Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmaceutics and Biopharmaceutics 58 (2004) 509–519
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

The effect of cholate on solubilisation and permeability of simple and protein-loaded phosphatidylcholine/sodium cholate mixed aggregates designed to mediate transdermal delivery of macromolecules

S.I. Simões[a], C.M. Marques[a], M.E.M. Cruz[a], G. Cevc[b], M.B.F. Martins[a,*]

[a]UNFAB, Departamento de Biotecnologia, INETI, Lisboa, Portugal, EU
[b]IDEA AG, München, Deutschland, EU

Received 9 March 2004; accepted in revised form 18 May 2004
Available online 3 September 2004

Abstract

Carriers for non-invasive administration of biologically important antioxidant enzymes Cu,Zn-superoxide dismutase (SOD) and catalase (CAT) were developed. Solubilisation and permeabilities of various soybean phosphatidylcholine/sodium cholate (SPC/NaChol) mixtures, mainly in the form of lipid bilayers, focussing on system properties relevant for non-invasive enzyme delivery were investigated in this work. Static and dynamic light scattering measurements gave information on the behaviour of the systems containing up to 40 mM NaChol and 30.6–1.2 mM SPC in the final suspension. The average size of such mixed aggregates was in the 100–200 nm range. Suspension turbidity decreased by 50% upon increasing nominal molar detergent/lipid ratio to NaChol/SPC = 7 and 1.25, in case of SPC = 1.2 and 19.6 mM, respectively. The effective NaChol/SPC molar ratio in bilayers saturated with the detergent was found to be: $R_e^{sat} = 0.70 \pm 0.01$; bilayer solubilisation point corresponded to $R_e^{sol} = 0.97 \pm 0.02$, independently of enzyme loading. Vesicles became very permeable to SOD when membrane bound NaChol concentration exceeded 13.7 mM, in case of total starting lipid concentration of 138 mM diluted to SPC = 19.6 mM. Specifically, we measured a 50% loss of SOD from the vesicles with an aggregate-associated molar detergent ratio NaChol/SPC ~ 0.7, which is near the saturation but well below the solubilisation limit. Calcein efflux from such vesicles was compared with SPC/NaChol/SOD mixed aggregates. Our results should contribute to the future design of vesicle mediated transdermal delivery of antioxidant enzymes.

Keywords: Antioxidant enzymes delivery; Drug delivery; Soybean lecithin; Sodium cholate mixed lipid vesicles; Bilayer permeability; Non-invasive transdermal drug delivery

1. Introduction

Interest in membrane solubilisation by bile salts (cholates) was originally driven by the desire to understand fat disintegration and digestion in the gastrointestinal tract [1–5]. Later on, the focus shifted to detergent dialysis for manufacturing small unilamellar vesicles for pharmaceutical usage [6–10]. More recently, the appeal of such systems has increased due to the use of lipid–cholate mixed aggregates for non-invasive transdermal drug delivery [11–13].

The first widely known ternary phase diagram for sodium cholate (NaChol)/phosphatidylcholine/water was published in 1966 [1]. The initial picture of solubilisation into disk-like mixed micelles was later refined, postulating co-existence of disk micelles with cholate rich bilayers [2,3,14]. More recently, long flexible micelles were detected microscopically [15] and with diffraction methods [16]. The disk-like intermediates were shown to form transiently, during the first 15 min of vesicle creation, after sufficient dilution of mixed lipid micelles. Total time for the micelle-to-vesicle transition in such systems is 4 h [16]. An intermediate solubilisation time was reported by Nagata and colleagues [17], compatible with the insertion rate constant of $8.6 \times 10^{-2}\ mM^{-1}\ s^{-1}$.

A wealth of information on lipid bilayer solubilisation by various bile salts, especially NaChol, is now available.

* Corresponding author. UNFAB, Departamento de Biotecnologia, INETI, Estrada do Paço do Lumiar, 22, Edifício F, 1649-038 Lisboa, Portugal. Tel.: +351-21-092-4734; fax: +351-21-716-3636.
E-mail address: barbara.martins@ineti.pt (M.B.F. Martins).

0939-6411/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2004.05.010

The following sequence of steps can be deduced: cholate first binds to the outer half of a lipid bilayer vesicle, at low cholate concentrations. Increasing surfactant concentration forces more detergent molecules deeper into lipid bilayers [18], against an increasing electrostatic repulsion caused by the vesicle-bound cholate molecules. Above certain concentration limits cholate nonetheless solubilises mixed lipid vesicles into mixed disk-like micelles, which then transform into spherical micelles and ultimately into smaller phospholipid–cholate complexes with further increasing cholate/lipid molar ratio [8,19–21]. The thermodynamics of such solubilisation was elucidated by Garidel [22].

Lichtenberg [14] contributed greatly to the quantitative understanding of lipid bilayer solubilisation. The key was to postulate that the effective surfactant-to-lipid ratio, which leads to saturation and solubilisation of mixed lipid bilayers, depends on the surfactant critical micelle concentration and on the bilayer/water distribution coefficient of the surfactant. This allowed successful and quantitative comparison of various detergents, by using an effective molar detergent/lipid ratio as the independent system characteristic. Phase behaviour of amphiphile mixtures also depends on absolute component concentration [23].

A mechanistic picture of vesicle solubilisation/formation was designed by Lasic [24] and, independently, by Fromherz [25]. Both workers concluded that surfactant containing bilayers, at certain lipid/surfactant ratio, spontaneously un/curve and open/close to optimise edge tension.

From the point of non-invasive drug delivery, the mixed lipid vesicles containing an amount of sub-solubilising detergent are especially interesting [11,12]; the proviso is that this amount is judiciously chosen. For example, Cevc and colleagues [12] found that increasing phosphatidylcholine–detergent ratio from 13 to 35 mol% abolishes good carrier performance, probably by decreasing aggregate stability below the necessary level. On the other hand, decreasing this ratio below 9 mol% minimises carrier performance due to inadequate carrier deformability.

High carrier adaptability and stability are both required for successful non-invasive drug delivery with ultradeformable carriers. Each of these properties may be drug sensitive as well. Carrier optimisation on a case-by-case basis is therefore important. It is unclear to date whether or not the performance limits, determined for insulin loaded ultradeformable carriers, mentioned in the previous paragraph, are generally valid.

We therefore studied solubilisation parameters of phosphatidylcholine–NaChol mixtures alone or containing a water-soluble protein component. To that effect, superoxide dismutase (SOD) and catalase (CAT) were used as two practically relevant macromolecules. In the beginning we investigated the stability of such mixed lipid bilayers, using soybean phosphatidylcholine (SPC), as a phospholipid of biological origin, and NaChol, as a biogenic, ionic detergent. We then explored several practical implications of cholate incorporation into (highly flexible) lipid bilayers yielding ultradeformable vesicles, with special focus on the agent's release from such vesicles. Specifically, we extended the previously published vesicle retention studies, which were typically done with uncharged solutes, to a charged fluorescent marker, calcein, and to a charged antioxidant protein, SOD. We finally compared information on suspension turbidity with the average vesicle size and polydispersity; all these were studied in parallel as a function of nominal detergent-to-lipid molar ratio. We rationalised the results in terms of bilayer-associated detergent concentration and derived the solubilisation parameters that describe the highly adaptable ionic aggregates. The information thus gained should facilitate the future design of related drug-loaded vesicle suspensions, especially for vesicle mediated protein delivery.

