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INTERACTIONS OF DRUGS WITH BIOLOGICAL MODEL MEMBRANES: A PHYSICOCHEMICAL APPROACH

Doctoral Dissertation

Marjukka Ikonen

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Abstract

The interactions of drugs with biological membranes affect the delivery of drugs to the target sites within the body. Usually, a drug has to pass through several membranes in order to reach the target location. Because of this, knowledge on the interactions of drugs with biological membranes is essential not only for the understanding of the therapeutic action of existing drugs, but also in the discovery process of new candidates.

This thesis explores the possible use of the physicochemical methods in the study of drug–membrane interactions. The emphasis of the thesis is on the various physicochemical approaches in determining the partition coefficient of drugs. As the properties of the drug carriers are equally important in drug delivery, attention is also focused on the physicochemical properties of drug carriers and their interactions with biological membranes.

The most widely used parameter in the assessment of membrane permeability is the lipophilicity of a drug, which is most often expressed as the partition coefficient between two phases. In this thesis, the liposome–water partition coefficients of drugs were determined using two different methods, isothermal titration calorimetry and a new electrochemical method, which utilizes an electrified liquid–liquid interface. In addition, the partitioning to the hydrocarbon core of the lipid bilayer was studied using contact angle measurements on three different hydrophobic model membranes.

The physicochemical properties of cationic polymer/plasmid DNA complexes and their interactions with the cell surface glycosaminoglycans (GAGs) were studied using dynamic light scattering, isothermal titration calorimetry and agarose gel electrophoresis. It was shown that the aggregation of polyethylene imine/plasmid DNA complexes can be controlled with the surfactant polyoxyethylene stearate, which also protects the complexes against the negative effects of extracellular GAGs. These findings are particularly relevant for ocular gene delivery, as the membranes in the eye have a very high content of GAGs.

As a whole, this research addresses a number of important aspects of drug–membrane interactions from the physicochemical perspective. Various approaches to the partitioning of drugs were explored and three different experimental methods were used to determine the partition coefficients of eight drugs. Furthermore, physicochemical explanations were presented for a broad range of phenomena from the electrostatics of the binding of drugs to the aggregation of the DNA complexes.

Keywords drug, liposome, electrochemistry, liquid–liquid interface, partition coefficient, plasmid DNA

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Tiivistelmä

Lääkeaineiden ja biologisten membraanien vuorovaikutukset vaikuttavat siihen, miten lääkeaineet kulkeutuvat elimistössä. Yleensä lääkeaineen täytyy läpäistä useita kalvoja, ennen kuin se päätyy oikeaan kohteeseensa. Tämän vuoksi lääkeaineiden vuorovaikutukset ovat olennaisia paitsi jo olemassa olevien lääkkeiden vaikutusmekanismin ymmärtämisen kannalta, mutta myös uusien lääkekandidaattien kehitystyössä.

Tässä väitöskirjassa selvitetään fysikokemiallisten menetelmien mahdollisuuksia lääkeaineiden ja membraanien vuorovaikutuksien tutkimuksessa. Työssä painotetaan erityisesti fysikokemiallisia lähestymistapoja lääkeaineen jakautumiskertoimen määrittämiseksi. Kuitenkin lääkeaineiden kantajat ovat yhtä tärkeitä onnistuneen lääkkeenannon kannalta, joten työssä kiinnitetään huomiota myös lääkeaineiden kantajien fysikokemiallisiin ominaisuuksiin ja vuorovaikutuksiin biologisten membraanien kanssa.

Lääkkeiden membraaniaktiivisuuden arvioinnissa yleisimmin käytetty parametri on lääkeaineen lipofiilisyys, jota usein kuvataan kahden faasin välisen jakautumiskertoimen avulla. Tässä väitöskirjassa käytettiin kahta eri menetelmää lääkeaineiden liposomi–vesi -jakautumiskertoimen määritykseen: isotermistä titrauskalorimetriä (ITC) ja uutta sähkökemiallista menetelmää, jossa hyödynnettiin polarisoituvaa neste–neste -rajapintaa. Lisäksi lääkeaineiden jakautumista lipidikaksikerroksen sisäisiin hiilivetyketjuihin tutkittiin mittaamalla lääkeaineliuoksen kontaktikulma kolmella eri hydrofobisella mallipinnalla.

Kationisista polymeereista tehtyjen DNA-kompleksien fysikokemiallisia ominaisuuksia ja vuorovaikutuksia solukalvon glykosaminoglykaanien (GAG) kanssa puolestaan tutkittiin dynaamisella valosironnalla, ITC:llä ja agaroosigeelielektroforeesilla. Tutkimuksessa osoitettiin, että polyetyleeni-imiini/DNA -kompleksien aggregaatiota voidaan rajoittaa polyoksietyleenistearaatilla, joka on pinta-aktiivinen aine. Samalla kompleksit suojataan solunulkoisten GAG:ien negatiivisia vaikutuksia vastaan. Nämä tulokset ovat tärkeitä etenkin silmälääkityksen kehittämisen kannalta, sillä silmän membraaneissa GAG:ja on erityisen paljon.

Kokonaisuudessaan tässä väitöskirjassa esitetään fysikokemiallinen lähestymistapa useisiin tärkeisiin lääkeaine-membraani -vuorovaikutuksiin. Työssä käsiteltiin lääkeaineiden jakautumista useasta näkökulmasta ja kokeellisesti jakautumiskertoimet määritettiin kahdeksalle lääkeaineelle kolmella eri menetelmällä. Lisäksi lukuisia ilmiöitä pyrittiin selittämään fysikokemiallisesti, kuten lääkkeiden elektrostaattinen sitoutuminen ja DNA-kompleksien aggregoituminen.

Asiasanat lääkeaine, liposomi, sähkokemia, neste–neste -rajapinta, jakautumiskerroin, plasmidi-DNA

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Preface

This work was carried out at the Department of Chemistry, Aalto University School of Science and Technology from November 2004 to March 2010. I gratefully acknowledge the financial support from the Finnish Foundation for Technology Promotion (TES).

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Marjukka Ikonen
Espoo, April 28th, 2010

Contents

List of Publications

This thesis consists of an overview and of the following publications which are referred to in the text by their Roman numerals.

I M. Ikonen, L. Murtomäki, K. Kontturi, An electrochemical method for the determination of liposome-water partition coefficients of drugs, *J. Electroanal. Chem.* **602** (2007) 189-194.

II M. Ikonen, L. Murtomäki, K. Kontturi, Controlled complexation of DNA with cationic polymers: Effect of surfactant on the complexation and stability of the complexes, *Colloids Surf., B* **66** (2008) 77-83.

III M. Ikonen, L. Murtomäki, K. Kontturi, Studying the interactions of drugs and hydrophobic model membranes using contact angle goniometry, *Colloids Surf., B* **71** (2009) 107-112.

IV M. Ikonen, L. Murtomäki, K. Kontturi, Microcalorimetric and zeta potential study on binding of drugs on liposomes, *Colloids Surf., B* **78** (2010) 275-282.

Author's contribution

Marjukka Ikonen carried out all the experimental work and most of the data analysis in Publications I-IV. She was also responsible for the writing of these manuscripts. The phosphorus analyses in Publications I and IV were performed by Mr. Hannu Revitzer. The Matlab® simulations presented in Section 2.1 (Partitioning of ionized drugs) were carried out by Dr. Lasse Murtomäki.

Professor Kyösti Kontturi
Espoo, April 28th, 2010

List of Abbreviations

ADME	adsorption, distribution, metabolism, and excretion properties of drugs
Caco-2	human colon carcinoma cell line
CMC	critical micelle concentration
dmfc	decamethylferrocene
DCE	1,2-dichloroethane
DNA	deoxyribonucleic acid
DPPTE	1,2-dipalmitoyl-*sn*-glycero-3-phosphothioethanol
DS	dextran sulfate
IAM	immobilized artificial membrane
ITC	isothermal titration calorimetry
ITIES	interface between two immiscible electrolyte solutions
NB	nitrobenzene
NMR	nuclear magnetic resonance
NPOE	nitrophenyl octyl ether
N/P	nitrogen-phosphate ratio
PAMPA	parallel artificial membrane permeation assay
pDNA	plasmid DNA
PEI	polyethyleneimine
PLL	poly-L-lysine
POES	polyoxyethylene (100) stearate
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)]
SAM	self-assembled monolayer
SWV	square wave voltammetry
TATB	tetraphenylarsonium tetraphenylborate

List of Symbols

a	radius
$a_{\mathrm{i}}^{\mathrm{o}}$	activity of ion *i* in organic phase
$a_{\mathrm{i}}^{\mathrm{w}}$	activity of ion *i* in aqueous phase
$a_{\pm}$	mean activity
A	area
c	concentration
c_{i}	concentration of drug
c_{dmfc}	concentration of decamethylferrocene
$c_{\mathrm{L,f}}$	concentration of free lipids
c^{l}	concentration of drug in lipid bilayer
c^{w}	concentration of drug inside aqueous cavity of liposome
$c_{\pm}$	mean concentration
c*	1.0 mol dm^{-3}
D	distribution coefficient
D_i	diffusion coefficient of ion *i*
e	elementary charge
$E^{0}_{\mathrm{dmfc}_o^{+}/\mathrm{dmfc}_o}$	standard redox potential of the couple dmfc/$dmfc^{+}$
E_{f}	formal redox potential
F	Faraday constant
h	thickness of bilayer
K	adsorption coefficient
K_{app}	apparent value of binding constant
K_{b}	binding constant
$K_{\mathrm{d}}^{\mathrm{w}}$	dissociation constant of weak electrolyte
K_{a}	acid dissociation constant
n^{l}	amount of drug in the bilayer
N_{A}	Avogadro constant

P_i	partition coefficient of ion *i*
P_i^0	standard partition coefficient of ion *i*
q	grand canonical partition function
R	ideal gas constant
t_p	pulse width
T	temperature
V^l	volume of lipid bilayer
V^o	volume of organic phase
V^w	volume of aqueous phase
x	distance
z	total number of drug molecules in a liposome
z_c	number of drug molecules in aqueous cavity of liposome
z_i	charge number of ion *i*
α	degree of dissociation
γ_i	activity coefficient of ion *i*
$\gamma_\pm$	mean activity coefficient
γ_LV	liquid–vapor interfacial tension
γ_SL	solid–liquid interfacial tension
γ_SV	solid–vapor interfacial tension
Γ	surface excess
ΔG	Gibbs energy of binding
ΔH	enthalpy of binding
Δi_p	peak current
ΔS	entropy of binding
$\Delta\psi_\mathrm{p}$	dimensionless peak current
$\Delta_\mathrm{o}^\mathrm{w} G_i^0$	Gibbs free energy of transfer
$\Delta_\mathrm{o}^\mathrm{w}\phi$	Galvani potential difference
$\Delta_\mathrm{o}^\mathrm{w}\phi_{1/2}$	half wave potential
$\Delta_\mathrm{o}^\mathrm{w}\phi_i^0$	standard transfer potential of ion *i*

ε_0	permittivity of free space
ε_r	relative permittivity
ζ	zeta potential
θ	surface coverage or contact angle
κ	reciprocal of Debye length
μ_i^o	chemical potential of ion *i* in organic phase
μ_i^w	chemical potential of ion *i* in aqueous phase
$\mu_i^{o,0}$	standard chemical potential of ion *i* in organic phase
$\mu_i^{w,0}$	standard chemical potential of ion *i* in aqueous phase
$\tilde{\mu}_i^o$	electrochemical potential in organic phase
$\tilde{\mu}_i^w$	electrochemical potential in aqueous phase
σ	surface charge
ϕ^o	Galvani potential in organic phase
ϕ^w	Galvani potential in aqueous phase
$e^{\pm\varphi}$	potential dependent part of P_i

1 Introduction

The interactions of drugs with biological membranes affect the delivery of drugs to the target sites within the body. Usually, a drug has to cross several membranes in order to enter the target location. As a result, the optimization of the delivery of drugs requires understanding of the interactions of drugs with biological membranes. Knowledge of these interactions is also of prime importance when predicting adsorption, distribution, metabolism, and excretion (ADME) properties of drugs already in the early phases of drug discovery process.

The most common physicochemical property used in the prediction of drug-membrane interactions is the lipophilicity of a drug, which is usually expressed as log *P*, the logarithm of the partition coefficient between two immiscible solvents. Traditionally, the partition coefficient has been determined using *n*-octanol and water. However, the ability of the octanol–water partition coefficient to describe drug partitioning has been questioned due to the major differences in the biophysical properties of octanol and phospholipid cell membrane. Due to this, alternative approaches, including both experimental and computational methods, have been developed. Because of the significance of the partition coefficient in the evaluation of drug-membrane interactions, a major part of this thesis is devoted to the development of new methods for the determination of the partition coefficient of drugs. The determination of the partition coefficient is approached experimentally using various physicochemical methods (Publications I, III and IV) as well as from a theoretical point of view.

Physicochemical properties of the drugs are of utmost importance when considering the delivery of drugs to the target site. Yet equally important are the properties of the carriers which are used to improve the delivery and effectiveness of drugs. To minimize premature drug degradation, prevent undesirable side effects and increase the bioavailability of the drug, various drug delivery and drug targeting systems have been established, such as liposomes, micelles, synthetic polymers, and microspheres.

Liposomes are phospholipid vesicles that form spontaneously in aqueous environments. Drugs can be encapsulated either inside the aqueous cavity or within the phopholipid bilayer of the vesicle. The interaction of liposomes and drug molecules has been of interest mainly for two reasons: On one hand, because of their resemblance with biological membranes, liposomes have been used as model membranes to study interactions of drugs and phospholipids at cellular level. On the other hand, biocompatibility has allowed the use of liposomes as delivery systems in drug targeting. In this thesis, both of these aspects were considered, when the ability of liposomes to encapsulate β-blockers was studied (Publication I).

Carriers of drugs are especially important in gene delivery. Various viral and non-viral gene delivery vehicles have recently been developed for the use of gene therapy. However, due to the safety concerns associated with viral vectors, such as their toxicity and potential for generating a strong immune response, non-viral DNA carriers have gained increasing interest. In addition to safety issues, the advantages of the non-viral gene delivery vehicles include the ease of their structure modification and low cost. Despite the advantages, a lot of research is still needed on non-viral vectors before they can be utilized in clinical applications as their efficiency is much lower when compared with the viral vectors. Publication II focuses on the physicochemical properties of the cationic polymer–plasmid DNA complexes of two commonly used polymers, their tendency to aggregate, and their interaction with the cell surface glycosaminoglycans (GAGs). The physicochemical origin of the complexation and aggregation described in Publication II may prove to be of great importance when the interactions of the complexes with biological membranes are evaluated and better gene delivery systems are developed.

