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Review

Phase boundaries in mixtures of membrane-forming amphiphiles and micelle-forming amphiphiles

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Abstract

The phase behavior of mixtures of phospholipids and detergents in aqueous solutions is an issue of basic importance for understanding the solubilization and reconstitution of biological membranes. We review the existing knowledge on the compositionally induced reversible transformation of phospholipid bilayers into lipid-detergent mixed micelles. First, we describe the experimental protocols used for preparation of such mixtures and emphasize the scope and limitations of the various techniques used for evaluation of the microstructures of the self-assembled amphiphiles in the mixture. Subsequently, we interpret the existing data in terms of the spontaneous curvature of the amphiphiles and the finite size of the mixed micelles. These considerations lead to a general description of the phase behavior, which forms the basis for a rational approach to solubilization and reconstitution experiments.

Keywords: Detergent; Phospholipid; Membrane; Vesicle; Micelle; Solubilization; Reconstitution

1. Introduction

The most common use of detergents in biomembrane studies is based on their ability to solubilize the membranes by forming mixed micelles with the membrane lipids and proteins [1–3]. The essence of this process is a compositionally induced transformation of nearly flat phospholipid bilayers (containing embedded proteins) into mixed micelles composed of a detergent, phospholipids and membrane-bound proteins [4–8]. This bilayer-to-micelle transition can be reversed by removing the detergent from the mixed micelles either by dialysis, absorption to biobeads or simply by diluting the solution [6–10]. The mixed membrane bilayers restored by this procedure are referred to as reconstituted membranes.

These phase transformations depend in a complex and interrelated fashion on many factors, including the molecular structure of the detergent, the composition of the membranes, the composition of the aqueous medium and the temperature. Furthermore, the structure of the mixed micelles formed upon solubilization of the membranes, and, to a greater extent, the structure of the reconstituted membranes, depend on the details of the experimental protocols, namely on kinetic factors [11,12]. Yet, 'the baseline' for any investigation of membrane-detergent interactions is the phase diagram that describes the thermodynamically stable aggregates present in detergent-phospholipid mixed systems as a function of the aqueous concentrations of the components.

The character of the phase diagram is determined

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by the molecular structure of the two components. Both the phospholipids and the detergents are amphiphiles and, consequently, tend to self-assemble in aqueous solutions [13]. However, the shape of the aggregates formed by the two components is very different and depends on the effective molecular shape of each of them. Specifically, the molecular shape of long chain phospholipids is nearly cylindrical, meaning that the area of molecular cross-section is about equal to the molecular volume/length ratio. Such molecules tend to aggregate so as to form flat surfaces [14–16]. By contrast, the molecular shape of most of the commonly used ('head-tail') detergents is conical, with relatively large head groups (i.e. their cross-section areas are larger than the volume/length ratio). Accordingly, these amphiphiles tend to aggregate along curved surfaces [14–16].

The tendency to curve is commonly described in terms of the spontaneous curvature of the amphiphile (C_o) [16], which can be seen as the curvature of the surface along which an amphiphile tends to pack [15,16]. Most phospholipids are characterized by a very low spontaneous curvature ($C_o \approx 0$) whereas the spontaneous curvature of most detergents is of the order of $1/l$, where l is the length of the hydrophobic chain. Accordingly, detergents form micelles whereas long chain phospholipids form lamellar structures in aqueous solutions.

In relation to mixed aqueous solutions of phospholipids and detergents, the first question to be addressed is whether the two amphiphiles form mixed aggregates or, alternatively, each compound self-assembles on its own, avoiding mixing with amphiphiles of different spontaneous curvature. If mixed aggregates are formed, we want to know their structure. Are they represented by flat bilayers, similar to pure phospholipid aggregates, or do they have a micellar structure, dictated by the detergent?

Many experimental studies, conducted by a large variety of methods, have shown that phospholipids and detergents form mixed aggregates in aqueous solutions and that the structure of these aggregates is either micellar or lamellar, depending on the concentrations of the two compounds [6,7]. For any given phospholipid concentration, its mixture with a detergent contains lamellar aggregates at relatively low detergent concentrations, micellar aggregates at relatively high detergent concentrations and mixtures of these two types of aggregates in an intermediate range of detergent concentrations denoted as the range of coexistence [17,18].

A typical phase behavior of aqueous solutions of phospholipid-detergent mixtures is illustrated in Fig. 1. The two solid lines in Fig. 1 depict the concentrations of detergent (D_t) required for the onset (D_t^{sat}) and completion (D_t^{sol}) of the solubilization of phospholipid as a function of the phospholipid concentration, L. The lines $D_t^{sat}(L)$ and $D_t^{sol}(L)$ represent the phase boundaries separating the phase of lamellar aggregates, the region of coexisting lamellar and micellar aggregates and the phase of pure micelles. In all regions of the phase diagram, the aggregates coexist with a non-aggregated (monomeric) detergent of concentration D_w. For most studied lipid/detergent systems, both these phase boundaries $D_t^{sat}(L)$ and $D_t^{sol}(L)$ appear to be linear functions of L over a relatively large range of lipid concentration L:

$$D_t^{sat} = D_w^{sat} + L \cdot R_e^{sat} \quad (1)$$

$$D_t^{sol} = D_w^{sol} + L \cdot R_e^{sol} \quad (2)$$

The intercepts of the latter two lines, D_w^{sat} and D_w^{sol}, can be regarded as the constant concentrations of detergent monomers at the lower and upper phase boundaries, respectively. The slopes R_e^{sat} and R_e^{sol} characterize the composition (in terms of detergent/lipid ratio) of the coexisting mixed bilayers (R_e^{sat}) and mixed micelles (R_e^{sol}), respectively. This interpretation implies that the compositions of the coexisting aggregates do not change throughout the whole range of coexistence.

This issue contains detailed reviews, written by several of the major contributors to this field of research, on the experimental methods used in studying lipid-detergent mixtures, and on the phenomenology of solubilization and reconstitution of lipid bilayers and protein-containing membranes. In this review, we limit ourselves to the presentation of those general concepts regarding the phase boundaries in lipid/detergent mixtures that we find essential for a rational approach to the use of detergents in membrane solubilization and reconstitution. Accordingly, we first attempt to familiarize the reader with the problematic nature of the experimental determination of the phase diagrams by describing briefly some of the experimental procedures used for the preparation of

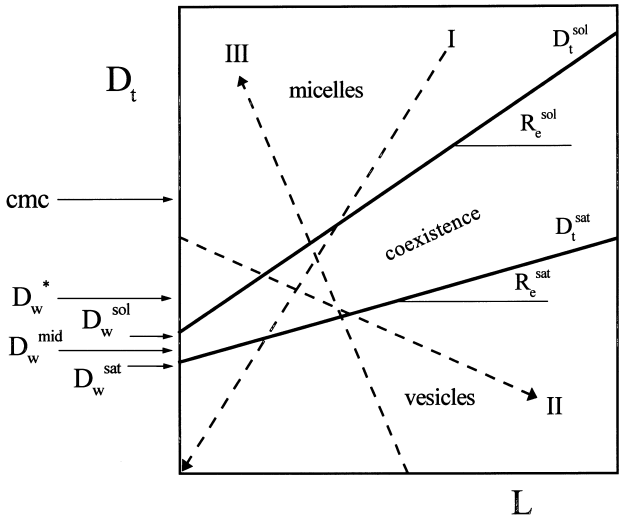

Fig. 1. Schematic phase diagram of a mixed lipid-detergent system. The bold lines depict the detergent concentration required for the onset of lipid solubilization (D_t^{sat}) and the detergent concentration required for complete solubilization (D_t^{sol}) as a function of the lipid concentration (L). Accordingly, these two lines are the boundaries of the range of lipid and detergent concentrations where mixed vesicles coexist with mixed micelles. In most of the published phase diagrams the straight lines intersect the detergent axis at different concentrations ($D_w^{sat} < D_w^{sol}$). The broken lines assigned I, II and III relate to three protocols used for preparation of lipid-detergent mixed systems, as described in the text.

mixed amphiphilic systems. Next, we relate to the general scope and to the limitations of the methods used in the investigation of the phase diagrams.