2. Materials and methods

2.1. Materials

SPC (>95% purity) was provided by Lipoid, KG (Ludwigshafen, Germany). NaChol and calcein were obtained from Sigma (St Louis, MO, USA), and the other chemicals were purchased from Merck (Darmstad, Germany). Poretics Corporation (Livermore, CA, USA) delivered the track-etched polycarbonate PCTE filters.

2.2. Methods

2.2.1. Transfersome preparation

Transfersomes®[1] were always made by mixing SPC with NaChol as a bilayer softener. In brief, to prepare a suspension of such highly adaptable vesicles, we mixed SPC with cholate in an appropriate volume of 50 mM sodium phosphate buffer, pH 7.4, to yield a suspension with 10% total lipid (total starting lipid concentration, TSL = 138 mM). The suspension was filtered through a track-etched filter, if required repeatedly, to obtain 100 ± 50 nm vesicles. After repeated freezing and thawing, the resulting fused vesicles were re-filtered to obtain bilayer aggregates with the size of approximately 150 ± 50 nm.

2.2.2. Vesicle size determination

The mean particle size and size distribution, in terms of polydispersity index, was measured by photon correlation spectroscopy (PCS) with a Malvern ZetaSizer 1000 using cumulant method of 3rd order for data analysis.

2.2.3. Lipid quantification

Lipid concentration was determined with the Trinder method. An enzymatic colorimetric assay kit

[1] A trademark of IDEA AG, Munich, Germany.

(Spinreact, Girona, Spain) was used to determine the absolute phosphatidylcholine concentration.

2.2.4. Protein quantification

Total protein amount was assessed with the method of Lowry, after vesicle disruption with 2% Triton X-100 and 20% sodium dodecyl sulphate.

2.2.5. Solubilisation studies

Bilayer solubilisation was monitored by static light scattering at 450 nm with a Shimadzu UV-160 spectrophotometer. In brief, suspension turbidity vs. time curve, i.e. a solubilisation profile, was determined for various suspensions with SPC concentrations ranging from 1.2 to 30.6 mM. An experiment with isothermal buffer was used as negative control. Typically, each turbidity value was independently determined ($n=3$), 10 min after detergent addition.

2.2.6. Solubilisation parameters

According to Lichtenberg [14], the surfactant/lipid ratio R_e, which ensures lipid bilayer solubilisation, depends on the CMC and bilayer/water distribution coefficient of the surfactant, K. If lipid is present at concentration L and detergent at concentration D, the surfactant distribution between lipid bilayers and the aqueous medium is given by

$$K = R_e/[D_w(1 + R_e)] \quad (1)$$

with $R_e = D_b/L$ and $D_b + D_w = D$, and D_b and D_w giving detergent concentration in bilayer and aqueous sub-phases, respectively.

Parameters R_e, K, and D_w are determined by linear regression analysis of the results, which relate the nominal total surfactant concentration D and lipid concentration L:

$$D = D_w + R_e L \quad (2)$$

It is customary to specify also total surfactant concentration at the onset of vesicle solubilisation D_t^{sat} and at process completion D_t^{sol}. The following relationships between the two then emerge:

$$D^{sat} = D_w^{sat} + R_e^{sat} L \quad (3)$$

$$D^{sol} = D_w^{sol} + R_e^{sol} L \quad (4)$$

R_e^{sat} and R_e^{sol} characterise the respective detergent/lipid ratios at the beginning and end of vesicle solubilisation. Likewise, D_w^{sat} and D_w^{sol} define the bulk detergent monomer concentration at the onset and completion of vesicle solubilisation, when all lipids are in a bilayer or micelles, respectively.

2.2.7. Determination of vesicle permeability

2.2.7.1. Fluorescent marker release. We determined the permeability of mixed lipid vesicles as a function of nominal detergent concentration by monitoring fluorescence intensity increase after the release of a fluorescent dye, calcein, from the vesicle interior upon detergent addition. We used two procedures to separate the vesicle-loaded calcein from non-encapsulated dye. (1) Ultracentrifugation (Beckman L8-60M ultracentrifuge; 180,000 × *g* for 2 h at 15 °C) after sample dilution and collection of the dye-loaded vesicles pellet. (2) Gel filtration (800 μl applied to a Sephacryl S-400 HR column, 30 cm × 1.6 cm) eluted with 50 mM sodium phosphate buffer, pH 7.4, with a medium containing 5 mM NaChol. The fraction that contained calcein in vesicles was collected.

Small aliquots of vesicle suspensions with a constant SPC (as given) and calcein (0.3 mM) concentration were used. This ensured that nearly all calcein fluorescence, originally, was self-quenched. The aliquots were supplemented with various detergent amounts, to yield increasing final cholate concentrations, and incubated for 10 min. This partially released calcein from vesicles, and due to the dye dilution, increased its fluorescence. The temporal dependency of the resulting fluorescence increase $If_c(t)$ was recorded in triplicate on a Hitachi F3000 spectrofluorimeter (excitation: 490 nm, emission: 520 nm) for 10 min. Finally, the vesicles were totally disrupted by the addition of 200 μl of 5% Triton X-100 to 1 ml suspension. Complete release of all originally entrapped calcein was set as the maximum fluorescence intensity (If_T). The latter was used to normalise all previously measured fluorescence intensity changes: $If_c(t)/If_T$.

2.2.7.2. Protein release. Small aliquots of a vesicle suspension with constant SPC concentration (19.6 mM) were contacted with the detergent suspensions of increasing concentration for 10 min. To separate the vesicle-associated and dissolved protein, the suspension was diluted 10-fold and then ultracentrifuged (Beckman L8-60M ultracentrifuge, 180,000 × *g* for 2 h at 15 °C). The resulting pellet was analysed for lipid and protein content. Empty vesicles did not sediment under the described centrifugation conditions.

3. Results

3.1. Effect of sodium cholate on stability of SPC/NaChol mixed vesicles

Visible-, neutron-, and X-ray-light scattering methods are powerful techniques for characterising surfactant systems in equilibrium. Such scattering measurements were used by other authors to characterise, quantitatively, structural parameters of mixed surfactant/lipid systems over a scale range almost down to molecular level [26]. Optical turbidity of a vesicle suspension does not have

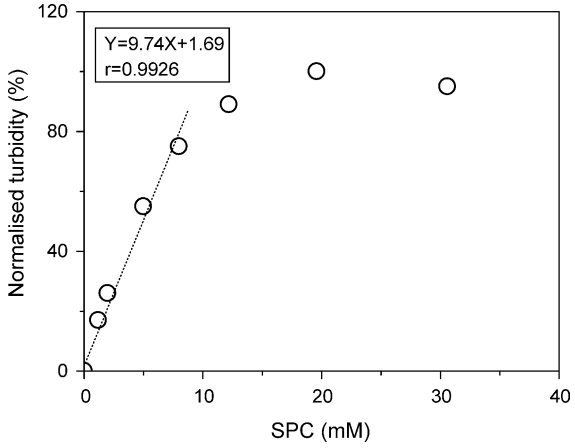

Fig. 1. Normalised turbidity of SPC/NaChol 3.75/1 mol/mol mixed aggregate suspensions with 138 mM starting total lipid concentration after dilution with simple phosphate buffer (50 mM, pH 7) to different final SPC concentrations given on the horizontal axis. The error bars are smaller than symbols.

such high resolution, but is a function of the average lipid aggregate size and of total vesicle number (total lipid concentration).

The short-term effect of suspension dilution (starting suspension with total lipid concentration, TSL = 138 mM) to a final SPC concentration ranging from 30.6 to 1.22 mM is shown in Fig. 1. A linear relationship was assumed between total phospholipid concentration and suspension turbidity in a concentration range of approximately 0–10 mM. At higher total lipid concentrations, the suspension turbidity ceased to change.

