

CHAPTER 7

EPILOGUE

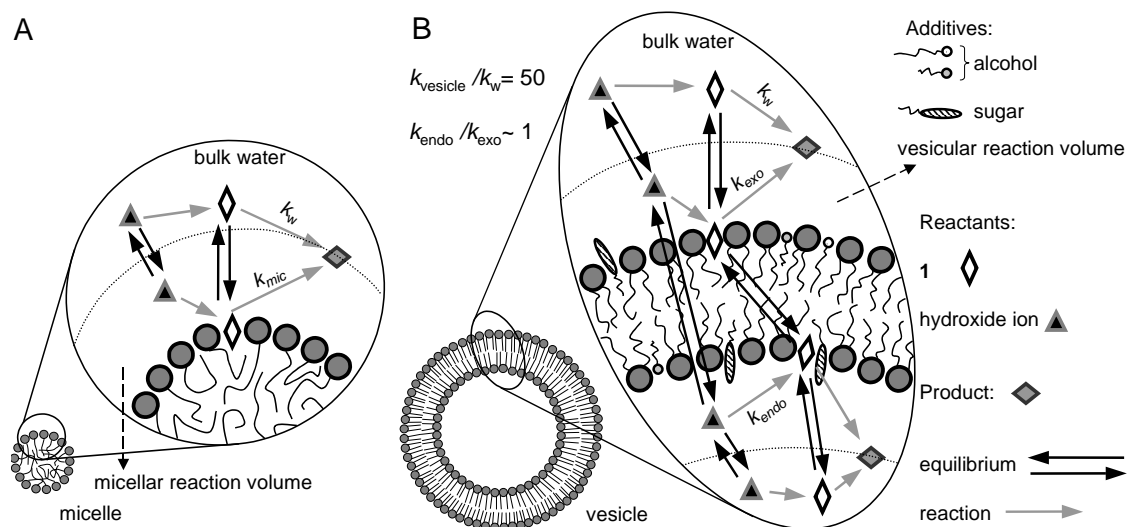
7.1 Introduction

This final chapter briefly discusses and evaluates the results obtained from the experimental work described in this thesis.^{1,2} These findings will be compared to the goal set out at the end of Chapter 1. On the basis of this discussion some suggestions for future work will be given.

7.2 Aims and Achievements

In Chapter 1 the properties of vesicles and biological membranes are presented. In addition, the use of vesicles as mimics for biological membranes is discussed, since their general properties are remarkably similar. They both enclose an aqueous compartment with a bilayer of amphiphile molecules. Among the most important parameters of the membranes are the phase of the tails and the permeability towards water, ions, and organic molecules. The cellular membrane consists of three main components, namely (glyco)lipids, steroids and proteins. In all three components there is a large variety in structures, which originates from a broad spectrum of functions (proteins) or from different conditions under which the membrane needs to retain structural integrity (lipids, steroids). Due to its complex composition there is only little understanding of the interactions in biological membranes on a molecular scale. On the contrary, studies of vesicles formed from synthetic amphiphiles are usually much less complicated because the chemical structure of the amphiphiles as well as the membrane composition (*i.e.* usually only one or two components) are much less complex.

