Affinity Two-Phase Partitioning of Liposomes and Membranes

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II. Barinaga-Rementeria Ramírez, I., S. Mebrahtu, and B. Jergil, *Affinity partitioning for membrane purification exploiting the biotin-NeutrAvidin interaction. Model study of mixed liposomes and membranes. J. Chromatogr. A* (**2002**) 971, p. 117-127(*).

III. Barinaga-Rementeria Ramírez, I., P. Abedinpour, and B. Jergil. *Purification of caveolae by affinity two-phase partitioning using biotinylated antibodies and NeutrAvidin-dextran.* **Submitted**.

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Abbreviations

CCD	Counter current distribution
D.L.	Debye Length
EM	Electron microscopy
ER	Endoplasmic reticulum
GPI	Glycosylphosphatidylinositol
LC	Liquid chromatography
MS	Mass spectrometry
MW	Molecular weight
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
PEG	Poly (ethylene glycol)
Tween 20	Polyoxyethylene-sorbitan monolaurate
SUV	Small unilamellar vesicles
WGA	Wheat germ agglutinin

1. Introduction

Advances in scientific research have always been closely tied to the invention and improvement of techniques since the beginning of the history of science. What we observe is limited by the detection system we use, whether it is simply our own eyes or very complex microscopes. Furthermore, by their very nature, all mixtures, from galaxies consisting of planets, stars, comets etc, soils with their many minerals and microorganisms to cells with thousands of proteins and lipids, are difficult to study and their individual components need to be isolated to determine their characteristics and function within the mixture. Hence, separation of such components is an essential and fundamental requirement. The objects we study depend strongly on the way we separate them from others, e.g. within biochemistry they depend on the protocols and/or methods of purification employed. Therefore, higher resolution techniques and purification techniques yielding products of higher purity are required to increase the understanding of the subject of study. Thus, development of scientific research goes hand in hand with progress in technology.

In 1665, Hooke described cells for the first time thanks to the invention of the light microscope. Through the seventeenth and eighteenth centuries there were some additional morphological descriptions of the cell but no further progress in biological research was achieved due to imperfections in the lenses of microscopes. Meanwhile, advances in chemical and physical techniques allowed Wöhler to synthesize organic molecules from inorganic precursors. This opened a new field of study: cell chemistry and physiology.

In 1923, Warburg developed the tissue slice-manometric technique. This technique was further utilized by his young assistant, Krebs, who systematically examined all the amino acids, and less than a decade later the ornithine cycle for mammalian urea formation was described [1]. Since then

different chemical mechanisms and cycles, forming the core of enzymology, have been discovered.

By the end of the nineteenth century many organic substances found in plants and animals had been isolated, identified and synthesized. In the 1930's integration of cell biology, genetics, physiology and biochemistry started, at the same time as investigations moved from morphology to biochemical and molecular studies. Once again this was partly a consequence of technique development. In 1930-45 Claude developed a technique for isolating and purifying cell parts by centrifugation [2,3]. A few years later Claude's fractionation protocol was improved by the use of the Potter-Elvehjem homogeniser in the homogenisation step [4] and the use of 0.25 M sucrose as iso-osmotic medium [5]. Another further improvement was the introduction of preparative ultracentrifuges in the early 1950's obtaining centrifugal forces of over 150 000 g and with them the use of gradient centrifugations [6]. Since then many proteins and cell organelles have been fractionated, isolated and analysed using centrifugation procedures.

Some years later, in the late 1960s, Giddings introduced a new technique, field-flow electrophoresis [7], to allow further separation of vesicles that could not otherwise be separated by means of centrifugation. Nevertheless, the use of this technique is still limited.

The use of labelled isotopes started during the course of World War II, and with their biochemical use and the development of chromatographic techniques a whole new world of discovery opened up to the biochemist. By about mid-century, most of the known vitamins (pantothenic acid, biotin among others) had been discovered and their modes of action made clear. At the same time, important hormones such as secretin, insulin and cortisone were isolated and their structures elucidated [1].

Another big step forward in biosciences came with the application of the electron microscope to biological materials, starting in the 1940's and allowing

the visualization of membranes. Yet another example illustrating that technique and progress in bioresearch goes hand in hand is the application of X-ray diffraction pattern analysis, using which, in 1953, Watson and Crick determined the structure of DNA. It was not until 1959 that the first X-ray structure of a protein was elucidated, that of sperm whale myoglobin [8]. In the years since then several thousand proteins have been purified and characterised and the pace of this endeavour is still accelerating. Determination of protein X-ray structures revolutionised the biochemical thinking and reshaped our understanding of the chemistry of life. From the DNA structure came the deduction of the genetic code, which was a major advance in the biochemistry of the 20th century. The complete sequencing of the genome revolutionized the manner in which biological research was being conducted [9]. This was partly permitted by advances in molecular biology techniques, DNA sequencing and cloning, and recombinant DNA technology. Fifty years ago, one or a few gene products were possible to study at a time, whereas now the use of microarrays has made possible the study of many gene products at a time, i.e. global analysis of cellular processes.

More recently, attention has turned from genomics to proteomics, the study of proteins and their activities. Traditionally, protein abundance has been examined using two-dimensional gel electrophoresis (2D-GE) [10]; in one case over 10000 proteins resolved in a single gel [11]. Still, 2D-GE has two intrinsic problems: the identity of expressed proteins remains unknown and the resolution is limited. Coupling 2D-GE to a newly introduced powerful technique, mass spectrometry (MS), has allowed the determination of molecular mass and identification of thousands of proteins.

The overall performance of the analysis strongly depends on the performance of the separation tool. Different separation techniques are now coupled to MS; the most well known combination to date is liquid chromatography (LC)/MS. Although LC/MS has been successfully applied in the identification of many proteins, there are still some difficulties with the detection of low-abundance proteins. The latest developments in chemistry are providing the market with

new isotope-coded affinity tags or with solid-phase isotope tags which permit the identification of such low-abundance proteins [12,13]. One problem, yet at the same time an exciting goal of proteomic research, is the purification and identification of membrane proteins. The number of membrane proteins identified so far is relatively low, although it is estimated that transmembrane proteins (not including peripheral membrane proteins) represent approximately 30% of the total proteins of a cell [14]. The lower the initial concentration of a substance, the more effort is required to isolate it in pure form. Many membrane proteins have a very low abundance, thus it is difficult to purify them with available techniques. Therefore, novel procedures to purify membrane fractions, specifically and avoiding cross-contamination, are of great interest.

In this thesis affinity two-phase partitioning in aqueous polymer two-phase systems will be presented as a technique for the purification of membranes. The aqueous two-phase systems used are composed of poly (ethylene glycol) (PEG) and dextran, in aqueous solution. The high water content of these systems, typically 80-95% in each phase, provides a mild environment for the purification of membranes. This technique is potentially useful for the rapid, high-yield purification of membranes, and domains and microregions of membranes, as well as of other subcellular material. The technique will be particularly useful for the purification of material that has so far been difficult or impossible to obtain in sufficient purity and yield for structural and functional examination. Specificity is introduced by an affinity ligand coupled to a phase polymer. The affinity ligand chosen in this work was NeutrAvidin which has been coupled to dextran. The technique could become one of more general use exploiting the avidin-biotin interaction.

1.1. Past and present techniques for membrane purification

An important part of biochemical investigations deals with the difficult task of isolating the subject material in sufficient purity and quantity to be properly characterised. There are few biochemical techniques developed for the isolation of cells, organelles and membranes compared to the large number available for the purification of soluble proteins. This chapter presents an overview of the most commonly used techniques for the isolation and purification of membraneous particles.

1.1.1. Centrifugation

Centrifugation is the most widely used technique for membrane purification [15-19]. Particles are separated by exploiting differences in size, shape and density (differential centrifugation) or in density (isopycnic centrifugation).

1.1.1.1. Differential centrifugation

Particles that differ in density, shape or size can be separated because they sediment at different rates in a centrifugal field. The rate of sedimentation of the major cell components decreases according to: whole cells > nuclei > chloroplasts > mitochondria > lysosomes > microsomes > ribosomes [20].

Differential centrifugation is the most commonly used method for the separation of a cell homogenate into different fractions. In differential centrifugation the cell lysate is centrifuged at a speed that sediments only cell components larger and denser than the desired organelle followed by another centrifugation at a higher speed that pellets the organelle of interest. The technique has been used for the fractionation of cell components from many tissues such as liver [21,22], kidney [23], cardiac [24] and skeletal [25]

muscle, lymphoid tissues [26] and brain [27]. Since the 1950's the use of differential centrifugation has greatly increased; the centrifuge has become a basic piece of equipment in biological/biochemical laboratories. The main advantages of the technique are: it is a rapid and simple technique; it can handle large homogenate volumes; the subcellular organelles are not stressed osmotically by exposure to hypertonic gradient media. The main disadvantage is that the separation depends only upon the sedimentation rate of the particles, leading to the isolation of heterogeneous material [28]. As particles move down to the bottom of the tube during centrifugation, the largest ones form a pellet. However, since the material was suspended homogeneously at the beginning of the centrifugation, particles of various sizes that were near the bottom will also sediment and subsequently contaminate the pellet.

1.1.1.2. Isopycnic centrifugation (density gradient centrifugation)

Isopycnic centrifugation separates particles mainly due to differences in their buoyant density. This separation is done in a density gradient through which the particles move under the effect of the centrifugal field until their density is the same as that of the surrounding medium. At this point of isodensity, no further sedimentation occurs regardless of how long the centrifugation proceeds.

After homogenisation, fragments of subcellular organelles such as mitochondria, lysosomes and peroxisomes, or fragments of them, do not separate well by differential centrifugation [28], presumably due to a combination of their sizes and densities. Nevertheless, it is possible to separate them based on their different densities by isopycnic centrifugation [29].

An ideal density-gradient medium should be inert and non-toxic to biological material, soluble in water, and have low viscosity and osmotic pressure.

Furthermore, the medium should have known physico-chemical properties to allow determination of the precise concentration of the gradient, it should be easy to separate from purified material and should not interfere with monitoring fractionated material within the gradient [28].