The temporal evolution of suspension turbidity, as a function of nominal phospholipid and detergent concentration (data not shown) reveals that the suspensions with two selected phospholipid concentrations (12 and 30 mM SPC) have a similar initial turbidity, as they are both in the saturation range. Optical density (OD) was nevertheless affected by absolute phospholipid concentration, when total cholate concentration increased, at least during the first few minutes of a 125 min observation period (data not shown). The characteristic OD decay time for the process was -0.003 and $-0.005\ \mathrm{min}^{-1}$ in case of 12 and 30 mM SPC concentration, respectively, and thus is roughly proportional to phospholipid content. When the bulk cholate concentration is sufficiently high to solubilise all vesicles (e.g. >20 (30) mM for 12 (30) mM SPC concentration), the suspension's OD fell rapidly below 0.1. These solubilisation studies suggest that different solubilisation profiles are achieved for various suspensions in turbidity saturation range.

In contrast, when a sub-solubilising detergent amount was added, NaChol distributed either into the aqueous subphase or into the mixed lipid bilayers, according to partitioning equilibrium. The effect of absolute cholate concentration on suspension turbidity during a 10 min

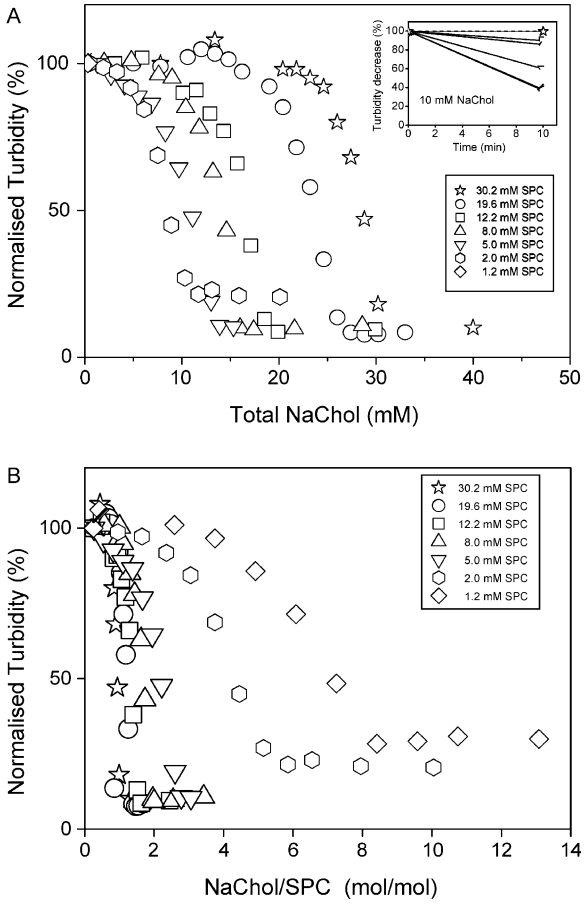

Fig. 2. Normalised turbidity of SPC/NaChol mixed aggregates with initial TSL 138 mM and final SPC concentration in the range 30.6–1.2 mM, as a function of nominal detergent concentration (A) or the detergent-to-lipid molar ratio (B). Turbidity was determined at 450 nm and 25 °C, 10 min after the addition of the external detergent. Inset: Suspension turbidity decreases after 10 min at constant NaChol concentration of 10 mM for different SPC concentrations.

observation window was studied. The representative results obtained are given in Fig. 2 for the suspensions with TSL = 138 mM and SPC concentrations between 30.6 and 1.2 mM. Fig. 2A represents the normalised turbidity in terms of nominal detergent concentration and Fig. 2B as a function of relative molar NaChol/SPC concentration. The inset in Fig. 2A shows the turbidity decrease at 10 min, measured at constant NaChol concentration. It shows that suspension turbidity decrease depends on SPC concentration, when the latter is below 8 mM. OD of the suspensions containing SPC concentrations above approximately 8 mM, first marginally increased and subsequently decreased with the increase of total NaChol concentration. In contrast, the turbidity of less concentrated suspensions generally decreased with the bulk NaChol concentration to a comparable, and low, final value. In our experiments, when SPC concentration exceeded 8 mM, the average vesicle size increased, presumably caused by cholate partitioning into lipid bilayers. When SPC concentration is between 2 and 5 mM, the average

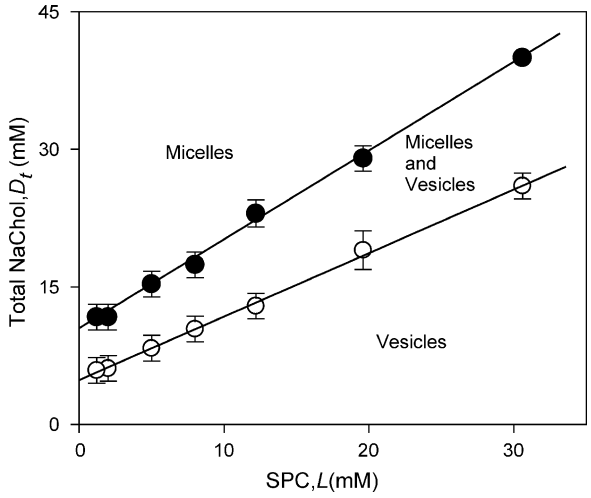

Fig. 3. Correlation between the total cholate concentration at the onset and end of bilayer solubilisation and total SPC concentration. Lines define the boundaries of SPC and NaChol concentration range in which the mixed lipid vesicles coexist with the mixed lipid micelles.

vesicle size increased moderately. For 1.2 mM SPC, the increase in vesicle size is minute.

Fig. 3 gives total detergent concentration at the point of bilayer saturation with NaChol or at solubilisation point, both as a function of SPC concentration. The detergent-to-lipid concentration ratio at the point of bilayer saturation with detergent, R_e^{sat}, and at the point of bilayer solubilisation, R_e^{sol}, thus can be derived from the slope of linear regressions ($D_t^{sat} = 0.6965L + 4.836$, $R^2 = 0.9984$; $D_t^{sol} = 0.9735L + 10.226$; $R^2 = 0.9975$) between the total detergent concentrations at which structural transformations occur (D_t^{sat} or D_t^{sol}) and L total lipid content. NaChol concentration in the mixed lipid aggregates and the bound/total NaChol concentration ratio, at the onset and end of SPC solubilisation, as a function of phospholipid concentration, are given in Fig. 4.

In Fig. 5, we compare the results of static (turbidity) and dynamic (PCS) light scattering experiments. We plot the average aggregate diameter and polydispersity index

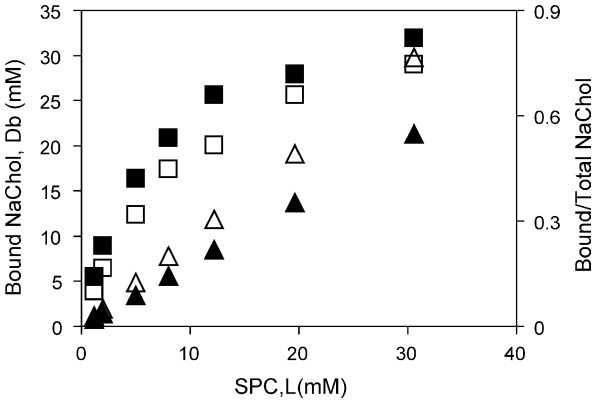

Fig. 4. Effect of SPC concentration on NaChol concentration in the mixed lipid aggregates at the onset (▲), and at the end (△) of lipid bilayer solubilisation and on the ratio bound to total NaChol at the onset (■), and at the end (□) of lipid bilayer solubilisation.

derived from the latter as a function of time. This reveals that both these parameters change approximately parallel to the corresponding OD variation. The comparison confirms the influence of absolute SPC and NaChol concentrations, as discussed for Fig. 2. The sharp increase of suspension polydispersity observed at the midpoint of vesicle solubilisation curve for the systems with SPC concentration higher than 2 mM is diagnostic of the co-existence of different aggregate populations—micelles and vesicles—with vastly different size (of the order of 10 and 100 nm, respectively). Below and above such a peak, mixed lipid vesicles and micelles prevail. Their shape and/or size depend on the relative as well as absolute concentrations of both amphipathic components.

