Polymer-surfactant interactions
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Interactions between bilayer vesicles and hydrophobically-modified polymers

Isothermal titration microcalorimetry and differential scanning calorimetry have been used to investigate the anchoring of hydrophobic side chains of poly(sodium acrylate-co-n-alkyl methacrylate)s and poly(acrylamide-co-n-alkyl methacrylate)s into vesicles formed from double-tailed surfactants with phosphate headgroups. Only those hydrophobes that do not significantly contribute to the stability of hydrophobic microdomains, or are not involved in the formation of these domains, anchor into bilayer vesicles. The bilayer is least disturbed when the length of the hydrophobic side-chain matches that of the alkyl chains forming the tails of the vesicle-forming surfactant.

3.1 Anchoring of polymers into membranes: a literature survey

Bilayer vesicles composed of mixtures of naturally occurring phospholipids and (< 5 mole% of) lipid molecules with covalently-linked poly(ethylene glycol) are receiving considerable attention, both experimentally and theoretically. The main reason for this strong interest lies in the enhanced (in vivo) circulation time of these stERICALLY-stabilized liposomes, which are ‘invisible’ to the human defence system. Such ‘Stealth’ liposomes are therefore of medical interest, as vehicles for drug targeting and transfection. Although studied in much less detail, polymers can also be anchored into membranes through their hydrophobic side chains. Indeed, small amounts of a hydrophobically-modified poly(acrylamide) containing 1.5% of n-dodecyl side chains (LMAM[1.5]) are sufficient to protect negatively charged, small unilamellar vesicles against Ca\(^{2+}\)-induced vesicle fusion (Figure 3.1).

The mechanism underlying this particular inhibition of vesicle fusion is strongly related to steric stabilization of lamellar dispersions by polymers with hydrophobic side chains. The anchoring of polymers into lamellar droplets is of considerable importance for the formulation of colloidally and physically stable concentrated liquid laundry detergents (Chapters 1, 4, 5). The aim of this study is to understand on a molecular basis the factors that influence these hydrophobic anchoring processes. Some fundamental questions will be raised: (i) can a hydrophobically-modified polymer anchor into a vesicle bilayer having the same charge?; (ii) is there an optimum alkyl chain length for anchoring efficiency, or should the anchoring side chains be as hydrophobic as possible?; (iii) why does anchoring occur?

We have studied using titration and differential scanning calorimetry the anchoring of hydrophobically-modified poly(acrylamide)s and poly(sodium acrylate)s into vesicles composed of sodium di-n-dodecyl phosphate (DDP) or its analog having a ‘cyclic’ headgroup (CDP); Scheme 3.1.
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**Scheme 3.1** Structures of the compounds that have been used. Vesicles composed of CDP and DDP were employed in the calorimetric studies. Vesicles from DDPBNS have been studied in fusion and aggregation assays, see Figure 3.1. The polymers PSA-CX[Y] and LMAM[Z] have been introduced in Chapter 2.

3.1.1 Anchoring of lipopolymers: a simple theoretical approach. Lipowsky\(^7\) presented a simple rationale for the anchoring of double-tailed lipids with covalently-attached PEO into bilayer vesicles. The polymer coverage is taken such that the mean separation of the anchored polymers is large compared to their linear size. In other words, the polymers are adsorbed as well-separated ‘mushrooms’.\(^8\) Another assumption is that the Gibbs energy of adsorption consists of two terms: (i) the Gibbs energy gained by
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inserting the anchor into the lipid membrane, and (ii) the entropy loss by constraining the polymer

Figure 3.1  (A) Rate of aggregation of DDPBNS vesicles as a function of [Ca$^{2+}$], as determined by a turbidity assay (see Scheme 1 for nomenclature). Aggregation is fully blocked in the presence of LMAM[1.5]. Poly(acrylamide) has no influence. (B) Final extent of fusion of DDPBNS vesicles as a function of [Ca$^{2+}$], as determined by a fluorescence self-quenching assay. Fusion is not affected by LMAM[0], but is fully blocked by LMAM[1.5]. [polymer] = 2.10$^{-3}$ wt.%; [DDPBNS] = 25 µM; T = 25 °C. Bottom: (i) diagrammatic representation of vesicle aggregation and fusion, and (ii) the absence thereof by anchoring polymers, which hamper close approach of the vesicles by steric stabilization. Adapted from ref. [6].
backbone by the membrane surface. We note that contributions of (de)hydration of polymers and lipids to the overall Gibbs energy are neglected. Thus, a self-avoiding chain anchored at a flat surface suffers an entropy loss:

\[
-T\Delta_{\text{confine}}S^0 = 1.2 \ln(\text{DP}) \quad \text{kJ mol}^{-1}
\]

where DP is the degree of polymerization of the hydrophilic backbone (expressed as the number of monomeric units). For a typical case, DP = 100, and the Gibbs energy of such a confined chain is \(\Delta_{\text{confine}}G^0 = 5.7 \text{ kJ mol}^{-1}\). On the other hand, as calculated from the critical vesicle concentration of phospholipids, the Gibbs energy of transfer of lipid chains from water into a bilayer is quite favorable, amounting to:

\[
\Delta_{\text{tr}(w/b)}G^0 = -4.2 n_C \quad \text{kJ mol}^{-1}
\]

where \((w/b)\) denotes the transfer of molecules from water to a bilayer; \(n_C\) is the number of carbon atoms per alkyl chain. For example, for dipalmitoyl phosphatidylcholine, \(n_C = 16\), and \(\Delta_{\text{tr}(w/b)}G^0 = -67 \text{ kJ mol}^{-1}\). The overall change in Gibbs energy corresponding to the anchoring of a PEO-lipid from water into a vesicle bilayer is:

\[
\Delta_{\text{anchor}}G^0 = \Delta_{\text{confine}}G^0 + \Delta_{\text{tr}(w/b)}G^0.
\]

In this case, neglecting configurational enthalpy, \(\Delta_{\text{anchor}}G^0 = -61 \text{ kJ mol}^{-1}\). Therefore, the affinity of the PEO-lipid for the bilayer is large, and the concentration of ‘free’ polymer can be ignored (\(K_{\text{anchor}} = 9.10^9\)).

### 3.1.2 Anchoring of single-chained surfactant molecules into bilayer vesicles.

In a first approximation, the anchoring of hydrophobic side chains into bilayers is modelled by the incorporation of single-tailed surfactants into vesicles. In other words, we neglect the contributions of configurational entropy and enthalpy, and focus on \(\Delta_G^0\).

Dilute lipid-surfactant mixtures can be treated as pseudo-binary systems. The liquid crystalline-micellar phase transition, when the composition of the mixture is varied from water+100% lipid to water+100% single-tailed surfactant, is described by the Lichtenberg model.\(^9\) Accordingly, single-chained surfactant molecules are incorporated into lipid membranes up to an effective critical surfactant/lipid ratio producing saturation, \(X_{\text{sat}}\). Then, lipid-saturated micelles with composition \(X_M\) start to coexist with surfactant-saturated vesicles of composition \(X_{\text{sat}}\). Herein, \(X\) refers to the ratio of bound surfactant to the total lipid concentration. Typically, \(X_{\text{sat}} = 1\) and \(X_M = 3\).\(^10\) Intuitively, since \(X_{\text{sat}}\) is about unity, one might predict that saturation occurs at a 1:1 surfactant-to-lipid ratio. In reality, at mM concentrations, the amount of surfactant required to saturate the bilayer vesicles is three times the lipid concentration. Thus, the affinity of single-chained surfactants for lipid bilayers is quite low, the binding constant is of the order unity.\(^10\) The conclusion is that the Gibbs energy of transfer of single-tailed surfactants from micelles to lipid bilayers is about zero.

Heerklotz et al.\(^11\) performed a thorough study of the thermodynamics of these saturation and
solubilization processes using isothermal titration calorimetry. Although transfer of a single-chained surfactant to a lipid bilayer is highly exergonic, transfer of a single-chained surfactant from aqueous solution to a micelle is even more favorable (near $X_{sat}$). The opposite was true for the transfer of lipid molecules from aqueous solution to micelles or bilayers. It appears that

$$\Delta G^0_{(m,b), surfactant} = -\Delta G^0_{(m,b), lipid} = 15 \text{ kJ mol}^{-1}. \tag{4}$$

Herein, $(m,b)$ refers to the transfer of molecules from micelles to bilayers. The transfer of lipid molecules from micelles to bilayers, and the transfer of single-chained surfactant from bilayers to micelles occur spontaneously.

3.1.3 Anchoring of hydrophobic side chains of polymers into bilayer vesicles. From the foregoing, we conclude that the binding of a hydrophobic side chain of a polymer into a vesicle bilayer must not be expected a priori to be strong. If the thermodynamics of anchoring can be described by the transfer of an alkyl side chain from a hydrophobic microdomain to a vesicle bilayer, the binding constant is expected to be smaller than unity, analogously to the transfer of a single-tailed surfactant from a micelle to a bilayer.

