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Review

Vesicle reconstitution from lipid–detergent mixed micelles

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Abstract

The process of formation of lipid vesicles using the technique of detergent removal from mixed-micelles is examined. Recent studies on the solubilization and reconstitution of liposomes participated to our knowledge of the structure and properties of mixed lipid–detergent systems. The mechanisms involved in both the lipid self assembly and the micelle–vesicle transition are first reviewed. The simplistic three step minimum scheme is described and criticized in relation with isothermal as well as a function of the [det]/[lip] ratio, phase diagram explorations. The techniques of detergent elimination are reviewed and criticized for advantages and disadvantages. New methods inducing micelle–vesicle transition using enzymatic reaction and T-jump are also described and compared to more classical ones. Future developments of these techniques and improvements resulting of their combinations are also considered. Proper reconstitution of membrane constituents such as proteins and drugs into liposomes are examined in the light of our actual understanding of the micelle–vesicle transition.

Keywords: Liposome formation; Detergent elimination; Micelle–vesicle transition; Surfactant; Membrane solubilization; Phospholipid

1. Introduction

There is a growing interest in the preparation of lipid vesicles able to encapsulate labile biological substances such as proteins, peptides or nucleic acids for the purposes of pharmaceutical, cosmetic and chemical applications (e.g., gene therapy, aroma or drug encapsulation). In this respect liposomes, because of their size, the biocompatibility of the lipids used, and the protection they provide against degradation of the encapsulated substances, are suitable for drug and diagnostic agent administration, whatever the route (oral, topic, parenteral or pulmonary). On the other hand, by providing the best cell membrane model known for biological studies, they are also helpful in exploring the basic mechanisms of membrane functions.

Since they permit to form vesicles without any degradation of their biological activity, techniques based on the detergent removal from lipid–detergent mixed micelles are, to date, the most widely used for liposome reconstitution when natural substances such as therapeutic proteins or nucleotides (proteoliposomes, nucleoliposomes) are encapsulated and/or incorporated. In this context, it appears of importance to understand the mechanisms of liposome reconstitution both at molecular and supramolecular scales as a prerequisite to the monitoring of vesicle formation. However, it is striking to notice that while this detergent removal process is widely used, the process, known as the micelle–vesicle transition (MVT) by which mixed micelles transform into vesicles, is still not completely elucidated.

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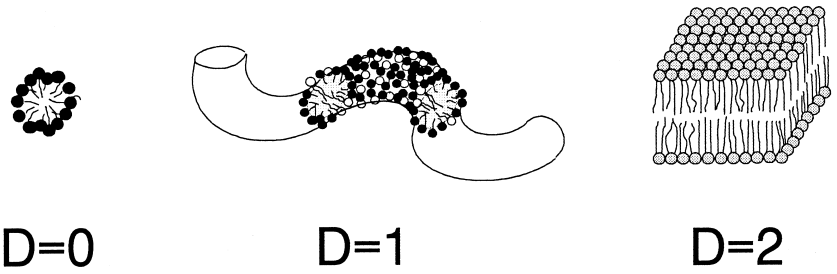

Fig. 1. Schematic drawing of aggregates illustrating their dimensionality (D). From left to right, spherical micelle (D = 0), cylindrical micelle (D = 1), lamellar phase (vesicles) (D = 2). The one-dimension cylindrical micelle is intentionally drawn bent and not straight and round-shaped to illustrate the possible deviations from ideality of aggregates occurring during MVT. The same type of deviation leads to relate vesicles to two-dimension lamellar phase. In both cases, respectively one and two curvatures have to be introduced in aggregate geometries to produce the deviations observed (note, in the second case, one deviation would have conducted to the formation of a tubule that correspond more or less to a cylindrical vesicle).

This review aims at providing insight into the mechanisms of lipid vesicle formation upon detergent removal as well as providing a critical analysis of the techniques used for its elimination. To this end, the mechanism of micelle–vesicle transition is examined below, while the principles as well as the respective advantages and disadvantages of the techniques used for vesicle formation are examined in Section 3 of this paper. The influence of the reconstituted/encapsulated constituents onto the structures formed will be examined in Section 4.

2. Mechanism of the vesicle formation

In excess water, both polar lipids and related hydrocarbon chain compounds such as detergents have in common the capacity to express molecular self-organization and amphiphilic properties. However, as a result of their differences in shapes and interactions these molecules self-pack with different curvatures at the oil–water interfaces. A large variety of supramolecular organizations results from the spatial delimitation of the oil and water compartments. These supramolecular organizations display dimensions in space ranging from 0 to 3D, when passing from aggregates such as micelles to a three dimensional structure such as cubic phase (Fig. 1). The direct influence of water and water-soluble compounds such as electrolytes on the curvatures of the oil–water interfaces, and the fact these organizations are formed even by amphiphile chains in the liquid state clearly demonstrates that these structures appear mainly as a result of the interface organization.

The packing parameter $p = v/al$ (p is dimensionless, v and l are total volume and the extended length of the amphiphile chain(s), and a is the surface occupied at interface by polar head group) has been introduced as an estimate of the amphiphile molecular shape at interface. This parameter allows for the rough prediction of the mean interfacial curvature [1].

What are the consequences of this interface-directed self-structuring by amphiphiles for MVT? Briefly, lamellar-phase-forming molecules such as phospholipids exhibiting a p value close to 1 self-assemble into flat or slightly curved bilayers like vesicles while most detergent molecules with $p \leq 0.5$ form micellar structures with highly curved interfaces (Fig. 1). It is of note here that the lamellar organization under certain conditions transforms into multilayered or single-walled closed vesicles by slightly deviating from thermodynamic stability [2]. On the other hand, micelles represent a thermodynamically stable state for medium chain length molecules. Taking into account that vesicles are related to the lamellar organization, then MVT corresponds to a packing parameter change from about 0.5 to 1.

The relative metastability/stability of the aggregates is in line with the respective molecular dynamics of these different types of amphiphiles. While for vesicles, the aggregate $\leftrightarrow$ water molecular exchanges are highly reduced due to the very weak solubility of phospholipid molecules in water ($2\text{–}4 \times 10^{-10}$ mol/l for DPPC) [3], the internal and internal/external molecular dynamical exchange of the detergent is very fast (in the μs range for medium chain detergent molecule). In micellar structures, short chain deter-