3.2. Solubilisation of enzyme loaded SPC/NaChol vesicles

To complement simple bilayer solubilisation with delivery relevant parameter data, the effects of NaChol on enzyme loaded vesicles with SPC = 1.2 and 19.6 mM were studied. The enzyme load was 4.2 mg/ml for SOD and 5.1 mg/ml for CAT. The results are given in Fig. 6, showing no significant difference between solubilisation of protein containing and simple bilayers. In either case, 50% turbidity change was observed at cholate/lipid ratio of approximately 7 mol/mol, for SPC = 1.2 mM, and at such ratio near 1.25, for SPC = 19.6 mM. The only marked difference is the approximately 20% higher turbidity of the fully solubilised bilayers in the presence of CAT, which may result from light scattering on the proteins or protein aggregates.

3.3. Detergent mediated SPC/NaChol vesicle permeabilisation

The integrity of mixed lipid bilayers was tested by monitoring the release of vesicle-encapsulated fluorescent marker, calcein, induced by cholate addition to a suspension. This was done for preparations with TSL = 138 mM, diluted to final concentration of 1 mM SPC. The resulting total lipid concentration was thus low enough to avoid concentration dependent fluorescence quenching.

To study the release-dependent fluorescence intensity increase, vesicles were separated from the non-loaded calcein with two different procedures: the dilution was either followed by a centrifugation or gel filtration step (the latter method was used to obviate the effect of ultracentrifugation stress on vesicle bilayers). Cholate was added to the suspension and the resulting blend was left to equilibrate for 10 min (this was the minimum time period needed to achieve a constant calcein release from vesicles). The studies were performed at a constant temperature of 25 °C.

Transient reorganisations of the mixed lipid bilayers upon surfactant addition were monitored by recording continuously the increase of fluorescence intensity.

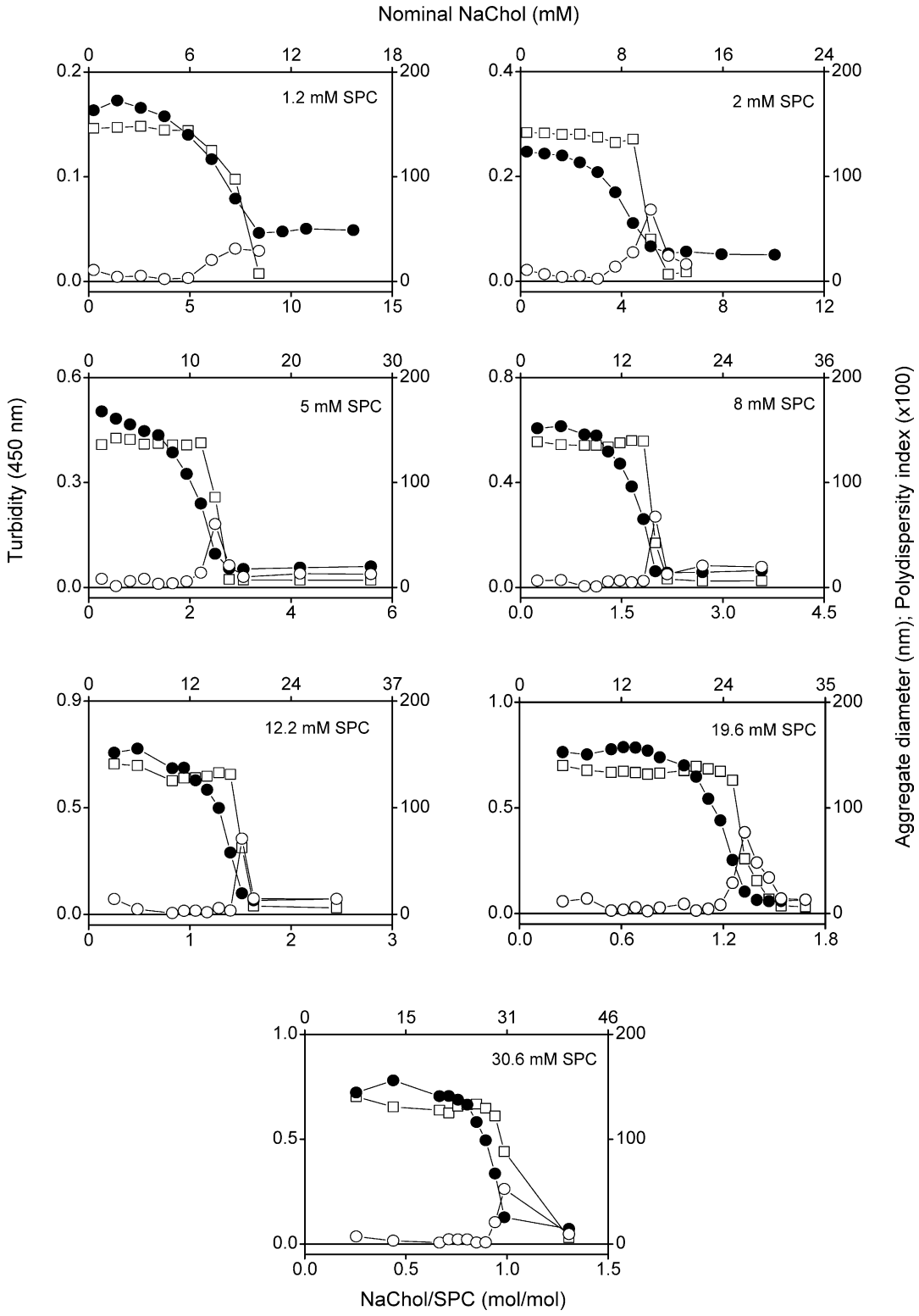

Fig. 5. Effect of detergent addition on NaChol/SPC aggregate size: absolute turbidity at 450 nm (●), aggregate diameter (□) and polydispersity index (○).

The detergent-dependent calcein release from the mixed lipid vesicles was thus related to structural changes in bilayers. This confirmed that calcein release from vesicles in the absence of surfactant is negligible and practically independent of the procedure used to remove the non-loaded calcein (see Fig. 7).

Calcein retention profiles can provide information on surfactant-induced membrane permeability increase.

