structure. $P>1$. Lipids having small polar head groups and a large bulky hydrocarbon chain, will form aggregates of so-called inverted micelles. $P=1$. Lipids that have a cylindrical appearance will form planar bilayers. $P<1$. Molecules that have a larger headgroup than the hydrocarbon chain will form micelles, where the hydrophobic tails are facing each other. As is illustrated to the far right, a mixture of different lipids having different shape parameters, can form bilayer structures.

So far, only aggregates of the same molecules have been discussed. However, the concept of the shape parameter can also be applied to mixtures of amphiphiles having different shape factors. For example, it is possible to obtain planar aggregates by mixing cone-shaped, inverted cone-shaped and cylindrical molecules. It has been hypothesized that the complex mixing of different kinds of lipids, all having different shape parameters, might be a way for the cell to facilitate complex shape changes and the creation of highly bent or curved structures. In these highly curved regions, the lipids having the most favourable shape factor might gather to relax the bending energy which is created (Lipowsky, 2002). Although simple in its character, the shape factor concept is quite useful when looking at pure surfactant systems where no phase segregation between species occurs. There are many different phases and structures formed from different lipid species in aqueous solutions, however, here focus will be on the lipid bilayer membrane and vesicles.

Lipid bilayer membranes

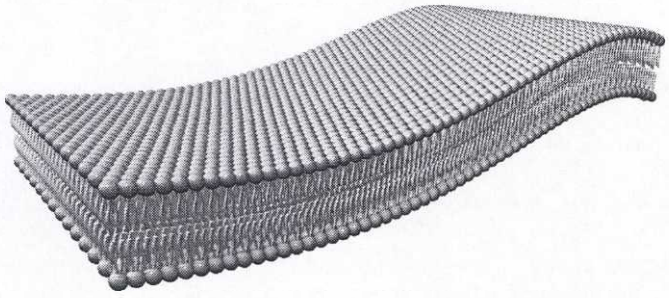


Figure 9. Schematic illustrating a lipid bilayer membrane. The lipids of the bilayer membrane form two monolayers that direct the hydrophobic alkyl chains towards each other, while the polar head groups are facing the surrounding water.

Bilayer membranes are formed by amphiphiles that have shape factors close to one, meaning they cannot form micellar-type aggregates because their headgroups are to small, or because the hydrocarbon backbone is to bulky to fit into such small aggregate structures. Micelle-forming amphiphiles usually have only one carbon chain and therefore amphiphiles having two hydrocarbon tails, having double the hydrophobic volume, are very likely to form bilayer membranes.

Besides increasing the probability of forming bilayer structures, the doubling of the hydrocarbon tails also leads to lower water solubility, compared to micelle-forming amphiphiles. The critical micelle concentration (CMC) or better critical association concentration or the solubility limit, for a bilayer forming amphiphile is 10^{-7} to 10^{-12} M (Israelachvili, 1992; Sackmann, 1995) compared to a micelle-forming amphiphile having CMCs of 10^{-2} to 10^{-4} M. The lower CMC arising from the doubling of hydrocarbon tails will also give longer residence times in the aggregates. In micelles, there is a rapid exchange of the amphiphiles in the aggregate and the free monomers in solution, though activation energy is needed to move a monomer from the aggregate and into solution. The residence times, τ_R, for micelle-forming amphiphiles is approximately 10^{-4} seconds and for bilayer-membrane forming amphiphiles, for example, glycerophospholipids, it is about 10^{4} seconds (Israelachvili, 1992).

In bilayers, there is also a possibility for a molecule to move from one monolayer to the other monolayer, a process called "flip-flop" (Wimley and Thompson, 1991). This

process is quite uncommon since the energy required to perform "flip-flop" is higher than for lipid exchange with the surrounding solution. The residence times, τ_R, of a monomer in an aggregate is determined by the length of the hydrocarbon chain, but the "flip-flop" mechanism is determined by the size and structure of the hydrophilic head groups of the amphiphiles. Because of the high barrier for the "flip-flop"-phenomenon to occur, it is possible to create a lipid bilayer membrane having different composition in the outer and inner monolayers of the bilayer, a fact that is very important for living cells. Interestingly, it has been seen that a high membrane strain, for example, caused by elastic deformation through pulling of nanotubes from vesicles might lead to an increase in the rate of "flip-flop" (Svetina *et al.*, 1998).

The properties of the lipid bilayer membrane can change with temperature since it is a thermotropic material that can display different phases. At physiological temperatures, the membranes are mostly in a liquid phase, in which the lipid molecules have a high degree of mobility, they can rotate around their own axis as well as diffuse laterally throughout the bilayer structure with a diffusion coefficients, D_l, ranging between 10^{-12} to 10^{-11} m^2/s (Almeida and Vaz, 1995). At low temperatures, the membrane attains a more ordered structure and finally solidifies to a crystalline phase. In bilayer membranes that are constituted from a pure component it is possible to determine a critical transition temperature, T_c, where the liquid and solid states are equally probable (Evans and Needham, 1987). When the liquid solidifies to the crystalline phase, sometimes intermediate crystalline phases can arise, so-called gel-phases, which display more disordering than the true crystalline phase. The coexistence of different phases in a bilayer membrane has been observed by fluorescent staining of two different phases, using confocal fluorescence microscopy (Korlach *et al.*, 1999)

Lipid bilayer vesicles

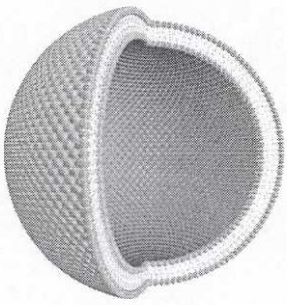


Figure 10. Schematic illustrating a lipid bilayer vesicle. Vesicles are formed when a lipid bilayer membrane self-closes and entraps an aqueous solution.

Lipid bilayer vesicles are spherical containers composed of a self-closed lipid bilayer which have trapped an aqueous solution in its interior space (Fig. 10). The aggregation of amphiphiles into a bilayer and the self-closing of this bilayer into a vesicle are thought to be the first steps towards a cell and the creation of life. This is because the bilayer provides a barrier between the internal solution and the external solution, thereby making it possible for the internal solution to be out of equilibrium with its surrounding environment, a key feature of life. Nowadays, artificially made vesicles are used for many different purposes, for example, in the cosmetics industry, the agro-food industry and as drug delivery systems in

medicine, they can be used as artificial cell models or as biomimetic reaction containers or to measure physical parameters of the membrane to mention a few application areas and research fields (Lasic, 1993; Lasic, 1995).

The vesicles can be classified into different groups according to their size and the number of lamellae constituting the walls of the vesicles (Fig. 11).

- Small unilamellar vesicles (SUVs) have sizes in the range up to 200 nm in diameter.
- Large unilamellar vesicles (LUVs) have diameters in the range of 200 and 1000 nm.
- Giant unilamellar vesicles (GUVs) range from sizes 1000 nm in diameters and up.
- If the walls constituting the vesicles have more than one lamella, they are called multilamellar vesicles (MLV).

The vesicles that were mostly used in this thesis were GUVs in the size range of 5 to 50 μm in diameter, but MLVs of the same sizes were also used. The most important characteristic of the GUVs and MLVs are that they can be observed under light microscopy, which makes it possible to handle and manipulate them, a vital characteristic for the techniques and methods developed in this thesis. This characteristic is also what fascinates scientists interested in membrane mechanics and membrane physics. The extremely small thickness of the membrane makes it possible to view it as a two-dimensional surface, the behaviour of which can be studied in three-dimensional space. Surface interactions, shape changes and thermal fluctuations can be studied under the microscope, as well as the response of the membrane under changes in the chemical environment or mechanical stress induced by different kinds of manipulation or adhesion effects.

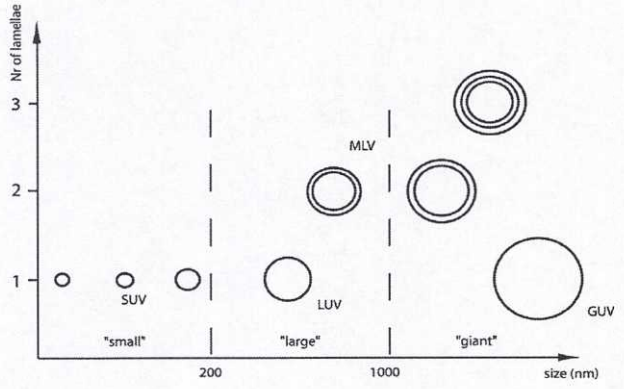


Figure 11. Schematic illustrating the definition of small, large and giant vesicles, as well as the distinction between unilamellar and multilamellar vesicles. The small unilamellar vesicles (SUVs) have diameters in the range up to 200 nm. The large unilamellar vesicles (LUVs) have diameters ranging between 200 and 1000 nm, while the giant unilamellar vesicles (GUVs) have diameters from 1000 nm and up. If the vesicles have more then one bilayer membrane constituting the wall, then they are called multilamellar.

Formation of giant vesicles

Unilamellar vesicles

Giant vesicles are usually prepared by hydrating a thin film of lipids, a technique which was developed by Reese and Dowben (Reese and Dowben, 1969). Lipids dissolved in an organic solvent like chloroform are dried to a thin lipid film on a substrate. The lipid film is then carefully hydrated by a water-saturated nitrogen gas and then swelled in an aqueous solution, thus forming vesicles.

When using this technique it is of utmost importance that the bilayer separates from each other during the swelling procedure. There are a few parameters that will affect the efficiency of vesicle formation; the temperature, the lipid composition and the ionic strength and composition of the aqueous solution in which the lipid film is swelled.

The temperature will decide in which state the lipids are. At temperatures below the melting temperature, T_c, the lipids will be in a solid gel-phase or crystalline phase and few or no vesicles will be formed upon swelling of the thin film of lipids. The melting temperature depends on the hydrocarbon chain length and degree of saturation of the chains. Above the melting temperature, the lipids are in a fluid state, sometimes referred to as liquid state and vesicles will be formed upon swelling. Higher temperature also leads to undulations of the lipid bilayer membrane, separating them from one another and increasing the probability of vesicle formation.

The lipid composition will also affect the swelling of the thin film of lipids. If some of the head-groups of the lipids are charged, say negatively as in phosphatidylserine, this will enhance the efficiency of bilayer separation and vesicle formation. When water is osmotically transported into the thin film of lipids, hydrating the small spaces left between the lipid bilayer, the negatively charged groups will repel each other and the bilayers will separate whereby vesicle formation is possible (Langner and Kubica, 1999). If the head-groups of the lipids are uncharged or neutral they will not repel each other to the same degree and vesicle formation is suppressed.

The ionic strength and composition of the aqueous solution in which the dried lipid film is swelled will also affect the efficiency of vesicle formation (Akashi *et al.*, 1996). The higher the ionic strength, the harder it is to form vesicles and normally ionic strengths of 10 – 50 mM are used. Multivalent cations will also affect the swelling procedure. When water hydrates the interbilayer spacings and tries to separate the bilayers, multivalent cations may be bound to negatively charged groups in the bilayers that are to be separated. The ions can work as a bridge, or an ionic glue, between the bilayers, holding them together and disturbing the swelling process. In this case the yield of vesicles will be low. However recently, higher concentrations of divalent cations like calcium or magnesium ions have proven to enhance vesicle formation. The explanation might be that the divalent cations bound to the negatively charged lipids change the charge of the membranes from negative to positive, establishing a repulsive force that leads to bilayer separation (Akashi *et al.*, 1998).

Another type of preparation method is the electroformation technique, which uses an electric field to stimulate bilayer separation and vesicle formation, a technique first developed by Angelova et al. (Angelova *et al.*, 1986). In order to create vesicles, lipids dissolved in an organic solvent are dried onto platinum electrodes, which are placed in the electroformation chamber. The chamber is filled with an aqueous solution and an electric field is applied across the electrodes. The electroformation procedure usually starts by applying a low field with potentials of 0,2 Volts and a frequency of 10 Hertz and then the voltage is increased up to 1 to 7 Volts. At the end, the frequency is lowered to 0,5 Hertz and finally the voltage is lowered to 0,2 Volts. This procedure is thought to give the vesicles a spherical appearance separated from the electrodes. Using this technique, vesicles having sizes in the range of 5-

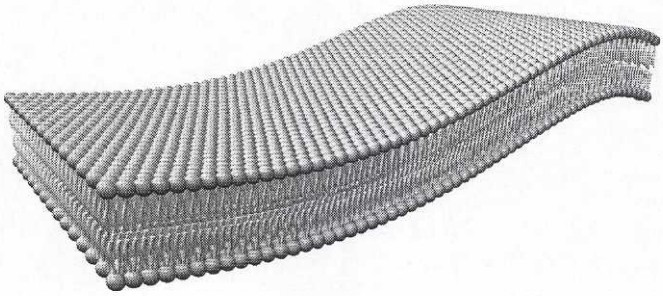
200 μm in diameter are formed. The membrane tension of the vesicles is thought to be dependent on the kinetics of the formation and normally the vesicles have an excess of membrane area. The mechanism behind the formation is thought to be that water is periodically moved by electroosmosis and that the bilayers are separated by the vibrations that are created perpendicular to the electrode surface, thereby promoting vesicle formation. It is also possible that the charges of the lipid bilayers themselves are repelled by the electrode surfaces. The vesicles bud off from the bilayer sheets and can remain connected to the bilayers by tethers, or lipid nanotubes, and can be detached by gentle agitation.

In this thesis a preparation technique based on the method developed by Criado and Keller was used (Criado and Keller, 1987), with modifications (Karlsson *et al.*, 2000) and one of the advantages with this technique is that it can generate vesicles even under physiological conditions. An aqueous lipid dispersion is dehydrated in order to obtain a lipid film which have entrapped the buffer salts of the aqueous solution. Upon rehydration, the osmotic pressure difference due to the high amount of salts trapped by the lipids will lead to an influx of water and a subsequent swelling of the lipid film. The bilayer structures of the lipid film were separated and in only a few minutes giant unilamellar vesicles were created in a high yield.

Multilamellar vesicles

Multilamellar vesicles can be produced by rehydrating dry lipid and then shake or vortex the solution. This creates a lipid suspension in which a range of vesicles having different number of lamellae and different sizes are formed. This suspension can then be processed in order to get a more evenly distributed vesicle population, both in size and in lamellarity. To get small unilamellar vesicles (SUVs), the lipid suspension can be sonicated, whereby the multilamellar vesicles break up and both the number of lamellae and size is reduced. The reduction in size and lamellarity can also be performed by extruding the suspension through polycarbonate filters having specific pore sizes. The multilamellar lipid suspension or the SUV solution can both be used to create giant vesicles by another dehydration step, as described above.

Mechanical properties of lipid bilayer membranes



It was realized that the behaviour of the supramolecular aggregate of the lipid bilayer membrane would be quite complex. In order to simplify the theoretical investigations, the first mechanical models for lipid bilayers treated the membrane as a "unit" structure, which could be viewed as a thin elastic shell (Canham, 1970; Helfrich, 1973; Evans, 1974). A theory was proposed by Helfrich in 1973 (Helfrich, 1973), in which an area of size A_0 is subjected to the different forces that generate the phenomenon of stretching, bending and shearing, all of which will influence the energy that is stored in the system (Evans and Needham, 1987).

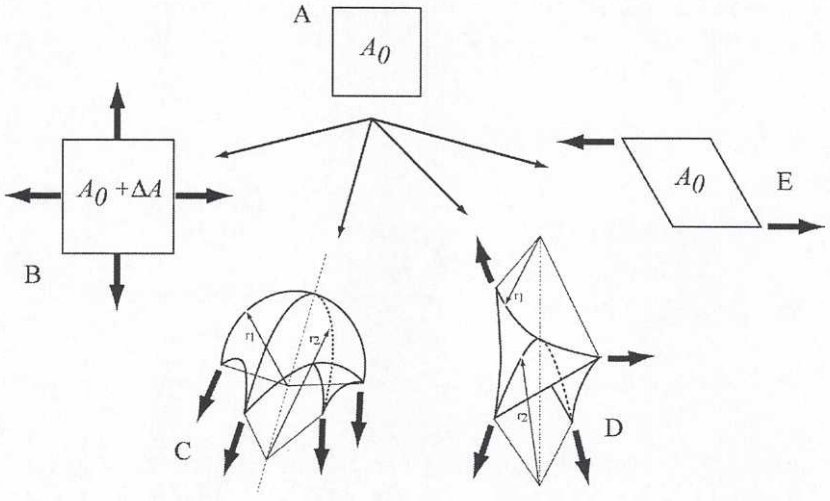


Figure 12. Schematic drawing illustrating the three basic shape deformations acting on an area element. (**A**) The starting shell having an area of A_0. (**B**) Forces acting perpendicular to the side of the membrane will stretch it symmetrically and increase the area by ΔA. (**C – D**) Different bending configurations, in C the surface is bent in the same direction, while in D the bending modes are in opposite direction from each other. (**E**) Tangential forces act in parallel with the side of the element, without change in area, causing a shear deformation.

Stretching

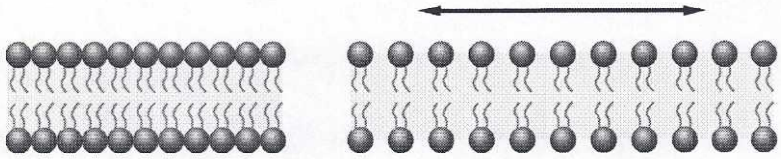


Figure 13. Schematic illustrating the stretching of a lipid bilayer membrane. (**left**) An unstretched membrane. (**right**) A stretched membrane.

In the case of stretching, the area A_0 is stretched symmetrically and expanded by an area of $\Delta A = A - A_0$ (Fig. 12), which will lead to higher energy according to

$$E_{stretch} = \frac{K_a}{2A_0}(A - A_0)^2 \qquad \text{(Equation 3)}$$

where K_a is the elastic stretching modulus, A is the stretched area and A_0 is the original area of the membrane. This term is a simple Hookean elastic stretching which illustrates the resistance to expansion of the surface. The value of K_a is typically 240 mN/m for a phospholipids bilayer membrane (Rawich *et al.*, 2000) and resistance to stretching is therefore quite large. The lipid molecules have a preferred intermolecular distance and any perturbation affecting this distance, like compression of stretching, will destroy the minimum energy situation. If the membrane is stretched too much, it can rupture. The rupture tension is normally 4 – 10 mN/m and the maximum area expansion before rupture is therefore astonishingly small varying between 2 and 5% (Kwok and Evans, 1981; Olbrich *et al.*, 2000).

Bending

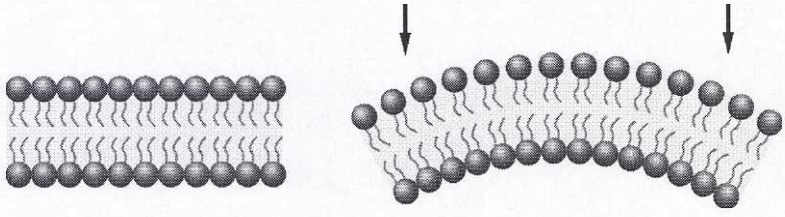


Figure 14. Schematic illustrating the bending of a lipid bilayer membrane. (**left**) An unbent membrane. (**right**) A bent membrane.

The shell can be bent, making it possible to define a radius, r, of the curved region (Fig. 12). The curvature, c, of the shell is simply the inverse of this radius, $c = 1/r$. In order to calculate the energy arising from bending, two principal curvatures, c_1 and c_2, are created for the surface. These curvatures are orthogonal to each other and can either be both positive if the surface is bent in the same direction or be of opposite signs if the shell is bent in different directions. For a spherical geometry, $c_1 = c_2 = 1/r$, whereas for a planar geometry $c_1 = c_2 = 0$. In a cylinder, one of the curvatures is the reciprocal of the cylinder radius and the other curvature is zero. The two principle curvatures are used to define a mean curvature, H, and a Gaussian curvature, K, according to

$$H = \frac{(c_1 + c_2)}{2} = \frac{\left(\frac{1}{r_1} + \frac{1}{r_2}\right)}{2} \qquad \text{(Equation 4)}$$

and

$$K = c_1 c_2 = \frac{1}{r_1 r_2} \qquad \text{(Equation 5)}$$

A spontaneous curvature, called c_0, is also defined, meaning that the surface might have a preferred curvature apart from the planar configuration and this term has to be subtracted from the mean curvature.

