THE JOURNAL OF PHYSICAL CHEMISTRY B

ARTICLE

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Use of Simple Kinetic and Reaction-Order Measurements for the Evaluation of the Mechanism of Surfactant−Liposome Interactions

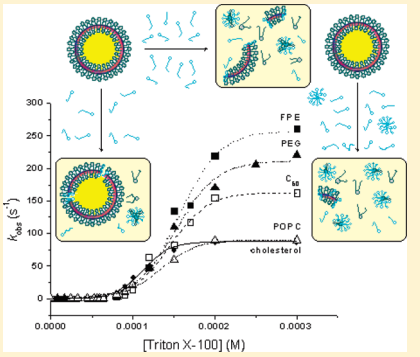
Diana Velluto,[†,§] Carla Gasbarri,[‡,§] Guido Angelini,[‡] and Antonella Fontana*[,‡]

[†]Institute for Bioengineering and Institute for Chemical Sciences and Engineering, École Polytechnique Fédérale de Lausanne (EPFL), Station 15, CH-1015 Lausanne, Switzerland

[‡]Dipartimento di Scienze del Farmaco, Università "G. d'Annunzio", Via dei Vestini, I-66013 Chieti, Italy

Supporting Information

ABSTRACT: Surfactant−liposome interactions have been previously studied through different methods and techniques. We present here a classical physical chemistry study on liposome solutions added to destabilizing agents at concentrations well above the solubilization concentration, which enable us to draw useful and interesting conclusions about the mechanism of surfactant-induced liposomal breakdown by simply exploiting the kinetics and the reaction order of the liposomal content release. In such excess of surfactant, the mechanism of surfactant-induced rupture of the liposomes has been demonstrated to be different from that proposed for low surfactant concentrations. Thus, depending on the surfactant concentration, two prevailing processes have been evidenced: (i) a cooperative mechanism that implies the assembly of a critical number of surfactant molecules to trigger the formation of a channel and therefore the release of the liposomal content and (ii) a mechanism driven by direct interaction of the surfactant molecules with the lipids that causes the complete solubilization of the liposomes. The former mechanism occurs at low surfactant concentrations, whereas the latter occurs at higher concentrations and above the CMC of the surfactants. The effect of different guests embedded into the liposomal bilayer on the mechanism of surfactant-induced liposomal breakdown has been compared by using the second-order rate constants measured for the liposome breakdown process.



INTRODUCTION

Liposomes are phospholipid vesicular systems that have come into widespread use both as simplified models of biological membranes and as delivery systems.[1] Liposomes have to meet precise requirements to be used as drug carriers, acting both as tight containers of a given drug and as releasers of the drug once the target has been reached. The release of the liposomal content could be either spontaneous or induced by different metabolites such as recently investigated positively charged antimicrobial peptides[2], or by destabilizing agents such as nonionic amphiphiles, often used as excipients of drug formulations. Therefore, a precise knowledge of the mechanism involved in the spontaneous and induced release of liposomal content becomes crucial. Previous studies on the process involved in surfactant-induced solubilization of liposomes had provided useful information on the interactions of the liposomes with amphiphilic molecules. The general scheme for lipid bilayer dissolution by nonionic surfactants has been described[3−5] by the so-called three-stage model. In the first stage, the surfactant partitions between the bilayer and the aqueous phase until a saturation of the bilayer is reached. In general, this concentration is identified by C_{SAT} or the corresponding, lipid independent R_{SAT}, the surfactant-to-lipid concentration ratio at the saturation point. C_{SAT} and R_{SAT} are generally associated with a maximum turbidity of the solution due to the swelling of the membrane or the fusion of liposomes. Upon further increasing of the surfactant concentration, the saturated bilayers coexist with mixed micelles of phospholipid−surfactant (second stage II). The surfactant-to-lipid ratio at which the transition from the lamellar phase to the micellar phase is complete, referred to as R_{SOL}, is indicative of the onset of the third stage where only surfactant−phospholipid mixed micelles exist.[6] The above-mentioned model pertains to surfactant solubilization of conventional phospholipids, but recently, the behavior of sterically stabilized[6] or guest-containing liposomes[7,8] upon addition of nonionic surfactants was also investigated by means of turbidity,[6] light scattering,[6] cryo-TEM,[6] optical microscopy,[8] and fluorescence techniques.[7,8] To describe properly the interaction between surfactants and liposomes, all of these studies paid overall regard[9] to the first and the second stages of the model, that is, to the stages in which low surfactant concentrations are added to liposomes and their membrane is either intact, although their original physical properties may be partially altered, or in equilibrium with mixed micelles. However, what happens when a large concentration of detergent over the lipid is used? What is the role of the surfactant once the concentration of solubilization of the membrane, C_{SOL}, has been reached?

