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The use of solvent relaxation technique to investigate headgroup hydration and protein binding of simple and mixed phosphatidylcholine/surfactant bilayer membranes

K. Rieber [b,1], J. Sýkora [a,1], A. Olżyńska [a], R. Jelinek [c], G. Cevc [b], M. Hof [a,*]

[a] J. Heyrovský Institute of Physical Chemistry of ASCR, v.v.i., 18223 Prague 8, Czech Republic
[b] IDEA AG, Frankfurter Ring 193 a, 80807 Munich, Germany
[c] Department of Chemistry Ben Gurion University of the Negev, Beersheva 84105, Israel

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Abstract

The subject of this report was to investigate headgroup hydration and mobility of two types of mixed lipid vesicles, containing nonionic surfactants; straight chain Brij 98, and polysorbat Tween 80, with the same number of oxyethylene units as Brij, but attached via a sorbitan ring to oleic acid. We used the fluorescence solvent relaxation (SR) approach for the purpose and revealed differences between the two systems. Fluorescent solvent relaxation probes (Prodan, Laurdan, Patman) were found to be localized in mixed lipid vesicles similarly as in pure phospholipid bilayers. The SR parameters (i.e. dynamic Stokes shift, $\Delta\nu$, and the time course of the correlation function, $C(t)$) of such labels are in the same range in both kinds of systems. Each type of the tested surfactants has its own impact on water organization in the bilayer headgroup region probed by Patman. Brij 98 does not modify the solvation characteristics of the dye. In contrast, Tween 80 apparently dehydrates the headgroup and decreases its mobility. The SR data measured in lipid bilayers in presence of Interferon alfa-2b reveal that this protein, a candidate for non-invasive delivery, affects the bilayer in a different way than the peptide melittin. Interferon alfa-2b binds to mixed lipid bilayers peripherally, whereas melittin is deeply inserted into lipid membranes and affects their headgroup hydration and mobility measurably.

Keywords: Time-resolved fluorescence; Mixed lipid/surfactant vesicles; Tween 80; Brij 98; Interferon alfa-2b; Melittin

1. Introduction

Transfersomes® are ultra adaptable lipid vesicles, which were developed to carry drugs for non-invasive pharmacotherapy [1–4]. The ability of such mixed lipid/surfactant vesicles to carry macromolecules across the skin [1,5] non-occlusively, non-invasively, and with low risk of skin irritation is unmatched to date and potentially could open a broad field of therapeutic applications, which are limited by the need to use injections. To obtain highly deformable, but stable vesicles, a lipid–surfactant ratio must be used, which depends on surfactant type and drug type loading [6]. Various lipid/surfactant combinations were therefore investigated, however, with different pharmacological effect [7].

With regard to applications, not only vesicle adaptability, but also the extent of drug loading is important. Therefore, the knowledge of surface properties of the deformable bilayer membranes is of eminent importance, as vesicle–vesicle or vesicle–drug interactions strongly depend on these characteristics [2,8]. We therefore decided to characterize two kinds of mixed lipid vesicles, containing either Brij 98 or Tween 80 as surfactants (see Fig. 1A) in terms of headgroup hydration and mobility of the phospholipid bilayer. The used lipid component soy phosphatidylcholine is of natural origin and consists of more than 97% phosphatidylcholine.

For the purpose we applied the time-resolved-fluorescence method based on the "solvent relaxation" phenomenon, which provides information on the amount of water and its mobility within a particular region in phospholipid bilayer [9–13]. In

* Corresponding author. Tel.: +42 0 266053264; fax: +42 0 286582677.
E-mail address: hof@jh-inst.cas.cz (M. Hof).
[1] Both authors contributed equally.