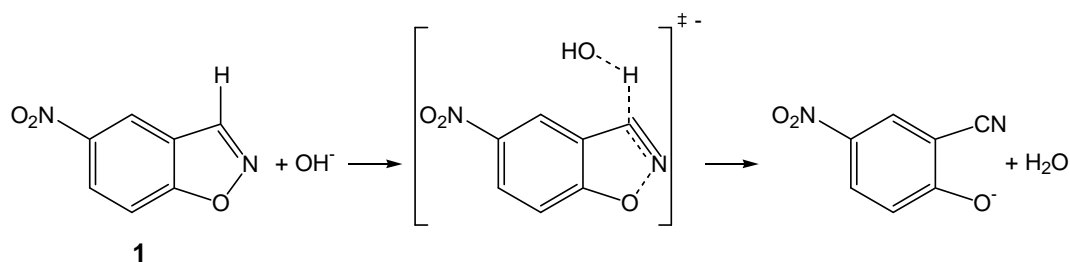
As a result of the above considerations, vesicular catalysis has mainly been studied using vesicles composed of structurally simple amphiphiles and the amphiphile is usually the only component. These notions lead to the main aim of the present thesis. We anticipate that by studying the effects of various additives on vesicular catalysis in cationic vesicles can lead to a better understanding of non-enzymatic catalysis taking place at the polar-apolar interface (Stern region) of biological membranes. The basic idea was that the use of four different classes of structurally simple additives would unravel some of the interactions that might be important in biological membranes. This would avoid the problem that in biological membranes phospholipids can undergo multiple interactions with their environment due to the large number of functional groups in these types of molecules. As a consequence, the use of structurally simple additives and amphiphiles would allow discussion of molecular interactions between additive and amphiphile. The additives are expected to change properties not only in the hydrophobic part of the membrane, but also in the Stern region. Properties that can be affected include the local polarity, counterion binding, head group hydration, local water concentration, and the phase of the tails.



Scheme 7.1. Schematic representation of micellar (A) and vesicular (B) catalysis. For details see text.

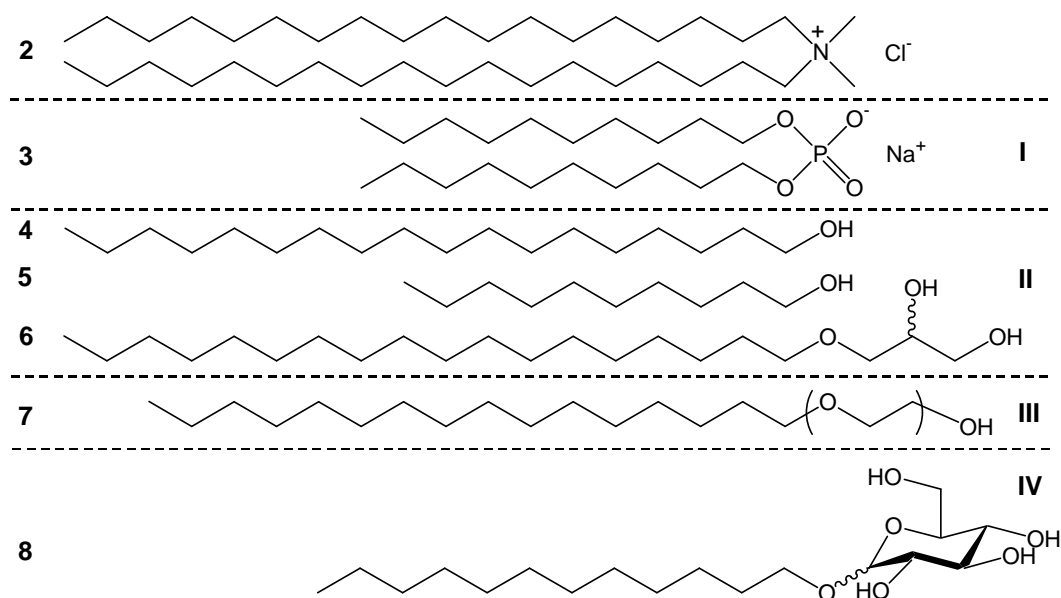
Before discussing to what extent this goal has been achieved, it is important to consider a number of parameters that are important in vesicular catalysis. A brief comparison with micellar catalysis is made, since micellar catalysis has been widely studied in the literature for a long time.³ In general, micelles and vesicles catalyse bimolecular reactions by bringing the two reactants together if they both bind to the micelle or vesicle. In this way, the two reactants are in close vicinity of each other, and hence their local concentration is high. In addition, due to a change in polarity between the aqueous and micellar or vesicular pseudophase, the micellar or vesicular rate constant might be different from the aqueous rate constant. In micellar catalysis only distribution of the reactants between the aqueous and the micellar pseudophase has to be considered (Scheme 7.1A). The observed rate constant is then a distribution-average of the micellar (k_{mic}) and aqueous (k_w) rate constant. Vesicular catalysis is considerably more complex (Scheme 7.1B), since also the distribution within the pseudophases has to be considered. This results from the fact that vesicles have an inner and outer leaflet, where the reaction proceeds with an endovesicular (k_{endo}) and exovesicular (k_{exo}) rate constant, respectively. In addition, the aqueous reaction that proceeds in the inner aqueous compartment might be different from the aqueous reaction in the bulk aqueous pseudophase. Little is known about the difference between the endo- and exovesicular rate constant, but they appear not to be so different.⁴ In addition, permeation of reactants has to be considered as well, depending on the solution preparation method (vesicles plus reactants) and the endovesicular rate constant relative to the rate constant of permeation. The rate of permeation is affected by the phase of the tails. However, the phase of the tails also affects the rate constant.^{5,6}