Subsequently, we present the experimentally obtained phase diagrams of several phospholipid-detergent mixtures. These results were originally interpreted in terms of the generally accepted model described above, which is based on the assumption that the effective ratio R_e governs not only the state of aggregation in phospholipid-detergent mixtures (hence, the phase boundaries) but also the equilibrium size and shape of the aggregates. The latter interpretation implies that the equilibrium state of aggregation in the lipid-detergent mixtures is independent of the absolute concentrations of the lipid and detergent, in apparent disagreement with some of the experimental results. To resolve this apparent inconsistency, we present a more general thermodynamic treatment of the phase behavior of lipid-detergent systems taking into account the finite size of the mixed micelles. This treatment predicts that at relatively low concentrations, the phase behavior of the mixture of amphiphiles depends not only on the effective ratio, R_e but also on the absolute concentrations of the components. This explains several experimental observations that have not been understood until recently.

Nevertheless, in spite of the considerable progress of our understanding of the basic questions concerning the phase behavior of phospholipid-detergent mixtures in aqueous solutions, many issues remain unclear. These include (i) the phase behavior of mixtures that contain few different phospholipids with varying chain length and head groups, (ii) the effects of proteins, lipoproteins and lipopolysaccharides on the phase behavior, and (iii) the partial (and maybe selective) solubilization of different membrane components by detergents in such heterogeneous dispersions. Much more research is required to clarify these issues. We have therefore chosen not to relate to these open questions in the present review, hoping that the effort devoted to them in many laboratories will clarify these issues in the foreseeable future.

2. Experimental procedures used for the preparation of mixtures of amphiphiles

Isothermal phase diagrams, in general, describe the phases present in a system composed of several components as a function of the concentrations of the various components at equilibrium. Preparation of equilibrium systems composed of phospholipids and surfactants is complicated by the very limited solubility of the phospholipids as explained below. To understand this complexity it is important to describe the experimental protocols that can be used to mix these two components in aqueous solutions. These include the following.

(1) Addition of a detergent solution to multilamellar phospholipid vesicles (MLV). MLVs are spontaneously formed upon hydration of a pure, semisolid phospholipid film (commonly prepared by evaporation of the solvent from a solution of the phospholipid in an organic solvent) [19–21]. The initial product of this procedure of adding a detergent solution to these aggregates is a co-dispersion of MLVs and detergent micelles and/or monomers. Subsequently, the detergent partitions between the outermost phos-

pholipid bilayers and the solution and if the detergent/lipid ratio in these bilayers exceeds a critical value, they transform into mixed micelles, exposing the next bilayer to detergent [11].

When the overall detergent/lipid ratio is higher than a critical value, a series of relatively slow sequential processes of 'peeling' of bilayers follows, via continuous repartitioning of the detergent between the aqueous solutions, detergent-containing lipid bilayers and lipid-detergent mixed micelles. Eventually, mixed micelles are formed at equilibrium, but this is likely to be a slow process, depending on the actual concentrations and on the lamellarity of the MLV [11]. This procedure is even more problematic when the total detergent/lipid ratio is insufficient for complete solubilization. In this case, the detergent to lipid ratio in the outermost bilayers may be sufficient for solubilization of these bilayers but the solubilized lipid may subsequently undergo revesiculation due to repartitioning of detergent to the next exposed bilayer. Thus, equilibration may be very slow and yield vesicles of different lamellarities and sizes, which do not necessarily represent a 'true' state of equilibrium.

(2) Dissolving the two components in a common organic solvent, so as to form a mixture on the molecular level, *and subsequently evaporating the solvent and dispersing the dried mixture in an aqueous solution* [11]. Mixtures of the two components formed by this procedure are likely to be closer to equilibrium than mixtures formed by other protocols. Notably, however, when water is added to the dry film, the initially formed aggregates may be multilamellar and equilibration of the system via subsequent 'extraction' of detergent from these aggregates into the diluting aqueous medium and repartitioning of detergent between bilayers and mixed micelles may, again, be slow.

(3) Mixing a detergent solution with a dispersion of unilamellar vesicles, preformed by any of the available methods [20,21]. When detergent is added to vesicles at sufficiently high concentrations to cause complete solubilization of the vesicle bilayers, the transformation of the lamellar into micellar structures is rapid and the resultant micelles are likely to represent a state of equilibrium [11]. By contrast, at subsolubilizing detergent concentrations the product of mixing the two components depends on the initial size of the vesicles. The general trend is that the detergent partitions between the bilayers and the aqueous solution according to a partition coefficient (see below) and that this partitioning results in growth of the mean vesicle size [22–30].

When the detergent is added to large unilamellar vesicles (LUV; diameter approx. 100 nm) this size growth can be fully accounted for by the increase in the surface area of the vesicles due to the introduction of detergent molecules into the vesicle bilayers [26]. A similar size growth was observed when low detergent concentrations were added to small unilamellar vesicles (SUV) [24]. However, at higher, yet subsolubilizing detergent concentrations many detergents have been shown to induce size growth of SUV into much larger vesicles, whose sizes depend on the detergent/lipid ratio [6–10,22–26,31]. Thus, addition of each of several detergents to SUVs of diameters of 25–35 nm made by sonication, extrusion or detergent removal from mixed micelles, resulted in LUVs of diameters of about 100 nm (Fig. 2).

The mechanism responsible for this detergent-induced vesicle size growth has been investigated for several detergent/lipid mixtures. As an example, addition of subsolubilizing concentrations of the nonionic detergent octyl glucoside (OG) to egg PC SUVs resulted in rapid size growth of the vesicles [24] via a 'disproportionation mechanism' [25]. This mechanism consists of a series of repartitioning processes through which the larger vesicles in the population grow in size by absorbing the lipid initially contained in the smaller vesicles in the population [24]. Following equilibration, the mean size of the vesicles exhibited a sigmoidal dependence on the OG/PC ratio in the vesicles. Notably, dilution of these equilibrated solutions resulted in extraction of detergent from the vesicles. As a result, the OG/PC ratio in the vesicles decreased but the size decreased only slightly (only due to the extraction of detergent). Similar results were obtained for several other detergent-lipid mixed systems, including PC/cholate mixtures. In the latter case equilibration was much slower than in PC/OG mixtures although the size growth probably occurs via the same mechanism [22].

One question that remains open thus far is whether the size of the vesicles formed upon detergent addition to unilamellar vesicles represents an

equilibrium size (see below). Yet, the occurrence of this size growth should be taken into consideration in any study of the phase behavior and particularly with enzymatic studies conducted with detergent/lipid mixtures (see below; [32]). Furthermore, the rate of detergent-induced vesicle size growth is an important determinant of the size of vesicles formed upon detergent removal from mixed micelles. Thus, infinite dilution of mixed micelles results in the formation of SUV because the R_e of the resultant vesicles is very low [33] so that detergent-induced size growth is limited. By contrast, when size growth is not much slower than removal of detergent (e.g. during dialysis) the initially formed small vesicles grow according to their initial (relatively large) R_e and when R_e decreases (upon continuing detergent removal) their size remains large [6,7].

Another interesting feature of the effect of detergents on the size of vesicles is that although at the onset of solubilization the vesicles are large, in the range of coexistence, small vesicles (SUV*) have also been observed [34–36]. In a recent publication, Ueno et al. [34] proposed that these vesicles are metastable and that the detergent/lipid ratio in them is higher than R_e^{sat}. The latter hypothesis is questionable because repartitioning of detergent between vesicle bilayers and the aqueous medium is likely to be rapid. An alternative explanation for the presence of small vesicles in mixtures of LUV and detergents is that such vesicles are formed via partial solubilization of LUVs followed by revesiculation of part of the resultant mixed micelles. The latter process probably yields SUVs, which tend to grow into LUVs but the latter process may be slow, so that SUV can be observed hours after preparation of the mixture.

(4) Titration protocols. In view of the problematic nature of the various procedures used for the preparation of mixed dispersions, we propose to adopt one or more of the following experimental protocols [37,38], schematically depicted in Fig. 1.

