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CALORIMETRIC STUDIES OF THE INTERACTIONS BETWEEN MICELLE-FORMING AND BILAYER-FORMING AMPHIPHILES

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Abstract

We develop a theoretical basis for detailed investigations of mixed aqueous solutions of amphiphiles by high resolution titration calorimetry (ITC). We review the phenomenology of phase behavior of these systems and formulate the questions addressed in thermodynamic studies. Based on the equations obtained, we introduce a general scheme of calorimetric measurements allowing to characterize comprehensively the energetics of the mixtures. We discuss the results of previous studies in relation to this scheme.

Keywords: energetics of the mixtures, phase diagrams, phospholipids, solubilization, surfactants (detergents), titration calorimetry

Introduction

The self-assembly of mixed, multicomponent systems of synthetic and biological origin is an issue of great interest in many disciplines. Of special importance is the state of aggregation and the composition-induced phase transitions in mixtures of bilayer-forming amphiphiles, such as naturally occurring phospholipids, which form the matrix of biological membranes, and micelle-forming amphiphiles (surfactants), which are in common use in solubilization and reconstitution of biomembranes [1–4].

Understanding of the interactions of various surfactants with phospholipid bilayers and of the factors which govern the phase transition between lamellar and micellar phases is important for the foundation of a rational approach to several biologically-significant issues, both basic and applied. These include:

1. Biochemical and biophysical studies of biological membranes, which require solubilization of the membranes for purification of their components, and subsequent reconstitution of purified proteins into well-defined phospholipid vesicles (proteoliposomes) for studies of their functions [5–8].

2. Solubilization of lipids (mostly phospholipids and cholesterol) by bile salts, which is of significance with respect to the formation of gallstones upon

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precipitation of cholesterol from mixed cholesterol–phospholipid–bile salts systems [9].

3. Solubilization of diatery phospholipids by bile salts, which is essential in the digestion of lipids by water–soluble pancreatic enzymes such as phospholipase A_2 [10].

4. Design of novel lipid-based drug carriers for parenteral and topical application, which is commonly based on lipid-surfactant mixtures [11].

In view of these considerations, much effort has been devoted to elucidation of the structure of the mixed assemblies formed in various phospholipid/surfactant mixtures. The use of a variety of microscopic, spectroscopic and chemical techniques of complementary nature resulted in comprehensive description of the phenomenology of the phase behavior in these mixtures. However, a quantitative understanding of the phase behavior of amphiphilic mixtures in terms of energetics of the interactions between phospholipids and surfactants still poses a challenge.

The aim of this paper is to propose experimental protocols by which high sensitivity isothermal titration calorimeters (ITC) can be used to collect detailed quantitative information on the thermodynamic properties of the lipid/surfactant mixtures in aqueous solutions.

For the reader to be able to follow this discussion, we first introduce, briefly, the phenomenological features of the phase behavior of mixed phospholipid/surfactant aggregates and the structure of these aggregates. Subsequently, we illustrate the complexity and formulate the problems associated with studying the self-assembly in mixtures of amphiphiles, in comparison to studies of the self-assembly of individual surfactants in aqueous solutions. We then give a theoretical foundation for ITC measurements that can be used to overcome these problems. At the end we shortly review previous calorimetric investigations that yield partial quantitative information on lipid/surfactant systems. While being neither comprehensive nor 'objective' (in terms of description of data) we show the relation of the previous measurements to the more complete scheme proposed in the present study and describe how realization of this scheme can improve our knowledge.

Phenomenology of phospholipid/surfactant mixed assemblies

Phase behavior of the mixtures

The phase behavior of a mixture of lipid and surfactant is schematically illustrated in Fig. 1 by a phase diagram obtained for a mixture of phosphatidylcholine (PC) and a surfactant [12]. For the sake of simplicity, we first describe 'solubilization' of phospholipid unilamellar vesicles by a surfactant. Specifically, when a surfactant is added to vesicles at a constant lipid concentration, L, micelles will be formed only if the surfactant concentration is higher than a characteristic con-

centration, D^{SAT}, and complete solubilization will occur only at a higher characteristic concentration of surfactant, D^{SOL}. Thus, below D^{SAT} the mixed aggregates are essentially lamellar, above D^{SOL} they are essentially micellar, whereas at intermediate surfactant concentrations, $D^{SAT}<D<D^{SOL}$, mixed micelles and bilayers coexist.

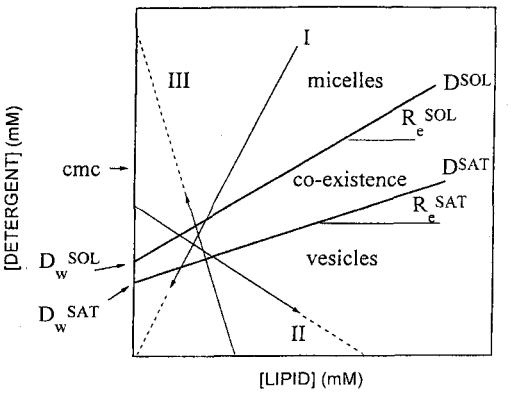

Fig. 1 Schematic description of the phase diagram of mixed lipid/surfactant systems. The bold lines describe the dependences of D^{SAT} and D^{SOL} on lipid concentration; The slopes of these lines represent the maximal value of R_e in vesicles (R_e^{SAT}) and the minimal value of R_e in mixed micelles (R_e^{SOL}), respectively; The intercepts of these lines (D_w^{SOL} and D_w^{SAT}) represent the respective (extrapolated) values of monomer concentrations; The lines denoted by I, II and III illustrate the three different protocols of ITC experiments as described in the text