However, it has been experimentally established that the anchoring of hydrophobically-modified poly(N-isopropylacrylamide)s (HM-PNIPAM) into phospholipid bilayers is associated with a negative standard Gibbs energy: the binding constant for mixture of HM-PNIPAM with dimyristoyl phosphatidylcholine liposomes at 300 K is 120 M$^{-1}$. Thus, the standard Gibbs energy of transfer of an n-octadecyl side-chain from a hydrophobic microdomain into a vesicle bilayer amounts to $-RT \ln (55.5K) = -22.0 \text{ kJ mol}^{-1}$: the affinity of the hydrophobic anchors for the bilayer is appreciable. A reasonable hypothesis involves the proposition that only those alkyl chains not involved in hydrophobic microdomains will be anchored into bilayer vesicles. In other words, the assumption is that anchoring of hydrophobes is thermodynamically equivalent to the (apparently favorable) transfer of alkyl side chains...
of the polymer from aqueous solution into a bilayer. We are not aware of literature critically exploring this idea, although we note one exception. Ringsdorf et al.\textsuperscript{14} found a decrease of non-radiative energy transfer (NRET) from naphthalene to pyrene labels attached to poly(N-isopropyl acrylamide)s with n-octadecyl acrylamide side chains when liposomes were added to aqueous solutions of the microdomain-forming polymers (Figure 3.2). The result of this particular experiment is best interpreted in terms of a break-down of most hydrophobic microdomains when liposomes are offered for anchoring. However, this phenomenon cannot be generalized to polymers not containing pyrene labels, because the affinity of pyrene for bilayer vesicles is much larger for bilayer vesicles than for micelles or hydrophobic microdomains. To be more specific, a partition constant $K_{py}$ is defined according to eq. 5:

$$K_{py (\text{pseudophase-water})} = \frac{\text{[py]_{ps phase}}}{\text{[py]_{aq}} \cdot [\text{surf}]}$$

where $\text{[py]_{ps phase}}$ and $\text{[py]_{aq}}$ refer to pyrene concentrations in the pseudophase (e.g., a microdomain, a micelle or a vesicle) and in the aqueous phase, respectively, expressed in moles per litre of solution; $[\text{surf}]$
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is the total concentration of surfactant forming the pseudophase. Using literature data, we calculate for the partitioning of pyrene from water into dimyristoyl phosphatidyl choline bilayer vesicles $K_{pwa}$, whereas for sodium tetradecyl sulfate micelles, $K_p = 5 \times 10^{-1}$ M. The affinity of pyrene for hydrophobic microdomains formed from alternating copolymers of disodium maleate and n-dodecyl vinyl ether is very similar to that for micelles, $K_p = 4 \times 10^{-1}$ M. Thus, solubilization of pyrene into bilayer vesicles is more favorable than in hydrophobic microdomains by a factor of $10^{-10}$. Hence, the large propensity of the fluorescent label for leaving the hydrophobic microdomains to enter bilayer membranes drives the break-up of the hydrophobic microdomains upon addition of vesicles.

We propose that only those hydrophobic side chains will anchor into bilayer vesicles which are not involved in, or least stabilize, hydrophobic microdomains. Hence, ‘dangling’ hydrophobes are not necessarily required for anchoring. It has been known for more than 30 years that the formation of hydrophobic microdomains is accompanied by a reduction of the number of available modes of chain configuration. The polymer backbone must adapt its conformation such that the hydrophobic side chains come together to form a micelle-like structure, however, upon anchoring, the backbone will be relaxed since a large number of chain configurations will allow the side chains to be anchored. Thus, an increase of the configurational entropy of the polymer is expected to contribute to the anchoring of its hydrophobic side chains into bilayer vesicles. Those side chains whose involvement in hydrophobic microdomains most severely restricts the configurational entropy of the polymer backbone bind most readily into bilayer vesicles.

3.2 The stability of vesicles in the presence of polymers

Vesicles formed from chemically pure surfactants are metastable structures for reasons related to spontaneous curvature; Chapter 1. When anchoring polymers are added, a bending moment is exerted onto the membrane due to the repulsive forces between the bilayer and the hydrophilic backbone. Thus, one could expect that addition of hydrophobically-modified polymers to dispersions of vesicles leads to an increase of the colloidal stability of the curved bilayers due to an induction of nonzero spontaneous curvature.

However, this is not the complete story. Polymer molecules are excluded from the inner water compartments of the vesicles. Hence, an osmotic pressure is exerted onto the vesicles. As a result, water flows from the inner compartment to the bulk solution, and the vesicles shrink. Vesicles may be even destroyed upon addition of polymers due to ‘osmotic shock’. The affinity of polymer for vesicles (bearing the same charge) may be low. Therefore, the fraction of polymer that is not anchored into bilayer vesicles may be appreciable, giving rise to depletion flocculation of the vesicles: free polymer molecules lose configurational entropy when they penetrate the (narrow) space between the interacting particle surfaces. As a result, the polymer is expelled from the space between the vesicles, and an osmotic flow of solvent from the interparticle space into the bulk solution drives the particles towards each other. The effect becomes more pronounced with increasing polymer concentration and/or molecular weight. Practical use of the mechanism of depletion flocculation is made in the induction of vesicle fusion by added poly(ethylene oxide).

Finally, some lipid material may become solubilized into polymeric hydrophobic microdomains. There are some indications that this problem may not be severe. Firstly, the problem is never mentioned
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in the (rather limited) literature on polysoap/vesicle interactions. Secondly, for efficient solubilization of
double-tailed surfactants in a micellar solution, the aggregation number of the micelle must be large. For
example, the affinity constant of micelles for egg lecithin phosphatidyl choline molecules is three times
larger for Triton X-100 (N = 100-150)\textsuperscript{26} as compared with SDS (N = 60-65)\textsuperscript{10a}. As a comparison, the
aggregation number of a relatively ‘hydrophobic’ copolymer poly(N-isopropyl acrylamide-co-N-octadecyl acrylamide), bearing 1.5% of octadecyl side chains, is only 14-17.\textsuperscript{27} A double-tailed amphiphile is unlikely to be solubilized in such small aggregates if the concentration of lipid molecules and polymer hydrophobes remains in the mM regime.

In view of these potential problems, we investigated prior to the calorimetric studies the physical
and colloidal stability of vesicle dispersions in the presence of PSA-CX\[Y\] and LMAM\[Z\], in
concentration ranges corresponding to experimental conditions. In the titration microcalorimetric
experiments, the concentration of double-tailed surfactant ranged between 0 and 2 mM; the concentration
of polymer in the cell was 160 unit mM. In case of DSC measurements, the maximum concentration of
polymer was 60 mM, and the concentration of vesicular DDP was 5 mM. Typical mixtures containing
vesicles and polymers were inspected visually, and were viewed using transmission electron microscopy.
The latter technique needs some introduction in order to understand the possible pitfalls.

3.2.1 Transmission electron microscopy of small vesicles. A relatively simple method for
observing vesicles in the electron microscope uses negative staining. A drop of the solution containing
vesicles is placed on a copper grid, and blotted off. A drop of solution containing a heavy metal salt is
added on top, and blotted off. After drying, the sample is inspected in the electron microscope.\textsuperscript{28} The heavy
metal stain surrounds the vesicles and provides an electron dense contrast agent for inspecting the lipid
dispersions. The advantage of negative stain is its simplicity and quickness in providing information on
the shapes and sizes of the lipid dispersions. Disadvantages include the fact that spherical liposomes are
flattened during desiccation. Moreover, the metal salts may introduce artifacts such as fusion of vesicles
(in analogy with Ca\textsuperscript{2+}-induced fusion of negatively charged vesicles).\textsuperscript{29}

Another important technique is cryo-transmission electron microscopy.\textsuperscript{30} A thin layer of a vesicle
dispersion on a copper grid is cooled to ca. -180 °C extremely rapidly in order to ‘freeze’ the molecular
motions in the bilayer. The specimen is observed directly, without staining agent. The observation that
the small water molecules are frozen into the glass state, without forming ice crystals, provides good
evidence that the much larger lipid molecules are really ‘frozen in’. The technique is quite difficult to
master, since the image contrast is low, and the ice layer is easily damaged by the electron beam.
Nevertheless, cryo-TEM does have the great advantage of direct observation of an undisturbed specimen,
and is the preferred technique to visualize lipid dispersions.\textsuperscript{31}

A third technique involves freeze-fracture electron microscopy.\textsuperscript{32} A drop of a lipid dispersion is
cryofixed ultrarapidly, and fractured. The fractured surface is shadowed using vapor of metallic platinum
and carbon. The resulting replica is imaged. This technique has been used to visualize concentrated
lamellar dispersions (Chapters 4 and 5), but we did not use it to investigate small unilamellar vesicles.

3.2.2 Stability of CDP vesicles as studied by TEM and visual inspection. A sonicated\textsuperscript{33}
dispersion of 5 mM CDP in water was checked for vesicles. The solution has a blueish appearance,
indicating the presence of small particles. Cryo-transmission electron micrographs unequivocally
demonstrate the presence of small unilamellar vesicles having an average diameter of 40 nm (Figure 3.3a).
As shown by Figure 3.3b, the vesicles do not flocculate, and are not compressed in the presence of 70 mM
of LMAM\[3\] (MW = \(2.10^8\)). No changes in visual appearance can be detected upon addition of the
polymer. As far as can be inferred from the micrographs, the concentration of vesicles is not reduced in the presence of polymer. Hence, no (significant) solubilization of vesicles occurs.

