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Microcalorimetric and zeta potential study on binding of drugs on liposomes

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A B S T R A C T

In this work, isothermal titration calorimetry (ITC) combined with zeta potential measurements was used to study the binding and partitioning of three β-blockers, alprenolol, labetalol and propranolol, and the local anaesthetic tetracaine into liposomes. The thermodynamic parameters of enthalpy, entropy, the Gibbs energy and the binding constant were determined using the one site model. Furthermore, the binding constants corrected for the electrostatic contribution were used to assess the partition coefficients for the drugs. Also, the effect of the concentration, ionic strength, temperature and membrane curvature on the interaction was included in the evaluation.

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1. Introduction

The delivery of drugs to the target sites within the body is affected by the interactions of drugs with biological membranes. Typically, a drug has to cross several membranes in order to enter the target location. Therefore, understanding of the interactions of drugs with biological membranes is crucial when optimising the delivery of drugs. Understanding of these interactions is also a deciding factor when predicting the adsorption, distribution, metabolism, and excretion (ADME) properties of drugs in the beginning of the drug discovery process.

Among the properties that affect the permeation of drugs through biological membranes are lipophilicity, charge, size and hydrogen bonding properties. Traditionally the partitioning between membrane and drug has been assessed using n-octanol–water partition coefficient, which is defined as the ratio of the activity of a species dissolved at equilibrium between two immiscible solvents. Because of their superior biomimetic properties, liposomes have been used as an alternative to octanol in drug partitioning studies. Various methods for the determination of liposome–water partition coefficients have been developed, including the distribution technique [1], equilibrium dialysis [2], potentiometric titration [3,4], NMR spectroscopy [5], chromatographic techniques [6,7] and an electrochemical method [8].

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 [9–12]. 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 co-workers have studied the interaction of liposomes with various amphiphilic compounds including calcium channel antagonists and local anaesthetics [13–16]. In the work of Matos et al. [17] 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. [18] 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. [19], 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 this work, ITC and zeta potential measurements are 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, are compared to check the reliability of the data. The enthalpy, entropy and Gibbs energy of binding are determined using the one site model and the electrostatic contribution to the binding is evaluated using the Gouy–Chapman theory. Furthermore, the binding constants are used to assess the partition coefficients for the drugs. Also, the effects of the concentration, ionic strength, temperature and membrane curvature on the interaction are included in the evaluation.

2. Materials and methods

2.1. Materials

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Alprenolol hydrochloride, labetalol hydrochloride, propranolol hydrochloride and tetracaine hydrochloride were purchased from Sigma Aldrich (Steinheim, Germany). The molecular structures of the drugs are shown in Fig. 1 and their physicochemical properties in Table 1 [20]. All other chemicals used were analytical grade or better.

2.2. Preparation of liposomes

Liposomes composed of POPC and POPG in a mass ratio of 7:3 were prepared using the extrusion method [21]. Solid POPC was dissolved in chloroform and POPG in a mixture of chloroform and methanol in a ratio of 1:1 by volume. A total of 15–30 mg of lipids in stock solutions was put in a test tube and the organic solvent was evaporated to dryness under a stream of nitrogen. The lipid film formed was hydrated with 1–1.5 ml of the buffer solution (20 mM Hepes + 150 mM NaCl, pH 7.4 or 2 mM Hepes + 15 mM NaCl, pH 7.4) at room temperature. In order to achieve a certain size distribution, the liposome suspension was extruded through two-stacked polycarbonate membranes with pore size of 50 nm, 100 nm, and 400 nm. The concentration of lipids was determined by phosphorus analysis using inductively coupled plasma spectrometry (ICP).