Both D^{SAT} and D^{SOL} depend linearly on the lipid concentration L (e.g. Fig. 1). Hence, the phase boundaries can be characterized by the ratios R_e^{SAT} and R_e^{SOL}, which are given by the slopes of the dependencies of D^{SAT} and D^{SOL}, respectively, on L. These slopes represent the ratios between the concentrations of surfactant and lipid within the aggregates at the phase boundaries: R_e^{SAT} is the highest possible surfactant–to–lipid ratio in mixed bilayers, whereas R_e^{SOL} is the lowest surfactant–to–lipid ratio in mixed micelles.

In the range of coexistence of vesicles and micelles, the mixture contains bilayers of a composition given by R_e^{SAT} and mixed micelles whose composition is given by R_e^{SOL}. Hence, when the system contains N_D surfactant molecules (N_D^m in micelles, N_D^b in bilayers and N_D^w in the aqueous medium as monomers) and N_L lipid molecules (N_L^m in micelles and N_L^b in bilayers, without any lipid in water, $N_L^w=0$), distribution of the components between the different phases can be found from the relationships $N_D=N_D^m+N_D^b+N_D^w$, and $N_L=N_L^m+N_L^b$ accounting for

$$R_e^{SOL}=\frac{N_D^m}{N_L^m} \text{ and } R_e^{SAT}=\frac{N_D^b}{N_L^b}$$

The consequence of thermodynamic equilibrium of three different phases is that the aqueous concentration of surfactant monomers has to be constant in the range of coexistence. Hence, the intercepts of the graphs that describe D^{SAT} and D^{SOL} as functions of the lipid concentration L (Fig. 1) can be expected to be equal and to give the monomer concentration in the range of coexistence D_w^*, so that $D_w^{SAT} \cong D_w^{SOL} \cong D_w^*$.

In intuitive terms, this expectation is that in the limit of vanishing lipid concentration, the surfactant concentration required for the onset of solubilization will be equal to the concentration required for complete solubilization. Yet, in many studies the (extrapolated) values of D_w^{SAT} and D_w^{SOL} were found to be different and it is not clear whether these differences can be attributed to the experimental errors.

Given the small differences between D_w^{SAT} and D_w^{SOL}, and the thermodynamic expectation for their equality, we have assumed in our analysis of calorimetric data (given below) that the monomer concentration in the range of coexistence is indeed constant and equal to D_w^*. Accordingly, the number of surfactant monomers in water in the range of coexistence, N_D^w, is given by $N_D^w = VD_w^*$, where V is the volume of the aqueous solution.

In the ranges of pure phases, the partitioning of surfactant between vesicles and monomers (in the vesicular range) or between mixed micelles and monomers (in the micellar range) is a complex function of the physical properties of the surfactant and the lipid as well as their concentrations.

For the micellar range ($R_e > R_e^{SOL}$), it has been previously assumed that the aqueous concentration of monomers, D_w, remains constant and equal to its value in the range of co-existence, $D_w = D_w^*$. The composition of the mixed micelles, R_e^m, changes with variations of the total concentrations of the components, L and D, according to $R_e^m = (D - D_w^*)/L$. However, this assumption can not be exact, since even the value of D^{SOL} is, in most cases, significantly lower than the critical micelle concentration (*cmc*) of the pure surfactant (Fig. 1). Hence, when the total concentration of the surfactant D increases, D_w must increase from its value at the phase boundary (D_w^*) to the *cmc*. This increase has been systematically investigated in our recent calorimetric study [13].

In the vesicular range, it has been previously assumed that the partitioning of surfactant molecules between bilayers and the aqueous medium obeys a partition coefficient K, defined as

$$K = \frac{D_b}{D_w(L + D_b)}$$

where D_b is the concentration of surfactant that resides in bilayers. In terms of effective ratio ($R_e = D_b/L$), the partition coefficient has a form

$$K = \frac{R_e}{D_w(1 + R_e)}$$

It has been often assumed that K is a constant coefficient throughout the vesicular range, which means that the values D_w and R_e are related through K. More recent works, however, revealed that for any given system, K is in fact dependent on the actual composition of the mixture and is, therefore, a function of R_e and D_w [14]. This finding makes the notion of K much less useful for applications.

Structure, shape and size of mixed vesicles and micelles

First, it should be emphasized that mounting evidence indicates that the structure of equilibrated mixed aggregates appears to be governed by their composition (R_e^A) and independent of the total concentrations of lipid and surfactant in the dispersion. Note that the superscript A denotes aggregates, which will be later replaced by m or b according to the specific range considered.