Using the negative staining technique, CDP vesicles can also be detected (Figure 3.3c). However, the vesicle shape is not reproduced accurately as a result of drying and flattening. More seriously, aggregates of vesicles are observed. Vesicle aggregation is brought about by the binding of positively charged uranyl ions to the vesicle surfaces.

In the presence of 160 unit mM of PSA-C12[4] (MW = 8200) the vesicles remain intact, as appears from Figure 3.3d (negative staining). There are no signs of depletion flocculation; indeed, the vesicle dispersion does not become turbid upon addition of the polymer. No indications for osmotic compression are obtained. The only effect of the hydrophobically-modified polymer is a beneficial one, apparently protecting the vesicles against aggregation, because no lumps of vesicle material are observed.

The effects of osmotic compression and depletion flocculation are demonstrated by using a poly(sodium acrylate) having a molecular weight that is at least an order of magnitude higher than that of the polymers that were employed in the calorimetric experiments (MW = 250,000). In the presence of 60 unit mM of this polymer, the vesicle dispersion becomes physically unstable, as is evidenced from the appearance of white flocs of material, sedimenting to the bottom of the vessel. The flocs are composed of densely packed, deformed vesicles, as shown by cryo-transmission electron microscopy (Figure 3.4).
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Figure 3.4  Cryo-electron transmission micrograph of a 5 mM sonicated dispersion of CDP in the presence of 60 unit mM poly(sodium acrylate), MW = 250,000. The vesicles are flocculated and deformed. Bar represents 100 nm.

3.3 Interactions between DDP vesicles and hydrophobically-modified poly(sodium acrylate)s studied by differential scanning calorimetry

In order to establish the anchoring of hydrophobically-modified poly(sodium acrylate)s into CDP bilayer vesicles, which have equal charge, recourse might have been taken to the wide variety of biochemical and biophysical techniques that are available to characterise protein-lipid interactions. These techniques include sedimentation, fluorescence spectroscopy, gel filtration and poly(acrylamide) gel electrophoresis (PAGE). Indeed, the electrophoretic mobility of CDP vesicles is reduced in the presence of PSA-C12[4], but is unaffected by poly(sodium acrylate). This result indicates the formation of a complex between the hydrophobically-modified polymer and the bilayer vesicles, despite unfavorable electrostatic repulsions.

Anchoring of PSA-CX[Y] into bilayer vesicles formed from di-n-dodecyl phosphate (DDP) was studied using differential scanning calorimetry (DSC). Using this technique, the influence of the anchoring polymer on the organization of the alkyl chains in the bilayer can be explored, through the thermal behavior of the bilayer vesicle dispersion. This information, which cannot be obtained using the biochemical methods mentioned above, aids in understanding, on a microscopic level, the principles that underlie the anchoring of hydrophobic side chains into membranes.
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Before presenting the results, we briefly introduce the phenomenon of a phase transition temperature, and how it can be measured using DSC. Moreover, the influence of hydrophobic solutes on the transition temperature will be summarized, as this provides the background necessary for understanding the effect of anchoring polymers.

3.3.1 Gel to liquid-crystal transitions in bilayer vesicles. Generally, at low temperatures, the alkyl chains in lipid bilayers are fully extended (all-trans conformation), ordered but tilted. The bilayer is in the gel state (L_g). As the temperature is raised beyond the main phase transition temperature (T_m), the structure ‘melts’ into a liquid-crystalline phase state (L_n). void volumes are created within the membrane, allowing enhanced rotational and lateral diffusion within the plane of the bilayer; the lateral diffusion coefficient of the double-tailed surfactant molecules increases by four orders of magnitude to $10^{-7} \text{ cm}^2 \text{s}^{-1}.^{14}$ In addition, the alkyl chains adopt some gauche conformations.

DSC determines $T_m$ and the enthalpy change associated with the phase transition ($\Delta H$). We consider here a single solute X in aqueous solution. At low temperatures the solute is in the form X(aq) whereas at high temperatures the solute is in the form Y(aq). At an intermediate temperature, a two-state equilibrium (with affinity constant $K$) is established:

$$X \rightleftharpoons Y.$$ 

Herein, X corresponds to the $L_g$ state, and Y corresponds to the liquid-crystalline state. Consequently, the isobaric heat capacity of such a solution, when plotted against temperature, forms a bell-shape, assuming that the heat capacities of the solute in the two states are the same. If the thermodynamic properties of the solution are ideal, then the shape of the curve is described by equation (6):

$$C_{p,m} = \frac{K}{(1 + K)^2} \cdot \frac{(\Delta H^\circ(vH))^2}{RT^2},$$

where $C_{p,m}$ is the molar heat capacity of the solute, and $\Delta H^\circ(vH)$ is the Van ‘t Hoff enthalpy of reaction. Experimentally, a plot is obtained of $C_{p,m}$ as a function of temperature. The area enclosed by the bell is the limiting calorimetric enthalpy of reaction, $\Delta H^\circ(\text{cal})$; alternatively, by fitting the shape of the experimental curve to eq. (5), the Van ‘t Hoff enthalpy of reaction can be obtained (Figure 3.5). In the textbook case described, $\Delta H^\circ(vH) = \Delta H^\circ(\text{cal})$.

The maximum in the plot corresponds to the temperature where the equilibrium constant equals unity, the standard Gibbs energy of reaction $(\Delta G^\circ)$ is zero and equal amounts of X and Y are present in the solution. We take this temperature as $T_m$; Figure 3.5.

In practice, two complications may arise. In most cases, the envelope recorded cannot be accounted for in terms of a simple two-state equilibrium. Instead, the DSC trace is satisfactorily described in terms of multiple, independent equilibria (m[X--Y]). The required curve fitting can be undertaken using the Origin software supplied with the DSC.

The second problem is slightly more complicated but prompted an important proposal for
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Figure 3.5 Development of the patch number concept: diagrammatic comparison of the dependence of the molar isobaric heat capacity on temperature as observed, and as calculated using equation (5) where the gel-to-liquid phase transition involves a single monomer unit (n_{DDP} = 1) or the total vesicle (n_{DDP} > 1000).

vesicular systems based on the concept of patch numbers. The measured dependence on temperature of an isobaric heat capacity cannot be transposed to that of the individual surfactant (i.e., DDP) molecules. Indeed, simulation of the DSC trace for a transition involving isolated monomers deviates substantially from the experimental data, the calculated trace being much broader. The opposite was observed when a transition involving the cooperative melting of all of the monomers within a given vesicle was modelled. Thus an iterative fitting procedure can be used to determine n_{DDP}, which is the size of the cooperative unit - a group (patch) of DDP monomers in the vesicle bilayer undergoing the transition cooperatively (Figure 3.5). That vesicles are comprised of a series of well-packed regions rather than one continuous-packed bilayer seems entirely reasonable. For example, this model accounts for the (albeit limited) permeability of bilayer vesicles to ions. Moreover, the concept of patch numbers has been confirmed by Peters who studied the melting of a hexane bilayer by molecular dynamics simulations. The calculation of patch numbers (or cooperativity units) as the ratio of the Van ’t Hoff enthalpy to the observed Δ_mH has been adopted by other researchers.

3.3.2 Partitioning of hydrophobic compounds between water and bilayer vesicles. The insertion of hydrophobic molecules within the lipid chains is a particularly facile process when the membrane exists in the liquid-crystalline phase. Incorporation of hydrophobic solutes (surfactants, alcohols, proteins, cholesterol, lindane) in the bilayer causes a lowering of T_m and a reduction of the melting enthalpy.

Plots of T_m vs. solute concentration are linear for the partitioning of oligo(ethylene glycol) n-alkyl
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ethers into phosphatidyl choline bilayer membranes. It was stated that the linear relation is similar to the well-known freezing point depression phenomenon. If this is true, then the partitioning coefficient \( K_p \) of the solute between the lipid vesicle (in the \( L_a \) phase) can be derived from the dependence of the main phase transition temperature on solute concentration according to the Van ‘t Hoff equation:

\[
-\Delta T_m = \frac{RT_{m,0}^2}{\Delta_m \Delta_H} 55.5 + \frac{K}{C_L} C_s.
\]

(7)

\( T_{m,0} \) denotes the main phase transition temperature of the bilayer in the absence of solute, \( C \) is the concentration of vesicle-forming surfactant and \( C_s \) is the solute concentration.

A prerequisite for this approach is that the solute is incorporated into the liquid crystalline state, but not into the gel state. The partition coefficient, as determined using eq. (7), can be grossly underestimated as the headgroup hydrophilicity decreases, or as the number of carbon atoms in the alkyl chain exceeds a certain limit (which is 7 in case of \( \omega \)-phenylalcohols). In view of the experimental data suggesting that hydrophobic solutes are dissolved surprisingly easily into the gel state, most likely due to the presence of defects in the bilayers, we contend that a thermodynamic analysis based on eq. (7) should be carried out with great care.