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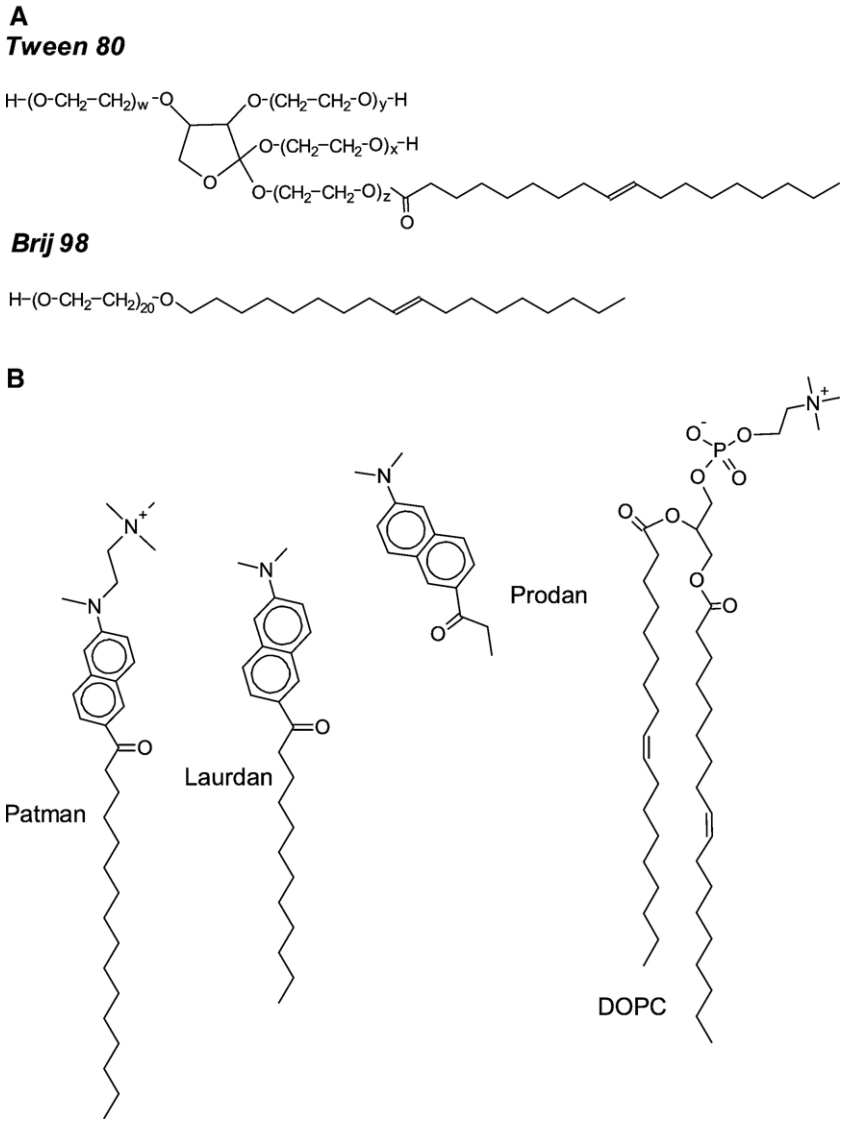

Fig. 1. (A) Structures of used surfactants, (Tween 80: $w+x+y+z=20$, and Brij 98). (B) A set of used fluorescent dyes (Prodan, Laurdan and Patman) and its relative membrane localization to the lipid molecule (DOPC) determined by parallax quenching method [16–18].

general, this approach takes advantage of the rapid change in the dipole moment of the fluorescent dye upon its excitation. This change causes that the surrounding dipoles of the solvent are no longer in the equilibrium state and start to reorientate. The reorientational motions decrease total energy of the system, which is reflected in the red shift of the recorded "time resolved emission spectra" (TRES). The analysis of TRES maxima $\nu(t)$ as a function of time, yields two basic parameters. First, the overall difference, referred to as the "dynamic Stokes shift", $\Delta\nu$, between the maximum of the initial, non-relaxed state, $\nu(0)$, and the final fully relaxed state, $\nu(\infty)$, is proportional to the micropolarity of the dye microenvironment. Second, the correlation function, $C(t)=(\nu(t) - \nu(\infty))/\Delta\nu$, reflects the mobility of the dye microenvironment [9–12]. As a matter of fact, the obtained data characterize the water organization in the vicinity of the dye in the bilayer. When applied to phospholipid bilayers, the dynamic Stokes shift, $\Delta\nu$, and the time course of the correlation function, $C(t)$, are related to the degree of bilayer hydration and to the extent of confinement of the water molecules at the position within the bilayer, at which the fluorophore is located, respectively.