Sucrose is the most common density-gradient medium mainly due to its cost-effectiveness. Serious drawbacks of sucrose are its high viscosity and hypertonicity, which causes dehydration, lysis and damage to or disruption of membranes [28,30]. Some membrane organelles are permeable to sucrose, hence altering their density [31]. Other gradient media have been developed which do not readily penetrate membranes, such as Ficoll, Metrizamide, Nycodenz, Percoll, sorbitol and mannitol. Although Ficoll solutions are osmotically inert below 20% (w/v), their osmolarity increases sharply at higher concentrations. Metrizamide and Nycodenz absorb in the u.v. region due to their tri-iodinated benzene ring, possibly interfering with enzyme assay detection systems.

One of the major problems encountered in centrifugation derives from intrinsic characteristics of the membranes to be separated, i.e., their sedimentation rates and densities. The heterogeneity of each membrane population results in an overlap in their density (Fig. 1). In practice, separations are often based on a combination of differential and isopycnic centrifugations. As nuclei have a high sedimentation rate, they can be pelleted by differential centrifugation, leaving the rest of the organelles in the supernatant. However, many organelles differ only slightly in size and density and they are not readily separated by centrifugation alone. Repeated washing and pelleting and/or density gradient steps are usually necessary to obtain high-purity fractions. However, such procedures lead to a considerable loss of material and consequently to lower yields. When the subject of purification is low-abundance membranes the number of purification steps required will be greater than for high-abundance ones to obtain fractions of similar purity. Thus, there may be problems in obtaining low-abundance membranes of sufficiently high purity and in reasonable quantities for further studies. In

addition, labile components may be damaged by repeated re-suspension and re-centrifugation. In light of this, centrifugations are usually used for the initial processing of heterogeneous mixtures prior to further purification steps. Although isopycnic centrifugation improves the fractionation of membranes a disadvantage of this technique, especially in today's society where we live against the clock, is that it is rather time-consuming.

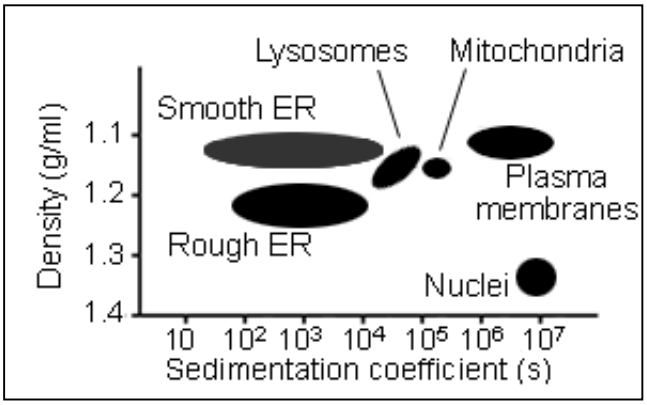

Figure 1. Sedimentation and density values for some organelles. Their overlapping profiles preclude their ready separation by means of centrifugation. Adapted from [32].

1.1.2. Immunoprecipitation

In immunoprecipitation, also called affinity adsorption, separation is based on a biospecific interaction between a membrane and a solid phase. The solid phase contains a bound ligand with affinity for specific membrane components. The solid phase can be Sepharose [33], polyacrylamide [34], cellulose [35] or magnetic beads [36]. There are two strategies for coupling the ligand to the support, either directly or via a sandwich procedure. In the first strategy the ligand, for instance an antibody, is bound covalently directly to the support. In the second strategy the ligand is linked to the support by a ligand-binding agent, which in the above example could be a secondary antibody or protein A.

Many different kinds of cells have been prepared by immunoprecipitation, for instance endothelial [37], mast [38] or B [39] cells. The number of membranes/organelles prepared by this technique is rather few and includes cholinergic synaptosomes [40] and microsomes [41]. The availability of more specific antibodies, including monoclonals, has allowed the purification of quantitatively minor membrane populations, such as vesicles derived from microdomains of the plasma membrane [36].

One of the major drawbacks of immunoprecipitation is that non-specific adsorption of contaminating membranes may occur, forming an aggregate of mixed membranes on the bead surface. In the case of immunoisolation of proteins this problem might be partially overcome by adding a non-ionic detergent (for instance, 0.1% Tween 20) both in the sample and in the washing buffer after isolation of the protein [42]. In addition to possible mechanical damage caused by such washing steps, elution of membranes from the solid support without damage is of great concern.

1.1.3. Two-phase partitioning

In 1896 Beijrinck noticed that when mixing aqueous solutions of gelatine and agar or starch two phases were formed [43]. However, it was not until the mid-1950s that Albertsson introduced aqueous two-phase systems as a separation tool in the study of biological material [44-46]. He found that two different polymers, when dissolved in water above a certain concentration, are immiscible and thus form two phases. A characteristic of such two-phase systems is that the main component (80-95%) of both phases is water. In addition to the low interfacial tension compared to organic/aqueous two-phase systems, the protective properties of the polymers against denaturation together with the high water content provide a mild environment for biological material.

The phases of a two-phase system are mixtures of both of the polymers and water, each phase being enriched in one of the polymers. For instance, in a PEG/dextran two-phase system each phase contains both PEG and dextran, the top phase being enriched in PEG and the bottom phase in dextran. The composition of two-phase systems is usually characterised by phase diagrams (Fig. 2) where the concentrations of the polymers are plotted against each other, usually expressed in % (w/w). A mixture of the polymers corresponding to a point "P" in the diagram situated above the binodial curve will separate into two phases. Conversely, a polymer mixture of concentration "M" situated below the binodial will be homogeneous forming only one phase.

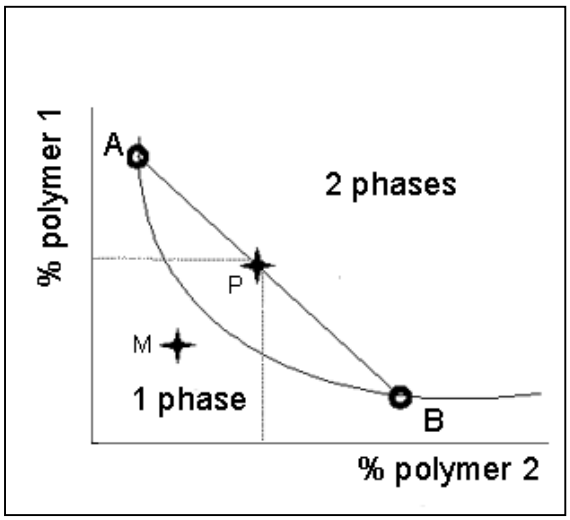

Figure 2. Schematic representation of a two-phase diagram. Polymer mixtures represented by points like "P" above the binodial curve yield two phases, while those represented by points like M below the binodial give only one phase.

From the phase diagram additional information may be obtained, such as the polymer concentration of each phase and the volume ratio of the phases. The concentration of each phase of a two-phase system with concentration P is given by the node points (A & B in Fig. 2), which are the cross-points of the tie line and the binodial curve. The volume ratio of the phases is described by [46]:

$$\frac{V_t}{V_b} = \frac{d_b}{d_t} \times \frac{BP}{AP}$$

where V_t and V_b are the volumes and d_t and d_b are the densities of the top and bottom phases respectively, BP is the distance from the node B to point P and AP from A to P. The phase diagram varies with the type of polymers used and with other system additives, such as salts, with temperature, etc. The distribution or partitioning of material between the phases of a two-phase system is usually expressed as the partitioning coefficient, K, defined as:

$$K = \frac{C^{TOP}}{C^{BOT}}$$

where C^{TOP} and C^{BOT} are the concentrations of the partitioned material in top and bottom phases, respectively. In preparative applications the distribution of the material between the phases is sometimes expressed in percentage found in one of the phases, in contrast to analytical applications where the partitioning coefficient, K, predominates.

1.1.3.1. Types of two-phase systems

The most commonly used polymer-polymer two-phase system is composed of PEG and dextran, PEG being the main component of the top phase and dextran of the bottom phase. Their molecular structures are depicted in Fig. 3. Dextran is commercially available in a range of molecular weight fractions from 10000 to 2000000. These fractions are usually polydisperse. For instance, the T500 fraction from Pharmacia, which is the most used fraction in two-phase partitioning, has a weight-average molecular weight (MW) of approximately 450000 to 500000. PEG is available from many suppliers and in a wide range of MWs. These include PEG 3350, PEG 8000 and PEG20000 (having corresponding MWs) often used in two-phase partitioning [47].

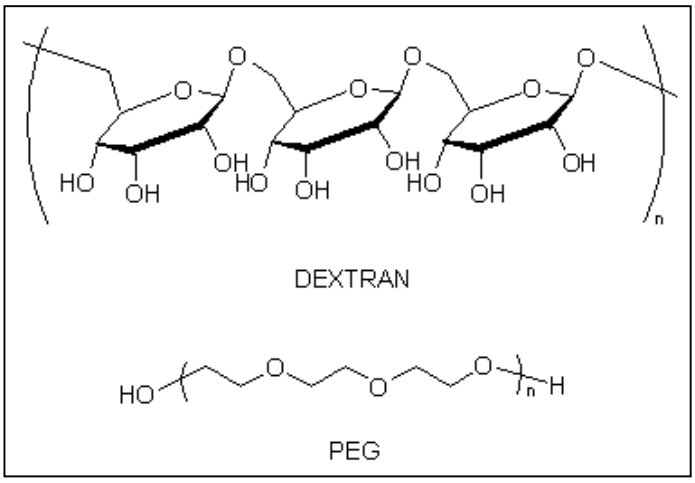

Figure 3. Molecular structures of the polymers PEG and dextran. PEG is an unbranched polymer of ethylene oxide units and dextran of glucose units linked by α-1, 6 bonds with some branches of α-1, 3 linkages.

Occasionally dextran and PEG have been substituted: some starch derivatives have replaced dextran [48,49] and PEG has been replaced by random copolymers of ethylene oxide (EO) and propylene oxide (PO), EOPO copolymers. The partitioning of proteins in EOPO/starch and in PEG/dextran systems is very similar [50]. Other alternatives for replacement of dextran are maltodextrins and pullulans [51,52]. The introduction of EOPO copolymers has led to a new feature in two-phase partitioning [50,53], i.e., the recovery of these polymers from solution by thermoseparation after phase extraction. Usually thermoseparation occurs in the temperature range of 32-50 ºC, this being too high to be ideally employed in the purification of membraneous material for which low temperatures are usually preferred.