Despite the limited applicability of the above-mentioned thermodynamic approach, DSC studies have yielded important insights into the influence of incorporated solutes on the organization of the alkyl chains in the bilayer. An important general finding is that the solute topology is important with respect to the effect on \( T_m \) and \( \Delta_H \). If the alkyl chain of the solute has the same length as that of the lipid molecules forming the bilayer, then the lowering of \( T_m \) and \( \Delta_H \) is only marginal. However, the decrease of \( T_m \) and \( \Delta_H \) is much more pronounced if the alkyl side chain lengths of solute and lipid do not match. Apparently, the packing of alkyl chains in the gel state is greatly disturbed upon incorporation of solutes that do not ‘fit’. This concept of hydrophobic match/mismatch is well-developed, and we make use of it in the interpretation of the data obtained for bilayer vesicles with anchoring polymers (vide infra).

3.3.3 Anchoring of PSA-CX[Y] into DDP bilayer vesicles. Firstly, we stress that DDP vesicles were used instead of the structurally similar CDP vesicles because the phase transition temperature of the latter compound is too low to be determined accurately (3 °C), whereas \( T_m \) of DDP bilayer vesicles (ca. 34 °C) can be conveniently measured.
Figure 3.6  Dependence of differential isobaric heat capacities on temperature for DDP (aq; 5 mM) containing different concentrations of PSA. In case of nonzero polymer concentrations, the curves report second scans. For clarity, the curves have been displaced on the heat capacity axis.

The phase transitions of DDP bilayer vesicles are essentially unaffected by poly(sodium acrylate);
Figure 3.7  Dependence of differential isobaric heat capacities on temperature for DDP (aq; 5 mM) containing different concentrations of PSA-C9[4]. In case of nonzero polymer concentrations, the curves report second scans. The curves have been displaced on the heat capacity axis for clarity.
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Figure 3.6. In sharp contrast, the influence of increasing amounts of added hydrophobically-modified poly(sodium acrylate) on the phase transition characteristics of DDP bilayer vesicles is pronounced; Figure 3.7. The line shapes in the thermograms are distorted and broadened relative to the rather sharp, bell-shaped curve observed for DDP in the presence of poly(sodium acrylate). These patterns strongly indicate that hydrophobically-modified poly(sodium acrylate)s interact with DDP vesicles, despite unfavorable Coulombic forces, through penetration of alkyl side chains of the polymer into the bilayer.

A typical plot of the dependence of differential isobaric heat capacities on temperature for DDP bilayer vesicles in the presence of hydrophobically-modified polymer is shown in Figure 3.8. As observed in many DSC experiments on vesicle/solute interactions, the pattern formed by the first scan differs from those recorded on subsequent scans over the range 13-80 °C. The observation that only the second and subsequent scans are reproducible has been made before, indicating that the system present after the first scan is in a metastable state. After equilibrating the sample contents for 11 hours at 13 °C, the small feature at $T \approx 35$ °C, which we also observed in the pattern recorded in the first scan, reappears. We attribute this feature to the inability of (at least part of) the polymer hydrophobes to penetrate into the gel state, but once the system is scanned through the gel to liquid crystal transition, and back to the gel state, the polymer is not expelled from the bilayer at temperatures below $T_m$. Thus, the hydrophobic side chains are (partly) insoluble in the gel state, but the expulsion of the anchors from the bilayer upon lowering the temperature to below $T_m$ is a slow process. Therefore, it is not possible to use eq. (7) in determining with satisfying accuracy the distribution of solutes over the aqueous and bilayer phase. Table 3.1 summarizes important characteristics of the DSC traces obtained for DDP in the presence of different amounts of polymeric additives. Unfortunately, we could not obtain polymers of identical molecular weight. It is expected that the molecular weight is an important parameter in the anchoring efficiency of the polymer, because it determines the number of anchors per polymer molecule (‘octopus’ or chelate effect, vide infra). However, the extent of anchoring could be verified qualitatively by evaluating the bilayer properties $n_{DDP}$, $\Delta_H$ and $m[X\sim Y]$ as a function of the concentration of added hydrophobically-modified polymer. These quantities are substantially altered only in case of the highest concentration of polymer. For this reason,
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Figure 3.8  Dependence of differential isobaric heat capacity on temperature for DDP (aq; 5 mM) containing 60 unit mM of PSA-C9[4], for first and subsequent scans.

the following analysis concentrates on the results obtained for the hydrophobically-modified polymers at the highest concentrations studied. We emphasize, however, that we cannot quantify the results obtained in terms of a contribution to the change of bilayer properties per hydrophobic side chain of the polymer, because the anchoring efficiency of the polymers is not comparable.

The original bell shape observed in the thermogram for pure DDP is significantly distorted upon addition of hydrophobically-modified polymer. The main phase transition temperature is essentially unaffected by PSA-C12[4], but addition of PSA-C9[4] or PSA-C18[4] lowers $T_m$ by ca. 2 °C. The thermal stability of a vesicle bilayer depends mainly on the alkyl chain packing. Hence the disordering of the chain packing in the bilayer vesicles is more pronounced the greater the hydrophobic mismatch of the alkyl chain of the polymeric solute incorporated into the bilayer. The effects of hydrophobic mismatch clearly show up in the anchoring of hydrophobic side chains into bilayers, as was found for other solutes (vide supra).

Similarly the enthalpy of melting is lowered when disorder in the bilayers is introduced. This effect is strongest for C$_{18}$, and least pronounced for C$_{12}$ chains, again reflecting the better fit of n-dodecyl groups into the di-n-dodecyl phosphate bilayer relative to n-nonyl or n-octadecyl.

Surprisingly, the patch number increases if hydrophobically-modified polymers are Table 3.1

<table>
<thead>
<tr>
<th>$T_m$ (°C)</th>
<th>$n_{patch}$</th>
<th>$\Delta H$ (cal)</th>
<th>$m_X$</th>
<th>$m_Y$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0</td>
<td>0.05</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>0.10</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td>0.15</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>35</td>
<td>3</td>
<td>0.20</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>40</td>
<td>4</td>
<td>0.25</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>45</td>
<td>5</td>
<td>0.30</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>50</td>
<td>6</td>
<td>0.35</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

Main phase transition temperature ($T_m$), patch number ($n_{patch}$), enthalpy of melting ($\Delta H$) and the mean number of independent two-state equilibria ($m[X-Y]$) corresponding to the gel to liquid crystal transition of DDP bilayer vesicles in the presence of added poly(sodium acrylate-co-n-alkyl methacrylate)s.
Interactions between bilayer vesicles and hydrophobically-modified polymers

<table>
<thead>
<tr>
<th>Additive</th>
<th>Molar Ratio DDP/polymer (DDP/anchors)</th>
<th>T_m/°C a</th>
<th>n_{DDP}</th>
<th>Δ_{m,H}/kJ.(mol DDP) b</th>
<th>m[X→Y]</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td></td>
<td>33.9</td>
<td>305 ± 14</td>
<td>13.4 ± 0.2</td>
<td>2</td>
</tr>
<tr>
<td>buffer a</td>
<td></td>
<td>34.5</td>
<td>125 ± 0</td>
<td>14.1 ± 0.04</td>
<td>1</td>
</tr>
<tr>
<td>PSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MW = 2000</td>
<td>2:1 (-)</td>
<td>34.4</td>
<td>569 ± 22</td>
<td>11.3 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1:4 (-)</td>
<td>33.7</td>
<td>334 ± 16</td>
<td>13.0 ± 0.3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1:12 (-)</td>
<td>33.7</td>
<td>564 ± 19</td>
<td>11.5 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>PSA-C9[4]</td>
<td>2:1 (48:1)</td>
<td>34.4</td>
<td>432 ± 29</td>
<td>11.6 ± 0.3</td>
<td>3</td>
</tr>
<tr>
<td>MW = 27,000</td>
<td>1:4 (6:1)</td>
<td>32.6</td>
<td>320 ± 7</td>
<td>12.5 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1:12 (2:1)</td>
<td>32.1</td>
<td>1343 ± 63</td>
<td>9.7 ± 0.2</td>
<td>7</td>
</tr>
<tr>
<td>PSA-C12[4]</td>
<td>2:1 (48:1)</td>
<td>33.7</td>
<td>478 ± 15</td>
<td>15.2 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td>MW = 8200</td>
<td>1:4 (6:1)</td>
<td>33.5</td>
<td>292 ± 18</td>
<td>14.7 ± 0.3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1:12 (2:1)</td>
<td>33.8</td>
<td>2522 ± 243</td>
<td>12.1 ± 0.5</td>
<td>8/10</td>
</tr>
<tr>
<td>PSA-C18[4]</td>
<td>2:1 (48:1)</td>
<td>34.1</td>
<td>471 ± 34</td>
<td>11.9 ± 0.3</td>
<td>3</td>
</tr>
<tr>
<td>MW = 7000 b</td>
<td>1:4 (6:1)</td>
<td>32.7</td>
<td>406 ± 13</td>
<td>11.4 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1:12 (2:1)</td>
<td>31.9</td>
<td>1889 ± 136</td>
<td>6.9 ± 0.2</td>
<td>6</td>
</tr>
</tbody>
</table>

a) 5 mM HEPES + 5 mM sodium acetate, pH 8. b) The DDP/polymer molar ratio is expressed as the ratio of the number of di-n-dodecylphosphate molecules to the number of unit monomers of the polymer present in solution, the DDP/anchor ratio refers to the number of DDP molecules relative to polymer hydrophobic side chains. The concentration of DDP is always 5 mM. c) The molecular weight of this polymer has been determined using viscometry with hydrophobically-modified poly(sodium acrylate) or poly(acrylic acid) as reference compounds, and lies in the range of 4500 to 9500. d) Error is 0.1 °C.