2.3. Isothermal titration calorimetry

The experiments were carried out using an isothermal titration calorimeter (Microcal VP-ITC, USA) at 25 °C or 37 °C and analysed with the accompanying software. The drug solutions for the ITC experiments were prepared using the same buffer as for the liposomes. In drug-into-liposome titrations, 10 µl injections of the drug solution were added to the liposome suspension at 4 min time intervals. The concentrations of the drugs were 3–10 mM and the lipid concentration of the liposome suspension 2–8 mM. In liposome-into-drug titrations, the drug concentrations were 0.4 mM and the lipid concentrations 17–28 mM. The volume of the cell was 1.44 ml and the stirring rate 450 rpm. The raw calorimetric data were corrected for the heat of dilution of the drug and lipid by subtracting blank titrations from the binding interaction data. The heats of dilution were small compared with the binding interaction heats.

2.4. Size and zeta potential measurements

The liposome size distribution and zeta potential values at various drug–lipid ratios were measured using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments Ltd, United Kingdom). The size distribution was determined both before and after the ITC measurement in order to observe the possible changes in the vesicle size caused by the solubilising effect of the drug. No changes in the size distribution, however, were detected and thus the solubilising effect of the drug was assumed to be negligible. The zeta potential measurements were performed at 25 °C using drugs and liposomes in 2 mM Hepes + 15 mM NaCl buffer solution. The measurements were planned to mimic the drug-into-liposome ITC titrations and thus they were performed at the same drug–lipid ratios, but due to the instrumental limitations the concentrations of drugs and lipids were two orders of magnitude lower. The initial volume of the lipid suspension was 1.4 ml into which 30 µl injections of the drug solution were added. After each injection, the solution was stirred for 4 min with a magnetic stirrer before the zeta potential measurement.

3. Results and discussion

3.1. Thermodynamics of the binding process

The interactions of drugs and liposomes were first studied at 25 °C using the 2 mM Hepes buffer containing 15 mM NaCl. The liposomes used were extruded through polycarbonate membranes of pore size 100 nm in diameter, as the packing density of the lipids in these liposomes resemble that of the phospholipids in the cell membrane [22]. The size of the liposomes was determined using dynamic light scattering and was found to be 110 ± 4 nm.

Fig. 2 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

<table>
<thead>
<tr>
<th>Drug</th>
<th>Molecular weight (g mol⁻¹)</th>
<th>Dissociation constant (pKₐ)</th>
<th>log Pₘet a</th>
<th>log Pₘet b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alprenolol</td>
<td>249.36</td>
<td>9.65</td>
<td>3.10</td>
<td>2.59</td>
</tr>
<tr>
<td>Labetalol</td>
<td>328.41</td>
<td>7.4; 8.7</td>
<td>3.09</td>
<td>2.18</td>
</tr>
<tr>
<td>Propranolol</td>
<td>259.35</td>
<td>9.45</td>
<td>3.56</td>
<td>2.75</td>
</tr>
<tr>
<td>Tetracaine</td>
<td>264.37</td>
<td>8.39</td>
<td>3.73</td>
<td>3.36</td>
</tr>
</tbody>
</table>

a Determined experimentally.
b Calculated using CLOGP version 3.54.
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. Thus the one site model according to the Langmuir adsorption isotherm, included in the Origin software for ITC was used to analyse the data. In the model, the fraction of sites occupied by the drug after the ith injection, \( \theta_i \), is calculated from the ratio of the cumulative heat produced after the ith injection, \( \sum \Delta H_i \), and the total heat produced during the titration, \( \Delta H_{\text{max}} \). The apparatus software determines the binding constant \( K_b \) using the Langmuir adsorption isotherm:

\[
\theta_i = \frac{\sum \Delta H_i}{\Delta H_{\text{max}}} = \frac{K_b c_f}{1 + K_b c_f}
\]

In Eq. (1), \( c_f \) is the concentration of free lipids calculated from the total concentration of lipids, \( c_T \), the number of binding sites, \( N \), and the lipid concentration, \( c_L \), as \( c_f = c_T - N c_L \).