Received: March 20, 2011
Revised: May 17, 2011
Published: May 23, 2011

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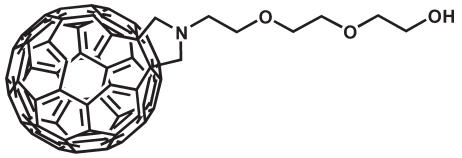
Chart 1. Structure of 2-[2-(2-Fulleropyrrolidin-1-ylethoxy)-ethoxy]-ethanol (FPE)



In the present study, we intend to use classical physical chemistry data such as the rate of release of a dye entrapped in liposomes or the reaction order of this release to get information on the mechanism of surfactant−liposome interactions. Our attention is focused on the consequences of the addition of destabilizing agents at concentrations well above C_{SOL} on the permeability and solubility of the bilayers. These conditions are particularly interesting and deserve to be investigated and understood in detail if one considers that many drug carrier formulations consist[10−12] of a mixture of phospholipids and nonionic surfactants. In particular, the extremely fast processes were monitored over time by using the stopped-flow technique. The kinetic data and the obtained rate law allow us to discriminate between (i) a cooperative mechanism that increases liposomal permeability via pores formation at surfactant concentrations below C_{SOL} and (ii) a mechanism in which surfactant molecules directly solubilize phospholipids into mixed surfactant−lipid micelles at surfactant concentrations above C_{SOL}. The investigated liposomes were made of pure 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) or of POPC with different guests such as cholesterol, C_{60} and an hydrophilic C_{60} derivative, 2-[2-(2-fulleropyrrolidin-1-ylethoxy)-ethoxy]-ethanol (FPE) (Chart 1) solubilized into the bilayer.

These guests are known to confer different features to the POPC bilayer and can therefore affect in various ways the mechanism of surfactant−bilayer interaction.[7,8,13−21] Indeed it has already been demonstrated that the presence of cholesterol alters the partition coefficient of Triton X-100 into the POPC bilayer.[22] The effects of the investigated guests have been compared with those inferred by the well-known 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanol-amine-*N*-[methoxy-(polyethylene glycol)-2000] ammonium salt (PEG), widely used for the preparation of stealth liposomes. As model solubilizing agents we have used the nonionic surfactants Triton X-100 and octaethylenglycol monododecylether ($C_{12}E_8$).

EXPERIMENTAL SECTION

Materials. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy-(polyethylene glycol)-2000] ammonium salt (a poly(ethylene glycol) polymer covalently bonded to a lipid molecule, PEG) were purchased from Avanti Polar Lipids (Alabaster, AL). Fullerene (C_{60}), cholesterol, 5(6)-carboxyfluorescein (CF) (95% purity), Triton X-100, and octaethylenglycol monododecylether ($C_{12}E_8$) were obtained from Sigma.

The synthesis of FPE was based on a 1,3-dipolar cycloaddition reaction, as previously reported.[23]

Preparation of Unilamellar Liposomes. Dry phospholipidic films were prepared by dissolving the pure phospholipid or the phospholipid added with the proper amount of guest in chloroform (or carbon tetrachloride in the case of C_{60}) and by removing the solvent by evaporation under vacuum. The film was kept in the refrigerator at 4 °C overnight and then rehydrated with 2 mL of an aqueous buffer. For turbidity measurements, the buffer (pH 7.4) was made of 174 mM NaCl, 105 mM Na_2HPO_4, and 20 mM KH_2PO_4, whereas for fluorimetric analysis, the buffer (pH 7.4) was made of 121.5 mM NaCl, 25.2 mM Na_2HPO_4, 4.8 mM KH_2PO_4, and 50 mM CF. Large unilamellar liposomes (LUV) were produced by multiple extrusion of the aqueous suspension through polycarbonate or polyester (in the presence of C_{60} and FPE) filters (Osmonics: pore size 200 nm) mounted on an extruder from Lipex Biomembranes, Vancouver BC, Canada. To get rid of unentrapped dye, the samples used for the fluorimetric analysis were run through a Sephadex G-50 column at room temperature. The concentrations of the guests in the final liposomal suspensions were 9.1 mol % for cholesterol, 3.9 mol % for PEG, 0.53 mol % for C_{60}, and 0.41 mol % for FPE, respectively.

Turbidity Measurements. Surfactant solutions (in the concentration interval 5.0×10^{-5} to 1.2×10^{-3} M for Triton X-100 and 5.0×10^{-5} to 8.2×10^{-4} M for $C_{12}E_8$) were added to the liposomal suspensions, and the resulting mixtures were left to equilibrate for 3 h. The final concentration of POPC was kept constant at ca. 3.3×10^{-4} M. Turbidity was monitored at 25 °C at a wavelength of 300 nm for liposomes of pure POPC, POPC-PEG, and POPC-cholesterol and at 400 nm for liposomes of POPC-C_{60} and POPC-FPE. The change in turbidity of the liposomal suspensions was plotted against the surfactant/lipid molar ratio, *R*.

Kinetic Measurements. For kinetic studies, the liposomal suspension was diluted with an isosmotic (578 mOsm) buffer made of 174 mM NaCl, 105 mM Na_2HPO_4, and 20 mM KH_2PO_4 (pH 7.4) and mixed with an equal volume of the nonionic surfactant solubilized in the same 578 mOsm buffer to achieve the final POPC concentration of 1.32×10^{-5} M. Surfactant concentrations were varied in the interval (0.8 to 3.0) $\times 10^{-4}$ M in the case of Triton X-100 and (0.3 to 1.0) $\times 10^{-4}$ M in the case of $C_{12}E_8$, and the liposome breakdown was monitored by measuring the release of entrapped CF at a fluorescence emission wavelength of 516 nm. The temperature was kept at 25.0 ± 0.1 °C. The rate of CF leakage was high in all measurements performed; therefore, the emission intensity was followed by using a fluo-stopped-flow instrument.

Instruments. A spectrophotometer model V-550 (Jasco-Europe, Milan, Italy) was used for turbidimetric measurements of R_{SAT} and R_{SOL}. A stopped-flow spectrofluorimeter (Tri-Tech, Manitoba, Canada) was used to monitor the release of CF entrapped in the liposomes. The osmolarity of the samples was checked by using a microosmometer model 330 (Advanced Instruments Inc., Norwood, MA, US).

