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Partitioning of Drugs and Lignin Precursor Models into Artificial Membranes

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ACTA
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UPSALIENSIS
UPPSALA
2006

ISSN 1651-6214
ISBN 91-554-6628-1
urn:nbn:se:uu:diva-7098

Dissertation presented at Uppsala University to be publicly examined in B42, BMC, Uppsala, Friday, September 29, 2006 at 10:15 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

Boija, E. 2006. Partitioning of Drugs and Lignin Precursor Models into Artificial Membranes. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 207. 53 pp. Uppsala. ISBN 91-554-6628-1.

The main aim of this thesis was to characterize membrane-solute interactions using artificial membranes in immobilized liposome chromatography or capillary electrophoresis. The partitioning of a solute into a cell membrane is an essential step in diffusion across the membrane. It is a valid parameter in drug research and can be linked to the permeability as well as the absorption of drugs. Immobilized liposome chromatography was also used to study partitioning of lignin precursor models. Lignin precursors are synthesized within plant cells and need to pass the membrane to be incorporated into lignin in the cell wall.

In immobilized liposome chromatography, liposomes or lipid bilayer disks were immobilized in gel beads and the partitioning of solutes was determined. Capillary electrophoresis using disks as a pseudostationary phase was introduced as a new approach in drug partitioning studies. In addition, octanol/water partitioning was used to determine the hydrophobicity of the lignin precursor models.

Electrostatic interactions occurred between bilayers and charged drugs, whereas neutral drugs were less affected. However, neutral lignin precursor models exhibited polar interactions. Moreover, upon changing the buffer ionic strength or the buffer ions, the interactions between charged drugs and neutral liposomes were affected. Hydrophobic interactions were also revealed by including a fatty acid or a neutral detergent into the bilayer or by using a buffer with a high salt concentration. The bilayer manipulation had only a moderate effect on drug partitioning, but the high salt concentration had a large impact on partitioning of lignin precursor models.

Upon comparing the partitioning into liposomes and disks, the latter showed a more pronounced partitioning due to the larger fraction of lipids readily available for interaction. Finally, bilayer disk capillary electrophoresis was successfully introduced for partitioning studies of charged drugs. This application will be evaluated further as an analytical partitioning method and separation technique.

Keywords: Bilayer disk, Capillary electrophoresis, Detergent, Drug, Electrostatic interaction, Hydrophobic interaction, Immobilized liposome chromatography, Lignin, Lignin precursor model, Liposome, Membrane model, Octanol/water partitioning, Partitioning, Phospholipid, Phospholipid bilayer, Sterol

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ISSN 1651-6214

ISBN 91-554-6628-1

urn:nbn:se:uu:diva-7098 (<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7098>)

*In memory of
Per Lundahl*

To Petter

Papers included in this thesis

This thesis is based on the following papers. They will be referred to in the text by their Roman numerals.

- I Boija, E., Lundquist, A., Martínez Pla, J.J., Engvall, C., Lundahl, P. (2004) Effects of ions and detergents in drug partition chromatography on liposomes. *Journal of Chromatography A* 1030: 273-278.
- II Lundquist, A., Engvall, C., Boija, E., Kurtovic, S., Chattopadhyaya, J., Lagerquist Hägglund, C., Lundahl, P. (2006) Interactions of drugs and an oligonucleotide with charged membranes analyzed by immobilized liposome chromatography. *Biomedical Chromatography* 20: 83-87.
- III Boija, E., Johansson, G. (2006) Interactions between model membranes and lignin-related compounds studied by immobilized liposome chromatography. *Biochimica et Biophysica Acta* 1758: 620-626.
- IV Boija, E., Lundquist, A., Edwards, K., Johansson, G. Bilayer disks can replace liposomes as model membranes for lignin precursor model partitioning. *Submitted*.
- V Boija, E., Johansson, G., Nilsson, M., Isaksson, R., Lundquist, A., Edwards, K. Bilayer disk capillary electrophoresis (BDCE): a novel method to study drug partitioning into membranes. *Manuscript*.

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Abbreviations and denotations

CE	capillary electrophoresis
DSPC	1,2-distearoyl- <i>sn</i> -glycero-3-phosphocholine
DSTAP	1,2-distearoyl-3-trimethylammoniumpropane
EPL	egg phospholipid
HPLC	high performance liquid chromatography
ILC	immobilized liposome chromatography
PC	phosphatidylcholine
PEG-Cer(5000)	N-palmitoyl-sphingosine-1-[methoxy(polyethylene glycol)-5000]
PEG-DSPE(5000)	1,2-distearoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000]
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
PS	phosphatidylserine
UV	ultraviolet

Introduction

The cell is the foundation of life and the membrane surrounding the cell is essential to keep the cell intact as well as to regulate the environment within it. To be able to regulate the transport into and out from the cell, the cell membrane is selectively permeable, where preferably hydrophobic solutes pass by diffusion due to the hydrophobic core of the membrane. Solute-membrane interactions are interesting for many fields, such as the pharmaceutical industry. The partitioning of a solute is the first and the last step in diffusion across the membrane. When xenobiotics, such as drugs, need to pass the membrane to perform their actions, diffusion is the most likely transport route. Therefore, the pharmaceutical industry is dependent on methods to determine diffusion. Lignin is a polymer in the cell wall surrounding plant cells. It is essential for the growth and survival of terrestrial plants. The monomers making up lignin are synthesized within the cell, but their transport route through the cell membrane is largely unknown and, thereby, interesting from a biological point of view. Diffusion is not easily determined, but the partitioning, being a substantial part of it, can be determined in several ways. In this thesis, work regarding partitioning of drugs and of lignin precursor models has been performed primarily with immobilized liposome chromatography using liposomes and lipid bilayer disks as membrane models. Bilayer disks were also used as a pseudo-stationary phase in capillary electrophoresis for drug partitioning studies. Furthermore, octanol/water partitioning was used for characterization of lignin precursor models.

The cell membrane

All cells are circumscribed by a 6-nm thick cell membrane composed of lipids and proteins. The lipids are glycerophospholipids (hereafter referred to as phospholipids), sphingolipids, glycosphingolipids, glycolycerolipids and sterols (Mathews *et al.* 2000). The phospholipids are the most common membrane lipids. They have a glycerol-3-phosphate derivative as a hydrophilic head group and two hydrophobic fatty acid chains. The sterol has a rigid hydrophobic four-ring structure and a small hydrophilic part. In an aqueous milieu the hydrophobic parts turn toward each other, which results in a bilayer structure where the hydrophobic parts are shielded from the

aqueous environment inside and outside of the cell by the hydrophilic parts (Fig. 1) (Singer & Nicolson 1972, Storch & Kleinfeld 1985).

The membrane proteins can either be integral, i.e., inserted into the bilayer, or peripheral, where they are associated with the surface of the membrane (Fig. 1). The integral proteins have their hydrophobic amino acids situated in the parts imbedded in the hydrophobic core of the bilayer, and their hydrophilic parts situated in the head group region of the membrane (Mathews *et al.* 2000).

As a result of the hydrophobic core in the lipid bilayers, the membrane is selectively permeable (Subczynski & Wisniewska 2000). Many non-polar molecules can partition into the membrane and pass through it by diffusion, as can a few polar ones. Since most polar and ionic solutes cannot pass the membrane by diffusion, they need to be transported via active or facilitated transport.

The components of the cell membrane have been known for a long time, but it was not until the 1970s that the now well-known and generally accepted fluid mosaic model was presented (Singer & Nicolson 1972). The membrane is described as a dynamic structure with continual lateral movement of lipids and proteins. In addition to lateral movement, the lipids can flip-flop, i.e., move from one to the other leaflet of the bilayer (Storch & Kleinfeld 1985, McConnell & Vrljic 2003). The flip-flop velocity is very slow unless specific enzymes belonging to the flippase family are involved (Devaux 1993).

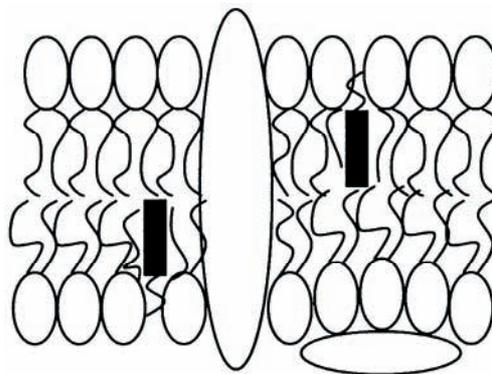


Figure 1. Schematic illustration of a lipid bilayer consisting of phospholipids, sterols, and integral and peripheral proteins.

The most common phospholipids in animal and plant cell membranes are the zwitterionic phosphatidylcholine (PC) and phosphatidylethanolamine, and the negative phosphatidylserine (PS) (Fig. 2). The animal cell contains considerable amount of cholesterol as opposed to the plant cell, where

cholesterol is scarce in most tissues, and the main sterols are stigmasterol, β -sitosterol (Fig. 3) and campesterol (Bohn *et al.* 2001, Quartacci *et al.* 2002).

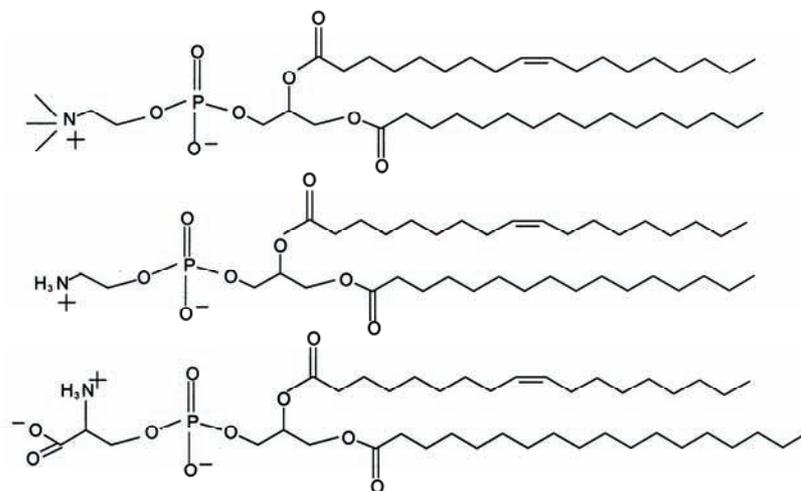


Figure 2. The structures of PC, phosphatidylethanolamine and PS, respectively.

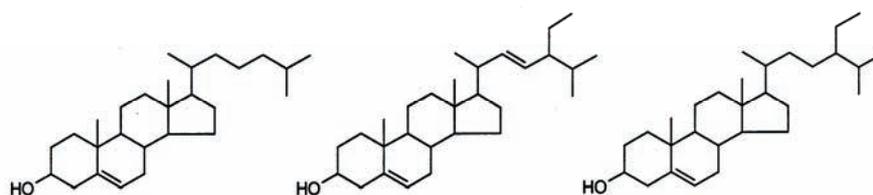


Figure 3. Cholesterol, stigmasterol and β -sitosterol, respectively.