Bending of the membrane will thus create energy according to the relation

$$E_{bend} = \frac{k_c}{2}(c_1 + c_2 - c_0)^2 + k_G c_1 c_2 \qquad \text{(Equation 6)}$$

where k_c is the bending modulus, c_1 and c_2 are the principal curvatures of the shell, c_0 is the spontaneous curvature and k_G is the Gaussian modulus. The last term, the Gaussian bending is included in the equation in order to take the energy of saddle-points into account. In a saddle-point, the two principal curvatures cancel out each other, since $c_1 = -c_2$. Bending is usually quite easily performed due to the extremely thin membrane, however, for nanoscale structures this energy term can become very important.

Shearing

When the area is subjected to shearing, this means that the surface is deformed in-plane while the surface area is constant. This leads to shearing of the shell and the energy which is created can be found in the relation

$$E_{shear} = \mu_s \beta \qquad \text{(Equation 7)}$$

where μ_s is the shear modulus and β describes the degree of shear deformation. However, in fluid membranes, that is, above the melting temperature, the shear modulus, $\mu_s = 0$ and this term therefore disappears.

Adding all these terms together and integrating over the entire surface, gives the total energy of the system.

$$E = \frac{K_a}{2A_0}(A - A_0)^2 + \frac{k_c}{2}\int (c_1 + c_2 - c_0)^2 dA + k_G \int c_1 c_2 \qquad \text{(Equation 8)}$$

This expression only recognizes the membrane as a thin elastic shell. However, the membrane is composed of two independent monolayers of lipid molecules.

Area difference elasticity or non-local bending

The bilayer is, as the name implies, not a single sheet, but two monolayers that are coupled together. The lipids constituting the monolayers are more or less free to move about, whereas exchange of lipids between monolayers, the so-called "flip-flop"-mechanism, is highly unusual. The number of lipids in the two monolayers is thus considered to be more or less constant. If there is a difference in the number of lipids between the two monolayers, this gives rise to a preferred area difference, ΔA_0, as opposed to the actual area difference that is described by

$$\Delta A = h \oint (c_1 + c_2) dA \qquad \text{(Equation 9)}$$

where h is the distance between the neutral surfaces of the bilayer (approximately half the bilayer thickness). It will cost energy to maintain a difference between the preferred and the actual area difference, and an area difference elasticity term arises (Miao *et al.*, 1994; Svetina *et al.*, 1998; Seifert, 2000; Döbereiner, 2000)

$$E_{ADE} = \frac{k_r}{2A_0 h^2} (\Delta A - \Delta A_0)^2 \qquad \text{(Equation 10)}$$

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

$$E = \frac{K_a}{2A} (A - A_0)^2 + \frac{k_c}{2} \int (c_1 + c_2 - c_0)^2 dA + \frac{k_r}{2A_0 h^2} (\Delta A - \Delta A_0)^2 + k_G \int c_1 c_2 \qquad \text{(Equation 11)}$$

Mechanics and shapes of vesicles

When studying the shapes and mechanics of vesicles it is important to know if the volume and area is constant during the study. For example, any osmotic imbalance will lead to a water flux across the membrane, since water is permeable to the membrane, to eliminate the imbalance. Also, an increase in temperature might lead to an expansion of the membrane area of the vesicle. If the area and volume can be viewed as constant (constant temperature and osmotic conditions) membrane mechanical parameters such as bending or stretching energy will instead determine the shape of the vesicle.

The shapes and shape transformation of vesicles have been investigated both experimentally and theoretically. Soon after the first experimental findings, theoretical studies of the shapes emerged and so-called curvature models were created (Canham, 1970; Helfrich, 1973; Deuling and Helfrich, 1976; Evans, 1974). By changes in temperature or pressure, addition of amphiphiles or adsorbents, mechanical, electrical or magnetic treatments, or adhesion to surfaces, it was possible to induce shape transformations of the vesicles. One example is the use of temperature changes to induce shape transformation and achieve a so-called budding transition. With increased temperature, the bilayer membrane was thermally expanded and almost spherical vesicles changed shapes, first to a prolate shape (cigar-shape) and then to a pear (Fig. 15). Finally, a small bud could be expelled from the vesicle and if the temperature was increased further, more buds could be formed. Most often, the buds remained connected to the mother vesicle by necks or narrow constrictions (Berndl *et al.*, 1990).

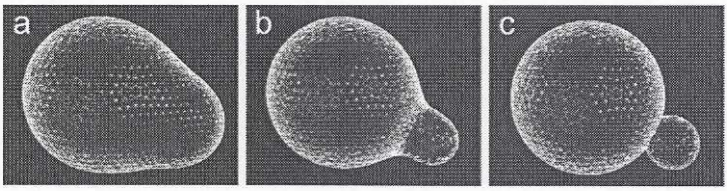


Figure 15. Schematic three-dimensional drawings showing the mechanism of temperature-induced budding. (**a**) A vesicle having the shape of a pear. (**b**) With an increase in temperature, a neck is formed. (**c**) Finally, this neck can close, forming a bud which is connected to the larger vesicle by a small pore-like structure.

The shape of the vesicles could be predicted by using the principle that the vesicle will achieve the shape that minimizes the elastic energy according to equation 11

$$E = \frac{K_a}{2A_0}(A - A_0)^2 + \frac{k_c}{2}\int(c_1 + c_2 - c_0)^2 dA + \frac{k_r}{2A_0 h^2}(\Delta A - \Delta A_0)^2 + \int k_G c_1 c_2$$

Since the vesicle is a topological sphere with a genus of zero, the so-called Gaussian curvature can be neglected. Integrated on the whole surface, the Gaussian term will not change, no matter what shape the vesicle will have.

More recently, the curvature models have been revisited (Svetina *et al.*, 1989; Berndl *et al.*, 1990; Miao *et al.*, 1991; Seifert *et al.*, 1991) and even for vesicles with non-spherical topology, that is, with one or more holes, the shape has been predicted (Seifert, 1991; Julicher *et al.*, 1993).

Forced shape transformation of vesicles

The shapes of free vesicles have been studied and many different shapes have been found to exist, for example spheres, toroids, starfish and nanotubes (Seifert, 1997; Döbereiner, 2000). However, investigations have also been performed on forced shape transformation, such as adhesion of vesicles to a surface or the pulling of nanotubes, or tethers, from vesicles.

Adhesion of vesicles

If a vesicle interacts with a surface, this will also affect the shape (Fig. 16). The adhesion of vesicles can be studied experimentally by using, for example, the micro-pipet aspiration technique (Evans, 1980) or by using reflection interference microscopy (Zilker *et al.*, 1987; Rädler and Sackmann, 1993). The forces that a vesicle experience close to a surface can be of various nature, for example, electrostatic, van der Waals or hydration forces, which can all range in the nanometer scale.

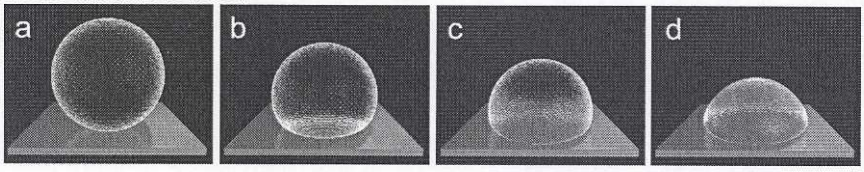


Figure 16. Schematic three-dimensional drawing illustrating increasing strengths in adhesion of vesicles. (**a**) If the contact potential is zero, the vesicle will only sit on the surface. (**b**) A weak contact potential will lead to a deformation of the vesicle, the adhesion energy competing and compensating for the energy created by the bending. (**c – d**) With an increase in the contact potential towards the high adhesion regime, the vesicle will be more deformed and instead of competing with the bending energy, the adhesion will be balanced by the stretching energy.

A contact potential of strength W can be stated, which takes all these forces into account (Seifert and Lipowsky, 1990). If the vesicle and the surface have a contact area A^*, the vesicle gains the adhesion energy

$$F_a = -WA^* \qquad \text{(Equation 12)}$$

which must be added to the energy expression for the vesicle.

$$E = \frac{K_a}{2A_0}(A - A_0)^2 + \frac{k_c}{2}\int(c_1 + c_2 - c_0)^2 dA + \frac{k_r}{2A_0 h^2}(\Delta A - \Delta A_0)^2 - WA^* \qquad \text{(Equation 13)}$$

The contact potential, W, have values in the range of 10^{-4} – 1 mJ/m^2. 1 mJ/m^2 gives a strong adhesion regime, 10^{-4} mJ/m^2 gives a weak adhesion regime. In the weak adhesion regime, bending will compete with the adhesion energy (Seifert and Lipowsky, 1990; Seifert and Lipowsky, 1991) and a balance between the gain in adhesion energy and the cost in curvature energy will be created. For strong adhesion, the energetic competition which determines the shape of the vesicle is not the balance between the curvature energy and the adhesion energy, but instead the balance between the adhesion energy and the elastic stretching of the membrane. In this strong adhesion regime, one can state the term effective contact angle (Fig. 17) (Seifert and Lipowsky, 1990).

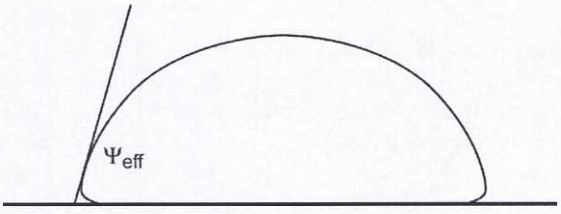


Figure 17. Schematic illustrating the definition of a contact angle. The definition of a contact angle can only be done in the so-called high adhesion regime.

It is important to realize that if the adhesion is to strong, this will stretch the membrane past the lysis tension (a few mN/m) and the membrane of the vesicle will rupture (Sandre *et al.*, 1999).

Creation of nanotubes from vesicles

Lipid membrane nanotubes can be formed spontaneously in bulk solution (Schnur *et al.*, 1993; Bar-Ziv *et al.*, 1998) but they can also be formed by applying a point load to a vesicle or a cell. This was first discovered by Hochmuth *et al.* (Hochmuth *et al.*, 1973) who noticed formation of lipid membrane nanotubes, or tethers, when red blood cells attached to a surface were exposed to a fluid shear force. The cells detached from the surface, but an attachment point remained and the cell was held back by an "invisible" tether.

To be able to study this phenomenon under a more controlled manner, Hochmuth et al. (Hochmuth and Evans, 1982; Hochmuth *et al.*, 1982) developed a micropipette technique, which allowed them to hold cells and form tethers by attaching a bead to the membrane which was pulled from the cell, for example, by gravity. They noticed that by measuring the applied force it was possible to calculate the radius of the tube. Later, Waugh *et al.* (Waugh

and Hochmuth, 1987; Bo and Waugh, 1989) used this technique for giant vesicles and were able to determine the membrane bending stiffness of a lipid bilayer membrane. Pulling of nanotubes has also made it possible to study other physical parameters of the lipid bilayer membrane, such as, for example, intermonolayer friction (Evans and Yeung, 1994) as well as local and non-local bending modulus (Waugh *et al.*, 1992).

Other techniques have been developed for pulling of tubes, for example, by optical tweezers (Dai and Sheetz, 1999), pulling of hydrodynamic force (Rossier *et al.*, 2003) and pulling by action of polymers (Fygenson *et al.*, 1997). One interesting example of how nanotubes can be pulled from a giant vesicle is the use of molecular motors associated with microtubules (Roux *et al.*, 2002). Roux et al. attached kinesin motor proteins to giant vesicles, using small polystyrene beads as linkers between the kinesin molecules and the lipid bilayer membrane of the vesicle. When these vesicles were incubated with microtubules and ATP, membrane tubes or even complex networks of membrane tubes were formed. As mentioned, it is believed that the endomembrane system, for example, the endoplasmic reticulum and the Golgi, as well as the recently found complex tubular networks, are maintained by the cytoskeleton, the membranes being attached to and moved about using molecular motor proteins, such as kinesin.

The nanotubes form when a point load is applied to the vesicle or cell and the mechanism behind this transformation is quite complex (Heinrich *et al.*, 1999), involving first-order shape transitions (Derényi *et al.*, 2002). As a simplification one can say that the system wants to reduce the surface free energy and it does so by optimizing the surface-to-volume ratio. The equilibrium force required for pulling a tube is

$$f_0 = 2\pi\sqrt{2\sigma k_c} \qquad \text{(Equation 14)}$$

where σ is the membrane tension and k_c is the bending modulus of the lipid membrane. Interestingly, it has been found that the force required for forming a tube from a vesicle has to overcome a barrier and the force is therefore somewhat higher than expected (Derényi *et al.*, 2002).

The radius of the lipid nanotubes will be decided by the force balance between the lateral membrane tension and the curvature energy (Evans and Yeung, 1994), according to

$$r_t^2 = \frac{k_c}{2\sigma} \qquad \text{(Equation 15)}$$

The tension wants to reduce the radius, while the curvature wants to increase it. A high degree of tension, for example, through a high adhesion, will therefore result in nanotubes of small diameters. As mentioned, the membrane is much more easily bent than stretched, which leads to the fact that the diameters of the nanotubes often are in the nanometer range.

The dynamics of the pulling of nanotubes, or tethers, from vesicles have also been studied (Evans and Yeung, 1994; Rossier *et al.*, 2003). Evans and Yeung studied slow extrusion of relatively short tubes and were able to measure, for example, the intermonolayer friction. In this case it was assumed that the radius was uniform across the tube, as well as the membrane tension, σ. Rossier *et al.* studied extrusion of relatively long tubes and at higher velocities. The friction force to the surrounding water will in this case become important and it is assumed that a gradient of surface tension can be generated across the length of the nanotube. Since the tension in the tube is related to the radius, this will mean that the tube radius of the nanotube is not uniform. Tube retractions experiments, as well as theoretical

predictions, were performed in order to explain how the tube would be retracted if not having uniform distributions of the tube radius and surface membrane tension (Rossier *et al.*, 2003).

Dynamical properties of the lipid bilayer membrane

Shear surface viscosity

Since the bilayer behaves as a two-dimensional fluid, the lipid molecules constituting the lipid bilayer have a high degree of both translational and rotational mobility. However, the lipid-lipid interactions give rise to a viscosity of the bilayer, called shear surface viscosity, η_s. The shear surface viscosity reflects the molecular interactions of the lipids constituting the membrane and it thereby differ for different membranes.

Measurements have been performed on lipid bilayers and lipid monolayers, as well as cell membranes, and the values obtained for η_s fall in the range 10^{-7} - 10^{-3} g/s (Meiselman *et al.*, 1978). There are several techniques for measuring the shear surface viscosity, one being the study of the lateral diffusion of lipids or for example fluorescent membrane probes inserted into the membrane. Estimates of η_s for biological membranes that are based on measurements of lateral diffusion give $\eta_s = 10^{-6}$ to 10^{-5} g/s (Meiselman *et al.*, 1978; Velikov *et al.*, 1997; Tocanne *et al.*, 1994; Galla *et al.*, 1979). Recently, methods for measuring the shear viscosity of giant unilamellar vesicles have been developed, such as tether pulling, falling ball viscometry or optical dynamometry (Velikov *et al.*, 1997; Dimova *et al.*, 2002). For example, in the falling ball viscometry method, a small solid particle, or bead (a few microns in size), is attached to the membrane of a spherical GUV. The bead is brought to the top of the vesicle by optical manipulation and then released. The motion of the bead under gravity is monitored and the friction experienced by the bead moving tangentially along the surface is determined.

The surface viscosity is, as the name implies, a two-dimensional parameter, acting in the plane of the membrane; however, the surface viscosity can be converted to a three-dimensional viscosity

$$\eta_3 = \eta_s / h \qquad \text{(Equation 16)}$$

where h is the thickness of the membrane. The large values of the surface viscosity are found in experiments that deform extended portions of a cell membrane. Such high values probably reflect additional forces from macromolecular structures, such as interactions between cytoskeleton and membrane. The lower end, $\eta_s = 10^{-7}$ g/s, is the measured microviscosity of the hydrocarbon interior of a bilayer membrane and corresponds to $\eta_3 = 1$ Poise= 1 g/s*cm, the viscosity of olive oil.

Intermonolayer friction

The assumption that the membrane can be treated as a thin elastic shell could only be true if the monolayers of the bilayer were chemically bonded together. In reality, the two monolayers are only weakly adhered and are free to move relative to each other. If the shape of the bilayer remains constant this relative movement can take place with very low input of local energy. However, if the shape of the bilayer changes rapidly, there will be an additional viscous resistance to lipid flow, besides the shear surface viscosity. This viscous resistance arises because the two monolayers will move relative to each other and a drag between the monolayers will be created. Evans and Yeung (Evans and Yeung, 1994) called this a "hidden dynamic coupling". The frictional interactions between monolayer leaflets as they slip past

each other have been studied by pulling tethers out of macroscopic-sized vesicles (Merkel *et al.*, 1989; Evans *et al.*, 1992; Evans and Yeung, 1994; Raphael and Waugh, 1996).

Rapid extraction of bilayer through the 1000-fold increase in curvature going from the vesicle geometry to a tube, creates an enormous relative motion between layers near the vesicle-tube junction (molecules in opposite layers slip past at rates approaching 10^5/s) and, in turn, an interlayer drag which is proposed to dominates the extraction force (Fig. 18) (Evans and Yeung, 1994).

When normalized to a monolayer thickness of $h = 2$ nm, η_r was found to be between $4*10^{-7}$ g/s (Evans and Yeung, 1994) and $1{,}8*10^{-6}$ g/s (Raphael and Waugh, 1996). However, in some cases viscosity has been measured to be significantly larger; pulling microtethers out of neuronal growth cone membranes yielded $\eta = 2*10^{-4}$ g/s (Dai and Sheetz, 1995).

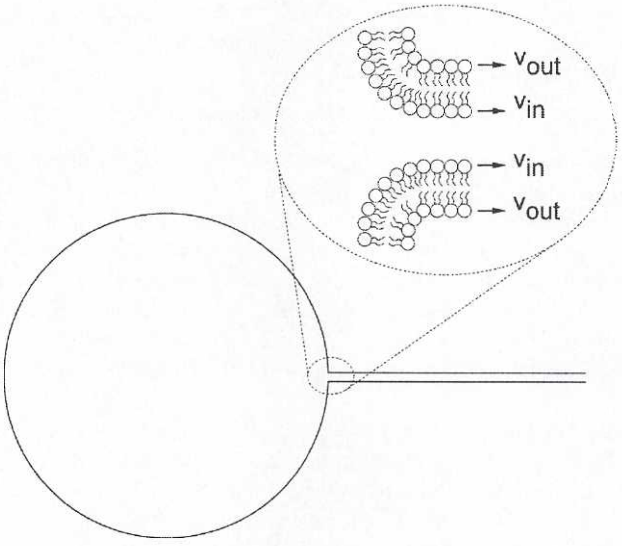


Figure 18. Schematic illustrating the difference in velocity between the two monolayers during extraction of a nanotube from a vesicle. The vesicle, having sizes in the range of tens of micrometers up to the size of hundreds of micrometers, leads to to fact that the membranes of the vesicles can be viewed as almost planar compared to the small nanotubes, which have sizes in the nanometer range. The monolayers in the nanotube will have different areas, the outer monolayer will have a larger area than the inner monolayer. Therefore, when a lipid nanotube is pulled out of a giant vesicle, there will be a difference in the velocities of the different monolayers flowing through the vesicle-nanotube junction. The outer monlayer will have to move with a higher velocity in order to compensate for the larger area in the nanotube. This difference in velocity will create an intermonolayer friction.