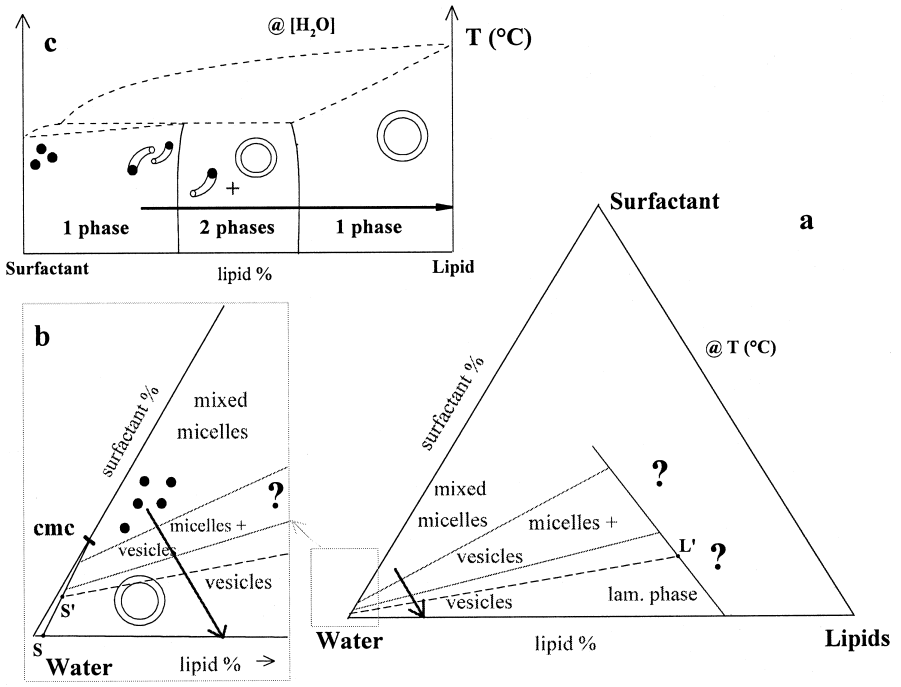

Fig. 2. Schematic phase diagrams in which micelle–vesicle transition takes place. (a) Isothermal lipid–surfactant–water ternary phase diagram. (b) Enlargement of the water corner. (c) A cut of the full lipid–surfactant–water ternary diagram vs. T; a hypothetical pseudo-diagram drawn at constant water concentration. In all three diagrams, the micelle–vesicle transition which is shown as an arrow drawn from mixed micelles to vesicles, crosses a domain in which vesicles and mixed micelles coexist. (@ sign indicates the constant parameter of the diagram). The intercept of the two planes corresponding to the pseudobinary and ternary phase diagrams is the MVT line. At the aggregate level, the MVT corresponds to a transition from 0 or 1D→2D aggregates depending from where it starts in the diagram. Both the solubility limit of the mixture of both types of amphiphiles and the excess water limit have been deliberately shown for simplification as straight lines, in the water-rich corner, joining the surfactant critical micellar concentration (cmc) and the limit of the lipid solubility (s) and, on the amphiphile-rich side, showing a hypothetical hydration limit of amphiphile-rich phases, noted ?, respectively. Then, in the ternary phase diagram when the MVT process (arrow) crosses two phases domains, equilibrium establishes between the phases corresponding to these limits. Such a tie-line, noted S′L′, allows for the determination of partition coefficient (a full series of such tie-lines have determined in [10], for the egg phosphatidylcholine–octylglucoside–water ternary phase diagram). note: all dashed lines at the top of c showing a peritectical behavior are drawn as an example of possible connections of the quasi-vertical boundaries, but do not correspond to a real phase diagram.

gent molecules exhibit fast internal as well as internal/external exchanges with the monomers of the surrounding medium due to the relatively high solubility (the shorter the chain length, the higher the critical micellar concentration) of the molecules (generally ranging from about 10^{-4} to 0.1 mol/l).

When lamellar- and micellar-forming molecules are mixed together they form various types of structures ranging from flat to highly curved interfaces depending on their proportions. Detergent-rich mixtures form highly curved structures, micelles, while long-chain lipid-rich ones form flat structures or vesicles. Their different structural behaviors are responsible for their limited miscibilities and their phase separation (Figs. 2 and 3). As usually observed in binary phase diagrams of partially miscible constituents, when one of the two constituents is in a much larger proportion than the other one, the structure of this main constituent is adopted (as illustrated in Fig. 3c). The latter, as a minor constituent, is only acting as an impurity in the structure but contributes in modifying it. When the proportions of the minor constituent increase, the changes that its insertion provoke may be acceptable by the structure or not, leading in the latter case to the formation of a new structure compatible with both molecules at their respective proportions. The mechanism of continuous change of structure and mean radius of curvature is comparable to the change in mesophase structure observed in the temperature–concen-

tration phase diagrams of surfactant water systems. In these phase diagrams the continuous water depletion at the head group of the surfactant by increasing proportions of surfactant results in a series of continuous and/or step-by-step changes that correspond to the evolution from 0 dimension aggregates to flat two-dimensional structures (Fig. 1) [4].

When mixing them in intermediate proportions, a series of mixed aggregates exhibiting intermediate radius of curvatures are formed [4,5]. As each molecule has its own packing characteristics, lipid–detergent mixtures display numerous packing combinations. In this respect, some of the intermediate aggregates exhibit two different radii of curvature.

The difference of the respective solubilities in water of the two types of constituents, lipid and surfactant, allow their separation. The more water-soluble molecule, i.e., the detergent, is physically removed, thanks to its partition between lipid phase and solution, by a variety of techniques including dialysis, gel exclusion chromatography, adsorption onto polymeric materials or by a biochemical reaction using enzymatic hydrolysis. The influence of temperature or concentration on the partition coefficient is also used to take advantage of an enhanced water solubility of the detergent using temperature jumps and/or dilution to provoke the micelle–vesicle transition.

2.1. Detergent partition

The main consequence of both the water solubility and the amphiphilic character of the detergent is that it partitions between the aggregates and the aqueous medium. Then, a quantitative description at the molecular and supramolecular levels of micelle–vesicle transition requires a knowledge of the molecular partition of the detergent. In this review, we use the following notation for detergent and lipid concentration [6–11]. The total detergent concentration, $[Det]_{tot}$, is the sum of the monomeric (in water), $[Det]_w$, and in aggregates (associated to either vesicles or micelles or both), $[Det]_{ag}$, detergent concentrations

$$[Det]_{tot} = [Det]_w + [Det]_{ag} \quad (1)$$

Assuming that at equilibrium the chemical potential of detergent in lipidic aggregates is the same as in water, each type of aggregate whatever its concentration is in equilibrium with a certain a unique $[Det]_w$ as the existence of tie-lines in the schematic ternary phase diagram shows (Fig. 2). Then, the detergent to lipid ratio in the aggregates being noted as $R_e = [Det]_{ag}/[Lip]_{ag}$ (note that $[Lip]_{tot} = [Lip]_w + [Lip]_{ag}$; as $[Lip]_w$ is neglected, then $[Lip]_{ag} = [Lip]_{tot}$ see above) [8,9,12,13]:

$$[Det]_{tot} = [Det]_w + R_e \bullet [Lip]_{tot} \quad (2)$$

Then a distribution/partition coefficient, K, between the aggregates and water is defined as

$$K = [Det]_{ag}/([Det]_w \bullet ([Det]_{ag} + [Lip]_{ag}))$$

Assuming $[Det]_w \ll [H_2O]$ and $[H_2O] = \text{constant}$, $K = (R_e/(R_e+1)) \bullet (1/[Det]_w)$ [10,13–15].

2.2. Structures involved and phase diagram exploration

Ideally, the method of liposome reconstitution using detergent removal is the reverse of the solubilization process. However, while solubilization of phospholipidic vesicles by detergent is easily obtained by its simple addition to the medium, the detergent removal is more difficult (see below). Moreover, the presence of additional constituent(s)/material(s) which are often required for detergent elimination results in more complex processes of vesicle formation and equilibria.