...added, suggesting that the cooperativity of the phase transition is enhanced. In this case, n-dodecyl anchors promote the transition cooperativity most favorably. This observation is related to the theoretically predicted[7, 8] and experimentally observed[41] phenomenon that anchoring polymers induce spontaneous curvature in normally flat bilayers by filling the gaps in the outer leaflet that originate from bending the bilayer. The model proposed by Safran for spontaneous vesicle formation illustrates the point.[62] As was stated in Chapter 1, a bilayer composed of identical monolayers is frustrated, because the monolayer curvatures are of equal magnitude and are of opposite sign. The bilayer has its lowest Gibbs energy when its spontaneous curvature is zero, and this explains why the formation of closed vesicles from chemically pure amphiphiles needs the input of mechanical energy. However, if the compositions of the two monolayers are allowed to differ then, possibly, the spontaneous bilayer geometry will be nonplanar, thus favouring the formation of finite size vesicles. The anchoring of the hydrophobic alkyl chains of the polymer into the outer monolayer clearly produces such a stabilizing effect. In addition, the polymer coils...
at the surface of the vesicle enhance the spontaneous bilayer curvature. It remains to explain why the patch number increases upon addition of hydrophobically-modified polymers. One model for a vesicle is a quilt composed of patches of monomers. Applying the concepts of spontaneous curvature to this model shows that also the individual patches composed of chemically pure double-tailed amphiphiles should be frustrated. Since part of the curvature strain in patches will be relieved by the anchored polymers, these patches are allowed to grow, which is indeed observed.

In summary, there is strong evidence that anchoring hydrophobically-modified poly(sodium acrylate)s disrupt bilayer vesicles (composed of sodium di-n-dodecyl phosphate amphiphilic molecules) depending on the extent of the hydrophobic mismatch. This conclusion follows from the consistent trends that $T_m$ and $\Delta_n H$ are hardly affected by the anchoring of poly(sodium acrylate)s containing n-dodecyl chains, for which no hydrophobic mismatch exists. In contrast, polymers bearing n-nonyl or n-octadecyl groups do significantly alter these thermodynamic variables, indicating the loss of bilayer thermal stability upon introduction of hydrophobic mismatch between the bilayer-forming surfactant and the polymeric anchors. In addition, we propose that the increase in phase transition cooperativity occurring upon adding hydrophobically-modified poly(sodium acrylate)s reflects the relief of curvature strain by the anchors filling the gaps between patches of surfactant molecules in the outer leaflet.

### 3.4 Polysoap-vesicle interactions studied by titration microcalorimetry

Further insight into the thermodynamics of anchoring of hydrophobic side chains into bilayer vesicles was
Interactions between bilayer vesicles and hydrophobically-modified polymers

Figure 3.9  Plots of the enthalpy per injection vs. CDP concentration for the titrations of CDP vesicles from water into aqueous solutions containing 160 unit mM of hydrophobically-modified poly(sodium acrylate)s.

Figure 3.10  Plots of the enthalpy per injection vs. CDP concentration for the titrations of CDP vesicles from water into aqueous solutions containing 160 unit mM of hydrophobically-modified poly(acrylamide)s.

obtained using titration microcalorimetry. In this study, 6-10 µl aliquots of 10 mM sonicated dispersions
Chapter 3

of CDP (aq) were consecutively injected into the sample cell containing 160 unit mM of hydrophobically-modified poly(sodium acrylate)s or poly(acrylamide)s. The observed enthalpies are a function of the amount of polymer bound to the vesicles and the standard enthalpy of transfer of a hydrophobic side-chain from a microdomain into a bilayer, $\Delta_{m\rightarrow b}H^\circ$. Therefore, plots of $\Delta H$ vs. [CDP] are difficult to interpret directly, and for a quantitative analysis of the data, we use models relating the change of composition of the solution in the sample cell to the observed enthalpy.

3.4.1 A brief glance at the enthalpograms for the titrations of LMAM[Z] or PSA-CX[Y] with CDP vesicles. Figures 3.9 and 3.10 are enthalpograms illustrating the interactions between vesicles of CDP and hydrophobically-modified poly(sodium acrylate)s and poly(acrylamide)s, respectively. The gross features of the plots reveal some important aspects.

Firstly, dilution of CDP vesicles into water is endothermic by ca. 1.5 kJ mol$^{-1}$, decaying to zero at higher concentrations. If water is replaced by a 160 unit mM solution of poly(sodium acrylate) or poly(acrylamide), the envelope of $\Delta H$ vs. [CDP] is transformed into a horizontal line, corresponding to an essentially athermal dilution of vesicles into non-anchoring polymer solutions. Thus, there is no need to take into account the dilution of CDP vesicles into polymer solutions in calculations of binding constants or standard heats of reaction. We mention that corrections have been made for the dilution of the polymer solutions with water.

Secondly, binding of hydrophobically-modified polymers to CDP bilayer vesicles is an exothermic process. The magnitudes of the heat effects decrease with increasing CDP concentrations as less and less hydrophobic side chains are available for binding. An envelope is observed that is reminiscent of a ‘binding isotherm’. However, no such dependency of $\Delta H$ on CDP concentration appears in case of LMAM[1.5] or PSA-C9[4]. Instead, the plots are featureless. This result is remarkable, since the binding of PSA-C9[4] to DDP bilayer vesicles has been demonstrated using DSC, and the anchoring capacity of LMAM[1.5] has been exploited in the steric stabilization of DDPBNS vesicles. The fact that a binding process cannot be detected using titration calorimetry could be due to the anchoring processes being athermal at the temperature of the experiment. An alternative explanation involves the notion that the affinity of the polymers for the bilayers is too small to result in a measurable ‘binding isotherm’.

The observation that hydrophobic anchoring is accompanied by exothermic heat effects is quite remarkable. As was discussed in Chapter 1, prototypical hydrophobic interactions are entropy-driven near room temperature, and $\Delta H^\circ > 0$. The observed discrepancy can be explained by the fact that insertion of hydrophobic solutes into bilayer vesicles involves the liberation of water molecules filling the interstitial spaces between the lipid chains near the headgroup. Analogously to host-guest interactions, the water molecules in the interstitial cavities may be oriented such that the number of hydrogen bonds is less than in bulk water. Hence, their displacement into bulk water is exothermic. Indeed, the incorporations of hydrophobic solutes such as amphiphilic drugs or nonionic surfactants into phospholipid bilayer vesicles have also been found to be exothermic.

On the other hand, the question arises whether the observed heat effects can be explained by changes in the hydrophobic hydration of the lipid chains in the membrane alone. As was discussed above, it is expected that the configurational entropy of the polymer backbone is a major factor in determining the anchoring efficiency of the polymer. At the same time, the increase of conformational freedom of the backbone upon anchoring will be accompanied by an increased hydration of the hydrophilic acrylate or acrylamide moieties. This process is expected to be exothermic, which is in line with the experimental findings.
3.4.2 A quantitative analysis of the anchoring of hydrophobic side chains into bilayer vesicles in terms of a partitioning equilibrium. In this section, we attempt to analyze the enthalpograms obtained for the titrations of CDP vesicles into polysoaps in terms of Gibbs energies and enthalpies of binding.

Following the \(i\)th injection of an aliquot of \(V_{\text{inj}}/\text{dm}^3\) of vesicle solution into the sample cell, \(\delta n\) moles of polymeric side chains are anchored into the bilayer vesicles. The associated heat of reaction is:

\[
\delta h_i = \delta n_i \Delta H^0_i,
\]

where \(\delta h_i\) is the heat of titration corrected for dilution effects and \(\Delta H^0_i\) is the standard enthalpy of transfer of a hydrophobic side chain from aqueous solution into a vesicle bilayer.

At the moment, we leave aside whether all hydrophobic side chains are involved, in which case \(\Delta H^0_i\) relates to the enthalpy difference of a mole of side chains as present in hydrophobic microdomains or anchored into bilayer vesicles, or that only those alkyl groups penetrate into the vesicle bilayer which are not involved in hydrophobic domains. The latter process is accompanied by an enthalpy of transfer from water into the vesicle bilayer.