Electrical properties of membranes, electroporation and electrofusion

As mentioned, the lipid bilayer membrane is impermeable for charged species such as ions, due to the hydrocarbon chain interior. When two aqueous and conducting solutions surround the membrane, it can store electrical charge on each side, that is, act as a capacitor, when subjected to an electrical field. The capacitance (C) depends on the dielectric thickness of the membrane (h_e), the area of the membrane (A) and the dielectric constant (ε) of the material in between (hydrocarbon, $\varepsilon = 2$), according to

$$C = \frac{\varepsilon \varepsilon_0 A}{h_e} \qquad \text{(Equation 17)}$$

where ε_0 is the permittivity in vacuum with a value of $8{,}85*10^{-12}$ F/m. (The dielectric constant is a measure of the polarizability of the membrane and the degree to which dipoles in the membrane responds to an electric field.)

Electroporation

When an electric field is applied across the membrane, ions in the solution will move towards the electrodes, positive towards the cathode and negative towards the anode, until they are held back by the membrane. The membrane can only be charged up to a certain threshold value, but then it has to discharge the stored electrical energy. It does so by forming pores in the membrane through which current can flow, a phenomenon called electropermeabilization or electroporation (Weaver and Chizmadzhev, 1996). The pores emanating from electroporation is thought to occur at lattice defects in the membrane. It is believed that pores can spontaneously form at defects, but an electric field enhances the probability of pore formation.

The formation of pores is thought to start with the creation of very small hydrophobic pores. The lipid molecules of the membrane has the same orientation as in the relaxed bilayer, however, the lipids lining the pore are exposing their hydrophobic tails to the water in the pore. The water will therefore adopt a more ordered structure in the pore. The process in going from a membrane to a hydrophobic pore is reversible and the pore can therefore close again unless energy is put in to hold it open. This hydrophobic pore can evolve into a hydrophilic pore, where the lipids lining the pore orient so that the polar head groups face the pore interior. The conversion between the hydrophobic and hydrophilic pores is thought to occur when a critical radius is reached (Fig. 19).

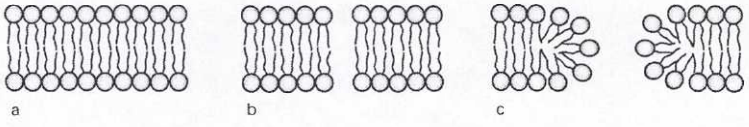


Figure 19. Schematic illustrating the formation of a hydrophobic pore and the evolution of such a pore into a hydrophilic pore. (**a**) An intact membrane. (**b**) A hydrophobic pore with the hydrocarbon chains exposed to the water in the pore. (**c**) A hydrophilic pore where the polar head groups of the lipids face the pore interior, thereby protecting the hydrocarbon chains from the water.

The energy needed for the formation of hydrophilic pores can be calculated from (Glaser *et al.*, 1988.)

$$E_i = 2\pi\gamma.r_p - \pi\sigma.r_p^2 \qquad \text{(Equation 18)}$$

where γ is the edge energy of the pore walls (line tension or one dimensional surface tension), r_p is the radius of the narrowest part of the pore, and σ is the membrane tension. The line tension can be seen as a 1-dimensional membrane tension which arises from the unfavourable bending and packing constraints of the lipids lining the pore. The first term in the equation reflects the edge energy, and if r_p is below γ/σ, this term will dominate and the pore will reseal again. The second term in the equation shows the energy arising from the membrane tension and if this term dominates, then the pore radius will grow and eventually the membrane will rupture. (Wilhelm *et al.*, 1993.)

Usually, pores are formed very fast, within μs if the electric field is above the critical potential needed for pore formation. As soon as the field is turned off, the pores will re-seal again, however this process is much slower, often requiring milliseconds to seconds to complete. There have also been reports concerning so-called meta-stable pores were the re-seal times can be even longer, but this has mainly been seen in cells, where defects between the lipid bilayer membrane and membrane proteins can be stabilized and remain open.

Ever since the phenomenon of electroporation was discovered people have wanted to see the pores formed in the membrane and various approaches has been made in order to do this. 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 visualized as "volcano-shaped" openings in the membrane.

The tension of the membrane will be one of the parameters deciding the fate of the pore, as can be seen in the equation above. A high tension in the membrane, for example, induced by a high degree of adhesion can open up the pore (Sandre *et al.*, 1999). It has also been shown that the pores induced by electroporation can be stabilized by tension in the membrane (Zhelev and Needham, 1993). A vesicle was held in a micropipette and by application of an electric field, pores were formed in the membrane. By controlling the membrane tension through the aspiration pressure, the pores could be made to remain open and the interior solution could therefore elute from the vesicle. This was also a way in which the pores could be visualized. By loading the liposomes with media having different refractive index than the outside solution, the pores could be visualized 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 will close rapidly 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). The dynamics and life-times of pores have also been investigated under the influence of addition of detergents or cholesterol to the lipid bilayer system. It was found that cholesterol increased the line tension and thereby reduced the life-time of the pores, while detergents showed the opposite effects with a decrease in line-tension and a concomitant increase in the pore life-time (Karatekin *et al.*, 2003).

The mechanical stress might also come from the electric field across the membrane, a phenomenon called electro-compressive mechanical stress (Needham and Hochmuth, 1989). The electrocompressive force acts normal to the plane of the membrane and leads to a decrease in the thickness of the bilayer. This force is proportional to the voltage drop across the membrane, V, and therefore to the strength of the applied electric field

$$\sigma_e = \frac{1}{2}\varepsilon_r\varepsilon_0\left(\frac{V}{h_e}\right)^2 \qquad \text{(Equation 19)}$$

where h_e is the length of the hydrophobic part of the lipid bilayer membrane. Taking this force into account, the differential overall mechanical work, dW, done on the lipid membrane is the sum of the electrocompressive stress, σ_e, and the isotropic membrane tension, which is controlled by the mechanical strain applied to the membrane

$$dW = \left[\sigma + \frac{1}{2}\varepsilon_r\varepsilon_0\left(\frac{V}{h_e}\right)^2 h\right]dA \qquad \text{(Equation 20)}$$

where h is the overall thickness and A is the surface of the lipid bilayer membrane.
In one of the techniques for creation of networks described later in this thesis and in paper II, both mechanical and electrical strain is used to insert the tip of a micropipette into a vesicle, a technique which can be explained by the theory that mechanical stress can reduce the transmembrane potential needed for permeabilization of the membrane.

In our research group, a minityrized electroporation set-up has been developed, making it possible to electroporate a single cell or a single vesicle (Lundqvist *et al.*, 1998; Ryttsén *et al.*, 2000; Åberg *et al.*, 2001). Carbon fiber microelectrodes, having diameters of 5 μm, controlled by micromanipulators, were used to create a highly localized electric field across the cell or vesicle of interest, without affecting the rest of the solution (Fig. 20). It was shown that this technique, for example, could be used to transfect living cells with DNA-plasmids or use the technique to initiate reactions inside vesicles.

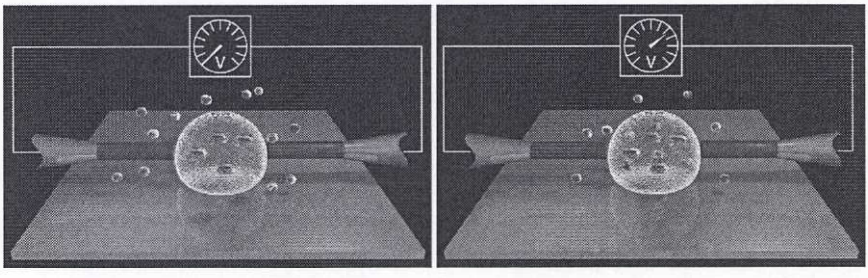


Figure 20. Schematic three-dimensional drawings showing the phenomenon of electroporation using a miniaturized technique for electroporating single cells and single vesicles. (**left**) The carbon fiber microelectrodes were place next to the cell or vesicle of interest using micromanipulators. (**right**) By applying an electric field across the electrodes, pores were formed in the membrane, whereby substances in the surrounding solution could penetrate the membrane and enter the interior of the cell or vesicle.

Electrofusion

If two membranes in close contact are exposed to an electric field, they can undergo fusion. There are different theories of the electrofusion phenomena, for example, the pore-induced fusion model. The pore-induced fusion theory has its foundation in the electroporation theory. When an electric field is placed across the two membranes, hydrophobic and hydrophilic pores are formed. When these pores in the two membranes are in close contact it might be energetically favourable to let the two membranes fuse instead of resealing the pores. Hydrophobic pores in the individual membranes can, for example, form a hydrophilic bridge

between the membranes instead of evolving into hydrophilic pores (Zimmermann, 1982). This theory also suggests that small vesicles which have an inside-out configuration might form in the fusion region, since several fusion bridges can be developed during the process (Fig. 20). The technique of membrane fusion is used in this thesis when creating closed or fully connected networks (Karlsson, M. *et al.*, 2002), a method which is described later.

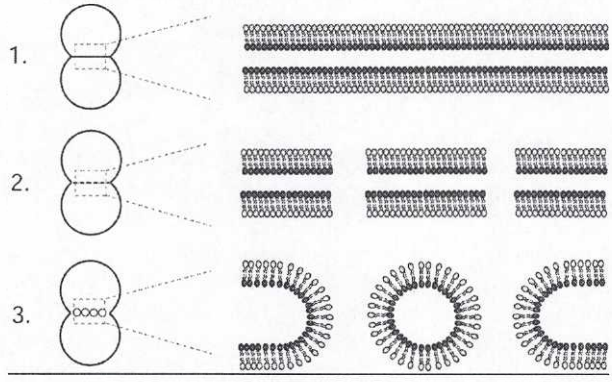


Figure 21. Schematic illustrating the pore-formation mechanism of electric-field induced membrane fusion. In the first stage the two membranes are brought into close contact by dielectrophoresis. In the second stage, electrical break-down leads to pore formation within the membrane contact zone. The third stage illustrates that the pores in the membrane can form hydrophilic bridges between the two membranes. This theory also predicts the formation of vesicles during the membrane fusion.

In our research group, a miniatyrized version for the electrofusion technique was developed (Chiu *et al.*, 1999a; Strömberg *et al.*, 2000). As in the miniatyrized electroporation set-up, the electrofusion was performed by using micromanipulator-controlled carbon fiber microelectrodes (Fig. 22). By placing the two species that were to be fused next to each other, using micromanipulation techniques, and then applying an electric field across them, using the carbon fiber microelectrodes, fusion was accomplished.

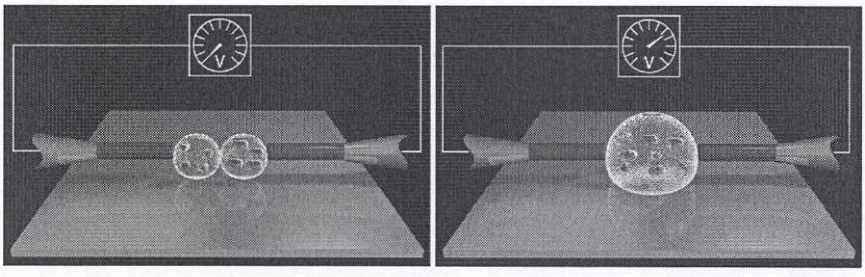


Figure 22. Schematic three-dimensional drawings showing the phenomenon of electrofusion using a miniaturized technique for fusing pairs of cells or pairs of vesicles together. (**left**) The two cells or vesicles that were to be fused, were placed next to each other using micromanipulation techniques. The carbon fiber microelectrodes were placed on opposite sides of the pair to be fused. (**right**) By applying an electric field across the pair, electrofusion allowed them to merge into a single cell or vesicle.

Formation of networks of vesicles and lipid bilayer nanotubes using forced shape transformations of vesicles

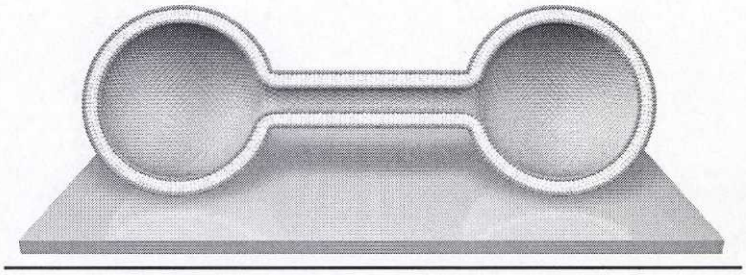


Figure 23. Schematic illustrating the creation of two vesicles interconnected by a lipid membrane nanotube.

In this thesis, networks of vesicles and lipid bilayer membrane nanotubes have been constructed using micromanipulation techniques to induce forced shape transformations of vesicles. Since the system is constructed from a continuous lipid bilayer, the shape having the least energy is a sphere. However, by attaching the nanotube-conjugated vesicles onto a surface, the spherical configuration cannot be obtained. The adhesion energy of the individual vesicles in the system keeps the vesicles apart and compensates for the cost in bending energy of the tubes. The shape of the system will instead be determined by a balance between bending, tension and adhesion energy, which favours a system of near-spherical surface-adhered vesicles connected by thin tubes.

Both multilamellar vesicles (MLV) formed by a rotaevaporative technique (Moscho *et al.*, 1996) or giant unilamellar vesicles (GUV) formed by a dehydration/rehydration technique (Karlsson *et al.*, 2000) were used as starting material. The vesicles had diameters in the range of 5 – 100 μm, which were attached to the underlying glass substrate. The connecting membrane nanotubes were 50 – 200 nm in diameter with lengths of 10 – 50 μm, but they could be made to be much longer. Networks of many containers with connecting nanotubes have been constructed with control of container-size, connectivity between containers, nanotube length and angle between nanotube connections. The complexity of the networks could be increased by forming three-way nanotube junctions or by fusing vesicles of open-ended networks together, creating closed networks or even fully connected networks.

Formation of networks of vesicles and lipid membrane nanotubes

Experimental set-up

We build networks of lipid membrane nanotubes using three different techniques of micromanipulation. Due to the small-scale of the network systems, having length-scales in the 100 μm range, all experiments were performed under a microscope (Fig. 24). The microscope was inverted, that is, the microscope objective (MO) was below the coverslip glass on which the networks were produced. When using the mechanical fission technique, two carbon fibers (CF) were controlled by micromanipulators (MM) and for the microelectoinjection technique that was used in the micropipette assisted technique, one of the carbon fibers was replaced by a microinjection pipette (MIP). The microinjection pipette was in this case connected to a pressurized-air driven microinjector (MI) and the voltage pulses that were used were applied by the use of a voltage generator (VG). For imaging, either Nomarski or fluorescence, the microscope objective (MO) collected the images which

were detected by a CCD-camera. For fluorescence imaging, usually a 488 nm laser light emitted from an Ar^+-laser was sent via a mirror (MR), a spinning disc (SD), a focusing lens, and a polychroic beam splitter through the objective. The images were digitally enhanced by an Argus-20 unit and then recorded by a digital video (DV). A monitor was used to show the output from the Argus-20. A similar setup was used when the single-nanoparticle detection experiments were performed with an avalanche photodiode detector replacing the CCD camera. In this case, a pinhole was used to filter out the fluorescence arising from other planes than the plane of focus. A lens was also used to focus the collected fluorescence onto the detector.

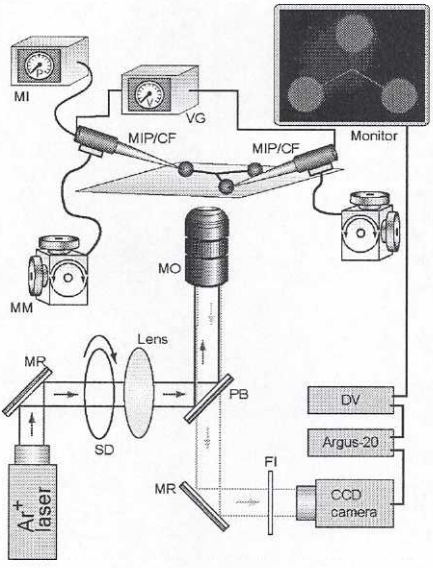


Figure 24. Schematic drawing showing the experimental set-up that was used for performing all the experiments. Shown are the micromanipulators (MM), the carbon fiber microelectrodes (CF), the microinjection pipettes (MIP), the voltage generator (VG), the microinjector (MI), which are all used for creating the networks. Also shown is the imaging tools, that is, the microscope objective (MO), the argon ion laser, the spinning disc (SD), the polychroic beamsplitter (PB), the mirrors (MR), the filter (F1), the CCD-camera (charge coupled device camera), the Argus-20 unit, the digital video (DV) and the monitor.

The mechanical fission technique

The mechanical fission technique described in paper I and V is the first technique that was developed for the construction of networks (Karlsson, A. *et al.*, 2001; Karlsson, A. *et al.*, 2003). The starting vesicle was usually 5-30 μm in diameter and a carbon fiber, controlled by a high-graduation micromanipulator, was used to divide the vesicle into two (Fig. 25). The carbon fiber was placed on the top of the vesicle in the desired position and was then translated axially in the z-direction until it touched the coverslip glass, whereby the vesicle was divided (Fig. 25 a-c). By placing the carbon fiber at the equator of the vesicle the result of the division would be two equally sized daughter vesicles, but it was also possible to divide it asymmetrically to create differently sized daughter vesicles. After division, the two vesicles were separated, again using the micromanipulator-controlled carbon fiber, but they remained connected to each other by a thin lipid tube, a bilayer membrane nanotube (Fig. 25 d). By further divisions of the vesicles and subsequent micromanipulation, networks of several vesicles connected by nanotubes could be created. This technique was mainly used

for multilamellar vesicles. Unilamellar vesicles could be divided once or twice, but then the membrane surface tension became too high for further division. The technique allowed for networks to be created with control of container size, angle between nanotube connections and length of connecting nanotubes. The precision with which daughter vesicles could be created was +/- 1 μm for a 10 μm diameter vesicle and the positional precision of the vesicles in the networks was within 5 μm. The efficiency of creating nanotube connections between separated daughter vesicles using the mechanical fission technique was close to 100%.

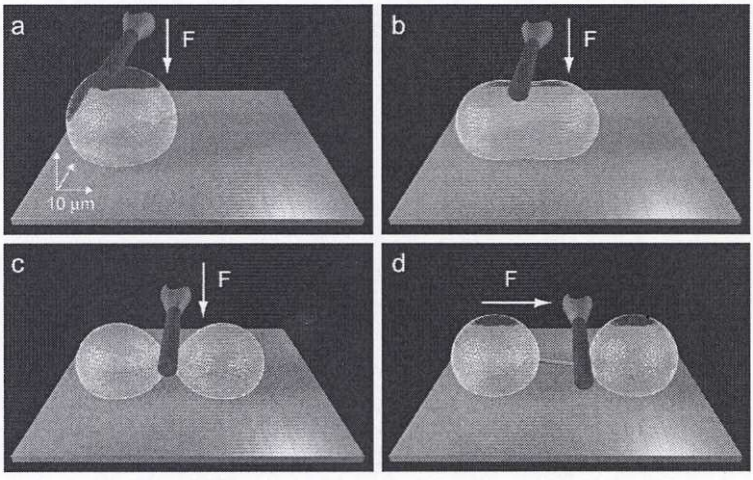


Figure 25. Schematic three-dimensional illustration of how the mechanical fission technique is used to create networks of vesicles and nanotubes. (**a**) The carbon fiber is placed at the equator of the vesicle. (**b**) By pushing the carbon fiber down towards the underlying surface, the vesicle was deformed and started to divide. F represents a force. (**c**) The carbon fiber was pushed down until it touched the surface and the vesicle was divided into two, however a small nanotube still connected the two vesicles. (**d**) With the help of the micromanipulator-controlled carbon fiber, the vesicles could be separated and a network of two vesicles interconnected by a nanotube was formed.