Another kind is the polymer/salt two-phase system. This is formed when the concentration of salt is increased sufficiently in the presence of a polymer solution. The most common two-phase system of this type is composed of PEG and sodium or potassium phosphate, although other salts have also been used [54,55]. The salt-rich bottom phase contains approximately 10-15% (w/w) salt. Some advantages are that 1) the hydrophobicity difference between the two phases is greater than in two-polymer systems thereby

resulting in a more extreme partitioning, 2) they are easier to use than polymer/polymer systems, 3) phase separation is more rapid, 4) the phase components are less expensive. For all the above mentioned reasons, these types of systems are the most commonly used for industrial protein purification applications [56-58]. The high contents of salts make such polymer/salt systems unsuitable for membrane purification, as membrane structure will be affected by the high salt concentrations.

A third type of system currently available is made up of detergents. Detergents may also form two-phase systems when mixed with polymers or alone above their cloud point temperature. One widely used detergent is Triton X-114 [59]. Although these types of systems have been extensively used for the extraction of membrane proteins [60,61], it is obvious that they are definitely not appropriate for the purification of membraneous structures.

In the area of membrane and cell research conventional two-phase systems consisting of dextran/PEG-type polymers seem to be the most adequate at the moment.

1.1.3.2. Factors affecting the partitioning of material

Most hydrophilic polymer pairs are incompatible in aqueous solutions. Such polymers yield two phases in equilibrium with each other, each phase containing predominantly water and one of the polymers. The phase separation is attributed to the high molecular weight of the polymers combined with interactions between the different polymers and salts in the system [46]. The driving force for the demixing process is the enthalpy associated with the interactions of the components, which is opposed by the entropy associated with the segregation of the components during phase separation. A detailed thermodynamic description of the phase separation process is complicated and beyond the scope of this thesis.

The mechanisms through which salts influence phase separation are still poorly understood. The distribution of particulate material or molecules in solution between the phases of the two-phase system can be manipulated by controlling the salts in the system. Negatively charged material is pushed up into the PEG rich-phase by cations in the order $Cs^+ \approx K^+ < Na^+ < Li^+$ and by anions in the order $Cl^- \approx H_2PO_4^- < HPO_4^{2-} \approx SO_4^{2-} \approx$ citrate. Therefore, to get a maximal distribution of negatively charged membranes into the top phase one should use for instance Li_2SO_4, or, to push them into the bottom phase, KCl. Another factor that alters the phase behaviour is the MW of the polymers used and their concentration. A lower MW of the polymer results in a greater tendency for particles to partition into that phase [46].

Furthermore, the distribution of a specific particle between the phases of two-phase systems can be manipulated by using affinity ligands. These bind to the specific target material pulling it into one phase, leaving the rest of the material in the other phase. This is the basis of affinity two-phase partitioning and will be discussed later in this thesis (see section 1.2).

1.1.3.3. Two-phase partitioning of membraneous material

Membranes, due to their different surface properties, partition differently between the phases of two-phase systems. In the case of PEG/dextran systems the preference for the upper phase increases in the following order: mitochondria, ER, Golgi membranes and plasma membranes [62,63]. When the partitioning behaviour of membranes is relatively similar, they can be separated by multiple-step extraction, for instance using a CCD apparatus. Different kinds of membrane vesicles have been subfractionated by CCD, such as ER vesicles from rat pituitary homogenates [64], synaptosomal membranes [65], and ER and vacuolar membranes from cauliflower [66]. In some cases different regions of a specific membrane have been separated, such as from rat liver plasma membrane [63] or the fractionation of thylakoid membranes from tobacco into “end membrane” and “stroma lamellae”

membrane [67]. In addition to membrane vesicles, whole cells have also been partitioned and several populations separated by this technique. Some examples include lymphocytes [68], reticulocytes [69], erythrocytes [70], and amoebae [71].

In some cases, cells and membranes have been successfully separated by a batch two-phase partitioning procedure. However, only in the case of material from plant tissues has this procedure become the isolation method of choice. Some examples include the isolation of chloroplast membranes from spinach [72-74] and of plasma membranes from spinach [75], barley [76-79], wheat [80,81], tobacco [82-86], carrot [87], rice [88-90], maize [91,92], pea [93-95], tomato [96-98], soybean [99], cauliflower [100], mung bean [101], and red beet [102,103]. One interesting observation is that plasma membrane vesicles can be turned inside-out (cytoplasmic side-out) by freezing and thawing, and also that two-phase partitioning is sensitive enough to separate the sealed inside out and right-side out vesicles [104].

Other types of material than plant material have also been partitioned: bacterial, fungal and animal cells and subcellular membranes. However, the number of studies that use two-phase partitioning and these types of cells/membranes is not as vast as in the case of membranes derived from plant sources, indicating that two-phase partitioning is still not a technique of choice for the separation of membranes from these sources. Combinations of different centrifugation techniques are still the most common method for the fractionation of membranes derived from animal tissues, although centrifugation combined with immunoprecipitation is increasing rapidly. Some reasons why two-phase partitioning is not widely used for the isolation of animal membranes could be the ease of use of standardised centrifugation protocols, the often low resolution of two-phase partitioning for these types of membranes, and the introduction of specificity by antibodies in immunoprecipitation.

The behaviour of membranes in two-phase systems is supposed to depend on surface properties that are still rather poorly defined, the net surface

charge of membranes might be one of these. Due to the lack of knowledge of how such surface properties affect partitioning, there are difficulties predicting the behaviour of membranes in two-phase systems, particularly those derived from animal tissues. This could be another reason why two-phase partitioning is not a widespread technique for membrane purification. So far, most partitioning protocols are based on empirical findings. A systematic examination of separation variables, intrinsic characteristics of membranes that affect their behaviour in two-phase systems, as well as introduction of thermodynamic models capable of accurately predicting phase equilibrium and membrane partitioning coefficients would stimulate a wider use of the method.

1.1.4. Other techniques

In sedimentation field flow fractionation the separation occurs by differential retention of components in a stream of liquid flowing through a thin channel that encircles the centrifuge axis like a belt. The separated components are eluted one at a time through a detector [105]. Their separation depends on differences in mass, volume, or in density between the particle and the carrier liquid, with the separation modulated by the centrifugal field used. Viruses [106] as well as abnormal erythrocytes have been fractionated from normal erythrocytes using this methodology [107].

Free-flow electrophoresis is also based on the separation of material in a flow, but in contrast to the technique described above, an electrical field is applied causing the material to separate depending on surface charge. Subfractions of endosomes [108] and melanosomes [109] are examples of membranes isolated by this technique.

The use of these methodologies has been limited, however, perhaps due to the special equipment required and/or because the techniques are difficult to master.

1.2. Affinity two-phase partitioning

Conventional two-phase systems often have a comparatively low selectivity for biological membranes. As a consequence, two-phase partitioning usually results in a rather low degree of purification in batch procedures unless the extraction steps are repeated several times. One way to increase the selectivity is to introduce affinity ligands. This process is generally known as affinity two-phase partitioning.

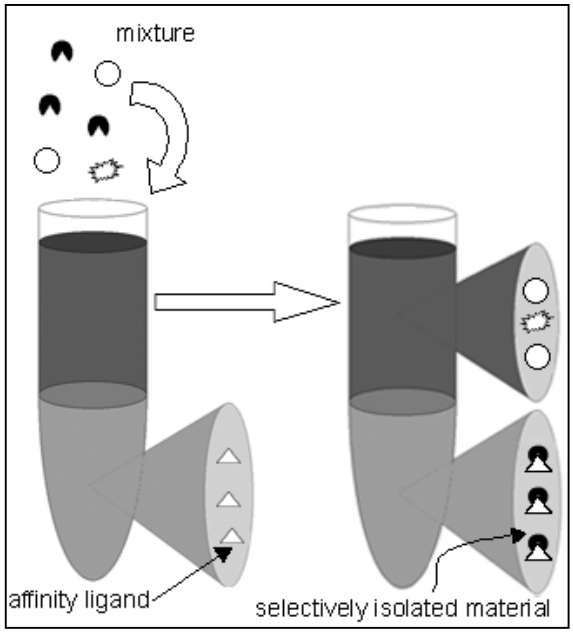

Figure 4. Affinity two-phase partitioning. The target material is selectively isolated from a mixture by interactions with an affinity ligand coupled to the bottom phase polymer.

The general idea of affinity two-phase partitioning is to partition the bulk of material (proteins or membranes) into one of the phases, and then to redistribute the material of interest (a specific protein or membrane) into the other phase by interactions with an affinity ligand coupled to the phase

polymer. The principle of affinity partitioning with an affinity ligand coupled to the bottom phase polymer is illustrated in Figure 4.

There has been a steady increase in the number of publications in the area of two-phase partitioning in contrast to affinity two-phase partitioning (Fig. 5). A reason for this might be that additional experimental steps are involved in affinity two-phase partitioning. The introduction of affinity ligands involves covalent coupling to one of the phase-forming polymers. Although protocols are available for attaching ligands to PEG or dextran, the coupling of each specific ligand may require a certain degree of modification of the procedure. In addition to this, the use of organic synthetic chemistry is often unfamiliar within the biological research community. Furthermore, the use of quite expensive ligands, to ensure a high specificity, in combination with the lack of standardised affinity partitioning protocols might be influential factors as well.

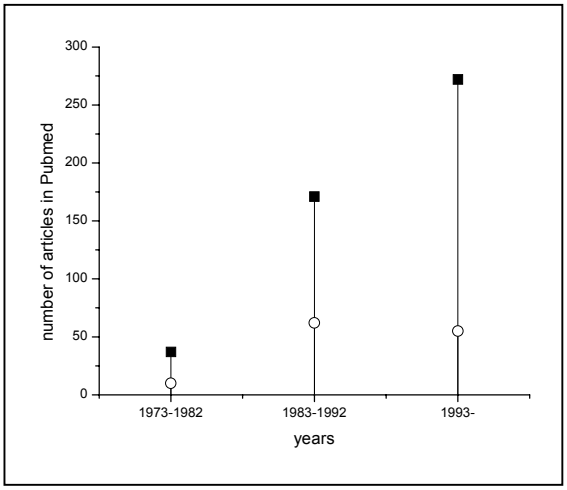

Figure 5. Number of publications in Pubmed. Concerning two-phase partitioning (■) and affinity two-phase partitioning (○).