This thesis aims to highlight the value of physicochemical methods when studying drug–membrane interactions, with emphasis on the various physicochemical approaches in determining the partition coefficient of drugs. As the properties of the drug carriers are equally significant in drug delivery, attention is also focused on the physicochemical properties of drug carriers and their interactions with biological membranes.

2 Partitioning of drugs

Before obtaining the therapeutic effect of a drug, the drug has to enter the body and reach the site of action. There are two main routes for drug permeation across the cell membrane: paracellular transport between the adjacent epithelial cells and transcellular route across the cells [1]. Transcellular processes can be further divided into passive diffusion and active transport, which requires specialized membrane proteins. Of these, the process of passive diffusion is in the focus of this thesis as it is the primary mechanism for most conventional drug molecules. Furthermore, the epithelial interface is usually assumed to act as a simple lipophilic barrier where the rate of absorption correlates with the lipophilicity of the drug. The drug lipophilicity is often measured by its partition coefficient P_i, which is defined as the ratio of the activity a species in two immiscible phases in equilibrium [2]:

$$P_i = \frac{a_i^{\mathrm{o}}}{a_i^{\mathrm{w}}} \tag{1}$$

where a_i^{o} is the activity in the oil phase and a_i^{w} the activity in the aqueous phase.

The partition coefficient is important not only in the absorption of the drug, but also in the other pharmacokinetic processes. Along with the structure of the drug and drug–receptor interactions, the pharmacokinetic ADME processes are the determining factors which govern the efficacy of the drug. In these processes, lipid solubility often plays a major role and thus the study of drug partitioning is a cornerstone for understanding the interactions of drugs with biological membranes.

2.1 Partitioning of ionized drugs

Understanding the dissociation equilibrium and partitioning of electrolytes is of fundamental importance in drug delivery, as most drugs exist as weak acids or bases in the body. However, earlier it was commonly accepted that only neutral and non-polar compounds are able to penetrate the phospholipid membrane [3]. Even though ionic species were observed to diffuse across the biological membranes, the popular explanation for the phenomenon was that the ionized drugs form lipophilic ion pairs and enter the membrane in the neutral form [4,5]. Yet many later studies have shown that also ionized drugs permeate biological membranes [6–10]. Therefore, it is now recognized that the partition coefficients need to be determined not only for the neutral drug, but also for the ionized species.

In the determination of partition coefficients for ionized drugs, the distribution of the ions between the aqueous phase and the lipid phase is of utmost significance. A general method for the calculation of the equilibrium values from the initial concentrations of the ions was first reported by Hung [11]. In the work of Hung, equations are presented for the determination of the Galvani potential difference when the concentrations, activity coefficients, standard Gibbs energies of transfer of ions, volumes of each phase and temperature are known. The theoretical treatment of ion partitioning, which takes into account the effect of the volume ratio of the two phases was continued by Kakiuchi [12]. The work of Kakiuchi examines the effect of complexation on the partition equilibria more closely and attention is also paid to the cases of limiting behavior when the volume ratio is extremely large or small. The works of Hung and Kakiuchi have previously been applied to the analysis of microemulsions, where the size of the droplet is comparable to the Debye length [13].

Here, the Galvani potential difference, phase volume ratio and pH are demonstrated to affect the partitioning of ionized drugs between two bulk phases. As in the general case of the equilibrium of ions in a system of two immiscible liquid phases, a Galvani potential difference across the aqueous and lipid phases is created when ions partition

into the biological membrane. This Galvani potential difference can be seen as a driving force for the partitioning of the ionized drugs and it can be related to the partition coefficients of the ionic forms of the drugs. Furthermore, the partition coefficient of an ionized drug is not an independent constant, but it is dependent on the volume ratio of the phases and the pH value of the surroundings. In addition, when studying the partitioning of ionized drugs, one has to keep in mind that the ionized form of the drug cannot penetrate the membrane alone, but a counter ion is always transferred with the ionized drug due to the electroneutrality condition. The purpose of the following sections is to show with straightforward examples and illustrative simulations how to evaluate the effects of the Galvani potential difference, phase volume ratio and pH in the case of partitioning of ionized drugs. As partitioning is defined between bulk phases, this treatment excludes the deviations from the electroneutrality, which take place in very thin electrical double layers at the interfaces of two phases.

2.1.1 Galvani potential difference

When ionized drugs partition into biological membranes, the Galvani potential difference is created. This is because the electrochemical potentials of the ion in the two phases, i.e. the aqueous phase outside the membrane and the lipid phase inside the membrane, are equal:

$$\tilde{\mu}_i^{\mathrm{w}} = \tilde{\mu}_i^{\mathrm{o}} \tag{2}$$

The electrochemical potential can be expressed as the sum of a chemical and electrical term:

$$\tilde{\mu}_i^{\mathrm{w}} = \mu_i^{\mathrm{w}} + z_i F \phi^{\mathrm{w}} = \mu_i^{\mathrm{w},0} + RT \ln a_i^{\mathrm{w}} + z_i F \phi^{\mathrm{w}} \tag{3}$$

where μ_i^{w} is the chemical potential of the ion, z_i the charge number of the ion, F the Faraday constant, and ϕ^{w} is the Galvani potential of the aqueous phase. On the right

hand side, $\mu_i^{w,0}$ represents the standard chemical potential and a_i^w the activity of the ion in the aqueous phase. *R* is the molar gas constant and *T* is temperature.

The electrochemical potential can be written for the lipid phase in a similar manner. From the equality of the electrochemical potentials in the two phases, the Galvani potential difference can be expressed as:

$$\Delta_o^w\phi = \phi^w - \phi^o = \frac{\mu_i^{o,0} - \mu_i^{w,0}}{z_i F} + \left(\frac{RT}{z_i F}\right)\ln\left(\frac{a_i^o}{a_i^w}\right) \tag{4}$$

Equation (4) can be rewritten as:

$$\Delta_o^w\phi = \Delta_o^w\phi_i^0 + \left(\frac{RT}{z_i F}\right)\ln\left(\frac{a_i^o}{a_i^w}\right) = \Delta_o^w\phi_i^0 + \left(\frac{RT}{z_i F}\right)\ln\left(P_i\right) \tag{5}$$

where $\Delta_o^w\phi_i^0$ is the standard transfer potential of ion *i*, and P_i is its partition coefficient. The standard transfer potential is related to the Gibbs free energy of transfer, $\Delta_o^w G_i^0$ as follows:

$$\Delta_o^w\phi_i^0 = -\frac{\mu_i^{w,0} - \mu_i^{o,0}}{z_i F} = -\frac{\Delta_o^w G_i^0}{z_i F} \tag{6}$$

The partition coefficient of a neutral drug can be calculated from the partition coefficient of the corresponding ionized form by subtracting the electrostatic contribution of the Gibbs free energy of transfer, which can be estimated as [14]:

$$\Delta_o^w G_{es}^0 \approx \frac{z^2 e^2 N_A}{8\pi\varepsilon_0 a}\left(\frac{1}{\varepsilon_r^w} - \frac{1}{\varepsilon_r^o}\right) \tag{7}$$

For an ion of the radius a = 0.3 nm Equation (7) gives ca. −20 kJ/mol*, which on log P scale means about −3.5 units. Hence, log P of an ionic species is usually negative.

From Equations (5) and (6), the partition coefficient of the ionized drug is solved as [15]:

$$P_i = \frac{a_i^{\mathrm{o}}}{a_i^{\mathrm{w}}} = \exp\left(\frac{\Delta_{\mathrm{o}}^{\mathrm{w}} G_i^0}{RT}\right)\exp\left(\frac{z_i F}{RT}\Delta_{\mathrm{o}}^{\mathrm{w}}\phi\right) = P_i^0 \exp\left(\frac{z_i F}{RT}\Delta_{\mathrm{o}}^{\mathrm{w}}\phi\right) \quad (8)$$

In Equation (8), P_i^0 is the standard partition coefficient, which depends only on the chemical structure of the ionized drug. Equation (8) shows, however, that the Galvani potential difference also affects the value of the partition coefficient of the drug between the two phases, and not just the chemical structure is responsible. Thus, it is evident that the partition coefficient of an ionized drug can be altered by changing the Galvani potential difference between the phases. Equation (8) for a neutral species (z_i = 0) reduces to the usual form. In the following sections, illustrative simulations are used to elucidate the effects of the counter ion and the phase volume ratio on the Galvani potential and partitioning.

2.1.2 Role of counter ion

Because of the electroneutrality condition, a single ion cannot partition across two phases alone. In order for the electroneutrality condition to be fulfilled, the same amount of the opposite charges must be transported simultaneously. This means that the ionized drug always carries a counter ion with it, and thus partitioning of an ionized drug depends not only on the properties of the drug, but also on the properties of the

*This is the Born model, where e = elementary charge, 1.6×10^{-19} C, N_A = Avogadro constant, 6.02×10^{23} mol^{-1}, ε_0 = permittivity of free space, 8.854×10^{-12} F m^{-1}, ε_r = relative permittivity, taken as 78.4 for water and 10.0 for the organic phase.

counter ion. Although deviations from the electroneutrality are found in very thin electrical double layers at interfaces, partitioning is defined between bulk phases.

Here, partitioning of 1-1 electrolyte is considered in order to exemplify the role of the counter ion. Writing Equation (2) for a cation and an anion gives:

$$\mu_{+}^{0,\mathrm{w}} + RT \ln a_{+}^{\mathrm{w}} + F\phi^{\mathrm{w}} = \mu_{+}^{0,\mathrm{o}} + RT \ln a_{+}^{\mathrm{o}} + F\phi^{\mathrm{o}} \tag{9}$$

$$\mu_{-}^{0,\mathrm{w}} + RT \ln a_{-}^{\mathrm{w}} - F\phi^{\mathrm{w}} = \mu_{-}^{0,\mathrm{o}} + RT \ln a_{-}^{\mathrm{o}} - F\phi^{\mathrm{o}} \tag{10}$$

As the electrochemical potential in one phase can be expressed as $\tilde{\mu}_{\pm}^{\alpha} = \tilde{\mu}_{+}^{\alpha} + \tilde{\mu}_{-}^{\alpha}$ (α is w or o), summing Equations (9) and (10) results in:

$$\mu_{\pm}^{\mathrm{w},0} + RT \ln\left(a_{\pm}^{\mathrm{w}}\right)^2 = \mu_{\pm}^{\mathrm{o},0} + RT \ln\left(a_{\pm}^{\mathrm{o}}\right)^2 \tag{11}$$

and

$$\left(P_{\pm}\right)^2 = \left(\frac{a_{\pm}^{\mathrm{o}}}{a_{\pm}^{\mathrm{w}}}\right)^2 = \left(\frac{\gamma_{\pm}^{\mathrm{o}}}{\gamma_{\pm}^{\mathrm{w}}}\frac{c_{\pm}^{\mathrm{o}}}{c_{\pm}^{\mathrm{w}}}\right)^2 = \exp\left(\frac{\Delta_{\mathrm{o}}^{\mathrm{w}} G_{\pm}^{0}}{RT}\right) = \frac{a_{+}^{\mathrm{o}}}{a_{+}^{\mathrm{w}}}\frac{a_{-}^{\mathrm{o}}}{a_{-}^{\mathrm{w}}} = P_{+}P_{-} \tag{12}$$

where $c_{\pm}$ is the mean concentration, $a_{\pm}$ the mean activity and $\gamma_{\pm}$ the mean activity coefficient that can be determined experimentally. This example shows the difference between the partitioning of a neutral and an ionized drug.

Furthermore, an expression for the Galvani potential difference can be found by subtracting Equation (10) from Equation (9):

$$\begin{aligned}\Delta_{\mathrm{o}}^{\mathrm{w}}\phi &= -\frac{1}{2}\frac{\Delta_{\mathrm{o}}^{\mathrm{w}}G_{+}^{0}-\Delta_{\mathrm{o}}^{\mathrm{w}}G_{-}^{0}}{F}+\frac{RT}{2F}\ln\left(\frac{a_{+}^{\mathrm{o}}a_{-}^{\mathrm{w}}}{a_{+}^{\mathrm{w}}a_{-}^{\mathrm{o}}}\right)\\ &\approx \frac{1}{2}\left(\Delta_{\mathrm{o}}^{\mathrm{w}}\phi_{+}^{0}+\Delta_{\mathrm{o}}^{\mathrm{w}}\phi_{-}^{0}\right)=-\frac{1}{2}\frac{RT}{F}\ln\left(\frac{P_{+}^{0}}{P_{-}^{0}}\right)\end{aligned} \tag{13}$$

In Equation (13), the approximation is due to the electroneutrality condition $c_{+}^{\mathrm{o}}=c_{-}^{\mathrm{o}}$ and $c_{+}^{\mathrm{w}}=c_{-}^{\mathrm{w}}$ and the fact that the ratio of the activity coefficients approaches unity much faster than any of the activity coefficients alone when the concentration approaches zero. The role of the counter ion in drug partitioning is clearly illustrated by Equation (13). It is not only the ionized drug that creates the Galvani potential difference, but the counter ion contributes to it as well. Thus the counter ion has a major effect on the measured value of the partition coefficient for an ionized drug.

Moreover, understanding the role of the Galvani potential difference in partitioning of ionized drugs provides the means to control the partitioning phenomenon. The potential between the two phases can be adjusted simply by a common ion present in both phases. If the aqueous phase contains, say, an electrolyte $C^{+}A^{-}$ and an organic phase an electrolyte $C^{+}B^{-}$, the common cation C^{+} fully determines the Galvani potential difference between the phases that is:

$$\Delta_{\mathrm{o}}^{\mathrm{w}}\phi=\Delta_{\mathrm{o}}^{\mathrm{w}}\phi_{\mathrm{C}^{+}}^{0}+\left(\frac{RT}{z_{i}F}\right)\ln\left(\frac{a_{\mathrm{C}^{+}}^{\mathrm{o}}}{a_{\mathrm{C}^{+}}^{\mathrm{w}}}\right) \tag{14}$$

In equation (14), the partitioning of the species A^{-} and B^{-} is assumed to be negligible. Adding some hydrophobic electrolyte into the organic phase is found to shift partition equilibria, which is often explained by ion-pairing in the organic phase, but as Girault and co-workers [15] have also pointed out, the reason more probably is the shift of the Galvani potential difference according to Equation (14).