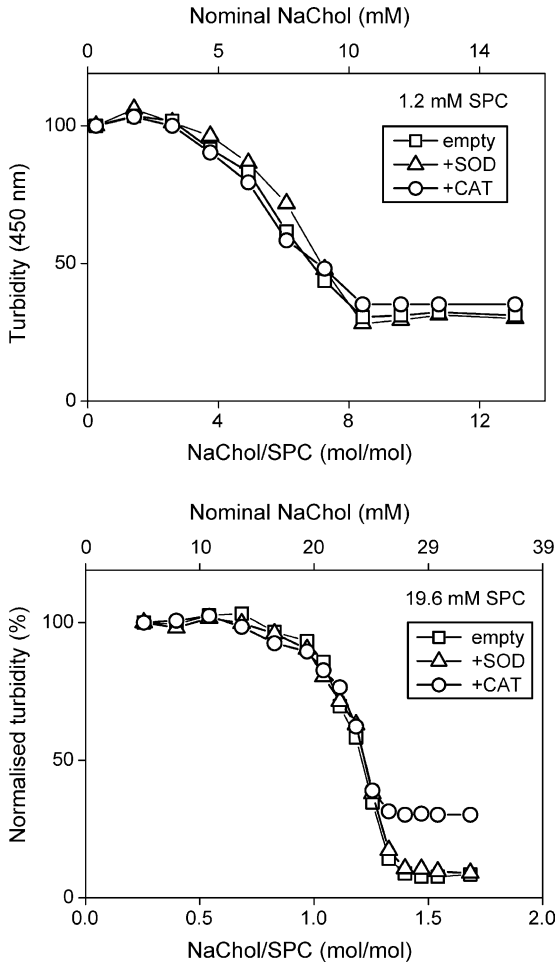

Fig. 6. Solubilisation of enzyme loaded mixed lipid aggregates with different SPC concentrations (1.2 and 19.6 mM). Solubilisation of empty vesicles with the same SPC concentration is illustrated for comparison. Mean turbidity (at 450 nm) at the start of the experiment: 1.2 mM SPC graphic empty vesicles 0.163; +SOD 0.167; +CAT 0.185; 19.6 mM SPC graphic empty vesicles 0.76; +SOD 0.78; +CAT 0.828.

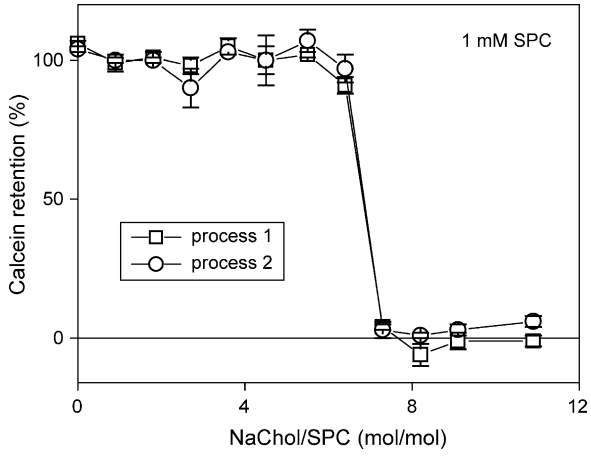

Fig. 7. Calcein retention in SPC/NaChol vesicles, with TSL 138 mM, diluted to final concentration of 1 mM SPC, after addition of various NaChol solutions 10 min earlier. Processes 1 and 2 is the ultracentrifugation- and gel filtration-based procedure for separating non-loaded calcein, respectively, as is described in Section 2.

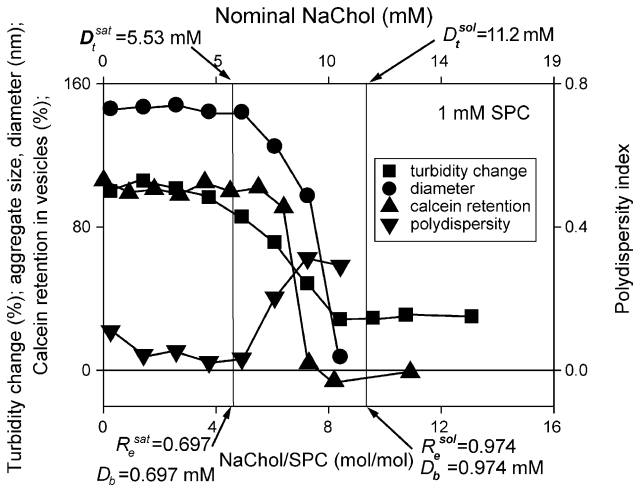

Fig. 8. Turbidity change, aggregate diameter, calcein retention and polydispersity of SPC/NaChol mixtures containing different cholate concentrations. The study was performed at constant lipid concentration. Error bars were smaller than 0.01 and were omitted for clarification. Upper axis gives the nominal cholate concentration and the total detergent at saturation and solubilisation (D_t^{sat} and D_t^{sol}), and lower axis gives the total relative cholate concentration and the aggregate associate relative cholate concentration at saturation and solubilisation (D_b^{sat}/L and D_b^{sol}/L).

Lipid bilayer leakage parallels cholate-concentration dependency of the other two main parameters: the suspension turbidity and the average lipid aggregate size, which are both a function of nominal NaChol concentration at constant SPC concentration (cf. Fig. 8).

Bilayer permeability to macromolecules as a function of detergent concentration, using SOD as the test protein, was also determined. For this purpose, mixed lipid vesicles were diluted to 19.6 mM SPC, which allowed usage of relatively high protein amounts and improved the results of protein quantification. After detergent addition, the aggregates were separated from the aqueous medium by ultracentrifugation. The results, expressed in terms of protein retention in vesicles, are presented in Fig. 9. For comparison, the suspension turbidity as well as the average vesicle size and polydispersity of the unloaded vesicles are given.

Vesicles are highly permeable to a macromolecule with 32 kDa molecular weight when the membrane bound cholate concentration is above 13.7 mM (cf. Fig. 9). Specifically, 50% of the vesicle-loaded SOD was released at this NaChol concentration, which corresponds to the aggregate-associated relative cholate molar ratio of ~0.7. Comparison of protein retention profiles with the average vesicle size and turbidity profiles confirmed that lipid bilayers are permeabilised, and that sufficiently wide transmembrane pores were created to accommodate macromolecules, before complete membrane solubilisation. The starting point appears to coincide with the first change in the sign of the derivative of turbidity change with respect to relative cholate concentration.

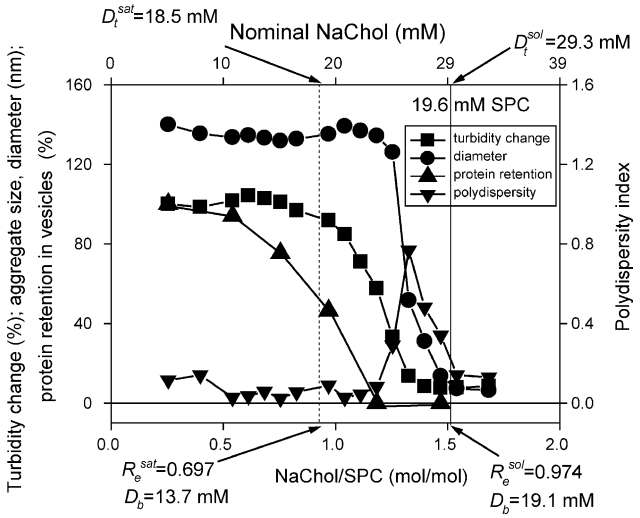

Fig. 9. SOD release from SPC vesicles after 10 min in a solution of sodium cholate yielding different final detergent/SPC concentrations expressed as retained protein content, turbidity change and the average vesicle diameter. Error bars were smaller than 0.01 and were omitted for clarification. Upper axis gives nominal cholate concentration. Lower axis gives total relative cholate concentration and aggregate-associated cholate concentration at saturation and solubilisation in relative terms.