Chromatography is another separation technique from which affinity chromatography has evolved by the introduction of affinity ligands coupled to the chromatographic resins. In comparison with affinity two-phase partitioning,

affinity chromatography is widely used nowadays. One reason why affinity chromatography has such a great acceptance among researchers could be the fact that many affinity resins are commercially available. There is also much more theoretical and practical knowledge of the chromatographic process in terms of ligand density, capacity, number of plates etc. Although affinity chromatography has been successful for the purification of proteins, the technique is not readily applicable for membrane purification. For instance, membranes often adsorb tightly to the resin and harsh conditions may be required for elution, which would probably damage the membranes. As an alternative affinity two-phase partitioning, avoiding solid supports, should be advantageous for the purification of biological membranes.

1.2.1. Affinity ligands used in affinity two-phase partitioning

In PEG/dextran affinity two-phase systems, PEG has been the most common choice of polymer as ligand carrier. In principle all phase-forming polymers are capable of carrying covalently bound affinity ligands. Some reasons as to why PEG has been the polymer of choice are that this polymer is available in different sizes, its low cost and because it appears easier to find conditions to steer proteins into the dextran phase [110]. Also, a number of methods for attachment of ligands to PEG molecules have been developed [111]. Consequently, there are abundant examples in which ligands coupled to PEG have been applied to the purification of proteins and also in some cases cells, by affinity two-phase partitioning. Examples of different types of ligands that have been used include dyes for protein partitioning [112-116] and peptides and proteins for partitioning of antibiotics or cells [117,118]. Advantages of using dyes are low cost, ease of coupling and high chemical and biological stability, while a disadvantage is that dyes are not particularly specific, often interacting not only with one protein but with several ones within the same class, for instance nucleotide-dependent enzymes [119]. Other ligands include metals used in the partitioning of both enzymes [120,121] and cells

[122-124]. An additional approach includes PEG-linked antibodies for affinity partitioning of cells [125-129].

Some protocols for coupling ligands to dextran are also available [130-132], but dextran-coupled ligands have not been applied as often as PEG derivatives in aqueous two-phase systems. Some examples include dyes [132,133] and ATP [130] used in the partitioning of enzymes. As to membraneous structures, plasma membranes from different animal tissues have been purified using the lectin wheat germ agglutinin (WGA) coupled to dextran [134,135]. WGA binds to polysaccharides containing sialic acid and N-acetylglucosamine mainly exposed on the outer surface of plasma membranes. Thus, when different membrane fragments are added into a PEG/dextran two-phase system, the plasma membrane fragments are pulled into the dextran-rich bottom phase by interaction of their surface carbohydrates with WGA-dextran, leaving other kinds of membrane fragments in the PEG-rich top phase. This approach is approximately 10 times faster than conventional centrifugal protocols, and results in at least a similar degree of purification and yield [135].

1.2.2. Factors affecting affinity partitioning

The partitioning depends on several factors, including the choice of ligand-carrying polymer, its MW and the concentrations of the phase polymers. The partitioning behaviour of a particle complexed to a ligand-polymer conjugate is determined to a large extent by the partitioning of the conjugate itself [136]. One way of increasing the partitioning of a particle-ligand-polymer complex would therefore be to increase the partitioning of the ligand-polymer conjugate. Usually, this conjugate partitions similarly to the phase polymer. As polymers partition more extremely in systems with longer tie lines [46], working with systems made up of higher polymer concentrations would presumably force the ligand-polymer conjugate more extremely into one

phase. Therefore, working with systems with longer tie lines would also increase the partitioning of the particle-ligand-polymer complex. The problem is that the further from the binodial curve, the more membranes tend to partition in the interface, a problem not encountered with proteins as these do not tend to collect at the interface. Thus, conditions yielding short tie-lines are desirable when working with membranes or cells, in comparison with affinity partitioning of proteins, in order to maintain the material in one of the phases and avoiding the interface.

As the ligand-polymer conjugate partitions similarly to the phase polymer itself, the phase polymer chosen to carry the ligand would ideally distribute with the smallest fraction possible in the other phase. In the case of the PEG/dextran system, the fraction of dextran in the top phase is usually much smaller than the fraction of PEG present in the bottom phase [46]. Therefore, it would appear to be advantageous to couple the affinity ligand to dextran rather than to PEG.

The partitioning of any particle is strongly dependent on its size: the larger the particle, the more extreme partitioning in the two-phase system [46]. When applied to a target-ligand-polymer complex, yet another way to increase the affinity partitioning of the target would be to increase the size of this complex by using a polymer of a larger MW coupled to the ligand, for instance, using Dextran T2000 instead of Dextran T500.

1.3. Why membranes?

A membrane defined the outer boundary of the first living cell nearly 4 billion years ago. Since then, membranes have evolved and with them their functions and roles in the cell. Membranes provide a means of communication between the inside and outside of the cell as well as between the compartments they delimit inside the cell. They allow passage of ions and

molecules, and they contain enzymes, which are involved in numerous fundamental cellular functions such as DNA replication, protein biosynthesis and secretion, and electron transport and ATP synthesis in mitochondria. They also allow passage of information, transmitted through conformational changes induced in membrane components. Transmembrane signalling is a primitive and ubiquitous function of membranes. Fossil evidence shows that cyanobacteria are the oldest known living cells, yet they are able to respond to their environment. Examples of biomolecules participating in transmembrane signalling in higher organisms include hormones, neurotransmitters and growth factors.

The key roles of membranes and membrane components in the life of all kinds of cells has turned the focus of biology, biochemistry, biophysics, medicine and other disciplines towards them: understanding how membranes work at the molecular level is a major goal of biological research of today.

1.3.1. History of membranes

The modern study of membranes began separately in 1925 with Fricke [137] and Gorter and Grendel [138]. Using different techniques, they concluded that cells are surrounded by a hydrocarbon layer approximately 5 nm thick, a lipid bilayer. This theory was further elaborated by Danielli and Davson [139] in 1935 with the “paucimolecular” model, a lipoid core covered by protein layers. In the 1950’s advances in electron microscopy (EM) allowed visualization of membranes as trilamellar structures. Later, with the arrival of NMR technology, came the first indications that membranes have a certain level of fluidity and in 1972 Singer and Nicholson [140] formulated the fluid-mosaic model. Since the 1970’s, stimulated by the Singer and Nicholson model, there has been a dramatic increase in our perception of membrane structure and function. Our understanding of membranes arises from development of new techniques, such as spectroscopy, use of detergents, application of antibodies and DNA cloning, to membrane research.

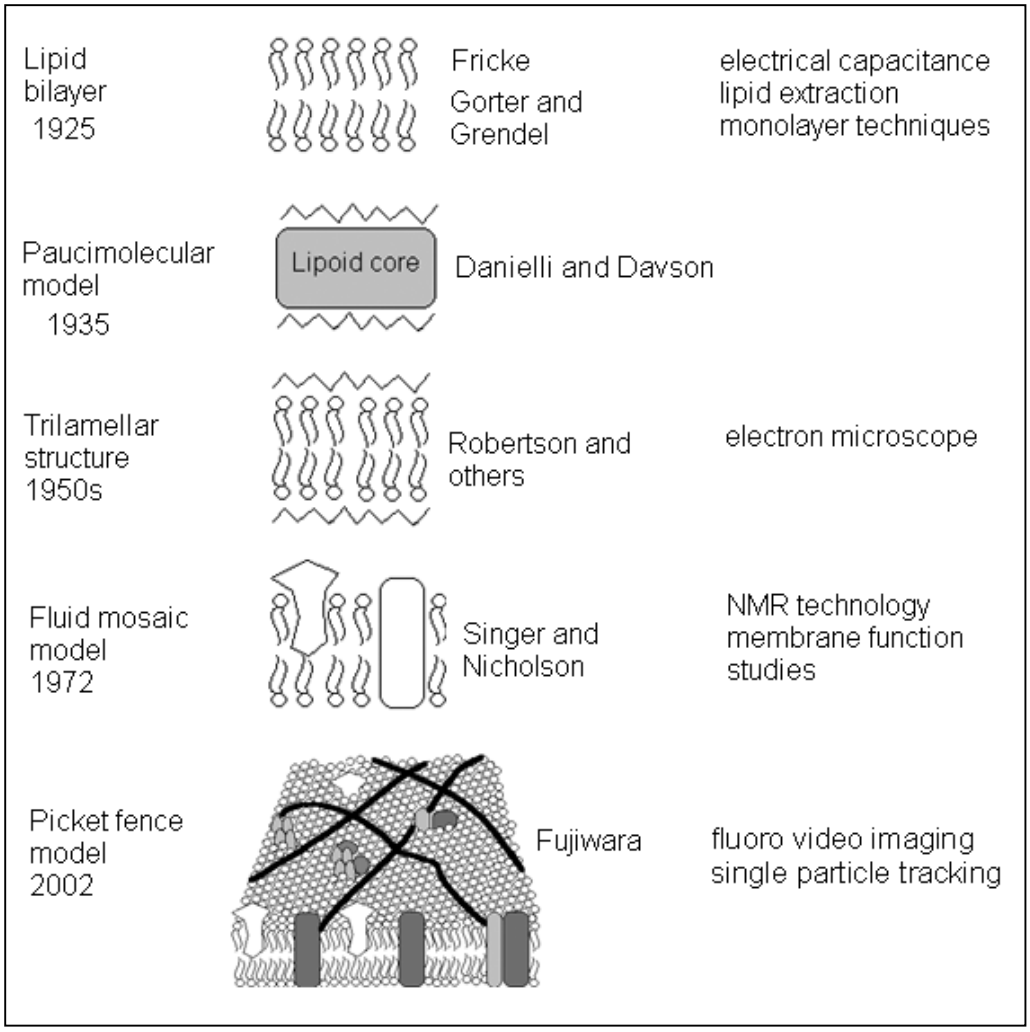

Figure 6. Membrane model evolution. Different models of membrane structure are sketched together with an indication of some techniques used at the time that helped design the models.

The Singer and Nicholson model assumes that membranes are largely homogeneous structures, but it is now clear that membranes are heterogeneous in different respects. Apart from transverse asymmetry assumed in the model, i.e., the inner and outer leaflet contain different sets of protein and lipid, there is also lateral asymmetry as in the case of the apical and basolateral domains of liver plasma membranes. In the last decade the existence of microdomains within membranes has also been documented [141]. Fujiwara *et al.* recently proposed a new model, an anchored membrane-protein picket fence model, based on calculations of diffusion rates of single molecules in a membrane [142]. Their model postulates that the cell

membrane is compartmentalised and that this compartmentalisation depends on an actin-based membrane skeleton. Transmembrane proteins anchored to the skeleton act as posts along the skeleton fence (Fig. 6).