Based on the above-mentioned considerations, the deprotonation of 5-nitrobenzisoxazole (**1**; Kemp elimination; Scheme 7.2) was selected to be studied in the presence of vesicles formed from a cationic amphiphile and in the presence of various additives. The choice of this reaction was made on the basis of the different interactions that both reactants have with vesicles. Whereas binding of the hydroxide ion is purely electrostatic, binding of **1** is largely driven by hydrophobic interactions. In addition, the rate constant is sensitive towards the local polarity. Permeation of both reactants through the bilayer is fast on the time scale of the reaction, which simplifies the kinetic analysis.



Scheme 7.2. Kemp elimination reaction.

In the presence of cationic vesicles the *observed vesicular catalysis* ($k_{\text{obs,max}}/k_{\text{obs,w}}$) of the base-catalysed reaction of **1** amounts to a factor of *ca.* 1000. The data was analysed using a modification of the pseudophase model with ion exchange as derived by Romsted.⁷ Parameters that can be obtained using this model are the vesicular rate constant (k_{ves}), the binding constant of **1** to the vesicles (K_{S}), the counterion binding (β) and the ion-exchange competition constant. This latter constant describes the competition of hydroxide and chloride ions to bind to the vesicular surface ($K_{\text{OH}^{\text{Cl}}}$). Application of this model to the catalysis in the presence of cationic vesicles formed from dimethyldi-*n*-octadecylammonium chloride (**2**; Scheme 7.3) shows that the *catalytic rate acceleration* as calculated from the bimolecular rate constants ($k_{\text{ves}}/k_{\text{w}}$) is *ca.* 50.



Scheme 7.3. Cationic amphiphile employed in the experiments (**2**) and some examples of the four classes (**I-IV**) of additives. Class **I** are anionic double-tailed amphiphiles, class **II** alcohols, class **III** ethylene glycol surfactants and class **IV** *n*-alkyl pyranosides. Dimethyldi-*n*-octadecylammonium chloride (**2**); sodium di-*n*-decylphosphate (**3**); *n*-octadecanol (**4**); *n*-decanol (**5**) batyl alcohol (**6**), eicosa-ethylene glycol mono *n*-hexadecyl ether (**7**) and *n*-dodecyl- β -glucoside (**8**).

A detailed error analysis in Chapter 3 reveals (1) that varying all four, or even only three, parameters at the same time leads to meaningless numbers⁸ and (2) that all parameters can compensate each other to a large extent, leading to large errors in the obtained values. This latter observation can be exemplified by considering that for a certain amphiphile concentration an increase in the observed rate constant can be accounted for by either increasing the vesicular rate constant, increasing the binding constant, increasing the counterion binding, or by decreasing the ion exchange constant. Especially, as is clear from the mathematical equations, K_S and k_{ves} are well able to compensate each other when K_S is smaller than 30 M^{-1} . Therefore, fitting the experimental data requires knowledge of at least one or two of the parameters in order to obtain reasonable fitted parameters. Attempts were made to measure each of these parameters independently. Despite some experimental limitations, the ion exchange constant could be estimated by measuring the observed rate constant as a function of externally added sodium chloride. The composition of the vesicles did not have an influence on the value of this parameter and therefore this parameter was kept constant throughout the kinetic analysis. Unfortunately, attempts to elucidate the influence of the additives on the other three parameters failed. These attempts included the use of isothermal titration microcalorimetry to measure the binding constant of counterions and UV/vis spectroscopy to measure the binding constant of the kinetic probe to the vesicles.⁹ Therefore, the experimental kinetic data was fitted allowing at most two of the remaining three parameters to vary, and using, if possible, educated guesses (from the literature) for the other parameter(s).