RESULTS

Determination of the Solubilization, R_{SOL}, and the Saturation, R_{SAT}, Surfactant-to-Lipid Ratio for Triton X-100 and $C_{12}E_8$. The solubilization of liposomes upon the addition of surfactants leads to the breakdown of liposomes and the formation of mixed micelles that, differently from liposomes, do not scatter light.[24] The breakdown of liposomes can therefore be monitored by turbidity variations upon the addition of surfactants. We are aware that to determine the phase boundaries of the studied liposomal systems and thus to draw accurate phase diagrams we should have performed these measurements at different concentrations of the constituent lipids;[6] nevertheless,

dx.doi.org/10.1021/jp2026187 |J. Phys. Chem. B 2011, 115, 8130–8137

the aim of the present study was not to construct phase diagrams[25] but rather to have a rough and relative indication of R_{SAT} and R_{SOL} of the different guest-containing liposomal systems investigated; therefore, we were satisfied in the present study with relative measurements performed at a POPC concentration of 3.3×10^{-4} M for all investigated systems.

In agreement with the three-stage model, the initial addition of the surfactant generally increases[26,27] the turbidity of the suspension because of the incorporation of the surfactant into the liposomal bilayer and the consequent increase in the liposomal dimensions. On further increasing the concentration of the surfactant, the turbidity decreases and finally is canceled out because of the complete transformation of liposomes into non-light-scattering micelles.

The turbidity curves highlight the presence of two critical values corresponding to the surfactant/lipid concentration ratios at the point of saturation of the liposomal bilayer (R_{SAT}) and at the onset of the complete solubilization of phospholipids into mixed micelles (R_{SOL}). As an example, Figure 1 reports the turbidity curve of the pure POPC liposomal system used for the determination of R_{SAT}, which corresponds to the maximum turbidity of the liposomal suspension, and of R_{SOL}, which corresponds to the point of achievement of an almost null turbidity.

Table 1 reports the obtained R_{SAT} and R_{SOL} values for the different liposomal systems. For the sake of comparison, Table 1 reports corresponding literature data obtained[7,8] by fluorimetry.

The rather poor agreement between R_{SAT} data obtained by fluorimetric and turbidimetric measurements is only apparent and depends on the fact that these techniques are indirect methods of monitoring the insertion of surfactant into the bilayer. Both techniques are quite good in determining the concentration of surfactant that solubilizes the bilayer, C_{SOL}, because this concentration involves either a relatively high increase in the CF release when the former technique is chosen or a complete disappearance of the turbidity of the liposomal dispersion when the latter method is performed. The determination of C_{SAT} is not straightforward. In the fluorimetric determination, C_{SAT} corresponds to the concentration of surfactant at which the rate of release of embedded CF is significantly higher than that monitored in the absence of the surfactant (i.e., spontaneous leakage). C_{SAT} could therefore be hampered, for example, by a relatively low rate of release of CF or by a bilayer in which the release of CF is concealed by linkages (i.e., hydrogen bonds) between CF and the bilayer constituents.[7,28] In the turbidimetric measurements, the C_{SAT} is the concentration at which a maximum increase in the dimensions of the vesicles is observed. However, the insertion of surfactant does not necessary involve an enlargment of the dimension of the aggregates, wheras sometimes a simple wrinkled surface of the bilayer may induce large turbidity or light scattering variations. For example, in Figure 1, the plateau region of high turbidity spans within the C_{SAT} value obtained by fluorimetry. A similar poor agreement of R_{SAT} values has been already highlighted for measurements reported by different authors for the same surfactant.[9] The data reported in the present study are in fairly good agreement with data previously published[29] on similar systems.

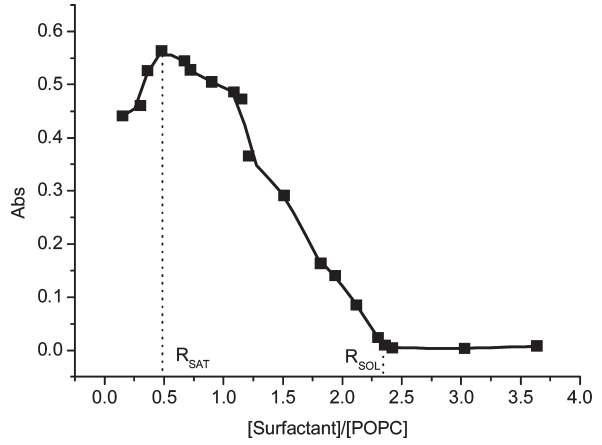


Figure 1. Turbidity curve for the solubilization of pure POPC liposomes upon addition of Triton X-100.

Kinetic Measurements. The process of liposome breakdown, induced by the addition of nonionic surfactants at concentration ratio above R_{SOL} to form mixed-micelles has been followed by spectrofluorimetrically monitoring the release of entrapped CF from liposomes at an emission wavelength of 516 nm by using the excitation wavelength of 490 nm. The initial fluorescence intensity was relatively low (see, as an example, Figure 2) because of the self-quenching effect due to the high concentration (50 mM) of CF used for re-hydrating the phospholipidic film. The final fluorescence intensity, that is, the intensity at infinite time, was experimentally monitored and inserted in the rate law equation.

The nonionic surfactant, either Triton X-100 or $C_{12}E_8$, was added to the liposomal suspension in a 1:1 mixing ratio, as shown in Scheme 1.