It has been found that the time scale of solvent relaxation (SR) kinetics varies widely. This kinetics is especially sensitive to the localization of dye along the z-axis of the bilayer [13], owing to the steep interfacial polarity profile [15]. For instance, in the headgroup region of a fluid phosphatidylcholine bilayer, the SR process measured with commonly used dyes, misses completely the ultrafast (sub)-picosecond solvation dynamics, typical for the neat solvents. The resolved SR kinetics occurs on the slower (sub)-nanosecond time scale [13,14], which indicates that there is no unbound water in the vicinity of the dye. The direct influence of the chemical composition of a bilayer on the solvation dynamics, moreover, implies that the reorientational motions of the hydrated groups (i.e. acyl, phosphate, or choline groups) are responsible for the "slowing-down" of the solvation dynamics compared to the bulk.

For the phospholipid headgroup region, a set of fluorescent dyes (Prodan, Laurdan, Patman—see Fig. 1B) is often used to probe SR process. All these probes contain the identical chromophore [13], which in addition to the possible comparison of the $C(t)$ function (independent of the structure of the chromophore), also enables to compare values of the dynamic Stokes shift. These dyes are located at different positions in the headgroup region, which makes them useful for mapping the solvation dynamics at various depths [10,12]. Addition of various surfactants, which can modulate interfacial properties, together with Prodan, Laurdan and Patman, thus enabled us to explore the changes in the solvation dynamics caused by different surfactant concentrations.

In addition to fundamental characterization of surfactant effect on hydration of the headgroup region of lipid bilayers, we also tried to study protein–membrane interactions with the same method. In general, High Performance Liquid Chromatography (HPLC) is a method routinely used to measure drug loading of lipid vesicles [19]. However, in the case of a reversible drug–vesicle interaction, this method may not be suitable or sensible enough to test the extent of drug loading in adequate detail. For protein therapeutics the problem is most notorious. Thus, we used SR to study interaction between melittin and a neutral membrane and a membrane containing negatively charged lipids. This small amphipathic peptide is already known to bind to lipid bilayers and served as a positive control [14,20–22]. Second, the hitherto unexamined interaction between a neutral bilayer and a water-soluble protein, Interferon alfa-2b (IFN alfa-2b), was investigated. The results were analyzed and compared with those pertaining to melittin.

2. Materials and methods

All fluorescent probes 6-hexadecanoyl-2-(((2-(trimethylammonium)ethyl) methyl)amino)naphthalene chloride (Patman), 6-propionyl-2-dimethylaminonaphthalene (Prodan), and 6-lauroyl-2-dimethylaminonaphthalene (Laurdan) were used as purchased from Molecular Probes (Karlsruhe, Germany). Dioleoylphosphatidylcholine (DOPC), palmitoyloleoylphosphatidylcholine (POPC), and dioleoylphosphatidylserine (DOPS) were supplied by Avanti Lipids (Alabaster, USA). Soy phosphatidylcholine (SPC) was purchased from Lipoid KG (Ludwigshafen, Germany), Brij 98 from Sigma-Aldrich (Munich, Germany) and Tween 80 from Synopharm (Barsbuettel, Germany). The investigated protein interferon alfa-2b (IFNα-2b) was supplied by Biochemie Kundl (Kundl, Austria) and melittin was synthesized using solid-phase peptide synthesis and purified to >97% using reverse-phase HPLC. Purity of melittin was confirmed with amino acid analysis and analytical HPLC [14].

The preparation of small unilamellar vesicles (SUVs) from the polydisperse, turbid suspension of lipids, here DOPC or POPC/DOPS, in 50 mM Tris–100 mM NaCl buffer pH=7.4 was performed by sonication as described previously [9,10,13]. In brief, for preparation of pure SPC vesicles, a homogeneous suspension of 10 wt.% SPC was prepared by stirring the lipid in 50 mM phosphate buffer pH=7.4 at room temperature for 3 days. Afterwards, the suspension was extruded several times through a series of polycarbonate filters (0.4 μm to 0.05 μm; Poretics, CA, USA) with progressively smaller pores to obtain unilamellar vesicles, also described as liposomes. The resulting average vesicle size was 100 nm as confirmed by dynamic light scattering. A stock solution of fluorescent dye in chloroform was first dried under nitrogen and then redisolved in a suitable volume of methanol. This solution was injected slowly into vesicle suspension (lipid/dye ratio 100/1 mol/mol), sonicated for 5 min to facilitate the dye distribution, and then allowed to anneal for 1 h. Mixed lipid vesicles, containing Tween 80, were prepared similar to liposomes. SPC and Tween 80 were mixed in 2/1 molar ratio, stirred in 50 mM phosphate buffer at room temperature for 3 days, extruded and fluorescently labelled as described for liposomes. Mixed lipid vesicles, containing Brij 98, were prepared analogously, except that an ethanolic lipid/surfactant solution was injected into the buffer under stirring to obtain the starting coarse vesicle preparation. The latter again was stirred for 3 days at room temperature and than extruded to obtain final vesicles. All further steps were done similar to pure SPC vesicle preparation. For the time-resolved fluorescence measurements, the vesicle suspension was diluted with the same buffer to 375 μM lipid (SPC+surfactant) concentration. For protein measurements, IFN alfa-2b was post-incubated with the fluorescently labelled vesicles to obtain a protein/vesicle ratio of 3000/1 mol/mol. Pure Brij micelles and mixed SPC/Brij micelles (1/4 mol/mol) were prepared in the same buffer. The components were mixed, stirred for 3 days, filtered and fluorescently labelled with Patman as mentioned above.