4. Discussion

It is generally accepted that the effective detergent concentration in a bilayer, reflected in the ratio R_e, is an important parameter in phospholipid–detergent aggregation/solubilisation. In contrast, the absolute lipid and detergent concentrations are not important, at least when the bulk detergent concentration is above CMC. In a more general thermodynamic description, the finite micelle size can be explained [23]. In a more refined model, the absolute concentration of each amphipathic molecule in the system becomes influential in the low concentration range. Phase behaviour of the NaChol/SPC mixtures studied in this work corroborates such a conclusion.

For example, Fig. 3 shows that the detergent-to-lipid molar ratio at which suspension turbidity drops sharply depends on total phospholipid concentration. This is expected on the basis of the relationship $R_e = D_b/L$. The detergent-to-lipid molar ratio at which suspension turbidity drops sharply depends on total phospholipid concentration as well (cf. Section 2.2).

The detergent-to-lipid concentration ratio at the point of bilayer saturation with a detergent, R_e^{sat}, and at the point of bilayer solubilisation, R_e^{sol}, thus can be derived from the slope of linear regression ($D_t^{sat} = 0.6965L + 4.836$, $R^2 = 0.9984$; $D_t^{sol} = 0.9735L + 10.226$; $R^2 = 0.9975$) between the total detergent concentrations at which structural transformations occur (D_t^{sat} or D_t^{sol}) and L, total lipid content. The corresponding SPC concentration is another model variable.

The relationship $R_e = D_b/L$ is given in Fig. 3 and in Table 1 where a comparison with the results obtained by other authors is presented. Briefly, it was deduced that relative cholate concentration (mol/mol) in each lipid bilayer is $R_e^{sat} = 0.70 \pm 0.01$ and $R_e^{sol} = 0.97 \pm 0.02$. These two values are 40–75% higher than the most frequently cited in the literature, obtained for the systems based on SPC-cholate ($0.17 \le R_e^{sat} \le 0.4$, $0.3 \le R_e^{sol} \le 0.7$ [8,10,15,19,20,27,29]). Further literature data for the related but not identical amphipathic molecule mixtures [18,22,27,28], include values ranging between $0.1 \le R_e^{sat} \le 1.3$ and $0.3 \le R_e^{sol} \le 3.6$. The discrepancy between our results and those from the literature is not surprising given that studies were typically done under different experimental conditions with special emphasis on total starting lipid concentration; the latter, in our study, was higher than in the previously reported investigations, causing an increase in R_e^{sol} [19]. Indeed, even a brief glance at Table 1 shows that several factors, such as temperature, buffer composition, or lipid chain composition, affect these parameters appreciably.

The dissolved detergent concentration in an aqueous subphase at the described two-phase boundaries is given by the intercept with the vertical (SPC = 0) axis. From Fig. 3,

Table 1
Compilation of data on phosphatidylcholine solubilisation in aqueous solvents by sodium cholate under different experimental conditions

R_e^{sat}	R_e^{sol}	Phospholipid	Concentration	T (°C)	Ref.
n.a.	0.3	Egg PC	TSL = 0.75 mM		[8]
n.a.	0.3	Egg PC	TSL = 0.75 mM		[19]
0.3	n.a.	Egg PC	TSL = 0.75 mM		[20]
n.a.	n.a.	Egg PC	TSL = 9 mM		[15]
n.a.	>0.28	Egg PC	TSL = 45 mM		[19]
0.3	0.4	Egg PC	TSL = 200 mM		[29]
(0.01)	n.a.	DMPC	TPL = 1.5 mM	23.1	[18]
0.11	0.15	DPPC	TPL = 6 mM in 0.1 NaCl	60	[22]
0.41	0.64	POPC	TPL < 30 mM		[28]
n.a.	0.3	Egg PC	TPL = 17 mM; ±cholesterol (9:1; 8:2; 7:3 mol/mol)		[10]
0.3	0.7	Egg PC	TPL = 2 mM; ±BSA (0–100 mg/ml)		[27]
0.7	1	Soy PC	TSL = 138 mM; ±SOD (4 mg/ml); CAT (5 mg/ml)	25	This study

Egg PC, egg yolk phosphatidylcholine; DMPC, 1,2-dimyristoyl-3-glycerophosphocholine; DPPC, 1,2-dipalmitoyl-3-glycerophosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-3-glycerophosphocholine; BSA, bovine serum albumin; SOD, Cu/Zn superoxide dismutase; CAT, catalase; TSL, total starting lipid concentration; TPL, total phospholipid concentration. n.a., not available; T, temperature.

it is thus obvious that $D_w^{sat} = 4.84 \pm 0.19$ mM and $D_w^{sol} = 10.2 \pm 0.33$ mM.

Lipid vesicles become highly deformable shortly before partial bilayer solubilisation [30]. In order to be simultaneously sufficiently stable, such vesicles should be used before major solubilisation, i.e. when $R_e \leq R_e^{sat}$ [30,31]. Our results given in Figs. 8 and 10 confirm that lipid bilayer integrity is practically uncompromised in the tested range of relative detergent concentrations, at least as far as bilayer permeability is concerned.

Lichtenberg [23] proposed that the suspension turbidity in vesicle–micelle co-existence range is diagnostic of vesicle fraction in such mixed lipid aggregates population. In 'pure' phase regions, conversely, any measured turbidity value contains information about the system's composition, R_e.

The steep decrease in turbidity curve does not coincide with the average aggregate diameter diminution when SPC concentration exceeds approximately 1.2 mM. The greatest turbidity change is measured around lytic detergent concentration and the greatest size change is measured just above such a concentration point. The decrease in suspension turbidity is a mirror of small mixed lipid micelle formation, as such aggregates scatter light less strongly than the corresponding mixed lipid vesicles. In addition to micelles, the mixed lipid bilayers in the form of partly open vesicles, bilayer sheets or long, cylindrical micelles (threads) were reported to exist in phospholipid/NaChol mixtures [15,20,23].

Among other factors, the vesicle fraction in various aggregate co-existence regions depends on NaChol as well as SPC concentration. Sample dilution with water for the dynamic light scattering experiments (2 to 20-fold), therefore, can decrease the average vesicle size and skew the size distribution results. We found, however, that such change is very small. Lichtenberg also observed a minor decrease of the average vesicle size following detergent extraction from the mixed lipid vesicles, after dilution of NaChol/egg–phosphatidylcholine mixtures [23].

According to our data given in Fig. 8, and concluding from a previously published phase diagram [32], vesicles and micelles may co-exist. For phosphatidylcholine/cholate mixtures in aqueous suspensions with a neutral pH and with nominal detergent concentration ranging from 6 to 11 mM, vesicles co-exist with the mixed lipid micelles. This corresponds to an increase of detergent associated lipid in aggregates from 0.7 to 1 mM.

Increasing nominal detergent concentration increases the R_e value. If this is done within the concentration range specified in the previous paragraph, the micellar pool increases at the expense of vesicles in a suspension. Partition coefficient values pertaining to the onset and the end of phospholipid solubilisation confirm the trend ($K_e^{sat} = 84.9\ \mu M^{-1}$ and $K_e^{sol} = 48.3\ \mu M^{-1}$). Our experimental data are hence in harmony with the current picture of mixed lipid aggregate evolution.

By expressing lipid solubilisation in terms of R_e [23], one can highlight small differences in aggregate solubilisation curves: in most published experiments, vesicle size and leakage increase roughly in parallel. Solute permeation through vesicle bilayer is thus enhanced by a rising R_e value, especially near complete solubilisation.