According to the three-stage model, the breakdown of liposomes is a potentially complex process because the reaction taking place is necessarily a multistep process. In an attempt to understand the kinetics of the breakdown and to compare the

Table 1. R_{SOL} and R_{SAT} Determined by Turbidimetry upon the Addition of Triton X-100 and $C_{12}E_8$ to Different Liposomal Systems[a]

	Triton X-100		$C_{12}E_8$	
liposome[b]	R_{SOL} (lit. data)[c]	R_{SAT} (lit. data)[c]	R_{SOL} (lit. data)[d]	R_{SAT} (lit. data)[d]
POPC	2.3 (2.1)	0.48(0.45)	1.5 (1.9)	0.50 (0.54)
POPC-PEG	2.4 (2.2)	0.61 (0.77)	1.7 (1.8)	0.40 (0.48)
POPC-cholesterol	2.1 (3.0)	0.48 (1.1)	1.7	0.50
POPC-C_{60}	1.7 (2.4)	0.67 (0.95)	1.7	0.50
POPC-FPE	1.8 (1.9)	0.67 (0.23)	1.7	0.65

[a] For the sake of comparison, previously determined data are also reported. [b] Concentration of POPC is 3.3×10^{-4} M in all investigated systems. [c] Value previously determined[7,8] by fluorimetry. [d] Value previously determined[6] by turbidity from EPC and EPC/PEG 95:5 liposomes obtained by thin layer hydration, followed by extrusion through 100 nm pores.

dx.doi.org/10.1021/jp2026187 |J. Phys. Chem. B 2011, 115, 8130–8137

data of different liposomal systems, we have therefore employed a phenomenological approach.

It has been evidenced that all experimental kinetic profiles approximate reasonably well to a single exponential (see, as an example, Figure 2), and for each experiment, it was therefore possible to define an apparent first-order rate constant, k_{obs}, following eq 1

$$\text{rate} = \mathrm{d}[\mathrm{CF}]/\mathrm{d}t = k_{obs}[\mathrm{CF}] \quad (1)$$

In particular, when the concentration of the surfactant is well above the C_{SOL} and an exhaustive release of CF is expected because of the complete breakdown of liposomes, as is the case in the present study, the following eq 2 holds

$$k_{obs}[\mathrm{CF}] = k'_{obs}[\text{liposomes}] \quad (2)$$

thus liposomal permeability and solubility processes do coincide.

The obtained k_{obs} can be used as a measure of the rate of breakdown of the liposomes and in turn can be defined as follows

$$k_{obs} = k[\text{surfactant}]^n \quad (3)$$

The obtained k_{obs} values are reported in Tables S1 and S2 of the Supporting Information. The rate of release of CF from the investigated liposomes depends on the molar concentration of the surfactant until a maximum plateau value is reached. (See, as an example, Figure 3.)

DISCUSSION

In previous papers,[7,8] all of the chosen guests were demonstrated to have a stabilizing effect toward the spontaneous and surfactant-induced leakage of CF from liposomes. Indeed, at low Triton X-100 concentrations (concentrations below 0.1 mM and thus below the CMC but not exceeding C_{SOL}), values of k_{obs} for Triton X-100-induced release of CF from guest-containing POPC liposomes are considerably lower than those from POPC liposomes. (See also Table S1 of the Supporting Information.) However, this stabilization was imputed to different effects depending on the considered guest. For example, fullerene-(C_{60})-[7] and cholesterol-[8] containing liposomes appeared to allow a higher uptake of Triton X-100 from the membrane in agreement with the higher determined R_{SAT} values with respect to that of pure POPC liposomes. Grafted-PEG stabilization was instead ascribed[7] to the reduction of the bilayer defects[28,31] and consequently to the reduction of the pore-coating activity of Triton X-100, induced by the presence of PEG. The effect of FPE had been previously imputed[7] to the capability of the pyrrolidine nitrogen (connecting the polyethylenoxide chain to the fullerene spheroid) to function as a good hydrogen bond (HB) acceptor and therefore forming strong hydrogen bonds with the neighboring phospholipids of the membrane.

Above 0.1 mM Triton X-100, the behavior of guest-containing liposomes is completely inverted with respect to that of pure POPC vesicles. Therefore, the aim of the present study is to investigate surfactant concentrations well above the solubilization ratio where the prevailing process, subsequent to the interaction between nonionic surfactants (in the present study Triton X-100 and $C_{12}E_8$) and pure or guest-containing POPC liposomes, should be the complete transition of liposome into mixed micelle. Somasundaran[32] studied a similar process in the presence of mixed phosphatydic acid/phosphatidylcholine liposomes and a large excess of sodium dodecylsulfate (SDS). The almost instantaneous process of liposome solubilization was in that case imputed to the large number of SDS molecules used. Our data are in agreement with these findings because the rate of CF release under the adopted conditions is more than 1 million times faster than that of the spontaneous leakage and, in the case of Triton X-100, on average 1 million times faster than the rate of CF release induced by a 10 times lower concentration of Triton X-100. Actually, in such excess of surfactant, the mechanism of

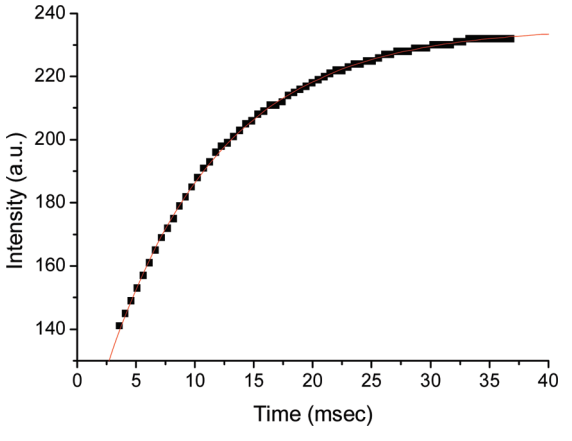


Figure 2. Kinetics of the release of CF from POPC−C_{60} liposomes induced by the addition of 1.7×10^{-4} M Triton X-100 at 25.0 °C. [POPC] = 1.32×10^{-5} M. The solid line is the single exponential fit of the experimental data.