The micropipette-assisted technique

The micropipette-assisted technique described in paper II and V was developed for constructing networks of unilamellar vesicles (Karlsson, M. *et al.*, 2001, Karlsson, A. *et al.*, 2003). The starting vesicle was often a unilamellar vesicle (GUV) connected to a multilamellar vesicle (MLV). The MLV worked as a lipid reservoir which could feed lipid material into the unilamellar system. A micromanipulator-controlled borosilicate-glass micropipette was back-filled with aqueous buffer solution and mounted on an electroinjection system (Karlsson et al., 2000). Using the micromanipulator, the tip of the micropipette was pushed against the membrane of the GUV at the same time as a carbon fiber electrode was placed on the opposite side of the vesice (Fig. 26 a). The micropipette was inserted into the unilamellar vesicle by the use of an electroinjection technique, in which a transient electric field was placed over the vesicle at the same time as the tip was mechanically pushed through the membrane using the micromanipulator. The field-strengths that was applied over the vesicle was 10-40 V/cm and the duration in time was 1-4 ms. When the tip had penetrated the membrane, it was allowed to reseal around the pipette tip (Fig. 26 b). The pipette-tip was then pulled out of the membrane, whereupon a lipid membrane nanotube was formed (Fig. 26 c). This tube could be made to be very long, up to hundreds of micrometers. By injecting aqueous buffer into the nanotube through the pipette tip using a pressurized-air-driven

microinjector, a new vesicle was formed at the tip and expanded, taking lipid material from the mother vesicle, originally coming from the MLV (not shown in the figure) (Fig. 26 d). By controlling the volume injected into the small daughter vesicle, the size could be controlled, typically vesicles having diameters of 5 – 15 μm was used. As the new daughter vesicle had reached a desired size it was allowed to attach to the surface again using the micromanipulators to position the vesicle (Fig. 26 e-f). As mentioned, the starting GUV most often had a multilamellar vesicle (MLV) attached to it, to ensure that enough lipid membrane material existed in the system. The method could also be used for solitary GUVs, but only one or two daughter vesicles could be formed, then the membrane tension became too high for further growth of the network. When using the GUV-MLVs as starting vesicles, networks having many containers and interconnecting nanotubes were possible to create.

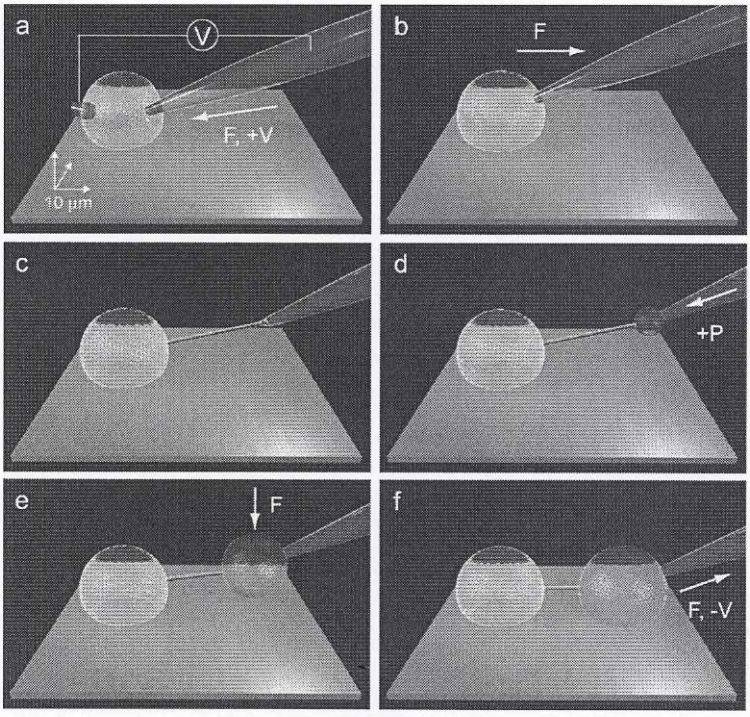


Figure 26. Schematic three-dimensional illustration showing how the micropipette-assisted technique is used to create networks of vesicles and nanotubes. (**a**) A micromanipulator-controlled micropipette was placed next to a unilamellar vesicle. The micropipette was filled with aqueous buffer solution and also connected to a pressurized-air driven microinjector. A micromanipulator-controlled carbon fiber was placed on the opposite side of the vesicle and then a short electric pulse was applied over the vesicle at the same time as the micropipette was pushed through the membrane. The electric field destabilized the membrane so it was more easily penetrated. F represents a force, V represents the application of an electric field and P represents the application of a pressure to the pipette. (**b**) The lipid membrane was allowed to re-seal around the pipette tip. (**c**) By pulling out the tip from the membrane, a nanotube was created. (**d**) By injection of buffer into the nanotube a daughter vesicle could be created. (**e**) The daughter vesicle grew, taking lipid material from the original vesicle, and when it had reached a desired size it was allowed to adhere to the surface. (**f**) The micropipette could be detached from the new vesicle by applying an electric field at the same time as it was pulled away. A network was now formed. By changing the solution of the micropipette, when creating the new vesicles, the network could be differentiated.

Since the vesicles used for this technique were unilamellar and since the daughter vesicles were created by injecting fluid from the micropipette tip it was very easy to differentiate the containers in a network, even during the creation of the networks. By switching of the injection solution between the formation of the daughter containers, networks could be created where the daughter vesicles had different internal solutions. Besides the use of unilamellar vesicles for creation of networks, the micropipette assisted technique had the advantage of higher control of daughter vesicle sizes and positioning.

The micropipette-writing technique

The micropipette-writing technique (not presented in this thesis) was developed mainly for multilamellar vesicles (Sott *et al.*, 2003). A large multilamellar vesicle (5-50 μm in diameter) was aspirated into the tip of a micropipette using a pressurized-air microinjection system (Fig. 27 a). The micropipette was controlled by a micromanipulator and was positioned near the surface were the first network vesicle was to be attached.

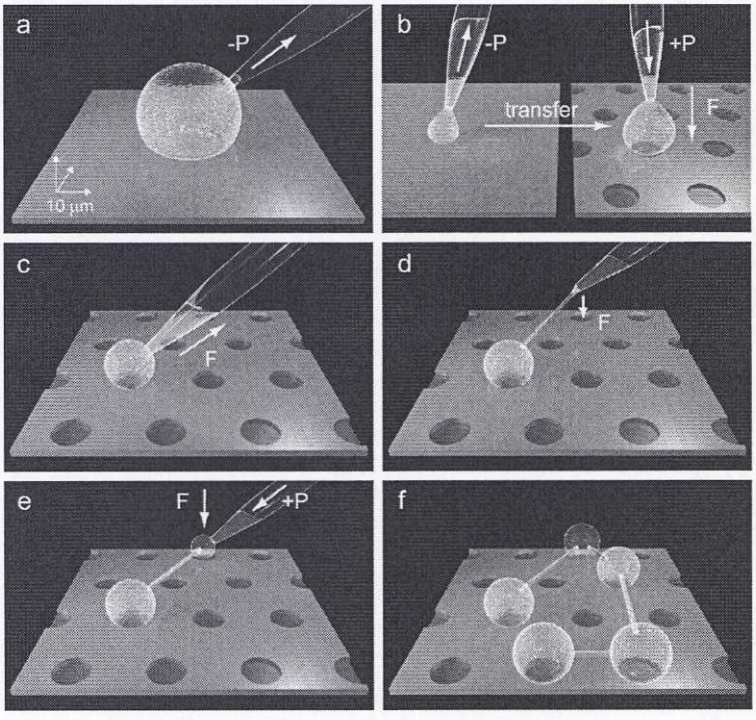


Figure 27. Schematic three-dimensional illustration showing how the pipette-writing technique is used to create networks of vesicles and nanotubes. (**a**) A large multilamellar vesicle was aspirated into the tip of a micropipette by applying a negative suction pressure. (**b**) The technique could be used both on untreated surfaces and on surfaces which had been modified. Here the technique is illustrated by having a modified surface on which spots are created, were the vesicle can be attached. Outside the spots, the vesicles have a very low degree of adhesion. By placing the micromanipulator-controlled pipette next to a spot and by applying a positive ejecting pressure to the pipette, a portion of the aspirated vesicle was ejected out from the tip. (**c**) The ejected portion was allowed to attach to the spot and the applied pressure was stopped. (**d**) The micromanipulator was then used to move the pipette to the next spot. The ejected portion stayed attached to the surface and was linked to the aspirated portion by a nanotube. (**e**) The procedure was repeated by ejecting another portion of the aspirated vesicle to the next spot. (**f**) After several repetitions, a network could be formed.

The aspiration pressure could be regulated both to negative pressure (suction) and positive pressure (ejection). A small portion of the multilamellar vesicle was pushed out of the pipette and this portion was allowed to attach to the surface (Fig. 27 b). The tip of the pipette was moved and the portion stayed on the surface but remained connected to the vesicle in the pipette by a nanotube (Fig. 27 c-d). The micromanipulator-controlled micropipette was moved to the next location where the second vesicle of the network was to be attached. Again, a portion of the MLV was pushed out of the pipette and was allowed to attach to the surface (Fig. 27 e). The procedure was repeated over and over, thereby creating a network (Fig. 27 f). This technique has advantages over the first technique, it was easier and faster to use, and it was realized that it should be easy to make the technique automated. However, it cannot be used for unilamellar vesicles, due to the lack of surplus membrane material. Using this technique it was possible to create networks of up to 25 vesicle containers and compared to the first mechanical fission technique it had the advantage of greater control of daughter vesicle size and positional precision. This technique was also used in conjunction with patterned surfaces, in which specific spots were created on the surface and by chemically modifying the surface, vesicle adhesion was only possible to occur on these spots. The spots were made of gold, onto which a biotin molecule was attached. NeutrAvidin binds hard to biotin and first the biotinylated gold spots were covered with it. The multilamellar vesicle was doped with a biotinylated lipid which then bound to the gold-biotin-NeutrAvidin surface. In order to hinder unwanted adhesion to regions outside the spots, the rest of the surface was covered with a phosphatidylcholine bilayer membrane.

Electrofusion and creation of closed and/or fully connected networks

All of the techniques for creation of vesicle and nanotube networks will lead to the generation of open-ended linear or branched networks. In order to create closed networks or even fully connected networks, where all vesicle containers are linked to all other vesicles by a nanotube, an additional technique had to be used, namely the electrofusion technique (Strömberg *et al.*, 2000; Karlsson, M. *et al.*, 2002). When having an open-ended network of say four vesicle containers, it was possible to generate a closed three-container network by fusing the two end-vesicles together (Fig. 28) (Karlsson, M. *et al.*, 2002). The two end-vesicles were placed next to each other and an electric field was placed across them (Fig. 28 d). The electric field created pores in the membranes and fusion could take place. The electric field could be generated by using two carbon fiber microelectrodes or one carbon fiber and one micropipette electrode, the same tools used for the micropipette-assisted network formation technique. The electric field strengths that was applied was in the range of 40 – 80 V/cm with durations of 1 – 4 ms. Not only could this technique be used for creating closed networks, it was also possible to link the network to external membrane structures such as solitary vesicles or even biological cells. To demonstrate the capability of linking a cell to a vesicle-nanotube network, a nanotube was fused to an adherent NG108-15 cell (Karlsson, M. *et al.*, 2002). Through the creation of these biohybrid networks many interesting possibilities arise. The biohybrid networks will be integrated with biological functions, giving them, for example, a sensing function. It might also be possible to study cells and cellular processes using the biohybrid networks.

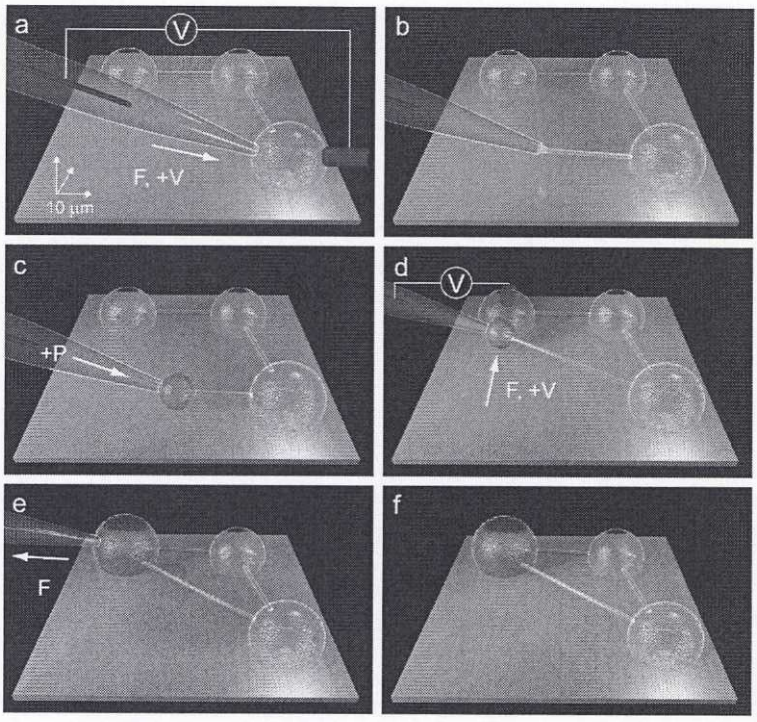


Figure 28. Schematic three-dimensional drawings showing how the electrofusion technique can be used for creation of closed network structures. (**a**) A linear, open-ended network was created by using the micropipette-assisted technique (in this case a linear network of three vesicles was used as the starting network). (**b**) By using the electroinjection technique, the pipette tip was inserted into one of the end-vesicles and a lipid nanotube was pulled out. (**c**) By injecting buffer into the nanotube orifice, a new vesicle could be created. (**d**) The micromanipulator was used to position this vesicle next to the other end-vesicle by which also a carbon fiber microelectrode was placed. An electric field was placed across the pipette-attached vesicle and the end-vesicle, whereby electrofusion merged these two vesicles into one. (**e**) The pipette was pulled away and any nanotube connecting the pipette to the network could be removed by applying an electric field. (**f**) A three container closed network was formed.

Network design, self-organizing networks and three-way junctions

As mentioned, the balance between bending, tension and adhesion energies will favour a system of near-spherical vesicles attached to the surface, interconnected by thin tubes. From geometrical considerations, the membrane in the tube segment will be at a higher state of energy, compared to the surface-adhered vesicles, due to higher bending energy contribution. The energy of the tube, E_{tube}, can be derived from the Helfrich equation of the elastic energy and when just considering bending energy one obtains

$$E_{tube} = \pi k_c \frac{L_t}{R_t} \qquad \text{(Equation 21)}$$

where k_c is the bending modulus, L_t is the length of the tube and R_t is the radius of the tube.

When also taking the stretching into account

$$E_{tube} = \left(\frac{k_c}{2R_t^2} + \sigma \right) 2\pi R_t L_t \qquad \text{(Equation 22)}$$

where σ is the membrane tension.

The energy is thus dependent on the dimensions of the nanotube and as the system of vesicles and interconnecting nanotubes will want to reduce the total elastic energy and it can do so by having as short tubes as possible. Since the lipid bilayer membrane behaves like a two-dimensional fluid, the vesicle-nanotube junctions are free to move across the vesicle surface. Therefore, when designing and creating the network, this has to be taken into account, since the nanotubes will always arrange themselves so that they connect the vesicles at the shortest distance possible, a fact which also holds for the third dimension. This pathway minimization phenomenon can also be used during the creation of the networks. It is possible to create a nanotube connected and pipette-attached vesicle at one location in the network and then translate the nanotube junction across the system to another place, before attaching the vesicle to the surface (Karlsson, M. *et al.*, 2001).

The tubes can also connect to each other, the tube-tube junction also being able to move over the lipid bilayer surfaces. The tube-tube connection will form a three-way nanotube junction and by the same energy minimization principles described above, the system wants to have as short tubes as possible (Karlsson, M. *et al.*, 2002). It can be found that the angle between the nanotubes in the three-way junction will be 120°. Interestingly, the problem of finding a point in a triangle (provided no angle exceeds 120°) that minimizes the sum of the distances to the vertices was investigated in the first half of the 17th century (Spain, 1996). The center of the vesicles would correspond to the vertices of the triangle and the junction point to the Fermat point.

When trying to create a four-way junction, this always led to two individual 120° three-way junctions, the four-way junction only existing as an unstable intermediate (Fig. 29).

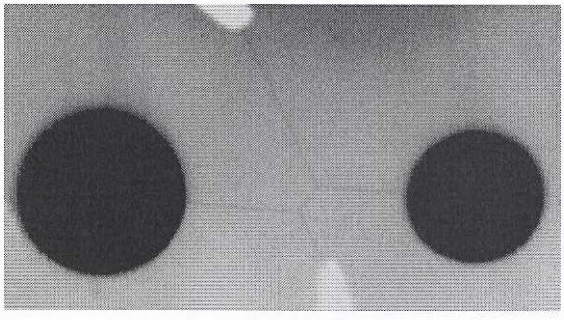


Figure 29. Negative fluorescence micrograph showing two three-way nanotube junctions. The black circular areas are vesicles and the white rods in the upper and lower part of the picture are two micromanipulator-controlled carbon fibers (5 μm in diameter). The carbon fibers were used to move the two three-way junctions towards each other in order to create a four-way junction. However, the four-way junction existed only as an unstable intermediate structure, the system rapidly crossed over to having two three-way junctions instead.

Three-way nanotube junctions having angles differing from 120° could be created by attaching the tubes to the surface, although the attachment points showed some instability (Karlsson *et al.*, 2003).

The highly dynamic character can also be used to create self-organizing networks. Firtst, a network is constructed with a specific starting geometry and this network can then be triggered to evolve into another geometry. The concept of self-organizing networks was demonstrated by building networks of vesicles and interconnecting nanotubes that had double nanotube attachment points on several of the vesicle containers. The nanotube attachment points on the vesicle were made to be very close together. The network could then be made to evolve and self-organize into another connecting geometry by allowing two such nanotube connections on one of the vesicles merge together (Derenyi *et al.*, 2002), thereby starting a chain reaction that spread throughout the network (Fig. 30). It is realized that this concept also can be used for creating geometries that might be hard to build by hand, for example complex three-dimensional structures. In this case, a geometry that is possible to build by hand, using the micromanipulation techniques, can first be constructed, whereby the system can be triggered to evolve into the desired geometry (Karlsson, M. *et al.*, 2002).

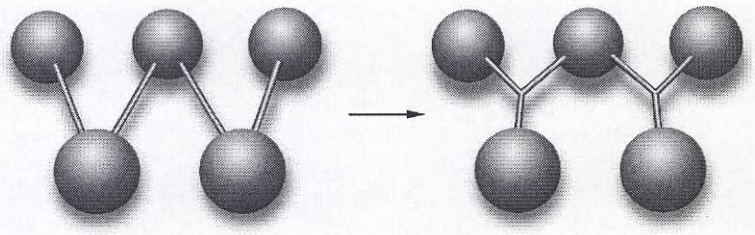


Figure 30. Schematic three-dimensional drawings, illustrating the self-organizing character of the networks. By merging two nanotube connections on the same vesicle, the system could evolve to instead form three-way junctions.

Use of the vesicle-nanotube networks as nanofluidic systems

The construction of unilamellar networks of vesicles and interconnecting nanotubes awakened the desire to use them as nanofluidic systems, where chemical and enzymatic reactions could be performed. However, in order to do this, a means to transport fluids in these small-scale systems had to be invented.

Fluid mechanics

Fluid mechanics, or hydrodynamics, was introduced in the mid 1750's by Leonard Euler (Happel and Brenner, 1983), who tried to explain the motion of liquids, which in some cases can be very difficult. One well-known example of the complex motion of liquids is the phenomenon of turbulence, a non-linear, chaotic motion.