The exact mechanism of vesicle formation is not well understood to date. Several theoretical models have been proposed which explain the bending properties of the mixed membrane in terms of curvature energy and elastic deformation [16–18]. Depending on the balance between interfacial edge energy and curvature energy, three stages are proposed to describe the process of vesicle formation and closure: (i) increase in the size of the mixed micelles, (ii) formation of flat lamellae, stabilized on the membrane edge by detergent molecules, and (iii) bilayer bending and closure when the interfacial edge energy becomes higher than the curvature energy [19–21]. On the other hand, a series of structures comprising globular micelles, flexible cylindrical micelles, open or rough vesicles and lamellar sheets, and finally intact vesicles have recently been identified by cryo-transmission electron microscopy for non-ionic, Triton X-100 [22], octylglucoside [11], dodecyloctaethyleneglycol

C12E8 [23], anionic sodium cholate [24] and cationic cetyltrimethylammonium chloride [25] surfactants.

First of all, it is of importance to remark that whatever the process used, detergent removal is not obtained directly at the aggregate level but only through the reduction of the monomeric concentration of detergent which in turn leads to aggregate composition re-equilibration. The major consequences of this process of detergent removal are (i) the formation of a detergent concentration gradient in aggregates along the different steps of its elimination and (ii) the rate of elimination depends tightly on detergent solubility. The subsequent detergent gradients and concentration-in-aggregate re-equilibration will be examined later in this paper. Then, in what follows, we will first describe the ideal detergent removal in which we assume that no such detergent concentration gradients exist.

Due to the large difference in packing parameters, chain lengths, etc., the lipid and detergent molecules have a limited affinity for each other. Ideal or even non-ideal miscibility of the two constituents is never observed. This is not surprising taking into account the chain length influence and keeping in mind that a four carbon difference in carbon number is enough to induce a phase separation of lipids [26]. Then, a continuous change of aggregate morphology allowing the progressive change from micelle to vesicle is never considered. However, such a scheme should not be definitely ruled out since some specific system might allow such a transformation.

The limited miscibility of each type of molecule into the structure made by the other leads to a minimum three-step scheme to describe the micelle–vesicle transition [27–31]. Usual description of the micelle–vesicle transition considers in a minimum scheme vesicles or mixed vesicles, mixed micelles and an intermediate step in which both mixed aggregates coexist (Figs. 2 and 3) by analogy to the reverse process of solubilization. Solubilization is achieved when enough detergent is added to reach the boundary which separates both mixed micellar domains. This boundary also corresponds, in the solubilization direction, to the vanishing of the last vesicle or its first occurrence in the reverse process of vesicle formation. To describe the vesicle formation mechanism, we shall start from almost lipid-depleted mixed micelles (e.g., mixed micelles containing, as an average, one or two lipid molecules per aggregate). Such mixed micelles display a large excess of detergent as compared to lipid ($R_e \gg 1$).

Then, in the simplified three-stages model, Eq. 2 (above) could be generalized by taking into account the existence of only two types of aggregates, mixed vesicles and mixed micelles (Fig. 3).

$$[\mathrm{Det}]_{tot} = [\mathrm{Det}]_w + [\mathrm{Det}]_{mic} + [\mathrm{Det}]_{ves} \quad (3)$$

then

$$[\mathrm{Det}]_{tot} = [\mathrm{Det}]_w + R_{mic}\bullet[\mathrm{Lip}]_{mic} + R_{ves}\bullet[\mathrm{Lip}]_{ves} \quad (4)$$

2.3. Minimum scheme

Fig. 3 illustrates the three steps in detergent removal by showing the various aggregates formed, their lipid and detergent concentrations, and the corresponding turbidity variation expected during MVT.

2.3.1. Mixed micellar domain

The size of the almost pure detergent mixed micelle which is generally not far from the theoretical minimal micellar size (diameter of the hydrophobic core is twice the detergent chain length) necessarily increases upon detergent removal assuming the lipid has a chain longer than that of the detergent. This increase which corresponds to the structural adaptation described above, is associated with the change in one of the radii of curvature of the micelle, leading to null curvature in one direction and formation of cylindrical micelles [11,23]. The aggregate adaptation is continued as far as possible by incorporating more and more lipid molecules in the structure. This results for instance in increased aggregation numbers, i.e., for mixed micelles the mean total number of molecules and the length of the cylindrical micelles [32]. When such an adaptation is not possible any more, unless micelle reaches infinite length which is not thermodynamically favored, such aggregate has no other issue than (i) deform in another direction, for instance by making ribbons, (ii) connect with neighboring micelles or itself forming interconnected micelles, or (iii) stop changing, leaving the possibility to another type of aggregate to develop beside the initial structure. In the case of strictly cylindrical micelles, the first issue is unlikely since the deformation in the second direction would have taken place

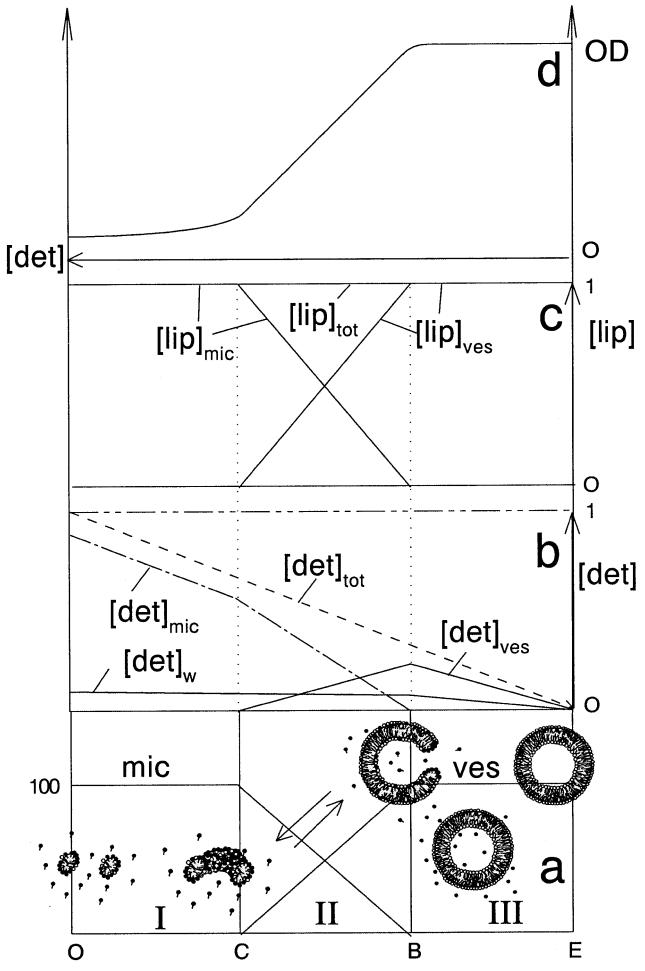

Fig. 3. Schematic drawing illustrating the 'minimal scheme' for the micelle–vesicle transition. The various types of aggregates found in the three-stages model, and their respective lipid and detergent concentrations are indicated as a function of decreasing (from left to right) detergent concentration in the system. From a to d: (a) Schematic drawing of aggregates, detergent-rich mixed micelles, lipid-rich mixed micelles, detergent-rich open lamellar aggregates, closed lamellar aggregates, detergent-depleted closed lamellar aggregates (liposomes), with their respective amounts, normalized to 100%, in the back. In stage I only detergent–lipid mixed micelles, noted mic, are present. In stage III only detergent–lipid mixed vesicles, noted ves, are present. In intermediate domain (II) both types of aggregates coexist in equilibrium as indicated.by arrows. (b,c) Total and 'in each compartment' concentrations of detergent and lipid, normalized to 1. At any point along the MVT, (b) total detergent concentration $[Det]_{tot} = 1 = [Det]_w + [Det]_{mic} + [Det]_{ves}$ is the sum of the detergent concentrations found in water as monomers, $[Det]_w$, mixed micelles, $[Det]_{mic}$, and in mixed vesicles, $[Det]_{ves}$. (c) Total lipid concentration $[Lip]_{tot} = 1 = [Lip]_w + [Lip]_{mic} + [Lip]_{ves}$ is the sum of the lipid concentrations found in water as monomers, in fact $[Lip]_w = 0$ (quasi-null solubility), mixed micelles, $[Lip]_{mic}$, and in mixed vesicles, $[Lip]_{ves}$. (d) Idealized variation of the optical density (turbidity) during the process of liposome reconstitution through detergent removal (note the non-linear variation in the OC domain (stage I). Drawing inspired by Fig. 7-1 from [3].
←