The important role of the counter ion in drug delivery can be further illustrated by a thought experiment on solubility. Salicylic acid is soluble in water as such, and is partitioned between an organic and an aqueous phase, but if the hydrogen ion in it is replaced by a more hydrophobic cation, say, tetraethylammonium ion, the resulting tetraethylammonium salicylate no longer dissolves in water in significant amounts. This demonstrates why it is crucial to evaluate not only the partitioning of the ionized drug, but also that of the counter ion. Because of the electroneutrality condition, a single ion does not partition across two phases alone, but the same amount of the opposite charges must be transported simultaneously.

2.1.3 Effect of phase volume ratio

Partitioning of drugs may also depend on the volume ratio of the aqueous and lipid phases. The volume ratio not only affects the determination of the partition coefficients of the drugs *in vitro*, but it can also be of importance when the drug partitions the biological membrane *in vivo* as the volume of the lipid membrane is very small compared to the surrounding aqueous media.

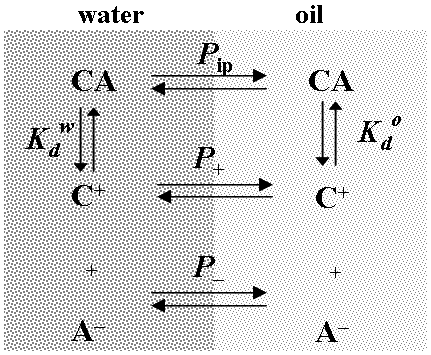

Figure 1. Scheme of partitioning of a weak electrolyte C^+A^- between an aqueous and an organic phase; 'ip' denotes an ion-pair.

The situation is illustrated by partitioning of a weak electrolyte according to the scheme in Figure 1, where dissociation and partitioning equilibria are accompanied by the

pertinent equilibrium constants. Due to electroneutrality*, the dissociation constant of a weak electrolyte in the aqueous phase, K_d^w, can be written as:

$$K_d^w = \frac{[C^+]^w[A^-]^w}{[CA]^w} = \frac{\left([C^+]^w\right)^2}{[CA]^w} \quad (15)$$

where C^+ denotes the cation and A^- the anion. If the initial concentration of a weak electrolyte CA in the aqueous phase is denoted by c_0, the mass balance at equilibrium is:

$$c_0 = [CA]^w + [C^+]^w + \left([CA]^o + [C^+]^o\right)/r \quad (16)$$

where $r = V^w/V^o$, the ratio of the phase volumes. Applying the dissociation and partitioning equilibria Equation (16) becomes:

$$c_0 = [CA]^w\left(1 + P_{ip}/r\right) + \sqrt{[CA]^w K_d^w}\left(1 + P_+/r\right) \quad (17)$$

where P_{ip} denotes the partition coefficient of an ion-pair. Equation (17) is a second order polynomial of $\sqrt{[CA]^w}$ and can thus be easily solved, after which $[C^+]^w$ is known from Equation (15). It is worth mentioning that in the case of partitioning of a single 1-1 electrolyte, the partition coefficients P_+ and P_- are equal, because the Galvani potential difference compensates the difference between P_+^0 and P_-^0, and $P_+ = P_- = \sqrt{P_+^0 P_-^0}$.

*From here onwards, concentrations are used instead of activities, because the estimation of activity coefficients, although feasible, does not change the picture qualitatively.

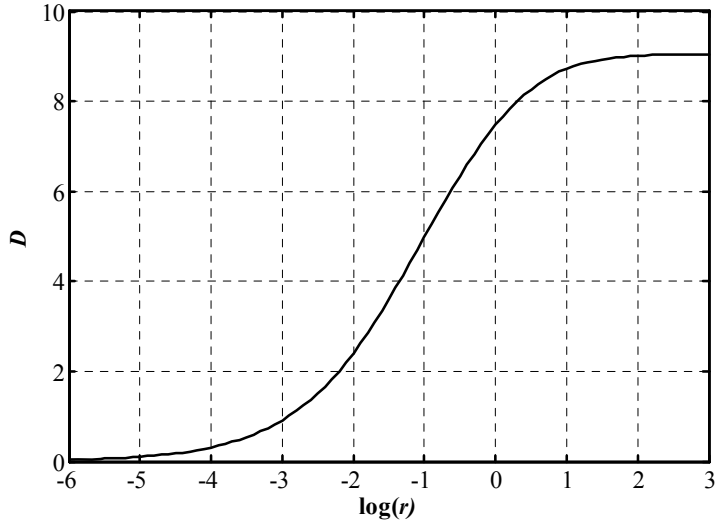

Figure 2. Distribution coefficient of a weak electrolyte as a function of the phase volume ratio. Parameters: c_0 = 1.0 mM, P_{ip} = 10, P_+ = 0.01 and K_d =10^{-5} M.

Figure 2 displays the distribution coefficient D as a function of the phase volume ratio.

$$D = \frac{[\mathrm{C}^+]^{\mathrm{o}} + [\mathrm{CA}]^{\mathrm{o}}}{[\mathrm{C}^+]^{\mathrm{w}} + [\mathrm{CA}]^{\mathrm{w}}} \tag{18}$$

With the chosen parameter values, D varies between 0.037 and 9.05. The origin of the dependence of D on the volume ratio is ion-pairing: if the electrolytes were completely dissociated in both phases, D would be equal to $P_+ = P_-$ at all volume ratios r.

2.1.4 Effect of pH

The effect of pH on the distribution coefficient has traditionally been taken into account using the following equation [6]:

$$D = \frac{P_{\mathrm{D}} + P_{\mathrm{DH}^+} 10^{\mathrm{p}K_{\mathrm{a}} - \mathrm{pH}}}{1 + 10^{\mathrm{p}K_{\mathrm{a}} - \mathrm{pH}}} \tag{19}$$

where P_D represents the partition coefficient of the neutral drug and P_{DH^+} the partition coefficient of the protonated species.

In the work of Avdeef *et al.* [6], the plots of log *D* as a function of pH, or the lipophilicity profiles, of four acidic and four basic drugs for octanol–water and liposomal membrane–water systems were compared. In their study, Avdeef *et al.* used the difference of pK_a values in the aqueous and organic phases to calculate the partition coefficients for the ionized species. The results showed that as the drugs ionize, the partitioning into liposomal membranes is significantly higher than into octanol. The difference between the two systems was explained by the electrostatic interactions between the ionized drug and the zwitterionic phospholipid, even though the electrostatic contributions of the partition coefficients were not considered as such. Recently, Elsayed *et al.* [16] developed the potentiometric determination of lipid membrane–water partition coefficient further and added electrostatic corrections to the analytical procedure.

Even though Equation (19) describes the changes in *D* as a function of pH quite accurately at some pH values, it fails to capture some aspects of the phenomenon as the effects of the Galvani potential difference and the counter ion have not been taken into account. Here, the effect of pH on the distribution coefficient is illustrated by considering the simultaneous partition dissociation equilibria depicted in Figure 3. A basic drug is added into the aqueous phase in the concentration c_D and assumed to remain constant and pH is fixed. In addition, the concentration of the counter ion in the water phase is assumed to be constant c_A, i.e. the aqueous buffer capacity is assumed to be very high. Proton partitioning is assumed to be negligible and ion-pairing in the oil phase is also neglected at first to allow a comparison with Equation (18).

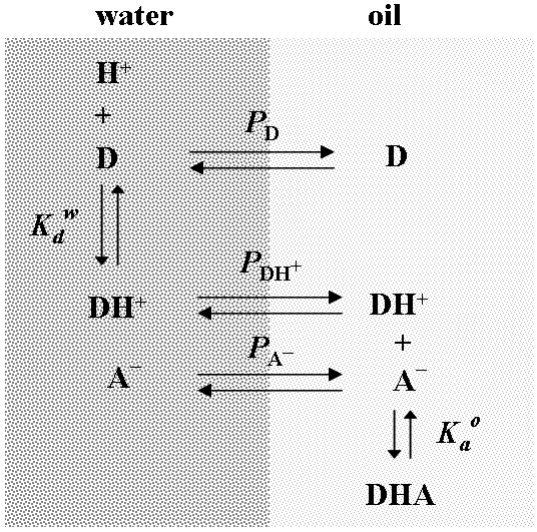

Figure 3. Partitioning of a drug that is a weak acid.

As the basic drug D dissociates, the concentrations in the aqueous phase in equilibrium are:

$$[\mathrm{D}]^{\mathrm{w}} = \alpha c_{\mathrm{D}} \text{ and } [\mathrm{DH}^+]^{\mathrm{w}} = (1-\alpha) c_{\mathrm{D}} \tag{20}$$

where α is the degree of dissociation and c_{D} is concentration of the drug. The concentrations of the counter ion A^- and the ionized drug DH^+ in the organic phase can be written as:

$$[\mathrm{A}^-]^{\mathrm{o}} = c_{\mathrm{A}} P^0_{\mathrm{A}^-} e^{-\varphi} \tag{21}$$

$$[\mathrm{DH}^+]^{\mathrm{o}} = [\mathrm{DH}^+]^{\mathrm{w}} P^0_{\mathrm{DH}^+} e^{\varphi} = (1-\alpha) c_{\mathrm{D}} P^0_{\mathrm{DH}^+} e^{\varphi} \tag{22}$$

where P_i^0 is the standard partition coefficient and $e^{\pm\varphi}$ the potential dependent part of P_i ($\varphi = F\Delta^{\mathrm{w}}_{\mathrm{o}}\phi / RT$). Due to electroneutrality in the organic phase $[\mathrm{A}^-]^{\mathrm{o}} = [\mathrm{DH}^+]^{\mathrm{o}}$, and e^{φ} can be solved from Equations (21) and (22) as:

$$e^{\varphi} = \left(\frac{c_{\mathrm{A}} P^{0}_{\mathrm{A}^-}}{(1-\alpha) c_{\mathrm{D}} P^{0}_{\mathrm{DH}^+}} \right)^{1/2} \tag{23}$$

For the neutral drug, the concentration in the organic phase is simply:

$$[\mathrm{D}]^{\mathrm{o}} = \alpha c_{\mathrm{D}} P_{\mathrm{D}} \tag{24}$$

Substituting Equation (23) into Equation (22) results in:

$$[\mathrm{DH}^+]^{\mathrm{o}} = \sqrt{P^{0}_{\mathrm{DH}^+} P^{0}_{\mathrm{A}^-} (1-\alpha) c_{\mathrm{D}} c_{\mathrm{A}}} \tag{25}$$

Now the distribution coefficient can be written as:

$$D = \frac{[\mathrm{D}]^{\mathrm{o}} + [\mathrm{DH}^+]^{\mathrm{o}}}{[\mathrm{D}]^{\mathrm{w}} + [\mathrm{DH}^+]^{\mathrm{w}}} = \alpha P_{\mathrm{D}} + \sqrt{(1-\alpha) P^{0}_{\mathrm{DH}^+} P^{0}_{\mathrm{A}^-} \frac{c_{\mathrm{A}}}{c_{\mathrm{D}}}} \tag{26}$$

Equation (26) can be rewritten using the Henderson-Hasselbalch equation as:

$$D = \frac{P_{\mathrm{D}}}{1 + 10^{\mathrm{p}K_{\mathrm{a}} - \mathrm{pH}}} + \sqrt{\frac{P^{0}_{\mathrm{DH}^+} P^{0}_{\mathrm{A}^-} \left(\frac{c_{\mathrm{A}}}{c_{\mathrm{D}}} \right) \times 10^{\mathrm{p}K_{\mathrm{a}} - \mathrm{pH}}}{1 + 10^{\mathrm{p}K_{\mathrm{a}} - \mathrm{pH}}}} \tag{27}$$

An acidic drug, such as salicylic acid, dissociates to D^- and H^+, and the counter ion of D^- is C^+. Now, knowing $\mathrm{p}K_{\mathrm{a}} = -\log K^{\mathrm{w}}_{\mathrm{d}}$, Equation (27) is modified into the following form:

$$D = \frac{P_{\mathrm{DH}} \times 10^{\mathrm{p}K_{\mathrm{a}} - \mathrm{pH}}}{1 + 10^{\mathrm{p}K_{\mathrm{a}} - \mathrm{pH}}} + \sqrt{\frac{P^{0}_{\mathrm{D}^-} P^{0}_{\mathrm{C}^+} \left(\frac{c_{\mathrm{C}}}{c_{\mathrm{D}}} \right)}{1 + 10^{\mathrm{p}K_{\mathrm{a}} - \mathrm{pH}}}} \tag{28}$$

Resemblance of Equations (27) and (28) with Equation (19) is obvious, and in Figure 4 these two equations are compared with varying the lipophilicity of the anion and cation via its partition coefficient. Parameters in Figure 4 are: $P_{\mathrm{D}} = P_{\mathrm{DH}} = 1000,\ P^0_{\mathrm{DH^+}} = P^0_{\mathrm{D^-}}\ 0.01,\ c_{\mathrm{A}} / c_{\mathrm{D}} = c_{\mathrm{C}} / c_{\mathrm{D}} = 3$. p$K_{\mathrm{a}}$ of the basic drug is 9 and that of the acidic drug is 3. The values of $P^0_{\mathrm{A^-}} = P^0_{\mathrm{C^+}}$ are indicated in Figure 4.