Cell membrane models

Due to the fact that studies with natural cell membranes can be rather complex, several artificial membrane models have been developed over the years. The liposome is a common membrane model that was discovered approximately 40 years ago (Bangham & Horne 1964, Bangham *et al.* 1965). The liposome is simply a spherical lipid bilayer array consisting of phospholipids and, if interesting in the present study, membrane proteins and sterols (Fig. 4), which makes it a highly suitable model for studies of specific functions of the components of cell membranes (De Gier 1988, Engvall & Lundahl 2003). The bilayer is approximately 4 nm thick (Lasic 1998) and, like cell membranes, selectively permeable (De Gier 1988) and fluid (Brekkan *et al.* 1995). Liposomes are not only interesting models for studies of membrane characterization and solute-membrane interactions, but also as

vehicles for transport of drugs in the blood and their release at appropriate targets in the body (Wallstén *et al.* 1989, Betageri & Parsons 1992, Gregoriadis 1995, Lasic 1998).

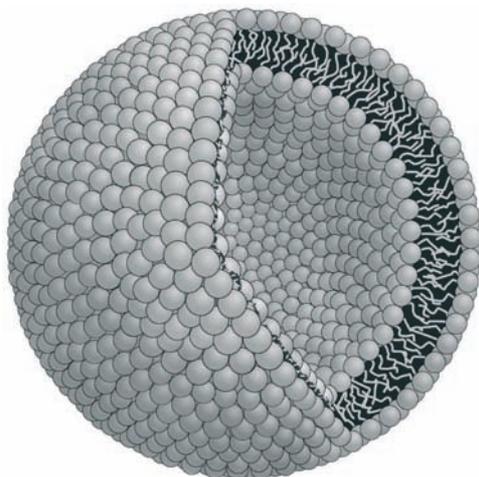


Figure 4. A liposome consisting of a phospholipid bilayer. © Göran Karlsson.

A more recent discovery is the lipid bilayer disk, where the bilayer is disc-shaped instead of spherical (Edwards *et al.* 1997). The disc-shape is stabilized by inclusion of cholesterol and polyethylene glycol-substituted lipids (Fig. 5). The cholesterol prevents the phospholipids from forming threadlike micelles and the polyethylene glycol keeps the bilayer stable. Polyethylene glycol has been used earlier in liposomes. However, upon increasing the amount of polyethylene glycol, more and more lipids fuse together to form disks (Edwards *et al.* 1997). By changing the polyethylene glycol concentration the size of the disks can be manipulated (Johnsson & Edwards 2003).

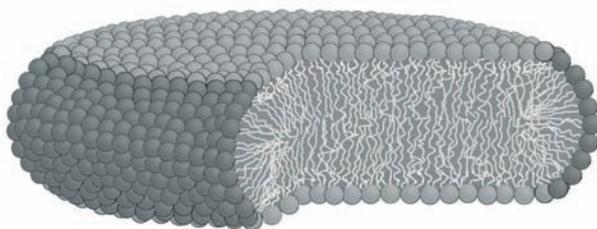


Figure 5. Illustration of a lipid bilayer disk consisting of phospholipids and cholesterol with polyethylene glycol-substituted lipids along the edge. © Göran Karlsson.

A more distant membrane model is octanol, which is used in octanol/water partitioning studies to evaluate the partitioning of solutes between a hydrophobic and a hydrophilic phase (Rogers & Wong 1980). Although octanol largely mimics hydrophobic interactions, it does not mimic polar interactions, which exist in membrane partitioning (Rogers & Wong 1980, Österberg *et al.* 2001).

Detergents are similar to phospholipids in having a hydrophilic head group region, but they have only one fatty acid chain (Fig. 6). In aqueous media they form micelles above a critical micelle concentration, where the chains that occupy the inside are effectively shielded by the head groups (Helenius *et al.* 1979). There is a great variety of detergents, both charged and neutral, which can be chosen according to the properties of the analytes studied. The micelles can be used as membrane models (Molero-Monfort *et al.* 2000) or the detergents can be included in liposomes to change their properties, such as charge and lipid packing (Heerklotz *et al.* 1997, Wenk & Seelig 1997, Kragh-Hansen *et al.* 1998).

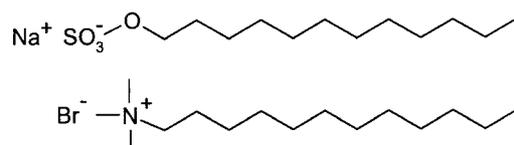


Figure 6. Two typical detergents, sodium dodecylsulfate and dodecyltrimethylammonium bromide, respectively.

Other models are cell membranes for *in vitro* studies, such as red blood cell membranes, intestinal brush border membranes and Caco-2 cell monolayers. Red blood cells can be used intact or prepared as ghosts or proteoliposomes. They are mainly used in studies of the mammalian glucose transporter GLUT1 (Gottschalk *et al.* 2003, Lagerquist Hägglund & Lundahl 2003), but have also been used for determination of drug partitioning (Beigi *et al.* 1998). Since the absorption of orally administered drugs occurs in the small intestine, the brush border membrane from the small intestine of rat or pig is a valuable membrane model. It can be prepared as membrane vesicles or liposomes and has been used in drug partitioning studies (Alcorn *et al.* 1993, Engvall & Lundahl 2004). Caco-2 cells are cell lines from human colon adenocarcinoma cells. They are distributed as monolayers on polycarbonate membranes, after which drug transport is studied to estimate partitioning, permeability and drug absorption (Camenisch *et al.* 1998, Sugawara *et al.* 2002, Tammela *et al.* 2004). In addition, there is extensive use of various isolated tissues from animals and also clearly more ethical computer simulations (Grass 1997).

Transport across cell membranes

There are two major classes of transport across cell membranes; namely, active and passive transport. In active transport, membrane proteins pump specific substances through the membrane. This requires energy, which is provided directly or indirectly from the hydrolysis of adenosine triphosphate or, for specialized cells, light energy. Ion pumps that pump specific ions at high rates and against the concentration gradient use direct adenosine triphosphate hydrolysis. Indirect adenosine triphosphate hydrolysis is used by protein cotransporters that transport different substances together (Engvall & Lundahl 2005).

Facilitated transport, which is passive, uses pores or carriers in the membrane to accelerate passage (Fig. 7). The pores are transmembrane proteins that form channels in the membrane for specific substances. Carriers are proteins that easily diffuse through the membrane encapsulating a specific substance. These proteins are typically hydrophobic on the outside and hydrophilic on the inside and transport polar or charged substances (Mathews *et al.* 2000, Engvall & Lundahl 2005).

Both active and facilitated transport systems can be saturated with their substances. This is not the case for the passive transport route diffusion (Fig. 7). The substances diffuse freely across the membrane at a rate proportional to the concentration gradient, according to (Eq. 1):

$$J = -\frac{K * D * (C_2 - C_1)}{l} \quad (1)$$

which is derived from Fick's first law of diffusion (Engvall & Lundahl 2005) where J is the transport rate, K is the partition coefficient, D is the diffusion coefficient, $(C_2 - C_1)$ is the concentration difference, and l is the membrane thickness. Since several of these parameters are difficult to measure, a permeability coefficient, P , is usually determined instead (Eq. 2):

$$J = -P * (C_2 - C_1) \quad (2)$$

Therefore, the permeability is linearly related to both diffusion and partitioning of a substance into a lipid phase (Ong *et al.* 1995). Due to the hydrophobic core of the cell membrane, many hydrophobic substances are able to diffuse, but only very few polar ones, such as water, manage this transport route (Engvall & Lundahl 2005). Water can also cross the membrane by facilitative transport or slip through transport proteins that are specific for other substances (Haines 1994). Yet another way for a substance to pass the cell membrane is by exocytosis or endocytosis, where the substance is simply trapped in a membrane vesicle which then buds off or

fuses with the main membrane, and the substance is ultimately released (Engvall & Lundahl 2005).

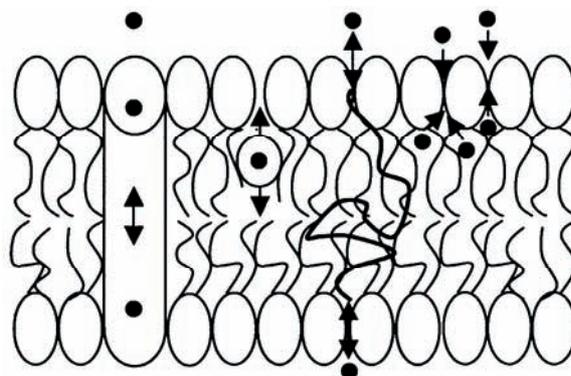


Figure 7. Some common transport routes. Facilitated transport, with proteins acting like pores or carriers, diffusion and partitioning, respectively.

The permeability of a substance over the cell membrane is an important factor for the transport. The selective permeability achieved due to the hydrophobic bilayer core and the specific transport proteins is one of the main purposes of the cell membrane, since it enables control over the environment in the cell. If xenobiotics, such as drugs, need to be taken up by the cell they have to be able to pass the membrane by diffusion, unless they mimic the structure of a nutrient that is actively transported (Artursson *et al.* 1996). Hence, the permeability of potential drug substances needs to be elucidated, which is very complex. This creates a need for alternative analysis methods that are easily performed *in vitro* as well as quick and accurate for screening of the tremendous number of prospective drugs produced by the pharmaceutical industry.

Absorption is the passage of a substance from its administration site to the plasma. Some of the factors involved in the absorption from the intestine are the permeability, solubility, stability and partitioning of the substance, as well as the enzymes, pH and surface area of the guts (Yu *et al.* 1996, Grass 1997, Burton *et al.* 2002), which makes absorption very difficult to determine. Since absorption is a crucial parameter for orally administered drugs, many methods to measure it have been developed, such as *in vivo* and *in situ* animal studies as well as *in vitro* studies of animal tissue (Grass 1997). Although these methods take many parameters into account, they only give approximations of human absorption.

The partitioning of a substance into a membrane can be determined easily by several methods. Furthermore, it correlates with the drug absorption provided that the drug is transported by diffusion (Liu *et al.* 2002). A pronounced partitioning is an essential step in diffusion (Tammela *et al.*

2004) and substances that cannot partition into a lipid bilayer cannot pass the membrane by diffusion. This may, e.g., be a first step to expel unsuitable drug candidates from further investigation.

Partitioning into cell membranes

The substance partitions into the membrane, diffuses through it and partitions out from it (Fig. 7). The partitioning, being determined by substance-membrane interactions, is a valuable parameter in studies of diffusion and permeability (Ong *et al.* 1995, Wang *et al.* 2004). Determination of the partition coefficient, K , can be done in several ways. The simplest way is by the concentration quotient of the substance between a hydrophobic (C_o) and a hydrophilic phase (C_w) (Katz & Diamond 1974, Alvarez *et al.* 1993, Engvall & Lundahl 2005) (Eq. 3):

$$K = \frac{C_o}{C_w} \quad (3)$$

which is Nernst's distribution law (Ramsden 1993). Another way is based on the Gibbs free energy, ΔG , of transfer (Eq. 4):

$$K = e^{\frac{-\Delta G}{R \cdot T}} \quad (4)$$

where R is the gas constant and T is the absolute temperature (Rogers & Wong 1980, Da *et al.* 1992).