before the end of the lengthening in the first, if energetically favored. The second possibility would lead to entangled micelles and ultimately to the formation of a network. Such a network would have the rheological properties of the intermediate phase observed at the second step of the micelle–vesicle transition. Whatever the evolution route adopted, the non-linear turbidity increase observed up to this limit is in favor of a dramatic size increase (Fig. 3a). The observation of a break point in the evolution of mixed aggregates properties suggests that the third possibility is effectively observed at this point and beyond. However, this does not rule out the second issue, both mechanism could be combined in MVT.

2.3.2. Mixed micelle and vesicle coexistence domain

The second step corresponds to the coexistence of two types of aggregates. A second population of aggregates develops to the detriment of the mixed micelle population which no longer change in morphology (size, shape, etc.). The occurrence of this second population is equivalent to the formation of a new phase developing beside the first. The Gibb's law imposes in the pseudo-binary phase diagram that the two phases have fixed compositions and only vary in proportions in this biphasic domain. In the usual description, lipid-saturated mixed micelles coexist with detergent-saturated lipid–detergent mixed vesicles [10,27–31]. The quasi-linear turbidity increase observed in this domain at low lipid content is in favor of the formation of large aggregates scattering about one order of magnitude more light than the mixed micelles (Fig. 3a).

In the direction of vesicle formation, the process starts when the mixed micelles are saturated with phospholipids and ends when the last mixed micelles have been transformed into vesicles.

2.3.3. Mixed vesicle domain

The mixed vesicles are progressively depleted of the detergent until vesicles only contain lipid molecules. The closure of the vesicle which occurs at the

beginning of this step imposes to consider several regimes of detergent elimination [8,33]. Before vesicle closure, there is almost unlimited diffusion of the detergent molecule from the interior of the vesicle towards the external medium, from which detergent is eliminated. In fact, this supposes that in all the steps considered above, we neglect the one corresponding to detergent release from the aggregate towards the aqueous medium (see below).

2.4. Formation of detergent concentration gradients and intermediate structures

While the vesicle opening occurring during the solubilization process has been studied by encapsulation of hydrophilic polymers of various molecular mass [33], the vesicle closure and the detergent concentration at which this closure operates during the process of the MVT have been studied by differential encapsulation of markers as a function of detergent removal [8]. It has been established that this closure was obtained at a $[Det]_w < [Det]_w$ observed at break point B, indicating that the last mixed micelle vanishes before the vesicle closure. This is correlated with recent time-resolved fluorescence investigation using dehydroergosterol, a fluorescent cholesterol analogue [34]. The consequence of the vesicle closure is that upon detergent removal, molecules entrapped in the internal compartment of the vesicle at its closing point and beyond, would not be released by simple diffusion as easily as those removed before this point. A series of detergent concentration gradients develop between the internal and external compartments of the vesicle as a function of the intermediate non-equilibria developing to counterbalance its removal from the external compartment (Fig. 4). These gradients get established as follows. The decrease of detergent concentration in the external compartment leads first to its depletion from the external leaflet of the vesicles, which in turn provokes a flip-flop from internal leaflet. Then, the internal compartment of the vesicle repopulates the internal leaflet. The local gradients and the overall one will then depend on the detergent propensity for diffusion between the different locations and especially upon the rate of flip-flop.

Above, we made the assumption that the micelle–vesicle transition was performed in three steps, and that a bilayer was first formed when leaving the mixed micelles stage. The study of pseudobinary phase diagrams constructed at constant lipid concentration but varying detergent/lipid ratio or temperature has recently shown that several intermediate pseudo-phases could be encountered before the biphasic bilayer-micelle equilibrium took place [36–38].

The type and number of intermediates depends on

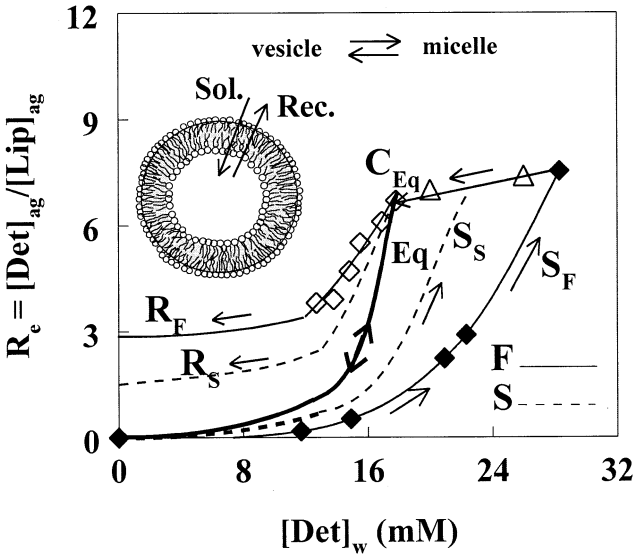

Fig. 4. Schematic drawing illustrating the possible 'hysteresis' of the micelle–vesicle transition. Original hysteresis plot (thin solid line with concentration marks, cycle F) has been inspired from the study of niosome solubilization/reconstitution by OG [39,40] and from the results of the PC–sodium taurocholate–cholesterol–water systems [35,91]. It has been drawn by plotting the detergent ratio in the aggregates $R_e = [Det]_{ag}/[Lip]_{ag}$ vs. its concentration in water ($[Det]_w$). Depending on the detergent and lipid natures as well as on the rate, fast (F) or slow (S) of detergent removal/addition, the reconstitutions (R_F, R_S)/solubilizations (S_F, S_S) processes are not ending at the same detergent concentrations $[Det]_w$ and R_e values: the faster the detergent addition, the higher the detergent concentration needed to reach a given R_e, while the faster the reverse process of detergent elimination, the higher the detergent concentration retained by the vesicles. The dashed line corresponds to slower rates of both processes. Both fast and slow processes whatever the direction are supposed out of equilibrium. The medium line (bold, noted Eq) represents the equilibrium path towards which both slower processes are tending. It ends at C_E, corresponding to at equilibrium $[Det]_w$ and R_e values delimiting the effective mixed micellar boundary. Then, any point out of this line would represent a system out of equilibrium. The surface of the hysteresis cycle will depends on the rate of addition/removal of detergent as well as bilayer permeability. The faster the rate the larger the surface. The two arrows drawn across the vesicle bilayer symbolize the orientations of the solubilization (Sol.) and reconstitution (Rec.) gradients responsible for the hysteresis.

the nature of the phospholipid and detergent, as well as on the temperature. For instance, DPPC multilayers form several intermediate mixed lamellar structures when mixed with octylglucoside (OG) or DM, whatever the temperature below the main transition and before the mixed lamellar–mixed micellar two-phase domain is reached. Then, the lamellar/vesicular structure in equilibrium with the mixed micelle is likely such an intermediate structure rather than the original pure bilayer. The existence of such intermediate structures is in agreement with the observation of up to seven break-points during the solubilization of vesicles by continuous addition of detergent [36,38].