Protocol I: Stepwise dilution of mixed micelles. Micellar systems are likely to equilibrate rapidly when detergent is extracted from the mixed micelles into a diluting medium. As long as the addition of the diluting medium does not cause transformation of mixed micelles into mixed vesicles, the change in the composition of mixed micelles results in relatively small and rapid changes in the size of the mixed micelles. Further dilution results in partial transformation of mixed micelles into vesicles. Subsequently, all the mixed micelles become transformed into vesicles whose size after equilibration of the dispersion depends not only on thermodynamic factors but also on kinetic factors. When the rate of extraction of detergent from the vesicles is slow, the vesicles are likely to undergo size growth (see below), which is not the case during rapid detergent removal.

Protocol II: Addition of unilamellar vesicles into detergent solutions containing detergent monomers and micelles. Under the conditions of this protocol, the added vesicles become exposed to an excess of detergent so that all the added vesicles become solubilized. Both the composition and the size of the resultant mixed micelles are likely to depend only on the composition of the dispersion, because equilibration of mixed micellar systems is rapid, so that kinetic factors cannot be expected to play a major role. Further addition of vesicles results in their micellization until the detergent/lipid effective ratio becomes lower than R_e^{sol}. Thereafter, the added vesicles become saturated with detergent (i.e. their composition is given by R_e^{sat}) whereas some of the mixed micelles, whose composition is given by R_e^{sol}, become transformed into detergent-saturated vesicles of a composition R_e^{sat}. Finally, after all the mixed micelles transform into mixed bilayers, further addition of phospholipid vesicles results in 'extraction' of detergent from detergent-lipid mixed vesicles into the added 'pure' lipid vesicles. This repartitioning can be expected to result in a heterogeneous population of vesicles because those vesicles that were formed at early stages, when the effective ratio was high (within or close to the range of coexistence), are likely to be large and remain large when detergent is 'extracted' from them, whereas the small vesicles added at later stages will have lower effective ratios and will therefore be smaller. Re-equilibration is likely to be very slow. Hence, the size distribution of vesicles formed through this procedure can be expected to be wide and dependent on the experimental details, particularly on the rate of mixing.

Protocol III: Addition of a detergent solution into a dispersion of unilamellar vesicles. This protocol has been described in terms of a three stage model [4,13]. According to this model, when subsolubilizing detergent concentrations are added to the vesicles,

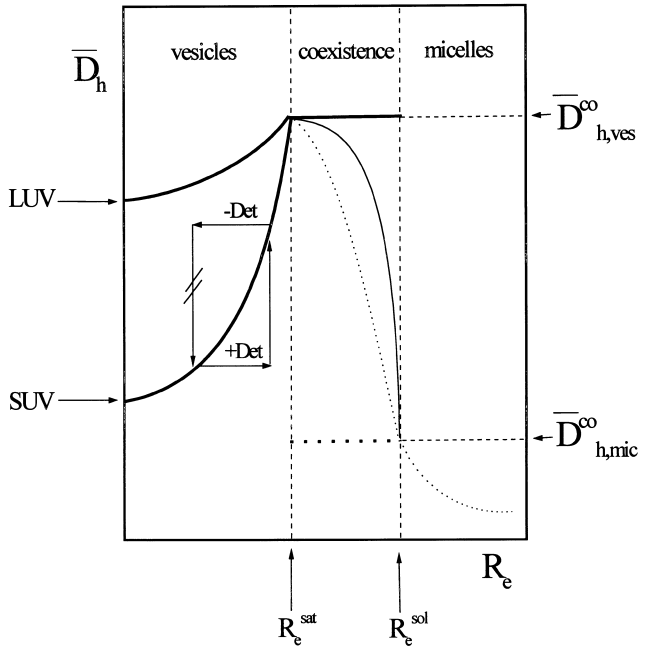

Fig. 2. Schematic description of the size of the lipid-detergent mixed aggregates as a function of their composition. The hydrodynamic diameters of the vesicles formed upon addition of a detergent to SUV and LUV are described as functions of R_e, the ratio of detergent to lipid in mixed aggregates $R_e = (D_b + D_m)/L$. Note that within the vesicular range addition of detergent to small vesicles results in size growth whereas extraction of detergent from detergent-containing large vesicles has only a slight effect on their size. Within the range of coexistence, vesicles of a composition given by R_e^{sat} and a constant hydrodynamic diameter $D^{co}_{h,ves}$ coexist with mixed micelles of a composition given by R_e^{sol} and a constant apparent hydrodynamic radius $D^{co}_{h,ves}$. Increasing R_e within this range results in increased concentration of micellar lipid, L_m, on the expense of vesicular lipid, L_b. This results in a reduction of the scattering of light, as illustrated by the dotted line and, at higher R_e values in reduction of the apparent size of the particles present in the dispersion, as detected by DLS and depicted by the full line.

the detergent partitions between the bilayer and the aqueous solutions; at higher detergent/lipid ratios, the vesicles disintegrate into micelles and at yet higher R_e values, the resultant micelles become smaller as they become poorer in phospholipid. As described above, detergent addition to small vesicles results in their growth. Hence, when detergent is slowly added to vesicles of any size and the effective ratio approaches R_e^{sat}, further addition of detergent results in partial transformation of large, detergent-saturated vesicles into mixed micelles, regardless of the initial size of the vesicles. Within the range of coexistence, the dispersion probably contains only vesicles of a composition R_e^{sat} and micelles of a composition R_e^{sol} but the dispersions are very heterogeneous with respect to the existing microstructures [39]. Furthermore, the aggregation state in this range is a function of the experimental details, being especially sensitive to the rate of detergent addition and to the time of equilibration. In all the cases, further addition of detergent results in complete solubilization. Thereafter, addition of detergent results in increased effective ratio, $R_e > R_e^{sol}$, and the resultant 'thread-like' micelles become progressively shorter.

3. Methods used in structural investigations, scope and limitations

While the various methods are addressed in several of the reviews in this volume, we wish to make a few general comments of relevance to the determination of the phase boundaries. First, it is essential to realize that the only data of a direct nature come from electron microscopy (EM). This underlines the importance of EM in the characterization of the microstructures that exist in any of the mixed detergent/lipid systems. However, fixation of a specimen for observation by EM may affect the system [40]. Although this is less likely to occur with specimens fixed by vitrification than with negatively stained or freeze fractured specimens, quantitation of EM results remains problematic [40]. Given these difficulties, less direct but more quantitative techniques should be used in conjunction with EM. Scattering of X-rays (SAXS) and neutrons (SANS) added much to the existing data on the microstructures of both vesicles and cylindrical thread-like micelles [41–43]. Yet, most of the data available on the phase boundaries is based on light-scattering measurements. Specifically, vesicles scatter, in general, much more light than micelles. Hence, most of the light scattered from mixed systems containing vesicles and micelles is scattered by vesicles. As a consequence, increasing R_e within the range of coexistence results in decreased intensity of scattered light while the apparent hydrodynamic radius, as observed by dynamic light scattering (DLS, QLS), remains almost constant (Fig. 2).

This makes DLS measurements particularly suit-

able for determination of the completion of solubilization because as long as the system contains large (non-solubilized) vesicles the DLS measurement is likely to indicate that such particles are present. By contrast, NMR measurements are very sensitive to solubilized (micellar) lipid, which yield narrow NMR resonances. This makes this technique particularly suitable for determination of the onset of solubilization because even when a very small fraction of the lipid becomes micellar, it results in easily detectable narrow NMR signals [43–45]. In relating to the results obtained by NMR spectroscopy, it is important to note that NMR measurements require higher concentrations than those necessary for light-scattering measurements. Yet, comparison is possible for millimolar phospholipid concentrations, so that phase diagrams based on the combined use of QLS and NMR can be regarded as being more precise than diagrams obtained by other methods.

The results based on other spectroscopic (mostly spectrofluorimetric [28,44–47]) and calorimetric [37,48–51] methods can also be used. Nonetheless, a combination of turbidity measurements, aimed at gaining approximated values for the phase boundaries, with QLS and NMR data is quite sufficient for precise determination of the phase boundaries of equilibrated systems.