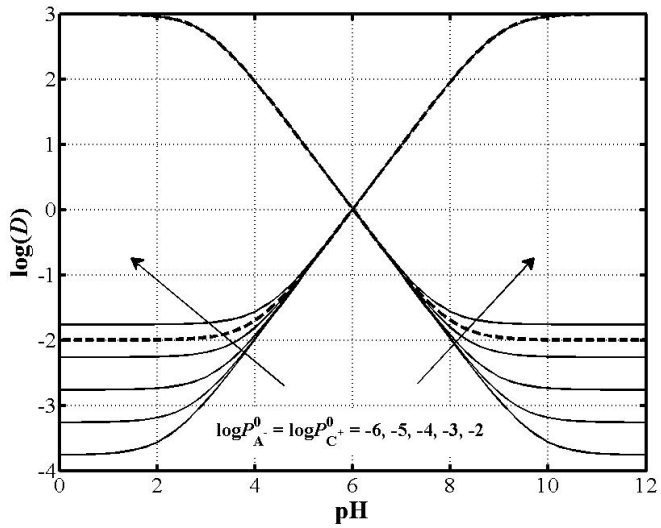

Figure 4. Distribution coefficient according to Eqs. (27) and (28): $P_{\mathrm{D}} = P_{\mathrm{DH}} = 1000,\ P^0_{\mathrm{DH^+}} = P^0_{\mathrm{D^-}}\ 0.01,\ c_{\mathrm{A}} / c_{\mathrm{D}} = c_{\mathrm{C}} / c_{\mathrm{D}} = 3$. Dotted lines represent Eq. (19) and the corresponding equation for an acidic drug.

The Galvani potential difference created by partitioning can be calculated from Equation (23) as:

$$\Delta^{\mathrm{w}}_{\mathrm{o}}\phi = \frac{RT}{2F}\ln\left(\frac{c_{\mathrm{A}}P^0_{\mathrm{A^-}}}{c_{\mathrm{D}}P^0_{\mathrm{DH^+}}}\right) + \frac{RT}{2F}\ln\left(1+10^{-\mathrm{p}K_{\mathrm{a}}+\mathrm{pH}}\right) \qquad (29)$$

Figure 5 shows the Galvani potential difference with the parameter values given above.

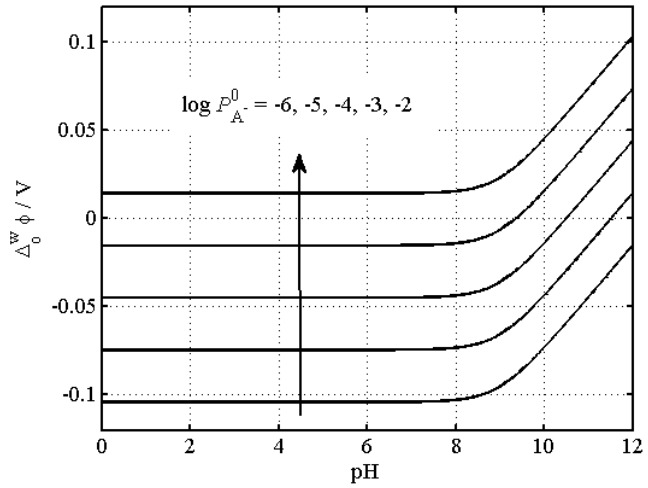

Figure 5. Galvani potential difference created by partitioning. $P_D = P_{DH} = 1000$, $P^0_{DH^+} = P^0_{D^-} 0.01$, $c_A / c_D = c_C / c_D = 3$.

Figures 4 and 5 show that for a basic drug, the deviation from the generally used Equation (19) is due to the Galvani potential difference that is created by the partitioning of the ionic species. The deviation naturally does not exist at high pH where ionization is negligible, although the Galvani potential difference is not zero, hence, the theory has relevance mainly in oral delivery due to the low pH of the stomach. The distribution coefficient can deviate from the expected value by two orders of magnitude, since a Galvani potential difference of 100 mV means about 1.7 units on log *P* scale. For an acidic drug, however, ionization does not really have significance, because it takes place outside the physiological pH range, unless the pK_a of the drug is very low, of the order of 1-2. Therefore, acidic drugs are not discussed any further.

To finalize this analysis, the effect of the phase volume ratio is included. Also ion-pairing in the oil phase is considered, which is substantial in a low permittivity medium. The concentration of the counter ion A^-, c_A, and pH are fixed due to high buffer capacity, but the drug concentrations depend on the mass balance; the initial drug concentration in the aqueous phase is c_D. The mass balance for the drug is:

$$V^w c_D = V^w \left([D]^w + [DH^+]^w \right) + V^o \left([D]^o + [DH^+]^o + [DHA]^o \right) \qquad (30)$$

Applying the partitioning equilibria, the dissociation equilibrium in the aqueous phase, and the ion-pairing equilibrium in the oil phase,

$$K_{\mathrm{a}}^{\mathrm{o}} = \frac{[\mathrm{DHA}]^{\mathrm{o}}}{[\mathrm{DH}^+]^{\mathrm{o}}[\mathrm{A}^-]^{\mathrm{o}}} \tag{31}$$

Equation (30) becomes:

$$c_{\mathrm{D}} = [\mathrm{DH}^+]^{\mathrm{w}}\left[1+\frac{1}{r}\left(P^0_{\mathrm{DH}^+}e^{\varphi}+K_{\mathrm{a}}^{\mathrm{o}}P^0_{\mathrm{DH}^+}P^0_{\mathrm{A}^-}c_{\mathrm{A}}\right)+\frac{K_{\mathrm{d}}^{\mathrm{w}}}{[\mathrm{H}^+]^{\mathrm{w}}}\left(1+\frac{P_{\mathrm{D}}}{r}\right)\right] \tag{32}$$

e^{φ} takes essentially the same form as in Equation (23):

$$e^{\varphi} = \left(\frac{c_{\mathrm{A}}P^0_{\mathrm{A}^-}}{[\mathrm{DH}^+]^{\mathrm{w}}P^0_{\mathrm{DH}^+}}\right)^{1/2} \tag{33}$$

Equations (32) and (33) contain only two unknowns, $[\mathrm{DH}^+]^{\mathrm{w}}$ and e^{φ}, hence the problem is solvable. A Matlab® script was written to simulate partitioning depicted in Figure 6. In the simulation shown in Figure 6, c_{A} = 0.03 M, c_{D} = 0.01 M, r = 0.01 and 100, $K_{\mathrm{a}}^{\mathrm{o}}$ = 5000 M^{-1}, and $P^0_{\mathrm{A}^-}$ = 0.01.

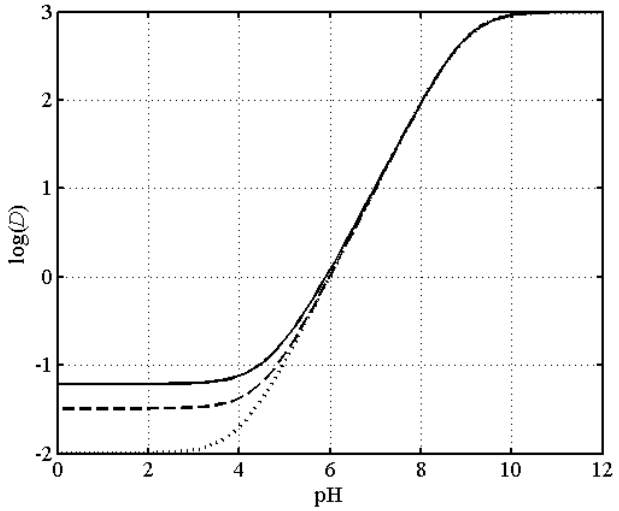

Figure 6. Effect of the phase volume ratio: r = 0.01 (solid) and 100 (dashed). Dotted line depicts Equation (19). c_A = 0.03 M, c_D = 0.01 M, r = 0.01 and 100, K_a^o = 5000 M^{-1}, and $P^0_{A^-}$ = 0.01.

Figures 2 and 6 illustrate that the phase volume ratio has a much smaller effect on the distribution coefficient than the Galvani potential. In Figure 7, the effect of the phase volume ratio on the Galvani potential is simulated with the same set of parameter values as in Figure 6.

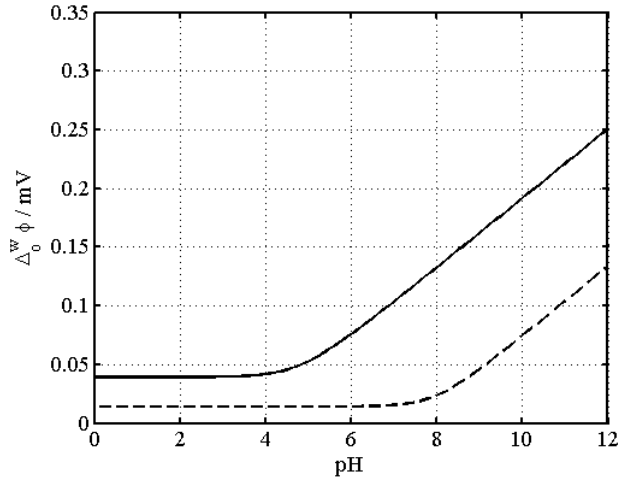

Figure 7. Effect of the phase volume ratio: r = 0.01 (solid) and 100 (dashed) on Galvani potential.

As can be seen, the Galvani potential is shifted only about 25 mV at low pH when the phase volume ratio is changed from 0.01 to 100.

To summarize, the simulations that take into account the Galvani potential difference, phase volume ratio and pH show that at low pH, the deviation of the distribution coefficient from the generally used formula can be as much as two units on the log *P* scale. It is thus clear that all of these effects must be considered when evaluating the partitioning of ionized drugs as changing one value changes the others as well. The next section will present an overview of the experimental methods that can be used to study the partitioning of drugs.

2.2 Experimental approaches

The traditional experimental approach to drug partitioning is the measurement of the octanol–water partition or distribution coefficient by determining the equilibrium concentrations of a drug in both of the phases of an octanol–water mixture. Even though some studies have shown fairly good correlations of the octanol–water distribution coefficients with biological permeation [17,18], poor correlations have also been reported [8,19], suggesting that the octanol–water system does not account for all of the aspects of biological permeation. Some of the shortcomings of an octanol–water system as a partitioning model have been explained by the hydrogen bonding of a drug, which is very different in *n*-octanol compared with a biological membrane [20]. Thus alternatives for the octanol–water partition coefficient have been proposed in order to improve the biomimetics of the partitioning studies. Many of the popular experimental approaches utilize various model membranes, such as cell culture monolayers, artificial membranes, or liposomes. The most frequently used cell cultures for passive drug transport studies are Caco-2 cultures, which are derived from human colon carcinoma cells [21]. In Caco-2 cultures, the monolayers of the polarised cells, which mimic the function of the small intestinal villus epithelium, are grown on permeable filter supports and the transport of drugs through the monolayer is measured. In the parallel artificial

membrane permeation assay (PAMPA), on the other hand, the two compartments are separated by a hydrophobic filter impregnated with an organic solution of lipid, which forms bilayer structures in the filter pores [22]. Even though both of these techniques are extensively used, both the Caco-2 system and PAMPA seem to suffer from interlaboratory variability [23,24].

Because of their excellent biomimetic properties, liposomes have become a popular alternative in membrane partitioning studies. Liposome–water partition coefficients have been measured using various methods, including the distribution technique [25], equilibrium dialysis [26], potentiometric titration [6,9], and NMR-spectroscopy [27]. Despite the better biomimetic properties of liposomes, most of the approaches based on liposomes as model membranes are not efficient enough to be used on a large scale as they are very tedious and time-consuming. To overcome the problem in efficiency, automated methods for the rapid screening of drug compounds have been developed, where the biomimetic properties of liposomes have been combined with chromatographic techniques [28,29].

In this thesis, a new electrochemical method was developed for the determination of the liposome–water partition coefficient (Publication I). Also, isothermal titration calorimetry was used to evaluate the drug–liposome interactions (Publication IV). Additionally, the adsorption coefficients of drugs on model surfaces determined using surface activity and contact angle measurements were correlated with the partition coefficients in Publication III. Thus this thesis aims at exploring the various possibilities of physicochemical and electrochemical methods in studying the interactions of drugs with model biological membranes.

2.2.1 Electrochemical methods

Electrochemistry is a useful tool especially when studying ionized drugs. The use of liquid–liquid electrochemistry as a means to determine the ionic partition coefficients of

drugs was first presented in 1992 by Kontturi and Murtomäki [30]. The method is based on the determination of the standard free energy of transfer of ionized drugs at the interface of two immiscible electrolyte solutions. Unfortunately, the ionic free energies of transfer in Equation (8) cannot be obtained directly from experimental results. Because of this, an extrathermodynamic assumption has to be made to define a scale for the standard Gibbs energy of transfer of a single ion. Kontturi and Murtomäki utilized the commonly used the TATB assumption, which states that the cation and the anion of tetraphenylarsonium tetraphenylborate ($TPAs^+TPB^-$ or TATB) have equal standard free energies of transfer for any pair or solvent:

$$\Delta_{\mathrm{o}}^{\mathrm{w}} G_{\mathrm{TPAs}^+}^0 = \Delta_{\mathrm{o}}^{\mathrm{w}} G_{\mathrm{TPB}^-}^0 \tag{34}$$

This assumption is based on the fact that both the cation and the anion have similar size and shape and are symmetrical. Thus their energies of solvation can be considered equal.

The standard transfer potential of an ionic drug can be obtained from a cyclic voltammogram as it is related to the half wave potential:

$$\Delta_{\mathrm{o}}^{\mathrm{w}} \phi_i^0 = \Delta_{\mathrm{o}}^{\mathrm{w}} \phi_{1/2} + \frac{RT}{z_i F} \ln\left[\left(\frac{D_i^{\mathrm{o}}}{D_i^{\mathrm{w}}} \right)^{\frac{1}{2}} \cdot \gamma_i^{\mathrm{w}} \gamma_i^{\mathrm{o}} \right] \tag{35}$$

where D_i and γ_i are the diffusion and activity coefficients of the drug in the appropriate phases respectively.

The traditional solvent used in the partition studies, *n*-octanol, cannot be used in electrochemical measurements because electrolytes do not dissolve in it. In their studies, Kontturi and Murtomäki [30] used an organic phase consisting of 1,2-dichloroethane (DCE). Indeed, the water/DCE system preferred in liquid–liquid electrochemistry has

been suggested to be a more useful system when compared with the traditional water/alkane in determining the interactions of solutes with biological membranes [31]. In the later studies, nitrophenyl octyl ether (NPOE) has also been used as an organic solvent because of its suitable viscosity, vapour pressure and hydrophobicity [32,33]. In addition, NPOE has been reported to be a suitable solvent for the permeability assays with the PAMPA technique [34].