After \(i\) injections the total amount of bound anchors is:

\[
n_j = \sum \delta n_i
\]

and the cumulative heat of reaction is:

\[
\sum \delta h_i = n_i \Delta H^0_i,
\]

Since the reaction takes place in a microcalorimeter cell of defined volume \((V_{\text{cell}})\), the concentration of bound anchors in the sample cell \((C_{\Lambda}^b)\) is given by

\[
C_{\Lambda}^b = \frac{n_j}{V_{\text{cell}}} = \frac{\sum \delta h_i}{\Delta H^0_i V_{\text{cell}}}
\]

or

\[
\sum \delta h_i = \Delta H^0_i V_{\text{cell}} C_{\Lambda}^b.
\]

The next step is to relate the concentration of bound anchors to the concentration of injected lipid through an equation containing otherwise only constants. In this way, the observed cumulative enthalpy can be related to the concentration of injected CDP (both of which variables are known), and by fitting procedures \(\Delta H^0_i\) and the other relevant constants can then be obtained.
A simple approach is to describe the hydrophobic anchor / CDP vesicle bilayer equilibrium in terms of a partition model where the number of moles of anchors bound into the bilayer \( (n_A^b) \) is proportional to the total amount of CDP 'lipid' \( (n_L^{tot}) \) and the concentration of free anchors in the aqueous phase \( (C_A^{wat}) \):\(^{66}\)

\[
(13) \quad n_A^b = K_p C_L^{tot} \frac{n_A^{f}}{V_{wat}},
\]

where \( K_p \) denotes the partition coefficient. Neglecting the volume fraction occupied by the vesicles, equation (13) reduces to

\[
(14) \quad \frac{C_A^b}{C_L^{tot}} = K_p \frac{C_A^{f}}{C_L^{tot}},
\]

which can be written in the following convenient form:

\[
(15) \quad C_A^b = C_A^{tot} \frac{K_p C_A^{tot}}{1 + K_p C_L^{tot}}.
\]

The total lipid concentration in the cell after the \( i \)th injection is related to the concentration of CDP in the syringe, the injection volume \( (V_{inj}) \) and the volume of the cell:

\[
(16) \quad C_L^{tot} = i \langle V_{inj} / V_{cell} \rangle C_{L,i}^{spr}.
\]

Combining eqs. (12), (15) and (16), we obtain:

\[
(17) \quad \Sigma \delta h_i = \Delta_v H^0 \langle V_{cell} \rangle \sum_i \frac{i K_p \langle V_{inj} / V_{cell} \rangle C_{L,i}^{spr}}{1 + K_p C_{L,i}^{tot}}.
\]

The same equation was used by Wenk \textit{et al.} \(^{67}\) for modelling the partitioning of single-tailed surfactant molecules into phospholipid bilayer vesicles. Since the Origin software supplied with the microcalorimeter automatically calculates the concentration of CDP in the cell \( (C_{L,i}^{tot}) \), we used the more convenient expression

\[
(18) \quad \Sigma \delta h_i = \Delta_v H^0 \langle V_{cell} \rangle \sum_i \frac{K_p C_{L,i}^{tot}}{1 + K_p C_{L,i}^{tot}}.
\]
Interactions between bilayer vesicles and hydrophobically-modified polymers

As an example, a plot of the cumulative enthalpy vs. CDP concentration (= C$_{L,i}^{tot}$) for LMAM[3] is shown in Figure 3.11. The solid line represents the fit of the data to eq. (17). The numerical results of the fit procedure, together with those obtained for the other polymers, are listed in Table 3.2. A striking finding is the fact that the experimentally found Gibbs energies of transfer have the same order of magnitude as the Gibbs energy of transfer of monomeric (octaethylene glycol) dodecyl ether from water into 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine bilayer vesicles, reported as -30.4 kJ.mol$^{-1}$ by Heerklotz et al. This result indicates that in case of our anchoring experiments, mainly those alkyl side chains that are ‘free’ in solution (i.e., not involved in hydrophobic microdomains) bind into bilayer vesicles. Thus, Δ$\text{H}^\circ$ truly reflects an enthalpy of transfer of the unaggregated polymeric side-chain from water into a bilayer. This conclusion is supported by additional circumstantial evidence. The reported binding constants are high, i.e. in the order of 10$^2$ - 10$^3$ M$^{-1}$. Now, if all polymeric Table 3.2
Partition coefficients, standard enthalpies and Gibbs energies of transfer of polymeric hydrophobic side chains from solution into bilayer vesicles.

Figure 3.11  Cumulative enthalpy vs. CDP concentration for the titrations of CDP vesicles into aqueous solutions containing polysoaps. Solid lines represent fits to a partitioning equilibrium. The upper four plots have been offset on the enthalpy axis for clarity (without offset, all plots go through the origin). Polymer concentrations are 160 unit mM, except for LMAM[3] - 40, where [polymer] = 40 unit mM.
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<table>
<thead>
<tr>
<th>Polymer</th>
<th>$\Delta_n H^0 / \text{kJ (mol side chains)}^{-1}$</th>
<th>$K_n / \text{M}^{-1}$</th>
<th>$\Delta_n G^0 / \text{kJ mol}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA-C12[4]</td>
<td>-0.33 ± 0.01</td>
<td>$(3.7 ± 0.2) \times 10^3$</td>
<td>-30.8 ± 0.1</td>
</tr>
<tr>
<td>PSA-C12[8]</td>
<td>-0.253 ± 0.001</td>
<td>$(1.5 ± 0.1) \times 10^3$</td>
<td>-28.6 ± 0.2</td>
</tr>
<tr>
<td>PSA-C18[4]</td>
<td>-0.463 ± 0.005</td>
<td>$(0.93 ± 0.02) \times 10^3$</td>
<td>-27.3 ± 0.1</td>
</tr>
<tr>
<td>EC7800$^b$</td>
<td>-0.25 ± 0.01</td>
<td>$(1.5 ± 0.2) \times 10^3$</td>
<td>-28.5 ± 0.3</td>
</tr>
<tr>
<td>LMAM[3]</td>
<td>-2.85 ± 0.01</td>
<td>$(1.08 ± 0.01) \times 10^3$</td>
<td>-27.71 ± 0.02</td>
</tr>
<tr>
<td>LMAM[3]$^c$</td>
<td>-1.58 ± 0.02</td>
<td>$(5.2 ± 0.2) \times 10^3$</td>
<td>-31.7 ± 0.1</td>
</tr>
</tbody>
</table>

$^a$) Polymer concentration in the sample cell is 160 unit mM. The concentration of CDP in the syringe is 10 mM. $^b$) EC7800 is a poly(sodium acrylate) containing a C$_{11}$/C$_{12}$ anchor at one terminus of the molecule (cf. Chapter 4). The number-average degree of polymerization is 82. $^c$) Concentration is 40 unit mM. partitioning into the bilayer.

hydrophobes were available for binding (including those involved in hydrophobic microdomains), then the ratio of bound side chains ($n_b$) to the number of lipid molecules ($n_{tot}$) as calculated from eq. (19) would be much too large for the vesicles to keep their integrity. As an example, $R_c = 2.8$ in case of a 2 mM CDP solution in the presence of 160 unit mM PSA-C12[4], assuming that all hydrophobic anchors (6.4 mM) are involved in the Under conditions of $R_c > 2$, dmyristoyl phosphatidylcholine vesicles are completely dissolved into micelles by n-octyl glucoside surfactant. Analogously, the CDP vesicles are expected to be disrupted by the adsorbed polymer at the calculated $R_c = 2.8$, particularly since adsorbed polymers additionally destabilize the bilayer through the introduction of lateral repulsive forces (see Chapters 4 and 5). However, under the experimental conditions the vesicles seem fully intact (Figure 3.3 D). We obtain a rough indication of the maximal number of anchors bound per polymer by relating the area occupied by a polymer molecule adsorbed on a surface (at maximal coverage) to the total surface area of the bilayer vesicles. The hydrated ionic radius of a phosphate headgroup is ca. 4 Å, which makes the effective headgroup area 5 $10^{19}$ m$^2$. Since approximately 60% of the total number of CDP molecules is available for binding (i.e., 60% of the lipid molecules reside in the outer bilayer leaflet in case of these small vesicles), the total surface area per mole of CDP is 0.6 $\times$ 6.10$^{23}$ $\times$ 5.10$^{19} = 180.10^3$ m$^2$. Hence, the area occupied by one hydrophobically-single-endcapped poly(sodium acrylate) EC7800 is at least 2.5$\times$10$^{18}$ m$^2$. Hence, the area occupied by 1 mole of polymer molecules is at least 1.5 $10^6$ m$^2$. Hence, the equivalent (vesicle) surface area is fully covered as the number of anchors per lipid molecule is 1:8.3. In other words, the maximal value of $R_c$, corresponding to 100% surface coverage, is 0.11. The value of $R_c = 2.8$, as calculated above, exceeds this theoretical maximum by a factor of 30. Thus, the number of polymeric side chains available for anchoring is at least 30 times less than the total number of hydrophobes in solution (in case of PSA-C12[4]).

The conclusion that only a fraction of the polymeric hydrophobes in solution is available for anchoring has important consequences for the reported values of $\Delta_n H^0$. In the example of PSA-C12[4], $\Delta_n H^0$ as reported in Table 3.2 has been underestimated by a factor of at least 30. Thus, absolute values of $\Delta_n H^0$ are quite uncertain and may not even be compared amongst different polymers. It is known with certainty, however, that the enthalpy of transfer of hydrophobic side chains from water to bilayer vesicles
is exothermic. Moreover, partition constants are independent of the concentration of available hydrophobes. Thus, reported values of $K_p$ and $\Delta_v C^0$ are expected to be in the right order of magnitude.

### 3.4.3 A quantitative analysis of the anchoring of hydrophobic side chains into bilayer vesicles in terms of a Langmuir adsorption model.