4. Equilibrium and metastable structures of the aggregates existing in 'pure' phases

Characterization of lipid-detergent mixed aggregates by any spectroscopic method commonly assumes that not only the phase boundaries but also the physical properties of the mixed aggregates are governed by their composition. Specifically, when mixtures of different compositions exhibit similar physical properties, it is assumed that the mixed aggregates present in them are of the same composition. As an example, when the molar absorbance (in OD units/M) is described as a function of the detergent concentration for different lipid concentrations (as illustrated for one lipid concentration in Fig. 2), the detergent concentration above which additional detergent causes marked reduction of turbidity can be described as a function of lipid to yield estimation of R_e^{sat}. In other words, this treatment assumes that the composition of vesicles at the onset of solubilization is given by R_e^{sat}, and that these vesicles exhibit the highest molar turbidity, independent of lipid composition. Similar treatment of the detergent concentration above which additional detergent has only a slight effect on the turbidity can be used to estimate R_e^{sol}. Furthermore, in the range of coexistence, the molar turbidity is assumed to reflect the fraction of vesicles in the population, whereas in the regions of 'pure' phases, any given molar turbidity is assumed to correspond to a given composition R_e. A similar approach has been used to evaluate the composition that relates to any other specific property of the mixed aggregates [6–10,44,52–55].

In the pure 'vesicular range', i.e. at $R_e > R_e^{sat}$, the vesicles have been characterized with respect to the composition dependence of their size, as well as of the motional state within the bilayers and of the integrity of the membranes, as reflected by leakage of entrapped solutes. All these composition-dependent properties may also depend on kinetic factors. As discussed above, kinetic factors are important in determining the size of detergent-lipid mixed vesicles prepared by any of the available methods. Furthermore, given the dependence of the physical properties of bilayers on their curvature, kinetic factors are likely to affect the other properties of detergent-containing vesicles.

In this context, it is important to note that based on the presently available data we cannot relate to the size of vesicles formed via detergent-induced size growth (e.g. Fig. 2) as representing a state of equilibrium. A reasonable alternative is that the 'real' state of equilibrium of phospholipids (in the absence of detergent) is that of LUVs, in which the curvature energy is negligible. SUVs, formed through investment of energy, are metastable, at least below the phase transition temperature (T_m) [20,56]. However, above T_m they are 'long lived', probably because there is no efficient mechanism through which they can grow. Detergent addition may provide such a mechanism if it is assumed that detergents enhance the 'off rate' of phospholipids from the vesicles, whereas size growth leads to reduction of the 'off rate'. Under these assumptions the finding of the dependence of size on R_e can be explained by competition between these two apposite tendencies, because increasing R_e yields larger vesicles until the 'off rate'

becomes sufficiently low to stop the size growth. It is also consistent with the finding that reduction of R_e by detergent removal does not have marked effects on the size of the vesicles.

Nonetheless, the large vesicles with little detergent may also be metastable and kinetically trapped by the lack of an efficient mechanism for size decrease. This possibility implies that the sigmoidal dependence of $\tilde{D}_h$ on R_e (Fig. 2) may still represent equilibrium systems. This possibility is supported by the fact that the dependence of size on R_e, as obtained for PC/cholate mixtures, was independent of the presence of calcium ions in the solution [23]. Specifically, in the presence of Ca^{2+}, the cholate-induced size growth of SUV occurs rapidly, via a fusion mechanism, as opposed to the slow disproportionation mechanism that is responsible for the size growth in the absence of calcium ions. Yet, the dependence of the mean size on R_e is very similar to that observed in the absence of Ca^{2+} [23], which supports the view that the size of the vesicles is governed by thermodynamic factors. The lack of effect of detergent removal on the size of the detergent-containing vesicles in equilibrated systems may therefore mean that the system remains 'kinetically trapped'.

The integrity of detergent-containing vesicles is another issue of apparent inconsistencies. The mere presence of any of the most studied detergents in bilayers results in fluidization of the bilayers, probably due to detergent-induced reduction of lipid-lipid interactions. Charged detergents may further interfere with the lipid packing, while also interfering with vesicle-vesicle interactions and by that contributing to the stability of the vesicles against aggregation and fusion. In many experiments, solutes entrapped within vesicles leaked out of them upon detergent addition. This is obviously an expected result of size growth via disproportionation (entrapped solutes leak out of disintegrated vesicles [24]). However, leakage also occurs under conditions where no size growth occurs (e.g. at low R_e or upon detergent addition to LUV). In the latter cases, the leakage appeared to depend on R_e not only in terms of the rate of leakage but also with respect to the apparent equilibrium of the retained, entrapped solute [57].

In our view, if the leakage induced by addition of detergent to the vesicles is only partial and a considerable amount of the entrapped solutes remain inside the vesicles for a long time, the mixed lipid-detergent membranes are not leaky in their equilibrium state. The membrane leakage occurs only in a transition state in the course of detergent insertion and equilibration in the membranes. In terms of this interpretation, the amount of solute that leaks out of the vesicles prior to equilibration is an increasing function of R_e because annealing of the bilayers after detergent addition is likely to depend on how much detergent became accumulated. All the entrapped solutes left in the vesicles by the end of this annealing process are likely to remain entrapped, unless the equilibrated bilayers are leaky. Perforated vesicles were in fact observed by EM not only in the range of coexistence of mixed vesicles and micelles but also in the vesicular range at relatively high R_e values (close to R_e^{sat}). Thus detergents form permanent 'holes' in the bilayer only when the bilayer is almost saturated with detergent.

The structure of 'pure' mixed micelles (at $R_e > R_e^{sol}$) is usually cylindrical. In fact, for many years it has been assumed that the most favorable mode of aggregation of detergent-phospholipid micelles is disc-like. The rational of this 'disc model' was that in such aggregates the 'curvophobic' phospholipids assemble as a flat discoidal bilayer, the perimeter of which is covered by the 'curvophilic' detergent [58,59]. While this arrangement has been first proposed for mixtures of phospholipids and bile salts, the mixed micelles composed of phospholipids and other detergents (e.g. Triton X-100) were similarly described as oblate ellipsoids in which the less curved surface is rich in phospholipids whereas the more curved surface is rich in detergent [59–61]. The original 'disc model' was later modified to a 'mixed disc model' [62] that 'allows' bile salts to reside in the disc (not only on its perimeter), which made the model more similar to the 'oblate model'. Both these models were supported by a close agreement between the theoretically derived dependence of micellar size on composition and QLS data. However, closer examination of scattering results yielded better agreement between the experimental results and a cylindrical model [63–65], in agreement with HPLC analysis [66] and with direct observation of long thread-like mixed micelles by cryo-TEM [36,37,67].

The preference of the cylindrical (over discoidal) structure can be understood by considering the interplay between the entropy of mixing the two amphiphiles within an aggregate, on one hand, and the bending elasticity of the amphiphilic monolayer, on the other [68]. This analysis shows that the entropy of mixing is sufficiently large to prevent a large gradient of detergent concentrations between the flat part and the strongly curved perimeter of a discoidal mixed micelles. As a result, formation of such mixed micelles is much less favorable than expected earlier. In contrast, a difference in shapes between the edges and the main body of a cylindrical micelle is less pronounced than that in a discoidal one. In addition, the unfavorable edges (end caps) of cylindrical micelles involve significantly fewer molecules than the rims of the disc-like micelles. As a result, under regular conditions, solubilization of lipid bilayers is predicted to yield cylindrical rather than disc-like micelles [68], in agreement with the experimental results.

Similar considerations can be raised to explain the decrease of the length of cylindrical micelles upon increasing the relative concentration of the detergent (increasing R_e). It is well established that the average aggregation number $\bar{n}$ of cylindrical micelles is determined by the interplay between the translational entropy of micelles in aqueous solution, on one hand, and the energy of end caps, on the other. The former factor tends to produce a large number of short micelles, whereas the latter favors a small number of larger micelles [69]. As a result, $\bar{n}$ and, hence, the average micellar length l, are proportional to the exponent of $2\varepsilon/kT$, where 2ε is the energy of creating two end caps of a cylindrical micelle, also called the scission energy [69,70]. Addition of 'curvophilic' detergent molecules favors formation of end caps, decreasing the end cap energy ε, and by that decreasing the micellar length l.