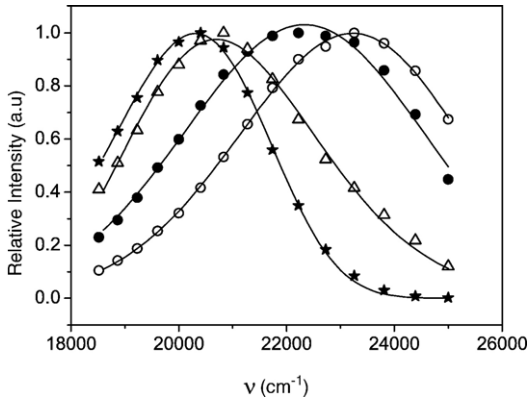

Fig. 2. An example of time resolved emission spectra of Patman in SPC/Brij (2/1 mol/mol). The spectra were reconstructed for 0.03 ns (○), 0.5 ns (●), 4.0 ns (△), and 15.0 ns (★). A shift towards shorter wavenumbers (red-shift) as well as TRES broadening at intermediate times is obvious. The time evolution of FWHM is depicted in Fig. 4.

Fluorescence decays and steady-state emission spectra were recorded with a IBH 5000 U SPC equipment (HORIBA Jobin Yvon, Edison, USA) and Fluorolog-3 steady-state spectrometer (HORIBA Jobin Yvon, Edison, USA), respectively, at 25 °C. Fluorescence decays were collected by using a picosecond laser diode (IBH Nanoled 11, 370 nm peak wavelength, 0.1 ns pulse width, 1 MHz repetition rate) and a cooled Hamamatsu R3809U-50 microchannel plate (Hamamatsu, Shizuoka, Japan) with 30 ps time-resolution.

The primary data consisted of a set of emission decays, recorded at wavelengths spanning the steady-state emission spectrum (390 nm to 550 nm). The time evolution profile of the spectral halfwidths (FWHM, full width at half maximum) and emission maxima, $\nu(t)$, of the reconstructed time-resolved emission spectra (TRES) (Fig. 2) were obtained as described in [9–11,13,23]. Absorption spectra were recorded on a Perkin-Elmer Lambda 19 spectrometer (Perkin-Elmer, Wellesley, MA 02481-4078, USA) at 25 °C. The time-zero spectra, and the corresponding $\nu(0)$ values, were determined as described elsewhere [11,23,24].

Correlation functions $C(t)$ were calculated from the emission maxima $\nu(t)$ of the TRES at time t after excitation as follows:

$$C(t) = \frac{\nu(t) - \nu(\infty)}{\nu(0) - \nu(\infty)} \quad (1)$$

where $\nu(0)$ and $\nu(\infty)$ are the emission maxima at time zero and infinity, respectively. The $\nu(\infty)$ value was always assessed by inspecting the reconstructed TRES. In most cases, the solvent response could not be satisfactorily described by a single-exponential relaxation model. To characterize the overall time scale of solvent response, an (integral) average relaxation time was calculated as follows:

$$\langle \tau_r \rangle \equiv \int_0^\infty C(t)\mathrm{d}t \quad (2)$$

3. Results and discussion

3.1. Probing SR in headgroup region of SPC/Brij mixed vesicles by the labels Prodan, Laurdan and Patman [9–14,25,30]

In previous publications, we have shown that the solvatochromic labels, Prodan, Laurdan and Patman, can give information on micropolarity and -viscosity in phospholipid headgroup regions at different distances from the lipid/water interface [9,14]. In simple phosphatidylcholine bilayers, Prodan is known to be located closest to the lipid/water interface, while the chromophore of Patman is located deepest (in comparison to Laurdan and Prodan) [12,13] at the level of the acyl-groups of the lipid molecules [18]. Laurdan has an intermediate position (Fig. 1B). To validate this statement also for highly adaptable bilayers, all three dyes were investigated in SPC/Brij (2/1 mol/mol) mixed vesicles. This revealed the Stokes shift ($\Delta\nu = \nu(0) - \nu(\infty)$) as well as the average solvent relaxation time, τ_r, to be in full agreement with the conclusions drawn for simple phospholipid vesicles (Table 1 and Fig. 3). The increasing $\Delta\nu$ and decreasing τ_r values for the series Patman, Laurdan, and Prodan qualitatively reveal a polarity and fluidity gradient within the mixed amphipathic headgroup region.

A comparison of the $\Delta\nu$ values determined from the time-zero spectrum estimation [23,24] with the corresponding results obtained exclusively by TRES reconstruction [9–11,13,23] shows that the solvent relaxation probed by Patman and Laurdan occurs exclusively on nanosecond timescale since approximately 90% of SR is captured with the given experimental 30 ps time resolution (Table 1). This conclusion is confirmed by the time evolution of the FWHM (Fig. 4). The profiles observed for Patman and Laurdan, featuring a distinct maximum at times close to the values of τ_r, prove that a substantial part of the solvent relaxation is captured by the used equipment and only a single relaxation process takes place. The absence of an "ultrafast"[2] component clearly demonstrates that the chromophores of both dyes are located in the headgroup region and are not in contact with the "bulk" water. The observed solvent relaxation process is likely to be guided by the reorientation of water molecules tightly bound to the polar groups of the studied amphipaths. In contrary, a considerable amount (>20%) of the SR probed by an ensemble of Prodan molecules occurs on a timescale faster than 30 ps (Table 1 and Fig. 4). Prodan, in contradiction to Patman and Laurdan, is soluble in water. Thus, a part of the observed ultrafast component may be assigned to Prodan molecules present in the bulk solution. The partition coefficient of Prodan between water and fluid phospholipid bilayer was determined in two consequent publications by Krasnowska et al. [25,26]. The obtained values of 10×10^4 [26] and 36.2×10^4 [25] can be used to estimate the contribution of Prodan molecules dissolved in water to the overall fluorescence signal for the given experimental conditions. This contribution of Prodan from the solution could be directly reflected in the amplitude of the ultrafast component gained from SR experiment. Thus, we performed additional SR measurements keeping Prodan concentration constant but varying the concentration of DOPC. We plotted the relative amplitude of the fastest component (fixed time $\tau_1 = 0.03$ ns) obtained by three-exponential fitting of $C(t)$ as well as the contribution of Prodan from water to the overall fluorescence for each lipid concentration calculated from the above mentioned partition coefficients. As obvious from Fig. 5, the experimental SR amplitudes indeed correlate with the calculated values gained from the partition coefficients. The visual inspection also indicates that the partition coefficient of 36.2×10^4 [25] is likely to be more realistic than the more recent value of 10×10^4 [26]. Please note that such an explanation for the origin of the ultrafast component holds only for water soluble molecules, and cannot be applied for chromophores attached to long aliphatic chains. As shown in [13,27], certain coumarin-based membrane labels do show a considerable amount of ps or sub-ps solvation dynamics in bilayers and are practically water insoluble. In that case, the ultrafast SR response might come from chromophores attached to the bilayer, which are in contact with the "bulk"-water molecules located at/close to the water/lipid interface. Please note, that a fluid phospholipid bilayer is a dynamic fluctuating system and broad statistical functions were found to be most suitable for describing the relative positions of the atoms along the z-axis of the bilayer. Similarly, the position of the dye molecules will also be spread over a certain region of the bilayer. Thus, at a certain time some part of the chromophore molecules might face an environment with a large amount of unbound free water which leads to the observed ps or even sub-ps solvation dynamics, whereas some ensemble of the dye molecules will be buried deeper probing nanosecond SR kinetics.