In the study of Scholz *et al.* [35], the limitations due to the low solubility of the electrolytes in some organic solvents have been overcome by the introduction of a three-phase electrode technique. The basis of the technique is an electrochemical system where three different phases, a solid electrode, an organic liquid, and an aqueous phase are brought into contact. A droplet of an organic solution of an electroactive compound is immobilized on the electrode surface and the electrode is then immersed into an aqueous solution containing the anionic form of a drug. By applying a potential difference between the working and the reference electrode, it is possible to study the electrochemical processes at the three phase junction.

At the three phase boundary, the electrochemical reaction of the electroactive species decamethylferrocene (dmfc) in the organic phase is coupled with the transfer of the anionic form of the studied drug across the organic solvent–water interface. This process can be described by the following reaction scheme:

$$\text{dmfc (o)} + \text{D}^- \text{(w)} \quad \rightleftarrows \quad \text{dmfc}^+ \text{(o)} + \text{D}^- \text{(o)} + \text{e}^- \qquad \text{(I)}$$

Now the standard transfer potential can expressed as:

$$E_{\mathrm{f}} = E^{0}_{\mathrm{dmfc}_o^+/\mathrm{dmfc}_o} + \Delta^{\mathrm{o}}_{\mathrm{w}}\phi^{0}_{i} - \frac{RT}{F}\ln(c_i) + \frac{RT}{F}\ln\left(\frac{c_{\mathrm{dmfc}}}{2}\right) \qquad (36)$$

where E_f is the formal redox potential of the couple dmfc/dmfc$^+$, $E^0_{\mathrm{dmfc_o^+/dmfc_o}}$ the standard redox potential of the couple in the organic phase, c_{dmfc} is the concentration of dmfc in the organic phase and c_i is the concentration of the anionic form of the drug in the aqueous phase. As the standard transfer potential is determined, the partition coefficient of a drug can be obtained using Equation (5).

The advantage of the three-phase electrode technique over the traditional liquid–liquid electrochemistry is that the measurements do not require an additional electrolyte in the aqueous phase, but the studied species can be present alone and in high concentrations. No electrolyte is necessary in the organic phase either. In the work of Gulaboski *et al.* [36], the partition coefficients of anionic drugs and model compounds were determined using the three electrode approach in nitrobenzene (NB) and NPOE and then compared with values determined in the *n*-octanol–water system [37]. The lipophilicity range for the studied anions was wider for NPOE and NB than for *n*-octanol, which was interpreted as a larger difference in the solvation energies of anions between water and NPOE or NB than between *n*-octanol and water. The effects of ionic radii, charge delocalization, and the molecular size on the lipophilicity of the compounds were also evaluated in the study, with unusually high lipophilicity values reported for compounds containing a pyrrole ring.

As an extension to the determination of the partition coefficient of various drugs, liquid–liquid electrochemistry has been used to construct ionic partition diagrams for solutes, which resemble the Pourbaix pH-potential diagrams for metals [15,38]. In these diagrams, the charged state and phase of a coumpound is presented as a function of the interfacial Galvani potential difference and pH of the solution. The purpose of the diagrams is to help to visualize the lipophilicity of different species present in a system. The diagrams are particularly useful in pharmacokinetics when determining which species of a multiprotic drug are present at a certain pH and potential and describing the transfer process of a drug. After the first pH dependence studies by Reymond *et al.* [15], the efficiency of the experimental setup has been improved first by the introduction of a

96-well microfilter plate system [33] and later by the use of a commercial immobilized pH gradient gel [39].

A major limitation of the partitioning studies at the liquid–liquid interface is that the organic solvents used in the studies do not mimic the properties of a biological membrane very well. After the first studies of Kontturi and Murtomäki [30], however, the biomimetic properties of liquid–liquid interface were improved by adding a monolayer of lipids at the interface [40–42]. The combination of Langmuir trough and electrochemistry allowed not only the control of the potential drop across the interface but also the control of the surface pressure. Using this system, Grandell *et al.* [40–42] studied the transfer of two model drugs, propranolol and picrate, across the interface. It was found that more energy was required to transfer picrate across the interface with the lipid compared with one without it. In addition, picrate was found to have a stabilizing effect on the lipid monolayer, whereas the transfer of propranolol was found to destabilize it. The water/DCE system was further improved in the works of Liljeroth *et al.* [43] and Mälkiä *et al.* [44,45]. In these studies, a biological membrane was modeled by depositing a lipid monolayer at the interface between the aqueous phase and an immobilized, gelled organic phase using the Langmuir-Blodgett technique. The Langmuir-Blodgett technique provided a better control of the surface pressure, as it was not controlled *in situ*, but the monolayer was transferred to the interface in its desired state. In addition, the gelled organic phase reduced the monolayer dissolution to the organic phase and the smaller interfacial area improved the quality of the electrochemical data. This set-up was used to study the membrane activity of six ionized drugs [44,45]. As a result, a preferential orientation of the drugs in the phospholipid monolayer was proposed for each drug.

In Publication I, an electrochemical method was developed for the determination of the liposome–water partition coefficients of drugs. Square wave voltammetry (SWV) was used to study the interactions of liposomes and β-blockers at a water–DCE interface created at the tip of a micropipette. Five different β-blockers, propranolol, timolol,

carteolol, nadolol and metoprolol were encapsulated in liposomes prepared using the extrusion method. The partitioning of the drugs between the cavity and the membrane of the liposome was determined using an electrochemical set-up shown in Figure 8.

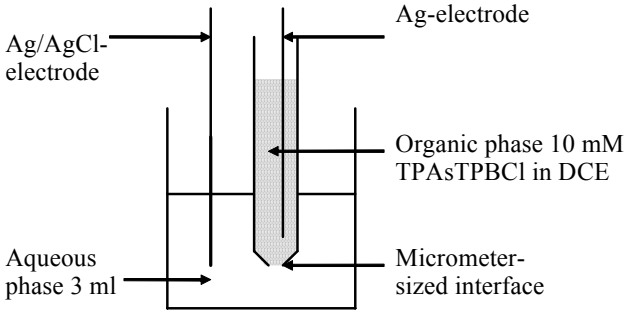

Figure 8. Schematic illustration of the experimental set-up. (Reprinted from Publication I with permission from Elsevier)

Within the available potential window, which was determined by the supporting electrolytes used, the β-blocker was the only species transferring across the liquid–liquid interface. Diffusion of the liposomes to the interface, their decomposition at the contact with the organic phase, the subsequent release of the drug, and the transfer of the drug across the interface was observed as a peak current.

The relationship between the peak current and the concentration of the ionic species for hemispherical diffusion is given by Equation (37) [46]:

$$\Delta i_{\mathrm{p}} = \frac{zFAD^{1/2}c}{\pi^{1/2}t_{\mathrm{p}}^{1/2}}\Delta\psi_{\mathrm{p}} \tag{37}$$

where Δi_{p} is the peak current, F is the Faraday constant, A the area of the micropipette tip, D the diffusion coefficient, $\Delta\psi_{\mathrm{p}}$ a dimensionless peak current, t_{p} the pulse width, c the bulk concentration of the transferring species, and z is the charge. The simplest

method for determining the dimensionless peak current is to compare the measurement with that of a known solution.

The liposome–water partition coefficient of the drug was determined from the peak current using the following model: Partition coefficient is defined as the ratio of the activity a species in two immiscible phases in equilibrium. In this work, drugs partition between the aqueous cavity of the liposome and the lipid bilayer. For simplification, the activities can be approximated by concentrations:

$$P = \frac{c^{\mathrm{l}}}{c^{\mathrm{w}}} \tag{38}$$

where c^{l} is the concentration of drug in the lipid bilayer and c^{w} is the concentration inside the cavity.

The concentration inside the cavity can be assumed to be that of the solution used in the preparation of the liposomes, and the concentration of drug in the lipid layer can be solved as the total amount of drug in a liposome is known:

$$c^{\mathrm{l}} = \frac{n^{\mathrm{l}}}{V^{\mathrm{l}}} = \frac{z - z_{\mathrm{c}}}{N_A V^{\mathrm{l}}} \tag{39}$$

where c^{l} is the concentration in the bilayer, n^{l} the amount of drug in the bilayer, z the total number of drug molecules in a liposome measured using square wave voltammetry, and N_{A} is the Avogadro constant.

The amount of drug in the cavity, z_{c}, can be calculated from the drug concentration inside the liposome and the size of the cavity:

$$z_{\mathrm{c}} = N_{\mathrm{A}} c^{\mathrm{w}} \frac{4}{3} \pi a^3 \tag{40}$$

where c^{w} is the concentration of the drug used in the preparation of liposomes and a is the radius of the liposome. V^{l} is the volume of the lipid bilayer given by:

$$V^{l} = 4\pi a^{2} h \tag{41}$$

where h is the thickness of the bilayer, which was taken to be 5 nm [47]. The log P values determined for each β-blocker are shown in Figure 9.

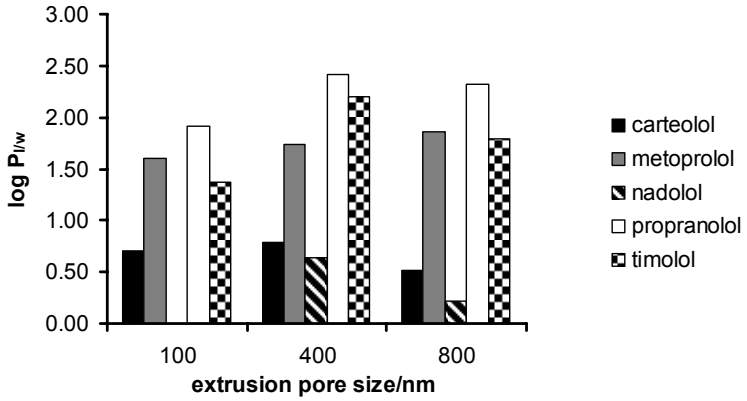

Figure 9. Liposome–water partition coefficients of the five β-blockers. (Reprinted from Publication I with permission from Elsevier)

The results shown in Figure 9 demonstrate that the partition coefficient of ionized drugs can be determined using the method described in Publication I. Compared to partition coefficients determined by other methods, the method presented here gives log P values of the same order of magnitude as liposome–water partition coefficients determined using liposome distribution studies [48] and equilibrium dialysis [10]. The results also suggest that the liposome–water partition coefficients are somewhat lower than the respective octanol–water partition coefficients. The comparison of the octanol–water partition coefficients and liposome–water partition coefficients is shown in Figure 10. The measured values shown are average values of the determined partition coefficients.

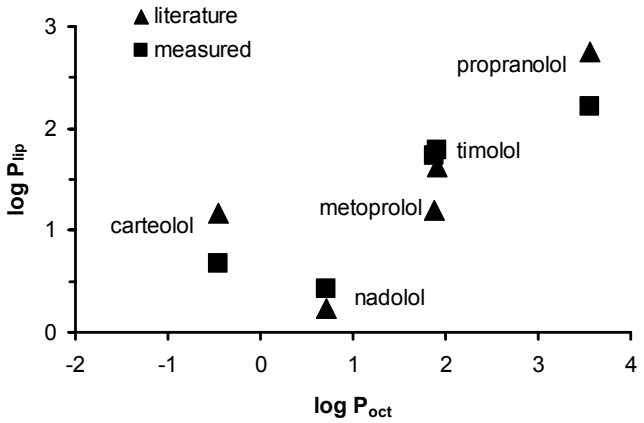

Figure 10. Comparison of log P_{octanol} values and log P_{liposome} values. (Reprinted from Publication I with permission from Elsevier)

The method presented in Publication I is a fast and easy way to determine the partition coefficient for ionized drugs. It provides some advantages for studying drug–membrane interactions compared with traditional methods: As the method utilizes electrochemistry, the system is easily controllable and the measurements can be carried out quickly. Also, because of the liposomes, the partitioning in the system mimics the real cell membrane partitioning quite accurately.

Liquid–liquid electrochemistry is a versatile approach in drug partitioning studies as the setup enables the control of the potential difference across the interface and the study of ion transfer. The most evident drawback of the approach has been that the properties of the organic phase deviate from those of the biological membrane. Earlier, the biomimetic properties of experimental setup have been improved by incorporating lipid monolayers in the interface of the two immiscible electrolytes. Publication I, however, solves the biomimetic problem by introducing liposomes in the aqueous phase. This method not only utilizes the rapidity of the electrochemical measurement, but also allows the determination of the liposome–water partition coefficient, which has been shown to correlate with the pharmacokinetic parameters in humans [25]. As the method can be applied to a wide range of ionized molecules with varying lipohilicity, it provides a new electrochemical tool for the drug partitioning studies.

2.2.2 Isothermal titration calorimetry

One of the available methods to study the interactions of drugs with liposomes is isothermal titration calorimetry (ITC), which is an effective method for the study of binding in biological systems, as it allows for the determination of the Gibbs energy (ΔG), the enthalpy (ΔH), and the entropy of binding (ΔS) in a single experiment [49–52]. The thermodynamics of interactions between drugs and membranes can be related to the structural details of the process. The formation or breaking of non-covalent bonds in the system is observed as ΔH, whereas ΔS gives a quantitative value of the change in order of the system. ITC has been applied to various drug–membrane systems. Seelig and coworkers [53–56] have studied the interaction of liposomes with various amphiphilic compounds including calcium channel antagonists and local anesthetics. In the work of Matos *et al.* [57], the thermodynamics of the membrane binding of anti-inflammatory drugs was evaluated in a broad concentration range. In addition to calorimetric studies on their own, ITC has been used to complement studies carried out mainly using other methods. Gerebtzoff *et al.* [58] used ITC combined with surface activity measurements to study the effect of halogenation of drugs on the membrane binding. In the study of Johansson *et al.* [59], calorimetric liposome partition data was used to validate the partition coefficients measured using an alternative model membrane, the sterically stabilized bilayer disks. In both of these studies, ITC data was reported to be in good agreement with the complementary data.

In Publication IV, ITC and zeta potential measurements were used to study the binding and partitioning of four β-blockers and one local anaesthetic into liposomes. Two types of titrations, drugs into liposomes and liposomes into drugs, were compared to check the reliability of the data. The enthalpy, entropy and Gibbs energy of binding were determined using the one site model and the electrostatic contribution to the binding was evaluated using the Gouy-Chapman theory. Furthermore, the binding constants were used to assess the partition coefficients for the drugs, and additionally the effects of the concentration, ionic strength, temperature and membrane curvature on the interaction were included in the evaluation.