2.5. Influence of detergent removal rate on vesicle size

It has been proposed by many authors and from the characterization of various systems that vesicle size obtained upon detergent removal is related to its rate of elimination and is 'thermodynamically' controlled [14,39,40]. Indeed, this appears to be somewhat contradictory, but it is not, reminding that at equilibrium, lamellar phase formation is thermodynamically controlled, however out-of-equilibrium process, kinetically controlled, deviates the system towards metastable vesicle structure. In other words, the system tends towards lamellar phase because of the thermodynamic but does not reach it at human time scale because of kinetic. It results that the faster the removal, the smaller the vesicles [7,41]. When a fast elimination process is conducted, small unilamellar monodisperse vesicles are formed. The fast detergent elimination from coexistence domain, i.e., from mixtures of open mixed vesicles and polymer-like mixed micelles, results in relatively monodisperse-in-size vesicles. The size of the vesicles obtained by fast detergent elimination as well as their sharp size distribution could be related to the existence of an 'equilibrium size'. Indeed, in the presence of detergent, small, likely-open vesicles undergo a process of growth until they reach such an apparent equilibrium size [10,42]. However, the size dependence upon dilution conditions and composition remains one of the more puzzling questions since most theoretical models predict an opposite behavior [43].Indeed, the growth mechanism of lamellar phase/vesicles is not yet clear, nor is the process by which they form. The discussion of long-lived metastable states which may develop due to a 'kinetic trap' in which they fall is out of the scope of this review [29].

3. Techniques used for vesicle reconstitution by detergent removal

In this section, we will present the various strategies proposed for vesicle reconstitution by detergent removal. The different methods for detergent removal are generally related to their physical properties, especially their critical micellar concentration (cmc), micelle size (aggregation number, n_{agg}) and critical packing parameter ($p = v/al$). Their respective advantages and disadvantages will be discussed below in this respect.

3.1. Dialysis

Dialysis of mixed micelles against a surfactant-free aqueous medium, which was among the first techniques proposed for vesicle reconstitution, is based on the selective retention by a porous membrane of lipidic aggregates (mixed micelles, vesicles) compared to detergent monomers, that favor elimination of the latter [44–46].

3.1.1. Principle

In the simplest form of the method, the lipid–detergent mixed micelles solution is placed in a dialysis bag (high permeability cellulose membrane) the cut-off of which is about 10 kDa and dialyzed against detergent-free aqueous medium under slow stirring. More sophisticated systems, including the use of flow-through dialysis cells in which the rate of detergent removal can be increased and the dialysis time decreased, have been developed to perform fast and controlled dialysis [47,48].

3.1.2. Advantages and drawbacks

The main advantage of dialysis is the simplicity of the technique and the low cost of the materials used. However, in its simplest set up, it suffers from numerous drawbacks among which we notice: (i) uncontrolled rate of dialysis/exchange, (ii) unknown final concentration resulting from osmotic pressure

differences, (iii) possible retention of molecules on the dialysis membrane, (iv) poor reproducibility of the experiments, and (v) long duration of the experiments due to the number of changes of buffer. These disadvantages are partially avoided using controlled dialysis.

In order to control the rate of exchange, two of the refinements of the technique consist of dialyzing against either a fixed or a variable concentration of detergent. Then, providing the rate of elimination from the dialyzed compartment is not too slow compared to the change of detergent concentration in the external compartment, it allows either a step by step in the former case or a continuous decrease of its concentration in the latter. The exposure of dialysis bag to known detergent concentration(s) also provides a convenient way to get perfectly defined detergent concentrations $[Det]_w$ in vesicular or micellar systems and full equilibration of the latter.

Detergents with high cmc such as octylglucoside, sodium cholate and Hecameg are easily removed by dialysis, while the technique is not appropriate for the complete removal of low cmc ones such as $C_{12}E_8$ or Triton X-100.

3.2. Dilution

The technique takes advantage of the partition coefficient of the detergent between the aqueous phase and the aggregates [8,41,49].

3.2.1. Principle

The dilution of mixed micelles by the components of the continuous phase depleted of the detergent allows to transform these mixed aggregates into mixed vesicles.

3.2.2. Advantages and drawbacks

The simplicity of the technique is evident. Since the total concentration is always precisely known, it is the most suitable technique to control detergent removal and monitor vesicle reconstitution. Dilution rates are variable in a wide range. Moreover, monitoring the addition rate of the diluting buffer is easily and accurately mastered for instance using syringe pumps powered by either step-by-step or synchronous motors. Monitoring the dilution provides control of even complex patterns of liposome formation. However, the technique suffers from numerous drawbacks which make it not suitable for large scale production. Among them, the addition of the diluting solution creates larger local inhomogeneities in the detergent concentration within the reaction vessel than dialysis. A concentration gradient develops within the preparation and at the addition point the mixed micelles are directly exposed to the concentration of the diluting solution. Full detergent removal cannot be attained since this would correspond to infinite dilution. Even reaching low detergent concentrations requires that the sample is highly diluted. In this respect, the use of detergents with high cmc is convenient since it limits the concomitant lipid dilution.

Dilution is especially suitable in the study of the effect of external parameters, since this technique is not affected by the presence of external materials susceptible to interact with the process of vesicle formation. The effects of bilayer composition and process parameters on liposome characteristics have been examined by Jiskoot et al. [41]. The presence of cholesterol has been especially considered. They have shown that the incorporation of more cholesterol yielded larger particles. The inhibition of bilayer curvature in relation with the increase of elastic modulus could be responsible for this size increase. The incorporation of either a negatively charged compound, phosphatidylserine or dimethyldioctadecylammonium bromide respectively, resulted in smaller particle formation upon dilution as compared to neutral liposomes at equal dilution rates. Both effects were attributed to the flip-flop of charged lipids during the bending step of vesicle formation [41]. Another explanation for size reduction could be that fusion rate is decreased by the repulsion between charged particles during the stage of aggregate growth. Lasic suggested that this reduction in collision frequency operates at the mixed micelles step. The comparison of vesicles sizes obtained by CHAPS or sodium cholate removal allowed to verify that zwitterionic mixed micelles, which must have a higher fusogenic ability than charged ones, yielded larger vesicle sizes [50]. Seras et al. suggested that the mechanism of growth might persist in the lamellar/vesicular domain [51].

3.3. Gel exclusion chromatography

Gel filtration of mixed micelles, which was also among the first techniques proposed for vesicle reconstitution [52–54], takes advantage of the different accessibility to the pores of a gel by mixed micelles as compared to the vesicles. It represents probably the smartest technique for both vesicle formation and removal of unencapsulated material.

