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SHORT COMMUNICATION

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Anchoring of hydrophobically modified poly(sodium acrylate)s into DDP vesicle bilayers: hydrophobic match and mismatch

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Abstract Differential scanning microcalorimetric thermograms have been recorded for aqueous solutions containing vesicles formed by sodium di-*n*-dodecyl phosphate, in the presence of different concentrations of poly(sodium acrylate-co-*n*-alkyl methacrylate), where *n*-alkyl = C_9H_{19}, $C_{12}H_{25}$, $C_{18}H_{37}$. The mole fraction of hydrophobic moieties in the copolymer is 0.04. The main phase transition temperature (T_m) is hardly affected by the presence of poly(sodium acrylate)s bearing *n*-dodecyl chains, whereas the anchoring of polymers bearing *n*-nonyl or *n*-octadecyl groups reduces the main phase transition temperature significantly from ca. 34 °C to ca. 32 °C. In parallel, the enthalpy of transition per mole of DDP monomer ($\Delta_m H_{int}$) is lowered upon adding polymer. Again, the polymer containing *n*-dodecyl moieties hardly affects $\Delta_m H_{int}$. These patterns are explained by the notion that the extent of the disruptive effect of alkyl chains incorporated into the bilayer depends on the extent of the mismatch between the chain lengths of the intruding alkyl chains and the hydrophobic moieties composing the vesicle bilayer. Added hydrophobically modified polymers increase the cooperativity of the melting process, as shown by the increase of n_{DDP}. We suggest that the anchoring poly(sodium acrylate-co-*n*-alkyl methacrylate) relieves the strain in the curved outer monolayer of a pure DDP bilayer by allowing the presence of larger "patches" characterized by low curvature.

Key words Differential scanning microcalorimetry – hydrophobic mismatch – anchoring – sodium di-*n*-dodecyl phosphate – vesicle bilayers – hydrophobically modified poly(sodium acrylate)s

Introduction

Synthetic vesicles have been recently used as vehicles for drug targeting and transfection [1]. There is considerable interest in stabilizing these vehicles in order to increase their lifetime in the human body [2]. In one approach the outer leaflet of these vesicle bilayers is coated with polymers containing hydrophobic moieties which anchor the polymers into the bilayer, while the hydrophilic backbone protrudes into the aqueous phase, providing the required steric stabilization [3].

In a previous study [4], we investigated using isothermal titration calorimetry the anchoring of "cyclic" sodium di-*n*-dodecylphosphate vesicles (**1**) by hydrophobically modified poly(sodium acrylate)s (**3**). We observed that binding of polymers to vesicle bilayers is most efficient if the anchors are *n*-dodecyl, whereas the efficiency decreases upon increasing or lowering chain lengths. We speculated that this observation might be related to a better fit of

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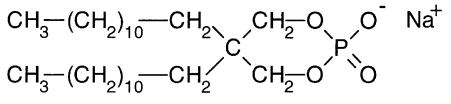
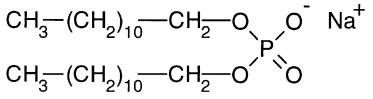
n-dodecyl anchors into a vesicle bilayer comprising hydrophobic moieties of the same length, compared to a situation where a "hydrophobic mismatch" occurs between anchoring moieties and bilayer-forming alkyl chains.

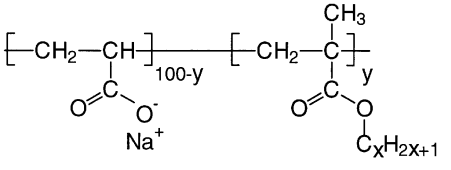
A differential scanning microcalorimetric study on the interactions between poly(sodium acrylate-co-*n*-alkyl methacrylate) and DDP (**2**) vesicle bilayers was undertaken to investigate this possibility. DDP vesicles were employed instead of the structurally similar CDP vesicles because the phase transition temperature of the latter compound is too low to be determined accurately (3 °C) [5], whereas T_m of DDP vesicle bilayers (ca. 34 °C) can be conveniently measured [6]. The hydrophobically modified poly(sodium acrylate)s are called PSA-C$X(Y)$, where X indicates the alkyl chain length as in C_XH_{2X+1}, and Y refers to the degree of hydrophobic modification. In this study, $Y = 0$ or 4 indicating zero or 4 unit mol% of hydrophobic anchors, respectively.



1 CDP



2 DDP



3 PSA-CX(Y)

Experimental

The synthesis of DDP has been described [7]. 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 were completely converted into their sodium salts [8]. 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, and the polydispersity index is about 2.5–3.

DDP was added to 5 cm^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 [9]. This "hot water + stirring" method was shown using electron microscopy to yield spherical vesicles [10a], which are unilamellar [11]. 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^{-1}.

We have shown previously [4] that small unilamellar vesicles formed from the structurally analogous CDP retain their integrity when hydrophobically modified poly(sodium acrylate)s are added, provided that the polymer molecular weight does not exceed a certain limit. This implies that osmotic shock or vesicle solubilization effects are insignificant.