The partitioning of a substance into a hydrophobic phase, such as a lipids or octanol, is straightforward to analyze and many different methods have been developed in this field. Generally, neutral substances partition more than charged substances into lipid bilayers and inclusion of sterols decreases the partitioning. The effect of membrane proteins on the partitioning depends on the protein structure (Engvall & Lundahl 2005). Some of the methods used are immobilized liposome chromatography (ILC), octanol/water partitioning and capillary electrophoresis (CE) with lipid bilayers.

Another method is immobilized-biomembrane chromatography, which is closely related to ILC, but uses complete biomembranes, such as red blood cells, instead of artificial membranes (Beigi & Lundahl 1999). Additional techniques available for determination of substance-membrane interactions are, e.g., immobilized artificial membrane chromatography, biopartitioning micellar chromatography, micellar electrokinetic chromatography, surface plasmon resonance analysis and pH-metric titration. Immobilized artificial membrane chromatography is used to determine substance retention and, thereby, partitioning. Furthermore, it has been used to predict drug transport through biomembranes (Alvarez *et al.* 1993, Ong *et al.* 1995 & 1996).

However, both lipid density and lateral movement is lower than for liposomes, since lipid monolayers immobilized on a surface are used (Taillardat-Bertschinger *et al.* 2002 & 2003).

In biopartitioning micellar chromatography detergents are adsorbed on the stationary phase and micelles are present in the mobile phase of a high performance liquid chromatography (HPLC) system. The retention of the substance allows calculation of its partitioning and the passive drug absorption can be estimated (Molero-Monfort *et al.* 2000, Quiñones-Torrelo *et al.* 2002, Martínez-Pla *et al.* 2003). Micellar electrokinetic (capillary) chromatography is a CE mode where micelles are included in the background electrolyte as a pseudostationary phase (Terabe *et al.* 1985, Wiedmer *et al.* 1997, Örnskov *et al.* 2005). The analytes partition into the micelles and separate according to their interaction with the micelles (Terabe *et al.* 1985, Cohen *et al.* 1987, Wiedmer *et al.* 1997). The different partitioning patterns help to separate analytes which would not separate in the background electrolyte alone (Cohen *et al.* 1987), such as neutral analytes (Landers 1993, Hong *et al.* 1998).

The surface plasmon resonance technique uses liposomes or biomembranes immobilized on a biosensor chip. By flowing the analyte across the liposomal surface their interaction can be determined and the kinetics calculated (Baird *et al.* 2002, Abdiche & Myszka 2004). In pH-metric titration the substance is dissolved in the aqueous phase and the pH around the substance pK_a is changed by titration. Then liposomes or octanol is added and the system is titrated back to the original pH. Due to the hydrophobic phase the pK_a -value shifts, which can be related to the partition coefficient (Takács-Novák & Avdeef 1996, Avdeef *et al.* 1998, Balon *et al.* 1999).

Background of immobilized liposome chromatography

The group of Per Lundahl at Uppsala University in Sweden has developed ILC, also called drug partition chromatography, which was introduced at a meeting in Nancy, France, in 1994 (Beigi *et al.* 1995). It is used to increase our understanding of membrane properties and to estimate permeability by elucidating the partitioning of various substances, such as drugs and peptides, into lipid bilayers using liposomes or proteoliposomes as membrane models (Yang & Lundahl 1994, Beigi *et al.* 1995, Zhang *et al.* 1995a, Brekkan *et al.* 1997). It can also be used as a separation technique for complex mixtures (Mao *et al.* 2002 & 2003). The (proteo)liposomes are entrapped in porous, yet rigid gel beads to serve as a stationary phase in liquid chromatography (Fig. 8) (Beigi *et al.* 1995, Lundqvist & Lundahl 1997). The immobilization of liposomes into gel beads can be done in several different ways, e.g., by freeze-thawing (Brekkan *et al.* 1997, Lagerquist *et al.* 2001), dialysis (Wallstén *et al.* 1989, Yang *et al.* 1990, Pattarino *et al.* 1997), covalent immobilization (Yang *et al.* 1999a,

Shimanouchi *et al.* 2000, Mao *et al.* 2003), and avidin-biotin coupling (Yang *et al.* 2000, Liu *et al.* 2001). By calculating the specific capacity factor from the analyte retention, the analyte-lipid interaction can be described as the partitioning of the substance into the liposomes (Beigi *et al.* 1995 & 1998, Yang *et al.* 1999b & 2000).

Different properties of the substance, such as charge and hydrophobicity, lead to different partitioning patterns. So do the properties of the lipid bilayer, including charge, membrane proteins and sterols (Beigi *et al.* 1998, Lagerquist *et al.* 2001, Liu *et al.* 2001). This can be utilized to study different components more closely (Zhang *et al.* 1995a). Liposomes in gel beads are stable for months (Brekkan *et al.* 1995, Beigi *et al.* 1995 & 1998, Yang *et al.* 1999b, Österberg *et al.* 2001, Liu *et al.* 2002), which makes ILC a convenient analysis method. The short analysis time required can be tuned by altering the amount of lipids immobilized (Beigi & Lundahl 1999, Österberg *et al.* 2001). The reproducibility of the results is good among different preparations, and ILC competes favorably with other methods in the determination of analyte-membrane interactions (Beigi *et al.* 1998, Yang *et al.* 1999b, Lagerquist *et al.* 2001, Österberg *et al.* 2001, Liu *et al.* 2002).



Figure 8. Illustration of liposomes immobilized in gel beads. Reprinted from *J. Chromatogr. B*, 699, Lundqvist, A., Lundahl, P. Chromatography on cells and biomolecular assemblies, 209-220, Copyright (1997), with permission from Elsevier.

Background of octanol/water partitioning

The partitioning of a substance between an organic phase, generally octanol, and an aqueous phase is a widespread method for comparing hydrophobicities of substances (Rogers & Wong 1980, Da *et al.* 1992, Avdeef *et al.* 1998). Especially in the pharmaceutical field it is used to elucidate whether

potential drugs have a possibility to pass cell membranes by diffusion (Da *et al.* 1992, Artursson *et al.* 1996, Avdeef *et al.* 1998, Perlovich *et al.* 2006). The partitioning can be determined in several ways, e.g., by a shake-flask method or by pH-metric titration.

The partition coefficient, P_{oct} , is determined for neutral substances and the apparent distribution coefficient, D_{oct} , is determined at pH 7.4 for charged substances (Artursson *et al.* 1996, Avdeef *et al.* 1998). The partitioning of highly hydrophobic substances cannot be related directly to the permeability because of their low solubility in the aqueous phase. This results in high partitioning into, but poor partitioning out from the membrane and, thereby, low permeability (Artursson *et al.* 1996). Although octanol is a hydrophobic solvent it can form hydrogen bonds to solutes, and since hydrogen bonding also occurs in membrane partitioning octanol is a more accurate model than other organic solvents (Rogers & Wong 1980, Artursson *et al.* 1996). However, the partitioning is mainly dependent on interactions with the hydrophobic fatty acid chains and it does not include the polar interactions exerted by the lipid head group region (Rogers & Wong 1980, Ramsden 1993, Ong *et al.* 1995, Avdeef *et al.* 1998). Therefore, partitioning methods including lipid bilayers are more relevant, especially when dealing with charged substances.

Background of capillary electrophoresis

Alexander Reuss first identified electrophoresis “borne by electricity” in 1807. He recognized that charged particles in a conducting solution moved towards the oppositely charged electrode when electricity was applied. Electrophoresis has received a lot of attention since then, with Arne Tiselius as one of the major pioneers and the inventor of an apparatus for free electrophoresis in the 1930s (Gray 1951, Landers 1993). The technique was further developed by Stellan Hjertén, who designed an apparatus for free zone electrophoresis wherein a horizontal revolving glass tube was used for zonal separation of analytes (Tiselius *et al.* 1963, Hjertén 1967).

A large problem with electrophoresis is the heat production, the so-called Joule heating, resulting from the high current (Landers 1993). In part to dissipate Joule heat thinner tubes were introduced (Jorgenson & Lukacs 1981), finally resulting in CE, which has grown to be a widely used separation technique. CE can be used for separation of everything from small molecules and ions up to macromolecules, liposomes and cells (Radko & Chrambach 1999 & 2002, Owen *et al.* 2005). The thin capillary with an inner diameter of a few micrometers reduces the amount of analyte and buffer needed, and gives sharp separation peaks. Furthermore, CE is a highly reproducible and easily automated technique (Landers 1993, Owen *et al.* 2005).

The capillaries are composed of fused silica, which exposes negative silanol groups over a wide pH-range. This charge causes an electroosmotic

flow, which is the net movement of fluid in the capillary. The flow depends on the current as well as the fluid interaction with the capillary walls. The movement of an analyte depends both on its behavior in an electric field and on the electroosmotic flow (Landers 1993, Srinivasan *et al.* 1997, Horvath & Dolník 2001). By coating the capillary walls with, e.g., neutral or charged polymers, both the analyte-wall interaction and the electroosmotic flow can be manipulated (Srinivasan *et al.* 1997, Horvath & Dolník 2001).

As mentioned earlier, the inclusion of micelles into the CE system can improve separation of various substances. To further mimic a biological system for determination of substance-membrane interaction, liposomes are sometimes included in CE, either in the running buffer as a pseudostationary phase (Zhang *et al.* 1995b, Roberts *et al.* 1996, Owen *et al.* 2005) or adsorbed to the capillary walls (Örnskov *et al.* 2002, Hautala *et al.* 2003, Owen *et al.* 2005). These methods can be used for separation of charged as well as neutral compounds (Hautala *et al.* 2003).

The plant cell wall

The plant cell is surrounded by a cell wall, which consists mainly of cellulose, hemicellulose, pectin, lignin and proteins (Heredia *et al.* 1995, Whetten & Sederoff 1995, Önerud *et al.* 2002). Cellulose is a glucose polymer (Gibeaut & Carpita 1994, Heredia *et al.* 1995), hemicellulose is a diverse group of polysaccharides, and pectin is a group of colloidal polysaccharides (Heredia *et al.* 1995). The proteins in the cell wall are divided into structural proteins and enzymes (Heredia *et al.* 1995, Whetten & Sederoff 1995).

The cell wall is divided into three zones, where the middle lamella consists mainly of pectins and the primary cell wall consists of polysaccharides, mostly cellulose. When the cells stop growing some plants, such as woods and grasses, but also specialized cells lining vascular and epidermal tissues in most plants, develop a secondary cell wall. It comprises lignin, cellulose and hemicellulose and provides mechanical support for the cell (Gibeaut & Carpita 1994, Heredia *et al.* 1995).

The composition and structure of the secondary wall is very important for industries using wood, such as the pulp industry. Many attempts have been made to modify these properties in order to adapt them to specific uses. To further enlighten the area of secondary wall formation and to modify those walls genetic engineering is used. Moreover, the use of the energy produced by the vast biomass of plants is very small today and the development of methods to use this energy is therefore very important for the future (Boudet *et al.* 2003).

Lignin

Lignin, which is one of the most abundant polymers in nature, is a large polymer present in the cell wall of woody plants and in the vascular tissue of virtually all terrestrial plants. Lignin has several functions among which the most important is the lining of the vascular tissue to make the tracheids/vessels impermeable and, thus, perfectly adapted for transport of water and nutrition throughout the plant. The mechanical support provided by lignin is essential for the growth of plants and without it all plants would be very small, e.g., trees would not exist (Whetten & Sederoff 1995, Hatfield & Vermerris 2001, Mellerowicz *et al.* 2001, Öennerud *et al.* 2002). Furthermore, lignin provides a defense against pathogens, like microorganisms and fungi, but also against herbivores (Hatfield & Vermerris 2001, Öennerud *et al.* 2002).