In Chapter 3, the addition of small amounts (< 50 mol%) of the first class of additives, anionic double-tailed amphiphiles (Scheme 7.3), is described. As anticipated, upon increasing amounts of additives the catalysis of the Kemp elimination is decreased. Above 50 mol% the reaction is inhibited. The anionic amphiphiles are sodium di-*n*-decylphosphate and sodium *n*-decyl-*n*-octadecylphosphate.

By using five different dyes, that have proven to report the polarity at the surface area of aggregates, pyrene, Nile Red, 1,8-ANS, Laurdan and the $E_T(30)$ dye, changes in the membrane polarity were examined. All dyes indicated that the polarity of the bilayer is comparable to the polarity of methanol, and no significant change in the polarity was observed upon increasing amounts of anionic amphiphile. A change in the local polarity of the membrane would affect both the vesicular rate constant and the binding constant of the kinetic probe. Upon going from the initial state of the two reactants to the activated complex the localised charge of the hydroxide ion is being delocalised in the activated complex. Due to the charge delocalisation a change in polarity leads to a change in Gibbs energy to different extents for the initial state and the transition state. The vesicular rate constant is a function of the difference in Gibbs energy between the initial state and the activated complex. Hence a change in polarity affects the vesicular rate constant. The binding constant of the kinetic probe depends on the difference in Gibbs energy of the kinetic probe (and not the hydroxide ion) between the initial state in the membrane and the aqueous phase. Since the Gibbs energy in the aqueous pseudophase does not depend on the membrane polarity, any change in the membrane polarity will lead to a change in the binding constant. In the fitting procedure of the kinetic data the vesicular rate constant and the binding constant were assumed to remain constant, since no change in membrane polarity was sensed. Therefore, the decrease in catalysis must be explained by a decrease in counterion binding. This can be explained as the result of two effects. The first effect is a

replacement of the hydrophilic counterions (including reactive hydroxide ions) from the Stern region, since the anionic amphiphiles themselves serve as excellent counterions, binding with high efficiency. The second effect comes from a dilution of the remaining cationic charges¹⁰ leading to a lower surface charge density, and hence an even further lowered counterion binding. A closer look at the charge density as a function of the bilayer composition reveals that the overall surface charge density remains constant up to 35 mol% of anionic amphiphile. In Chapter 2 the zeta (ζ) potential of these mixed vesicles is discussed and the magnitude and sign of the potential is in accord with the previous discussion.

In the previous discussion two observations, described in Chapter 2, that complicate the kinetic analysis were not taken into account. Cryo-EM pictures reveal that the shape of the vesicles changes as a function of bilayer composition. Whereas in the absence of anionic amphiphile lens-shaped vesicles are formed, spherical vesicles are observed at 40 mol% of anionic amphiphile. As a result, the packing of the amphiphile might be different, which can lead to changes in the vesicular rate constant and the binding constant. However, the observation of a constant polarity indicates that either the dyes are not sensitive towards these changes, or that the change in shape has no effect on the polarity. The second observation concerns the number of peaks in the DSC scans between 5 and 50 mol% of anionic amphiphile. The presence of more than one peak can either indicate the presence of more than one type of transition, or the existence of domains of different composition. In the former case, the first peak is usually small in enthalpy and can be assigned to a transition to the rippled phase. However, since in our experiments the first peak is usually large we anticipate that domain formation occurs, with domains rich in cationic amphiphile and other domains mainly consisting of 1:1 cationic and anionic amphiphiles. This conclusion was derived on the basis of the temperature of the transitions. The presence of domains leads to a significant complication of the pseudophase model and cannot be accounted for if these domains are large. Under those circumstances in the neutral domains inhibition would occur, whereas in the cationic domains there would be catalysis similar as that for cationic vesicles. In such a situation the distribution of hydroxide ions throughout the Stern region would be inhomogeneous. However, if the neutral domains are small, the distribution of hydroxide ions is expected to be homogenous, although it can be argued that due to the lack of electrostatic attraction the hydroxide ions are not close enough to the vesicular surface in the domains to react with membrane-bound **1**.