5. Thermodynamic considerations

Phase diagrams have been constructed to describe the self-assembly of many detergent/phospholipid mixed systems. Common to all these systems, solubilization of a given phospholipid at a given concentration L begins when the system contains a critical concentration of detergent D_t^{sat}. Below this concentration, the system consists of detergent-containing vesicles in equilibrium with detergent monomers in the solution. The partitioning of detergent molecules between the bilayers and the solution is given by a partition coefficient

$$K = \frac{D_b}{D_w(L + D_b)} \tag{3}$$

where D_b and D_w are the concentrations of detergent within the bilayer and in monomeric form, respectively [24]. Eq. 3 can be rewritten as

$$\frac{1}{K} = D_w\left(\frac{1}{R_e} + 1\right) \tag{4}$$

where R_e is the detergent/lipid ratio in the vesicles $R_e = D_b/L$. This equation defines the relationship between the concentration of the monomers in the solution and the composition of the vesicles

$$KD_w = \frac{R_e}{R_e + 1} \tag{5}$$

If it is assumed that micelle formation occurs when the bilayer reaches the maximal level of detergent that it can accommodate (given by R_e^{sat}), it follows that the onset of solubilization occurs when

$$D_w^{sat} = \frac{1}{K}\frac{R_e^{sat}}{1 + R_e^{sat}} \tag{6}$$

Accordingly,

$$R_e^{sat} = \frac{KD_w^{sat}}{1 - KD_w^{sat}} \tag{7}$$

Alternatively, micellization may be expected to begin when the solution becomes saturated with respect to detergent monomers, i.e. when D_w reaches the critical micellar concentration (cmc). For most detergent/lipid systems studied thus far $D_w^{sat} < \text{cmc}$, which means that the lipid promotes micellization of the detergent [6]. This also means that the value of R_e^{sat} indeed represents the maximal accommodation of detergent in the bilayer because the saturation of the bilayers by detergents occurs at sub-cmc detergent concentrations.

The completion of solubilization can also be described in similar (saturation) terms, namely R_e^{sol} may be understood as being that value of R_e where the detergent micelles are saturated by phospholipids. A

given surfactant at a given concentration can solubilize up to a maximal phospholipid concentration, given by $L = D_b^{sol}/R_e^{sol}$. At higher detergent concentrations (higher R_e values), all the phospholipid will reside in lipid/surfactant mixed micelles of varying composition, size and shape, whereas just below R_e^{sol}, mixed micelles of a composition given by R_e^{sol} (the minimal value of R_e in micelles) will coexist with vesicles of a composition given by R_e^{sat} (the maximal value of R_e in vesicles). Using the notion of a partition coefficient of detergent between mixed micelles and the aqueous solution, the critical composition of the micelles can be related to the corresponding concentration of the detergent monomers, D_w^{sol}:

$$R_e^{sol} = \frac{KD_w^{sol}}{1-KD_w^{sol}} \tag{8}$$

It has been shown for many systems that not only D_w^{sat} but also D_w^{sol} is smaller than the cmc. This indicates that the maximal solubility of monomeric detergent in the water, the cmc being larger than the D_w^{sol}, cannot be the determining factor of R_e^{sol}. This must mean that R_e^{sol} is determined by a more basic, fundamental property of the system.

According to the commonly accepted phenomenological concept described above, the phase state of the mixture as well as the aqueous concentration of the surfactant monomers D_w are determined solely by the ratio R_e between the concentrations of surfactant, D_A, and of lipid, L_A, inside the aggregates (bilayers and/or micelles)

$$R_e = \frac{D_A}{L_A} \tag{9}$$

Within this model, the lower phase boundary corresponds to saturation of the bilayers at a constant ratio $R_e = R_e^{sat}$ given by Eq. 1 whereas the upper phase boundary corresponds to mixed micelles saturated by lipid at constant ratio $R_e = R_e^{sol}$ given by Eq. 2. According to this interpretation, the compositions of both the mixed bilayers R_e^{sat} and the mixed micelles R_e^{sol} are given by the slopes of the straight lines in Fig. 1, whereas the related aqueous concentrations of the surfactant monomers D_w^{sat} and D_w^{sol} are given by the intercepts of the two apparently linear phase boundaries.

To explain the thermodynamic background of this phenomenological model, we first define the three possible subsystems that constitute the mixture: (i) mixed vesicles (considered separately from the aqueous solution) indicated by a superscript 'b', (ii) mixed micelles (also considered separately from the water) indicated by the superscript 'm', and (iii) the aqueous solution of the surfactant monomers indicated by the superscript 'w'. Assuming that each of these subsystems is a real thermodynamic phase, it follows that the chemical potentials of the surfactant, μ_D, and of the lipid, μ_L, in each of the subsystems are determined only by the intensive thermodynamic variables of this subsystem [68,71,72]. The intensive variables characterizing the compositions of the three subsystems are the detergent-to-lipid ratios R_e^b and R_e^m for the vesicles and the micelles, respectively, and the concentration D_w for the aqueous solution of the surfactant monomers.

This consideration means that the vesicles are regarded as one extended closed bilayer with composition R_e^b, while all the micelles are represented by just one thread-like micelle with composition R_e^m, which is so long that the inhomogeneity in its structure due to the two end caps can be neglected [68,71,72]. Under these assumptions we neglect all the possible effects of separating the bilayer and the micelle into smaller aggregates and of repartitioning of the aggregates in the available aqueous volume.

These assumptions result in several desirable relationships. In the lower part of the phase diagram mixed vesicles coexist with surfactant monomers so that the chemical potential of surfactant is equal for these two subsystems,

$$\mu_D^w(D^w) = \mu_D^b(R_e^b) \tag{10}$$

Solution of Eq. 10 determines the function $D^w(R_e^b)$ for the vesicular phase.

Analogously, equality of the chemical potential of surfactant in the micellar phase

$$\mu_D^w(D^w) = \mu_D^m(R_e^m) \tag{11}$$

yields the relationship $D^w(R_e^m)$ for the upper part of the phase diagram.

And, finally, consideration of the conditions of coexistence between mixed vesicles and micelles, corresponding to the composition induced transition be-

tween them, requires the equality of the surfactant chemical potential in all three subsystems,

$$\mu_D^w(D^w) = \mu_D^b(R_e^b) = \mu_D^m(R_e^m) \quad (12)$$

while the chemical potential of lipid must be equal in the vesicles and micelles (it is commonly assumed for simplicity that lipid is not solvable in water),

$$\mu_L^b(R_e^b) = \mu_L^m(R_e^m) \quad (13)$$

Solution of the three independent equations given by Eqs. 12 and 13 yields the values of D^*_w, $R^{b*}_e = R_e^{sat}$ and $R^{m*}_e = R_e^{sol}$ which represent the compositions of the three phases in the range of coexistence between them. Hence, the major features of the phenomenological model can be explained by simple assumptions. The model based on these assumptions predicts that the phase boundaries are characterized by constant ratios R_e^{sat} and R_e^{sol} and, accordingly, are represented by straight lines on the phase diagram.

However, one important issue that still poses a challenge relates to the aqueous concentrations of the surfactant monomers at the phase boundaries, D_w^{sat} and D_w^{sol}. These values have been evaluated from measurements of the phase behavior of different combinations of amphiphilic compounds using various experimental methods. In all these studies, the phase boundaries were experimentally determined from measurements over a limited range of lipid concentrations L and the data were interpreted under the assumption that both D_t^{sat} and D_t^{sol} depend linearly on L (Eqs. 1 and 2, respectively). The intercepts of the straight lines that describe these dependences to $L=0$ were assumed to represent D_w^{sat} and D_w^{sol}. In most of these studies D_w^{sat} was lower than D_w^{sol} (Fig. 1). The difference between D_w^{sat} and D_w^{sol} was always small compared to their absolute values, the actual difference varied between experiments, but the finding that $D_w^{sol} > D_w^{sat}$ was reproducible. For example, for the mixture of PC and OG, $D_w^{sol} \approx 16$ mM whereas $D_w^{sat} \approx 15.5$ mM [18].