Asymmetric binding of cholate above CMC to a lipid bilayer can induce transient membrane holes through a bilayer [8]. Permanent 'holes' in lipid bilayers were observed by electron microscopy [19,23], most often in the vesicle–micelle co-existence range, but also in purely vesicular suspensions. In the latter situation, a relatively high R_e value had to be used to achieve almost complete bilayer saturation with the detergent ($R_e \rightarrow R_e^{sat}$).

Schubert and Schmidt [8] published data on labelled vesicle solubilisation and inulin release through a bilayer during solubilisation. The conclusion was that solubilisation of phosphatidylcholine vesicles for TSL = 0.75 mM commences around $R_e = 0.3$ and is nearly independent of the choice of bile salt or phosphatidylcholine. The midpoint for such an uncharged solute release, according to Schubert, is $R_e = 0.24$. In contrast, we observed no significant leakage of the charged calcein from SPC vesicles at SPC = 1 mM, if the relative cholate concentration was below $R_e = 0.7 \sim R_e^{sat}$. Membrane solubilisation in our experiments did not begin before $R_e \sim 0.8$, requiring 6 mM bulk NaChol concentration that is needed for ~0.5 mM phosphatidylcholine solubilisation. A small difference between the total phospholipid concentrations used by Schubert and us, 1 vs. 0.75 mM, respectively, does not explain the difference.

One possible explanation for the above-mentioned discrepancy is different phospholipid composition, which for the natural phosphatidylcholines can vary between batches, manufacturers, etc. Another more likely explanation is that the difference may be due to the charge on calcein molecules. Electrostatic interactions between the negatively charged calcein and the negatively charged cholate molecules on a bilayer certainly lower the probability for calcein leakage in comparison with a non-charged solute, such as inulin. Bilayer permeabilising cholate concentration appears to be sensitive to permeant charge as well: less cholate is needed to release various uncharged oligo-saccharides (MW = 594 and 5000) from a lipid vesicle than to help a charged chemical (MW = 622.5) leak through a NaChol containing mixed lipid bilayer [19].

According to the literature [33,34], SOD or CAT does not affect lipid solubilisation despite their charge and surface activity. SOD encapsulation data measured with liposomes moreover suggest that the enzyme does not adsorb to phosphatidylcholine vesicles [35]. This indicates that the proteins used in our study neither adsorb nor strongly interact otherwise with the tested mixed lipid bilayers. The observation is in accord with the need to encapsulate SOD and CAT in SPC/NaChol mixed lipid vesicles to achieve protein carrier association.

In contrast, when bovine serum albumin (BSA) is combined with SPC/NaChol bilayers, the protein shifts bilayer solubilisation upwards, i.e. towards higher cholate concentrations [27]. However, BSA is known to adsorb to phosphatidylcholines [36] and to bind water-soluble lipids directly. Bilayer solubilisation sensitivity to BSA thus could be a consequence of either of these phenomena. Cholate binding to BSA, for example, would lower the dissolved detergent concentration and thus diminish the effective R_e value. Cholate adsorption to a bilayer could stiffen the membrane and thus increase the required R_e^{sat} value. In our view, the latter explanation is more probable.

Membrane-bound cholate always (partially) destabilises lipid bilayer and makes it more prone to poration. Macromolecular passage is thus facilitated, which has already been used to load lipid vesicles with enzymes [37]. The well-known alterations in membrane structure at sub-solubilising detergent concentrations are a reflection of this [27]. Stress of ultracentrifugation, imposed on the mixed lipid bilayers with increasing detergent content, may support similar structural changes as well, and thus promote lipid vesicle disruption. The decrease in lipid amount recovered from ultracentrifugation sediment (results not shown) points in a similar direction.

Phosphatidylcholine–NaChol mixed bilayers obviously undergo strong shape and/or size changes during solubilisation; the increased mixed lipid aggregate polydispersity in the solubilisation region demonstrates this clearly (cf. Figs. 5 and 9). The conclusion is in accord with the results of computer simulations of vesicle solubilisation/fluctuations, published by Kroll and Gompper [38]. These researchers found that each soft lipid vesicle forms bilayer protrusions. The frequency and/or length of protrusions are enhanced by bilayer elasticity, which was shown independently to increase with detergent concentration in a bilayer [11]. Such protrusions can detach from the original vesicle, and then transform into a (thread-like) mixed lipid micelle. The process must go hand in hand with trans-bilayer pore ('hole') formation, which increases bilayer permeability. Bilayer permeability, indeed, is typically augmented just before or during lipid bilayer solubilisation.

5. Conclusions

In this study, new information about the ability of NaChol to alter permeability and to solubilise unilamellar mixed lipid bilayer vesicles in the presence of large molecules was gained. Mixed lipid aggregates suitable for non-invasive delivery of small and large molecules were characterised. In particular, for SOD and CAT, our results showed no measurable effect of such macromolecules on phosphatidylcholine mixed aggregate solubilisation by NaChol. Lack of structurally influential lipid–protein interaction is reflected in the nearly constant values of all parameters that describe NaChol partitioning into lipid bilayer. Our corresponding experimental results, $R_e^{sat} = 0.7$ and $R_e^{sol} = 1$, are essentially the same for the protein-loaded and simple phosphatidylcholine mixed vesicles. A slightly higher cholate concentration is required to make lipid bilayers containing phosphatidylcholine leaky to SOD (MW = 32 kDa) compared with calcein (MW = 622.5). The time and concentration dependency of vesicle interactions with NaChol were studied in sub-lytic and lytic concentration range. In contrast to several previous publications, which deal with the conventional liposomes stabilised and stiffened by cholesterol [32,39] we paid attention only to destabilised vesicles in the presence and absence of SOD and CAT. This afforded the data that will be useful for designing and improving carriers for such proteins delivery, aiming specifically at non-invasive and targeted drug application.

Acknowledgements

This work was partially supported by the project POCTI/1999/FCB/35787 from Fundação para a Ciência e a Tecnologia. The authors acknowledge Dr M.J. Costa Ferreira for revising the manuscript and Dr M.L. Corvo for the helpful discussion of results.

References

[1] D.M. Small, M.C. Bourges, D.G. Dervichian, The biophysics of lipidic associations. I. The ternary systems: lecithin–bile salt–water, Biochim. Biophys. Acta 125 (1966) 563–580.
[2] D.M. Small, in: P.P. Nair, D. Kritchevsky (Eds.), The physical chemistry of cholanic acids The Bile Salts vol. I, Plenum Press, New York, 1971, pp. 340–349.
[3] N.A. Mazer, G.B. Benedek, M.C. Carey, Quasielastic light-scattering studies of aqueous biliary lipid systems. Mixed micelle formation in bile salt–lecithin solutions, Biochemistry 19 (1980) 601–611.
[4] N.A. Mazer, M.C. Carey, Mathematical model of biliary lipid secretion: a quantitative analysis of physiological and biochemical data from man and other species, J. Lipid Res. 25 (1984) 932–953.
[5] N.A. Mazer, P. Schurtenberger, Quasi-elastic light-scattering studies of aqueous biliary lipid systems and native bile, Hepatology 12 (1990) 587–606.
[6] M.H.W. Milsmann, R.A. Schwendener, H.G. Weder, The preparation of large single bilayer liposomes by a fast and controlled dialysis, Biochim. Biophys. Acta 512 (1978) 147–155.
[7] D. Schwarz, D. Zirwer, K. Gast, H.W. Meyer, U. Lachmann, Preparation and properties of large octylglucoside dialysis/adsorption liposomes, Biomed. Biochim. Acta 47 (1988) 609–616.
[8] R. Schubert, K.H. Schmidt, Structural changes in vesicle membranes and mixed micelles of various lipid compositions after binding of different bile salts, Biochemistry 27 (1988) 8787–8794.
[9] R. Naeff, V. Pliska, H.G. Weder, Pharmacokinetics of small unilamellar liposomes and incorporated lonazolac after i.m. administration, J. Microencapsul. 7 (1990) 95–103.
[10] R. Schubert, H. Wolburg, K.H. Scmidt, H.J. Roth, Loading of preformed liposomes with high trapping efficiency by detergent-induced formation of transient membrane holes, Chem. Phys. Lipids 58 (1991) 121–129.