In the analysis, it is taken into account that the model compounds are weak basic drugs. Therefore, they are mostly in their ionised form and unlikely to penetrate to the inner leaflet of the membrane. Thus the concentration of lipids available for binding for the ionised drugs is 50% of the lipid concentration used in the experiments. In the case of labetalol, however, the first \( pK_a \) of which is 7.4 (Table 1), it is possible that the neutral form of the drug penetrates to the inner side of the membrane. Thus the whole amount of lipid was assumed to be available for binding.

The results of the analysis of the binding isotherms are summarised in Table 2. The tabulated errors are the errors of the fit given by the software package. From the data in Table 2 it can be seen that for all of the drugs enthalpy is the driving force of the interaction. The enthalpies of binding for all of the drugs are of the same order of magnitude as those reported in the literature. Seeleg and co-workers [13–15] have investigated the membrane location and binding mechanism of calcium channel antagonists using ITC. For the calcium channel antagonist amlodipine, the binding process was observed to be enthalpy driven and \( \Delta H \) was measured to be \(-9.2 \text{ kcal mol}^{-1}\), which corresponds to \(-38.5 \text{ kJ mol}^{-1}\) [13]. For another calcium antagonist, flunarazine, \( \Delta H \) was somewhat smaller \(-22.2 \text{ kJ mol}^{-1}\) [14]. The interaction of calcium channel antagonist verapamil with lipid membranes resulted in enthalpies ranging from \(-13.8 \text{ kJ mol}^{-1}\) to \(-5.9 \text{ kJ mol}^{-1}\) depending on the salt concentration and the charge of the lipid [15]. Seeleg and Ganz [16] extended the study to various amphiphilic compounds and reported negative \( \Delta H \) also for the local anaesthetic dibucaine, for which the measured \( \Delta H \) was considerably smaller, \(-7.9 \text{ kJ mol}^{-1}\).

The largest negative binding enthalpy was measured for the anticancer drug paclitaxel, \(-105 \text{ kJ mol}^{-1}\) [23]. In the study of Matos et al. [17] the enthalpies of binding for anti-inflammatory drugs were from \(-19.7 \text{ kJ mol}^{-1}\) to \(-14.2 \text{ kJ mol}^{-1}\). When comparing the enthalpies reported in the literature, one has to keep in mind that the enthalpies of binding are largely dependent on the \( pH \), temperature and the type of the liposomes used in the study. Therefore, direct comparison of the magnitude of the parameters is rather difficult. In all of these studies, however, the negative enthalpies of binding were explained by the van der Waals interactions between the non-polar parts of the drugs and the hydrocarbon core of the membrane. Additionally, the electrostatic effects on the binding were considered in the case of charged drugs.

### 3.2. Zeta potential measurements

In this study, to account for the electrostatic effects of the binding, the zeta potential values of the 100 nm liposomes were measured at the same drug to lipid ratios as used in the ITC experiments. A typical result of the zeta potential measurements is illustrated in Fig. 3, which shows the effect of the addition of tetracaine on the liposome zeta potential. Similar curves were measured with all of the drugs studied. Surprisingly, the results suggest that the zeta potential remains practically constant throughout the ITC experiments.