These differences are inconsistent with the above thermodynamic background which implied that the aqueous concentration of surfactant D^*_w has to be constant throughout the whole region of the vesicles-micelles coexistence, including the phase boundaries. In other words, according to the model above $D_w^{sat} = D_w^{sol} = D^*_w$, which in terms of the phase diagram (Fig. 1) must mean that upon extrapolation of the straight lines that represent the phase boundaries to $L=0$, these lines should intersect the detergent concentration axis in the common point D^*_w, in disagreement with experimental results.

As noted above, both D_w^{sat} and D_w^{sol} are smaller than the cmc of the detergent (Fig. 1). This can be intuitively explained as being due to the presence of lipid which constitutes an additional driving force for micellization. It also means that the lines describing D_t^{sat} and D_t^{sol} as a function of lipid concentration, L, are meaningful only when L exceeds a minimal value above which bilayers can be formed. Irrespective of the behavior of lipid-detergent mixtures below this extremely low lipid concentration, the main challenge is to explain why the two apparently linear lines that describe the phase boundaries do not intersect the detergent axis at the same point. In other words, the challenge is to explain why D_w changes in the range of coexistence.

In an attempt to resolve this challenge, we have further developed the treatment of aqueous mixtures of lipids and surfactants by removing one of the main assumptions of the previous model, namely that the micelles are a separate thermodynamic phase of a constant composition. We have explicitly accounted for the finite size of the thread-like micelles, as well as for their repartitioning in the aqueous volume and for the effects of the end caps. These considerations [69] lead to a different physical picture regarding the effects of the translational entropy of micelles in the aqueous solution. According to this picture, the chemical potentials in the micellar phase depend not only on the surfactant-to-lipid ratio R_e^m, but also on the absolute aqueous concentrations L^m and D^m of the amphiphiles constituting the micelles. We have shown that the phase boundaries deviate from straight lines and that this deviation becomes pronounced in the range of low lipid concentrations where the micelles become relatively short. We have also demonstrated that the real shape of the phase boundaries deviates from straight lines and that this deviation is the reason for the apparent difference between D_w^{sol} and D_w^{sat} observed in the previous studies.

These theoretical considerations are briefly described below. In the range of high lipid concentrations, the mixed micelles that are formed upon par-

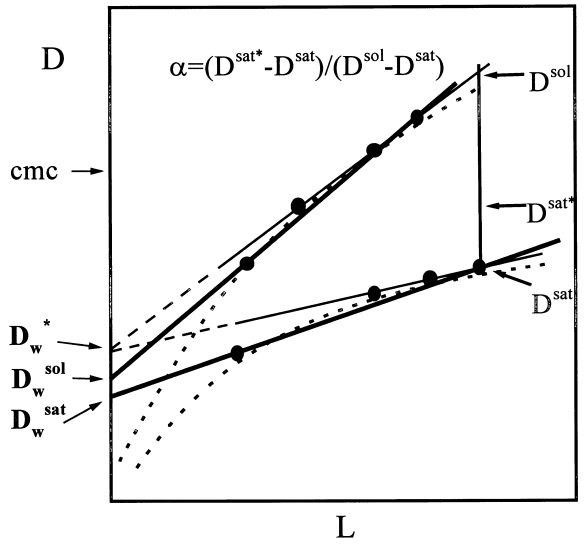

Fig. 3. Illustration of the phase boundaries in lipid-detergent mixed dispersions. The detergent concentrations required for the onset (D^{sat}) and completion (D^{sol}) are plotted as functions of the lipid concentration. Note that the bold lines intersect the detergent axis (at D_w^{sat} and D_w^{sol}) below D^*_w, which is the common concentration of monomers in aqueous solutions at high absolute concentrations of lipid and detergent. The broken lines illustrate the expected non-linear dependence (see text for details).

tial solubilization of the vesicles by added detergent have a composition given by R_e^{sol} and are very long so that the contributions of both the excess energy of the end caps and the translational entropy of the micelles can be neglected. Only under these conditions the micelles can be regarded as a true phase, the concentration of monomer throughout the range of coexistence of micelles and vesicles will remain constant and extrapolation of the two lines that describe D_t^{sat} and D_t^{sol} to zero lipid concentration will yield one value for D_w (D^*_w in Fig. 3).

In the range of lower lipid concentrations the micelles formed upon solubilization are shorter and the contributions of their translational entropy and of the excess energy of the end caps (2ε per micelle) have to be considered. A model-independent theoretical treatment [69] based on general thermodynamic considerations reveals that at low lipid concentrations, where the mixed micelles are of a finite size, the concentration of monomeric detergent can be expected to be lower than D^*_w. At the onset of solubilization of a given lipid concentration L, the monomer concentration is

$$D_w^{sat} = D_w^* - \frac{A_0}{\alpha \cdot \sqrt{L} \cdot e^{2\varepsilon/kT}} \quad (14)$$

whereas upon complete solubilization

$$D_w^{sol} = D_w^* - \frac{A_0}{\sqrt{L} \cdot e^{2\varepsilon/kT}} \quad (15)$$

In both these equations, A_0 is a constant and α (in Eq. 14) is a fraction ($0 < \alpha < 1$).

These results can be expressed in terms of the concentration-dependent deviations of D_w^{sat} and D_w^{sol} from D^*_w, which is the value expected for D_w when the mixed micelles are infinity long cylinders. Both these deviations (ΔD_w^{sat} and ΔD_w^{sol}) are inversely proportional to $\sqrt{L}$ and both decrease upon increasing the end cap energy ε (e.g. upon increasing the detergent's chain length). However, since α is a frac-

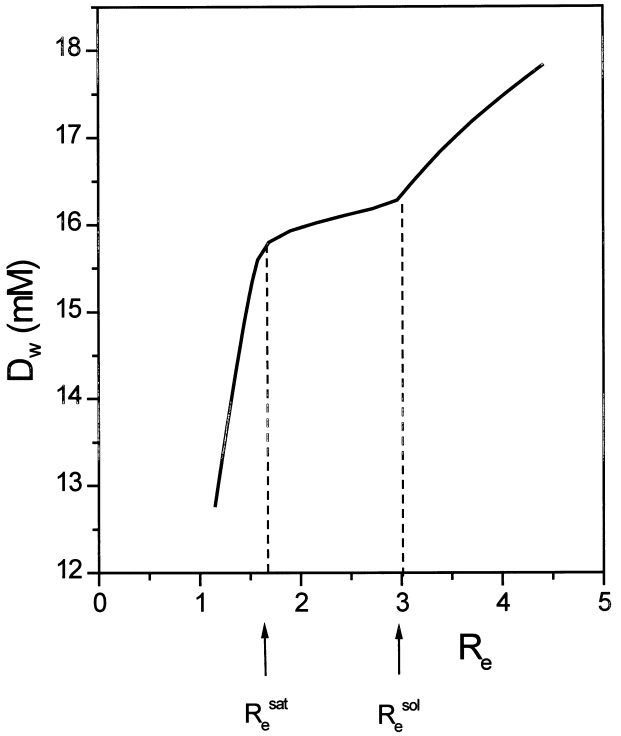

Fig. 4. An approximate dependence of the concentration of detergent monomers (D_w) in mixtures of PC and the non-ionic detergent OG on the OG/PC ratio in mixed aggregates (R_e). The dependence is based on isothermal calorimetric titration of OG/PC mixed micelles according to protocol I (Fig. 1), as interpreted [18] under the assumption that the main contribution to ΔQ is due to extraction of OG monomers from OG/PC mixed aggregates (with constant $\Delta H_D^{a \to w}$).