In the vesicular range, increasing R_e^b results in surfactant-induced size growth of the vesicles, through a lipid transfer (disproportionation) mechanism [15], or, in the case of calcium-containing systems of phospholipid and cholate, fusion [16]. This general result is rather surprising for many reasons (for more comprehensive discussion see [17]).

Mixed micelles formed in the micellar range of most systems studied thus far have the shape of flexible cylinders. The mean length of these 'thread-like' micelles appears to be a decreasing function of R_e^m [18]. An alternative structure of lipid-rich mixed micelles can be that of discoidal, surfactant-containing fragments of bilayers whose perimeters are covered by surfactant molecules [19]. However, discoidal micelles can be expected in phospholipid–surfactant mixtures only under special conditions [20] although such micelles were observed in PC–cholesterol–bile salt mixtures [Kaplun *et al.*, in preparation].

Methodological aspects of vesicles–micelles transition

Upon hydration, most phospholipids form multilamellar vesicles (MLV). When these concentrically-arranged ('onion skin') bilayers are exposed to a surfactant, the surfactant molecules can initially partition only between the outermost bilayer and the aqueous medium. Subsequent equilibration can be very slow, particularly, at low surfactant concentrations [21]. For calorimetric studies, such slow processes are difficult to follow.

By contrast, rapid equilibration of surfactant–phospholipid mixtures can be achieved when the lipid is used either as mixed micelles with surfactant or as unilamellar vesicles, either small (SUV) or large (LUV). A more detailed description of these protocols is given below.

Thermodynamic studies of mixed systems of surfactants

The physics of self-assembly of surfactants in aqueous solutions can be studied at different levels of sophistication.

The major factor, driving this process for all amphiphiles and their mixtures is known for many decades as the hydrophobic effect [22]. At a qualitative level of understanding, the free energy of an amphiphilic molecule in water is higher than inside an aggregate, where the hydrophobic parts of all the molecules are shielded from aqueous solution by a layer of polar heads. As soon as the concentration of amphiphile monomers in water reaches a particular value, called *cmc*, a gain in the free energy due to aggregation prevails the related lose of the entropy of dilution of the monomers. As a result, aggregates effectively start to form.

Hence, the process of self-assembly is determined by the relationship between the free energy of a surfactant molecule in aqueous solution, μ_w^o, and inside an aggregate, μ_A^o. A quantitative information about this energetics is given by measurements of *cmc*, what has been performed for most of known surfactants. The value of *cmc* is a direct measure of $\delta\mu^o=\mu_A^o-\mu_w^o$ [22].

However, the *cmc* proved to be a convenient quantitative characteristics of self-assembly only for aqueous solutions of individual surfactants. Indeed, an individual surfactant forms aggregates of a particular type and can be characterized by a specific value of $\delta\mu^o$. By contrast, in an aqueous solution of a mixture of several surfactants, formation of mixed aggregates requires much more extended measurements and related analyses of energetics.

As we described above, self-assembly in mixed systems of surfactants is characterized by a phase diagram in terms of the concentrations of all components, rather than by just one value of *cmc*, as illustrated in Fig. 1.

Obviously, by contrast to the aqueous solution of an individual surfactant, the self-assembly in a mixed system is determined by the relationships between the energies of all components in all possible types of aggregates. In addition, these energies can depend on the compositions of the aggregates. A comprehensive description of this energetics requires joint experimental and theoretical research. Although some work has been done in this direction a complete characterization has not yet been achieved.

Thermodynamic characteristics of the mixture

We consider an aqueous solution of a mixture of two surfactants, one of which will be called lipid, and the second detergent. We will assume, according to the phase diagram (Fig. 1), that the lipid molecules are not soluble in water and can reside only in vesicles or mixed micelles. By contrast, the molecules of detergent have a markable solubility in water and, therefore, can exist as monomers in the aqueous solution, as well as form micelles and/or enter the mixed vesicles.

Let us recall that the three phases, present at the phase diagram are characterized by their compositions: the aqueous solution of surfactant monomers by its concentration D_w, the mixed micelles and vesicles by the ratios $R_e^m=N_D^m/N_L^m$ and $R_e^b=N_D^b/N_L^b$, respectively.

The equilibrium of the mixture is determined by an equation of state, relating the intensive thermodynamic variables. The variables changing along the phase

diagram and, therefore, relevant for the present study, are the compositions. Therefore, the equations of state determining the behavior of the mixture are given by the functions $R_e^m(D_w)$ in the micellar range of the phase diagram, $R_e^b(D_w)$ in the vesicular range and the relationships between the compositions of all three phases in the range of coexistence of micelles and vesicles.

The energetics of the process of self-assembly is determined by the energies of transition of the different components between the different phases. As our analysis relates to calorimetric measurements, we will consider the changes in enthalpy as characteristics of transition. The values relevant for our analysis are: the molar enthalpy of transition of detergent from mixed micelles to water, $\Delta H_D^{m-w}(R_e^m, D_w)$, the molar enthalpy of transition of detergent from mixed micelles to mixed vesicles, $\Delta H_D^{m-b}(R_e^m, R_e^b)$ and the molar enthalpy of transition of lipid from the mixed micelles to the mixed vesicles, $\Delta H_L^{m-b}(R_e^m, R_e^b)$. Note that all these enthalpies depend on the compositions of the corresponding phases and, consequently, can have different values for different points of the phase diagram.