---

**Table 2**

Thermodynamic parameters for the binding of drugs into 100 nm liposomes at 298 K. Buffer used was 2 mM Heps + 15 mM NaCl.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Titration method</th>
<th>( K_b \times 10^{-3} \text{ (M}^{-1})</th>
<th>( \Delta H \text{ (kJ mol}^{-1})</th>
<th>( \Delta S \text{ (J mol}^{-1} \text{ K}^{-1})</th>
<th>( \Delta G \text{ (kJ mol}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alprenolol</td>
<td>Drug-into-liposome</td>
<td>13 ± 1</td>
<td>−9.9 ± 0.2</td>
<td>45.6</td>
<td>−23.4</td>
</tr>
<tr>
<td></td>
<td>Liposome-into-drug</td>
<td>6 ± 2</td>
<td>−3.3 ± 0.3</td>
<td>62.6</td>
<td>−21.9</td>
</tr>
<tr>
<td>Labetalol</td>
<td>Drug-into-liposome</td>
<td>67.2 ± 0.2</td>
<td>−36.1 ± 0.2</td>
<td>−47.7</td>
<td>−21.8</td>
</tr>
<tr>
<td></td>
<td>Liposome-into-drug</td>
<td>43 ± 0.6</td>
<td>−4.6 ± 0.1</td>
<td>53.9</td>
<td>−20.7</td>
</tr>
<tr>
<td>Propranolol</td>
<td>Drug-into-liposome</td>
<td>20 ± 4</td>
<td>−18.8 ± 0.08</td>
<td>18.4</td>
<td>−24.3</td>
</tr>
<tr>
<td></td>
<td>Liposome-into-drug</td>
<td>11 ± 2</td>
<td>−7.0 ± 0.3</td>
<td>53.8</td>
<td>−23.1</td>
</tr>
<tr>
<td>Tetracaine</td>
<td>Drug-into-liposome</td>
<td>19 ± 3</td>
<td>−13.3 ± 0.4</td>
<td>37.2</td>
<td>−24.3</td>
</tr>
<tr>
<td></td>
<td>Liposome-into-drug</td>
<td>10 ± 2</td>
<td>−4.2 ± 0.2</td>
<td>62.6</td>
<td>−22.9</td>
</tr>
</tbody>
</table>

---

**Fig. 2.** Titration of alprenolol (●), labetalol (■), propranolol (□) and tetracaine (▲) into 100 nm liposomes at 25 °C. The buffer used was 2 mM Heps + 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.
titrations. Since the addition of the drug does not change the ionic strength of the solution significantly, the most probable explanation for the phenomenon is that at this high salt concentration the degree of ion binding of sodium ions to the liposomal membrane is quite high, and they are just replaced by the added drug molecules. Thus the changes in the surface charge of the liposome and hence their effect on the zeta potential remains minimal during the titration.

3.3. Determination of the partition coefficient

The one site model 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 ionised drugs can be evaluated by multiplying the binding constant with the concentration of free lipids [11]:

$$P_{on} = K_{b}c_{L}$$

(2)

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 [13,15,18], although other methods have also been proposed [17]. The Gouy–Chapman theory assumes that a smooth surface has a charge density smeared out uniformly, as electrons do in metals [24]. 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:

$$c_{l}^{s} = c_{l}^{b} \exp \left( -\frac{z_{i}F}{RT} \phi_{0} \right)$$

(3)

where ‘s’ denotes the interfacial and ‘b’ for the bulk concentration, and $\phi_{0}$ is the Galvani potential at the interface, $z_{i}$ is the ionic charge, $F$ = 96486 Cmol$^{-1}$, $R$ = 8.314 J K$^{-1}$ 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:

$$K_{app} = K_{b} \left( -\frac{z_{i}F}{RT} \phi_{0} \right)$$

(4)

In the evaluation, the solution is 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 can be calculated to be approximately 2500, which means that less than 5% of the lipids are charged, although 30 w-% of the lipids are anionic (POPG). 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.

Table 3

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_{app} \times 10^{-3}$ (M$^{-1}$)</th>
<th>Zeta potential (mV)</th>
<th>log $P_{b \rightarrow L}$</th>
<th>log $P_{L \rightarrow D}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpenolol</td>
<td>33</td>
<td>-24</td>
<td>2.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Labetalol</td>
<td>24</td>
<td>-33</td>
<td>2.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Propranolol</td>
<td>85</td>
<td>-37</td>
<td>2.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Tetracaine</td>
<td>62</td>
<td>-30</td>
<td>2.4</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Here, the Gouy–Chapman theory is used to estimate the surface potential $\phi_{0}$ from the zeta potential, $\zeta$. The Galvani potential profile at the interface is calculated using the following equation [24]:

$$\tanh \left( \frac{z_{i}F}{4RT} \zeta \right) = \exp(-\kappa x_{2})$$

(5)

where $\zeta$ is the relative permittivity of water, 78.4, and $\epsilon_{0}$ is the permittivity of free space, 8.854 $\times$ 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.