Membrane models will probably continue to undergo modifications as new techniques become available providing new data leading to a reassessment of our view of these intriguing structures that are membranes.

1.3.2. Structure of membranes

The major components of membranes are protein and lipid where the protein part may constitute from 20 to 80% (dry weight) of the membrane. In addition, membranes may contain carbohydrate, in some cases as much as 10%, usually in the form of glycolipid or glycoprotein [32]. Although virtually all membranes share structural similarities, i.e., they are composed of a lipid bilayer with embedded proteins, they also exhibit very different functions, from the mitochondrial membrane with the highest protein to lipid ratio to neuronal membranes with the lowest ratio. Even when their protein to lipid ratio is very similar, membranes have different functions due to the different types of proteins and/or lipids they contain. Each membrane is a unique world, in which different processes and functions take place.

Membrane proteins are associated with the bilayer by a variety of means. Some span the membrane, integral proteins, interacting through nonpolar surfaces with the hydrophobic core of the bilayer. Others are associated with the membrane surface, peripheral proteins, through a combination of electrostatic and hydrophobic noncovalent interactions. Others again have covalently bound lipids that facilitate association to membranes [143].

All membranes have two faces, each exposed to a different environment, for instance, the cytoplasmic and the lumenal surface of intracellular membranes or the cytoplasmic and the exterior ones in the case of the plasma membrane.

The monolayer halves of the bilayer are different in composition, conferring a transverse asymmetry to the membrane.

In addition to transverse asymmetry, membranes show lateral asymmetry. Many membranes possess distinct macroscopic and microscopic domains. Macroscopic domains are morphologically visible and often separated by molecular barriers, e.g., the apical and basolateral domains of the liver plasma membrane or the stacked and stroma-exposed regions of the thylakoids [144]. Each domain contains its unique set of proteins. Other macroscopic domains can be formed due to protein aggregation as in the case of connexin in gap junctions [145].

Lateral asymmetry can also be found in the prokaryote kingdom. For example, gram-negative bacteria contain adhesion zones that appear to connect the inner and outer membranes [146].

Microscopic lipid domains are defined as small regions within the bilayer with distinct physical properties and composition. Plasma membranes have been extensively investigated biochemically and biophysically and the coexistence of two major kinds of microdomains within the plasma membrane are recognised [147]. These domains are usually called lipid rafts. One type of raft is enriched in glycosylphosphatidylinositol (GPI)-anchored proteins and the other in caveolin, these latter structures being termed caveolae.

In the picket fence model, Fujiwara *et al.* proposed that compartmentalisation into such microdomains of the eukaryotic cell membrane depends on transmembrane proteins anchored to the actin skeleton acting as posts along the skeleton fence. They measured the diffusion rates of phospholipids within these compartments and how these rates decreased when the lipids hopped to the next compartment.

The existence of lipid microdomains is not limited to plasma membranes, but might be a general feature of biomembranes. For instance, the rough endoplasmic reticulum (ER) has been shown to be laterally heterogeneous in

several subfractionation studies [148,149]. Other reports have also indicated the heterogeneity of the smooth ER, and Gierow *et al.* in the late 1980´s studied this heterogeneity by two-phase partitioning. They succeeded in further fractionating smooth microsomes by CCD into at least five populations having different proportions of marker enzymes [150].

Lipid microdomains might be advantageous for cell function. Enzymes within the same membrane could be sequestered in unique environments where their activities may be optimised or regulated by specific protein or lipid interactions. At the same time the boundaries between domains would present packing discontinuities as in lipid phase transition where gel phase and liquid-crystalline phase coexist. From model studies it seems that such co-existence of phases will enhance passive transport several-fold [151]. Signal transduction and budding and fusion in vesicle transport are some cellular processes that might be explained in terms of presence or formation of microdomains [152,153].

1.3.2.1. Plasma membrane microdomains: rafts and caveolae

Many important functions of cellular membranes are closely associated with various specialised domains in the membrane. These domains include macrodomains such as tight junctions and microdomains like clathrin-coated pits, lipid rafts and caveolae.

Rafts and caveolae are microdomains within the plasma membrane of higher organisms. One shared structural characteristic is that they are detergent-insoluble. Caveolae are cell surface invaginations stabilised by structural proteins (caveolins) and deficient in GPI-anchored proteins, whereas lipid rafts do not invaginate, are enriched in GPI-anchored proteins and depleted in caveolin. Both types of microdomains have some common constituents, such as cholesterol, whose distribution between the two compartments is dynamic. Rafts have an average size from 25 to several hundred microns while

caveolae have a diameter of 60-80 nm [147]. Both types of microdomains are supposed to be involved in signalling. Some caveolin-interacting proteins involved in signalling are G-protein-coupled receptor kinase, protein kinase A, adenylyl cyclase and PKCα [154]. In contrast, rafts are associated to other transmembrane signalling proteins such as *Rho-A* and the interleukin receptor [155,156], thus possibly functioning in other signalling pathways than caveolae. Both microdomains are dynamic within the cell surface and might interact with each other. It has been reported that GPI-anchored proteins (usually found in rafts) may enter caveolae upon cross-linking with antibodies [157]. The relationship of all components involved in the regulation of signalling through these compartments is still poorly known. Only a few years ago, in 1998, Iwabuchi and co-workers managed to separate these microdomains from each other [158]. The lack of techniques to purify rafts and caveolae and the difficulty to observe them in vivo has impeded an understanding of their functions. Advances in light microscopic techniques have allowed the visualization of rafts. Single particle tracking microscopy allows the study of particle movements with submicron spatial resolution on the surfaces of living cells [159]. A combination of results from this new biophysical technique with those from in vitro subfractionation studies indicates that rafts occur in vivo and thus are not preparation artefacts.

1.3.3. Liposomes as model membranes

Liposomes have been widely used as model membranes because they are simplified lipid bilayers, lacking other components as proteins or carbohydrates present in biological membranes. Liposomes have also been used for the introduction of genetic material into cells [160], the co-presentation of antigens to lymphocytes [161], and in chemotherapy [162] and immunotherapy [163] as a drug delivery system.

Liposomes are vesicles in which an aqueous volume is enclosed by a lipid bilayer. Liposomes can be prepared from extracted constituents of biological

membranes to obtain the same lipid composition as in the membranes. Hence, the value of liposomes as model membrane systems.

Liposomes can be unilamellar or multilamellar (MLV) vesicles. These latter are usually 100-1000 in nm diameter with 5 or more concentric lipid bilayers. Unilamellar liposomes are usually classified according to their size into small unilamellar vesicles (SUV), approximately 20 nm diameter, and large unilamellar vesicles (LUV) with diameters of 1000 nm. Intermediate unilamellar vesicles have a size in between those of SUVs and LUVs [164].

There are different methods for preparing liposomes and depending on the preparation method chosen, liposomes can be obtained in different sizes and consisting of a single bilayer or multiple concentric layers. A common characteristic of the methods for liposome preparation is that the first step is usually to dry the lipids from organic solvents followed by dispersion in aqueous solution. When lipids are dispersed in an aqueous solution, multilamellar structures form spontaneously. Some examples of preparation methods are sonication, French Press treatment and membrane extrusion. Sonication of MLVs yields vesicles of the smallest size possible and is the most widely used method for producing SUV. The French Press technique yields intermediate sized liposomes (30-80 nm) by extrusion of preformed liposomes under high pressure. Membrane extrusion is a gentler method of reducing the size of liposomes by passing them through a membrane filter of defined pore size. This can be achieved at lower pressures than those of the French Press and defined sizes can be obtained depending on the pore size of the filter used [164].

It is also possible to reconstitute membrane proteins, such as transporters and receptors, into liposomes where their characteristics can be more easily investigated than in their original membranes [165]. Proteins and other types of molecules such as biotin can be covalently attached to the surface of liposomes. Protein-conjugated liposomes are used in diagnostic applications and as targeted drug delivery systems. Biotinylated liposomes have been

used in the work presented in this thesis (papers I, II and IV) as models for membranes in affinity separation studies.

2. Present investigations

The objective of this thesis work was to develop affinity two-phase partitioning to become a general biospecific purification tool for membranes. The method should be particularly useful for less abundant membranes or membrane subfractions. As is evident from the discussion in the previous sections, different techniques are used for the purification of membranes, each of them having advantages and drawbacks. No technique alone is sufficient for the isolation of a specific highly purified membrane fraction; rather, combinations of two or more techniques usually have to be employed. This is particularly evident in the purification of less abundant membranes. It is therefore of interest to have at hand as many fractionation techniques as possible, based on different separation principles. In contrast to centrifugation, the most common and rather unspecific fractionation technique, affinity two-phase partitioning introduces specificity by the use of an affinity ligand. In addition, separations are performed in a mild aqueous environment, also avoiding the shear effects of centrifugation or the non-specific adsorption to solid phases of immunoprecipitation. As an example, caveolae, a microregion of the plasma membrane, was purified by combining centrifugation steps with affinity two-phase partitioning in paper III.

When this work started there were some reports concerning affinity two-phase partitioning for the purification of membraneous material, but little was known about why the method works and the limitations of the technique. Thus, examination of separation variables was imperative before the method could be generally applied.

The first objective was to define basic parameters required for affinity partitioning of membranes. This was done using a model system of biotinylated liposomes with NeutrAvidin-dextran as affinity ligand (papers I & II). A next step was to partition biotinylated biological membranes instead of liposomes with the same affinity ligand (paper II). Then, based on the

conditions found for affinity partitioning of biotinylated membranes, caveolae were purified. In this case antibodies were used as immunoaffinity ligands. The introduction of a primary antibody provided specificity and the combination of biotinylated secondary antibody interacting with NeutrAvidin-dextran intended to make the technique generally applicable (paper III). Finally, the possibility of performing micro-scale affinity two-phase partitioning was investigated in paper IV.