Determination of the equation of state of the mixture and of the three enthalpies of transition in the whole range of compositions completely characterizes the energetics and equilibrium of the self-assembling mixture, and is, therefore, the aim of our research.

Protocols of isothermal calorimetric titration measurements

A major feature of the calorimetric titration measurement is that, at each step of titration, the composition of the mixture is changed in a controlled way. Accordingly, the system transforms to a new equilibrium, described by the new point on the phase diagram, as prescribed by the equation of state. This is accompanied by repartitioning of the components of the system between the available phases. The heat evolution resulting from these transformations is measured directly.

Three possible protocols of titration [23–26] are indicated by arrows in Fig. 1.

– Protocol I consists of continuous dilution of surfactant–lipid mixed micellar systems and consequent extraction of surfactant molecules from the mixed micelles into the diluting media and a consequent reduction of R_e^m. This, in turn, first leads to formation of longer thread-like mixed micelles, a subsequent phase transformation and, finally, continuous extraction of surfactant molecules from the resultant unilamellar vesicles. Under this protocol, a volume V_t of pure buffer is injected into the experimental cell of volume V_c. After equilibration and measurement of the heat, the same volume V_t is removed so that the initial volume V_c is restored. Since the result of this titration is a simple dilution of the mixture, the total concentrations of lipid and detergent, denoted by L and D, respectively, change as a function of the number n of the titration step according to

$$L = L_o\left(\frac{V_c}{V_c + V_t}\right)^n, \; D = D_o\left(\frac{V_c}{V_c + V_t}\right)^n \tag{1}$$

where L_o and D_o are the initial lipid and surfactant concentrations. The volume of one injection is much smaller than the volume of the cell, $V_t/V_c \ll 1$. The same volumes V_t and V_c (per one injection and of the experimental cell) are used also in the following protocols.

– Protocol II involves continuous addition of unilamellar vesicles into surfactant solution. This first results in solubilization (micellization) of the added phospholipid vesicles and a subsequent transformation of the mixed micelles into mixed vesicles due to simultaneous increase of lipid concentration and decrease of surfactant concentration.

Under this protocol the titrant contains lipid vesicles of a concentration c_L, so that the total concentration of lipid increases according to

$$L = L_o\left(\frac{V_c}{V_c + V_t}\right)^n + c_L\left[1 - \left(\frac{V_c}{V_c + V_t}\right)^n\right] \tag{2}$$

The detergent is diluted and the change of its concentration is given by (1).

– Protocol III consists of continuous titration of phospholipid unilamellar vesicles by a surfactant solution. This first results in mixed vesicles of increasing content of surfactant, subsequent solubilization of the surfactant-containing vesicles and further increase of R_e^m in the mixed micelles.

Under this protocol, the titrant contains detergent of a concentration c_D. Hence, the concentration of the lipid decreases according to (1), while the concentration of detergent in the cell increases according to

$$D = D_o\left(\frac{V_c}{V_c + V_t}\right)^n + c_D\left[1 - \left(\frac{V_c}{V_c + V_t}\right)^n\right] \tag{3}$$

Repartitioning of the molecules of detergent resulting from titration in the micellar and vesicular ranges

The main result of titration of the mixture in the micellar and vesicular ranges of the phase diagram is transfer of the molecules of detergent between the aqueous solution and the aggregates. Therefore, to evaluate the heat accompanying one step of titration, we have to determine the number of molecules of detergent transferred from the aggregates to water. The results of the following calculation apply to analysis of both micellar and vesicular ranges.

The main equation we use states that the total number of molecules of detergent N_D consists of $N_D^w = D_w V$ of monomers in water, where V is the total volume

of the system, and of the number of surfactant molecules in aggregates N_D^A, given by $N_D^A=N_L R_e^A$ where N_L is the total number of molecules of lipid. Hence,

$$D_w V + N_L R_e^A = N_D \tag{4}$$

One injection of titrant results, in the general case, in a change of the volume and of the total numbers of molecules of the two components

$$dV = V_t,\ \ dN_L = c_L V_t,\ \ dN_D = c_D V_t \tag{5}$$

Differentiating (4) and taking into account (5) we obtain a variation of aqueous concentration of surfactant monomers

$$dD_w = \frac{V_t}{V_c} \frac{c_D - D_w - R_e^A c_L}{1 + L\dfrac{dR_e^A}{dD_w}} \tag{6}$$

Using (5) and (6) we obtain that the change of the number of the molecules of detergent in the aggregates, $dN_D^A=d(R_e^A N_L)$, is given by

$$dN_D^A = V_t \left[(c_D - D_w) \frac{L\dfrac{dR_e^A}{dD_w}}{1 + L\dfrac{dR_e^A}{dD_w}} + c_L R_e^A \frac{1}{1 + L\dfrac{dR_e^A}{dD_w}} \right] \tag{7}$$