The apparent values of the binding constant, the average zeta potentials and the partition coefficients are summarised in Table 3. Also, it is possible to evaluate the degree of ion binding using the equation for surface charge $\sigma$ [24]:

$$\sigma = \left( \frac{8RT\epsilon_{0}}{\epsilon_{f}\epsilon_{0}R} \right)^{1/2} \sinh \left( \frac{z_{i}F}{2RT} \phi_{0} \right)$$

(6)

In the evaluation, the solution is 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 can be calculated to be approximately 2500, which means that less than 5% of the lipids are charged, although 30 w-% of the lipids are anionic (POPG). 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.

In the literature and also in the Microlab software, the amount of bound injectant is usually calculated from the cumulative enthalpy divided by the molar enthalpy of binding, obtained from the first injections assuming that all injectant is bound. This method inherently assumes that binding is non-cooperative, like in the Langmuir model. The fact that an adsorption isotherm, bound ligand vs. free ligand, thus obtained has a curved shape can be explained either by an electrostatic effect or by the Langmuir isotherm. As explained above, in our case, the electrostatic correction remains constant during the titration; hence, applying the Langmuir model used in the Microlab software is justified.

To check that the applied partitioning model gives meaningful values, the experiments were also performed as liposome-into-drug titrations. Fig. 4 shows the titration of 100 nm liposomes into 0.4 mM alpenolol solution. Comparing Figs. 2 and 4, it can be seen that the quality of the raw ITC data is much better in the drug-into-liposome titrations. This is because the measured heats in the liposome-into-drug titrations are only about one third of those in the drug-into-liposome titrations. Nevertheless, the one site binding model can be readily applied to the liposome-into-drug-titrations as well. The results of the analysis of the binding...
isotherms are shown in Table 2 and the corresponding partition coefficients in Table 3. The thermodynamic parameters differ from those of the drug-into-liposome titrations because they are calculated per mole of injectant, i.e. per mole of drug for the drug-into-liposome and per mole of lipid for the liposome-into-drug titrations. The partition coefficients determined in the liposome-into-drug titrations are, as expected, close to those gained from the drug-into-liposome titrations and thus they support the use of the one site model in the analysis.

From the ratio of the enthalpies of binding per mole of lipid and per mole of drug it is possible to estimate the stoichiometry of the binding reaction. The lipid–drug ratio \( N \) determined from the ratio of the enthalpies of binding is compared to the \( N \) given by the one site model in Table 4. It can be seen that the values correspond to each other reasonably well, noticing that the elementary analysis of phosphorus from the liposome solutions is at the brink of its sensitivity; no better method, however, is available for us. This gives support that the two titration procedures are comparable and it is justified to use either of the procedures in the further experiments. Since the quality of the raw data is much better in the drug-into-liposome titrations, this procedure was chosen to be used for the rest of the titrations. For alprenolol, propranolol and tetracaine the lipid–drug ratio is close to 3, which actually agrees with the POPC:POPG ratio in the lipid formulation. Hence, it is plausible that electrostatic contribution to the binding equilibrium is dominating for these drugs, i.e. each POPG molecule binds one drug molecule.

### 3.4. Effect of concentration

As the partitioning of the drugs into liposomes represents a thermodynamic equilibrium, the concentration of the species involved should not have an effect on the determined thermodynamic parameters. In the literature, however, fewer drugs have been reported to be taken up by the liposomes at higher concentrations than expected for simple partitioning [17]. This has been explained by the saturation of the membrane. To test the effect of the concentration on the binding parameters, the titration of 10 mM propranolol into liposomes was performed at two different concentration ranges. For the lower concentration of lipid (2 mM), the obtained parameters were \( K_b = (21.2 \pm 0.8) \times 10^4 \text{ M}^{-1} \), \( \Delta H = (20.2 \pm 0.2) \text{ kJ mol}^{-1} \), \( \Delta S = 15.1 \text{ J mol}^{-1} \text{ K}^{-1} \), which are very close to the values reported for the lipid concentration of 6 mM in Table 2. The slight deviation is most probably due to the inaccuracy in the phosphorus analysis, not in the binding model. Nevertheless, the obtained parameters at the two different concentration ranges were so similar that further studies on the effect of the concentration were not found necessary.