Lignin is built up primarily from the monolignols *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Fig. 9) (Whetten & Sederoff 1995, Hildén *et al.* 2000, Boudet *et al.* 2003), but also other phenolic components are incorporated (Ralph *et al.* 2001). Lignin biosynthesis starts with phenylalanine and goes along the phenylpropanoid pathway to the monolignols, which are transported out of the cell to the cell wall where they are added to the lignin web (Mellerowicz *et al.* 2001). Neither the reaction order, the roles of the enzymes, the storage form nor the transport through the cell wall is fully understood (Whetten & Sederoff 1995, Mellerowicz *et al.* 2001), although the precursors are suggested to be transported to the cell wall by exocytosis (Mellerowicz *et al.* 2001).

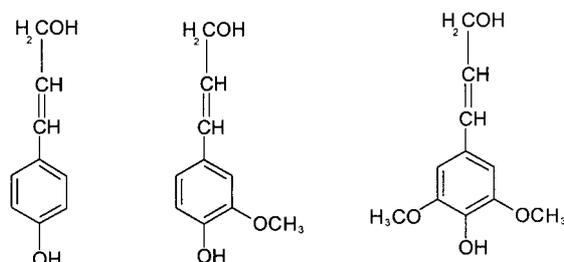


Figure 9. The monolignols *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, respectively.

The lignin molecule expands continuously to circumscribe many cells. The monolignols are added to the growing lignin web by a so far unknown mechanism. What is clear is that the monolignols are oxidized to radicals by H_2O_2 -dependent peroxidases or O_2 -requiring laccases (Heredia *et al.* 1995, Nose *et al.* 1995, Whetten & Sederoff 1995, Hatfield & Vermerris 2001). The monolignol radicals presumably connect to radical sites on the growing

lignin molecule in a random fashion (Adler 1977, Hatfield & Vermerris 2001, Önerud *et al.* 2002), although there are theories about a so-called dirigent protein helping in the actual coupling (Davin & Lewis 2000). Due to the apparently random building of lignin, its composition and the degree of lignification differs among different tissues and different plants (Heredia *et al.* 1995, Mellerowicz *et al.* 2001).

Present investigation

Aims of the study

The main aim of my thesis was to further characterize and interpret interactions between lipid bilayers and various analytes by partitioning studies with ILC and CE.

The ILC studies have been performed with charged and neutral liposomes to elucidate electrostatic and hydrophobic interactions with charged and neutral drugs (Papers I & II). The effects of the buffer ionic strength and the nature of the buffer ions were also studied (Paper I). Furthermore, the interaction between an oligonucleotide and liposomes was investigated (Paper II).

The partitioning of neutral lignin precursor models into charged and neutral liposomes was determined (Paper III). This study was a first attempt to use ILC for studies of lignin-related molecules and to try to elucidate whether these molecules can pass the cell membrane by diffusion. Furthermore, to elucidate the significance of bilayer shape, ILC was used to study the partitioning of the lignin precursor models into both liposomes and lipid bilayer disks (Paper IV). The plant cell membrane lipid composition was mimicked to determine the significance of using defined lipid compositions in partitioning studies.

CE with lipid bilayer disks as a pseudostationary phase was introduced as new a method (Paper V). Both negative and neutral disks were used in the system and by studying drug partitioning the method could be evaluated. Since both charged and neutral disks were used, as well as positive drugs, both electrostatic and hydrophobic interactions could be studied.

Methods and calculations

Preparation and immobilization of liposomes and disks

The liposomes (Papers I-IV) were prepared essentially as in (Brekkan *et al.* 1997). The appropriate lipids were dissolved in chloroform, which was rotary evaporated. The resulting lipid film was rehydrated in buffer A (10 mM Tris, 150 mM NaCl, 1 mM Na₂EDTA, adjusted to pH 7.4 with HCl at

room temperature). To be able to rehydrate lipid films including sterols (Paper IV), deep freeze-quick thawing cycles (N₂ (l)/water bath 70°C) were performed. The lipid solution was mixed with dried Superdex™ 200 prep grade beads and left for swelling. Lipids in solution spontaneously form liposomes (Bangham *et al.* 1965, Lasic 1998). Upon mixing the solution with porous gel beads the liposome formation takes place within the beads, which results in sterically immobilized multilamellar liposomes. Since small liposomes can escape from the porous gel beads, five freeze-thawing cycles (-70°C/25°C) were applied. This is a method developed by Lundahl and coworkers (Yang & Lundahl 1994) based on knowledge of lipid fusion upon sudden temperature change (Morris & McGrath 1981, Pick 1981, Mayer *et al.* 1985). The lipid fusion gives larger liposomes and, hence, larger amounts of entrapped lipids. When preparing unilamellar liposomes (Paper II) no freeze-thawing was done, the liposome-gel suspension was instead extruded more than 20 times through a polycarbonate filter to form uniform sized unilamellar liposomes (MacDonald *et al.* 1991). Free lipids were washed away and the liposome-gel solution was packed into glass columns and equilibrated with the running buffer. When detergents were to be included (Papers I & II), they were added to the running buffer and thereby became included in the liposomes during equilibration.

The lipid bilayer disks (Papers IV & V) were prepared essentially as in (Johansson *et al.* 2005). The lipids were dissolved in chloroform, which was evaporated under N₂ (g) and vacuum. The lipid film was then either rehydrated in buffer A, mixed with the gel beads, swelled, washed and packed into columns, as described above (Paper IV), or rehydrated in the CE buffer (10 mM Tris, 50 mM NaCl, pH 7.4) (Paper V). When preparing neutral disks (Papers IV & V) liposomes appeared. To remove them, dextran was added to the rehydrated lipids and the sample was sonicated to improve lipid mixing. The latter procedure was also applied to disks mimicking plant cell membranes (Paper IV). Finally, the liposomes entrapping dextran were separated from the neutral disks by centrifugation.

Immobilized liposome chromatography

The substances (0.1 mg/ml in buffer A or water and ≤10vol% ethanol (the oligonucleotide 0.5-4 mg/ml in buffer A), injection volume 10 or 20 µl) were run at 0.1-1 ml/min at room temperature or as a series at 10-50°C, and detected by ultraviolet (UV) light at 220 or 270 nm using a HPLC system (Papers I-IV) (Fig. 10). The interaction between analytes and liposomes was expressed as a specific capacity factor, K_s (M⁻¹) (Eq. 5) (Papers I-III):

$$K_s = \frac{V_E - V_0 - V_G}{A} \quad (5)$$

where V_E is the elution volume of the analyte, V_0 is the elution volume of $\text{Cr}_2\text{O}_7^{2-}$ that does not interact with the liposomes and the gel, and V_G is the retention volume of the analyte on a liposome-free gel bed (Lagerquist *et al.* 2001). The phospholipid amount, A , was determined by phosphorus analysis (Bartlett 1959) after the experimental series. The known weight or molar fraction of non-phosphorus lipids was included in the A -values. The equation was slightly modified (Paper IV), since analyte-gel interactions are generally negligible (Beigi *et al.* 1998) (Eq. 6):

$$K_S = \frac{V_E - V_0}{A} \quad (6)$$

The specific capacity factor is closely related to the partition coefficient, K , (Paper IV) where the latter includes the volume of the lipid phase (Yang *et al.* 1999b & 2000) (Eq. 7):

$$K = \frac{K_S}{V_{\text{app}}} \quad (7)$$

where V_{app} is the apparent lipid molar volume, set at 0.6 M^{-1} (Paper IV), which represents a good approximation for a 60/40 molar ratio of phospholipids and sterols.

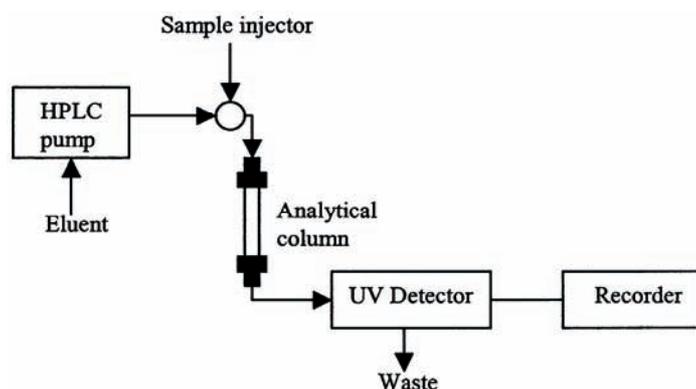


Figure 10. Apparatus arrangement for ILC.

Octanol/water partitioning

The shake-flask method (Rogers & Wong 1980, Da *et al.* 1992) was used to determine the octanol/water partitioning of the lignin precursor models

(Paper III). The study was done at room temperature using 1-octanol and buffer A saturated with each other as hydrophobic and aqueous phase, respectively. The model substances were dissolved in both the octanol and the buffer to construct standard curves. The standard curves were linear, giving an equation according to Beer's law for concentration calculations (Gargadennec *et al.* 2005). The substances dissolved in the octanol phase were shaken with the buffer for 24 hours, by which time equilibrium was assumed to have been reached (Da *et al.* 1992). The absorbance in each phase was measured with a UV spectrophotometer to determine the concentrations of the substance in the phases. The partitioning between the octanol and the aqueous phase was calculated according to (Eq. 3).

Bilayer disk capillary electrophoresis

Both negative and neutral disks were used (Paper V). Fused silica capillaries (50 μm inner diameter, effective length 24.5 cm) were coated with the neutral polymer poly(vinylpyrrolidone) according to (Srinivasan *et al.* 1997). The capillary was etched with NaOH, washed with water, leached with HCl and then washed again. Thereafter, the capillary was flushed with γ -methacryloxypropyltrimethoxysilane/acetic acid solution, after which the poly(vinylpyrrolidone) solution was added. The capillary was finally washed with water and flushed with the degassed and filtered running buffer. Mesityl oxide was run to determine the electroosmotic flow.

The disks were diluted in the running buffer and served as a pseudostationary phase (Zhang *et al.* 1995b, Wiedmer *et al.* 2000 & 2001, Burns & Khaledi 2002). They were hydrodynamically injected by partial filling (Chien & Helmer 1991, Heintz *et al.* 1999, Nilsson *et al.* 2004) to form plugs of various lengths (50, 100 or 150 seconds), after which the drug (50 μM in buffer) was injected (5 seconds, 34.5 mbar). Injection of the drug alone was used as a null sample. The positive drugs migrated through the disk solution due to partitioning into the disks and ionic attraction, whereas the disks were moved only by the electroosmotic flow. All runs were preceded by a buffer wash and performed in triplicate at 45 μA , 25°C and positive polarity. The buffer was changed between runs using automated replenishment. A diode array detector was used for absorbance detection at 204 or 214 nm (Fig. 11).