In Chapter 4 the effects of the second class of additives, which comprises four long linear alcohols containing 10 to 18 carbon atoms, is discussed. The alcohols are *n*-octadecanol (**4**), *n*-decanol (**5**), batyl alcohol (**6**) and oleyl alcohol. These alcohols were chosen on the basis of their match and mismatch in size with the *n*-octadecyl tails of the amphiphile, the number of hydroxyl groups and the double bond in the tail, respectively. In the plots of the observed rate constant versus the amphiphile concentration the maximum observed rate constant decreases by at most a factor of *ca.* 2. This relatively small change complicates a detailed discussion of the origin of these effects.

Similarly as for the addition of anionic amphiphiles, addition of the alcohols should lead to a decrease in counterion binding. However, since alcohols are nonionic, they cannot act as counterions and hence they can only "dilute" the charges of the cationic amphiphile. Therefore, their effect on the counterion binding is expected to be significantly smaller. The DSC scans show several peaks indicating a variety of domains (transitions), but no

information has been obtained on the nature on these domains. On the basis of the polarity experiments no change in vesicular rate constant and binding constant is expected. However, only allowing the counterion binding to change could not satisfactorily fit the data. In addition, it led to significantly different results for the alcohols, which seems a bit unexpected considering the similar head group area for the different alcohols. Strikingly, addition of oleyl alcohol even leads to an *increase* of the observed rate constant, which cannot be explained by changing the counterion binding. Therefore, the counterion binding is assumed to be smaller assuming that four alcohol molecules take up the space of one cationic-anionic amphiphile pair. Then the vesicular rate constant and binding constant were allowed to vary in order to fit the experimental data. Although there are no indications that the membrane polarity changes, we anticipate that the changes in membrane polarity are too small to be detected using the fluorescent dyes, but large enough to be detected kinetically. In general, the effects on the vesicular rate constant and the binding constant are less than a factor of *ca.* 4, whereby the binding constant decreases and the vesicular rate constant increases. The effects on the catalysis are least pronounced in the case of *n*-decanol, and most pronounced in the case of batyl alcohol.

Chapter 5 describes the influence of the addition of the third class of additives, ethylene glycol surfactants. This class contains two surfactants, namely eicosa-ethylene glycol mono *n*-hexadecyl ether (**7**) and a PEG-ylated SAINT-2 amphiphile. This latter amphiphile has been used as steric stabiliser in transfection experiments. Their structure consists of a pyridinium ion with two oleyl tails in the position *para* to the nitrogen and a PEG-5000 unit attached to the nitrogen. The influence of these surfactants on the catalysis of the Kemp elimination reaction is surprisingly small. Above 35 mol% of **7** care has to be taken since single-tailed surfactants can solubilise vesicles into mixed micelles. In fact, such behaviour is observed. Hence, the kinetic model is complicated similar as for domain formation. Despite that the DSC scans and light scattering experiments, described in Chapter 2, indicate that a vesicular solution containing 35 mol% of **7** contains mainly mixed micelles, the observed rate constants are still about 60% of the values in the absence of **7**. This agrees with experiments where the observed rate constants measured in the presence of vesicles formed from **2** were compared with micelles formed from *n*-octadecyltrimethylammonium chloride. In these experiments the maximum observed rate constant in the presence of micelles is approximately half the value measured in the presence of vesicles.