3.3.1. Principle

Gel exclusion chromatography of mixed micelles using surfactant-depleted buffer for elution leads to an immediate formation of vesicles and the removal of non-encapsulated material in a single step. The dilution, resulting from both molecular diffusion and faster access of the smaller aggregates (monomers, mixed micelles) to the gel pores, results into formation of vesicles and their exclusion from the gel pores. This size exclusion process self-accelerates vesicle formation and closure as well as their separation from surrounding materials. Because of the large difference in size of the vesicles compared to that of the materials to be encapsulated it allows direct entrapment and separation of a variety of materials as large as macromolecules. Gel type should selected accordingly for pore size selectivity.

3.3.2. Advantages and drawbacks

The technique is simple, efficient in detergent removal and fast, elution using pre-packed G25 Sephadex short columns takes about 5–10 min and is operated at low cost, especially since columns can be detergent-cleaned for reuse. Non-encapsulated material is readily separated from the vesicles and could be eventually reused. The technique has some disadvantages including (i) the dilution of both vesicles and non-encapsulated materials; (ii) contact with and possible retention by the gel of both vesicle-constituting lipid and encapsulated materials; (iii) not precisely known dilution and retention factors contribute to render the method semiquantitative. However, the use of adequate amounts of gel in relation to the volume eluted allows limited dilution ($\times 3$). Again, elution with controlled detergent concentrations also allows a step-by-step control of detergent removal.

3.4. Adsorption onto polymeric materials

This technique also allows a single-step, full detergent removal [55,56].

3.4.1. Principle

The physical adsorption of detergent onto the surface of hydrophobic polymer beads (Biobeads SM_2 or Amberlite XAD) allows its semi-selective removal.

3.4.2. Advantages and drawbacks

Although the physical process of detergent elimination is different, this technique offers more or less the same advantages, but also suffers from the same disadvantages, than gel exclusion. In addition: (i) the beads have to be removed from the vesicle suspension and (ii) lipid losses are hardly avoided since adsorption selectivity depends on the lipid type and its concentration as compared to that of the surfactant. Then, the amount of polymer beads used should be adjusted precisely to reduce both lipid losses and selective retention of encapsulated material. It is also recommended that the concentration of polystyrene beads must be adjusted so as to promote the whole MVT process in not less than about 3 h [57]. The main advantages, as compared to gel filtration are: (i) the virtual absence of dilution of the materials and (ii) the complete removal of detergent that allows reconstitution of vesicles even from those detergents whose cmc is very low. As for gel filtration, beads can be reused after methanol washing. In spite of limited adsorption onto polymer beads, the resulting vesicle concentration is rather high. The whole process, when adequately set up, is quasi-quantitative.

3.5. Enzymatic reactions

Although the process of phospholipid bilayer formation via enzymatic reaction is the major way of membrane synthesis in living systems, vesicle formation using enzymes at the laboratory scale has only been recently introduced ([58], see also [59] for a review).

3.5.1. Principle

Here, rather than being physically eliminated as above, the surfactant is enzymatically converted

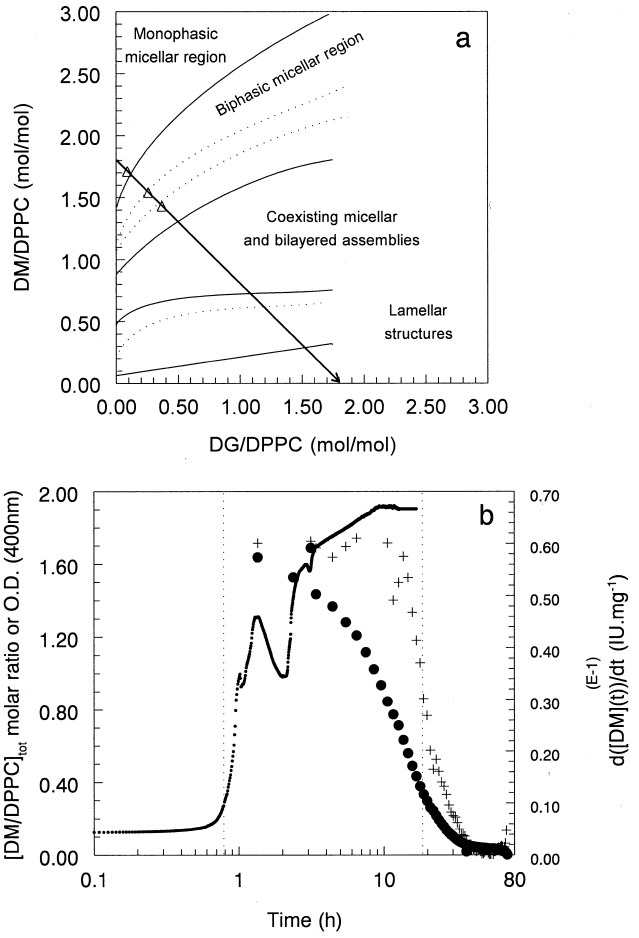

Fig. 5. Enzyme-mediated formation of liposomes. (a) Partial pseudo-ternary DPPC–DG–DM phase diagram in excess buffer at 37°C (redrawn from [38]). DM, dodecylmaltoside; DG, dodecylglucoside. The solid lines delimit the main phase domains and the dotted lines indicate secondary aggregation state transitions. The oblique arrow shows the enzymatic reaction pathway on which are reported the compositions corresponding to the turbidity break points. (b) Time-resolved turbidity and enzyme reaction monitoring. Variations of DM/DPPC molar ratio (●), turbidity (•) and reaction rate d([DM](*t*))/d*t* (+) vs. time recorded during DM hydrolysis by amyloglucosidase starting from mixed micelles and ending into the vesicular domain (redrawn from [60]) ([DPPC] = 2.5 mM, [E] = 0.6 μM). Vertical dashed lines indicate the starting and ending points of lamellar formation. The set of variations of the turbidity monitoring illustrates the complex mechanism of the vesicle reconstitution even in the absence of any added substance or material and at rate of detergent removal that favors approaching equilibrium.

into molecules which are no longer surface-active agents. The enzymatic reaction is frequently chosen to hydrolyze one of the covalent links between the hydrophobic and hydrophilic moieties of the detergent molecule. The lipid-solubilizing power of the detergent is progressively decreased, leading to self-formation of vesicles (Fig. 5).

3.5.2. Advantages and drawbacks

The main advantage of the process is that vesicle formation can be obtained extemporaneously after simple mixing of two solutions (that in turn could be obtained by simple hydration of the lyophilized components, or by pouring a mixed micelle solution onto a lyophilized enzyme). One of the conditions for vesicle formation from mixed micelles is that the detergent is an enzymatic substrate. Vesicle formation is obtained at constant mass since no external component is needed once enzyme is added to the mixed micelle solution.

A limitation comes from the fact that enzyme and by-products of the enzymatic reaction are not removed from the medium, so that they should be compatible with use to be given to the vesicles. In this respect, cholesterol derivatives have been envisioned as enzymatic substrates in order to get advantage of the vesicle impermeability offered by the presence of free cholesterol in the bilayer. Fig. 5 shows an interesting feature in that the rate of enzymatic reaction is sensitive to the substrate availability, in the form of dodecylmaltoside monomeric molecules. Then, the decrease in enzyme rate after about 15 h of reaction (right vertical line in Fig. 5b) is interpreted in terms of a decreased availability of the substrate as a result of vesicle closure [38,60].

3.6. Temperature and pressure jumps

The influence of temperature on micelle–vesicle transition and its application to vesicle formation was only recently introduced [61–63].