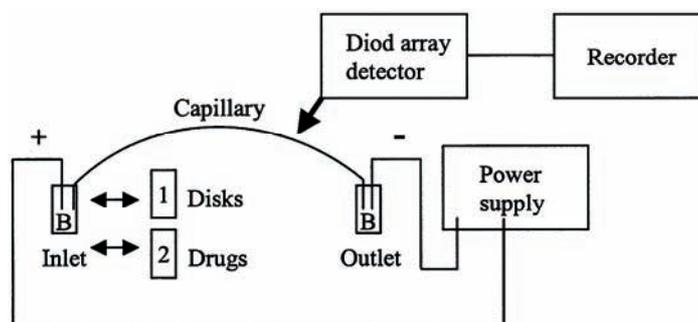


Figure 11. Apparatus arrangement for CE with disks as a pseudostationary phase, where B is buffer.

The partition coefficient, K , was calculated (Eq. 8) for determination of the drug partitioning into the disks using a modified version of an algorithm derived for biomolecular interactions (Nilsson *et al.* 2004):

$$K = \frac{\pi * r^2 * l_{\text{det}} * \frac{d\Delta t}{dV_{\text{lip}}}}{t_{0,\text{det}}} \quad (8)$$

where r is the inner radius of the capillary, l_{det} is the length to the detection window, $t_{0,\text{det}}$ is the retention time for the null sample, Δt is the change in retention time when disks are present, and V_{lip} is the total volume of the lipid phase in the capillary, which was calculated according to (Eq. 9):

$$V_{\text{lip}} = C_{\text{lip}} * V_{\text{inj}} * V_{\text{app}} \quad (9)$$

where C_{lip} is the molar lipid concentration, V_{inj} is the injected volume of the lipid, and V_{app} is the apparent lipid molar volume, 0.6 M^{-1} .

Thermodynamics

The temperature dependence shown for the partitioning of lignin precursor models into lipid bilayers was used to determine the water-lipid transfer in the systems (Papers III & IV). The changes in Gibbs free energy, ΔG , enthalpy, ΔH , and entropy, ΔS , (Eq. 10) could be calculated from the retention according to (Eqs. 4 & 11) (Ong *et al.* 1995, Yang *et al.* 2000, Perlovich *et al.* 2006).

$$\Delta G = \Delta H - \Delta S * T \quad (10)$$

$$\ln K = \frac{\Delta S}{R} - \frac{\Delta H}{R} * \frac{1}{T} \quad (11)$$

Results and discussion

Partitioning of drugs and an oligonucleotide as studied by immobilized liposome chromatography (Papers I and II)

The drug partitioning into liposomes was determined by ILC (Papers I & II). In addition, the partitioning of an oligonucleotide was determined (Paper II). The liposomes were varied with respect to charge and hydrophobicity by introducing charged or neutral detergents (Papers I & II), charged lipids (Paper II), or a fatty acid (Paper I). Both unilamellar (Paper II) and multilamellar (Papers I & II) liposomes were used. Moreover, upon changing the buffer ionic strength or the buffer ions the electrostatic effects changed.

Detergent effects

Detergents were introduced into egg phospholipid (EPL) liposomes by equilibration with detergent containing buffer A below the critical micellar concentration (Papers I & II). This allows partitioning of the detergents into the liposomes, where they stay due to hydrophobic interactions and hydrogen bonding (Heerklotz *et al.* 1997, Wenk & Seelig 1997, Kragh-Hansen *et al.* 1998). Sodium dodecylsulfate being negative, since the sodium ions disperse in the buffer, decreased the partitioning of the negative drugs owing to repulsion and increased the partitioning of positive drugs due to attraction (Papers I & II). These effects are in agreement with earlier results on charged liposomes (Beigi *et al.* 1998, Krämer *et al.* 1998, Liu *et al.* 2001 & 2002) and were used to separate differently charged drugs that co-eluted on neutral bilayers.

Dodecyl-, tetradecyl-, and hexadecyltrimethyl-ammonium bromide, where bromide disperses in the buffer leaving the detergents positive, were introduced into EPL liposomes (Paper I). The retention of negative drugs increased considerably and the retention of positive drugs decreased. This effect was accelerated with increasing chain length of the detergents, which even gave a slight increase in the partitioning of neutral drugs. These findings were used for separation of negative, positive and neutral drugs that co-eluted on neutral bilayers.

The introduction of the neutral detergent octaethyleneglycol monododecyl ether did not affect the partitioning of any drug significantly. Therefore,

electrostatic interactions are significant for the partitioning of charged drugs into charged bilayers, in addition to hydrophobic effects (Krämer *et al.* 1998, Liu *et al.* 2001 & 2002).

Fatty acid effects

The insertion of the fatty acid arachidic acid (Paper I) decreased the partitioning of negative and neutral drugs slightly, but had a negligible effect on positive drugs. Only partial separation of the drugs was achieved (Fig. 12), although as much as 50 mol% of the arachidic acid was present in the EPL liposomes. The fatty acid should modulate both the hydrophobic and the hydrophilic region of the liposomes due to its saturated, hydrophobic chain and its carboxyl end. Although the carboxyl group is expected to be ionized at pH 7.4 (Diaz *et al.* 2005) the effects on the partitioning were small. Hence, it is possible that the effect of the arachidic acid on the bilayer core dominated over its effect on the polar head group region, in accordance with insertion of neutral detergents into lipid bilayers (Heerklotz *et al.* 1997).

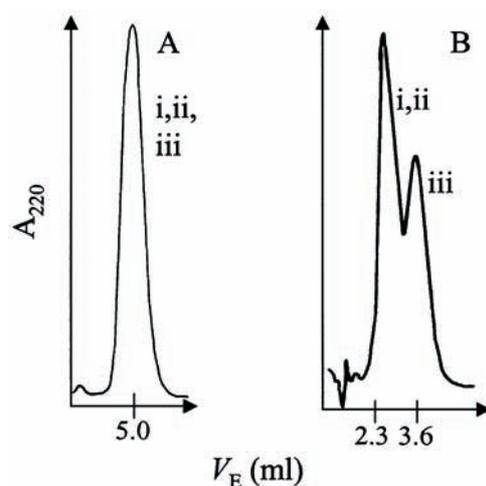


Figure 12. The effect of inserting arachidic acid into EPL liposomes. A) EPL liposomes. The drugs co-eluted, $\log K_S$ 2.43, and B) EPL including arachidic acid (50 mol%). $\log K_S$ 2.03 and 2.24. The drugs were gemfibrozil (i, negative), corticosterone (ii, neutral) and alprenolol (iii, positive).

Buffer effects

The partitioning of charged drugs was altered by changing the ionic strength of the buffer (0.0003-0.20) (Paper I). The partitioning of positive drugs into EPL liposomes decreased, and that of negative drugs increased at first to decrease later as the ionic strength increased further. The effect of ionic strength has also been shown for the permeability of charged compounds through Caco-2 cells (Sugawara *et al.* 2002). In contrast, the positive drugs

were unaffected and the retention of negative drugs decreased upon using 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomes. Although both EPL and POPC bilayers are zwitterionic, since the phosphate group is deprotonated above pH 3 (Krämer *et al.* 1998), the primary amino group in the head group of phosphatidylethanolamine present in EPLs is probably easier to reach than the quarternary amino group in the PC head group (Fig. 2) (Österberg *et al.* 2001), which may explain the difference in partitioning. Possibly the negative drugs can reach the quarternary amino group in POPC at low ionic strength, but as it increases the buffer anions form a shield around the amino group. Hence, the retention of the negative drugs decreased, since they are larger than the buffer ions, making it more difficult to penetrate the region around the lipid head groups due to sterical hindrance. The partitioning of the negative drugs into the EPL bilayer was not as affected, possibly since the primary amino group is accessible. Hence, the competition between the drugs and the buffer ions was not as prominent. The effect on the positive drugs showed an electrostatic repulsion from phosphatidylethanolamine in the EPL liposomes, whereas the POPC liposomes kept their positive groups effectively hidden. Neutral drugs were not affected by the change in ionic strength, which is in accordance with the permeation of neutral drugs through Caco-2 cells (Sugawara *et al.* 2002).

Also, the nature of the buffer ions was shown to affect the partitioning of charged, but not neutral, drugs into POPC liposomes (Paper I), where Li^+ caused an increase in the partitioning of negative drugs compared to Na^+ . The partitioning of positive drugs increased slightly upon exchanging Cl^- for F^- , whereas negative drugs were unaffected. Thus, Li^+ and F^- possibly competed harder with the drugs for the positions close to the head groups or shielded the counter ions in the head group from the drugs.

Phospholipid charge effects

The liposome charge was altered by inclusion of the negative phospholipid PS, negative detergent sodium dodecylsulfate, or the positive artificial lipid 1,2-distearoyl-3-trimethylammoniumpropane (DSTAP) into PC or EPL bilayers (Paper II). The retention of an oligonucleotide was studied on multilamellar PC/DSTAP liposomes. At pure PC or low concentrations of DSTAP the oligonucleotide eluted in the void volume. Upon increasing the amount of DSTAP the oligonucleotide retention increased. Since the oligonucleotide is a large polyelectrolyte it does not partition into liposomes (Akhtar *et al.* 1991, Dass 2002), but due to its multiple negative charges it is electrostatically attracted to positive liposomes (Maurer *et al.* 2001, Dass 2002), which probably enables multipoint interaction (Garcia-Chaumont *et al.* 2000). By increasing the oligonucleotide concentration it was shown that the surfaces of the liposomes could be saturated (Akhtar *et al.* 1991), since the retention decreased with increased oligonucleotide amount.

The drug partitioning was affected by charged liposomes (Paper II). Negative liposomes decreased the partitioning of negative drugs due to repulsion and increased the partitioning of the positive drugs due to attraction. The opposite was shown on positive liposomes. The neutral drugs were slightly affected, showing a decrease in partitioning, so some polar interactions probably occur even here. The electrostatic effects are in agreement with earlier results on charged lipid bilayers (Pattarino *et al.* 1997, Beigi *et al.* 1998), and since most natural cell membranes have a negative net charge, the partitioning into them does include electrostatic interactions in addition to hydrophobic ones (Krämer *et al.* 1998). Drugs have been shown to distribute between the hydrophilic head groups and the hydrophobic chains (Fig. 13) (Wang *et al.* 2004), similarly as analytes distributes in micelles (Wiedmer *et al.* 1997 & 2001). The partitioning was more affected as the liposomal charge increased, indicating that the electrostatic interactions increased in a concentration-dependent manner.

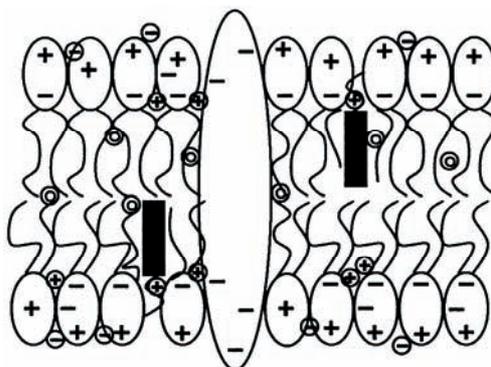


Figure 13. A schematic illustration of possible positioning of charged and neutral drugs in lipid bilayers.