In Chapter 6 it is reported that the maximum observed rate constant increases up to a factor of 7 upon the addition of 50 mol% of *n*-alkyl pyranosides, the fourth class of additives. Like the third class, this series contains two compounds, *n*-dodecyl- β -glucoside (**8**) and *n*-dodecyl- β -maltoside. The head group of the latter compound consists of two glucose units. Due to its high Krafft temperature, **8** is barely soluble in water, but *n*-dodecyl- β -maltoside can be easily solubilised. As a result, the single-tailed surfactant *n*-dodecyl- β -maltoside is able to solubilise vesicles into micelles, as is described in Chapter 2. When present above 25 mol% in vesicles (or micelles) formed from **2**, addition of sodium hydroxide leads to immediate precipitation. The material can be redissolved upon shaking. At 25 mol%, in the absence of sodium hydroxide, the vesicles aggregate loosely. Dynamic light scattering experiments indicate the presence of vesicles, but micelle formation cannot be excluded. In fact, the observed rate constants are considerably higher in solutions

containing **8**, which is unable to solubilise vesicles. This agrees with the observation made in Chapter 5 that vesicles have a higher efficiency than micelles to catalyse the reaction. Analysis of the kinetic data reveals that for both pyranosides the binding constant increases, but to different extents. The vesicular rate constant also increases, but it decreases again for *n*-dodecyl- β -maltoside when micelle formation becomes significant. The origin of the increased catalysis is difficult to determine. Based on the membrane polarity experiments no significant change is expected, although the use of Nile Red seems to indicate that the polarity decreases slightly upon increasing amounts of incorporated pyranosides. The change in polarity is comparable to a change in $E_T(30)$ value from 56 to 54.¹¹ A decrease in polarity leads to an increase in the vesicular rate constant and binding constant. The decrease could be a result of water being replaced from the Stern region by the sugar moieties as has been reported in the literature.¹² A decrease in the water concentration in the Stern region would also lead to a decrease in the hydration of the hydroxide ion, explaining the increase in the vesicular rate constant. In addition, **1** would be dehydrated as well. The effect of dehydration of **1** on the vesicular rate constant is not so large as for dehydration of the hydroxide ion, but it does stabilise **1** leading to an increase in the binding constant. The importance of the hydration of the reactants was already shown in Chapter 3 where a large increase in rate constant was observed in mixtures of water and an organic solvent. A similar change in polarity in these solvent mixtures, compared to the change in polarity as sensed by Nile Red, leads to an increase of the rate constant by a factor of two. However, a five-fold increase in vesicular rate constant is observed upon the addition of pyranosides. This probably reflects the inability of water/organic solvent mixtures to correctly mimic the Stern region rather than that kinetic medium effects have been overlooked.

Experiments to study specific or preferential binding of hydroxide ions to pyranoside-containing vesicles show that these processes do not occur, since the ion-exchange constant is not affected, and no catalysis is observed in phospholipid vesicles containing 25 mol% of **8**. This indicates that if dehydration of the hydroxide ion were the origin of the increase in vesicular rate constant, the chloride ion is also dehydrated to a similar extent, since otherwise the ion exchange constant would have decreased.

Deprotonation of the sugar hydroxyl groups can account for the changes in observed rate constants as well. However, on the basis of literature data it appears unlikely.

Throughout the kinetic analysis we assumed the counterion binding to be constant, since we have no accurate knowledge of (the extent of the change in) the counterion binding. However, in the literature¹² it has been suggested that the counterion binding decreases upon pyranoside addition,¹³ but even at very low water concentrations it is still about 30%. If changes in the counterion binding had been taken into account the fitted vesicular rate constants would even be higher, which is consistent with dehydration (and decrease in polarity).

In summary, the experiments, which are described in this thesis, reveal that even in relatively simple bilayer mimics of biological membranes, interactions can be highly complex. This is especially true for sugar-containing surfactants.

7.3 Some Comments on Aspects of Vesicular Catalysis

Few studies have dealt with vesicular catalysis involving additives that were incorporated into the vesicular membrane.^{6,14-16} As discussed in Section 7.2, the two most important differences between micellar and vesicular catalysis are (1) the possibility that the tails can be in two phases and (2) the presence of a bilayer leading to an inner and outer leaflet. As will be discussed, additives affect these properties, and hence they affect rate constants as well.