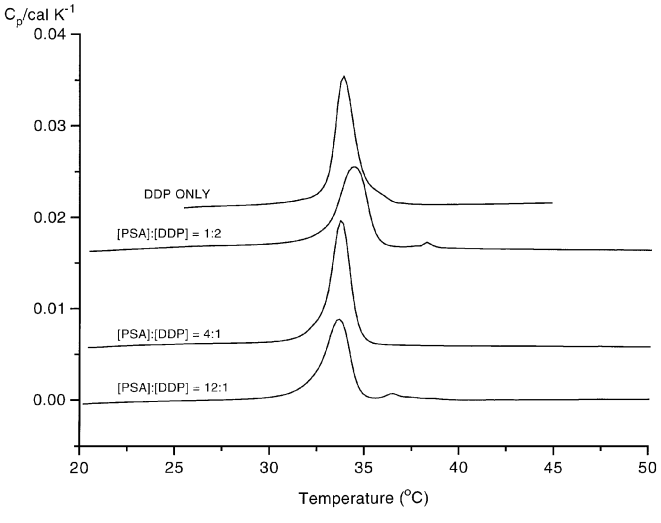
A MicroCal differential scanning microcalorimeter was operated in the manner previously described [10]. The output from the DSC produced plots showing the dependence of differential heat capacity on temperature. We found that the transition near 35 °C could be accounted for in terms of two independent 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. The thermodynamic analysis has been reported in the literature [12]. The second problem is slightly more complicated but prompted an important proposal for vesicular systems based on the concept of patch numbers. As has been described in ref. [11], 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 very much broader and less intense. 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 determine the size of the cooperative unit – a group (patch) of DDP monomers in the vesicle bilayer [11]. 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 vesicle bilayers to ions. Moreoever, the concept of patch numbers has been confirmed by Peters [13] who studied the melting of a hexane bilayer by molecular dynamics simulations.

Results and discussion

The phase transitions of DDP vesicle bilayers are essentially unaffected by poly(sodium acrylate); Fig. 1. In sharp contrast, the influence of increasing amounts of added hydrophobically modified poly(sodium acrylate) on the phase transition characteristics of DDP vesicle bilayers is pronounced; Fig. 2. 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 PSA. This pattern points to strong polymer–bilayer hydrophobic interactions.

A typical plot of the dependence of differential isobaric heat capacities on temperature for DDP vesicle bilayers in the presence of hydrophobically modified polymer is shown in Fig. 3. As is 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 [14]. After equilibrating the sample contents for 11 h at 13 °C, the small feature at $T \approx 35$ °C, which was also observed in the pattern recorded in the first scan, reappears. We attribute this feature to the inability 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

Fig. 1 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.)



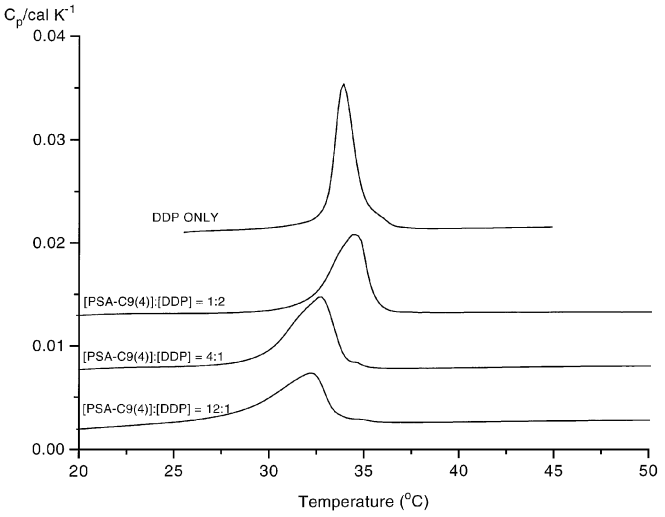


Fig. 2 Dependence of differential isobaric heat capacities on temperature for DDP (aq; 5 mM) containing different concentrations of PSA-C9(4). (In case of nonzero polymer concentrations, the curves report second scans. The curves have been displaced on the horizontal axis for clarity.)

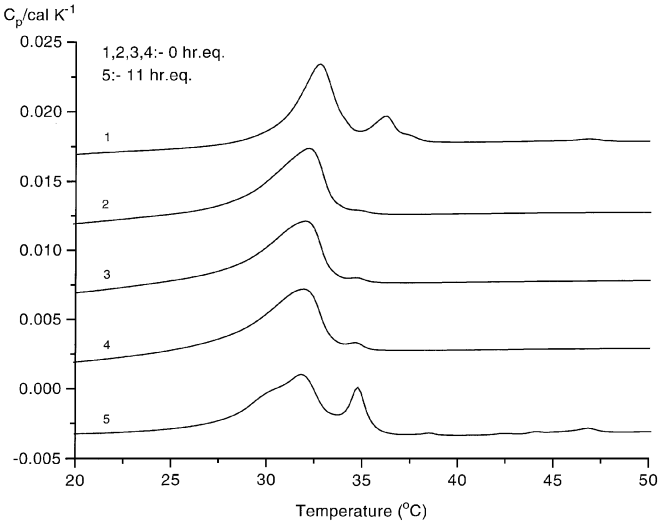


Fig. 3 Dependence of differential isobaric heat capacity on temperature for DDP (aq; 5 mM) containing 60 unit mM of PSA-C9(4), for first and subsequent scans