2.1. Affinity two-phase partitioning of membraneous material

2.1.1. Model membranes: biotinylated liposomes (papers I and II)

From the various kinds of liposomes that can be prepared (see review in [164]), we chose to work with SUV prepared by sonication as model for membranes. These liposomes are uniform in size and easy to prepare. The affinity pair chosen for the affinity partitioning experiments was avidin/biotin, because their affinity constant is extremely high (K_a = 10^{15} M^{-1}) [166] and should not be a limiting factor. Biotin was introduced into the liposomes by incorporation of biotinylated phosphatidylethanolamine (PE). As for avidin, the deglycosylated form NeutrAvidin was used to avoid unspecific binding without affecting the interaction with biotin [167]. In paper I, NeutrAvidin was coupled to Dextran T500, which was also the bulk bottom phase polymer in the PEG/dextran two-phase system. In paper II NeutrAvidin was coupled to Dextran T2000 instead and with Dextran T40 as bulk phase polymer. In both types of systems the liposomes partitioned equally well to the top phase in the absence of affinity ligand. In its presence, however, a slight increase in partitioning towards the bottom phase was observed when the ligand-coupled polymer was noticeably larger than the phase polymer. Therefore NeutrAvidin was coupled to Dextran T2000 and Dextran T40 was chosen as phase polymer.

In a previous study, biotinylated liposomes composed solely of phosphatidylcholine (PC) had been extracted by NeutrAvidin-dextran into the bottom phase of a PEG/dextran two-phase system [168]. Membranes biotinylated in the same manner could not be extracted, however. Therefore, in paper I, a more complex membrane model was introduced to investigate various partitioning parameters, as well as to optimise the system for future applications with biological membranes. Liposomes containing a mixture of two different lipids, PC together with one of the phospholipids phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI) or PE were partitioned in the two-phase system. PC liposomes are electroneutral, while membranes carry a negative net surface charge. Introduction of the negatively charged PS, PG and PI into PC liposomes were therefore thought to make these liposomes resemble biological membranes more.

Conditions were chosen to partition all material into the top phase of the two-phase system in the absence of NeutrAvidin-dextran. In the presence of NeutrAvidin, instead, the biotinylated liposomes were expected to partition in the bottom phase due to interactions between NeutrAvidin and biotin. Conversely to expectations, this behaviour was only observed with biotinylated liposomes containing less than 10% (w/w) of negatively charged phospholipids; biotinylated PC liposomes including 10 % or more of PS, PG or PI were found in the top phase. However, PC liposomes containing up to 75% of the electroneutral PE distributed in the bottom phase in a NeutrAvidin-dependent manner similar to liposomes consisting of PC only. These results indicated that the introduction of negative charges in the liposomes weakened the otherwise very strong interaction between biotin and NeutrAvidin.

The effect of charge on the biotin-NeutrAvidin interaction was further investigated by altering the net surface charge of either liposomes or NeutrAvidin. To assure that the behaviour of the liposomes was not inflicted by the polar head group of the phospholipids, the charge of electroneutral PC liposomes was this time altered by introduction of the negatively charged

surfactant sodium dodecyl sulphate (SDS) or the positively charged dodecyltrimethylammonium bromide (DTAB). The charge of NeutrAvidin was modified by changing the pH of the affinity two-phase system in a pH interval where the surface charge of the liposomes was unaffected. It was found that biotinylated PC liposomes, negatively charged by inclusion of SDS, distributed in the PEG-rich top phase, both in the presence or absence of NeutrAvidin, in the same manner as PC liposomes including negatively charged PS did (Fig. 7 A). When the negative charges of these liposomes were neutralised by inclusion of DTAB these liposomes instead partitioned into the bottom phase (Fig. 7 B). When the charges on NeutrAvidin were modified by lowering the pH of the affinity two-phase system below its isoelectric point (pI=6.3), the negatively charged PC liposomes containing PS partitioned in the bottom phase (Fig. 7 C).

An explanation for these findings comes from the structure of avidin and its biotin-binding site. The avidin molecule forms a beta-barrel and the biotin-binding site is situated deeply in a pocket close to one end of the barrel, 1-1.2 nm below the avidin surface [169]. Due to this structure biotin was attached via a 0.9 nm long spacer arm to avoid steric hindrance and allow biotin to reach and bind to the binding site properly. This was also the case for neutral PC liposomes [168]. The effect of negative charges of the liposome surface on the biotin-NeutrAvidin interaction was highly unexpected, however, as the avidin-biotin interaction is extremely high, in fact the highest affinity found between biological molecules. An interpretation is that acidic amino acids in the loops at the rim of the biotin-binding pocket of NeutrAvidin may repel negatively charged residues of the closely apposed liposome surface affecting the affinity interaction.

The effect of the charges of the surroundings may not only be crucial in affinity two-phase partitioning employing the biotin-avidin interaction, but also in other affinity techniques where similar ligand interactions are involved. Wider implications of such electrostatic effects might be in model binding studies and in the regulation of natural processes. Electrostatic repulsion

could be part of regulatory mechanisms in protein-protein interactions if at least one protein is embedded in a membrane, such as in receptor-effector interactions, or when interacting proteins are embedded in two different membranes, such as in the docking of transport vesicles. Regulation of the surface charge of a vesicle, for instance by phosphorylation reactions, could be a means of regulating the permitted proximity and thus, in the latter example, docking.

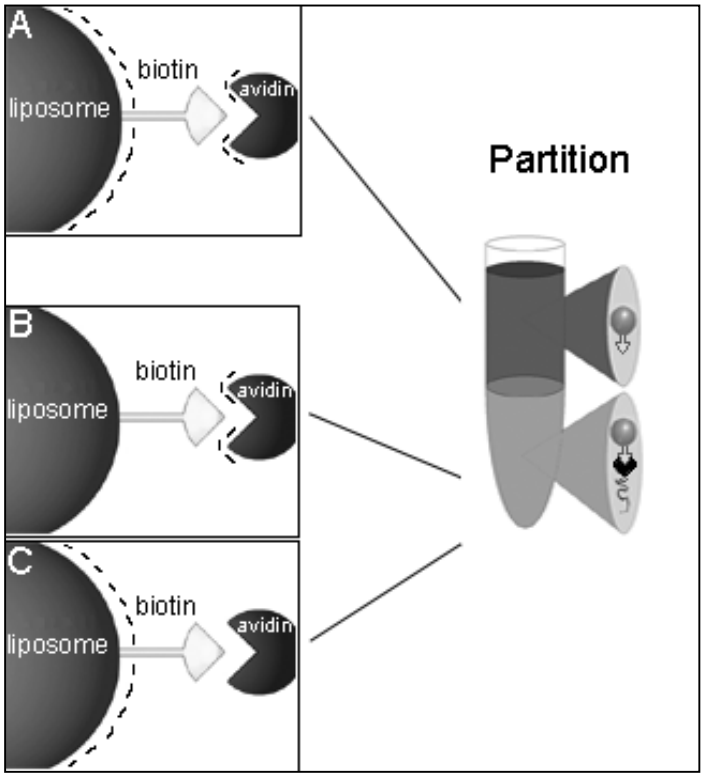

Figure 7. Affinity partitioning of liposomes under different charge conditions. A) Both liposome and NeutrAvidin are negatively charged and liposomes partition in the top phase. If either liposome (B) or affinity ligand (C) has no net negative charge, interactions between biotin and avidin are allowed and the liposomes are pulled down into the bottom phase.

Results from paper I indicated that charge limitations of the affinity interaction could be overcome by three approaches. The first and second approaches involve the alteration of the net charge of either liposomes or NeutrAvidin,

respectively, while the third one involves the physical separation of the charged surfaces of the liposome and NeutrAvidin, thereby decreasing the repulsive force. As liposomes are used merely as membrane models, alteration of their net surface charge or performing the affinity two-phase partitioning at low pH values were not ideal solutions. The third and most attractive approach involves the use of a longer spacer to keep liposome and NeutrAvidin sufficiently apart to avoid not only steric hindrance but also electrostatic repulsion. This approach could feasibly also be applied to membrane fractions with a more or less negative net surface charge. Consequently, the use of a longer spacer would facilitate the development of affinity partitioning as a general tool for membrane purification.

In paper II, the use of a hexanamidohexanoyl spacer (1.7 nm), instead of the earlier amidohexanoyl one (0.9 nm) in negatively charged PC liposomes including PS increased the partitioning of the liposomes into the bottom phase in the presence of the affinity ligand. This corroborated the previous hypothesis that an increase in the distance between the charged liposome surface containing biotin and the charged loops of NeutrAvidin would favour their interaction. These results were further interpreted in terms of the Debye Length (D.L.) [170], which is the distance between two charged species where the repulsion is attenuated (Fig. 8). In other words D.L. represents the restrictive distance between two charged species. The D.L. is strongly influenced by the ionic strength of the solution. At 20 mM Li_2SO_4 the negatively charged liposomes carrying the short spacer were found predominantly in the top phase, indicating that the affinity interaction was not fully achieved (Fig. 8 A). Under these conditions, the D.L., i.e., the restrictive distance between the liposome surface and the loops of the NeutrAvidin pocket, was such that the biotin moiety did not reach the binding site at the end of the pocket. When the spacer length was increased, 50-65% of the liposomes partitioned to the bottom phase. A possible explanation for this observation is that the biotin moiety penetrates further into the pocket but does not reach the binding site completely (Fig. 8 B). Thus, taking into account that avidin is a dynamic structure, it is a matter of probability to find

50-65% of the liposomes bound to avidin. Increasing the Li_2SO_4 concentration decreased the restrictive distance (D.L.) and liposomes with biotin attached via the long spacer were then found predominantly in the bottom phase of the two-phase system, indicating that the biotin moiety reached the binding site and optimum binding occurred (Fig. 8 C).