In the partition model, a linear relationship is assumed between polymer (anchors) added and distributing in the lipid phase. In other words: the partition coefficient is independent of the degree of advancement of the reaction. This assumption is strictly not correct (but will hold if the average number of side chains bound per vesicle is so small that the adsorbed polymers do not ‘feel’ each other). As was mentioned above, the number of polymers that can bind to a vesicle is limited to a maximum which relates to complete surface occupancy. Therefore, it is likely that the chemical potential of the adsorbed polymers ($\mu_{\text{pol}}(\text{ads})$) is a function of the degree of surface occupancy ($\Theta$). Langmuir established the following relationship for the adsorption of gas molecules $j$ to solid surfaces:\(^{71}\)

$$\mu_j(\text{ads}) = \mu_j^0(\text{ads}) + \ln\frac{\Theta}{1 - \Theta}. \quad (20)$$

As described by Blandamer et al.,\(^{72}\) the Langmuir isotherm can also be used in titration calorimetry to describe observed enthalpies in terms of a change in composition of the sample cell due to adsorption of solute molecules to surfaces in solution. Let us consider the equilibrium between free and adsorbed polymer, characterized by a dimensionless equilibrium constant $K$:

$$K = \frac{\Theta}{1 - \Theta} \frac{V_{\text{cell}} C_r}{n_{\text{pol}}^\text{tot} - n_{\text{pol}}^j}. \quad (21)$$

where $C_r = 1 \text{ mol dm}^{-3}$. The degree of surface occupancy is a function of the area occupied by bound polymers and the total surface area provided by the vesicles:

$$\Theta = \frac{n_{\text{pol}}^j a_{\text{pol}}}{n_{\text{pol}}^\text{tot} a_{\text{ves}}} \quad (22)$$

where $a_{\text{pol}}$ is the area occupied by the polymer per mole of hydrophobic side chains, and $a_{\text{ves}}$ is the total surface area of the vesicles per mole of CDP. As was calculated above, the ratio $\text{SR} = a_{\text{pol}} / a_{\text{ves}} \geq 8.3$. Importantly, the total surface area of the vesicles available for binding increases with the injection number. We make some progress by introducing the variable $\pi$:

$$\pi = \frac{a_{\text{pol}}}{n_{\text{pol}}^\text{tot} a_{\text{ves}}} \frac{\text{SR}}{C_{\text{pol}}^{\text{tot}} V_{\text{cell}}} \quad (\text{SR} \geq 8.3). \quad (23)$$

By definition, $q = (V_{\text{cell}} C_r / K)$. We now rearrange eq. (21) into the form

$$q \pi n_{\text{pol}}^j = (1 - \pi n_{\text{pol}}^j) (n_{\text{pol}}^\text{tot} - n_{\text{pol}}^j) \quad (24)$$
or

\[(25) \quad \pi \left( n_A^b \right)^2 - n_A^b (1 + \pi n_A^{tot} + q \pi) + n_A^{tot} = 0.\]

Solving this equation, making the appropriate substitutions for \(q\) and \(\pi\), yields the concentration of bound anchors as a function of the concentration of added CDP \((C_i^{tot})\):

\[(26) \quad n_A^b = \frac{1 + \frac{SR}{C_L^{tot}} \left( C_A^{tot} + \frac{C_f}{K} \right)}{2 SR \left( C_L^{tot} V_{cell} \right)^{-1}} - \left[ \left( 1 + \frac{SR}{C_L^{tot}} \left( C_A^{tot} + \frac{C_f}{K} \right) \right)^2 - \frac{4 SR C_A^{tot}}{C_L^{tot}} \right].\]

(Only one solution has physical significance.) For those who are not prepared to go through the tedious algebra, a dimensional analysis confirms the derivation of eq. (26). By substitution of \(n_A^b\) from eq. (26) into eq. (12), writing \(V_{cell} C_a^b = n_A^b\), we obtain an equation to which we can fit the experimentally obtained plot of \(\Sigma \delta h_i\) vs. \(C_i^{tot}\):

\[(27) \quad \sum_i \delta h_i = \frac{\Delta_v H^0}{2 SR \left( C_L^{tot} V_{cell} \right)^{-1}} - \left[ \left( 1 + \frac{SR}{C_L^{tot}} \left( C_A^{tot} + \frac{C_f}{K} \right) \right)^2 - \frac{4 SR C_A^{tot}}{C_L^{tot}} \right].\]

As an example, a plot of the cumulative enthalpy vs. CDP concentration \((= C_L^{tot})\) for LMAM[3] is shown in Figure 3.12, the data being fitted to the Langmuir isotherm (27). We did not use SR as a dependent fit parameter. Instead, we fixed its value at 8.3 in order to maintain geometrically reasonable conditions regarding the size of the polymer related to the size of the vesicle. For the calculation to converge, we had to reduce the concentration of hydrophobic anchors available for binding by three orders of magnitude as compared with the total concentration of hydrophobic side chains in solution. This confirms the hypothesis that only a fraction of the polymeric side chains is available for anchoring into bilayer vesicles. The numerical results of the fit procedure are listed in Table 3.3. From Figure 3.12, one may obtain a visual impression of the quality of the fits.

As mentioned in Section 3.5.3, the magnitudes of the calculated enthalpies of transfer strongly depend on the concentration of ‘free’ side-chains available for binding. Hence, absolute magnitudes of \(\Delta_v H^0\) are not reliable. To compare the values reported in Table 3.3 with those in Table 3.2, the latter must be multiplied by a factor of 1000.

**Table 3.3**

| Standard enthalpies of transfer and binding constants calculated for the binding of hydrophobically-modified poly(sodium acrylate)s and poly(acrylamide)s onto CDP bilayer vesicles according to a Langmuir adsorption isotherm.

<table>
<thead>
<tr>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\Delta_v H^0)</td>
<td>(J/mol)</td>
</tr>
<tr>
<td>(\Delta_v S^0)</td>
<td>(J/mol K)</td>
</tr>
<tr>
<td>(\Theta)</td>
<td>(^\circ C)</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Polymer</th>
<th>$\Delta_n H^0$ / kJ.(mol anchors)$^{-1}$</th>
<th>$K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA-C12[4]</td>
<td>-362 ± 14</td>
<td>$(18 \pm 2) \times 10^3$</td>
</tr>
<tr>
<td>PSA-C12[8]</td>
<td>-316 ± 23</td>
<td>$(6.0 \pm 0.1) \times 10^3$</td>
</tr>
<tr>
<td>PSA-C18[4]</td>
<td>-669 ± 14</td>
<td>$(3.2 \pm 0.1) \times 10^3$</td>
</tr>
<tr>
<td>EC7800</td>
<td>-439 ± 36</td>
<td>$(6.1 \pm 0.7) \times 10^3$</td>
</tr>
<tr>
<td>LMAM[3]</td>
<td>-4246 ± 43</td>
<td>$(3.5 \pm 0.1) \times 10^3$</td>
</tr>
<tr>
<td>LMAM[3], 40 unit mM</td>
<td>-1792 ± 13</td>
<td>$(21.9 \pm 0.3) \times 10^3$</td>
</tr>
</tbody>
</table>

Corrected values of $\Delta_n H^0$ as determined using the partition model are in excellent agreement with those obtained using the binding isotherm approach. Analogously, $K$ and $K_p$ as calculated using the different models are in good agreement. The observed trends are explained in the following section. Depend on the concentration of ‘free’ side chains available for binding, so that absolute values of $\Delta_n H^0$ are not reliable. In order to compare the values reported in Table 3.3 with those in Table 3.2, the latter must be multiplied by a factor of 1000.

3.4.4 An interpretation of the observed binding constants. The observed differences between the binding constants of the polyoaps are difficult to explain. Some trends can be discerned, however. At the outset, we expected that the ‘octopus’ or chelate effect would be a dominant factor in determining the anchoring efficiency of polyoaps. The chelate effect refers to the strong increase in binding constant of a ligand to (for example) a metal center as the number of coordinating groups of the ligand is increased. The rationale is that as one of the ligand sites is coordinated to the metal, the other groups can bind much more easily since the entropy loss due to binding has been already paid for by the group that was first complexed. In our study of the binding of poly(sodium acrylate)s to bilayer vesicles we seem to observe such effects. The binding constant of the low-molecular weight polymer PSA-C12[8] (MW = 3000, 4 anchors per molecule) is lower than the binding constant of PSA-C12[4], which has a molecular weight of 27000 (18 anchors per molecule). However, the affinity of the hydrophobically-endcapped polymer for the bilayer is the same as that of PSA-C12[8], despite the lack of a favorable octopus effect (the number of anchors per polymer is only one). It is tempting to explain the discrepancy using a statistical argument.
Figure 3.12  Cumulative enthalpy vs. CDP concentration for the titrations of CDP vesicles into aqueous solutions containing polysoaps. Solid lines represent fits to a Langmuir isotherm. The upper four plots have been offset on the enthalpy axis for clarity (without offset, all plots pass through the origin). Polymer concentrations are 160 unit mM, except for LMAM[3] - 40, where [polymer] = 40 unit mM.