Upon comparing the charge effect of PS and dodecylsulfate, it was evident that dodecylsulfate had a greater effect on the partitioning, even at low concentrations. This may be due to the positional effects, since PS was integrated during liposome preparation and sodium dodecylsulfate was added afterwards, thus, possibly occupying a more exposed position with more easily accessible negative charges. Moreover, since dodecylsulfate ions have a slow flip-flop rate, probably most of them were positioned in the outermost leaflet (Kragh-Hansen *et al.* 1998), whereas PS probably was evenly distributed throughout the multilamellar liposomes. Hence, the effective concentration of dodecylsulfate was higher than accounted for in the most accessible lipid regions.

Upon comparing the partitioning into unilamellar and multilamellar liposomes supplemented with sodium dodecylsulfate (Papers I & II) there

were some differences in the $\log K_S$ -values, but the partitioning pattern was the same. Therefore, the difference is probably related only to the lipid amount accounted for, since less of the present lipids are readily available for interaction in multilamellar than in unilamellar liposomes (Liu *et al.* 2001, Johansson *et al.* 2005).

Summary

To summarize, the partitioning of charged drugs was affected by charged lipid bilayers. Changing the ionic strength or the nature of the buffer ions could also alter the partitioning of charged drugs. The introduction of a neutral detergent left the retentions unchanged. Therefore, electrostatic effects are significant for the partitioning of charged drugs. The neutral drugs were largely unaffected by the bilayer type and the buffer. This was probably due to the fact that they partition into the hydrophobic core, where hydrophobic interactions dominate.

Partitioning of lignin precursor models as studied by immobilized liposome chromatography (Papers III and IV)

Lignin precursor model substances were studied by ILC to reveal their partitioning into multilamellar liposomes (Papers III & IV) and lipid bilayer disks (Paper IV). The models were monomeric or dimeric and mimicked monolignols and dilignols, respectively, concerning overall structure and hydrophobicity. Monolignols were not used due to their instability and toxicity (Whetten & Sederoff 1995).

Ammonium sulfate effects

To elucidate whether the models exerted any electrostatic interactions, although they are neutral at the pH used (7.4 in buffer A and 7.6 in buffer B, the latter was buffer A including 1 M $(\text{NH}_4)_2\text{SO}_4$), the liposomes either included the negative PS or the positive DSTAP, in addition to the main phospholipid PC, which was also used alone as a neutral bilayer (Paper III). The purpose of the ammonium sulfate was to examine the degree of hydrophobic interactions, which are revealed at high salt concentrations at the same time as electrostatic interactions decrease (Hammar *et al.* 1975, Scopes 1994). As expected, the partitioning of all model substances increased in the presence of ammonium sulfate. Therefore, the hydrophobic interactions between the models and the lipids were significant and exceeded the electrostatic ones. The change in retention was dependent on the liposome charge, revealing that electrostatic effects occurred to some extent. Moreover, the dimeric substances partitioned more than did the monomers into all kinds of liposomes and both buffer systems, which probably was related to their higher hydrophobicity and, thereby, increased hydrophobic interactions

with the lipids. This is in accordance with earlier data on the partitioning of phenolic compounds of increasing size into liposomes (Rogers & Davis 1980).

Octanol/water partitioning

The hydrophobicity of the model substances was determined by octanol/water partitioning (Paper III) and the values were compared to the partitioning into lipid bilayers (Papers III & IV). The pronounced $\log P_{\text{oct}}$ -values for most models showed that they were predominantly hydrophobic. The correlation with the $\log K_{\text{S}}$ -values was good (Paper III). However, there was not a 1:1 relation between the results, which may be explained by the electrostatic interactions present in liposome partitioning (Österberg *et al.* 2001). Therefore liposomes better mimic the partitioning into cell membranes. The $\log P_{\text{oct}}$ -values were consistently lower than the $\log K$ -values derived from the partitioning of the model substances into disks mimicking the lipid composition of plant cell membranes, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)/cholesterol/N-palmitoyl-sphingosine-1-[methoxy-(polyethylene glycol)-5000] (PEG-Cer(5000)) disks, and DSPC/cholesterol liposomes (Paper IV). This further supports the importance of electrostatic interactions present in lipid bilayers.

Temperature dependence

The partitioning decreased gradually with increasing temperature (Papers III & IV) (Fig. 14). This may be explained by the increased solubility of hydrophobic substances into the aqueous phase, which should decrease the partitioning into the bilayer (Liu *et al.* 2001). The temperature effect on the partitioning into PC, PC/PS, and PC/DSTAP liposomes (Paper III) was greater than into plant cell membrane disks, DSPC/cholesterol/PEG-Cer(5000) disks, and DSPC/cholesterol liposomes (Paper IV). This discrepancy is possibly related to the sterols included in the latter cases, being stigmasterol and β -sitosterol in the plant cell membrane disks and cholesterol in the model disks and liposomes. The rigid ring structure of sterols has an ordering effect on the lipid bilayers (Subczynski & Wisniewska 2000), and increasing amounts of sterols have also been shown to decrease the partitioning (Lagerquist *et al.* 2001). The sterol effect on partitioning was more pronounced for the dimeric models than for the monomers (Paper IV). This agrees with earlier results on drug partitioning, where the more hydrophobic drugs were more affected, probably due to deeper penetration, allowing more contact with the cholesterol than occurs with less hydrophobic drugs (Liu *et al.* 2001).

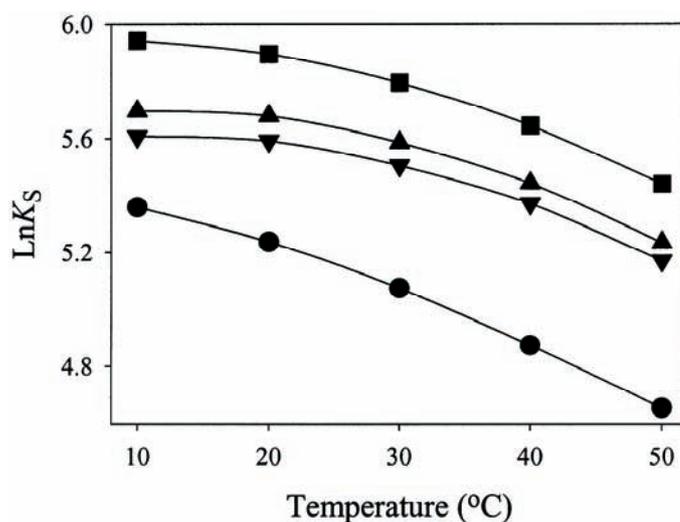


Figure 14. The temperature effect on the partitioning of lignin precursor models into multilamellar PC liposomes as shown by $\ln K_S$ -values versus temperature. The dimers were 4-acetoxy-3,5-dimethoxy- α -(2,6-dimethoxyphenoxy) acetophenone (■), 4'-hydroxy-3',5'-dimethoxy-2-(o-methoxyphenoxy) acetophenone (▲), 1-(4-hydroxy-3-methoxyphenyl)-2-(2,6-dimethoxyphenoxy) acetophenone (▼), and 3-hydroxy-3',4'-dimethoxy-2-(o-methoxyphenoxy) propiophenone (●).

Bilayer structure effects

The correlation was excellent between the plant cell membrane disks and the DSPC/cholesterol/PEG-Cer(5000) disks (Paper IV). Although the partition coefficients was lower in the first case, which may be explained by the plant cell membrane disks being slightly negative and the model disks being neutral, the difference was consistent (Fig. 15). Therefore, the model disks can be used as suitable membrane models, being easier to prepare than the more complex mixture in the plant cell membrane disks.

The partition coefficients differed quite markedly between the DSPC/cholesterol liposomes and the corresponding bilayer disks (Paper IV) (Fig. 15), which is in agreement with earlier results on drug partitioning into liposomes and disks (Johansson *et al.* 2005). The difference in K -values is probably an artifact caused by the difference in the fraction of lipids actually participating in the interaction. All lipids are readily available in the disks as opposed to the liposomes, where the lipids of both the inner leaflet as well as the inner liposomes are hidden from interaction if not equilibrated long enough (Liu *et al.* 2001, Johansson *et al.* 2005). However, the correlation between the liposomes and the disks was very good.

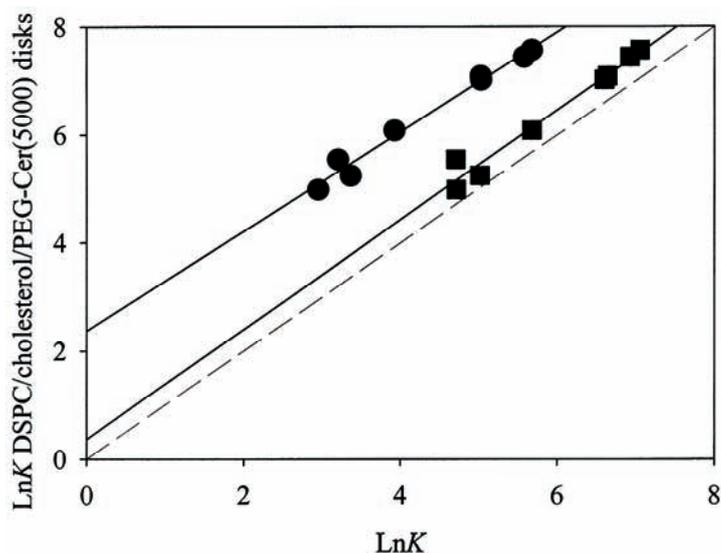


Figure 15. Correlation between DSPC/cholesterol/PEG-Cer(5000) disks and DSPC/cholesterol multilamellar liposomes (●) and plant cell membrane disks (■). Slope 1 is marked.

Thermodynamics

To elucidate the lipid-water transfer, thermodynamic parameters were calculated from the partitioning into liposomes and disks at five different temperatures (Papers III & IV). Since the partitioning into the lipid bilayer was temperature dependent, especially in the absence of sterols (Paper III), there should be a contribution to the interaction energy from the enthalpy. The ΔH -values were negative in all cases except for monomers in PC/DSTAP and DSPC/cholesterol liposomes, which most likely is due to experimental error. All ΔG -values were negative and the entropy changes were positive (Papers III & IV). This shows that the system has enthalpy dependence and hydrogen bonding is likely to be present (Rogers & Wong 1980). A liposome system below the transition temperature is entropy driven (Rogers & Davis 1980). Therefore, the studies were probably performed above the transition temperature for all lipid compositions (Papers III & IV). This is further supported by the fact that cholesterol decreases the phase transition temperature, which at high sterol amounts is undetectable (Morris & McGrath 1981, Chong & Choate 1989).

Summary

In conclusion, the lignin precursor models partitioned well into both neutral and charged liposomes and disks. Although the models were clearly hydrophobic, as shown by the octanol/water partitioning, there were differences when comparing the specific capacity factors from the partitioning into

charged and neutral liposomes, as well as the partition coefficients from the partitioning into charged and neutral disks. The pronounced partitioning due to the strong interactions exerted by the hydrophobic models, and the few hydroxyl groups (Tammela *et al.* 2004) present in the models suggest that diffusion is a possible transport mechanism for the lignin precursor models.

Partitioning of drugs as studied by bilayer disk capillary electrophoresis (Paper V)

Preliminary evaluation of bilayer disk CE was performed using drug partitioning. Earlier work with liposomes has shown that their effect on drug retention in CE is better correlated to drug permeability than CE with micelles (Örnskov *et al.* 2005). By using bilayer disks even better correlation may be achieved, since all lipids are accessible for drug interactions in contrast to the lipids in liposomes (Johansson *et al.* 2005).