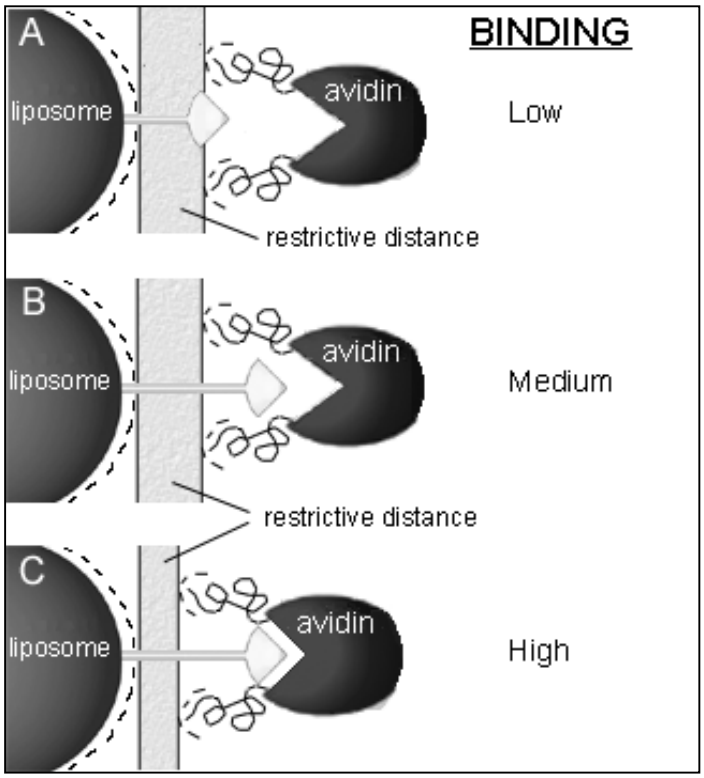

Figure 8. Influence of D.L. (restrictive distance) and spacer length on binding. At 20 mM Li_2SO_4 (A & B) the D.L. is longer than at 35 mM Li_2SO_4 (C). The biotin moiety does not reach the binding site with a short spacer (A). A longer spacer (B & C) allows it further into the pocket. Only when the D.L. is decreased and the long spacer used (C) does biotin reach the binding site at the bottom of the pocket properly, allowing optimum binding.

Addition of Li_2SO_4 was not only critical for shielding the electrostatic repulsion (decreasing the D.L. distance), but also to distribute negatively charged biotinylated mixed liposomes to the top phase of the two-phase system. A minimum of 5 mM Li_2SO_4 was required to push the negatively charged

liposomes into the top phase but up to 35 mM was necessary for the affinity ligand to redistribute the liposomes into the bottom phase. An optimum working concentration was found between 35 and 60 mM Li_2SO_4. In this range *ca.* 90% of the liposomes partitioned to the top phase in the absence of affinity ligand and 95% to the bottom phase in its presence. A rough thermodynamic interpretation of this observation could be as follows: from a thermodynamic point of view, the two main factors determining the partitioning of the liposomes in the two-phase system are the contribution of hydrophobic interactions between liposomes and phase components and the contribution of electrostatic interactions. When no Li_2SO_4 is present, negatively charged PC liposomes containing PS distribute to the bottom phase presumably due to their preference for dextran molecules as these are more hydrophilic than PEG. Adding salt to the system increases electrostatic interactions. As sulphate ions prefer the dextran-rich bottom phase and lithium ions the PEG-rich top one [46], the negatively charged liposomes are forced into the top phase. It appears that 35 mM salt produced the ionic strength required to decrease the D.L. and allow interaction between NeutrAvidin-dextran and biotin. At higher salt concentrations the liposomes distribute again in the bottom phase with and without affinity ligand. This could be explained by the effect of higher concentrations of salts on the binodial curve; the position of the binodial curve in the phase diagram moves towards higher polymer concentrations under such conditions. Consequently, the overall composition of the phases of the system become more similar to each other and the contribution of hydrophobic interactions seems to dominate over electrostatic ones, possibly explaining the observed partitioning of negatively charged liposomes at high salt concentration.

A conclusion to be drawn from this analysis is that an increase of the salt concentration to decrease the D.L. is a good solution to avoid charge repulsion between closely apposed surfaces as long as this salt concentration does not affect the binodial curve of the phase system considerably.

The effects of the concentration of NeutrAvidin-dextran and the density of biotin on the surface of the liposomes were investigated to establish minimal requirements for affinity partitioning of liposomes using the biotin-NeutrAvidin interaction. At the highest amount of NeutrAvidin-dextran tested, 60 μg, the affinity ligand is in 10-fold excess over the amount of liposomes added to the system. Under these conditions, liposomes carrying the short or long spacers distributed similarly in the two-phase system. It was only when less NeutrAvidin-dextran was used that liposomes having the longer spacer distributed slightly more into the bottom phase than those with the short one. At all NeutrAvidin concentrations, the increase of biotin on the surface of the liposomes (from *ca.* 15 to *ca*. 30 biotin residues per liposome) increased their distribution into the bottom phase.

Once the conditions had been established for the affinity partitioning of liposomes having different charges, liposomes composed of a more complex mixture of phospholipids to reflect the lipid diversity in biological membranes were tested. These liposomes had a similar lipid composition to that of rat liver microsomes with a total of 11.8% of negatively charged phospholipids (58% PC, 29% PE, 3.6% PS, 1.2% PG and 7% PI). These liposomes were found in the top phase in the absence of affinity ligand, while 98% distributed in the bottom phase with NeutrAvidin-dextran.

2.1.2. Biological membranes: biotinylated microsomes (paper II)

Having established conditions for the affinity partitioning of complex model membranes, the next step was to examine whether biological membranes behave in the same way in affinity two-phase systems under similar conditions. Rat liver microsomes were isolated by centrifugation and biotinylated in the same manner as liposomes by inclusion of a biotinylated lipid. The distribution of biotinylated microsomal membranes in the two-phase system was followed by monitoring the total protein and specific markers for

two main membrane populations of this fraction, i.e. 5'-nucleotidase for plasma membranes and arylesterase for ER.

With the exception that 35 mM salt was required to distribute them into the top phase, all three markers (protein, plasma membranes and ER) partitioned similarly to the negatively charged liposomes in the studied salt range and with long and short spacers. This indicates that the negatively charged PC liposomes containing PS are the simplest models to predict membrane partitioning, in contrast to the liposomes containing solely PC.

In view of the fact that liposomes can predict the partitioning of membranes fairly well, the lipid constituents of biological membranes seem to have an important role in their partitioning behaviour. The fact that liposomes do not predict the exact behaviour of microsomes in two-phase systems indicates that other membrane components than lipids also play a role. Proteins, for instance, could affect the partitioning of membranes by introducing extra charges or altering the local distribution of the lipids on the membranes.

The specificity of the technique was assessed by partitioning a mixture of biotinylated microsomal membranes and non-biotinylated liposomes. All the material stayed in the top phase in the absence of NeutrAvidin but only biotinylated membranes distributed in the bottom phase in the presence of the affinity ligand, indicating that the separation of biotinylated membranes from liposomes was due to the specific interaction of biotin and NeutrAvidin.

In conclusion, we have developed a system in which we can evaluate the influence of different parameters on the affinity two-phase partitioning of liposomes and furthermore affinity partitioning of biotinylated membranes exploiting the avidin-biotin interaction has been made possible.

2.1.3. Membrane microdomains: caveolae (paper III)

Although affinity partitioning of membranes was first introduced in 1975 by Flanagan *et al.* [171], the technique has only become a useful method for the isolation of plasma membranes utilising the lectin wheat germ agglutinin as affinity ligand [134,135,172,173]. Receptors suitable for affinity partitioning are less common in many membranes, however, impeding a general use of the method. We have, therefore, examined the possibility of exploiting antigen-antibody interactions as a basis for affinity partitioning.

To make the affinity method readily applicable to various antibodies an immunoaffinity sandwich approach was explored, using a specific primary antibody, a biotinylated secondary antibody and NeutrAvidin-dextran as affinity species. Reasons for introducing a biotinylated secondary antibody, rather than biotinylating a primary antibody are their commercial availability and because it obviates the need to separately biotinylate each primary antibody to be tested as an affinity ligand. In addition, conditions required for two-phase affinity partitioning based on the biotin-NeutrAvidin interaction have already been established in papers I and II.

We used anticaveolin-1 as primary antibody which interacts with caveolin, an integral protein and marker of caveolae vesicles. Caveolae are often-studied microdomains of the plasma membrane. We chose to purify caveolae by affinity two-phase partitioning not only because they presumably are involved in important cellular processes, such as signal transduction, but also because they are difficult to isolate with the range of fractionation techniques available today. They also have a defined size and are readily recognised by EM. The two-phase system chosen was that used previously in paper II but with borate buffer instead. Using this system all membrane material will distribute in the PEG-rich top phase in the absence of one or all affinity ligands. The use of borate buffer causes plasma membranes to distribute slightly more into the top phase than when HEPES buffer with Li_2SO_4 was used. In addition, the choice of borate obviates the need of extra salt in the system.

As an initial experiment this immunoaffinity sandwich approach was tested on caveolae-enriched fractions prepared by Triton X-100 treatment of plasma membranes followed by centrifugation in sucrose gradients. This fraction partitioned in the top phase in the absence of the affinity ligands. In the complete affinity system, containing primary and secondary antibodies as well as NeutrAvidin-dextran, the material distributed instead predominantly in the dextran-rich bottom phase indicating that the interactions in this immunoaffinity sandwich approach were sufficiently strong to redistribute caveolin-containing material from the top to the bottom phase.

Another microdomain of the plasma membrane, besides caveolae, that is also resistant to detergent treatment is lipid rafts. These rafts are therefore a likely contaminant of the caveolae-enriched fraction obtained by sucrose gradient centrifugation. Lipid rafts are characteristically bigger as visualised by EM and enriched in the GPI-linked 5'-nucleotidase. The selectivity of the immunoaffinity system and purity of the obtained material was assessed by EM visualisation and determination the caveolin/5'-nucleotidase ratio of the vesicles obtained in top and bottom phases.

EM revealed an uneven distribution of vesicles of different sizes between the phases of the affinity system. The bottom phase presented small vesicles resembling the size of caveolae given in literature and a high caveolin/5'-nucleotidase ratio. This ratio was similar to that of the caveolae-enriched fraction prepared by centrifugation, or higher in the case of the liver material. This difference presumably reflects the more pronounced heterogeneity of the liver fraction as seen in the EM pictures. The top phase contained larger vesicles, presumably representing lipid rafts as their size is in agreement with values given in literature for lipid rafts.

Selectivity of the immunoaffinity system was further investigated by following the distribution of the endoplasmic reticulum marker NADH-ferricyanide reductase of extrinsically added microsomes to a caveolae-enriched fraction. The ER marker partitioned in the top phase, as when microsomes were added

to the system alone, while caveolae were pulled to the bottom phase by the affinity components.