When studying fluid mechanics it is impossible not to encounter the so-called Navier-Stokes equations, which describe the motion of liquids according to

$$\rho\left(\frac{\delta v}{\delta t} + v \bullet \nabla v\right) = -\nabla P + \eta \nabla^2 v \qquad \text{(Equation 23)}$$

The left hand side of the equation shows the product of the fluid density ρ and the acceleration of the fluid, while the right hand side represents forces per unit volume due to a pressure gradient (∇P) and viscosity ($\eta \nabla^2 v$). These equations represent Newtons second law for fluids, which states that the acceleration of a fluid in a certain system will depend on the sum of the forces acting on it.

Another expression you are bound to encounter in fluid mechanics is the Reynolds number, Re. In simple words, the Reynolds number reflects the ratio of the inertial forces and the viscous forces in a fluid system and is defined as

$$\mathrm{Re} = \frac{\rho v l}{\eta} \qquad \text{(Equation 24)}$$

where ρ is the density of the fluid, v is the velocity with which the fluid is moving, l is the length scale of the system and η is the viscosity of the fluid. The Reynolds number can be used to check which flow regimes are possible for a certain system. From the equation it is possible to see that at high Reynolds numbers, the fluid system is dominated by inertia forces. At Re>2000 a very complex flow pattern occurs involving interacting vortices, and this regime is called the turbulent flow regime. If the Reynolds number is low instead, viscous drag forces will dominate and at Re<<1 the flow regime is called laminar flow, which is characterized by a predictable and smooth flow.

Fluids in small-scale systems, microfluidics and nanofluidics

In a conventional macroscale flow system, the phenomenon of turbulence is often desired. If two fluids are to be mixed in order to, for example, start a chemical reaction, the turbulence leads to an efficient mixing due to convection. But what happens when down-scaling a fluid system from the macroscale towards the micro- or even the nanoscale? A clue to this might be to use the Reynolds number relation above. As can be seen in the equation, the Reynolds number depends on the length scale of the system, the smaller the scale, the more will the viscous drag forces dominate. The nanotube channels in our systems have diameters in the 100 nm range, and since we work with aqueous solutions with density, ρ, of 1000 kg/m^3 and viscosity, η, of 0,001 Pa*s at velocities of 50 μm/s, the Reynolds number is quite small, around $5*10^{-6}$, meaning that the fluid system is in the laminar flow regime.

Typical microfluidic systems display Reynolds numbers in the range of 0,01 – 0,5 and they are consequently also under the influence of laminar flows. For a person used to working with a conventional macroscale flow system, this might seem very unpromising, since when a fluid is in the laminar flow regime, the mixing will take place, not by convection, but instead by diffusion. In macroscale systems it would not work to have diffusion as the driving force for mixing, since diffusion is a very slow mixing process when working on the macroscale. This can be realized by examining the relation between the diffusion transport time, t, the length scale of the system, l_D, and the diffusion coefficient for the molecule that diffuses, D_0,

$$t = \frac{l_D^{\,2}}{D_0} \qquad \text{(Equation 25)}$$

This means it would take hours to days to achieve mixing in a macroscale system of centimeter scales and the design of conventional flow reaction system is therefore not applicable to microsystems since they work at different flow regimes. Luckily, the micrometer-sized channels in micro-scale system not only lead to the fact that laminar flows dominate, the reduced length scale also makes it possible for diffusion to be an efficient mixing technique.

Yet another relation that one encounters when studying flows and flow behaviour, especially important in microscale systems, is the Peclet number, Pe, which describes the ratio of the time scales for convective and diffusive transport, respectively

$$\mathrm{Pe} = \frac{v l_D}{D_0} \qquad \text{(Equation 26)}$$

where v is the flow velocity, l_D is the length scale of the system and D_0 is the diffusion coefficient. The Peclet number can thus be used to estimate the length, L, of a channel with uniaxial flow, before complete mixing has occurred according to

$$L = \mathrm{Pe} l_D = \frac{v l_D^{\,2}}{D_0} \qquad \text{(Equation 27)}$$

From this, it can be seen that high velocities will give rise to longer channel lengths before mixing is complete, and since one wants to have as small systems as possible, this is a negative drawback. A simple solution would be to reduce the velocity, but this would mean that each analysis would take longer times, which is also not optimal.

In order to enhance the mixing, new designs of the fluidic systems have been created, with purpose to disturb the laminar flow profiles (Strook *et al.*, 2002; Vijayendran *et al.*, 2003).

What happens if the dimensions are reduced even further, towards the nanoscale? New fabrication techniques and materials have made it possible to create nanoscale channels (Tas *et al.*, 2002) and it is realized that when shrinking the size and reducing the volume of the system, the more importance will the surface and the subsequent surface interactions have. The study of fluid flow in nanoscale channels, or nanofluidics, is therefore becoming more and more important (Ramsey *et al.*, 2002; Foquet *et al.*, 2002) Looking at an aqueous solution confined in a small channel, it is first important to know that the size of water is approximately 0,3 nm. It is believed that the water closest to the surface will adopt a more ordered structure with a more restricted rotational and translational

mobility. This ordering and surface association is thought to affect physical parameters such as the viscosity and density of water. The smaller the channel is the higher will the fraction of ordered water be and it is then easily realized that in a channel approaching the size of 10 nm, the ordered water will have a greater effect on how the fluid behaves under flow. Due to the small sizes of the nanoscale fluid flow, even below the optical diffraction limit, it is hard to perform experimental studies of how fluids behave in these nanoscale channels, however some work have been made in this field (Pfahler *et al.*, 1989).

It has been realized that for a confined fluid, classical mechanics does not apply, instead molecular simulations must be used in order to better explain the properties and behaviour of the fluid. Most theoretical work performed on fluids near surfaces has been performed in the field of surface and colloid science, while less has been made on fluid in nanochannels. These theoretical studies have for example investigated the continuum hypothesis, that is, that the water would behave as in bulk solution even when close to a surface. It was found that the continuum approximation breaks down gradually when going down in size, but also that is can be used down to very small dimensions, even down to 10 molecular diameters (Travis *et al.*, 1997; Travis and Gubbins, 2000).

When treating the small-scale systems with continuum mechanics, boundary conditions have to used in conjunction with the general equations, the boundary condition at the wall being of particular importance. The no-slip condition states that the velocity closest to the wall is zero, implying that the fluid closest to the wall is tightly associated with it. Recently, it has been argued that the anomalous behaviour of liquids in small scale systems does not arise due to ordering of the liquid outside the wall. Instead, it is suggested that the so-called no-slip condition is not valid when going down in scale towards the nanometer-range (Yang and Kwok, 2003). Using computer simulations, the no-slip condition has been investigated and as the dimensions decrease, non-zero mean molecular velocities near the boundary wall have been observed (Thompson and Trojan, 1997; Barrat and Bocquet, 1999). Other investigations show contradictory results finding that the no-slip boundary condition actually holds despite the smallness of the confining geometry (Heinbuch and Fisher, 1989; Koplik and Banavar, 1998; Khare and de Pablo, 1997; Travis and Gubbins, 2000). The differences in observations probably reflect the details of the molecular wall-fluid interactions. Non-wetting fluids are more prone to display partial slip, whereas wetting fluids seem to follow the no-slip boundary condition. Of course, more work will have to be performed to elucidate the true behaviour of liquids in dimensions reaching the molecular scale.

It goes without saying, that another factor that will have to be considered when shrinking the dimensions and increasing the surface-to-volume ratio is possible electrostatic interactions between a charged wall and a charged species in the solution. The so-called Debye-length describe the length of electrostatic interactions and depends on the ionic strength of the solution and the potential or the surface charge density of the wall. The Debye length can extend tens of nanometers from the wall, meaning that it could span the entire width of a nanoscale channel.

Transport of fluids

But how can transport of fluids be performed in these small scale systems? The most widely used technique for transporting a fluid in a macroscale system is by generating a pressure difference across the system with the help of mechanical pumps. Conventional pumps have been used in conjunction with microfluidic systems, but the MEMS technology has also provided means to design and construct micropumps, which can be integrated onto the chip (Unger *et al.*, 2000).

However, when down-scaling the fluid system more and more is demanded from the pumps driving the flow, for example, since the volumes of a microfluidic system is so small, even a very small difference in the outflow from the pump might lead to huge differences in the velocity of the fluid.

Other techniques for fluid transport have been developed in order to transport liquids in small scale system, for example, through electrochemistry (Gallordo *et al.*, 1999), electrocapillary pressure (Prins *et al.*, 2001), electrowetting (Beni and Tenan, 1981) and thermocapillary coupling (Burns *et al.*, 1996). However, one of the most important techniques for transporting fluids in a microfluidic system is the generation of an electroosmotic flow (Jorgenson *et al.*, 1981). When an electric field was placed across a thin glass capillary containing an electrolyte, the fluid moved. The explanation for this phenomenon is that when an electrolyte is placed in the capillary, the silica groups of the walls can lose a proton, whereby the surface becomes negatively charged. Positive ions are attracted to this negatively charged surface and a so-called electrical double layer is formed. Upon application of an electric field, the mobile layer of positive ions moves towards the negative cathode and by viscous coupling the fluid contained in the channel will be dragged along the positive layer. This type of motion can be seen as a shear-driven kind of flow, which has a more or less plug-like appearance. This technique has recently been used to perform electrophoretic separations in sub-micrometer channels (Woods *et al.*, 2001).

Interestingly, it has been shown that it is possible to generate a stable fluid flow through micrometer-sized channels by a shear force, without the need for a pressure or a voltage gradient. The phenomenon of shear-driven flows has its foundation in the fact that every fluid has an intrinsic viscosity. It is therefore possible to drag fluid into, through, and out of a channel using the viscosity effect to transmit the applied impulse force to the entire fluid. To show this, a rectangular channel was constructed where the top wall was mobile. When the top wall moved with a velocity v_{wall}, the fluid was dragged along the wall with a linear velocity profile having velocity of $v_a=v_{wall}$ at the top moving wall and $v_a=0$ at the bottom wall by assuming a no-slip condition. (Desmet *et al.*, 2000; Desmet *et al.*, 2002)

In order to reduce the length scale for a fluid flow, without having to reduce the length scale of a channel, the phenomenon of a so-called hydrodynamic focusing has been used. The channel containing the fluid flow, which was to be focused, was designed to merge with two other channels coming from opposite sides. The flows from the three channels merged into one and continued to flow into a fourth, shared channel. The flows were all laminar and the flows coming in from the sides focused the first flow into a very small scale flow, down to widths of 100 nm and less (Knight *et al.*, 1998).

Another interesting method to manipulate fluid flows in microchannels is by modifying the surface properties in certain parts of the channel structures. This lead to a surface-directed liquid flow, in which aqueous solutions were restricted to hydrophilic pathways. However, upon increased pressure, the aqueous flows could be forced through other pathways (Zhao *et al.*, 2001).

Creation of tension-driven lipid flow and concomitant fluid flows across the nanotubes

How could we transport fluid between our vesicles through the thin nanotubes? We started to investigate the possibility of generating a pressure difference across the system and we discovered a very interesting phenomenon. The pressure difference that was to be applied across the nanotube was generated by mechanically deforming one of the vesicles using micromanipulator-controlled carbon fibers. We then noticed that due to the unique material properties of the bilayer membrane, behaving as a two-dimensional liquid, the system responded, not by a change in volume, but instead by a change in surface area. The change in surface area led to the creation of a difference in tension across the system and the phenomenon of tension-driven lipid flow. This had also been observed during construction of the networks, when daughter vesicles grew in size, taking lipid material from the mother vesicle.

Tension-driven lipid flow

In a system of two fluids separated by an interface, the interfacial tension must be constant as required by mechanical equilibrium. However, if there is a difference across or along the surface of a fluid system, this can give rise to a flow (Finkelstein, 1987; Bloom *et al.*, 1991). Interfacial tension-driven flows are generally called Marangoni flows and examples of this flow behaviour are spreading of a film on an air-water interface or wetting of a dry solid substrate by a film (de Gennes, 1985; Probstein, 1994). In a bilayer membrane, forces either chemically or mechanically produced, can generate surface tension gradients (force/area) (Probstein, 1994) which leads to membrane flow. Examples of forces that can induce membrane flow are fluid convection, temperature gradients, electric fields, laser light or mechanical forces.

This means that if the surface membrane tension is increased in a point in the membrane, the lipid membrane system will respond to this perturbation by transporting lipid material from regions of lower tension to the point of higher tension, and instead increase the tension isotropically, that is, over the entire surface.

The tension-driven lipid flow has been investigated by Chizmadzhev *et al.* (Chizmadzhev *et al.*, 1999) among others. Chizmadzhev *et al.* studied lipid flow through fusion pores connecting membranes of different tensions and by assuming a toroidal geometry of the fusion pore they were able to theoretically calculate the expected velocity and area flux of lipid through the fusion pore, driven by a difference in tension between the two connected membranes. Due to the geometry of the toroid and the fact that the bilayer is constituted by two monolayers, they had to take into account that there was a difference in velocity between the two monolayers. This gave rise to an intermonolayer friction factor, creating another viscous resistance to flow, besides the shear surface viscosity.

As mentioned, it has also been hypothesized that tension-driven lipid flows can be used in the intracellular endomembrane system as a means for transporting lipid material and vesicles along tubular structures that extend between the organelles. The proposed mechanism is that there is a difference in surface membrane tension between the membranous structures between which transport is to be achieved (Sciaky *et al.*, 1997; Iglic *et al.*, 2003).

Tension-controlled lipid flow in nanotubes

In order to show the tension driven lipid flow across the nanotubes, a simple system, consisting of two surface-immobilized vesicles interconnected by a lipid nanotube was constructed. Micromanipulation techniques were then used in order to dynamically disturb the energy balance of the system (Karlsson, R. *et al.*, 2002) (Fig. 31).

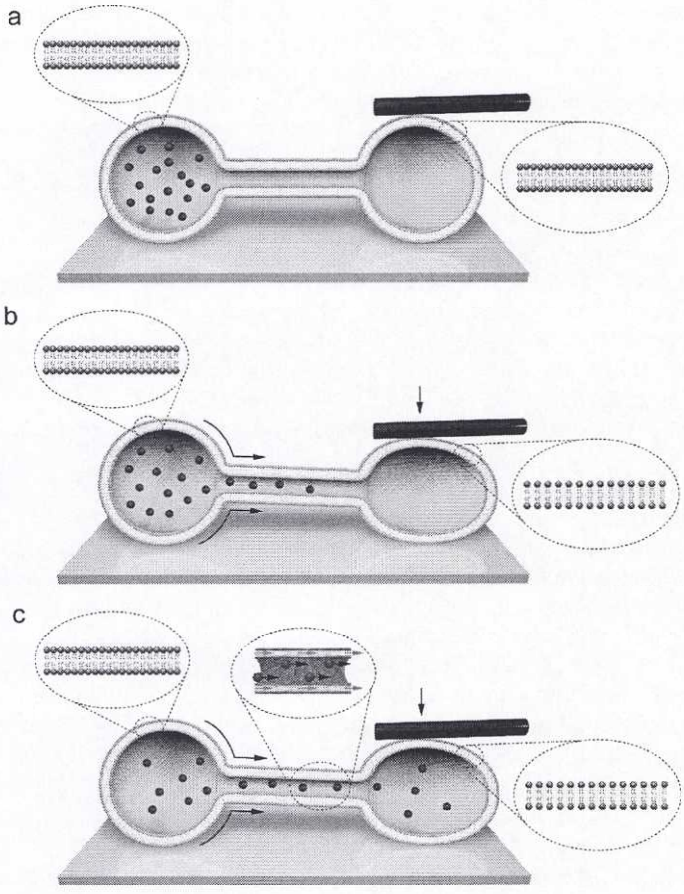


Figure 31. Schematic showing the vesicle-nanotube system used for demonstrating the tension driven lipid flow. The system was composed of two surface-immobilized vesicles interconnected by a lipid nanotube (**a**) A cross-section view of the system, showing that it is composed of a continuous lipid bilayer membrane. When unperturbed, the membrane tension is the same in the two containers (insets) and no lipid flow occurs. One of the vesicle containers are filled with particles (dots). (**b**) The vesicle to the right was deformed by using micromanipulator-controlled carbon fibers, whereby a tension difference was created across the system. This difference in tension induced a flow of lipid bilayer membrane across the nanotube, having a velocity of v_l. (**c**) Fluid and particles contained inside the tube was dragged along this lipid flow, the lipid flow setting an upper limit for the velocity of the fluid, v_a and the velocity of the particles, v_p. Zero or small pressure differences across the nanotube will result in flat (plug-like) flow profiles, meaning that the transport will take place as a solid-body translation of the tube element and the contained fluid and particles.

The geometry of the experimental system can be described as two surface-immobilized vesicles with near-spherical geometry, conjugated by a cylindrical tube. It is important to note (i) that the walls of the nanofluidic systems we are considering are in a fluid state,

consisting of a single, continuous lipid membrane bilayer; that is, they are spheres from a topological point of view, and (ii) that the terminal vesicles in these systems are anchored to the surface. In figure 31a the system is unperturbed and the surface free energy in the two nanotube-interconnected containers is the same. Consequently, there is no lipid flow between the vesicle containers in this instance, aside from diffusive lipid motion. Figure 31b-c illustrates how lipid flow can be driven by a difference in membrane tension along a nanotube by increasing the surface-to-volume ratio using ellipsoidal deformation of one of the vesicles. This results in lipid flow from a source (vesicle in a state of lower membrane tension) to a sink (vesicle in a state of higher membrane tension). The flow of the lipid membrane is represented by a lateral translation of a cylindrical wall, translating with velocity v_l, a quantity that can be experimentally determined. This velocity depends on the balance of the *tension forces work per unit time* and viscous dissipation due to the shear deformation of the lipid flow: $v_l \propto \Delta\sigma/2\eta$, where η is a coefficient of the viscous resistance of the system. Using our experimental set-up, it was impossible to perform an instant deformation and see the resulting flow, because of the very short time needed to achieve steady-state fluxes (Chizmadzhev *et al.*, 1999). Therefore, the shape deformations performed could almost be seen as continuous by the system and as soon as the deformation was stopped, the flow of lipids came to a halt. As expected a high viscous resistance to lipid flow across the nanotube was found, due to the surface viscosity and the intermonolayer friction created in the junction between the nanotube and the vesicle.

Intratubular liquid flow created by a moving lipid wall

When generating the flow of lipid bilayer membrane (constituting the wall of the system) across the nanotube, fluid which was contained inside the nanotube was dragged along the membrane wall, due to the small dimension of the tube and the viscous coupling of the water to the membrane wall. The flow of fluid is driven uniaxially through the nanotube channel by the moving lipid wall. Inertia forces vanish, leaving a fluid velocity, $v_a(r)$, that in the absence of any embedded particles or macromolecules assumes the following inverted Poiseuille profile:

$$v_a = v_l - v_0\left(1-\left(\frac{r}{r_t}\right)^2\right) \qquad \text{(Equation 28)}$$

where

$$v_l - v_0 = v_l - \left(\frac{\Delta p}{L_t}\right)\times\left(\frac{r_t^{\,2}}{4\eta_w}\right) \qquad \text{(Equation 29)}$$

is the fluid velocity on the centerline of the cylinder, v_l, as already mentioned, is the lipid velocity, v_0 is the amount to which the centerline velocity lags v_l. The radial distance from the centerline is denoted by r, and r_t is the tube radius, L_t is the tube length, η_w is the viscosity of water, and Δp is the pressure difference between the cylinder exit and entrance.