3.6.1. Principle

Under certain conditions of detergent and lipid concentrations, a temperature jump may induce a detergent solubility change which in turns induces a MVT. The process is reversible and takes advantage of the change in solubility of the detergent as a function of temperature. The direction and importance of the temperature jump depends on the nature of the detergent, as well as on lipid and detergent concentrations. Depending on the mechanism by which de-

tergent polar head groups are water-solvated, either ion solvated or hydrogen-bonded, a positive or negative T-jump is used to induce MVT. A temperature increase leads to increased solubility (cmc↑) for ionic detergents while it induces a solubility decrease (cmc↓) for non-ionic ones. Induction of MVT requires an increase in solubility. This increased detergent solubility will be counterbalanced by fast aggregates-solution re-equilibrations according to the partition coefficient at the new temperature and aggregate detergent depletion.

3.6.2. Advantages and disadvantages

The detergent depletion induced by T-jump, that depends on the detergent cmc and partition coefficients, is rather limited. Then, the use of this technique is reserved for specific applications in which: (i) detergent cmc is high, (ii) lipid concentration is low, and (iii) the mixed micelle composition is close to breakpoint C. The main advantage of the technique is the perfect control of the process through the T-jump, its reversibility and the fact that it operates in a closed system (absence of any compound addition or removal). To our knowledge, the possibility to use pressure to induce MVT was not yet demonstrated.

3.7. Future directions

In principle, any physical, chemical or biological process that either decreases the detergent concentration available for solubilization or increases the amount of lipid is acceptable for induction of the MVT. In this respect, ionic detergents are better candidates than nonionic ones, because of the possibilities offered by pH and ionic strength jumps.

Direct addition of lipid is not recommended since they are necessarily added in the form of preformed systems (e.g., vesicles) that will keep their characteristics in the medium (addition of mixed micelles to a water-rich medium is not considered either since it would be equivalent to a dilution process). However, molecular addition of lipid via an enzymatic process would be interesting to consider.

3.8. Combined techniques

The combined use of several techniques, while making the preparation more complex, allows to improve vesicle formation by detergent removal in that it helps to overcome some of the drawbacks of the techniques listed above. For instance, when full detergent removal is requested, either dialysis or gel filtration nicely complements the techniques such as dilution or enzymatic reactions for which residual detergent elimination is not possible. In this respect, Schurtenberger et al. have shown, using egg phosphatidylcholine and sodium glycocholate, that the combination of dilution and dialysis represents a convenient method for the preparation of unilamellar, monodisperse and detergent free vesicles with a desired diameter that can be chosen between 24 and 100 nm [49].

The one-step or two-step combinations of dialysis and polymer adsorption are also convenient techniques [64]. The one-step addition of Biobeads in the external compartment of a dialysis process allows: (i) not to change the dialyzing solution (in which case an excess of polymer is recommended), (ii) an easy removal of the latter, and (iii) step-by-step and limited additions of adsorbing polymer will permit controlled detergent removal. It is worth noting that this combination of techniques is especially efficient when the detergent displays a low cmc not favoring its elimination. It also avoids direct contact of the polymer with the material to be reconstituted reducing possible losses of material.

Dilution was reported as the most suitable procedure to monitor the reconstituted vesicle size through the control of detergent elimination rate, while dialysis results in heterogeneous liposome populations [29,39,41,65]. However, as discussed above, dilution does not allow complete elimination of the detergent. Then, the perfectly controlled process of dilution has to be complemented once the vesicle size distribution is set by a non-controlled technique of detergent elimination such as dialysis or its combination with the use of an adsorbing polymer.

3.9. Elimination of residual detergent from the vesicles

Efficiency of detergent removal by dialysis depends upon the detergent. In principle, the larger the detergent solubility in water (cmc), the more efficient and fast its removal. Allen et al. examined detergent removal efficiency of sodium cholate and deoxycholate

as well as Triton X-100 for gel filtration, dialysis and biobeads SM-2 [53]. For most of the detergents examined, removal was more efficient using gel filtration than extensive dialysis. However, even for sodium deoxycholate, a detergent with a high cmc value, the removal was not complete. On the other hand, octylglucoside is known to be easily removed from vesicles whatever the technique used [66]. Schurtenberger et al. have shown that dialysis allows to almost completely remove the bile salt molecules from mixed vesicles prepared by dilution without changing the vesicle size by more than 10% [14,49].

4. Implications of the detergent removal methods for the reconstitution of protein and encapsulation of drugs

The above considerations on the mechanisms of the micelle to vesicle transition and the techniques used for detergent removal have important consequences on the structures and the properties of functional vesicles reconstituted by these procedures, i.e., functional reconstituted vesicles with membrane proteins, peptides, nucleic acids or drugs. These consequences, as well as the opportunities offered by specific detergents and techniques to reach functional liposomes are discussed below [67,68]. Specific needs are often to be met in this field. For instance, for drug delivery it is essential that the maximal rate of encapsulation is reached, while for membrane protein incorporation its orientation within the liposomal membranes may be crucial.

Among all the techniques available for liposome preparation, those using detergents are most efficient to reconstitute 'proteoliposomes'. This comes from the fact that in order to reconstitute a membrane protein into artificial membranes the first step is its purification, which can be essentially achieved by solubilizing the natural membrane by detergents. Moreover, the broad variety and choice of available amphiphilic molecules makes them appropriate to maintain the structure and the activity of the protein even when it is extracted from its natural environment.

It has been demonstrated [9,69] that the detergent used in reconstitution procedures is not only important regarding the protein integrity and activity but also crucial for the mechanisms by which the protein is incorporated in the membrane of liposomes. As a consequence, the choice of the detergent greatly influences the reconstitution products in terms of: (i) proteoliposome morphology, size and homogeneity, and (ii) incorporation rate, distribution and orientation of the protein. More precisely, reconstitution experiments were performed with bacteriorhodopsin (bR) using three different detergents, octylglucoside, Triton X-100 and sodium cholate. Considering the 'three-step' model for the vesicle to micelle transition (see above, Section 2.3), octylglucoside was able to mediate reconstitution in the mixed vesicle domain, i.e., in absence of mixed micelles, Triton X-100 in the coexistence domain, and sodium cholate in the mixed micellar domain. Interestingly, the resulting proteoliposomes obtained under the various conditions were different:

- with octylglucoside the rate of incorporation was high, homogeneous, the protein was fully oriented within the membrane and the liposomes were homogeneous in size;
- with Triton X-100 the incorporation was a slow process and in the best conditions the rate of incorporation was high, homogeneous, the protein partly oriented within the membrane and the liposomes were homogeneous in size;
- with sodium cholate the incorporation was only achieved after previously achieving total solubilization of the different components (lipids and protein) and although the incorporation was homogeneous, the protein was randomly oriented within the membrane.

After this original study, a few other proteins have been incorporated using the same strategy to find the optimal incorporation conditions. Interestingly, whatever the membrane protein used these conditions depend strictly on the chosen detergent, and in particular for octylglucoside, Triton X-100 and sodium cholate the optimal reconstitution conditions found were similar to those established for bacteriorhodopsin [70–75].

Moreover, similar studies have been performed with other detergents like C12E8, CHAPS, CHAPSO, or dodecyl maltoside, and also indicate that the incorporation of membrane proteins by procedures

involving the use of detergents is determined by the molecular and supramolecular mechanisms of the vesicle-to-micelle transition [76,77].