For the following analysis we need to express dN_D^A in terms of the changes of all the variables as functions of the injection number n. For that we insert $R_e^A=(D-D_w)/L$ into (7) and obtain

$$dN_D^A = V_t \left[(c_D - D_w) \left(1 - \frac{\dfrac{dD_w}{dn}}{\dfrac{dD}{dn} - \dfrac{D-D_w}{L}\dfrac{dL}{dn}} \right) + c_L \frac{D-D_w}{L} \frac{\dfrac{dD_w}{dn}}{\dfrac{dD}{dn} - \dfrac{D-D_w}{L}\dfrac{dL}{dn}} \right] \tag{8}$$

Using (8) together with (1)–(3), according to the protocol under consideration, we can express the heat resulting from each step of titration in terms of the molar enthalpies, $\Delta H_D^{m-w}(R_e^m, D_w)$, $\Delta H_D^{m-b}(R_e^m, R_e^b)$ and $\Delta H_L^{m-b}(R_e^m, R_e^b)$.

Determination of the equation of state and the specific heats of transfer in the micellar and vesicular ranges

In this section we introduce a scheme of determination of the thermodynamic properties of the mixture in the micellar and vesicular ranges by means of calorimetric measurements. To avoid using complicated equations, we will con-

sider separately the expected results for Protocols III and II. (Note that the results for Protocol I are just a special case of those for Protocols II and III).

Protocol III

According to this protocol, a mixture in the vesicular range of the phase diagram is titrated with a solution of detergent. For simplicity we first consider titration of the vesicles with a surfactant solution of a concentration c_D smaller than the *cmc*. As a result of equilibration, part of the injected molecules enter the mixed vesicles. The heat per mole of the molecules inserted into the aggregates is $-\Delta H_D^{A-w}(R_e^A, D_w)$, so that the resulting heat of one injection is

$$\Delta Q = -\Delta H_D^{A-w}(R_e^A, D_w)\mathrm{d}N_D^A \tag{9}$$

Equation (9) in fact describes the heat measured in the micellar as well as in the vesicular range of the phase diagram. The value of $\mathrm{d}N_D^A$ is given by (8), where c_L=0 has to be taken. The dependences of the total concentrations of lipid L and detergent D entering (8) are given by (1) and (3), respectively. Accounting for (8), (1), and (3), Eq. (9) can be rewritten in a form of a differential equation for the aqueous concentration of detergent as a function of the injection number,

$$\frac{\mathrm{d}D_w}{\mathrm{d}n} = \left[\frac{\mathrm{d}D}{\mathrm{d}n} - \frac{D - D_w}{L}\frac{\mathrm{d}L}{\mathrm{d}n}\right]\left[1 - \frac{\Delta Q(n)}{\Delta H_D^{A-w}(R_e^A, D_w)}\frac{1}{(D_w - c_D)}\right] \tag{10}$$

where $R_e=(D-D_w)/L$

Combining Eq. (10) with the results of calorimetric measurement, one can obtain the equation of state, $R_e^A(D_w)$, and the heat of transition of the detergent from aggregates to the aqueous solution $\Delta H_D^{A-w}(R_e^A, D_w)$ in the micellar and the vesicular ranges of the phase diagram, as follows:

i) The heat evolution resulting from one injection has to be measured as a function of the number of the titration step n at different detergent concentrations in the titrant, c_D. This yields a function $\Delta Q(n, c_D)$, which has to be inserted into Eq. (10).

ii) Although the function $\Delta H_D^{A-w}(R_e^A, D_w)$ is unknown in the beginning, a tentative form on this function has to be assumed and inserted into Eq. (10).

iii) Using the latter assumption and the experimentally-determined dependence $\Delta Q(n, c_D)$, (10) has to be solved to obtain the aqueous concentration of detergent after each injection $D_w(n, c_D)$.

iv) Using the result of the latter calculation, the composition of the mixed micelles $R_e^A(n, c_D)$ can be computed from the equation $R_e^A(n, c_D)=[D(n)-D_w(n, c_D)]/L(n)$.

v) Excluding the variable n from the obtained functions for D_w and R_e^A, we obtain the equation of state in a preliminary form $R_e^A(D_w, c_D)$. In a final form of the equation of state, the dependence on the concentration c_D must vanish. This can be achieved by improving the form of the tentative function $\Delta H_D^{A-w}(R_e^A, D_w)$ and repeating the whole calculation. The iterations of the function $\Delta H_D^{A-w}(R_e^A, D_w)$ have to be performed until the equation of state in its final form becomes independent on the surfactant concentration in the titrant c_D.

The resulting function $R_e^A(D_w)$ and $\Delta H_D^{A-w}(R_e^A, D_w)$ give a major contribution to a complete thermodynamic description of the system in the micellar and vesicular ranges.

The equation obtained can be easily generalized for the case of high detergent concentrations in the titrant, $c_D > cmc$.

Protocol II

Titration under Protocol II provides us with additional information to that obtained from the results gained through the use of Protocol III.