Figure 11 shows the titration of 10 mM alprenolol, labetalol, propranolol and tetracaine into 100 nm liposomes. The binding of these drugs into liposomes is an exothermic process as the titration peaks are negative. The heat produced decreases continuously until all of the binding sites in the liposomes are occupied and the heat of interaction is observed to be close to zero. The monotonous decrease of the amount of heat produced after each injection suggests that there is only one type of binding site in the liposomes. In addition, the zeta potential measurements mimicking the titrations revealed that the effect of the addition of the drugs on the zeta potential is very small and thus the zeta potential is practically constant during the titration.

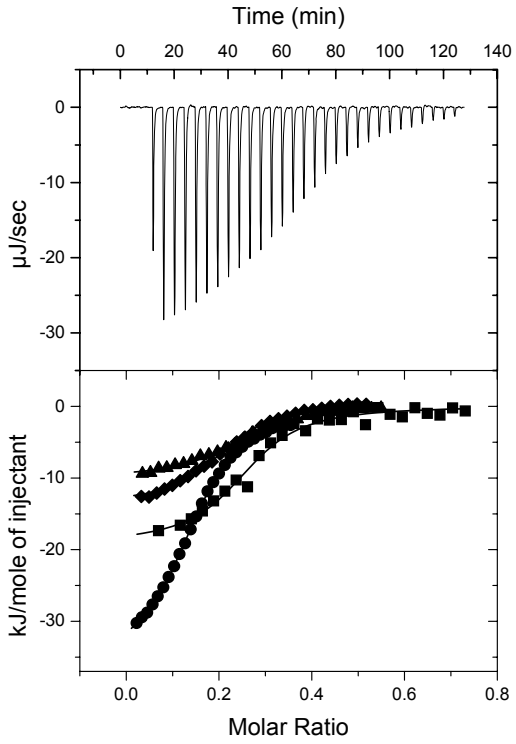

Figure 11. Titration of alprenolol (▲), labetalol (●), propranolol (■) and tetracaine (♦) into 100 nm liposomes at 25 °C. The buffer used was 2 mM Hepes + 15 mM NaCl, pH 7.4. The upper panel shows the raw ITC data for alprenolol and the lower panel the integrated enthalpies of interaction as a function of the ratio of drugs to lipids. The solid line represents the best fit using the one site model. (Reprinted from Publication IV with permission from Elsevier)

The one site model provided by the ITC software and used in the determination of the thermodynamic parameters is equivalent to the chemical equilibrium, where one drug reacts with a cluster of *n* lipids forming a complex of DL_n. Therefore, the partition coefficients of the ionized drugs can be evaluated by multiplying the binding constant with the concentration of free lipids [51]:

$$P_{\text{ion}} = K_{\text{b}} c_{\text{L,f}} \tag{42}$$

These partition coefficients describe drug partitioning as an overall process, which consists of various contributions, including the electrostatic effects, steric hindrance and hydrophobic interactions.

The usual practice in binding studies is to separate the electrostatic contribution from the other factors. The most widely used method for this is to correct for the increased or decreased concentration of drugs near the charged surface. This is usually done with the Gouy-Chapman theory [53–58], although other methods have also been proposed [57]. The Gouy-Chapman theory assumes that a smooth surface has a charge density smeared out uniformly, as electrons do in metals [46]. This surface charge is the origin of the electrostatic potential field, in which point charges, i.e. ionic sizes excluded, are distributed according to the Boltzmann distribution, assuming the solvent as a continuous dielectric medium. Even though the continuum is a very simplistic model of the liposome surface, it can be used to correct for the 'true' interfacial concentration:

$$c_i^s = c_i^b \exp\left(-\frac{z_i F}{RT}\phi_0\right) \tag{43}$$

where '*s*' denotes the interfacial and '*b*' the bulk concentration, and ϕ_0 is the Galvani potential at the interface, z_i is the ionic charge, $F = 96486\ \text{Cmol}^{-1}$, $R = 8.314\ \text{JK}^{-1}\text{mol}^{-1}$ and T is the absolute temperature.

The apparent value of the binding constant, K_{app}, which takes into account the electrostatics of the binding, is:

$$K_{app} = K_b\left(-\frac{z_i F}{RT}\phi_0\right) \tag{44}$$

In Publication IV, the Gouy-Chapman theory was used to estimate the surface potential ϕ_0 from the zeta potential, ζ. The Galvani potential profile at the interface was calculated using the following equation [46]:

$$\frac{\tanh\left(\frac{z_i F}{4RT}\zeta\right)}{\tanh\left(\frac{z_i F}{4RT}\phi_0\right)} = \exp(-\kappa x_2) \tag{45}$$

In Equation (45), the distance x_2 at which the potential corresponds to the zeta potential is taken to be 2.8 Å, which is the diameter of a water molecule.

The reciprocal of the double layer thickness κ (Debye length) is given by:

$$\kappa = \left(\frac{2c^b z^2 F^2}{\varepsilon_r \varepsilon_0 RT}\right)^{1/2} \tag{46}$$

where ε_r is the relative permittivity of water, 78.4, and ε_0 is the permittivity of free space, 8.854×10^{-12} Fm^{-1}.

As the zeta potential values were almost unaffected by the addition of the drugs and no concentration dependency could be deduced, the average value of the zeta potential was used for each drug. Also, it was possible to evaluate the degree of ion binding using the equation for surface charge σ [46]:

$$\sigma = \left(8RTc^{\mathrm{b}}\varepsilon_{\mathrm{r}}\varepsilon_0\right)^{1/2} \sinh\left(\frac{z_i F}{2RT}\phi_0\right) \tag{47}$$

In the evaluation, the solution was taken to be 1:1 electrolyte with the concentration of 17 mM. Taking into account the size of the vesicle, the charge number of a liposome was calculated to be approximately 2500, which means that less than 5 % of the lipids are charged, although 30 w-% of the lipids are anionic. The calculation shows that the degree of the ion binding of sodium ions is high and explains why the effect of the addition of drugs on the zeta potential value is only minimal during the titration.

As the electrostatic interactions play a major role in the interaction of cationic drugs with negatively charged membranes, the effect of the ionic strength was studied by carrying out the experiments at a different salt concentration, in 20 mM Hepes buffer containing 150 mM NaCl. The measurements revealed that the binding constant K_{b} is significantly lower at the higher ionic strength for all drugs. Lower values of K_{b} at higher ionic strength can be explained by the ion binding phenomenon. In the case of the high NaCl concentrations, Na^+ ions compete with the drugs in binding the negatively charged membrane. The system can be analyzed by means of statistical thermodynamics. The grand canonical partition function for competitive binding is:

$$q = 1 + K_1 c_1 + K_2 c_2 \tag{48}$$

where K_1 and K_2 are the binding constants for the drug and for the Na^+ ion, respectively, and c_1 and c_2 are the respective concentrations.

Writing the surface coverage of a drug (θ_1) in terms of the two binding constants gives:

$$\theta_1 = \left(\frac{\partial \ln q}{\partial \ln c_1}\right) = \frac{K_1 c_1}{1 + K_1 c_1 + K_2 c_2} \tag{49}$$

Figure 12 shows a simulation of the effect of salt concentration on the surface coverage of the drug. The simulation compares the salt concentrations of 0.01 M, 0.1 M and 1.0 M. The binding constant for the drug is taken as 1000 M^{-1}, and the binding constant for the Na^+ ion as 10 M^{-1} [60].

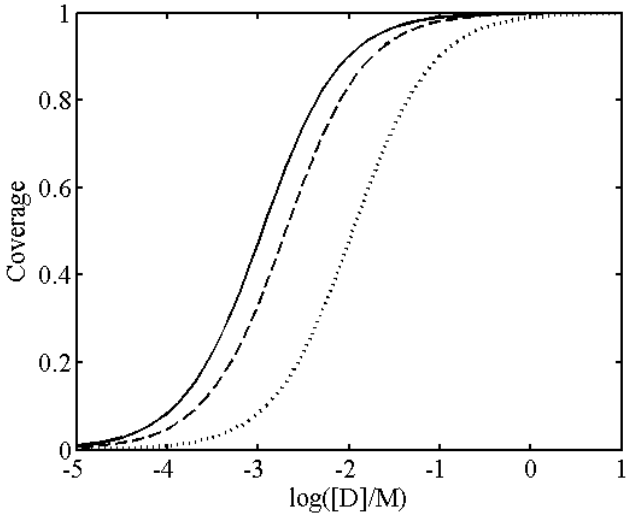

Figure 12. Effect of salt concentration on the binding constant. Concentrations of NaCl 1 M (···), 0.1 M (---) and 0.01 M (solid line). (Reprinted from Publication IV with permission from Elsevier)

From Figure 12, it is evident that the increase of the salt concentration by a factor of ten increases the equivalence point, i.e. the reciprocal of the binding constant, approximately by a factor of two and thus decreases the binding constant accordingly. As the effect of ionic strength on the binding of drugs in the ITC measurements was even greater than this, ion binding explains the results only partially. The structural organization of the lipids may also account for the phenomenon, as the ionic strength affects not only the electrostatics of the binding of the drugs, but also the organization of the lipids in the bilayer [61,62]. Moreover, it has been shown that phospholipid bilayers become more resistant to penetration as the ionic strength increases [63,64].

The apparent values of the binding constant determined in 2 mM Hepes, 15 mM NaCl at 25 ^oC, the average zeta potentials and the calculated partition coefficients are summarised in Table 1. The liposome–water partition coefficient of propranolol is very

close to the value reported in Publication I (Figure 10). The consistent values given by the two different methods give support to the fact that ITC and the electrochemical method presented in Publication I are both reliable tools in drug partitioning studies.

Table 1. Apparent binding constant, zeta potential and the logarithm of the partition constant for the binding of drugs into 100 nm liposomes at 298 K. The subscript D→L denotes the drug-into-liposome and L→D the liposome-into-drug titration (Reprinted from Publication IV with permission from Elsevier)

Drug	$K_{app} \times 10^{-3}$ (M^{-1})	ζ–potential (mV)	Log $P_{D\rightarrow L}$	Log $P_{L\rightarrow D}$
Alprenolol	33	−24	2.2	2.0
Labetalol	24	−33	2.0	1.8
Propranolol	85	−37	2.5	2.4
Tetracaine	62	−30	2.4	2.1

2.2.3 Surface chemistry

As the partitioning studies with lipid bilayers are usually rather time consuming, methods based on the surface active properties of drugs have been proposed for the determination of the partition coefficient. As an alternative to the lipid–water partition coefficient, Seelig and coworkers [65] have developed an approach based on the Gibbs equation of a drug. In this method, an air–water partition coefficient is determined by measuring the surface pressure of a drug monolayer at the air–water interface and applying the Szyszkowski equation [66]. The basis of the approach is that the partitioning of drugs at the air–water interface is assumed to be similar to the partitioning at the lipid–water interface, as the partitioning into both interfaces is driven by the hydrophobic effect, and because the dielectric constant for air is close to that for the hydrocarbon region of the lipid membrane [65]. Using the surface activity measurements, the ability of a drug to reach the central nervous system was predicted using three parameters derived from the adsorption isotherm: the minimum concentration at which surface activity is induced, the surface area of a molecule, and

the critical micelle concentration (CMC). Based on these parameters, drugs were divided into three groups: i) very hydrophobic that cannot pass the blood-brain barrier, ii) less hydrophobic that cross the blood brain barrier with ease and iii) hydrophilic drugs that cross the barrier only if applied at high concentrations. Later the method was also used to evaluate the effect of halogenation of drugs on membrane permeation [58]. It was found that the increased hydrophobicity due to halogenation increases the air–water partition coefficient.

Even though the surface activity measurements were suggested to be suitable for fast screening of drug candidates, the experiments presented by Seelig and coworkers were rather time consuming. The measurement of a Gibbs adsorption isotherm took from 45 minutes to four hours depending on the size of the trough used [65]. A new platform for the surface activity measurements was introduced in the work of Suomalainen *et al.* [67], where the measurements of drugs were carried out using a multichannel microtensiometer, which allowed fast measurement of surface activity of drugs with small sample volumes.

Despite the comparable dielectric constants of air and the hydrocarbon core of a membrane, air is no more than a very crude model for the hydrocarbon region. To improve the biomimetics of the surface activity measurements, hydrocarbon–water interfaces were used in Publication III to study partitioning of drugs. As in the studies of Fisher *et al.* [65] and Suomalainen *et al.* [67], the Gibbs equation was used to determine the partition coefficient for the adsorption of the drugs. However, instead of measuring the surface pressure at air–water interface and applying the Szyszkowski equation to determine the partition coefficient, the Langmuir adsorption isotherms were constructed using surface tension measurements combined with contact angle data measured at the hydrocarbon–water interface (Figure 13). The hydrocarbon region of the lipid membrane was modeled using three different self-assembled monolayers (SAMs) on Au(111) surfaces, two of which were formed of alkanethiols of different chain lengths and one of the thiolipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphothioethanol (DPPTE). The

adsorption coefficients of six β-blockers were determined and compared to the octanol–water partition coefficients as well as liposome–water partition coefficients.

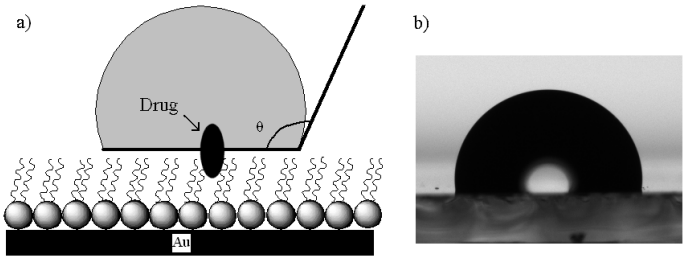

Figure 13. A schematic illustration of the experimental set-up in contact angle measurements (a) and an image of a drop of 1 mM propranolol solution on DPPTE SAM (b). (Reprinted from Publication III with permission from Elsevier)

Partitioning of β-blockers into the interface can be described by the Gibbs equation, which relates the surface excess (Γ) to the chemical potential (μ) of the drug:

$$\Gamma = -\left(\frac{\partial \gamma_{SL}}{\partial \mu}\right) \tag{50}$$

where R is the gas constant, 8.314 J mol^{-1} K^{-1}, T is temperature and γ_{SL} is the solid–liquid interfacial tension.