Experimental

The disks were composed of DSPC, cholesterol and either 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (PEG-DSPE(5000)) or PEG-Cer(5000), rendering negative and neutral disks, respectively. They were used as a pseudostationary phase, similarly to earlier work with liposomes (Wiedmer *et al.* 2000 & 2001, Burns & Khaledi 2002, Örnskov *et al.* 2005), and injected as a plug by partial filling (Chien & Helmer 1991, Heintz *et al.* 1999). The positive drugs used presumably exhibited both electrostatic and hydrophobic interactions with the disks. Positive disks were not used since they would have a fast migration towards the detection window, which would interfere with the detection of the drugs.

Results and discussion

The migration times of the drugs increased with increasing amounts of disks (Fig. 16), whether negative or neutral, which is in accordance with earlier results on liposomes (Burns & Khaledi 2002). The increase was linear for all drugs in the system. The partitioning of the drugs into the negative disks correlated well with earlier results on ILC (Johansson *et al.* 2005). Repeated runs showed that the results on both negative and neutral disks were reproducible. In the solution with neutral disks, a residue of dextran might remain after washing away the liposomes produced during preparation, which possibly increased the solution viscosity. Hence, the amount of lipids present in the injection plug of neutral disks was probably overestimated.

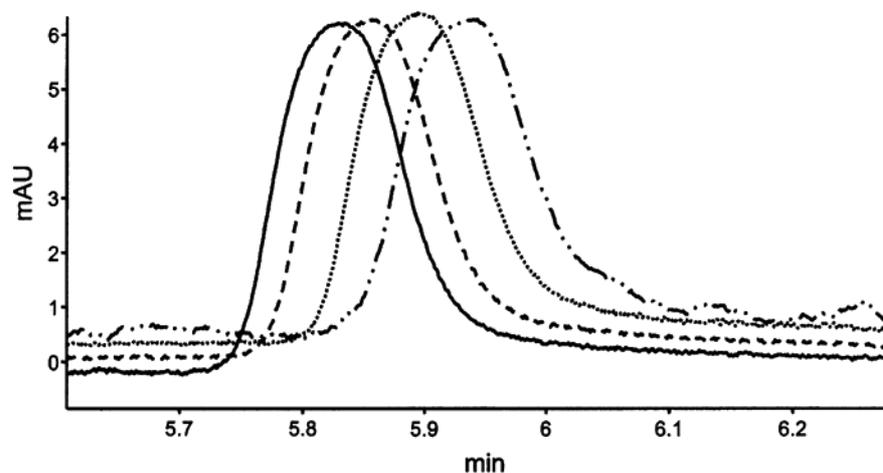


Figure 16. An overlay of electropherograms showing the increased migration time of pindolol as the amount of DSPC/cholesterol/PEG-DSPE(5000) disks increased.

Bilayer disk CE can utilize disks with different compositions and the shelf life of the disks is several months. The running conditions can be easily manipulated to fit the analytes by the choice of disks, the injection volume, and the coating of the capillaries. Bilayer disk CE outperforms ILC in several aspects, e.g., no immobilization procedure is needed and no matrix is present, which eliminate the possibility of analyte-gel interactions. Furthermore, CE requires less analyte and buffer volumes and the analysis time is shorter.

Summary

The good reproducibility together with the fact that the retentions increased linearly with the lipid amount suggests that lipid bilayer disks are well suited as pseudostationary phase in CE. However, the capillary had to be coated with a neutral polymer to avoid adsorption of the disks onto the capillary walls.

Concluding remarks and future aspects

The analytes were subjected to both hydrophobic and electrostatic interactions during partitioning into lipid bilayers. By including charged lipids in liposomes or disks, the bilayer attained a negative or positive net charge. The charge could also be altered by inclusion of charged detergents below the critical micellar concentration. The length of the alkyl chain of the detergents affected the partitioning of drugs, as did the inclusion of a fatty acid.

Hence, drugs clearly exerted both electrostatic and hydrophobic interactions with lipid bilayers. Upon increasing the ionic strength the buffer ions increasingly competed with the charged drugs for positions around the polar lipid head groups, thus affecting the drug partitioning. The effects on neutral drugs were always small due to their deeper penetration of the bilayer. They were more affected by the hydrophobic lipid chains than the hydrophilic head group region. The retention of an oligonucleotide was concentration dependent, and the interaction sites of the oppositely charged bilayer could be saturated.

Neutral lignin precursor models interacted mainly hydrophobically with charged and neutral liposomes and disks. Differences were seen between the partitioning into negative, positive and neutral liposomes, which indicates some electrostatic contribution. This was also seen upon including ammonium sulfate in the buffer. Considering the pronounced partitioning due to the strong interactions exerted by the hydrophobic models, as shown by octanol/water partitioning, and the few hydroxyl groups in the models, diffusion is a possible transport mechanism for the models. Increased temperature decreased the retention of the model substances into both liposomes and disks. The effect was larger into liposomes without sterols, which suggests that sterols affect the bilayer structure. The temperature dependence indicates an enthalpy effect, and the negative ΔH -, and ΔG -values achieved, together with positive ΔS -values, further supported this.

Lipid bilayer disks were for the first time used in a CE system. Negative or neutral disks were introduced as a pseudostationary phase by partial filling, followed by injection of positive drugs. The drugs were retained in the presence of disks, thus showing that partitioning studies are possible with bilayer disk CE in addition to ILC as previously shown. The advantages of bilayer disk CE over ILC are, e.g., the absence of gel, and thereby analyte-gel interactions, the easily adjusted lipid amount, the small amounts of analyte and buffer needed, and the shorter analysis time required.

In the future, ILC with liposomes or disks and bilayer disk CE can be used as convenient methods for quick assessment of partitioning of various solutes into lipid bilayers. The bilayers are simple, but sophisticated models of cell membranes. The quick and reproducible preparations and results are feasible alternatives to studies with natural membranes, and ILC and bilayer disk CE are clearly usable as screening methods in, e.g., the pharmaceutical industry. The present investigations should be complemented with studies of liposomes as vehicles for drugs and oligonucleotides in the blood system. Moreover, more studies on lignin precursor model transport through plant cell membranes are needed to elucidate an important step in lignin biosynthesis. Finally, more extensive studies of bilayer disk CE should be performed for development of this method as an analytical tool.

Summary in Swedish

Fördelning av läkemedel och ligninbyggstensmodeller in i konstgjorda membraner

Introduktion

Alla organismer är uppbyggda av celler vilka omges av ett cellmembran, som avgränsar cellen från dess omgivning. Cellmembranet består främst av fosfolipider, steroler och membranproteiner. Fosfolipider är fettmolekyler som utgör själva basen för membranet. De har en hydrofil, vattenlöslig, huvudregion med en negativ fosfatgrupp samt en yttre liggande grupp som kan vara oladdad eller laddad (Fig. 2). Huvudgruppen sitter ihop med två oladdade fettsyresvansar (Fig. 2), som alltid är hydrofoba, vattenavvisande. I och med att svansarna är hydrofoba vänder de sig emot varandra i vattenlösning. Detta resulterar i ett lipidbilager med svansarna inåt och de hydrofila huvudgrupperna ut mot omgivningen som skydd (Fig. 1). Kolesterol är den dominerande sterolen i djurceller, emedan växtcellerna domineras av stigmasterol, sitosterol (Fig. 3) och campesterol. Sterolerna har en stel ringstruktur och en liten hydrofil grupp.

Membranproteinerna är antingen genomgående, integrala, eller ytliga, perifera, och utgör en viktig del i transport av molekyler över membranet. De är dock oftast specifika och kostar energi, det vill säga aktiva transportörer, men en del bildar porer som fler än ett ämne kan ta sig igenom, så kallad faciliterad transport (Fig. 7). Alla andra molekyler som måste ta sig in i, eller ut ur, cellen gör det enklast genom diffusion över cellmembranet (Fig. 7). De tråcklar sig alltså emellan lipiderna. Diffusion begränsas främst av hur hydrofoba molekylerna är, just på grund av den hydrofoba kärnan i lipidbilagret. Hydrofila ämnen kan, med några få undantag såsom vatten, ej passera genom diffusion.

Växtcellerna är unika på så sätt att de har en cellvägg. Cellväggen ligger utanför cellmembranet och stöttar cellerna och därmed hela växten, samt bildar ett skydd mot attacker av andra organismer. En av de viktigaste molekylerna i denna cellvägg är lignin, som är vanlig framförallt i vedartade

växter. Det är en väldigt stor och stark molekyl som binder samman inte bara cellväggen i en cell, utan tusentals celler. Utan lignin hade vår vegetation varit totalt annorlunda eftersom stora växter inte hade funnits. Växterna är beroende av stödet från lignin samt av dess viktigaste funktion; att omgärda kanalerna som transporterar vatten och näring i växten. Lignin finns i större eller mindre mängder i de flesta växter. Det är uppbyggt av främst tre olika monolignoler (Fig. 9), som är giftiga och instabila.

Min forskning

Jag har jobbat med fördelningsstudier, vilka visar på hur mycket ett ämne interagerar med de olika membrankomponenterna. De konstgjorda membranstrukturer som användes var främst liposomer, vilka är lipidbilager som sluter sig spontant till sfärer när de kommer i en vattenlösning (Fig. 4). Liposomerna gjordes av framrenade lipider och deras sammansättning reglerades genom att till exempel tillsätta laddade fosfolipider eller steroler. Den andra membranmodellen som användes var diskar (Fig. 5), som också är lipidbilager, fast flata utan inre volym. De måste alltid innehålla steroler och polyetylenglykol-substituerade lipider för att kunna bildas, då sterolerna påverkar strukturen på den flata delen och polyetylenglykol-lipiderna sätter sig runt kanterna och stabiliserar hela disken.

Både neutrala och laddade liposomer och diskar användes, liksom liposomer med eller utan steroler. För att ladda liposomerna tillsattes antingen laddade fosfolipider under preparationen (Artiklar I-IV) eller så tillsattes laddade detergenter (Fig. 6) i bufferten (Artiklar I & II). Dessutom användes olika saltmängder samt olika saltjoner i bufferten för att se om det påverkade fördelningen (Artikel I). Steroler tillsattes till liposomer i ett fall (Artikel IV), när det undersöktes om en sammansättning med få lipidsorter kunde ge samma resultat som en sammansättning med många lipidsorter, där det senare var lipidsammansättningen i cellmembranet hos majsrot.

Jag har jobbat med läkemedelsfördelning in i liposomer och diskar (Artiklar I, II & V) och fördelning av ligninbyggstensmodeller in i liposomer och diskar (Artiklar III & IV). De flesta läkemedelssubstanserna som finns är okända för kroppen, vilket gör att det inte finns transportproteiner för dem, utan de i många fall måste diffundera över cellmembranet för att komma in och utgöra sin verkan. Om en substans inte kan fördelas in i membranet, kan det inte heller diffundera över det och verka. Fördelningsstudier är därför intressanta för läkemedelsindustrin då de testar oräkneliga substanser varje år i sin jakt på nya läkemedel. Fördelningen av ligninbyggstensmodeller är intressant rent biologiskt sett eftersom processen som sker från bildandet av monolignoler till uppbyggandet av lignin till stor del är okänd. Då pappersindustrin mest är intresserad av att bryta ner lignin, eftersom det stör papperstillverkningen, så är denna del bra mycket mer undersökt än ligninbildning.