The questions now are: (1) why and under which conditions the molecular and supramolecular mechanisms of the vesicle-to-micelle transition govern incorporation? and (2) why are the mechanisms different for each detergent?

Concerning the first question, in the reconstitution procedures the membrane protein is generally diluted in lipids at a ratio lower than those found in natural membranes. The general lipid/protein ratios used for reconstitution range from 80 to 150 (w/w), roughly equivalent to a lipid/protein molar ratio between 3000 and 5000 (considering an average molecular mass of 1 kDa for lipids and 30 kDa for proteins) (e.g., reconstitution of synaptosomal ATPase [78,79], or of vesicular stomatitis virus envelope using octyl β-D-glucopyranoside, [80]). Indeed in natural membranes the lipid/protein molar ratios are of the order of a few hundreds. This means that when the mixture of detergents, proteins and lipids is prepared, the lipid is in great excess against the protein, and proportionally almost all of the detergent interacts with the lipids and very little with the protein. In these mixed systems the proteins can be therefore considered as an 'infinitely diluted' solute having almost no influence on the detergent and lipid interactions. Moreover, because the quantity of protein added to the detergent and lipid mixture is low, the detergent bound by the solubilized protein is small enough to have almost no effect on the vesicle to micelle transition, i.e., the transition is not shifted by the detergent added with the protein [81]. This situation also allows the use of different detergents, e.g., one for the solubilization of the protein, and another for the lipids. Indeed, the proportion of the 'protein adapted' detergent is very low compared to the 'lipid adapted' one. This possibility is very important: for example a protein loosing its activity after contact with octylglucoside, may be solubilized in another more suitable detergent, and the reconstitution can then be performed using the properties of the octylglucoside mediated vesicle to micelle transition.

Concerning the second question, it is obvious that there are at least three classes of detergents with respect to the incorporation mechanisms: octylglucoside-like detergents, allowing the incorporation of protein into detergent-saturated liposomes; Triton X-100-like detergents, allowing the incorporation of protein into detergent-saturated liposomes but in the presence of a few mixed micelles, and cholate-like detergents, allowing protein incorporation only from totally solubilized material [71]. From the literature it appears that CHAPS and CHAPSO belong to the latter class [71,82]. These detergents derived from bile salts have a rigid backbone, very different from most of the detergents having an aliphatic chain and a polar head group. These rigid detergents like cholate, CHAPS and CHAPSO induce the vesicle to micelle transition at much lower detergent-to-lipid ratios than the aliphatic ones. In fact the molecular and supramolecular mechanisms of the transition are in this case very different from those induced by aliphatic detergents [68–71,82]. Indeed, these rigid detergents induce membrane splitting in chunks in which detergent is not much penetrating, whereas the others induce a progressive change in the lipid organization. It has been proposed that steric factors prevent access of the rigid planar surfactant molecules to the hydrophobic protein regions [82]. These different solubilization mechanisms may be at the origin of the differences of the reconstitution processes. Concerning the two other classes of incorporation inducing detergents, it is more difficult to understand the reason why some detergents require mixed micelles to induce the protein interaction with the detergent-saturated vesicles, while others do not. Moreover the case of octylglucoside is almost unique, most the aliphatic detergents belonging to the second class. The peculiarity of octylglucoside as compared to the other detergents is its very high CMC, and this may be at the origin of its peculiar behavior.

We have seen that membrane protein reconstitution mechanisms depend on the lipid and the detergent used, i.e., on the molecular and supramolecular mechanisms of the vesicle-to-micelle transition. As a consequence, depending on the lipids and the detergents, the protein has to be mixed with lipids and detergent at different ratios. Therefore, in order to reach these precise ratios and control the elimination of detergent from mixed micellar systems, the techniques of detergent removal have to be adapted in each case. For example, for high CMC detergents, dilution and dialysis can be used to control the final

detergent-to-lipid ratio in the sample. The addition of a small volume of the solubilized lipids in the dialysis bag and its dialysis against a large volume of buffer complemented with the monomer detergent concentration that is known to be in equilibrium with, for example, the detergent-saturated vesicles (as recommended for dialysis plus gel exclusion above) will allow a step-by-step, perfectly controlled detergent elimination rate. However, the situation is more delicate with low CMC detergents like Triton X-100 or C12E8. In these cases, it is more appropriate to hydrate a lipid film with the desired detergent concentration, to add the solubilized protein and to remove the detergent by an appropriate technique like direct contact with Biobeads SM2 (see Section 3.8, above).

The influence of 'encapsulated' (meaning here, encapsulated at interface and/or inserted in the bilayer) molecules on the MVT is directly exerted at the phase diagram boundaries as a result of the amphiphilic or hydrophobic character of the foreign molecules added. Thus, shifts of these boundaries as well as structural modifications resulting from molecule insertion are expected. The determination of such boundary and structure shifts induced by the presence of the molecules to be encapsulated is a preliminary requirement in the understanding of their proper reconstitution [83–85]. Very little has been done in this direction even in the leading edge of protein reconstitution domain, probably because of the time-consuming studies implied and of the protein-in-lipid dilution question (see above).

Reciprocally, MVT studies in the presence of foreign molecules susceptible of interfering with the process could be a tool for investigating their interactions with MVT components. The influence of triacylglycerols (triglycerides) on the MVT of the eggPC/Triton X-100 system allowed both to demonstrate the coexistence of phospholipid vesicles and emulsion droplets in the mixtures used for parenteral feeding and to provide a new technique for the determination of triglyceride solubility into phospholipids [86].

5. Conclusions and perspectives

Recent reviews give an estimate of existing knowledge of the micelle-to-vesicle (or vesicle-to-micelle) transition of phospholipid based systems [27–31]. Indeed, the main phase boundaries have been already evidenced and equilibrium properties seem to be well understood. Then, there is an agreement among authors about the minimum scheme description. The pseudo-binary phase diagram presentation of the transition allows to explain both limits of solubility of the detergent in vesicles and lipid in mixed micelles as well as the coexistence domain of the two types of aggregates at saturation. However, as revealed by recent phase diagram studies, it is likely that MVT is much more complex than actually described and that, depending on the system studied, more steps are involved in the process. Moreover, as both reconstitution and vesicle formation implies that the systems are out of equilibrium during and after the MVT process, the kinetic influences on the outcome have to be taken into account and explained. In this respect, the combination of time-resolved monitoring of the transition by continuous recording of physical parameters such as turbidity, and the determination of pseudodiagrams and phase diagrams describing out-of-equilibrium systems at both the molecular an supramolecular levels, reveal themselves as powerful and promising techniques of investigation [8,87,88] that may provide quantitative information (e.g., aggregate composition [89], partition coefficient [90], etc.) of intermediate and unstable aggregates.

Fortunately, the lack of precise knowledge of the mechanism by which mixed micelles assemble to transform into vesicles, which is one of the central points that are not fully understood yet, does not prevent us from making liposomes by using one of the techniques described above. In this respect, the more promising methods of vesicle formation seem to include a combination of techniques of the kind that allow both controlled vesiculation and full detergent removal.

The presence of foreign molecules, such as proteins or drugs renders even more complex an understanding of the phenomena encountered during the MVT, since they necessarily interfere with MVT components as soon as they exhibit an amphiphilic or hydrophobic character. In those cases it is necessary to take into account such an influence by determining the shift of the properties resulting

from their insertion/interaction with the MVT structures.

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