Since the solid–liquid interfacial tension cannot be obtained directly from the contact angle measurements, the Young equation can be used to rewrite the Gibbs equation in terms of the contact angle θ and the liquid–vapor interfacial tension γ_{LV}. The Young equation represents the mechanical equilibrium of a drop under the action of the three interfacial tensions:

$$\gamma_{SL} + \gamma_{LV} \cos\theta = \gamma_{SV} \tag{51}$$

where γ_{SV} is the solid–vapor interfacial tension. Substituting γ_{SV} into the Gibbs equation, assuming γ_{SV} constant, gives:

$$\Gamma = \frac{\partial(\gamma_{LV}\cos\theta)}{\partial\mu} \tag{52}$$

Thus, the surface excess can be determined from the graph of $\gamma_{LV}\cos\theta$ versus μ by taking the derivative at each data point. The chemical potential of the drug can be approximated by the infinite dilution limit as:

$$\mathrm{d}\mu = RT\mathrm{d}\ln\left(c/c^*\right) \tag{53}$$

where c is the concentration of the drug and c^* is 1.0 mol dm^{-3}.

In Publication III, the surface excesses of the drugs at each concentration were determined using equation (52). The Langmuir adsorption isotherm was used to evaluate the partition coefficients of the drugs. The Langmuir isotherm describes the equilibrium between the drug molecules in the aqueous solution and those partitioned at the surface: D + S $\rightleftarrows$ DS. The adsorption coefficient for the equilibrium is K = [DS]/([D][S]), where [DS] is the concentration of the drug at the surface, [D] is the concentration of the drug in the aqueous phase, and [S] is the concentration of unoccupied sites at the surface. The adsorption coefficients for the partitioning of the drugs were determined by fitting the Langmuir isotherm to the data, as the surface excess at each drug concentration was known:

$$\Gamma = \frac{\Gamma_{max}Kc}{1+Kc} \tag{54}$$

where Γ_{max} is the maximum surface excess and c is the concentration of the drug.

The maximum surface excess values determined from the fit varied from 8×10^{-11} mol cm^{-2} to 1.6×10^{-9} mol cm^{-2}. These values are comparable to the ones reported in the study of Nguyen *et al.* [68], where a novel deep-ultraviolet spectroscopic method was used to study the association of drugs to the planar supported lipid bilayers. The values determined in this study using the Langmuir isotherm were from 10^{-12} mol cm^{-2} to 10^{-11} mol cm^{-2}, which are only slightly smaller than the ones determined in Publication III. The difference in the magnitude of the surface excesses of different drugs has been suggested to be due to the repulsive interactions between drug molecules, which are stronger for some drugs than the others [68].

The adsorption coefficient K determined using Equation (54) can be compared to the traditional octanol–water partition coefficient P_{oct}, as both of the coefficients describe the equilibrium between the phase mimicking the hydrocarbon region of the membrane and the aqueous phase. When the logarithm of K was compared to the logarithm P_{oct}, it was evident that log $K_{octadecanethiol}$ correlated very well with log P_{oct} (Figure 14a, R^2 = 0.95), whereas no correlation was observed between log $K_{decanethiol}$ and log P_{oct} (Figure 14b). The difference is probably due to the less-ordered structure of the decanethiol SAM compared with the octadecanethiol SAM. As the lipid molecules in the phospholipid bilayer of the biological membranes are relatively tightly packed and well-ordered [69], the results suggest that the ability of the decanethiol surface to serve as a model membrane is not as good as that of the octadecanethiol SAM.

Interestingly, log K_{DPPTE} correlated fairly well with log P_{oct} (Figure 14c, R^2 = 0.86), even though the maximum surface excess of the drugs determined at DPPTE SAMs were somewhat different from those determined at the alkanethiol SAMs. Compared with the octadecanethiol SAM, the correlation of log K_{DPPTE} with log P_{oct} was slightly lower and the structural properties of the SAMs may account for this difference. The isotropic hydrocarbon phase in the octadecanethiol SAM is very similar to the octanol phase, whereas the DPPTE SAM is more anisotropic due to the phosphorus moiety of the DPPTE molecule. Because of this, it was interesting to compare the log K values not

only to the log P_{oct} values, but also to the log P_{lip} values, the liposome–water partition coefficients.

To compare the adsorption coefficients and the liposome–water partition coefficients, the log P_{lip} values for metoprolol, nadolol, propranolol and timolol were taken from Publication I and log P_{lip} for alprenolol was obtained from the literature [10]. Unfortunately, log P_{lip} for labetalol was not available. Comparison of log $K_{octadecanethiol}$ and log P_{lip} is shown in Figure 14d (R^2 = 0.69), and that of log K_{DPPTE} and log P_{lip} in Figure 14e (R^2 = 0.70). No correlation was found between log $K_{decanethiol}$ and log P_{lip}, and this lack of correlation is probably due to the less-ordered structure of the decanethiol monolayer as discussed above. As can be seen from Figures 14d and 14e, there is no difference in the correlation of log $K_{octadecanethiol}$ and log K_{DPPTE} and both log $K_{octadecanethiol}$ and log K_{DPPTE} correlate better with log P_{oct} than with log P_{lip}. Poor correlation with log P_{lip} can be explained by the anisotropy of the liposomal membrane.

Due to the excellent biomimetic properties of liposomes, log P_{lip} describe well the whole partitioning process of the drug into the membrane, which includes multiple barriers for the drugs. In addition to the hydrocarbon core of the membrane, the two polar headgroup interfaces act as diffusion barriers. Because of the simplicity of the model membranes used in this study, the log K values mainly describe the barrier properties of the hydrocarbon core of the bilayer membrane and should not be interpreted as mimicking the partitioning process as a whole. Moreover, good correlation with the traditional log P_{oct} supports the interpretation that the method presented in Publication III gives insight into the interaction of drugs with the hydrophobic core of the biological membrane.

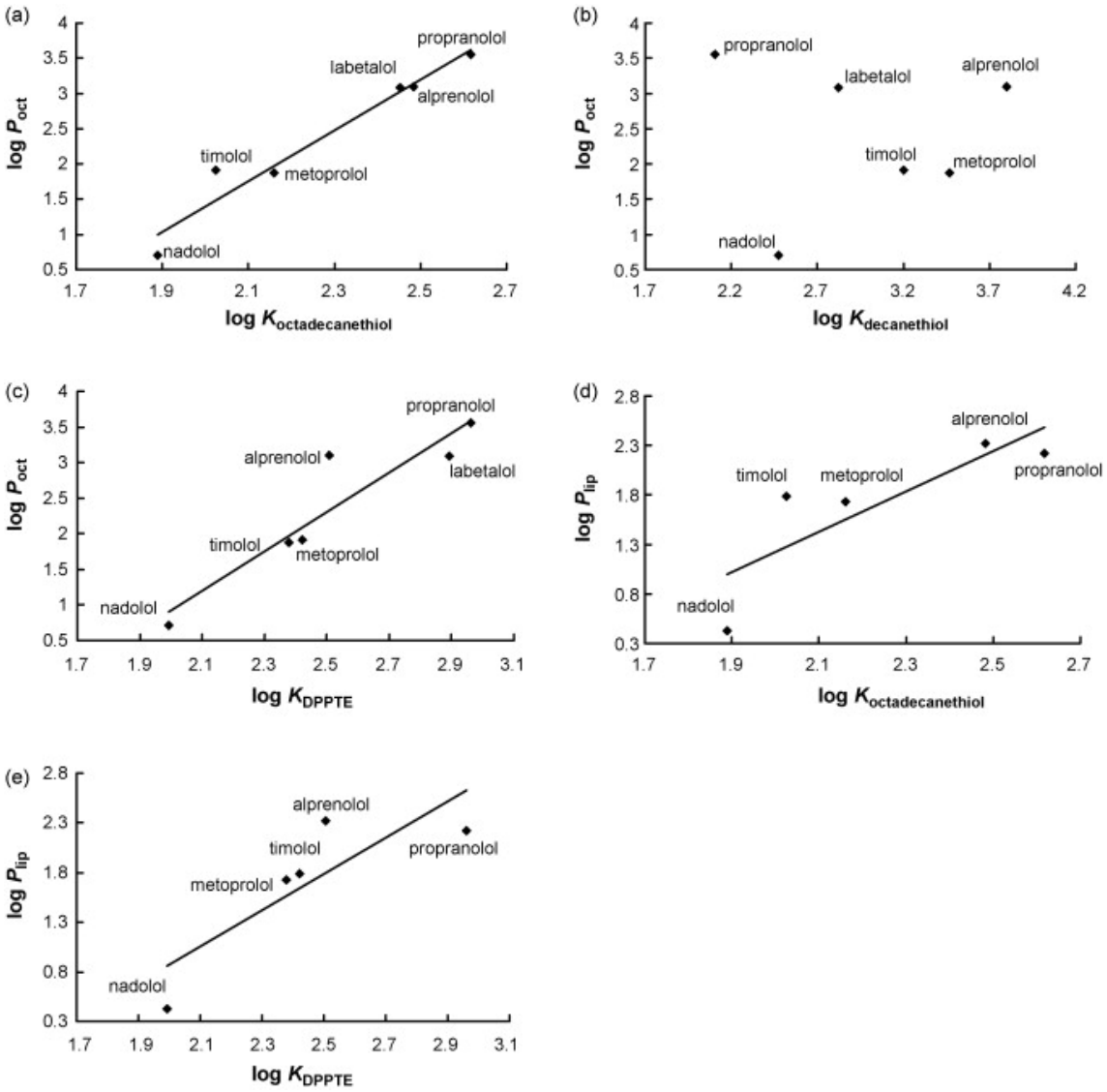

Figure 14. Correlation of log $K_{\text{octadecanethiol}}$ with log P_{oct} (a), log $K_{\text{decanethiol}}$ with log P_{oct} (b), log K_{DPPTE} with log P_{oct} (c), log $K_{\text{octadecanethiol}}$ with log P_{lip} (d), and log K_{DPPTE} with log P_{lip} (e). (Reprinted from Publication III with permission from Elsevier)

The purpose of Publication III was to demonstrate how surface tension and contact angle measurements on different SAMs can be used to assess drug–membrane interactions. When data obtained using the pendant drop method and contact angle goniometry were fitted to the Gibbs equation and the Langmuir isotherm, it was possible to determine the adsorption coefficient K. It was found that log K determined on the octadecanethiol and DPPTE SAMs correlated very well with log P_{oct} and fairly

well with log P_{lip} whereas no correlation was found between log K on the decanethiol surface and log P_{oct} or log P_{lip}. It was concluded that the ability of the decanethiol SAM to model the hydrophobic core of the membrane is not as good as that of the octadecanethiol SAM or DPPTE SAM due to its less ordered structure. In addition, comparison of the relative magnitudes of the surface excesses of the drugs on the alkanethiol SAMs versus those on DPPTE SAMs revealed that it is not only hydrophobicity, but also the conformational effects that are decisive factors in drug-membrane interactions.

3 Physicochemical properties of drug carriers

3.1 Liposomes

Liposomes are used as pharmaceutical carriers for a wide variety of drugs, including antitumor and antimicrobial agents, chelating agents, peptides, proteins and genetic material [70]. Depending on the physicochemical properties of the drug, it can be captured in the aqueous cavity of the liposome, interact with the bilayer surface or be taken up by the bilayer structure. The pharmacological properties of a liposomal drug formulation depend on the physicochemical properties of the liposomal carrier and the drug as well as the strategies used for drug encapsulation and retention [71].

In Publication I, the ability of liposomes to encapsulate and carry drugs was utilized in an electrochemical determination of the partition coefficient. Five β-blockers with varying lipophilicity were encapsulated in POPC/POPG liposomes and the amount of drug in the liposomes was determined using square wave voltammetry. Negatively charged liposomes were chosen because of their resemblance to biological membranes [72] and because the preliminary experiments showed that more of a drug was encapsulated into these liposomes compared with the neutral ones. The better encapsulation efficiency is most probably due to the electrostatic contribution that dominates the binding of cationic drugs into the negatively charged membrane as explained in Publication IV. The higher encapsulation efficiency of cationic drugs into the negatively charged liposomes due to the electrostatic interaction has been reported also in the literature [73].

Comparing the amounts of drugs incorporated into liposomes in Publication I, where the drugs were both in the bilayer and the aqueous cavity of the liposomes, and in Publication IV where the drugs were interacting with the surface of preformed liposomes, it is clear that less drugs were sequestered within the membrane in the latter case. When the drugs are added to the preformed liposomes, they do not penetrate

through the bilayer to the aqueous cavity, but reside on the surface of the liposome or partition into the hydrocarbon core of the membrane [74,75]. Various gradient driven methods have been developed to improve the loading efficiency when drugs are loaded after the formation of the membranes [76–78]. In this thesis, however, high drug loading was essential only in Publication I, where it was accomplished by passive loading during the hydration stage of the liposome preparation [79].

3.2 DNA complexes

Carriers of drugs are especially important in gene delivery, as naked DNA crosses various barriers of the cell very inefficiently because of its size and charge. To facilitate the entry of DNA into the cell and the subsequent expression in the nucleus, a wide variety of carriers have been designed for gene delivery purposes. Due to the safety issues related to the viral vectors, more and more research has focused on nonviral vector systems including cationic lipids, polymers, dendrimers and peptides [80].

Various polymers have been tested as carriers of DNA, and their structures usually include protonable amines, the number and pK_a of which is different in each carrier [81]. Two of the most commonly used polycations are poly-L-lysine (PLL) and polyethyleneimine (PEI). Both of these cationic polymers form small toroidal complexes with DNA [82], however, in vitro studies have shown that compared to PLL, the transfection efficiency of PEI is much higher [83,84]. The higher efficiency in gene transfer has been explained by its ability to buffer endosomes [83,85]. The buffering capacity of PEI results from its unique structure, as only a part of the amino groups in PEI are protonated at physiological pH.

One major problem when using polymeric carriers is that the complexes tend to aggregate in aqueous solutions. The preparation conditions of polycation-DNA complexes influence the aggregation behavior. Generally the aggregates grow bigger in higher ionic strength, and as a result, the aggregation is more severe under physiological

conditions whereas in glucose solution the complexes aggregate to a lesser extent. Also, PEI/DNA complexes formulated in 5 % glucose have been shown to be more efficient *in vivo* than complexes prepared in 150 mM sodium chloride [86]. It has been proposed that the difference in efficiency is due to the difference in their aggregation behaviour in different media. Nevertheless, the aggregation phenomenon exists even in 5 % glucose, so a detailed knowledge on the phenomenon is crucial when improving the efficiency of gene carriers.