The transport is initiated by generating a tension-difference across the system, which responds by changing the surface area at constant volume. Therefore, there will not be any substantial pressure buildup across the nanotube and the dominant driving force for moving fluid inside the nanotube will consequently be the moving lipid wall. For vanishingly small pressure differences there is no relative motion between the fluid and the wall; this situation corresponds conceptually to a "solid body", lateral translation of the cylinder with the entrapped fluid, relative motion being confined to the exterior of the nanotube bordering the

surrounding bulk fluid. For small pressure differences, the flow profile is flat, essentially plug-like. For larger pressure differences, such that $v_l/v_0<1$, the centerline velocity is negative, implying backflow along the center of the tube; however, for nanotubes with $L_t>>r_t$, we expect $v_l/v_0=4\eta_w L_t v_l/(\Delta p r_t^2)$ >>1 under relevant conditions. Another factor that might affect the hydrostatic pressure in the system is the amount of fluid that is transported to the receptacle vesicle along with the lipid flow. From geometrical considerations, it can be seen that the net volume of fluid transported across the nanotube is extremely small compared to the relative amount of membrane material that drives the flow (Goveas *et al.*, 1997).

Tranport and routing of fluids and materials in large networks

Using the technique of membrane tension lipid flow for inducing transport in a network having more than two containers might present difficulties. When increasing the surface tension in one of the containers, lipid membrane material would be transported from all the other containers, across all interconnecting nanotubes. For example, when having a symmetric network of four containers, one central and three vesicles connected to it, an increase in tension in the central container would result in flow from all three periferal containers across the three interconnecting nanotubes (Fig. 32 a). However, in a more complex network it is important that transport can be achieved between two selected containers without affecting the rest of the network. Therefore, a two-point perturbation technique was developed, meaning that both containers between which transport was to be achieved were manipulated (Karlsson *et al.*, 2003a). The membrane tension in one surface-immobilized vesicle (from which material was to be taken) was decreased at the same time as the membrane tension in another surface-immobilized vesicle (to which material was to be transported) was increased. The decrease in membrane tension was achieved by donating membrane material from a donor vesicle to the target vesicle, simply by letting the two vesicles merge together. Simultaneously, the increase in membrane tension was created by shape deformation using carbon fibers as described above. With the two-point perturbation technique, we created a difference in tension between the two target containers that was much larger than for all other connected containers and therefore, the only transport taking place during the manipulation was between these two containers (Fig. 32 b-c). The technique therefore opens up the possibility to use the phenomenon of tension-driven lipid flows in complex networks having many containers with an intricate connectivity going from open-ended networks to fully connected networks or even three-way nanotube junctions.

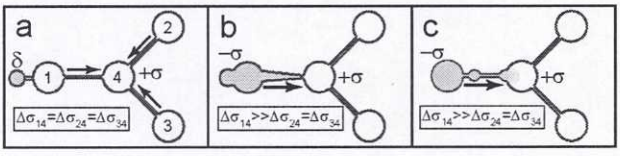


Figure 32. Schematics showing the principles of the two-point perturbation technique used for controlled and directed transport in networks having more than two containers. (**a**) A symmetric network of four containers, one central vesicle being connected to three periferal vesicles by nanotubes. If the membrane tension, σ, is increased in the central vesicle (vesicle 4), lipid and concomitant fluid flow will be created from all peripheral vesicles connected to it, across all nanotubes. (**b – c**) By instead using the two-point perturbation technique, lowering the tension in one vesicle, while simultaneously increasing the tension in another container, transport will mainly take place between these two containers. The decrease in tension was achieved by merging a membrane donor vesicle (denoted by δ in (a)) to the surface-adhered vesicle, while the increase in tension was performed by shape deformation of the other surface-adhered vesicle, using micromanipulator-controlled carbon fibers as mechanical tweezers.

Creation and transport of nanotube-integrated vesicles

The small volumes transported inside the nanotubes can in some applications and studies actually be too small. Therefore, we developed techniques to create small vesicles integrated on the nanotubes conjugating the surface-adhered giant unilamellar vesicles (GUVs) of a network (Karlsson *et al.*, 2003b). It was also shown that these vesicles could be transported along the nanotubes using the principle of creating tension driven lipid flows. The shape of the system, as mentioned, be determined by a balance between bending, tension and adhesion energy, which favours a system of near-spherical surface-adhered vesicles connected by thin tubes. Since the whole system demonstrates a pronounced fluidity and highly dynamic character, the stability of the system can be disturbed by changing the surface tension, σ, which is competing with the curvature of the membrane. In order to create the nanotube-integrated vesicles, we used the two-point perturbation technique, in which the surface tension was decreased in one of the surface-adhered GUVs, while it was increased at another one. As before, the decrease in surface membrane tension was achieved by donating membrane material from a donor vesicle to the target vesicle, by letting the two vesicles merge together (Fig. 33 c). By the sudden addition of excess membrane material, the product vesicle and the remaining nanotube connected to it were momentarily destabilized. The junction between the vesicle and the nanotube was dilated and both membrane material and internal fluid flowed into the unstable deformed nanotube. This can be explained by noticing that bending will dominate over membrane tension, subsequently leading to an increase in diameter of the thin tubes. Typically, tube diameters grew up to 10-fold (from 0,2 μm to 2 μm) at the largest-diameter position of the tube.

After the system has been destabilized, it always strives to minimize its energy and tries to reproduce the system of the two surface-adhered vesicles and the interconnecting nanotube. The spherical shape of the deformed vesicle is re-established and membrane material flows from the deformed nanotube out onto the surface-adhered vesicles. By energy minimization principles, the tube diameter is decreased again, but since internal fluid has entered the deformed nanotube, the volume is too large for the small nanotube to accommodate and undulations or varicosities on the destabilized nanotube therefore instead transform into vesicular structures. This transformation is usually completed within a few seconds. Due to the fact that the system consists of a continuous lipid wall, the small vesicles are thus integrated into the nanotube with both ends open (Fig. 33 d).

Vesicles integrated on lipid membrane nanotubes have been described by others, and the phenomenon is commonly called pearl-chain vesicles. However, these pearling states arise due to other situations than the one described by our system. It has been shown that pearl-chain vesicles can be formed by exciting the tubular structure, using optical tweezers (Bar-Ziv *et al.*, 1994; 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). When using the optical tweezing technique, it was believed that the pearl-chain vesicles formed because of an induced tension in the tubular structures. The optical tweezers trapped lipid membrane material, thereby inducing tension which competed with the bending energy of the tube, vesicles were created on the nanotube (Bar-Ziv *et al.*, 1994; Bar-Ziv *et al.*, 1998). The use of polymers to induce the pearling phenomenon had a different underlying mechanism. The pearling could be explained by area difference elasticity models and spontaneous curvature models (Tsafrir *et al.*, 2001). Interestingly, the vesicle-nanotube structures have been found in cells, a fact which strengthens the desire to investigate the formation and dynamics of these structures (Kosawada *et al.*, 1999). It is believed that the lipid nanotubes might have a role in the transport of the vesicular structures (Iglic *et al.*, 2003) and our system might be used to investigate this phenomenon.

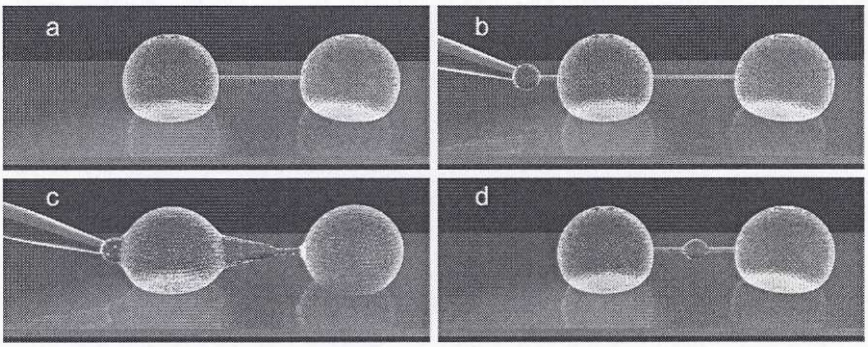


Figure 33. Schematic three-dimensional drawings showing the creation of nanotube-integrated vesicles. (**a**) The starting network consisted of two surface-immobilized vesicles interconnected by a nanotube. (**b**) A new vesicle was created, using the micropipette-assisted technique. (**c**) The pipette-attached vesicle was allowed to re-merge with the vesicle from which it originated. This could be done in two ways, either by letting the pipette-attached vesicle grow or move it towards the surface-adhered vesicle, both leading to a shortening of the nanotube until a structure resembling a fusion pore remained. When the two vesicles re-merged, the energy balance of the system was disturbed, due to the addition of surplus membrane area and the junction of the nanotube connected to the perturbed vesicle was opened up. Both lipid material and fluid entered the deformed nanotube during the destabilization phase. (**d**) Due to energy minimization principles, the system wanted to regain the geometry of surface-immobilized vesicles interconnected by a nanotube and lipid material flowed from the nanotube to the deformed vesicle, which regained the spherical shape. However, the fluid which had entered the nanotube became trapped and instead vesicles integrated onto the nanotube were formed. These vesicles were mobile and could be created, loaded and transported between selected target containers.

Detection of lipid, fluid and particle transport in vesicle-nanotube-networks

When performing the experiments illustrating transport of fluids and particles through the nanotubes, as well as when following enzymatic reactions inside the confined structure of a vesicle or even a nanotube, it is important to realize that the number of species to be detected can be very low. The small scale nanofluidic systems display promising characteristics that make them suitable for working with single molecules and therefore detection techniques of high sensitivity are needed. One example of a detection technique that meets these demands is the confocal laser induced fluorescence.

Fluorescence is a phenomenon occurring in certain molecules when illuminating them with light (Lakowicz, 1999). The phenomenon of fluorescence can be explained by noticing that molecules have different energy levels, which can be of electronic, vibrational or rotational character. If the energy of the light by which the molecule is irradiated matches the energy gap between two such energy levels, absorption of energy can occur, whereby the molecule reaches an excited state. The excited state normally only exist for a very short time, in the nanosecond range, before the molecule returns to the ground state. There are several processes by which the molecule can return to the ground state and lose the surplus excitation energy, for example by generation of heat, however two of these relaxation processes involves the emission of light, namely fluorescence and phosphorescence. All of these processes are best described by the use of a Jablonski diagram (Fig. 34).

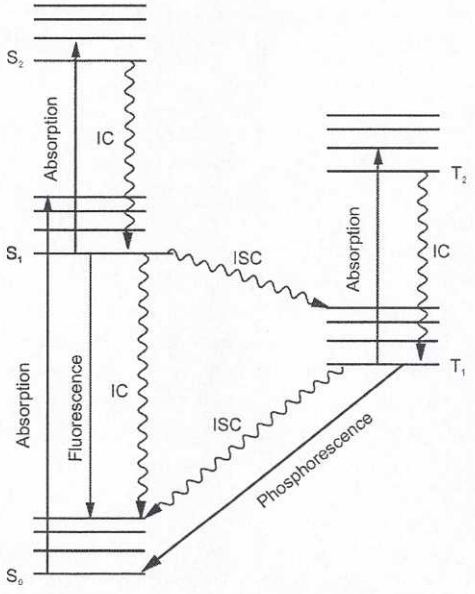


Figure 34. Schematic illustrating the so-called Jablonski diagram. Shown are different electronic states and overlapping vibrational states. The ground electronic state is the S_0-state, the S standing for singlet, meaning that the electrons in this state is paired, having opposite spin directions. Two singlet excited electronic states, S_1 and S_2 are also shown, as well as two triplet electronic states, T_1 and T_2. Triplet means that the electron in this state have the same spin direction as the electron left in the ground state. Absorption can take place between the various energy levels if the energy gap matches the energy which is put into the system, for example, by light of a certain wavelength. Internal conversion (IC) and intersystem crossing (ISC) are two non-radiative processes which allow the system to relax from excited states through the generation of heat. The system can also return from an excited state to the ground state via emission of light. The process of fluorescence takes place when the system returns from a singlet excited state to the ground state. Phosphorescence is a process taking place on a much longer time-scale, because it is spin-forbidden. The electron in the triplet state has to change spin-state

before it returns to the ground state, since two electrons occupying the same orbital must have opposite spin directions.

The ground state is termed S_0, the S standing for a singlet state, meaning that the electrons are paired and have opposite spin states (up and down). The first electronic state is called S_1 and it is between these two levels that the relaxation phenomenon of fluorescence takes place. In order for phosphorescence to take place, the molecule must go through a intersystem crossing whereby the singlet excited state is converted to a triplet excited state, called T_1. Triplet means that the electron in the triplet state has the same spin state as the electron in the ground state. When the molecule tries to relax from this state, this transition is forbidden since it is impossible for two electrons of the same spin to occupy the same energy level. Therefore, the electrons have to change spin state during relaxation and this is why the phenomenon of phosphorescence have longer life-times than fluorescence.

The advantage of using fluorescence as a detection technique lies in the very high sensitivity that can be accomplished compared to other detection techniques. The sensitivity can be explained by the fact that the fluorescence signal is measured against a dark background. This can be compared to the absorption process, in which a beam of light is passed through the solution of interest and where the intensity of the incoming light is compared to the outgoing light. The difference between these intensities is usually small and it is very hard to distinguish very small changes, due to the much higher background noise. Fluorescence can also be very selective, since it is possible to use one wavelength for excitation and then select another wavelength as a detection wavelength.

Confocal laser-induced fluorescence

In normal fluorescence microscopy, the excitation light will also excite molecules that are above or below the plane of focus and the probe volume in which molecules are excited can therefore be relatively large. The signal that creates the image from the plane of focus will therefore be disturbed by the out-of-focus light. The problem was solved by creating the confocal fluorescence microscope, which means that two pinholes were inserted into the optical pathway. One pinhole was inserted before the objective, at the illuminating side and another pinhole was placed at the detector to prevent out-of-focus light to reach the detector. This way, the probe volume is reduced down to the femtoliter range (Hill and de Mello, 2000) with a concomitant reduction of blurring from out-of-focus light. However, it also leads to a reduction in total light intensity, sometimes up to 95%. At low absorbances and irradiances there is a linear relationship between fluorescence intensity I_F and the intensity of the incident light I_0. Therefore, lasers have been used in conjunction with confocal fluorescence microscope set-ups, since lasers have the advantage of higher intensity, among others, compared to lamp-based light sources

The high sensitivity and the small probe volume of the confocal fluorescence microscope have opened up the possibility to use this technique for the study of single molecules (Xie and Trautman, 1997). The first studies were performed in bulk solutions and thereby quite high concentrations were needed, due to the low probability of a molecule entering the small probe volume. However, by confining the molecules in a small volume, for example, in a (microscale) channel or even a vesicle, the concentration can be lower. Our channels are in the nanoscale dimensions and also nanotube junctions can be used as a detection container, having volumes down to the attoliter volume range (10^{-18} L).

When using the confocal fluorescence set-up in conjuction with low concentrations, aiming at the single molecule level, very sensitive detectors have to be used. Examples of detectors used for this purpose is the charge coupled device cameras (CCD-cameras) and avalanche photodiode detectors. The CCD-cameras produce an image of the sample by using

an array of photodiodes. In avalanche photodiodes, a single photon gives rise to a photoelectron which in itself gives rise to new electrons through a cascade effect, enhancing the signal. In our experiments, we most often used a CCD-camera for imaging purposes and for the imaging of single nanoparticles we used the CCD-camera in conjunction with an intensified photon counting unit (or VIM-camera), controlled by an Argus-20 image processor. In order to detect single particles flowing through the nanotubes, we used an avalance photodiode detector.

Creation of a nanofluidic network for detection of single particles under flow

The networks we used for this purpose were designed to facilitate a good starting ground for detection of particles through the nanotubes (Karlsson, A. *et al.*, 2003) (Fig. 35). The criteria that had to be fulfilled were as follows:

1. Due to the small detection volume of the set-up, it was important that the nanotube did not move out of focus during the measurements, for example by convective motions. This was achieved by using as short nanotubes as possible, while still being capable of only having a nanotube segment in the detection probe volume.
2. The nanotube had to be close to the surface in order to help locating it and matching the coordinates of the nanotube in space with the point of detection. This was achieved by having as small vesicles as possible as anchors for the detection nanotube.
3. A continuous flow of lipid and concomitant fluid flow across the nanotubes were needed in order to record the flow of fluorescent particles across the nanotubes using LIF-detection. The continuous flow was created by injecting buffer in a vesicle connected to the vesicle-nanotube-vesicle detection system. The injection of buffer lead to an increase in membrane tension and a subsequent flow of lipids, fluids and particles across the detection system.

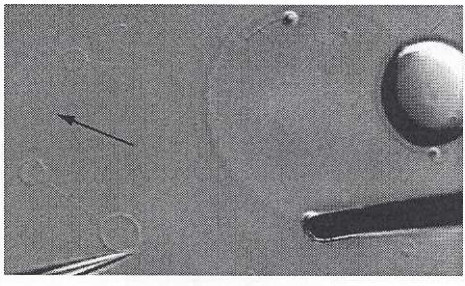


Figure 35. Photomicrograph showing the network design for the detection of single nanoparticles flowing through the nanotube. The first vesicle was used for sample introduction and therefore it was filled with a solution containing the nanoparticles. Two smaller vesicles were used to form anchors for the nanotube in the detection focus (black arrow) and the fourth vesicle was connected to a micropipette. By injecting buffer into the fourth vesicle, a tension-driven lipid flow was created across the vesicle-nanotube system, the lipid originally coming from the multilamellar protrusion of the first vesicle.

Use of vesicle-nanotube networks as cell-models

An important question in the field of the origin of life and evolution concerns the structure of early cells and it is clear that they must have been much simpler than the ones existing today. Scientists are exploring what they call the minimal cell model (Luisi, 2002), that is, what are the minimum components and structural complexity needed to display life?

In this line of thinking, vesicles are the natural starting ground for this research, since many believe that the formation of a vesicle was the first step towards a cell (Segré *et al.*, 2001; Monnard and Deamer, 2002). One example of this research area has been performed on oleate vesicles (Walde *et al.*, 1994). Oleic acid is a fatty acid, which is capable of forming bilayer membrane vesicles in a certain pH range. It was observed that if pre-formed oleate vesicles were exposed to an oleate anhydride, they grew in size. This was explained by the fact that the oleic anhydride, which was insoluble in water, interacted with the oleate vesicles in such a way that the vesicles catalyzed the hydrolyzation of the anhydride to form more oleic acid/oleate molecules. These free oleate molecules could be inserted into the vesicles, thereby increasing their size. When the vesicles had reached a critical size (also having excess membrane area), daughter vesicles could be budded of the mother vesicles, with input energy, such as shaking or stirring the solution and the system thus displayed self-reproduction characteristics. These self-replicating vesicles have also been used to trap enzymes and nucleic acids inside the vesicles, an example being the incorporation of the enzyme Q-beta replicase. Besides the Q-beta replicase, the vesicle also contained an RNA-template and free nucleotides. The Q-beta replicase can form copies of the RNA-template from the free nucleotides. By also using the self-reproducing oleate vesicles as containers for this reaction it was possible to create a system, where the vesicles were reproducing themselves and where the daughter vesicles also got copies of the RNA-strand as well as the Q-beta replicase (Oberholzer *et al.*, 1995).

In this field of research, the term artificial cell has been coined (Pohorille and Deamer, 2002), one example being based on ribozymes (Szostak *et al.*, 2001).

Incorporation of membrane proteins into vesicles

In order to increase the biomimetic character of a vesicle, membrane proteins can be incorporated into the lipid bilayer membrane (Fig. 36) (Eytan, 1982).

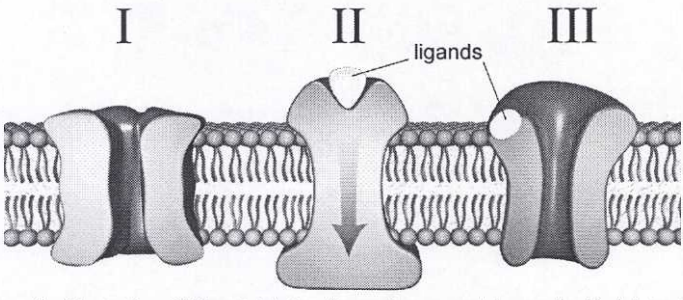


Figure 36. Schematic illustrating different kinds of membrane proteins embedded into the lipid bilayer membrane. **I** A transporter. Transporters carry substances into and out of the cell. **II** A signal receptor. Signal receptors transmit a signal across the membrane through the binding of a ligand. **III** An ion channel. When a ligand is bound to an ion channel it can open up and allow transport of certain ions across the membrane.