temperatures below T_m. Thus, the common features of colligative properties do apply for solutes in vesicle bilayers, as suggested by Kawamura et al. [15]. However, since the expulsion of the anchors from the bilayer upon lowering the temperature to below T_m is a slow process, it is not possible to use cryoscopy in determining with satisfying accuracy the distribution of solutes over the aqueous and bilayer phase.

Table 1 Main phase transition temperature (T_m), patch number (n), enthalpy of melting ($\Delta_m H_{int}$) and the mean number of independent two-state equilibria ($m[X \leftrightarrow Y]$) corresponding to the gel to liquid crystal transition of DDP vesicle bilayers in the presence of added poly(sodium acrylate-co-*n*-alkyl methacrylate)s

Additive	Molar ratio DDP/polymer (DDP/anchors)[a]	T_m/°C	n	$\Delta_m H_{int}$/ kJ.(mol DDP)$^{-1}$	$m[X \leftrightarrow Y]$
None		33.9	305 ± 14	13.4 ± 0.2	2
Buffer[b]		34.5	125 ± 0	14.1 ± 0.04	1
PSA	2:1 (−)	34.4	569 ± 22	11.3 ± 0.2	3
MW = 2000	1:4 (−)	33.7	334 ± 16	13.0 ± 0.3	2
	1:12 (−)	33.7	564 ± 19	11.5 ± 0.1	3
PSA-C9(4)	2:1 (48:1)	34.4	432 ± 29	11.6 ± 0.3	3
MW 27 000	1:4 (6:1)	32.6	320 ± 7	12.5 ± 0.1	3
	1:12 (2:1)	32.1	1343 ± 63	9.7 ± 0.2	7
PSA-C12(4)	2:1 (48:1)	33.7	478 ± 15	15.2 ± 0.2	3
MW 8200	1:4 (6:1)	33.5	292 ± 18	14.7 ± 0.3	2
	1:12 (2:1)	33.8	2522 ± 243	12.1 ± 0.5	8/10
PSA-C18(4)	2:1 (48:1)	34.1	471 ± 34	11.9 ± 0.3	3
MW ~ 7000[c]	1:4 (6:1)	32.7	406 ± 13	11.4 ± 0.1	3
	1:12 (2:1)	31.9	1889 ± 136	6.9 ± 0.2	6

[a] 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.
[b] 5 mM HEPES + 5 mM sodium acetate, pH 8.
[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 4500–9500.

Table 1 summarizes the most important characteristics of the DSC traces obtained for DDP in the presence of different amounts of polymeric additives. It is rather unfortunate that we could not obtain polymers of identical molecular weight. The molecular weight is an important parameter in the anchoring efficiency of the polymer, since it determines the number of anchors per polymer molecule ("octopus" or chelate effect). However, the extent of anchoring could be verified qualitatively by evaluating the bilayer properties n_{DDP}, $\Delta_m H_{int}$ and $m[X \leftrightarrow Y]$ as a function of the concentration of added hydrophobically modified polymer. It appears that these quantities are substantially altered only in case of the highest concentration of polymer. For this reason, the following discussion 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 vesicle bilayers 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 have been clearly demonstrated for the incorporation of surfactants and alkanols into vesicle bilayers [16]. 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*-dodecylphosphate bilayer relative to *n*-nonyl or *n*-octadecyl.

Surprisingly, the patch number is increased if hydrophobically modified polymers are 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 [17] and experimentally observed [18] 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 [19, 20] for spontaneous vesicle formation clearly illustrates the point. A bilayer is composed of two monolayers (bilayer leaflets), stuck together through their hydrophobic interfaces. Each

monolayer is characterized by an equilibrium spontaneous curvature which is in general nonzero. Therefore, 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 favoring 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, we propose that the polymer coils at the surface of the vesicle enhance the nonzero spontaneous bilayer curvature. The question still remains how to explain the patch number increase that is observed upon addition of hydrophobically modified polymers. As mentioned above, one model for a vesicle is a quilt composed of patches of monomers [6]. Applying the concepts of spontaneous curvature to this model yields the insight that also the individual patches composed of chemically pure double-tailed amphiphiles should be frustrated. Since part of the curvature strain in the 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 vesicle bilayers (composed of sodium di-*n*-dodecylphosphate amphiphilic molecules) to the extent of the hydrophobic mismatch. This conclusion follows from the consistent trends that T_m and $\Delta_m H_{int}$ 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 phase transition cooperativity increase that occurs 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.

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