According to Protocol II, a micellar solution composed of surfactant and lipid is titrated with a solution of lipid vesicles. Each injection results in transition of all the lipid molecules of the titrant from the pure vesicles into mixed aggregates and in transfer of part of the surfactant from water to mixed aggregates (mixed micelles or mixed vesicles, depending on the range of the phase diagram). The resultant heat of one titration is

$$\Delta Q = -\Delta H_L^{A-b}(R_e^A, 1)c_L V_t - \Delta H_D^{A-w}(R_e^A, D_w)dN_D^A \tag{11}$$

where the number dN_D^A is given by (8) with $c_D=0$ and the total concentrations of the lipid L and detergent D are given by (2) and (1), respectively. For the micellar range the term $\Delta H_L^{A-b}(R_e^A, 1)$ represents the molar heat of transfer of lipid molecules from the mixed micelles to the pure lipid vesicles, $\Delta H_L^{m-b}(R_e^m, 1)$, while for the vesicular range this term relates to the molar heat of transition of the lipid molecules between mixed vesicles and pure lipid vesicles, $\Delta H_L^{b-b}(R_e^b, 1)$.

Analogously to the precedent case, we present (11) in the form of a differential equation for the aqueous concentration of surfactant monomers,

$$\frac{dD_w}{dn} = \left[\frac{dD}{dn} - \frac{D - D_w}{L}\frac{dL}{dn}\right]\left[\frac{\Delta H_D^{A-w}(R_e^A, D_w)D_w - \Delta H_L^{A-b}(R_e^A, 1)c_L - \dfrac{\Delta Q}{V_t}}{\Delta H_D^{A-w}(R_e^A, D_w)\left[D_w + c_L\dfrac{D - D_w}{L}\right]}\right] \tag{12}$$

Equation (12) can also be used for determination of the equation of state, $R_e^A(D_w)$, and of the molar heat of transition of detergent from the mixed aggregates to water, $\Delta H_D^{A-w}(R_e^A, D_w)$. Specifically, ΔQ has to be measured at several dif-

ferent lipid concentrations in the titrant c_L, and the data can then be treated as in the previous case of Eq. (10). If addition to the functions above (which can be taken from the results of Protocol III), Protocol II provides us with determination of the molar heat of transition of lipid between mixed aggregates of the two types and the pure lipid vesicles $\Delta H_D^{A-b}(R_e^A, 1)$.

Determination of the specific heats of transfer in the range of coexistence

Titration of the mixture in the range of coexistence results in transfer of surfactant molecules between the aqueous solution and the aggregates, as well as in a considerable exchange of the two amphiphiles between mixed micelles and mixed vesicles. Yet, the assumption that the aqueous concentration of the detergent remains constant and equal to D_w^*, simplifies the analysis. We derive below an expression for the heat of one titration for a general case of titrant containing both the lipid at a concentration c_L and detergent at a concentration c_D.

Let us recall that the compositions of mixed vesicles and mixed micelles in the range of coexistence are constant and denoted as R_e^{SAT} and R_e^{SOL}, respectively. The equations determining the distribution of the components between the different phases have the form

$$N_L^b + N_L^m = N_L \tag{13}$$

$$D_w^* V + R_e^{SAT} N_L^b + R_e^{SOL} N_L^m = N_D \tag{14}$$

According to (13), (14) and accounting for the changes in the total volume, and the total numbers of lipid and detergent molecules, as given by (5), we obtain for the changes in the numbers of molecules of lipid and detergent inside the micelles and the vesicles

$$dN_L^m = \frac{c_D - D_w^* - c_L R_e^{SAT}}{\Delta R} V_t \tag{15}$$

$$dN_L^b = \frac{c_L R_e^{SOL} + D_w^* - c_D}{\Delta R} V_t \tag{16}$$

$$dN_D^m = R_e^{SOL} \frac{c_D - D_w^* - c_L R_e^{SAT}}{\Delta R} V_t \tag{17}$$

$$dN_D^b = R_e^{SAT} \frac{c_L R_e^{SOL} + D_w^* - c_D}{\Delta R} V_t \tag{18}$$

where $\Delta R = R_e^{SOL} - R_e^{SAT}$.

The resulting heat accompanying one injection is

$$\frac{\Delta Q}{V_t} = [\Delta H_D^{m-w}(R_e^{SOL}, D_w^*)D_w^* - \Delta H_D^{m-w}(1, D_w^*)cmc] +$$

$$+ \frac{1}{\Delta R}[\Delta H_L^{m-b}(R_e^{SOL}, R_e^{SAT}) + R_e^{SAT}\Delta H_D^{m-b}(R_e^{SOL}, R_e^{SAT})D_w^* +$$

$$+ c_L\left[\Delta H_L^{b-b}(1, R_e^{SAT}) + \frac{R_e^{SAT}}{\Delta R}[\Delta H_L^{m-b}(R_e^{SOL}, R_e^{SAT}) + R_e^{SOL}\Delta H_D^{m-b}(R_e^{SOL}, R_e^{SAT})]\right] +$$

$$+ c_D[\Delta H_D^{m-w}(1, D_w^*) - \Delta H_D^{m-w}(R_e^{SOL}, D_w^*)] -$$

$$- c_D\frac{1}{\Delta R}[\Delta H_L^{m-b}(R_e^{SOL}, R_e^{SAT}) + R_e^{SAT}\Delta H_D^{m-b}(R_e^{SOL}, R_e^{SAT})] \quad (19)$$

In the first term of (19) we take into account that the concentration of the detergent in the titrant c_D can be higher than the *cmc*, so that after injection the pure micelles undergo monomerization, which is related to the molar heat of transition $\Delta H_D^{m-w}(1, D_w^*)$.