### 3.5. Effect of the membrane curvature

In our previous paper [8], we have reported the partition coefficients for five \( \beta \)-blockers determined using an electrochemical method. In the electrochemical measurements, the magnitude of the partition coefficients seemed to be greater for liposomes extruded through 400 nm pores compared with those extruded through 100 nm pores. Because of this, the titrations were also performed using liposomes extruded through membranes with 400 nm pores. The average diameter of these liposomes was measured to be 230 ± 30 nm. The thermodynamic parameters determined for the binding of drugs into these liposomes are summarised in Table 5. When compared to the data in Table 2, it can be concluded that the increase of size does not have a significant effect of the binding parameters. In order to obtain further evidence for this conclusion, the effect of curvature was also studied by titrating 10 mM propranolol solution into smaller liposomes with a mean diameter of 70 nm (extruded through 50 nm membranes), but as before, the size of the liposomes had little effect on the binding. The binding parameters for this interaction were determined as \( K_b = 2.0 \times 10^4 \text{ M}^{-1} \), \( \Delta H = -19.16 \text{ kJ mol}^{-1} \), and \( \Delta S = 18.0 \text{ J mol}^{-1} \text{ K}^{-1} \), which are very close to those for 100 nm and 230 nm liposomes.

In the literature, the role of vesicle curvature in membrane binding has been studied with amphiphatic peptides [22,25] and calcium ions [26]. For the peptide–lipid interaction, the binding enthalpies and entropies were significantly different for 30 nm liposomes compared with 100 nm liposomes [22]. In the Gibbs energy of binding, however, the difference was observed to be insignificant. Differences in the thermodynamic parameters were explained by the higher curvature and thus less dense packing of the lipids in the liposomes with a diameter of 30 nm. Similar effect of curvature was not observed in our studies probably because the liposomes used were somewhat greater in diameter and thus the membrane is more planar. Also, it is likely that the effect of the peptide mangainin on the curvature is greater than that of the drugs because of the difference in the molecular weight. Most probably the penetration of the peptide, which is significantly larger in size, alters

![Fig. 4](image-url) Titrations of 100 nm liposomes into 0.4 mM alprenolol solution 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 ITC data for alprenolol and the lower panel the integrated enthalpies of interaction.

Table 4

<table>
<thead>
<tr>
<th>Drug</th>
<th>( N_{ADF} )</th>
<th>( N_{ADF-into-liposome} )</th>
<th>( N_{liposome-into-drug} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alprenolol</td>
<td>3.04</td>
<td>3.94</td>
<td>3.0</td>
</tr>
<tr>
<td>Labetalol</td>
<td>7.80</td>
<td>6.45</td>
<td>7.10</td>
</tr>
<tr>
<td>Propranolol</td>
<td>2.69</td>
<td>3.85</td>
<td>2.35</td>
</tr>
<tr>
<td>Tetracaine</td>
<td>3.16</td>
<td>4.74</td>
<td>2.71</td>
</tr>
</tbody>
</table>

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the conformation of the lipids in the membrane more notably. In the study of Arseneault and Lafleur [26] on the other hand, the differences in the thermodynamic parameters of the association of calcium ions were more related to the method of fabrication than to the average size of the vesicles. In their study, thermodynamic parameters for 50 nm vesicles were similar to those of 100 nm vesicles when the vesicles were prepared by extrusion, whereas for the 50 nm vesicles prepared by sonication the association was observed to be more endothermic. According to them, the differences in the thermodynamics are not only due to the small size of the vesicles, but also a result of the method of preparation. Sonication produces a population of vesicles with a large polydispersity index whereas extrusion results in more monodisperse populations. In polydisperse populations prepared by sonication there is a fraction of vesicles with a very small radius of curvature which are prone to endothermic transformations. Even though polydispersity of the vesicle populations did not seem to have an effect on the thermodynamic parameters in our studies, 100 nm liposomes were used for the rest of the experiments because they appeared to be less polydisperse compared to the liposomes greater in size.