Metoder

Tre olika metoder användes för bestämning av fördelning. I immobiliserad liposomkromatografi (ILC) (Artiklar I-IV) sätts liposomer eller diskar fast i gelkolor (Fig. 8), som sedan packas in i en kolonn och kopplas till ett högpresterande vätskekromatografisystem (Fig. 10). Fördröjningen av substanser i närvaro av lipidbilager kan räknas om till en specifik kapacitetsfaktor (Ekv. 5 & 6), som är direkt proportionell mot fördelningen. Även fördelningskoefficienten kan beräknas (Ekv. 7).

Oktanolvattenfördelning användes (Artikel III) för att bestämma hydrofobiciteten av ligninbyggstensmodellerna samt för att jämföras med ILC resultat. Substanserna löstes i oktanol och skakades med vattenfas, därefter mättes absorbansen i varje fas och man kunde beräkna fördelningen (Ekv. 3).

Diskar injicerades som en pseudostationär fas i kapillärelektrofores (CE) (Artikel V) (Fig. 11). Utifrån ökningen av läkemedelsfördröjningen med ökande mängd diskar beräknades fördelningskoefficienten (Ekv. 8).

Resultat och diskussion

Både negativa och positiva läkemedel påverkades av laddningar i liposomer och diskar (Artiklar I & II). De första kom ut tidigare om det var negativa lipidbilager, på grund av elektrostatisk repulsion, och senare vid positiva lipidbilager på grund av elektrostatisk attraktion med de hydrofila huvudgrupperna (Fig. 13). Det motsatta hände för positiva läkemedel. Neutrala läkemedel påverkades mycket lite av laddningarna i liposomerna och diskarna. Detta beror på att de kommer längre in i lipidbilagret (Fig. 13) och påverkas mest av hydrofoba interaktioner. Dessa kunde förändras något om man tillsatte en fettsyra (Fig. 12) samt detergenter, men ändringarna var inte signifikanta. Vid ILC med positiva liposomer visade det sig att en oligonukleotid inte fördelade sig in i membranet (Artikel II). Det var ett förväntat resultat på grund av dess storlek och dess många negativa laddningar, vilka båda talar emot fördelning. Den interagerade dock elektrostarktiskt med utsidan av liposomerna.

Ligninbyggstensmodellerna är neutrala vid fysiologiskt pH och påverkades bara till en mindre del av elektrostatiske interaktioner med laddade liposomer. De hydrofoba interaktionerna dominerade, vilket kunde ses både genom tillsats av en hög koncentration ammoniumsulfat till bufferten och vid oktanol/vattenfördelningen (Artikel III). Fördelningen in i växtlipiddiskar var mycket lik fördelningen in i diskar med färre lipidtyper (Artikel IV) (Fig. 15), därför kan man lika gärna använda de senare som är lättare att göra. Vid jämförelse av liposomer med samma sammansättning som de enklare diskarna kan man se att fördelningskoefficienterna är högre för diskarna (Fig. 15). Detta beror snarare på beräkningen än på att fördelningen skulle vara annorlunda i olika bilagerstyper. Lipidmängden som är tillgänglig för interaktioner är större i diskar än i liposomer. Lipiderna på det

inre bilagerbladet samt inre liposomer om multilamellära, flerskiktade, liposomer används är svåra att komma åt för substanserna, därmed blir den lipidmängd man räknar med för hög och fördelningen per lipidmängd blir underskattad. Möjligheten till korrekta beräkningar är fördelen med diskar i jämförelse med liposomer. Fördelningen av ligninbyggstemsmodellerna minskade med ökad temperatur (Artiklar III & IV) (Fig. 14). Temperaturberoendet var större när lipidbilagren inte innehöll steroler (Artikel III), vilket tyder på att sterolerna påverkar bilagrets struktur (Artikel IV).

Vid studier av diskar som pseudostationär fas i CE, vilket är en ny tillämpning av CE, visade det sig att positiva läkemedel fördelades sig in i både negativa och neutrala diskar (Artikel V). Fördelningen ökade linjärt med mängden diskar (Fig. 16). Det var en liten skillnad i fördelningen in i negativa respektive neutrala diskar, vilket troligen speglar skillnaden i elektrostatiske interaktioner. Fördelningen in i de negativa diskarna stämde väl överens med tidigare studier gjorda med ILC.

Slutsatser

Lipidbilagrets sammansättning påverkar fördelningen. Laddade fosfolipider samt detergenter ökade fördelningen av motsatt laddade substanser på grund av elektrostatiske attraktioner utöver hydrofoba interaktioner. Det senare är den viktigaste interaktionstypen för neutrala substanser, som inte påverkas nämnvärt av laddningar i lipidbilager. När steroler tillsattes ändrades bilagrets struktur vilket påverkade fördelningen. Både liposomer och diskar kunde användas i ILC-studier. De resulterande fördelningskoefficienterna skiljde sig dock åt, vilket härrör från mängden lipid tillgänglig för interaktion, vilken överskattas när liposomer används. Diskar kunde användas som pseudostationär fas i CE, vilket visades genom att studera läkemedelsfördelning in i både negativa och neutrala diskar. Dessa försök var preliminära och kan utvecklas mycket mer.

Avslutningsvis kan sägas att ILC och CE är enkla metoder som kan användas i fördelningsstudier av till exempel läkemedelssubstanser och det första också med ligninbyggstemsmodeller. Metoderna är snabba och reproducerbara och kan därför vara lämpliga när många substanser ska undersökas på begränsad tid, såsom i jakten på nya potentiella läkemedel.

Acknowledgements

This work was supported by the O.E. and Edla Johansson Science Foundation, the Swedish Research Council, the Swedish Fund for Research without Animal Experiments, and the Swedish Research Council for Engineering Sciences.

First of all I would like to acknowledge my supervisors Per Lundahl & Gunnar Johansson. Per, your faith in me I will never forget. Thank you for everything you taught me, and the warm caring person you were. Gunnar, thank you for letting me into your group and for all ideas awoken. Your brilliant mind is amazing and I wish you all the best with future projects. Thank you Christine Lagerquist Hägglund for being my co-supervisor. Your support has been indispensable.

I would like to thank the membrane group; Caroline, for all nice times both at work, during teaching and outside of work; Christine, for chats, nice conference trips and good co-teaching; Anna, for being a fun person to have around and for getting the work done; Ingo, you are truly original, as is Shusheng, nihao for trying to teach me some Chinese words, and both of you, thanks for good collaboration in the course lab; Sanela, for many nice talks about work and life, and Juanjo, for good collaboration and for the nice stay in Valencia. I would also like to thank my new colleagues in the cellulase group; Anu, for many laughs and nice chats in the doorposts; Jing, for always sharing a smile, and Lars for good co-teaching and for two of the model substances. My co-authors, in addition to the above mentioned, Katarina Edwards, Mikael Nilsson, Roland Isaksson and Jyoti Chattopadhyaya are acknowledged for fruitful collaborations.

My thanks also goes to the fika-room gang especially; David, for reading and correcting all of my papers as well as this thesis; Ulla, for administrative help; Lilian for excellent assistant during teaching, and Per-Axel for being friend with all apparatus. People in “the other corridor”, especially Ylva, Birgitta E, Göran, Carolina and Ann-Christin for collaboration in the course lab; the party committee members over the years, Christine, Cynthia, Nisse, Lilian, Ylva, Thomas, Anna, Malena, Göran, Carolina and Henrik; and also Dan, for your friendship, Anton, for talks and chocolate, and Birgitta T for excellent supervision during teaching.

At IMBIM I would like to thank Göran Akusjärvi, for giving me a change to try out to work as a scientist, Catharina Svensson for encouragement over the years, Vita for being an excellent teacher in the lab, and of course Cecilia, Camilla, Anette,

Margaret, Saideh, Christina and Tanel among many others for good times in the lab as well as in our limited free time.

I would also like to thank Hans-Erik Claesson at Karolinska Institutet for my intriguing examensarbete, Helén for help in the lab, and Stina my nice roommate.

My Karlstad friends, especially Maria A, Simsim, Maria Jo, Ia, Åsa, Maria Ja, Anna, Karin, Anne-Maj and Malin among many others. Our years in Karlstad were some of the most fun years of my life, thank you!

Från min gymnasietid vill jag tacka Ann-Cathrine, Maria, Andrea och Annika för glada stunder och mycket skitsnack i omklädningsrummet efter gympan.

Grundskolan lägger grunden. Anna, Ellinor, Beatrice, Gun-Britt, Camilla och Eva, tack för många roliga lekstunder. Therése, du gick från ovän till bästis, tack för allt vi gjorde ihop. Josephine, tack för många skratt och mycket prat om allt mellan himmel och jord. Sofia, tack för slitet under danskurser och i aerobicssalen och för innovativa håruppsättningar.

Tack mormor och morfar för ert stöd genom åren och moster för ditt engagemang i mitt liv. Tack farmor och farfar för er omsorg under våra sommarvistelser i brukstugan, jag saknar er! Tack faster Agneta, Kjell och Hans för trevliga sammankomster; farbror Pelle, Agneta, Lena, Christian, Emelie, Regina, Lennart, Jeanette, Niklas, Nina, Nelly, Linus och Linn, för mycket trevliga jular ihop; faster Kajsa och Peter för goda middagar; farbror Anders, Susanne och Fanny, för goda kakor och för vår första bil; faster Maria, för din starka personlighet och framåtanda och Göran som verkar vara en trevlig prick.

Gammelmoster Gunhild, du har varit som en extrafarmor och en väldigt god vän i ett, tack för att du tog så väl hand om mig under min tid i Karlstad och för vår fortsatta vänskap! Märith, Jari, Sara, Erika och Marie, tack för att ni varit min extrafamilj och för många glada skratt. Gammelfaster Kerstin, tack för att jag fick bo hos dig under mitt examensarbete. Du tog väldigt väl hand om mig och vår vänskap är värdefull för mig! ”Svärmor” Eleonor och ”svärfar” Lennart, Jenny, Jörgen, Jonathan och Jesper, tack för att ni välkomnat mig i er familj, för vänskap och lekstunder.

Mamma och Pappa, ni är otroliga på alla sätt och vis. Ni gjorde min uppväxt till en underbar tid, och jag är säker på att ni kommer att göra detsamma för min framtid! Lillasyster Susanne, även om jag ibland blir frustrerad på dig, så hoppas jag att du aldrig ändrar dig, du är underbar! Jörgen, tack för att du tar hand om Sussi, för din vänskap och för dina galenheter. Lycka till båda två med min lilla systerdotter! Lillasyster Thérèse, du håller familjen samman. Din energi och pratsamhet är smittande och avundsvärd. Tack för att du är den du är! Pelle, ta hand om henne så gott du kan, hon är krävande, men hennes kärlek är väl värt arbetet.

Min Petter, mitt liv vore alltför tomt utan dig! Jag älskar dig för den du är, och älskar dig för att du står ut med mig. Puss och kram, flickan din.

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