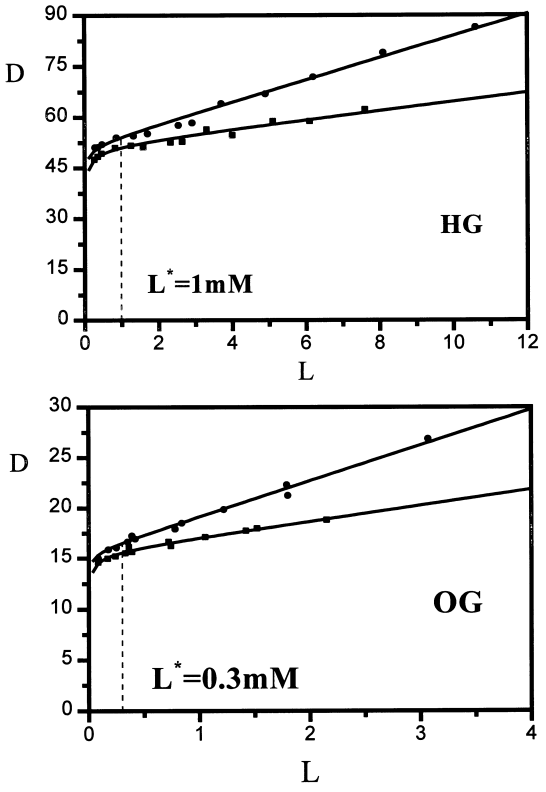

Fig. 5. Phase diagrams of HG/PC (upper panel) and OG/PC (lower panel) as obtained from calorimetric measurements [68]. The lines were fitted according to Eqs. 14 and 15 (see text for details).

tion, ΔD_w^{sat} is larger than ΔD_w^{sol} (Fig. 3). This means that when the phase diagram is based on measurements in the range where the effects of finite size of micelles cannot be neglected, the apparent value of D_w^{sol} can be expected to be larger than that of D_t^{sat}.

This is apparent even at relatively high lipid concentration, as shown in Fig. 4 for about 2.5 mM PC, in which we present the dependence of D_w on R_e for OG/PC mixtures on the basis of our calorimetric studies [18].

This prediction of the theoretical results is consistent with all the published phase diagrams. Furthermore, the theory predicts that the deviation from straight lines depends through the end cap energy ε on the effective molecular curvature of the surfactant, which for detergents with the same head group is determined by the length of the alkyl chain. Specifically, the lipid concentration below which the effects of the finite size will become pronounced (L^*) is predicted to become lower upon increasing the chain length. This prediction was verified experimentally for the two alkyl glucosides heptyl glucoside (HG) and OG (Fig. 5).

6. Possible relevance of the phase behavior for the use of detergents in biochemical and biophysical studies

The kinetic and structural aspects described above should be considered in the design of any biochemical and biophysical experiment in which detergents are used for solubilization of phospholipids, membrane proteins and biological membranes as well as for the preparation of reconstituted membranes (proteoliposomes). A few points of concern are briefly discussed below.

First, we wish to consider the choice of detergent. In studying issues of relevance to lipids in the digestive system the obvious choice is to use bile salts, which are the naturally occurring detergents of the native system of relevance [59] and the only question is which bile salt to use. By contrast, when a detergent is to be used for solubilization and reconstitution of membranes, the choice is much larger and many aspects have to be considered.

The most important consideration in the choice of a detergent for solubilization of a given membrane is whether the detergent causes denaturation of the membrane proteins. In general, non-ionic detergents are considered less harmful to proteins than either anionic or cationic surfactants. Alkyl saccharides and alkylpolyethoxyethylenes are the most commonly used non-ionic surfactants as they are considered 'mild' with respect to their effect on proteins. However, quite unfortunately, the choice between these detergents is still empirical and in several cases a given detergent is used because past experience with this detergent was relatively successful and other detergents were not even considered.

Another factor to be considered is the 'solubilizing power' of the detergents, which can be expressed through the number of detergent molecules required for solubilization of one phospholipid molecule. In these terms, the solubilizing power is inversely related to R_e^{sol}. Categorization of a detergent as being a 'strong' or a 'weak' detergent on the basis of R_e^{sol} (or R_e^{sat} [73]) is of very limited value with respect to the question of which detergent is the best [73]. First

of all, in dilute dispersions of membranes, the detergent concentration required for solubilization is governed by D_w^{sol}, which is close to the cmc of the detergent. Secondly, a low value of R_e^{sol} means that solubilized membrane proteins reside in mixed micelles with relatively little detergent (and much naturally occurring phospholipids). This, however, does not necessarily mean that a detergent with relatively low R_e^{sol} is less harmful to proteins.

In fact, several of the most commonly used detergents are characterized by a very high R_e^{sol} and high cmc. As an example, the cmc of the non-ionic detergent OG is about 24 mM, D_w^{sol} of this detergent is about 16 mM and R_e^{sol} (for egg PC) is about 3. This means that solubilization of 1 mM PC (in mixed micelles of OG/PC ratio of 3:1) requires at least 19 mM OG. Yet, OG is one of the most commonly used detergents in solubilization and reconstitution of biological membranes. This can be attributed to the 'mild nature' of OG with respect to its effects on membrane proteins and to the ease of its removal from detergent-lipid-protein mixed systems.

The ease of detergent removal is of special importance in reconstitution experiments. Specifically, studying a membrane protein is commonly based on a sequence of four consecutive steps: (i) solubilization of the membrane, in the form of detergent-lipid-protein mixed micelles under conditions that each micelle contains either one protein molecule or none, (ii) purification of the studied protein, which is most commonly conducted by affinity chromatography, (iii) enrichment of the mixed micelles made of the studied protein, endogenous phospholipids and detergent with exogenous phospholipids of choice, added to the protein-containing mixed micelles as phospholipid-detergent mixed micelles, and (iv) reconstitution of the studied protein into phospholipid-protein vesicles (proteoliposomes). The latter step, which is essential for evaluation of both the function of membrane proteins and of the lipid-protein interactions, requires removal of the detergent, either by extensive dialysis or by detergent absorbency to biobeads. In this respect, a high cmc, which makes the complete removal of the detergent easier, is a great advantage.

The unavoidable conclusion is that the choice of a detergent for solubilization and reconstitution experiment must still be largely empirical. Yet, several generalizations can be made.

(i) Only 'mild' detergents, which are not likely to cause denaturation of proteins, should be considered.

(ii) Among these detergents, those with low R_e^{sat} but relatively high cmc, which are easy to remove, should be preferred.

(iii) When a detergent is to be used for solubilization of membranes, its concentration should be only slightly higher than that required for obtaining mixed micelles with the lowest possible R_e^{sol}. Using higher detergent concentrations results in mixed micelles in which the protein resides in a detergent-rich environment. To ensure that the minimal (yet sufficient) concentration of the detergent is employed, it is possible to titrate the membrane preparation with detergent until all the membranes are solubilized, as can be judged from light-scattering measurements (preferably DLS).

(iv) To reduce protein denaturation by the detergent used for solubilization, it is important to avoid prolonged exposure of the protein to the detergent. Accordingly, following solubilization, it is important to employ rapid purification methods, mix the purified protein-containing mixed micelles with phospholipid-detergent mixed micelles and remove the detergent from the resultant (rapidly equilibrated) mixed micelles as quickly as possible. This can be done either by adding biobeads to the mixed micellar system, which absorb the detergent, or simply diluting the system so as to reduce the effective ratio (R_e) from its initial value (preferably just above R_e^{sol}) to values below R_e^{sat} [7]. Infinite dilution (i.e. dilution in a large volume of the medium) will result in proteoliposomes with low R_e values. Hence, this is likely to result in small but rather homogeneous proteoliposomes [33]. By contrast, if the dilution or removal of detergent by biobeads is to R_e values just below R_e^{sat}, the resultant proteoliposomes grow in size. A second step of dilution or removal of residual detergent by dialysis does not cause reduction of the size of the proteoliposomes. Detergent removal by dialysis of mixed micellar structures (unlike removal of residual detergent from detergent-containing proteoliposome) is likely to result in a heterogeneous population of proteoliposomes because the initially formed liposomes (formed at R_e just below R_e^{sat}) may or may

not undergo size growth, depending on the rate of detergent removal [7].