3.6. Effect of temperature

ITC experiments were also carried out at 37 °C. At the higher temperature, the enthalpies of binding were greater in magnitude compared with those at 25 °C. The effect of the temperature on the binding of alprenolol is shown in Fig. 5 and the thermodynamic parameters for the binding of all drugs at 37 °C in Table 5. The increase of the temperature results in more exothermic and less entropic interaction between the drugs and the liposomes. Also, molar heat capacities can be determined from the temperature dependence of the thermodynamic parameters. For the drugs studied, ∆C_p values range from −1226 J mol⁻¹ K⁻¹ to −159 J mol⁻¹ K⁻¹ (Table 5). In general, ∆C_p can be interpreted as a result of the changes in the degree of the surface hydration of the interacting species [27], and in the case of protein binding, the largest contribution is often attributed to the burial of the hydrophobic areas [28,29]. As for the drug interactions, large negative ∆C_p values have been observed for the interaction of the drugs tacrine and amiloride with the glycosaminoglycan dextran sulphate [30]. In the study of Santos et al. [30], the results were explained by strong hydrophobic interactions and conformational changes upon binding. For the drug–liposome interactions, ∆C_p values reported in the literature are somewhat smaller in magnitude and opposite in sign compared to the values determined in this study. For the calcium flux inhibitor verapamil, ∆C_p values of 50 J mol⁻¹ K⁻¹ and 59 J mol⁻¹ K⁻¹ were measured depending on the liposome composition [15].

3.7. Effect of the ionic strength

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. For this purpose, the binding isotherms were measured in 20 mM Hepes buffer containing 150 mM NaCl. The effect of the ionic strength on the binding of alprenolol is shown in Fig. 6. From Fig. 6 it can be seen that whereas the heats produced in the interaction are of the same magnitude as those at the lower ionic strength, the effect of the ionic strength on the shape of the curve is remarkable. The lower gradient of the curve at the higher ionic strength implies weaker interaction, and fitting the one site model to the data reveals that the binding con-
constant \( K_0 \) is significantly lower at the higher ionic strength. The effect of the ionic strength was similar for the other drugs. The thermodynamic parameters for all the drugs are summarised in Table 5. The enthalpies of interaction are somewhat more negative and the entropies are smaller at higher ionic strength for all the drugs. Thus the interactions are even more enthalpy driven.

Lower values of \( K_0 \) at the 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 analysed by means of statistical thermodynamics. The grand canonical partition function for competitive binding is

\[
q = 1 + K_1c_1 + K_2c_2
\]

In Eq. (8), \( K_1 \) 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 (\( \theta_1 \)) in terms of the two binding constants gives

\[
\theta_1 = \frac{\partial \ln q}{\partial \ln c_1} \frac{K_1c_1}{1 + K_1c_1 + K_2c_2}
\]

(9)

Fig. 7 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\(^{-1}\) [31]. From Fig. 7, 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

\[
\text{Effect of salt concentration on the binding constant. Concentrations of NaCl 1M ( ), 0.1 M ( ) and 0.01 M (solid line).}
\]

4. Conclusions

In this study, ITC combined with zeta potential measurements was used to study the binding and partitioning of three \( \beta \)-blockers, alprenolol, labetalol and propranolol, and the local anaesthetic tetracaine into liposomes. The interaction of these drugs with liposomes was found to be enthalpy driven, 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. These findings add to the knowledge on the interactions of drugs and biomembranes and they may prove to be useful not only when studying the transport of these drugs in the body, but also when designing new drugs.

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References