The first two terms in the total heat (19) are independent of the concentrations in the titrant (c_D and c_L), the third contribution is proportional to the concentration of lipid c_L, and the last two contributions are proportional to the concentration of detergent c_D in titrant. This feature of (19) allows to design experimental investigations of all the molar heats of transitions of components inside the range of coexistence through determination of ΔQ for several values of c_L and c_D, according to the Protocols II and III, respectively. Consideration of the slopes and intercepts of the dependencies of ΔQ on c_L and c_D, allows to determine the values of the molar heats. It is important to recall that in addition to the heat of transfer of detergent between aqueous solution and the aggregates, in the range of coexistence we can determine also the heat of transition of lipid and detergent between the mixed vesicles and the mixed micelles, $\Delta H_L^{m-b}(R_e^{SOL}, R_e^{SAT})$, and $\Delta H_D^{m-b}(R_e^{SOL}, R_e^{SAT})$.

Overview of previous results

The results obtained in previous work, conducted with low sensitivity ITC did not contribute much to our understanding of the energetics of the phase behavior of lipid–surfactant mixtures [27–31].

More recently, some parts of the general scheme described above have been studied by high sensitivity ITC. Heerklotz *et al.* [24, 25] studied the heat evolution for a mixture of palmitoyl oleylphosphatidylcholine (POPC) lipid bilayers and a series of nonionic surfactants $C_{12}E_n$. Both amphiphiles in the mixture where practically insoluble in water, so that their partitioning between aggre-

gates and the aqueous solution has been neglected (i.e. D_w=0). According to Eqs (10) and (12), under these conditions there is no need to solve differential equations and the molar heats of transition of the two amphiphiles between the different aggregates can be obtained from the measurements at just one set of values of concentrations c_L and c_D in the titrant. For this specific case, the molar heats have been determined as functions of the chain length of the surfactant. The measurements with $C_{12}E_8$ [24] revealed that the values of the molar heats of transition of lipid, ΔH_L^{m-b}, and detergent, ΔH_D^{m-b}, from micelles to vesicles have opposite signs. Specifically, transformation of lipid from curved micelles to flat bilayers is exothermic whereas transition of the surfactant into flat bilayers is endothermic. This result is a quantitative proof of a qualitative expectation based on a tendency of surfactant and lipid to form different types of aggregates: lipid molecules have lower energy inside the flat bilayers, while molecules of surfactant tend to form spontaneously strongly curved micelles. This tendency is often expressed in terms of an effective shape of amphiphilic molecules [32]. The changes in all molar heats of transition have been studied as functions of the chain length n in the series $C_{12}E_n$ [25].

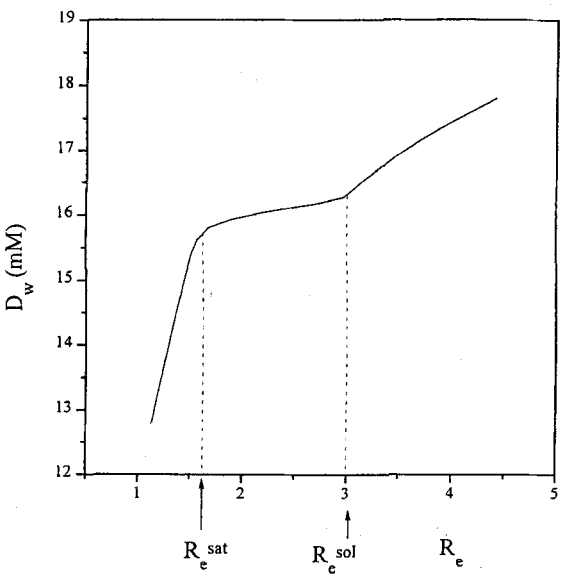

Fig. 2 The dependence of D_w on R_e obtained by Opatowski *et al.* [13]. It has been assumed that the heat evolution is essentially due to extraction of OG from OG/PC mixed aggregates and that the heat associated with this process is equal to ΔH=1700 cal mol^{-1} [33] and is independent of the shape and composition of the mixed aggregates;
The dependence of D_w on R_e has been computed for different values of monomer concentration in the PG/PC mixed micellar system prior to dilution according to protocol I. The line given in this figure was chosen so as to yield the best fit with the experimetally-observed values of R_e^{SAT} and R_e^{SOL}