Another issue of importance is the use of detergent in the solubilization and reconstitution of lipids for preparation of mixed micelles, mixed vesicles or pure phospholipid unilamellar vesicles. For all these experimental protocols, it is advisable to avoid using multilamellar vesicles because equilibration of a mixture of these aggregates with added detergent is slow and likely to result in heterogeneous mixed aggregates. The preferred simplest alternatives for preparing mixed micelles are either to mix the detergent solution with unilamellar vesicles made by sonication or extrusion, or to co-sonicate the lipid-detergent mixtures. Mixed micelles of varying detergent/lipid ratios (varying R_e values) and varied size can be made either by first preparing mixed micelles with relatively low R_e values (just above R_e^{sol}) and subsequently adding more detergent or by preparing mixed micelles with relatively high detergent content (high R_e) and subsequently removing detergent from the mixed aggregates (e.g. by dilution). As long as R_e is kept above R_e^{sol} the system will contain only mixed micelles whose size is determined by R_e, namely by the detergent/lipid ratio in the mixed micelles.

Accordingly, when enzymatic reactions are studied in which the micellar phospholipid is the substrate, it is impossible to assess separately the effects of size and composition because the size is governed by the composition [32]. However, in such studies, it is important to investigate the dependence of the reaction kinetics on the concentration of the substrate. For such investigations, it is important to prepare mixed micelles with a constant composition (R_e) and size, and varying phospholipid concentration. This can be done by diluting a mixed micellar solution of a given composition in a detergent-containing medium. Specifically, a given mixed micellar solution contains micelles of a given composition (R_e) and a given concentration of detergent monomers (D_w). If this mixture is diluted in a medium containing only monomers at the same concentration D_w, the composition (and size) of the mixed micelles will be retained and the mixed systems will only differ in the concentration of the mixed micelles, namely in the concentration of the solubilized lipid [32].

Investigation of the kinetics of enzymatic reactions in which the substrate is vesicular phospholipid is more complex because in such vesicles it is possible to investigate separately the effects of the detergent content of the vesicles (R_e), the effect of vesicle size (in the absence or presence of detergent) and the effect of the lipid concentration [32]. Protocols that can be used for these investigations are schematically illustrated in Fig. 6. Each of the experimental protocols depicted in this figure consists of a sequence of two steps of dilution of a mixed micellar solution.

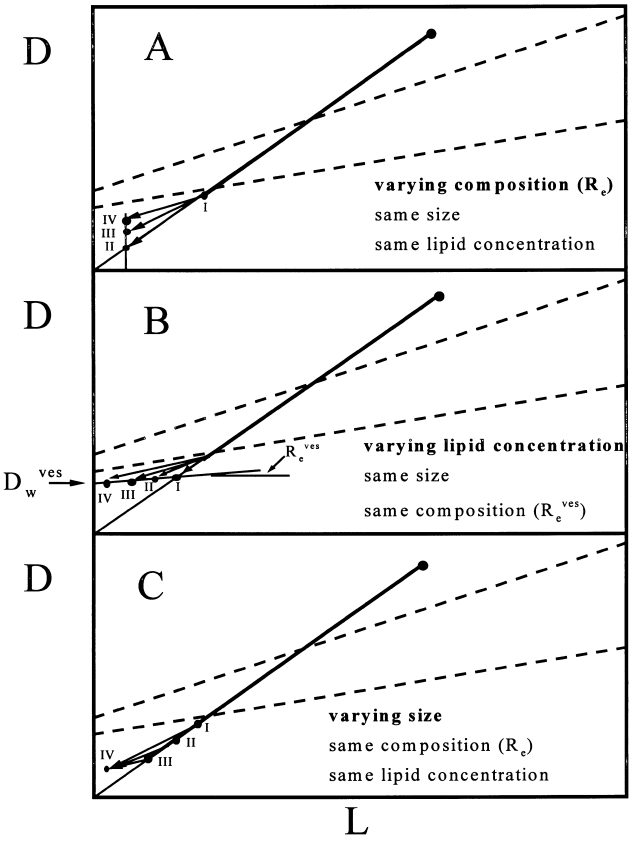

Fig. 6. Schematic illustration of experimental protocols that can be used to study detergent-containing vesicles of varying composition (A), lipid concentration (B) or size (C). Note that each of the protocols is designed so as to vary the factor of interest without alteration of other characteristics of the vesicles (see text for details).

To study of the effect of composition (Fig. 6A), the mixed micellar solution is diluted to an R_e value below R_e^{sat} and equilibrated, to form a dispersion of vesicles of a relatively large size (I). Subsequent dilution of these vesicles in a medium containing no detergent will result in the formation of a dispersion of vesicles (II) of the same size but lower R_e value. Similar dilution of the initially formed vesicles in

media of different detergent concentrations, all with lower detergent concentrations than the concentration of monomers in the initially formed vesicles (Fig. 6A_I), yields dispersions (e.g. III and IV) of vesicles of the same size and lipid concentrations but different R_e values.

A similar protocol can be used for studying the effect of lipid concentration (Fig. 6B). Here, the monomer concentration in the initially formed dispersion of vesicles can be computed from the partition coefficient K using Eq. 14 for the known values of D_t and L

$$K = \frac{D_t - D_w}{(L + D_t - D_w)D_w} \quad (16)$$

The computed value of D_w can then be used to prepare vesicles of the same composition (R_e^{ves}) but varying concentrations of lipids (e.g. II, III and IV) by simply diluting sample I in different volumes of a detergent solution of a concentration D_w^{ves}.

Vesicles of varying size but an equal composition (e.g. the composition given by point IV in Fig. 6C), can be made by first preparing vesicles of various sizes and compositions (e.g. I and II in Fig. 6C) from a mixed micellar solution by diluting the micellar solution by different volumes of the solvent. Subsequent dilution of all these dispersions to the same composition (e.g. IV) yields vesicles of different sizes but equal lipid concentration and R_e value.

A similar procedure can also be used to prepare lipid vesicles of various sizes without detergent. As an example, detergent removal by dialysis of samples I, II and III in Fig. 6C yields dispersions with phospholipid vesicles of different sizes. These vesicles can then be diluted by a medium containing no detergent to the same phospholipid concentration. This procedure may be of value in studying the effect of vesicle size on various properties of the vesicles.

7. Concluding remarks

The self-assembly of detergent-phospholipid mixtures in aqueous solutions is a key issue in many fields of research. These include: (i) *Lipid digestion*: bile salts, the detergents of our digestive system, play a role in lipid digestion by solubilizing the lipids in the form of mixed micelles, thus promoting phospholipolysis by the pancreatic phospholipase A_2. (ii) *Drug, delivery systems*: solubilization of lipid vesicles by bile salts interferes with the development of liposomal drug delivery systems for oral applications. (iii) *Gallstone formation*: compositionally induced vesicle ↔ micelle transformations play a major role in the pathogenesis of gallstones. (iv) *Phospholipases*: studying (or utilizing) reactions between water-soluble agents (particularly enzymes) and the water-insoluble phospholipids most commonly involves solubilization of the phospholipids in phospholipid-detergent mixed micelles. Hence, much of the research on phospholipases has been conducted on micellar phospholipids as substrates. (v) *Liposomes*: preparation of liposomes of various sizes and compositions, either for biophysical studies or for application of various liposome-associated (or entrapped) chemicals (drugs, cosmetics, etc.) can be based on detergent removal from mixed dispersions containing phospholipid-detergent mixed micelles. (vi) *Membrane biochemistry and biophysics*: isolation, purification and characterization of membrane proteins and their function all require solubilization of membranes by detergents as discussed above.

Accordingly, questions relating to mechanistic and structural aspects of mixed detergent-phospholipid systems had to be addressed with respect to each research project in these fields. As a consequence, our understanding of these systems matured quite slowly mostly because many of the important contributions were of researchers whose main interest was in questions other than the self-assembly of mixtures of micelle-forming and bilayer-forming amphiphiles but realized that questions in this field have to be addressed, to gain better understanding of the systems in which they had interest.

These include the pioneering contributions of the late Dr. Racker and his colleagues and of Dr. Small and his colleagues as well as Drs. Litman, Hellenius, Simons, Dennis and Blumenthal and his many students (including many of the contributors to this volume).

Acknowledgements

Our thinking on the issues addressed above has developed through interactions with many of these

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