The size of the DNA complexes formed using synthetic polymers has to be controlled in order to reach higher efficiency in gene delivery. Much research has already been done concerning the control of the size of the complexes and the prevention of the aggregation process. In most of the studies, the attempts to control the aggregation behaviour of polycations have either involved the right choice of environment and protocols of complexing [87,88] or the covalent attachment of protecting groups [89–91]. In the work of Lee *et al.* [92], aggregation of complexes was prevented by electrostatic attachment of cationic fusogenic peptides conjugated with poly(ethylene) glycol to negatively charged PEI/DNA complexes. Bromberg *et al.* [93] modified PEI with a diblock copolymer to protect the DNA complexes from aggregation in the presence of serum proteins. Also, biomaterial systems for controlled delivery of DNA have been used to overcome the aggregation problem [94,95]. Furthermore, it was proposed that the aggregation could be prevented by surfactants [96]. Sharma *et al.* [96] showed that the addition of the surfactant polyoxyethylene inhibits the aggregation process of PEI/DNA complex during 24 hours of storage. Control of aggregation is not only important during the storage of the gene medicine, but more importantly, controlling the size and charge of the complex is crucial when delivering the vector into a cell.

Prior to entering the cell, DNA complexes may interact with various extracellular matrix components. Extracellular matrices contain sulfated proteoglycans consisting of a core protein covalently linked to one or more sulfated glycosaminoglycans (GAGs): heparin, heparin sulfate, dermatan sulfate, chondroitin sulfate or keratin sulfate [97].

These negatively charged GAGs may bind to the positively charged DNA complexes on its way to the cell. GAGs have been shown to have a dual role in gene delivery. Whereas membrane associated GAGs have been suggested to mediate the binding and delivery of cationic complexes to the cell [98], many studies have also shown that extracellular GAGs can actually decrease the transfection efficiency of the non-viral carrier by blocking the access of the DNA complexes to the target cell [99–101]. If DNA is released from the complex upon interaction with GAGs, GAGs may be internalized into the cell with the free carrier instead of DNA [102]. Because of this, release of DNA by GAGs in the extracellular matrix is not desirable when designing new gene carriers.

Publication II presents a comparison of the size and charge of the cationic polymer-plasmid DNA (pDNA) complexes of two commonly used polymers, PEI and PLL. The complexation was studied in 5 % glucose solution at 25 °C using dynamic light scattering and isothermal titration calorimetry. The aggregation of the complexes was controlled by addition of the surfactant polyoxyethylene stearate (POES). In addition, the stability of the complexes in the presence of GAGs was evaluated using dextran sulfate (DS) as a relaxing agent. The relaxation of the complexes was studied using agarose gel electrophoresis. DS is a highly charged polyanion and because of its resemblance with heparin, it can be used as a model molecule for extracellular matrix GAGs. Publication II elucidates the role of surfactant in controlling the size of the PEI/DNA complex and reveals the differences of the two polymers as complexing agents.

In Publication II, it was found that at a certain nitrogen to phosphate (N/P) ratio POES reduced the size of the PEI/DNA complexes and prevented the aggregation over time. However, the addition of POES did not prevent the aggregation over the whole range of N/P ratios studied, but instead, the addition of POES shifted the aggregation process to a lower N/P ratio (Figure 15). It was also found that the higher the POES concentration, the more the aggregation peak was shifted to the direction of lower N/P ratios. Moreover, POES affected not only the size of the complexes, but also the zeta potential.

The measurements confirmed that the point of zero zeta potential was also shifted to the lower N/P ratios as the highest diameter of the complexes was measured to correspond to the zeta potential closest to zero.

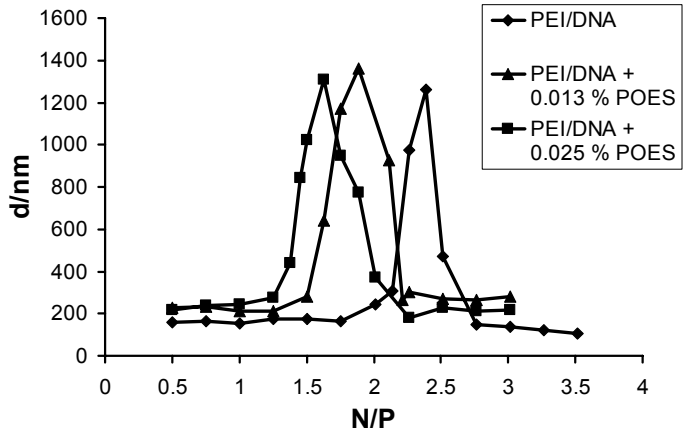

Figure 15. Size of PEI/DNA complexes as a function of N/P ratio. The effect of POES. (Reprinted from Publication II with permission from Elsevier)

The peaks observed in the size-N/P curve were related to the apparent pK_a of the polycation and it was found that the addition of POES affected the extent of the protonation of PEI increasing its pK_a value significantly. The shift in the pK_a of PEI can be explained in terms of the change in the Gibbs energy. As the surfactant molecule is in the vicinity of the polycation, the shielding effect of the like charges is increased and the distance between the charges is altered. As the surfactant screens the charges, a greater fraction of the polycation can be protonated. From Coulombs law, the change in the Gibbs energy as the change in distance between the charges is:

$$\Delta G = -\frac{e^2 N_A}{4\pi\varepsilon_0\varepsilon}\left(\frac{1}{r_1}-\frac{1}{r_2}\right) \tag{55}$$

where e is the elementary charge, N_A the Avogadro constant, ε_0 the permittivity of free space, ε the permittivity of water, and r_1 and r_2 are the separation distances of the charges.

On the other hand, the Gibbs energy is:

$$\Delta G = -RT \ln K_{\mathrm{a}} \tag{56}$$

And the change in the Gibbs energy

$$\Delta(\Delta G) = -RT \ln \frac{K_{\mathrm{a},1}}{K_{\mathrm{a},2}} \tag{57}$$

which can be rewritten as:

$$\Delta(\Delta G) = 2.303 RT \Delta pK_{\mathrm{a}} \tag{58}$$

Combining the equations (55) and (58) gives:

$$\Delta(\Delta G) = -\frac{e^2 N_{\mathrm{A}}}{4\pi\varepsilon_0\varepsilon}\left(\frac{1}{r_1} - \frac{1}{r_2}\right) = 2.303 RT \Delta \mathrm{p}K_{\mathrm{a}} \tag{59}$$

Assuming the distance between the charges of the polycation is 5 Å without the surfactant, the measured pK_{a} change of 0.2 units by the addition of the surfactant results in r_2 = 7.4 Å. Likewise, if the separation distance of the charges is 10 Å, r_2 = 27.9 Å. Thus it is evident that very small changes in the distance between the charges shift the pK_a value significantly. Unlike the PEI/DNA complexation, the PLL/DNA complexation was almost unaffected by the addition of POES.

To study the aggregation process further, the thermodynamic parameters of the complexation were determined using ITC. ITC data revealed that the enthalpy changes related to the complexation process are minute. For PEI/DNA complexation, ΔH = –640 cal mol^{-1} = –2678 J mol^{-1}, which is of the same magnitude as the thermal energy RT. For PLL, the change in enthalpy was so small that it could not be detected. As the

change in entropy for the PEI/DNA complexation was measured to be 27 cal mol^{-1} K^{-1} = 113 J mol^{-1} K^{-1} and the term $T\Delta S$ 8046 cal mol^{-1} = 33.6 kJ mol^{-1}, hence over ten-fold to the enthalpy contribution, the complexation process of pDNA with the polycation must be entropy driven. The results are also consistent with the values given in the literature for DNA binding with other polycations. The binding enthalpies determined for the complexation of DNA with (dimethylamino)ethyl methacrylate homopolymer varied from 0 kJ mol^{-1} to –6 kJ mol^{-1} of polymer, depending on the pH of the solution [103], whereas the binding enthalpies of the interaction of linear polyaminoamine polymer with DNA were from 0 kJ mol^{-1} to –3.5 kJ mol^{-1} [104]. Also binding enthalpies of the same magnitude, but of an endothermic nature have been observed: For the binding of DNA with trivalent cations cobalt hexamine and spermidine the enthalpies of binding ranged from 1 kJ mol^{-1} to 10 kJ mol^{-1} of cation [105] and for the binding of DNA with a cationic graft copolymer from 0 kJ mol^{-1} to 3 kJ mol^{-1} of copolymer [106]. The binding entropy of 113 J mol^{-1} K^{-1} is in accordance with the previously reported values for DNA binding with various polycations. In the entropy driven processes, the measured values of $T\Delta S$ varied from 28 kJ mol^{-1} to 42 kJ mol^{-1} for the complexation of DNA with trivalent cations [105], and from 11 kJ mol^{-1} to 26 kJ mol^{-1} for a cationic graft copolymer [106].

Plasmid DNA complexation and the stability of the complexes in the presence of DS was studied using gel electrophoresis. Only the free negatively charged pDNA was observed to migrate in the electric field whereas fully complexed pDNA could not be seen in the gel (Figure 16). Electrophoresis confirms the stabilizing effect of POES on the PEI/DNA complexes: PEI/DNA complex was relaxed upon addition of DS (lane 4), while PEI/DNA complexes stabilized by POES did not show this relaxation (lane 5). The results complement the findings of Sharma *et al.* [96]: POES not only prevents the aggregation of PEI/DNA complexes, but also stabilizes the complexes in the presence of extracellular GAGs. The GAG resistance developed with POES could be used as an alternative to the detergent removal method, where PEI/DNA complexes are protected against GAGs using a negatively charged lipid coating [107].

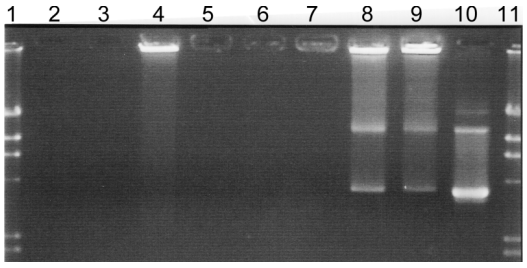

Figure 16. Effect of dextran sulfate on the stability of PEI/DNA and PLL/DNA complexes. Marker (lanes 1 and 11), PEI/DNA (lane 2), PEI/DNA/POES (lane 3), PEI/DNA + DS (lane 4), PEI/DNA/POES + DS (lane 5), PLL/DNA (lane 6), PLL/DNA/POES (lane 7), PLL/DNA + DS (lane 8), PLL/DNA/POES + DS (lane 9), plasmid DNA alone (lane 10). (Reprinted from Publication II with permission from Elsevier)

As a whole, Publication II presents a detailed study on the size and charge of the DNA complexes with two commonly used polycations PEI and PLL. The study also examines the effect of the surfactant POES on the complexation and elucidates the origin of the stabilizing effect of the surfactant. This area of research is important because knowledge on the interactions of surfactants with DNA complexes may help to adjust the size and charge of the gene carrier complexes to a desired value when designing new gene carriers.

4 Conclusions

The purpose of this thesis was to study the interactions of drugs with biological model membranes using physicochemical and electrochemical techniques. The topic included not only the interactions of traditional drugs with various membrane models, but also those related to carriers of drug, as they are equally important in controlling the delivery of the drug to the target site of the body.

When the ability of the drug to penetrate the cell membrane is assessed, one of the most widely used parameters is the partition coefficient of the drug. Publication I presents a novel electrochemical method for the determination of the liposome–water partition coefficient. The method utilizes square wave voltammetry in the determination of the amount of the drug encapsulated in the liposomes. A mathematical model was established to solve for the concentrations of drugs in the aqueous cavity and within the bilayer membrane, and as a result, the liposome–water partition coefficients for five β-blockers were reported in Publication I. The study demonstrates that the electrochemical measurements combined with the biomimetic properties of liposomes provide a rapid method to assess the interactions of drugs with the phospholipid membranes using only minute quantities of reagents.

In Publication IV, the binding of drugs onto liposomes was studied using ITC and zeta potential measurements. The thermodynamic parameters of enthalpy, entropy, Gibbs free energy and binding constant were reported for three β-blockers and one local anesthetic. The interaction of these drugs with liposomes was found to be enthalpy driven, with the electrostatics being the dominating factor. Increasing the ionic strength lowered the binding constant significantly, which was attributed to ion binding, although other contributions were also taken into account. Furthermore, the liposome–water partition coefficients obtained from the binding constants corrected for the electrostatic effect were determined to be somewhat lower that the corresponding octanol–water partition coefficients. Comparing the partition coefficients of Publication

I and IV, the liposome–water partition coefficient of propranolol determined using ITC in Publication IV corresponded very well with the electrochemically obtained value reported in Publication I.

The interactions of drugs with the hydrophobic part of the biological membrane were assessed using contact angle goniometry and surface tension measurements (Publication III). The hydrophobic core was modeled with two different alkanethiols and one thiolipid attached onto a gold surface. The adsorption coefficients of drugs on these hydrophobic model surfaces were found to correlate better with the octanol–water partition coefficients than with the liposome–water partition coefficients determined in Publication I. Because of the simplicity of the contact angle and surface tension measurements, it would be interesting to apply this approach also to other biomimetic surfaces, such as supported lipid bilayers.

The interactions of drugs with its carriers were explored in Publication II, which focuses on the properties of plasmid DNA-polycation complexes. It was shown that the aggregation of PEI/pDNA complexes can be controlled with the surfactant POES, which also protects the complexes against the negative effects of extracellular GAGs. These findings are particularly relevant for ocular gene delivery, as the membranes in the eye have a very high content of GAGs.

As a whole, this thesis addresses a number of important aspects of drug-membrane interactions from the physicochemical perspective. Various approaches to the partitioning of drugs were explored and three different experimental methods were used to determine the partition coefficients of eight drugs. Furthermore, physicochemical explanations were presented for a broad range of phenomena, ranging from the electrostatics of the binding of drugs to the aggregation of the DNA complexes. Understanding these interactions may prove to be useful not only when studying the transport of drugs in the body, but also when designing new drugs.

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