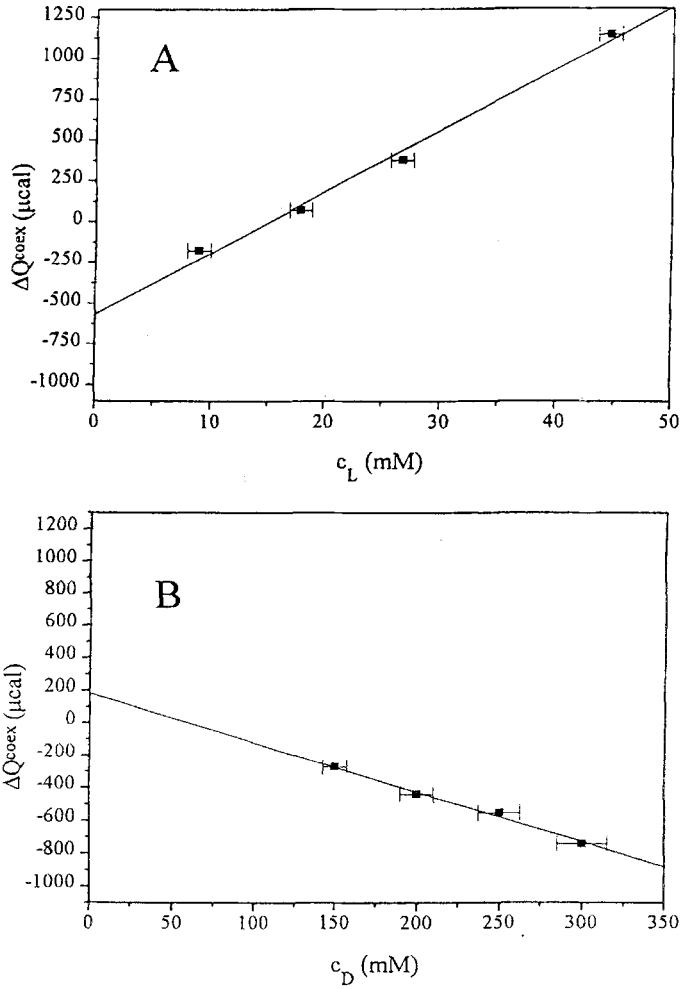

Fig. 3 According to Eq. (19) and under the simplifying assumptions of our previous studies, the dependence of ΔQ on c_L (Protocol II) is given by

$$\frac{\Delta Q}{V_t} = \Delta H_D^{m-w} D_w^* + \frac{1}{\Delta R}(\Delta H_L^{m-b} + R_e^{SAT}\Delta H_D^{m-b}) D_w^* + c_L \frac{R_e^{SAT}}{\Delta R}(\Delta H_L^{m-b} + R_e^{SOL}\Delta H_D^{m-b})$$

whereas the dependence of ΔQ on c_D (Protocol III) is given by

$$\frac{\Delta Q}{V_t} = (D_w^* - cmc)\Delta H_D^{m-w} + \frac{1}{\Delta R}(\Delta H_L^{m-b} + R_e^{SAT}\Delta H_D^{m-b}) D_w^* - c_D \frac{1}{\Delta R}\Delta H_L^{m-b} + R_e^{SAT}\Delta H_D^{m-b}$$

The experimentally obtained dependencies were

$\Delta Q = -563\ \mu cal + c_L 37.48\ \mu cal\ mM^{-1}$ and $\Delta Q = 182\ \mu cal - c_D 3.03\ \mu cal\ mM^{-1}$

for Protocols II and III, respectively;

The experimentally obtained slopes and intercepts were used to evaluate the four unknown thermodynamic characteristics of the mixture given in the text

In our previous study [13], we have used protocol I (titration with pure buffer, c_D=0, c_L=0) to evaluate the equation of state of the mixture of the lipid phosphatidylcholine (PC) and the nonionic surfactant octyl glucoside (OG). Assuming that the major contribution to the measured heat is due to extraction of OG from OG/PC mixed aggregates and that the heat associated with extracting OG molecule from these mixed aggregates is independent of their shape and composition, we obtained the dependence D_w on R_e^A described in Fig. 2. The function $D_w(R_e^A)$, obtained in this study, has a reasonable form, increasing in the micellar and vesicular ranges and remaining nearly constant in the range of coexistence. However, a more exact form of the equation of state derived as indicated in the present study is needed for detailed analysis of the energetics of the mixture.

In another study [26] we have used Protocols II and III to investigate the molar heats of transition in the range of coexistence in PC/OG mixture according to the model given by Eq. (19). However, in that work, we have made several simplifying assumptions, that might be significant. First, we have neglected the heat of transition of the lipid molecules between pure and mixed vesicles $\Delta H_L^{b-b}(1, R_e^{SAT})$. Second, we assumed that the heat of transition of detergent molecules from the micelles to the aqueous solution does not depend on the composition of the micelles, so that $\Delta H_D^{m-w}(R_e^{SOL}, D_w^*) = \Delta H_D^{m-w}(1, D_w^*)$. The resulting values of the heat of transition in the range of coexistence (Fig. 3) are $\Delta H_L^{m-b}(R_e^{SOL}, R_e^{SAT})$= -592 cal mol^{-1}, $\Delta H_D^{m-b}(R_e^{SOL}, R_e^{SAT}) = 645$ cal mol^{-1} and $\Delta H_D^{m-w}(R_e^{SOL}, D_w^*)$= -1732 cal mol^{-1}. These values support the qualitative expectation in terms of the effective shape of the amphiphiles. In addition, the values of *cmc* of the pure detergent has been found to be *cmc*=23.5 mM, in accord with previous measurements [33–35].

The scheme proposed in this work allows to improve the results of the precedent studies lifting the main approximations and assumptions involved in the analysis, and to obtain a considerable amount of the new information.

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