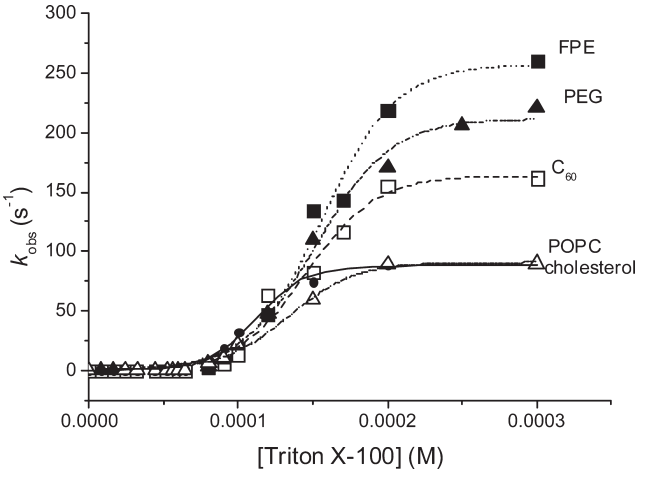
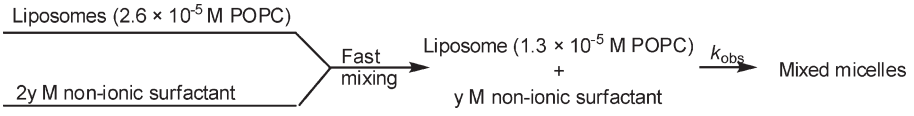


Figure 3. Plot of k_{obs} against the molar concentration of Triton X-100 for the five liposomal systems investigated: POPC (●), POPC-cholesterol (△), POPC-C_{60} (□), POPC-PEG (▲), and POPC-FPE (■).

Scheme 1. Stopped-Flow Mixing Ratio



dx.doi.org/10.1021/jp2026187 |J. Phys. Chem. B 2011, 115, 8130–8137

surfactant-induced rupture of the liposomes could be different from that proposed for low surfactant concentrations. In the latter case, the surfactant-induced release of CF had been ascribed to the surfactant-induced formation of additional hydrophilic pores or the stabilization of the transient holes of the liposomal membrane. The formation of these channels is associated with an unfavorable exposure of the hydrophobic chains of POPC to the aqueous solution. The surfactant approaches this interface and coats the hydrocarbon region, thus stabilizing the pores and increasing the rate of release.[7,8,33] This mechanism had been confirmed[8,13,34] by the nonlinear dependence of the rate of CF release on the concentration of CF "as a critical number n of surfactant molecules has to assemble in the membrane to trigger the formation of a new channel or to adsorb to the pre-existing channels, and the leakage rate will be proportional to the surfactant concentration to the power of n." In the range of surfactant concentrations investigated in the present study, instead, the dependence of the rate of CF release on the surfactant concentration is linear up to a critical value, close to the surfactant CMC value (see infra). Indeed, in the concentration interval $0.8 \times 10^{-4} \leq [\text{Triton X-100}] \leq 2.0 \times 10^{-4}$ M or $0.3 \times 10^{-4} \leq [C_{12}E_8] \leq 1 \times 10^{-4}$ M respectively, the value of n in eq 3 could be taken as unit, and the equation can be simplified as follows

$$k_{obs} = k[\text{surfactant}] \quad (4)$$

This same behavior has been previously turbidimetrically detected[35] for the breakdown of vesicles from synthetic surfactants due to the addition of single chain surfactants. The initially turbid suspension becomes clear upon vesicles → mixed micelles transitions, and the rate of reaction correlates linearly with the surfactant concentration leveling off once the surfactant CMC has been reached.

The linearity of this equation could therefore stem from the assumption that Triton-X-100-induced release of CF at high surfactant concentrations represents a complete breakdown of liposomes rather than a simple variation of CF entrapped permeability. This linearity confirms the soundness of the previously reported eq 2. The hypothesized mechanism of liposome breakdown is depicted in Figure 4.

Turbidity measurements on these systems did not enable us to obtain proper rate constants and thus to confirm the fluorimetric evidence, probably because of the transformation of liposomes upon the addition of surfactant into different aggregates with unpredictable turbidity before reaching the final mixed-micellar system. Recently, the sequence "bilayers in vesicles - bilayer fragments (flat mixed micelles) - tubular mixed micelles - globular mixed micelles" was indeed suggested for the solubilization mechanism of 1,2-dioleoylphosphatidyl-choline (DOPC) vesicles by *N*-dodecyl-*N*,*N*-dimethylamine-*N*-oxide (LDAO) from combined turbidimetric and small-angle neutron scattering (SANS) measurements,[36] whereas Stuart[37] and Kragh-Henderson[38] supported a mechanism of lipid bilayer transformation into mixed micelles that involves open vesicular intermediates.