Different observed rate constants between the inner and outer leaflet can be obtained, due to a potential non-random substrate distribution between the leaflets. For example, Ellman's reagent, which is dianionic, is expected to permeate only slowly or not at all through membranes formed from cationic amphiphiles, and hence depending on the method of vesicle preparation, the distribution of the reactants can be random or non-random.¹⁷ Attention has been focussed on this leaflet discrimination in vesicular catalysis including catalysis by cholesterol-containing vesicles,^{14,15} since cholesterol decreases the permeability of membranes. Unfortunately, similar experiments in the absence of cholesterol have been subject of misinterpretation¹⁸ and poor reproducibility,^{19,20} which largely clouds the influence of the cholesterol. Therefore, the details of the redistribution of charged organic substrates over the leaflets remains unclear. As a result, there is only one report that claims to have measured the endo- and exovesicular rate constant independently.⁴ In addition, most experiments have not been performed as a function of the amphiphile concentration, which leads to the inability to calculate the vesicular rate constant. Therefore, rough trends rather than, more valuable, detailed information was obtained.

On the contrary, the reaction of **1** with hydroxide ions has been studied as a function of the amphiphile concentration in the presence and absence of cholesterol.¹⁶ The distribution of the nonionic **1** was expected to be random irrespective of the method of solution preparation, age of the solution, and the presence of cholesterol. The rate of permeation of hydroxide ions through the bilayer was assumed to be faster than the vesicular rate constant. At 25 °C in vesicles formed from dimethyldi-*n*-octadecylammonium chloride or bromide the presence of up to 14 mol% of cholesterol leads to an increase in the vesicular rate constant. Above 14 mol% of cholesterol the observed rate constants decrease again as a result of a decreased counterion binding due to a lowering of the surface charge density. The initial increase was explained in terms of a change in the phase of the tails from gel-like to liquid-crystalline. This explanation is supported by the observation that (vesicular or observed) rate constants are not affected upon addition of 12 mol% of cholesterol to vesicles formed from di-*n*-dodecyldimethylammonium bromide, which are already in the liquid-crystalline phase.

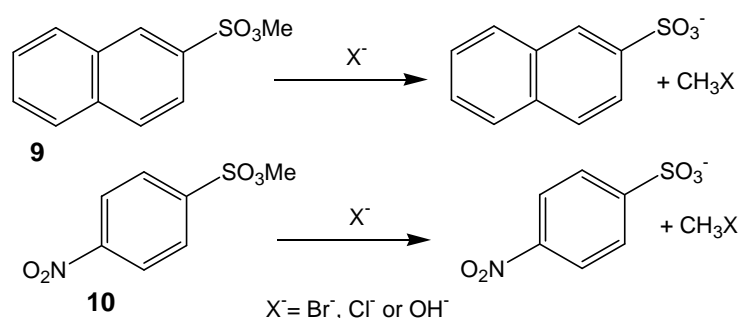
The importance of the phase of the tails on the vesicular rate constant was also observed in earlier studies of vesicular catalysis.^{5,6} An attempt was made to unravel the underlying thermodynamic activation parameters ($\Delta^\ddagger H^\ominus$ and $\Delta^\ddagger S^\ominus$), but instead of measuring the *vesicular* rate constant as a function of the temperature, *observed* rate constants were measured. This does not take into account that other parameters, such as the counterion binding, the binding constant of the substrate(s) and the aqueous rate constant, are also affected by a change in temperature. It should be noted that the enthalpy and entropy of

activation are usually difficult to interpret, since they depend on many parameters, such as restructuring of water (hydration and/or dehydration) and a local reorganisation of the aggregate. However, it might still reveal some valuable information if experiments are performed in a series of related vesicular systems.