The art of reconstituting membrane proteins into lipid bilayer membranes started when the desire grew to study proteins in a less complex environment compared to the living cell. Water-soluble proteins could more or less easily be separated and purified, whereby

their function and structure could be studied. However, membrane proteins were a different matter. Since the natural environment of the membrane proteins are in the hydrophobic lipid bilayer, either in part or wholly, they cannot be solubilized in aqueous solutions without the use of detergents. However, the use of detergents can also lead to denaturation of the protein. Therefore, the best way to study a membrane protein is to insert them into their natural environment, a lipid bilayer membrane.

The procedure for isolation of membrane proteins involves finding a membrane source that is rich in the specific protein of interest and then extract the protein, for example, using detergents. After the protein is purified, the suspension that is formed contains mixed micelles containing lipid, detergent and protein. The detergent is then removed, whereby so-called proteoliposomes are formed, that is, vesicles containing membrane protein. The critical step in this procedure is almost always the use of detergent for solubilizing the membrane protein. The detergent must not be to strong, since then there is the risk of denaturation and at the same time it must be easily removed in order to insert the proteins into the lipid bilayer membrane.

The re-insertion of membrane proteins into membrane structures was first investigated in the early 1970's by Racker *et al.* The membrane proteins that were studied were the mitochondrial proton-translocation ATP-ase, cytochrome oxidase also proton-translocating, and the (Ca^{2+} + Mg^{2+})-ATP-ase that uses ATP to transport Ca^{2+} across the membrane (Kagawa and Racker, 1971; Hinkle *et al.*, 1972; Racker, 1972).

The early reconstitutions of membrane proteins used bilayer membranes on solid supports or very small vesicles as the model system and these techniques are still widely used. However, research has been performed in order to insert membrane proteins into giant vesicles (GUVs) (Davidson *et al.*, 2003; Kahya *et al.*, 2001). GUVs have the advantage that the bilayer membranes are free standing, that is, the membrane proteins inserted into the membrane are not experiencing the risk of attaching to or even be denatured by a solid support. Another great advantage is that the GUVs can be studied under optical microscope, which enables techniques such as single-molecule detection and fluorescence labelling.

The contribution from our research group to this field of research is that we have incorporated membrane protein into our vesicle-nanotube-network (Davidson *et al.*, 2003). The membrane protein used to demonstrate this was the anion exchanger 1 (AE1) from human erythrocytes. After the protein had been extracted and purified, the detergent was removed and a solution of small unilamellar vesicles (SUVs) containing the AE1- protein was formed. The incorporation of membrane protein into our netoworks was performed in two different ways (Davidson *et al.*, 2003). In one experiment, the membrane protein was inserted into the lipid bilayer membrane constituting the wall of the network system. Giant unilamellar vesicles were created by the dehydration/rehydration procedure. A two-container network was then created using the micropipette-assisted technique. In another experiment, a solution of SUVs functionalized with the same membrane protein was injected into selected vesicle containers in a network. Since these SUVs have functionalized membranes they can therefore be seen as artificial organelles. The presence of the AE1-protein in the vesicles could be detected by using binding of fluorescent dye to the membrane protein.

Use of artificial cells as models for membranous processes

Vesicles are not only a predecessor for ancient cells, they are still very much important in biology. Vesicles are used as transporter compartments inside the cells, shuttling material between various organelles. In neurons, synaptic vesicles are transported to the plasma membrane and the two membranes of the vesicle and the plasma membrane undergo fusion, whereby neurotransmitter is released to the outside of the cells throught the process of exocytosis. This membrane fusion is one of several fusion events taking place in the eukaryotic cells. Both lipid bilayer membrane and proteins are involved in the fusion mechanism, and to be able to study these processes in a more controlled manner, scientists have used vesicles that are free of proteins to see to what extent the lipid bilayer membrane contributes to the process. One example is the study of fusion pores between a vesicle and a planar bilayer membrane (Chantariya *et al.*, 1997). It was proved that the phenomenon called "flickering", that is, a switching between an open and a closed state of the fusion pore, could take place in the protein-free system.

Our contribution to this field is that we have created a vesicle model system in order to mimick the late stages of exocytosis (Cans *et al.*, 2003). A small vesicle was created inside another larger vesicle, using the micropipette-assisted technique (Karlsson *et al.*, 2000; Karlsson, M. *et al.*, 2001). The small vesicle was held in place by the micropipette that was used for creating it and it was connected to the larger vesicle surrounding it by a nanotube. The nanotube connecting the two vesicles could be seen as an elongated fusion pore in the late stage of exocytosis. The small vesicle was allowed to remerge to the other membrane by shortening of the nanotube until forming an unstable toroid structure. Upon merging, the nanotube opened up and the material contained in the small vesicle was released to the outside of the larger vesicle. This release was shown both by having a fluorescent dye, fluorescein, and also by having catechol in the vesicle. The catechol could be measured by electrochemical means by placing a carbon fiber electrode at the fusion pore.

Another contribution to this field is the fact that the vesicle-nanotube networks hopefully can be used for modelling the intracellular network of membranous compartments. As mentioned in chapter 2-the cell, tubular elements connecting various compartments in the cell has been found, especially in the Golgi and between the ER and the Golgi, but also there might be connections between the plasma membrane and the Golgi (Sciaky *et al.*, 1997). Evidence points towards the fact that some of the transport might take place along these tubes, induced by tension-driven lipid flow. Since we have shown that we can achieve tension-driven transport along our nanotubes connecting our vesicles containers (Karlsson, R. *et al.*, 2002), our system seems to be a beautiful model system for the study of tension-driven material transport in living cells and hopefully it will be used more in the future for this purpose. For instance, it might be elucidated if the difference in tension between the containers has a mechanical or chemical origin.

Not only can we provide a model system of membranous compartments interconnected by lipid membrane nanotubes, we have also shown the creation and transport of vesicles integrated on nanotubes (Karlsson *et al.*, 2003b), which is also a very common phenomenon in the intracellular endomembrane system. In the cell, vesiculo-tubular structures (VTCs) are transported between different membrane compartments and this phenomenon can be studied by observing how our mobile vesicles are "budded off" from one compartment, move along interconnecting nanotubes and then finally fuse or remerge with other connected compartments (Iglic *et al.*, 2003).

Reactions in vesicle-nanotube networks

The insertion of enzymes and substrates into vesicles is not only interesting in the research of minimal cell models, it is also interesting to study chemical and enzymatic reactions in the confined and biomimetic environment provided by the vesicles from a pure kinetic view.

As realized, the simple view of the cytoplasm being an ordinary fluid contained by the plasma membrane is far from correct. For example, in order to study biochemical and enzymatic reactions inside the cell, several assumptions have to be made. In the first attempts to explain cellular biochemistry, it was assumed that the reaction volume was infinite, the solution was dilute, the concentrations of substrates was much higher than their enzymes, the solution was well-defined and that the solution was homogeneous. When learning more about the cell, one understands that the situation is far more complex (Pagliaro, 2000; Luby-Phelps, 2000). For example, the volume is not infinite, it is actually very small and the number of molecules can be quite small, while still being present in a high concentration. The cytoplasmic solution is not dilute, in fact, the concentration of, for example, proteins is very high leading to substantial interactions between the molecules.

Also, when realizing the great structural complexity of the inside of the cell, one can see that the cytoplasm is not well-defined or homogeneous. As mentioned, the endomembrane system compartmentalizes and divides the cytoplasm into even smaller volumes were specific reactions can take place. The endomembrane system also provides a vast membrane surface area, besides the plasma membrane surface. The complexity of the cells interior is further increased by the structural protein network of the cytoskeleton which criss-crosses throughout the whole cell, also providing surfaces to the system. The endomembrane system and the cytoskeleton thus both give rise to the fact that the water and the solvated proteins of the cytoplasm are always close to a surface. The surface effect has been investigated and it is known that water has to adopt a more ordered structure close to a surface, leading to changes in, for example, viscosity etcetera. Also, the enzymes and substrates involved in a biochemical reaction might be attracted or repelled by the surfaces. The conclusion is that the enormous amount of surface leads to a reduction in the dimensionality, a fact which will affect the kinetics of a biochemical reaction.

When the reaction volume is reduced to submicron scale, an exciting phenomenon of enzyme kinetics arises. The diffusive transport and mixing times becomes shorter than the times needed for an enzyme to convert a substrate to a product, that is, the turnover time. The reaction rate is thus limited by the time needed for a conformational transformation of the enzyme to take place. Theoretical studies have been performed which show that under these conditions it might be possible for a so-called molecular network to be established. By molecular network it is meant that the enzymes in the confined volume can synchronously convert the substrates into products (Stange *et al.*, 1998).

Our contribution to this field of research is to study enzymatic reactions inside small vesicles (Chiu *et al.*, 1999a; Chiu *et al.*, 1999b; Karlsson *et al.*, manuscript in preparation). For example, in one paper, two techniques for initiation of reactions in the small vesicles, having sizes in the range of 5 μm in diameter, were investigated. Micromanipulator-controlled carbon fibers (5 μm in diameter) were used to perform single vesicle electroporation or single vesicle pair fusion (Chiu *et al.*, 1999a). In another paper an enzymatic reaction occurring in these small vesicles was studied (Chiu *et al.*, 1999b). The enzyme alkaline phosphatase and the substrate fluorescein-diphosphate were mixed in a small vesicle and since the product of this enzymatic reaction was fluorescein, which is highly fluorescent, the product formation could be followed in time using laser-induced fluorescence (LIF). Also, a theoretical study characterized by a Brownian motion Monte Carlo simulation

was performed on the collisional environment between a single enzyme and substrate inside a vesicle (Chiu *et al.*, 1999b).

Another technique for initiation of reactions have recently been developed using the elegant technique of forming vesicle-nanotube networks (Karlsson *et al.*, manuscript in preparation). The first step was a unilamellar vesicle (GUV) attached to the surface, also having a multilamellar protrusion (MLV) as a lipid reservoir connected to it. A daughter vesicle was created using the micropipette-assisted network forming technique. By having a solution of a substrate in the micropipette, the daughter vesicle was filled with the substrate solution. The solution of the micropipette was changed to a solution containing enzymes for the enzymatic reaction and a new daughter vesicle, containing this enzyme solution, was created. Since the nanotube connections were free to move over the entire system of a continuous bilayer membrane, the nanotube connected to the substrate vesicle could slide over the surface of the other nanotube to the daughter vesicle containing enzymes. A nanotube connection between the substrate and enzyme vesicle was thus created. Since the nanotube resembled the structure of an elongated fusion pore, all which was needed for the reaction to be initiated was to bring the two vesicles together, whereby the nanotube opening mediated and completed the merging.

Reactions in three-way nanotube junctions

We have showed that we can induce flow of fluids and particles through our nanoscale channels by the use of tension-driven lipid flow, a moving-wall-driven movement. We have also showed that it is possible to create three-way nanotube junctions, where two nanotubes meet and merge into one. It is realized that by combining the three-way nanotube junction with the principle of tension-driven lipid flow, an elegant nanofluidic system can be created. The flow of fluid through the system can be designed so that two flows meet in the junction and then mix while continuing into the third channel. By having, for example, an enzyme in one of the channels and substrate in the other channel, an enzymatic reaction can be initiated and studied in the third channel. By stopping the flow immediately after the two flows have met, the enzyme reaction can be studied in the extreme small scale of the nanotube having volumes down to the attoliter (10^{-18} L) volumes (Fig. 37).

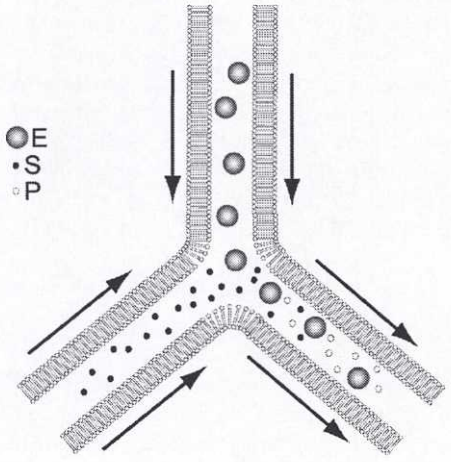


Figure 37. Schematic illustrating a cross-section of the three-way nanotube junction. By using the principle of tension-driven lipid flows in a three-way nanotube junction it is possible to let a flow of, for example, enzyme solution meet a flow of substrate solution. When the two flows meet in the junction point, they start to mix and continue into the third, shared nanotube, whereby product is formed.

We have started this line of work by using the technique of formation and transport of nanotube-integrated vesicles as transporters instead of using the in-tube transport. This was done in order to detect the transported material more easily. The nanotube-integrated vesicles were loaded with a solution of fluorescent dextrane and were then moved along the nanotube to the three-way nanotube junction, using the principle of tension-driven lipid flows (Fig. 38).

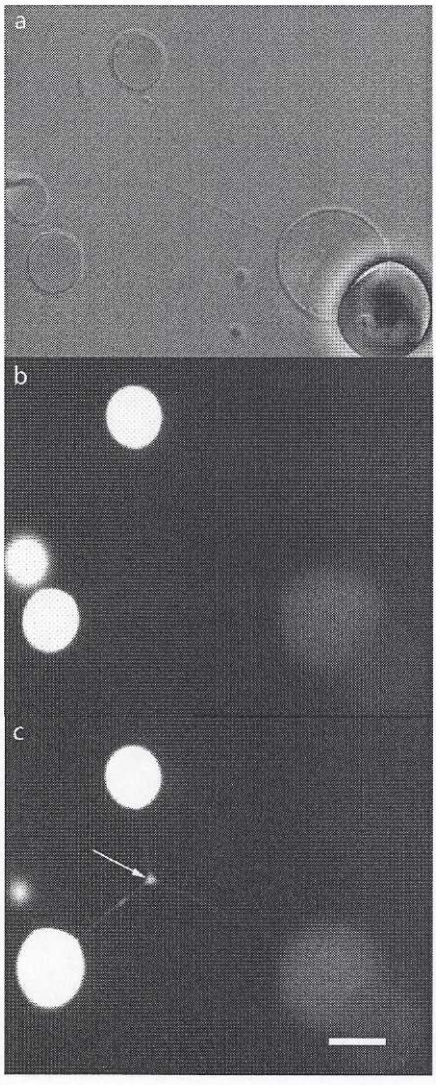


Figure 38. Photomicrographs showing injection of nanotube-integrated vesicles into a three-way nanotube junction. (**a**) Bright-field image of the starting network. A three-way nanotube-junction was created using the micropipette-assisted technique. The nanotubes interconnected three vesicles, two of which were filled with a solution containing fluorescent dextrane. A new vesicle was created, which acted as a membrane donor vesicle. (**b**) The corresponding fluorescent image. (**c**) The donor vesicle, which also contained fluorescent dextrane was allowed to remerge with one of the fluorescent vesicles in the network. Nanotube-integrated vesicles were formed on the nanotube, which could be moved into the nanotube junction using the principle of tension-driven lipid flows. Scale bar 10 μm.

Conclusion, discussion and future outlook

We use micromanipulation techniques to create networks of vesicles (5 – 50 μm in diameter) and lipid membrane nanotubes (diameters in range of 50 – 200 nm). The walls of the network systems are made of the same material as the major building constituent in living cells, a phopholipid bilayer membrane. The lipids constituting the monolayers and the monolayers themselvers are held together by non-covalent interactions giving the membranes a dynamic and fluid character and since it has a thickness of only 5 nm, it can be viewed as a two-dimensional fluid. The membranes thus have very special properties with low resistance to bending but high resistance to stretching.

These material properties did not only make it possible for us to create vesicle networks having nanoscale nanotubes, the fluid character also provided a means to transport material through these small-scale channels, namely the phenomenon of tension-driven lipid flow. When disturbing the energy balance by inducing a tension difference across the nanotubes, a lipid flow was created, and due to the small dimensions of the nanotube, fluid and particles were dragged along the moving lipid walls, due to viscous coupling. In a larger and more complex network it is important that transport can be achieved between two selected containers, without disturbing the rest of the network. Therefore a two-point perturbation technique was developed, meaning that both vesicles between which transport was to be achieved were manipulated. By decreasing the membrane tension in the container from which material was to be taken and at the same time increasing the membrane tension in the container to which material was to be transported, the transport preferentially took place through the nanotube connecting these two containers. The two-point perturbation technique could also be used to create vesicles having sizes of micrometers or even submicrometers integrated on the nanotubes. These vesicles could be loaded with material, transported along the nanotubes, using the principle of tension-driven flow and then made to release their material into the selected target container. Interestingly, our system of membranous compartments interconnected by lipid membrane nanotubes on which vesicles can be integrated shows similarities to many membranous processes occurring in living cells. For example, exocytosis and intracellular tension-driven membrane transport shows parallels to experiments and studies performed using our networks.

It is realized that these networks have great potential in many different areas of research and technology. The two major characteristics that open up all these possibilities are (**1**) the small-scale and (**2**) the biomimetic character and in summary we can conclude a few application areas

i) The use of the small-scale characteristic of the network for studying single molecule behaviour, anomalous fluid behaviour and possible anomalous kinetics of enzymatic reactions in a very confined geometry. For the enzymatic reactions, it would be interesting to study a low population number of enzymes working on substrates in a very confined geometry. As has been hypothesized, the reactions might deviate from normal kinetics, since the diffusion is not limiting the reaction, rather the conformational changes required of the enzyme for converting substrate to product. In this case, an oscillatory behaviour of product formation is possible, the enzymes working as a single unit. The biomimetic character also makes it possible to insert membrane proteins into the system, providing a reaction site or a means for delivery of substrate, whereby the crowded space of the network mimicks the complex cellular environment even more.

ii) The use of the networks as nanofluidic devices, showing parallels to the previous section. The use of tension-driven lipid flow has given us the possibility to handle

and transport volumes of liquid down to the range of attoliters, the transport taking place through the nanotubes between the connected vesicle containers. The two-point perturbation technique provided a means to use the phenomenon of tension-driven lipid flows for transport in large networks between selected containers without disturbing the rest of the networks. The creation of nanotube-integrated vesicles gave us the possibility to construct a system capable of performing femtoliter titrations into vesicle volumes of picoliters.

iii) The use of networks as templates for solid-state structures. Small-scale structures such as nanotubes can be contructed having dimensions down to 20 nm, dimensions which are hard to fabricate using conventional methods. It has been shown that lipid nanotubes can be used as templates for solid-state structures (Evans *et al.*, 1996) by metallization procedures (Schnur, 1993) or by protein crystallization (Wilson-Kubalek *et al.*, 1998). This way, it is realized that our small-scale structures constructed of soft lipid bilayer membranes can, for example, be metallized and used in microelectronics applications. Another great advantage of our network systems is the possibility to create structures in three dimensions. Hurtig et al have recently shown that it was possible to create networks of vesicles and interconnecting nanotubes in three dimensions by using microfabricated substrates (Hurtig *et al.*, Manuscript in preparation).

iv) The use of the networks as sensor devices. Sensor devices can be constructed in two ways. The unique material properties of the lipid bilayer membrane can in itself be used, for example, in order to make the networks respond to mechanical changes such as pressure. Another possibility of using the networks as sensor devices is to insert membrane proteins in the lipid bilayer membrane constituting the systems. This can give the network a sensing function, for example, responding to light gradients or detecting chemicals or biomolecules by molecular recognition.

v) The use of the networks as model systems for cells and membrane phenomena occurring in cells. We have shown that our systems could be used for studying membrane phenomena occurring in living cells, such as exocytosis or tension-driven lipid flow-mediated transport between organellar systems such as the ER and the Golgi. It is suggested that the controlling protein units present in cells might not be needed for the late stages of exocytosis, instead the mechanism might solely be driven by membrane processes (Cans *et al.*, 2002). It has been demonstrated that tension-driven lipid flows can be created in lipid nanotubes connecting intracellular compartments such as the Golgi and the ER (Sciaky *et al.*, 1997) and that the lipid nanotubes might be used as transport highways for vesicles (Iglic *et al.*, 2003)

Acknowledgements

First, I would like to thank Professor Owe Orwar, who gave me the opportunity to work in such an exciting field of science. Together, we have accomplished to create our own niche in science, networks of vesicles and nanotubes!