Therefore, by plotting k_{obs} values as a function of surfactant concentration and by excluding data residing in the plateau regions at the lowest and at the highest investigated surfactant concentrations (see Table 2 and Figures 5 and 6), second-order rate constants, k, for the breakdown of each liposomal system can be calculated. The latter constants, reported in Table 2, differently from first-order rate constants, represent the susceptibility of each system toward the addition of surfactant. The k value is independent of the concentration of surfactant once the concentration of the surfactant remains within the interval in which the dependence of k upon surfactant concentration is linear (i.e., $(0.8 \text{ to } 2.0) \times 10^{-4}$ M and $(0.3 \text{ to } 1) \times 10^{-4}$ M, for Triton X-100 and $C_{12}E_8$, respectively), and the higher it is, the easier it is for the surfactant to induce liposome breakdown.

By comparing data reported in Table 2 and Figures 5 and 6, it turns out that in agreement with the breakdown of vesicles from synthetic surfactants[35] the rate of breakdown is strongly correlated to the concentration of the added nonionic surfactant. The surfactant concentration therefore provides the "driving force" for liposomal breakdown; the higher the concentration of the

Table 2. Second-Order Constants for the Breakdown of Different Guest-Containing Liposomal Systems Induced by Triton X-100 and $C_{12}E_8$

	10^5k (s^{-1} mol^{-1} dm^3)	10^5k (s^{-1} mol^{-1} dm^3)
liposomes	Triton X-100	$C_{12}E_8$
POPC	9.25(±0.52)	9.07(±1.21)
POPC-PEG	14.7(±0.8)	20.5(±2.5)
POPC-cholesterol	7.03(±0.30)	9.71(±0.82)
POPC-C_{60}	13.6(±1.0)	13.1(±2.1)
POPC-FPE	18.6(±1.3)	14.9(±2.0)

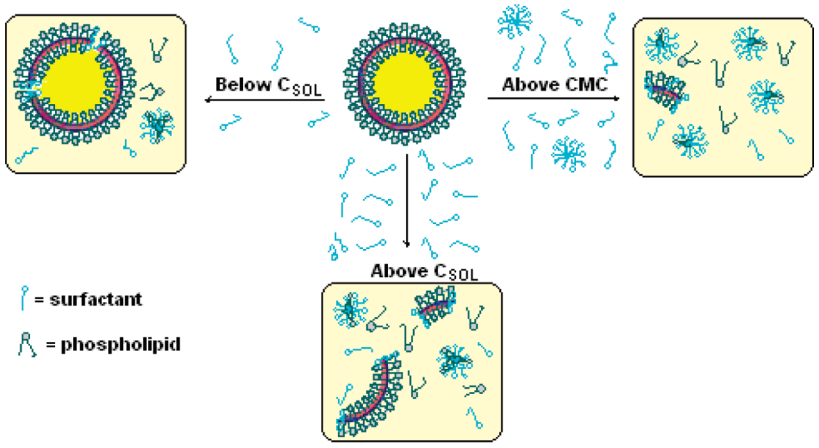


Figure 4. Mechanisms of surfactant-induced liposomal content release below and above C_{SOL} and above the CMC of the surfactant.

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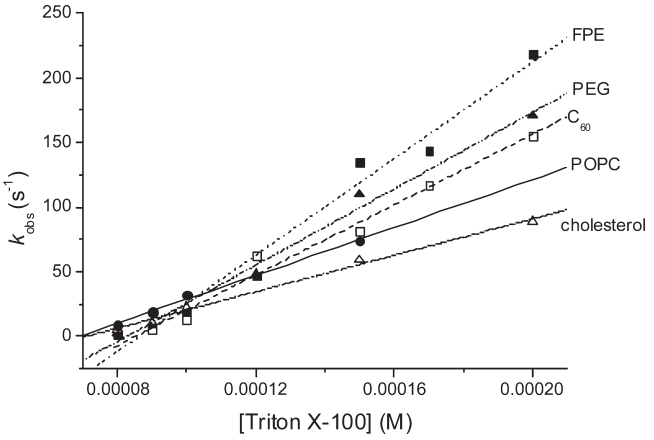

Figure 5. Breakdown of liposomes of pure POPC (●), POPC-PEG (▲), POPC-cholesterol (△), POPC-C_{60} (□), and POPC-FPE (■) upon the addition of Triton X-100.

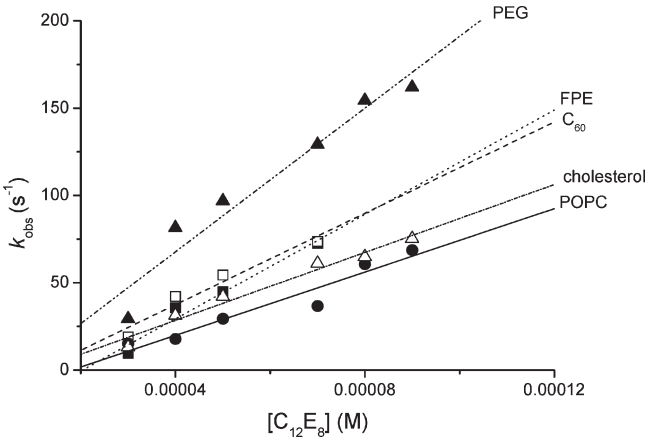

Figure 6. Breakdown of liposomes of pure POPC (●) and of POPC-PEG (▲), POPC-cholesterol (△), POPC-C_{60} (□), and POPC-FPE (■) upon the addition of $C_{12}E_8$.

surfactant, the larger the driving force, and the faster the rate of the breakdown.