The next step was the purification of caveolae from a cruder fraction, such as Triton X-100-treated plasma membranes, more heterogeneous in vesicle size and with a 20 times lower caveolin/5’ ratio. The caveolin marker distributed in the same manner as in previous experiments, i.e. predominantly in the bottom phase, and EM examination of the phases showed a similar uneven distribution of vesicles, i.e. with smaller vesicles in the bottom phase and larger ones in the top.

When the material was extracted from lung sources, electron micrographs and the caveolin/5’-nucleotidase ratio were similar in the caveolae-enriched fraction obtained by gradient centrifugation before as well as after affinity partitioning and also in the Triton X-100-treated plasma membranes after affinity partitioning. The vesicles were relatively small and they had the similar high caveolin/5’-nucleotidase ratios. A conclusion is that caveolae had been purified and that affinity two-phase partitioning yielded a fraction similar in purity as the one obtained by centrifugation. In the case of fractions prepared from liver tissue the caveolin/5’-nucleotidase ratio of Triton X-100-treated plasma membranes increased only 1.5-fold upon density gradient centrifugation and 3-fold after affinity two-phase partitioning, indicating that the affinity method provides a more pure fraction in just one step, also supported by the electron micrographs. The lower ratio found in liver compared to the lung material is in agreement with the relative quantity of caveolin and 5’-nucleotidase in plasma membranes of those tissues. Caveolin is more abundant in lung plasma membranes and 5’-nucleotidase in liver ones. Nevertheless, it would be interesting to examine whether the relative distribution of 5’-nucleotidase between rafts and caveolae would be different in lung and liver.

Also, the Triton X-100-treated plasma membrane fraction from lung was used to investigate the effect of the concentration of ligands as well as the loading

capacity of the affinity system. The material distributed to the bottom phase in a NeutrAvidin dependent manner similar to that observed for liposomes previously, although higher concentrations were required here. The distribution was also dependent on the concentration of the antibodies, where high concentrations appear to aggregate the membranes, decreasing their preference for the top phase in the absence of NeutrAvidin. As to the loading capacity of the system, it was found to be approx. 25 µg membrane protein / g system at the set concentrations of affinity ligands.

Finally, affinity two-phase partitioning might be useful for the purification of caveolae from other sources as, for instance, adipocyte tissue as indicated by some preliminary tests done in the laboratory, and for the isolation of other membrane fractions using this immunosandwich approach but with other suitable primary antibodies to membrane proteins as affinity ligands.

2.2. Miniaturisation of affinity partitioning in levitated drops
(paper IV)

Miniaturisation is a trend in many fields, including chemistry, which has generated considerable interest over the last decade and it has become very much a hot topic recently. Some reasons for moving to smaller-scale synthesis, fractionation and analysis include high sample throughput, cost reductions and, particularly within biochemistry, that fractionation and analysis on a smaller scale requires less biological material.

The possibility of performing affinity two-phase partitioning in a miniaturised system was examined in paper IV. Two-phase systems similar in composition to those used on a larger scale (1 ml) in paper II were used, containing NeutrAvidin-dextran as affinity ligand and with biotinylated PC liposomes as model material. The liposomes were heavily tritiated to allow the detection of small quantities of material.

Drops of approximately 1 µl can be trapped in a node of a standing ultrasonic wave in a levitator [174-176]. The open environment of the levitator, together with the small volume of the drop, causes significant evaporation. This was followed by measuring the drop volume aided by a microscope and a computer program. The evaporation could be compensated for by addition of picolitre droplets of water by a specifically made dispenser. Thus, changes in the polymer concentration of the two-phase drop and consequently in the affinity partitioning could be avoided. Drops were kept levitated up to 15 min without significant decrease in volume, but presumably drops could stay in a node for longer periods of time unaltered. Consequently, a drop can be kept levitating unaffected by evaporation, thus allowing incubation of ligands or components in the drop prior to phase separation.

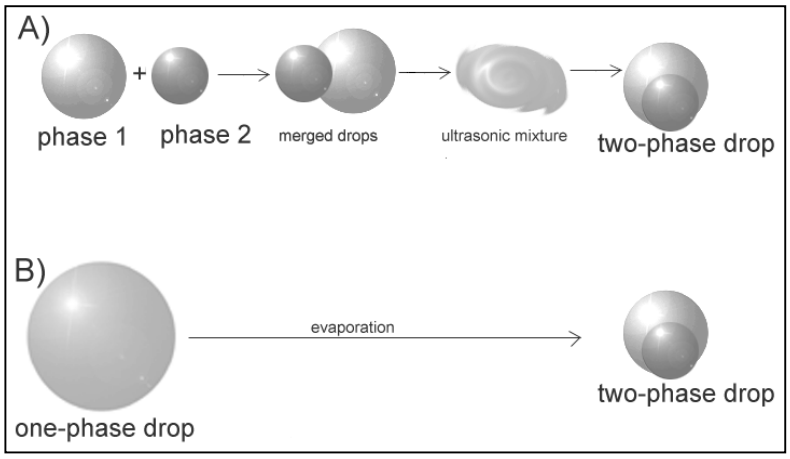

Figure 9. Formation of levitated two-phase drops. A) One drop of each phase of a pre-equilibrated two-phase system is placed in a levitator's node and mixed by adjusting the ultrasonic field and allowing phase separation in the levitator. B) By controlled evaporation of a one-phase drop of a polymer mixture.

Two alternatives were explored to prepare miniature two-phase systems in levitated drops (Fig. 9). As a first alternative one drop of each phase of a pre-equilibrated two-phase system was added to the node. The drops merged

immediately, and after mixing by altering the ultrasonic setting, phase separation was followed visually aided by a microscope (Fig. 9 A). Although such a manual addition of the phases works well, a future automation of the process would require addition, for instance, by a dispenser coupled to some kind of pump system. Due to the small size of the nozzles of the dispenser and the high viscosities of the phases a problem was that significant clogging occurred. As a second alternative, one two-phase drop was formed by controlled evaporation of a sufficiently diluted polymer mixture forming a one-phase drop (Fig. 9 B). Not only less manipulation is required in this approach, but it would also be suitable for automation as the phase system can be diluted sufficiently to avoid clogging of the dispenser.

Two alternative approaches were used to add the liposomes to drop. In the first one liposomes were pre-incubated in the pre-made two-phase system to allow interaction with the affinity ligand and added to the levitator together with the drop. In the second approach liposomes were added to the already levitated drop by a flow-through dispenser, and incubated in the drop kept at constant volume by water addition through the dispenser. The phases were removed from the drop by specifically made micropipettes. Radiometric analyses showed a similar liposome partitioning as in large-scale systems. Any other component or ligand required in other applications might be added in this way and incubated in the levitated drop.

The results of paper IV indicate the wide useful range of affinity two-phase partitioning, from 1 µl in levitated drops to larger preparative systems at least up to 30 g [177], or possibly even larger in industrial applications. They also indicate that affinity two-phase partitioning potentially can be applied to a wide range of purposes from industrial preparative fractionations to miniaturised analysis.

3. Conclusions and perspectives

The objective of this thesis work was to develop affinity two-phase partitioning to become a general tool for the biospecific purification of membranes. Caveolae from lung and liver have been isolated as an example of the application of this method. As a further development of the immunoaffinity sandwich approach presented in this thesis, it should be possible to isolate other membrane fractions by using other suitable primary antibodies to membrane proteins as affinity ligands. Hence, the main goal of this work has been largely fulfilled.

Several concrete conclusions can be drawn from the work presented in this thesis:

1. The otherwise extremely strong interaction between avidin and biotin is weakened by charges present on the surface of negatively charged biotinylated liposomes presumably due to electrostatic repulsion. This has implications for other systems utilising the biotin-avidin interaction.
2. By combining the use of a spacer, such as the hexanamidohexanoyl one and appropriate ionic strength in the two-phase system, the biotin moiety of the liposomes could interact properly with its binding site inside avidin. Consequently, affinity partitioning of negatively charged biotinylated liposomes and membranes using NeutrAvidin-dextran as affinity ligand became possible.
3. The introduction of biotin-NeutrAvidin as an affinity pair is a promising approach to make the method generally applicable as the biotin-avidin interaction is well studied and conditions required for two-phase affinity partitioning based on this interaction were established in papers I, II and III.
4. Purification of caveolae by affinity two-phase partitioning was not only remarkably faster than the gradient centrifugation method but it yielded a caveolae fraction as pure as in centrifugation in the case of lung and with even higher purity in the case of liver material.

5. The immunosandwich technique is a good approach to make the method generally applicable because it relies on biotinylated secondary antibodies, which are commercially available, obviating the need to separately biotinylate each primary antibody to be tested as an affinity ligand.
6. In addition to preparative fractionations affinity partitioning can be done on microscale suitable for analytical purposes.

Perspectives:

Model experiments:

Proteins and other membrane components should be reconstituted into liposomes to further investigate factors determining the affinity partitioning of membranes. Is the partitioning mainly determined by membrane lipids, membrane proteins, the protein/lipid ratio, or perhaps by net surface charge or distribution of charges?

The immunoaffinity approach:

The selectivity and general applicability of the method should be further investigated by using other primary antibodies for the isolation of other intracellular membrane fractions. Further questions to be addressed are the minimum number of binding sites on a membrane vesicle required for affinity interaction, the best way of separating purified membranes from the polymer-antibody-complex and the minimum abundance of a membrane population possible to isolate from a complex membrane mixture. A quantitative comparison between affinity two-phase partitioning and immunoadsorption to beads in terms of structure/function/stability/yield/purity of the purified material would also be interesting.

The miniaturised system:

A next step would be to test affinity partitioning with membranes, for instance using WGA as affinity ligand to extract plasma membranes. Detection systems must be developed for such small quantities of material; one approach would be the use of fluorescence tags. Another step would be to

test the immunoaffinity system and to use the miniaturised system for screening separation conditions and different ligands.

Affinity two-phase partitioning in levitated drops coupled to sensitive analytical detection techniques could open very interesting possibilities in the field of single cell biochemistry. A single cell could be levitated in a drop and disrupted *in situ* in order to fractionate membranes by affinity two-phase partitioning for further characterisation of cell components. Currently, little is known about differences between single cells in terms of structure and/or function. Miniaturised affinity two-phase partitioning might allow the study of components of individual cells and a comparison between cells from the same tissue.

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