I would also like to thank my examinator, Professor Daniel Jagner, for showing interest to my work during my studies, and for help and support, especially the last hectic weeks.

Thanks to all past and present members of the Owe Orwar research group, who are all very bright and intelligent. When we have worked together, combining all our knowledge, nothing has seemed impossible!

Thanks to Daniel Fagerlund for help with images!

Collaborators throughout the years, Professor Andrew Ewing, Professor Evan Evans, Professor Daniel Chiu, Doctor Wolkmar Heinrich, Professor David Needham, with special thanks to Professor Marina Voinova and Professor Johan Bergenholtz.

Also thanks to all at the Department of Analytical and Marine Chemistry for creating a nice working environment.

Special thanks to Ingrid Kubista, who have always lent a helping hand throughout the years and to Leo Huovinen, who have, to the best of my knowledge, every day asked me the question "Läget?".

Thanks to Eskil Sahlin and Bertil Axelsson for your "grumpy-old-men-style".

Thanks to my room-mates Charlotte Eliasson, Tobias Ankner and former room-mate Pia Engström, as well as phone-mate Anders Lorén, especially for putting up with never-ending pop-music on the radio.

Thanks to Katarina Abrahamsson for morning chats and for being the best course leader I have worked with.

Thanks to Stefan Hulth for always having time for small-talk, for help, support and showing interest to my work.

Thanks to Niklas Strömberg for help and support throughout the years and for always performing cool and inspiring experiments.

Also thanks to relatives and friends who have wondered what I have been doing the last years, when reading this you might understand more…

Finally, I would like to thank my loving family; my mother, Gunvor, my father, Kent and my brother, Anders. We always have fun together and I hope you know that you are the light of my life!

Summary of papers

Construction of networks of vesicles and interconnecting lipid nanotubes
(Papers I,II and V)

Transport of fluids and particles through nanotubes using lipid flows
(Paper III and V)

Construction of a model system for exocytosis using vesicle-nanotube networks
(Paper IV)

Formation and transport of nanotube-integrated vesicles
(Papers VI and VII)

Paper I
Networks of Nanotubes and Containers

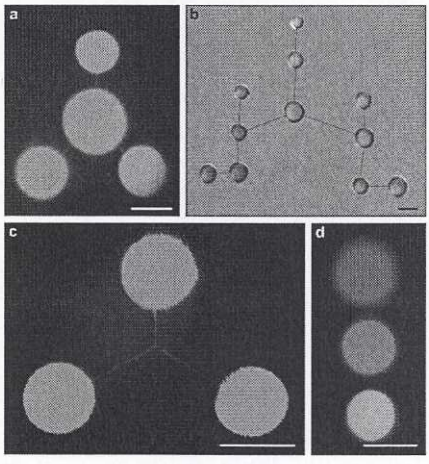


Figure from paper I

This paper demonstrates the formation of networks of vesicle containers interconnected by lipid nanotubes, using a mechanical fission technique. It was demonstrated that vesicles could be mechanically cleaved by using a micromanipulator-controlled 5-μm-diameter carbon fiber. Upon fission, a thin nanotube was formed, interconnecting the resulting two vesicles. The mechanical fission technique proved to be best suited for multilamellar vesicles, since unilamellar lacked some of the required surplus membrane material needed for formation of the interconnecting nanotubes. However, one or two consecutive divisions of a unilamellar vesicle were possible. A four- and eleven-container network interconnected by lipid nanotubes was shown, in bright-field and in fluorescence. The sizes of the vesicle containers were in the range of 5-20 μm in diameter and the diameter of the nanotubes was approximately 30-300 nm. Also shown was the formation of three-way nanotube junctions and that it was possible to differentiate the containers after network formation. Briefly, it was also demonstrated that fluid and particles could be transported between two vesicle containers through the interconnecting nanotube. Most suitable for multilamellar vesicles, the mechanical fission technique opened up the possibility to construct complex networks of vesicles and interconnecting nanotubes. The realization was made that the multilamellar

vesicle networks could be used as templates for solid-state devices with potential applications in microfluidics and microelectronics.

Paper II
Micropipette-Assisted Formation of Microscopic Networks of Unilamellar Lipid Bilayer Nanotubes and Containers

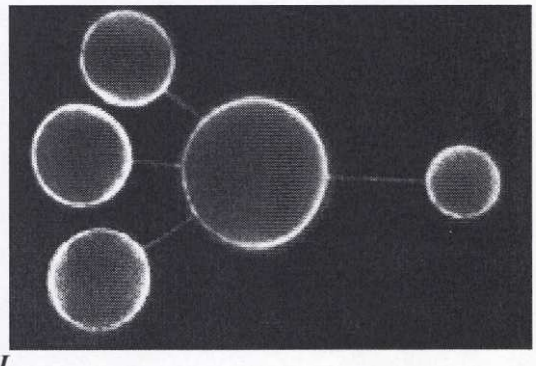


Figure from paper II

This paper demonstrates a micropipette-assisted technique for the formation of unilamellar networks of vesicles and lipid nanotubes. A micropipette was inserted into a unilamellar vesicle using an electroinjection technique, that is, the membrane of the vesicle was destabilized by a transient electric field and then penetrated using a mechanical force. The lipid membrane was allowed to reseal around the pipette, which was then pulled out of the vesicle, forming a lipid nanotube. By injecting buffer into the nanotube orifice it could be expanded, taking lipid membrane material from the originating vesicle across the nanotube. Therefore, unilamellar vesicles twinned to a multilamellar vesicle was always chosen as starting vesicle, the multilamellar vesicle working as a lipid reservoir, feeding membrane material into the unilamellar system during formation of the network. When the vesicle had reached the desired size it was allowed to attach to the surface. By repeating the procedure, forming more nanotube-connected vesicles, networks were formed, and it was also possible to create three-way nanotube junctions. It was realized that it was possible to create networks in three dimensions and since the new vesicles are formed by injecting buffer solutions the networks could easily be differentiated with respect to content, during formation of the network or afterwards. Advantaged of this technique is that it not only also provide the ability of creating nanoscale templates for solid-state devices or open up the third dimension, it can also be used as a small-scale fluidic device. The fact that fluids of different composition can be injected into the unilamellar containers and that there is fluid contact between the containers make the realization of a small bioanalytical and biomimetic system possible.

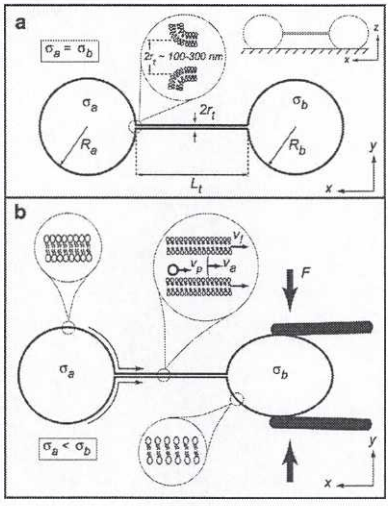


Figure from paper III

A technique for transporting fluids, particles and lipid membrane material between two interconnected vesicle containers through the lipid nanotubes was developed. The nanotubes have diameters of 100-300 nm, but can be made to have sizes down to 30 nm. The transport in these very small channels would be very hard to control in a conventional way, but here we use tension-driven lipid flows, moving the walls of the cylindrical nanotube channels. First, one has to realize that the system of near-spherical vesicles on a surface, interconnected by thin tubes, is energetically favoured due to the unique material properties of the lipid bilayer membrane. The membrane is very easy to bend but hard to stretch in the plane of the surface and therefore a delicate balance between bending and surface tension is established. In a system of two vesicles interconnected by a lipid nanotube, the surface membrane tension of the vesicles is the same. If a force is applied to one of these vesicles, deforming it in shape, the membrane tension is locally increased and a difference in tension across the nanotube is established. The system responds to this perturbation by transporting membrane material from regions of lower tension to the point of increased tension. Since the system is constructed of a continuous bilayer membrane, lipid material flows across the nanotube to the deformed vesicle in order to eliminate the difference in tension. Due to the small dimensions of the nanotube, the lipid flow, that is, the moving walls of the nanotube, dragged along fluids and particles trapped in the nanotube. The transport was illustrated by both a large particle seen in bright-field image and a small fluorescent particle (30 nm). The small dimensions of the nanotubes make it possible to use these nanoscale fluidic systems for handling and analysis of nanoparticles or even single molecules, as well as study enzymatic reactions in confined spaces.

Paper IV

Artificial Cells: New Insights into Exocytosis using Liposomes and Lipid Nanotubes

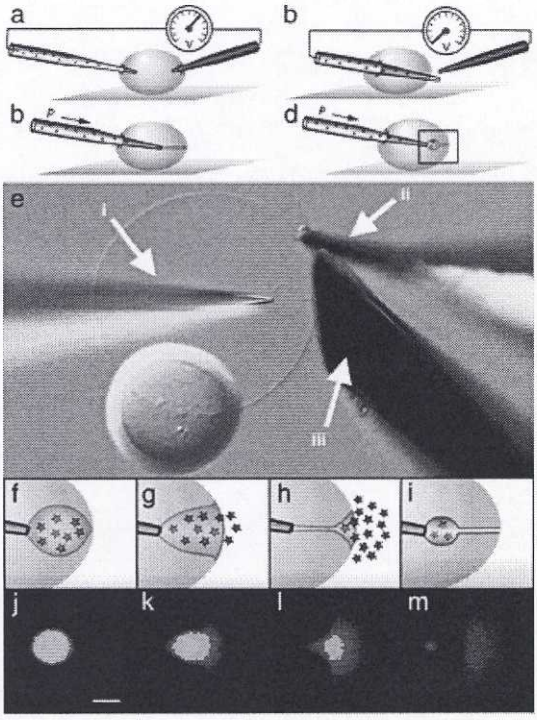


Figure from paper IV

In this paper, a vesicle model system was created in order to study the process of exocytosis. The starting point was a giant unilamellar vesicle on a surface. A micropipette was inserted into the membrane using the electroinjection technique, but instead of pulling out a tube, the tip of the micropipette was made to exit the membrane on the opposite side of the membrane, again using the electroinjection technique. When the tip was pulled into the vesicle, a nanotube was formed and by injection of buffer, a small vesicle was formed. This way a small vesicle inside a larger vesicle could be created, the two vesicles connected by a nanotube. The nanotube could be seen as an elongated fusion pore, thereby making the system resemble living cell with synaptic vesicles undergoing the process of exocytosis. The membrane of the small vesicle was then allowed to merge with the membrane of the surrounding larger vesicle, whereupon it released its contents to the outside of the system. The release was observed either by fluorescence or by electrochemical measurements. In the case of fluorescence detection, the small pipette-attached vesicle was filled with a solution containing fluorescein. For electrochemical detection, the vesicle was filled with catechol and a carbon fiber microelectrode was used to detect the release. The vesicle model system is free from the controlling protein units normally present in cells and it is suggested that the proteins might not be needed for the last stage of exocytosis, instead it could be driven by minimization of the membrane elastic energy.

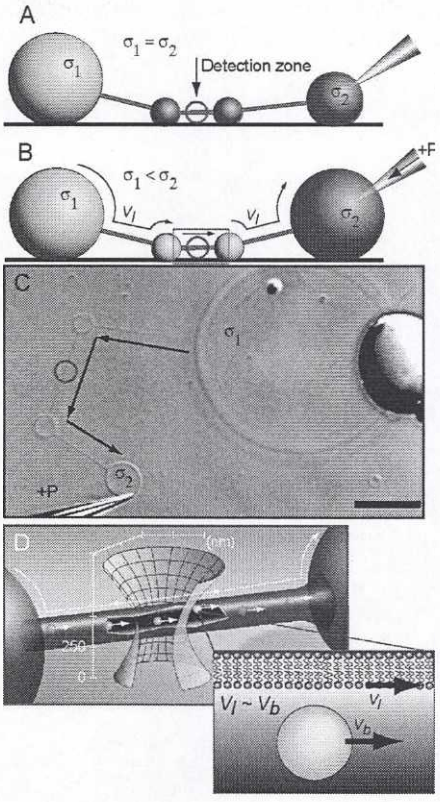


Figure from paper V

In paper V we review the two techniques used for creating the networks of vesicles and connecting nanotubes, mechanical fission and micropipette-assisted formation. We also investigate more about the energy of the system and explore the adhesion effects that arise. The nanotube will always be located at the smallest distance between the containers, that is, the equator of the vesicles. With higher adhesion, the vesicles will change shape from a spherical to a more dome-like configuration and therefore the closer to the surface will the nanotube reside. For very high adhesion, the nanotube might even attach to the surface and there is also the risk of the vesicles rupturing and spreading on the surface. A nanofluidic network was designed and created for the purpose of detecting single 30-nm-diameter fluorescent beads flowing through the nanotubes, using laser-induced fluorescence as a detection technique. A linear system of four nanotube-integrated vesicles was created, the end-vesicles being larger (10-μm-diameter) than the middle ones having a diameter of only 2 μm. The first end-vesicle was used as a sample injection container and it was filled with a solution containing the beads. The second end-vesicle was injected with buffer and upon expansion a lipid flow was created towards it across the interconnecting nanotubes, the lipid material originally coming from the multilamellar protrusion connected to the first end-vesicle. The two middle vesicles worked as anchors for the nanotube used for the detection purposes and in order to minimize the risk of convective movements out of the detection

probe volume, the distance between these two vesicles were relatively short. These anchoring vesicle were also made to be small, thereby matching the coordinates of the interconnecting nanotube with the detector probe volume focus, which resided close to the surface. Tension-driven lipid flows were created across the nanotubes with a concomitant flow of fluorescent particles, which could be detected by a photoavalance detector.

Paper VI
Formation and Transport of Nanotube-Integrated Vesicles in a Nanoscale Bilayer Network

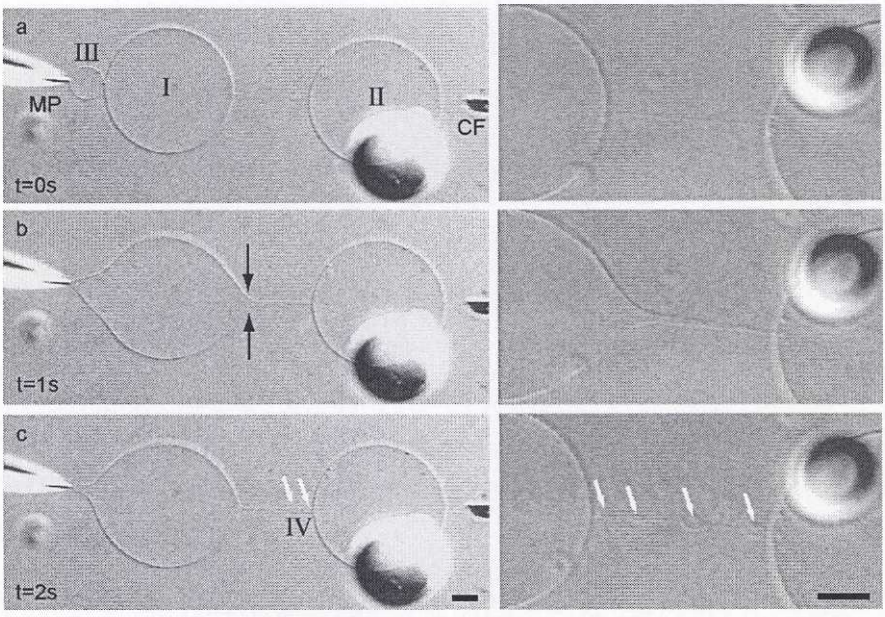


Figure from paper VI

In this paper we present a method to create small vesicles integrated on a nanotube. The vesicles have sizes in the range of 500 nm to 5 μm in diameter and the walls of the vesicles are continuous with the nanotube walls, which make them two-fold open-ended. Since the vesicles are created on suspended nanotubes, conjugating giant surface-adhered vesicles, they are free to move. We show that we can transport the nanotube-integrated vesicles along the nanotubes, using the principle of tension-driven lipid flows. The nanotube-integrated vesicles could be loaded with, for example fluorescent dextrane, during the process of creating them and then transported along the nanotube to a target vesicle. When reaching the end of the nanotube, the small, mobile vesicles could be made to coalesce and release their contents into the surface-immobilized target vesicle. As an example, 40 consequtive injections of micrometer-sized mobile vesicles containing fluorescent dextrane was shown to increase the fluorescence of a giant surface-adhered vesicle.

The interesting characteristic of this system is that it is capable of handling and transporting very small volumes of liquid, from picoliters down to attoliters (10^{-12} to 10^{-18} liters). We therefore see possibilities for use of these networks in, for example, the field of bioanalytical chemistry. Small packets of fluid, containing, for example, enzymes or substrates can be transported and mixed whereby a reaction can take place in the constricted and biomimetic environment provided by the bilayer membrane network. This idea opens up

the possibility to create micro- and nanofluidic devices able to create environments where detection of very few, perhaps even single molecules, is possible. Besides this exciting capability of using the vesicle-nanotube network as a bioanalytical system, the phenomenon of vesicles on nanotubes interconnecting membrane compartments is interesting in itself. In cells, nanotubes interconnect membrane compartments such as the Golgi and the ER and it has been suggested that vesicular transport can take place along the nanotubes.

Paper VII
A Nanofluidic Switching Device

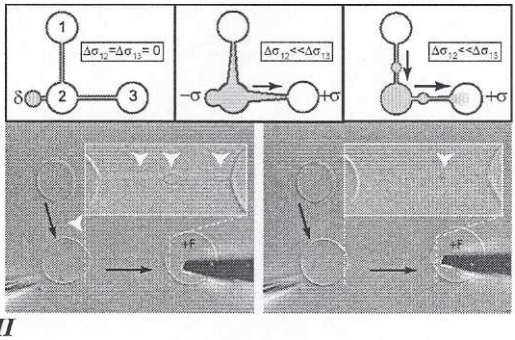


Figure from paper VII

In this paper, we demonstrate the concept of a nanofluidic switching device. When using the principle of tension-driven lipid flows for transporting fluids and substances in a large and complex network a problem might arise. If the surface tension is increased in a specific target container, lipids will start to flow from all containers in the network, through all tubes in the network and finally through all of the tubes connected to the target vesicle. This phenomenon arises from the fact that the whole system is made of a single, continuous lipid bilayer membrane. It would thus be impossible to achieve controlled transport between two selected containers in a complex network. However, this controlled delivery can be created by using a two-point perturbation technique, decreasing the tension in one container (from which material is to be taken), while simultaneously increasing it at the target container (to which material is to be delivered). The decrease in tension could be achieved by merging a donor vesicle with the selected surface-adhered vesicle. Because of the merging of the vesicles, the resulting vesicle will have a surplus of membrane material giving it a low membrane tension. The increase in tension could be achieved by mechanically pinching or perturbing the selected surface-adhered vesicle, using micromanipulator-controlled carbon fibers. This switching function was illustrated by donating membrane material to a surface adhered vesicle having two nanotubes emanating from it, connecting it to two target vesicles. First, material was routed to one of the target vesicles in the form of small vesicular structures, simply by increasing the membrane tension of this vesicle. Then, after another donation of membrane material, by increasing the tension in the other vesicle, material could be routed to this container instead. In a network having more than two vesicle containers, it is absolutely vital to have this controlled routing capability. Molecules can be shuttled through a network, however complex the connectivity is, making it possible to perform several sequential operations on them. The small scale of the nanotubes makes these systems suited for working with a few, or even single, molecules.

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ISBN 91-628-5870-X