Eventually, the comparison between the second-order rate constants measured for the liposomal breakdown allowed us to draw interesting conclusions on the effect of the different guests inserted into the lipid bilayer on the breakdown process. From data reported in Figures 5 and 6 and in Table S1 and S2 of the Supporting Information, it appears that Triton X-100 is less efficient as a destabilizing agent compared with $C_{12}E_8$; the rate of CF leakage is from 2 to 40 times lower at the same surfactant concentration. By considering the corresponding CMC values (CMC of Triton X-100 = 3×10^{-4} M;[30] CMC of $C_{12}E_8$ = 8×10^{-5} M)[39] and the well-known relation between CMC and lipophilicity, this evidence suggests that the hydrophobicity of the added surfactant contributes very significantly to the breakdown of liposomes, similarly to what had been previously observed for cetyldecyldimethylammonium bromide vesicles.[35] Analogously, the higher hydrophilicity of SDS with respect to $C_{12}E_8$ had been previously considered[38] to be the cause of its slower solubilization of DOPC liposomes.

Nevertheless, data reported in Table 2 highlight that Triton X-100 and $C_{12}E_8$ behave similarly when considering the susceptibility of pure POPC liposomes toward the addition of surfactants. Analogously, the presence of C_{60} in the liposomal bilayer increases the capacity of both of the surfactants to induce liposome breakdown with respect to pure POPC liposomes. It is likely that the hydrophobic C_{60} that has been demonstrated[7] to aggregate into the bilayer has a high affinity for the lipophilic portions of both of the surfactants, thus favoring a high bilayer loading of surfactant at low surfactant concentrations[7] and a high susceptibility at high surfactant concentrations. In particular, at surfactant concentrations below the C_{SOL}, the presence of C_{60} would favor the insertion of surfactant molecules into the aggregated C_{60}, thus hampering their coating of the transient pores of the membrane. As a matter of fact, an increase in R_{SAT} of POPC-C_{60} liposomes had been measured in the case of Triton X-100 (0.67 against 0.48 for POPC-C_{60} and pure POPC liposomes, respectively), thus allowing these liposomes to tolerate a higher amount of Triton X-100 with respect to pure POPC vesicles. Vice versa, when the concentration of surfactant is above the C_{SOL}, the added surfactants are able to solubilize completely C_{60} aggregates, thus disrupting the ordering and cohesion of the lipids in the bilayer and favoring liposomal breakdown.

The presence of FPE in the POPC bilayer seems to favor the destabilizing activity of both of the investigated surfactants, with Triton X-100 being more effective than $C_{12}E_8$. The higher susceptibility of POPC-FPE liposomes toward both surfactants as compared with POPC-C_{60} liposomes is particularly peculiar if one considers the fact that FPE is simply a functionalized C_{60}, and similar behavior for the two similar guests was therefore expected. Nevertheless, it has already been observed[7] that FPE, owing to its amphiphilic character, tends to intercalate in the monomeric form into the bilayer and does not substantially alter the physical properties of the membrane. In this respect, FPE molecules can be viewed as well-dispersed membrane constituents that simply favor liposome−surfactant interactions, as evidenced by the higher R_{SAT} measured for this liposomal system as compared with that of pure POPC liposomes (0.67 and 0.65 against 0.48 and 0.50, for Triton X-100 and $C_{12}E_8$, respectively). The kinetics highlight a higher affinity of POPC-FPE liposomes for Triton X-100 with respect to $C_{12}E_8$ that may be due to $\pi-\pi$ interactions between C_{60} and the phenyl ring of Triton X-100. This higher affinity could not manifest itself when the surfactant interacts with aggregated C_{60} because the lower affinity of $C_{12}E_8$ for the fullerene sphere could be compensated by the higher capacity of the linear, and therefore the nonhindered, hydrophobic portion of $C_{12}E_8$ to penetrate into the grooves of C_{60} aggregates.[40]

The high susceptibility of POPC-PEG liposomes toward both surfactants could, at first sight, appear to be unexpected. As a matter of fact, it is recognized that when covalently linked to the headgroup of lipids, PEG shields the surface from the close approach to liposome of micelles or globular macromolecules, such as protein,[41,42] thus providing, for example, long-lived liposomes for drug delivery. Nevertheless it has already been reported[43] that the PEG barrier obtained by incorporation of 5 mol % PEG_{2000} into the bilayer and which should represent full surface coverage, did not prevent poly(ethylacrylic acid) from inducing liposomal content release, demonstrating that highly hydrated polymeric layers are not effective barriers for other water-soluble polymers. This may point to some association between the two polymers overall when the concentration of the added polymeric surfactant is relatively high, as is the case in the present study. It might therefore happen that the interaction

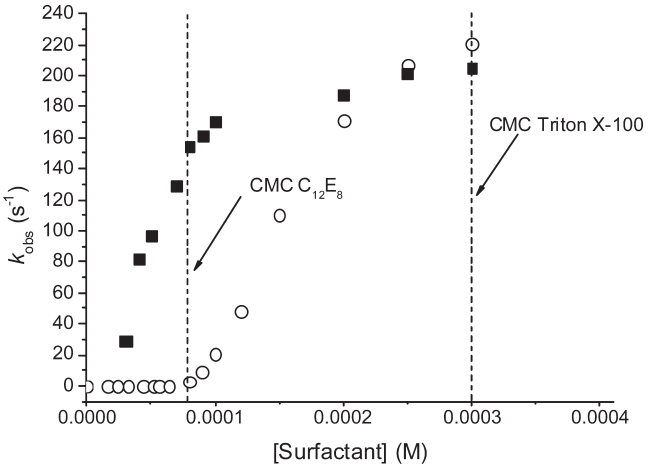

Figure 7. Comparison of Triton X-100 and $C_{12}E_8$ efficiency in breaking POPC-PEG liposomes down. Release of CF induced by the addition of Triton X-100 (○) and $C_{12}E_8$ (■) for POPC-PEG liposomes. The POPC and PEG concentrations were kept constant at 1.32×10^{-5} and 5.1×10^{-7} M, respectively.