7.4 Suggestions for Future Work

The work presented in this thesis leaves plenty of room for future work. In fact, more questions have been raised than have been solved. Especially the origin of the rate acceleration in the presence of *n*-alkyl pyranosides is intriguing. However, parameter compensation in the mathematical model leads to problems in interpreting the experimental data. Therefore, it would be wise to solve those problems first. This can be achieved by studying a system where independent determination of at least some of the parameters is possible. Using isothermal titration microcalorimetry, the binding constant of chloride and bromide ions to mixed vesicles of POPC and **2** could be measured. However, measuring the binding constant of hydroxide ions failed as a result from small heat effects that, in addition, depended on the age of the vesicular solution.²¹ Therefore, choosing a probe reaction that involves reaction with bromide or chloride ions solves two problems at once. Firstly, one can measure the binding constant of these ions and hence, the concentration of bound counterions can be calculated. Secondly, the number of unknown parameters decreases as the ion exchange constant and the counterion binding can be replaced using the binding constant of bromide ions. As a result, the model used to analyse the experimental data in this thesis simplifies since it only contains three parameters of which two remain unknown. The use of an additional reaction between a substrate that does not bind to vesicles and bromide ions could be used to confirm the concentration of bromide ions in the aqueous pseudophase.

In addition, it would be particularly helpful if a kinetic probe would be chosen, such that its binding constant is larger than 30 M^{-1} , since then compensation of the binding constant and the vesicular rate constant is much less important. Choosing a probe reaction that has been well-studied in the literature, already gives some clues of binding constants and the vesicular rate constant. The $\text{S}_{\text{N}}2$ reaction of **9** and **10** with bromide ions meets all of these criteria (Scheme 7.4).²²⁻²⁵



Scheme 7.4. $\text{S}_{\text{N}}2$ reaction of methyl naphthalene-2-sulfonate (**9**) and methyl 4-nitrobenzenesulfonate (**10**) with a nucleophile.

The binding constants of **9** and **10** to CTAB micelles are around 1500 and 75 M⁻¹, respectively. This difference in binding ability can be used to determine whether differences upon changing the system under study involve an effect of a change in vesicular rate constant or binding constant, since upon increasing the amphiphile concentration the amount of bound **9** will remain about constant, whereas the mole fraction of bound **10** will increase. A fully bound probe allows an easier determination of the vesicular rate constant, since the reaction in the aqueous phase can be neglected. Similarly as for the reaction of **1** with hydroxide ions, this reaction is sensitive towards changes in polarity and dehydration of the reactants.^{23,24}

In order to study the dehydration of ions by sugar moieties, a series of compounds with increasing number of hydroxyl groups could be studied. For example, *n*-dodecanol, 3-*n*-dodecyloxy-propane-1,2-diol, *n*-dodecyl- β -glucoside (**8**) and *n*-dodecyl- β -maltoside. To avoid problems with (slow additive concentration-dependent) permeation of bromide ions through the membrane, it might be wise to add 10 mol% of C₁₆EO₂₀ (**7**), so that under all circumstances permeation of all reactants is fast. In addition, problems with aggregation of the mixed vesicles under basic condition can be avoided.²⁶ This is important when this vesicular system is extended to the reaction of **1** at a later stage in the future.

Other factors that are worth to investigate are the size-dependence of the vesicular rate constant and binding constant of substrate and counterion, especially if various reactions are considered. In addition, studying the rate constants as a function of the temperature for a number of reactions in a number of comparable systems (described above) might reveal some additional valuable information.

7.5 References

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- (8) This is exemplified by a counterion binding larger than unity, or an ion exchange constant close to unity.
- (9) These experiments have not been described in this thesis.
- (10) The combination of an anionic and cationic amphiphile can be considered as a neutral pair, since both charges are compensated. The lifetime of such a pair is not important, since each anionic amphiphile can always form a pair with a cationic amphiphile in its vicinity, leading to the constant presence of a catanionic amphiphile pair.
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 - (17) A random distribution of Ellman's reagent over the leaflet is achieved by cosonication of amphiphile and substrate, whereas a non-random distribution is obtained by addition of substrate after sonication of the solution containing amphiphile (substrate bound to the outer leaflet), or filtration of a cosonicated solution over a column (substrate bound to inner leaflet).
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