[11] G. Cevc, Material transport across permeability barriers by means of lipid vesicles. In: R. Lipowsky, E. Sackman (Eds.), Handbook of Biological Physics vol. I, Structure and Dynamics of Membranes, Elsevier Science BV, North Holland, 1995, pp. 465–490.
[12] G. Cevc, D. Gebauer, J. Stieber, A. Schätzlein, G. Blume, Ultraflexible vesicles, transfersomes, have an extremely low permeation resistance and transport therapeutic amounts of insulin across the intact mammalian skin, Biochim. Biophys. Acta 1368 (1998) 201–215.
[13] G. Cevc, Transfersomes®—innovative transdermal drug carriers in: M. Rathbone, M. Robert, J. Hadgraft (Eds.), Modified Release Drug Delivery Technology, Marcel Dekker, New York, 2002, pp. 533–546.
[14] D. Lichtenberg, Characterization of the solubilisation of lipid bilayers by surfactants, Biochim. Biophys. Acta 821 (1985) 470–478.
[15] A. Walter, P.K. Vinson, A. Kaplun, Y. Talmon, Intermediate structures in the cholate–phosphatidylcholine vesicle–micelle transition, Biophys. J. 60 (1991) 1315–1325.
[16] S.U. Egelhaaf, P. Schurtenberger, The micelle-to-vesicle transition—a time-resolved structural study, Phys. Rev. Lett. 82 (1999) 2804–2807.
[17] M. Nagata, T. Yotsuyanagi, K. Ikeda, A two-step model of disintegration kinetics of liposomes in bile salts, Chem. Pharm. Bull. 36 (1988) 1508–1513.
[18] T.M. Bayerl, G.D. Werner, E. Sackmann, Solubilization of DMPC and DPPC vesicles by detergents below their critical micellization concentration: high-sensitivity differential scanning calorimetry, Fourier transform infrared spectroscopy, and freeze-fracture electron microscopy reveal two interaction sites of detergents in vesicles, Biochim. Biophys. Acta 984 (1989) 214–224.
[19] R. Schubert, K. Beyer, H. Wolburg, K.H. Schmidt, Structural changes in membranes of large unilamellar vesicles after binding of sodium cholate, Biochemistry 25 (1986) 5263–5269.
[20] J. Lasch, Interaction of detergents with lipid vesicles, Biochim. Biophys. Acta 1241 (1995) 269–292.
[21] D.E. Cohen, G.M. Thurston, R.A. Chamberlin, G.B. Benedek, M.C. Carey, Laser light scattering evidence for a common worm-like growth structure of mixed micelles in bile salt- and straight-chain detergent–phosphatidylcholine aqueous systems: relevance to the micellar structure of bile, Biochemistry 37 (1998) 14798–14814.
[22] A. Hildebrand, R. Neubert, P. Garidel, A. Blume, Bile salt induced solubilization of synthetic phosphatidylcholine vesicles studied by isothermal titration calorimetry, Langmuir 18 (2002) 2836–2847.
[23] D. Lichtenberg, E. Opatowski, M. Kozlov, Phase boundaries in mixtures of membrane-forming amphiphiles and micelle-forming amphiphiles, Biochim. Biophys. Acta 1508 (2000) 1–19.
[24] D.D. Lasic, A molecular model for vesicle formation, Biochim. Biophys. Acta 692 (1982) 501–502.
[25] P. Fromherz, Lipid-vesicle structure: size control by edge-active agents, Chem. Phys. Lett. 94 (1983) 259–266.
[26] S.U. Egelhaaf, U. Olsson, P. Schurtenberger, Time-resolved SANS for surfactant phase transitions, Physica B 276–278 (2000) 326–329.
[27] D. Meyuhas, D. Lichtenberg, The effect of albumin on the state of aggregation and phase transformations in phosphatidylcholine–sodium cholate mixtures, Biochim. Biophys. Acta 1234 (1995) 203–213.
[28] M. Treyer, P. Walde, T. Oberholzer, Permeability enhancement of lipid vesicles to nucleotides by use of sodium cholate: basic studies and application to an enzyme-catalyzed reaction occurring inside the vesicles, Langmuir 18 (2002) 1043–1050.
[29] S. Almog, T. Kushnir, S. Nir, D. Lichtenberg, Kinetic and structural aspects of reconstitution of phosphatidylcholine vesicles by dilution of phosphatidylcholine–sodium cholate mixed micelles, Biochemistry 25 (1986) 2597–2605.
[30] G. Cevc, A. Schätzlein, H. Richardsen, Ultradeformable lipid carriers can penetrate the skin and other semi-permeable barriers unfragmented. Evidence from double label CLSM experiments and direct size measurements, Biochim. Biophys. Acta 1564 (2002) 21–30.
[31] G. Cevc, A. Schätzlein, H. Richardsen, U. Vierl, Overcoming semi-permeable barriers, such as the skin, with ultradeformable mixed lipid vesicles, transfersomes®, liposomes or mixed lipid micelles, Langmuir 19 (2003) 10753–10763.
[32] M. Almgren, Mixed micelles and other structures in the solubilisation of bilayers lipid membranes by surfactants, Biochim. Biophys. Acta 1508 (2000) 146–163.
[33] M.B.F. Martins, M.E.M. Cruz, Characterization of bioconjugates of L-asparaginase and Cu–Zn-superoxide dismutase Proceeding of Third European Symposium on Controlled Drug Delivery (1994) pp. 188–191.
[34] C. Aguiar, M.L. Corvo, M.E.M. Cruz, M.B.F. Martins, Synthesis and characterization of chemically modified catalase Proceeding of the III Spanish–Portuguese Conference on Controlled Drug Delivery (1998) pp. 190–191.
[35] M.L. Corvo, J.C.S. Jorge, R. van't Hof, M.E.M. Cruz, D.J.A. Crommelin, G. Storm, Superoxide dismutase entrapped in long-circulating liposomes: formulation design and therapeutic activity in rat adjuvant arthritis, Biochim. Biophys. Acta 1564 (2002) 227–236.
[36] G. Cevc, L. Strohmaier, J. Berkholz, G. Blume, Molecular mechanism of protein interactions with the lipid bilayer membrane, Stud. Biophys. 138 (1990) 57–70.
[37] T. Oberholzer, E. Mayer, I. Amato, P. Lustig, P.A. Monnard, Enzymatic reactions in liposomes using the detergent-induced liposome loading method, Biochim. Biophys. Acta 1416 (1999) 57–68.
[38] D.M. Kroll, G. Gompper, The conformation of fluid membranes: Monte Carlo simulations, Science 255 (1992) 968–971.
[39] A. Moschetta, P.M. Frederik, P. Portincasa, G.P. vanBerge-Henegouwen, K.J. van Erpecum, Incorporation of cholesterol in sphingomyelin– phosphatidylcholine vesicles has profound effects on detergent-induced phase transitions, J. Lipid Res. 43 (2002) 1046–1053.