of both Triton X-100 and $C_{12}E_8$ molecules at sufficient high concentrations to form micelles competes with the tendency of unimers to insert into the PEG barrier of POPC-PEG liposomes and favor their destabilizing activity. A similar tendency of Triton X-100 to solubilize into the micellar corona of PEG chains of poly(ethylene glycol-*bl*-propylene sulfide) triblock copolymer micelles had already been pointed out.[44]

The two surfactants have different effects on POPC-cholesterol liposomes. Cholesterol seems to reduce the capacity of Triton X-100 to destabilize liposomes, whereas it has almost no effect on $C_{12}E_8$. Needham reported[14] that the presence of a certain amount of cholesterol is the single most influential factor in increasing bilayer cohesion of lipids, and we have previously demonstrated[8] that the inclusion of cholesterol in the liposomal bilayer has a quantitatively determined stabilizing effect on the corresponding liposomes. Therefore the anomalous behavior concerns $C_{12}E_8$, and to explain it, it is important to remember that liposomal solubilization is the result of a "balance of bending energy frustrations".[45] As a matter of fact phospholipids are "curvophobic",[45] namely, their spontaneous curvature is either zero or very small, whereas surfactants are "curvophilic";[45] that is, because of their conical molecular shape, they have a positive spontaneous curvature. Thus, the entropically driven partitioning of surfactant into bilayers[46] results in a state of "frustration"[45] for surfactant molecules forced to reside in flat bilayers. Similarly, when a solubilized phospholipid molecule resides in a phospholipid−surfactant mixed micelle, it is frustrated because it is forced to reside in a curved monolayer. In this context, cholesterol has a highly negative spontaneous curvature[47] it opposes in the bilayer, preferring to interact with the positively curvophilic surfactants and therefore, because of its non-hindered lipophilic chain, with $C_{12}E_8$ rather than with the less conical, and therefore less curvophilic, Triton X-100. A similar conclusion has been drawn by Stuart et al.,[37] who proposed that the packing parameter of the detergent and the consequent overall packing parameter of the detergent-saturated bilayer determines the solubilization mechanism that occurs under specific conditions. As a matter of fact, R_{SOL} values (1.7 and 2.1, for $C_{12}E_8$ and Triton X-100, respectively) highlight a higher efficiency of $C_{12}E_8$ with respect to Triton X-100 in determining liposomal solubilization.

At concentrations of surfactant ca. the CMC the rate of liposomal breakdown does not increase (Figure 7), thus indicating that micelles are not decisive for determining the rate of liposomal breakdown and that surfactants interact with liposomes in their monomeric form. This fact highlights that the CF release at the investigated concentrations is not determined by the saturation of the bilayer - the bilayer, depending of its composition, can accept much more of the surfactant - but by the onset of micelle formation as reported also by Almgren[48] and Sadaghiani.[49]

It must be stressed that the present data are in disagreement with previously published data[38] highlighting high cooperativity during DOPC liposomal solubilization by Triton X-100 or $C_{12}E_8$. This disagreement may be imputed to the different investigated lipid or the different protocol of liposome preparation and the consequent different packing of phospholipids in the bilayer. As a matter of fact the same authors[38] evidenced different mechanisms of vesicle solubilization that are cooperative or noncooperative and pointed out that their evidence "raise questions concerning the existence of different pathways for membrane solubilization with kinetic barriers, depending on the state and nature of the detergent and of the membrane".

CONCLUSIONS

In conclusion we have evidenced that a simple kinetic study of the breakdown of liposomes can shed light on the mechanism of liposome breakdown, helping in differentiating between cooperative effects, occurring at low surfactant concentrations and affecting liposomal permeability, and complete vesicle−micelle transformations, occurring at high surfactant concentrations and via open vesicular intermediates,[37] depending of the correlation of the rate of liposomal breakdown and the concentration of surfactants. If the correlation is linear, then the liposomal breakdown mechanism involves fast solubilization of phospholipids and occurs via open bilayer fragments; if the correlation is exponential, then the release of liposomal content depends on the formation or stabilization of transient pores of the membrane. The two behaviors presented in this study are models for what is likely to occur in liposomes made of different lipids exposed to surfactants of different hydrophobicity. Although punctual description of liposome breakdown has been already proposed by employing a variety of methods such as cryo-TEM,[6] freeze-fracture electron microscopy,[50] SANS measurements,[36] or laser light scattering,[6,38] the possibility presented here to discern the mechanism of liposome breakdown from the order of the process is extremely helpful and simple. Besides, this study highlights that the presence of guests into the lipid bilayer could significantly affect the rate and the susceptibility of the bilayer for the adopted solubilizing agent, and the effect depends strongly on the destabilizing agent concentrations.

ASSOCIATED CONTENT

Supporting Information. Observed rate constants, k_{obs}, for the five investigated liposomal systems at different Triton X-100 or $C_{12}E_8$ concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: fontana@unich.it. Fax: +39 0871 3554791. Tel: +39 0871 3554790.

Author Contributions

§These authors contributed equally.

ACKNOWLEDGMENT

We thank Prof. Michele Maggini and Dr. Stefano Schergna for providing a sample of FPE. This work has been supported by MIUR (PRIN 2008, prot. 20085M27SS).

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