Thus, if only a fraction of the hydrophobic side chains are available for anchoring, then chances are low that, for polymers bearing a ‘small’ number of side chains per molecule, more than one hydrophobe belonging to the same polymer will be involved in the partitioning equilibrium. We contend that this is an ad hoc hypothesis, and we need much more experimental evidence and a thorough statistical analysis for this matter.

The degree of hydrophobic match (or mismatch) does appear to play an important role. The partition constant of PSA-C18[4] (MW = 7000) is more than three times lower than that of PSA-C12[4] (MW = 8200). This result is in agreement with the conclusion drawn from differential scanning calorimetric studies that the vesicle bilayer is least disturbed as the length of the hydrophobic anchor matches that of the alkyl chains forming the bilayer.

Finally, the role of electrostatic forces in determining the anchoring efficiency is not immediately obvious. The partition constant of the electrically neutral LMAM[3] (MW ≈ 18000, 9 anchors per molecule) at 160 unit mM is only moderate. However, as the polymer concentration is lowered to 40 unit mM of LMAM[3], $K_p$ reaches the highest value reported amongst the series. These results signal that the affinity of an alkyl side-chain for a vesicle bilayer is reduced as the polymer to which it is attached is more extensively involved in hydrophobic microdomains (the hydrophobic microdomains are likely to become more interconnected with increasing polymer concentration. This is the basis of the ‘associative thickening’ effect observed for polysoap solutions at concentrations exceeding $c^*$ - see Chapter 2). Moreover, if this conclusion is valid, then the influence of electrostatic repulsions on the anchoring process of hydrophobically-modified poly(sodium acrylate)s as compared with poly(acrylamide)s is best
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reflected by a comparison of the partition coefficients of LMAM[3] at 40 mM and of EC7800 (this polymer cannot form a ‘network’ of hydrophobic domains). We conclude that the affinity constant of the electrically neutral polymer is 3-4 times higher than that of the polymer bearing the same charge as the vesicle bilayer.

3.5 Conclusions

Under experimental conditions where the concentration of hydrophobic anchors exceeds the lipid concentration by a factor of 2, the vesicles retain their structural integrity. In view of the high binding constants found for the anchoring of n-alkyl side chains into bilayer vesicles, we conclude that only a fraction of the total amount of hydrophobes takes part in the anchoring process, otherwise the vesicles would likely be destroyed, as can be inferred from studies on the incorporation of surfactants into membranes. Analogously to the observation that the binding of single-tailed surfactants to micelles is thermodynamically more favorable than to bilayers, we contend that only those hydrophobes that least contribute to the thermodynamic stability of the polymeric microdomains will be anchored into the bilayer vesicles.

The issue of vesicle structural integrity is re-addressed upon interpretation of the observed increase of the patch number, or cooperativity parameter characterizing the phase transition of DDP bilayer vesicles, when hydrophobically-modified poly(sodium acrylate)s are added. The observed phenomenon reflects a relief of curvature strain by the anchors filling the gaps between patches of surfactant molecules in the outer leaflet.

Differential scanning and isothermal titration calorimetry provided unambiguous structural information regarding the role of the anchor chain length in determining the affinity of the hydrophobe for the vesicle bilayer. Thus, in case of vesicles composed of surfactants bearing di-n-dodecyl chains, the binding of hydrophobic side chains is optimal for the anchor comprising 12 carbon atoms. The rationale behind this observation is that the bilayer is least disturbed if there exists a ‘hydrophobic match’ between the length of the alkyl side chains of the polymer and of the hydrophobic tails of the vesicle-forming surfactants.

Appreciable indications have accumulated that binding of polysoaps to bilayer vesicles is not hampered by Coulombic forces, but the binding constant for anchoring of hydrophobically-modified poly(acrylamide)s into bilayer vesicles formed from the anionic surfactant CDP is larger as compared with binding of the negatively charged polysoaps of general structure PSA-CX[Y].

Acknowledgements

I wish to thank Barbara Briggs and Prof. Michael J. Blandamer (University of Leicester) for performing the DSC experiments. In addition, Prof. Blandamer’s contribution to the thermodynamic analyses is highly appreciated. Dr. John van de Pas and Dr. Wilfried Blokzijl (Unilever Research) are acknowledged for fruitful discussions. Prof. A.D.R. Brisson (Dept. of Biophysical Chemistry, Interfacultary Institute for Electron Microscopy, University of Groningen) provided helpful comments on the electron micrographs. Last but certainly not least I warmly thank Mr Jan van Breemen (Dept. of Biophysical Chemistry, Interfacultary Institute for Electron Microscopy) who taught me the basics of cryo-EM, being always prepared to help.
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3.6 Experimental part

**Materials.** The syntheses of CDP\textsuperscript{90} and DDP\textsuperscript{24} have been described. Poly(sodium acrylate-co-n-alkyl methacrylate)s were obtained from National Starch and were used as received. The pH of the supplied polymer solutions was adjusted to 9 using sodium hydroxide so that the acidic groups are completely converted into their sodium salts.\textsuperscript{75} The polymers were characterized by aqueous gel permeation chromatography using an ultraviolet detector set at 215 nm. Fractionated poly(sodium acrylate) standards were used to construct a calibration graph. The molecular weight distribution can be described by Schulz-Flory theory,\textsuperscript{76} and the polydispersity index is about 2.5-3.

**Preparation of CDP vesicles for titration microcalorimetry.** Small CDP vesicles were prepared by sonifying approximately 3 mL of aqueous dispersions of the double-tailed surfactant (typically 10 mM) using a Branson Cell Disruptor B15 for 5 minutes (40 \% tip limit, 35\% duty cycle). During sonication, the temperature of the sample was allowed to rise from \textit{ca.} 25 to \textit{ca.} 40°C.

Importantly, the vesicles are unilamellar, as evidenced from electron microscopy. If the vesicles were multilamellar, only the \textit{outer} bilayer would be affected by the anchoring process. Experimentally determined changes of bilayer properties upon addition of anchoring polymer are averaged over the total number of moles of surfactant, including those that are buried inside the multilamellar aggregate. Only in case of SUVs, the influence of the anchoring process on the bilayer properties will be reflected honestly.

**Preparation of DDP vesicles for scanning calorimetry.** DDP was added to 5 cm\textsuperscript{3} of water to produce a surfactant concentration of 5 mM. The aqueous DDP suspension was heated to above 55°C, with stirring for 30 min.\textsuperscript{77} Using electron microscopy, this ‘hot water + stirring’ method was shown to yield spherical vesicles, which are unilamellar.\textsuperscript{78} The resulting vesicle solution was cooled to room temperature, and the appropriate amount of polymer was added. These solutions were degassed by pumping with stirring and scanned in the DSC from 13°C to 80°C several times after (a) immediate cooling to 13°C and (b) standing at 13°C for several hours. Temperature was increased at 60 K h\textsuperscript{-1}.

**Differential Scanning Calorimetry.** A MicroCal differential scanning microcalorimeter was operated in the manner previously described.\textsuperscript{79} The output from the DSC produced plots showing the dependence of the differential heat capacity on temperature. We found that the transition near 35°C could be accounted for in terms of two independent equilibria in aqueous solution. This was not always the case when hydrophobically-modified poly(sodium acrylate)s were added (see below). In some cases, the ORIGIN software was used to fit the dependencies of heat capacity on temperature to more than two independent two-state equilibria.

**Electron Microscopy.** A Jeol JEM EX1200 or Philips CM20 transmission electron microscope was used to characterize CDP vesicles, before and after the addition of polymer. In the case of cryoelectron microscopy, a 5\mu l aliquot of a vesicle solution (10 mM) was transferred onto bare copper grids (400 mesh) which were plunged into liquid ethane, and then observed at about -170°C using a Gatan cryotransfer specimen holder. The accelerating voltage was 80 kV (JEM) or 200 kV (CM20).

Negatively-stained images were obtained using 1\% uranyl acetate (aq), following the procedure outlined in Section 3.2. In all cases, exposures were made using the minimum dose system of the microscope to reduce specimen radiation damage as much as possible. The photographic plates (Agfa Scientia) were developed in full-strength Kodak D19 for 12 minutes.
3.7 Notes and references


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33. Input of mechanic energy, e.g. by sonication, is needed to form closed spherical bilayers (see also Experimental): Fendler, J.H. *Membrane Mimetic Chemistry*; Wiley: New York, 1982.


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39. Unpublished results. Courtesy to Dr. Irene van der Woude.
40. For a thorough survey of bilayer membranes and their physical properties, see Bloom, M.; Evans, E.; Mouritsen, O.G. *Quart. Rev. Biophys.* **1991**, *24*, 293.
56. For instance, the affinity constant for the binding of dipyramidole into phospholipid bilayers is reduced by only a factor of two upon decreasing the temperature below $T_m$, from 1000 M$^{-1}$ (L) to 500 M$^{-1}$ (L$_0$): Nassar, P.M.; Almeida, L.E.; Tabak, M. *Biochim. Biophys. Acta* **1997**, *1328*, 140.
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77. This method of vesicle preparation is preferable to the ethanol-injection method, since in the latter case traces of ethanol appear to be incorporated into the vesicle bilayer so that T_m is shifted to lower temperatures.60