The time evolution of TRES width (FWHM) brings a qualitative idea about the dye environment heterogeneity. It was shown that the time evolution of FWHM passes a distinct maximum in neat solvents and supercooled liquids. The theory clearly proves that this broadening of FWHM at intermediate times is caused by the fact that each solvation shell of each particular fluorophore differs and responds at different relaxation rate to the change in the local electric field invoked by the

Table 1
Characteristics of the solvent relaxation in SPC/Brij (2/1 mol/mol) mixed bilayers probed by three membrane labels (Prodan, Laurdan and Patman)

	$\Delta\nu$ (cm^{-1}) [a]	τ_r (ns) [b]	Observed SR (%) [c]
Prodan	4 100±50	0.63±0.10	78
Laurdan	4 000±50	0.92±0.03	92
Patman	3 400±50	1.16±0.03	95

[a] $\Delta\nu$ ($\Delta\nu = \nu(0) - \nu(\infty)$); $\nu(0)$ was determined by time-zero spectrum estimation [24].

[b] $\langle\tau_r\rangle \equiv \int_0^\infty C(t)\mathrm{d}t$

[c] Obtained by comparison of the $\Delta\nu$ values determined by using the $\nu(0)$ values from the time-zero spectrum estimation [24] with those obtained exclusively by TRES reconstruction.

[2] In the context of this work we mean by the term "ultrafast" processes which are not resolvable with the time-resolution of the experiment (~30 ps).

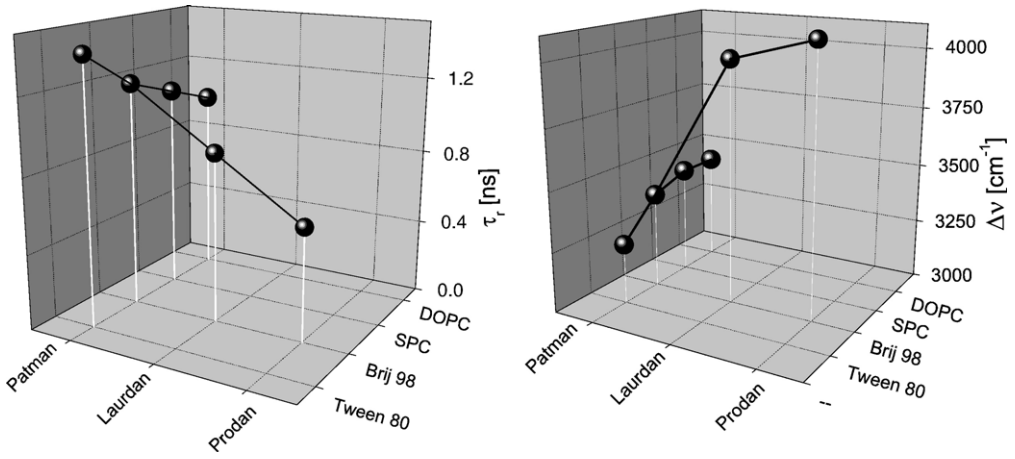

Fig. 3. Solvent relaxation time and Stokes shift data from Tables 1 and 2 plotted in 3D-Graphs.

fluorophore excitation [23,28,29]. The observed macroscopic pattern of emission widths then shows the characteristic maximum. An identical picture is observed in a bilayer. The extent of FWHM broadening is directly related to the heterogeneity of the dye environment. Simply said, the higher absolute values on the FWHM profile, the more heterogeneous environment the dye faces. The data for Patman yield the lowest FWHM value of all of the investigated dyes (see Fig. 4), suggesting that Patman is located in the most homogenous microenvironment. On the other hand, Prodan molecules are located in the most heterogeneous microenvironment indicating the broadest distribution along the *z*-axis of the bilayer, which is illustrated in Fig. 4 and has also been confirmed by independent quenching studies [18].

Our results taken together, thus, show that the SR method is suitable for following changes in the hydration of the headgroup region of simple [13] as well as mixed bilayers. The surfactant does not significantly change solvation dynamics profile relatively to that of the pure phospholipid bilayers [13]. The mobility of the bound water molecules in the vicinity of the dyes Patman, Laurdan and Prodan incorporated in any of these bilayers decreases with depth in the bilayer. The measured micropolarity (i.e. amount of water molecules), in contrast, increases when moving away from the water/lipids interface.

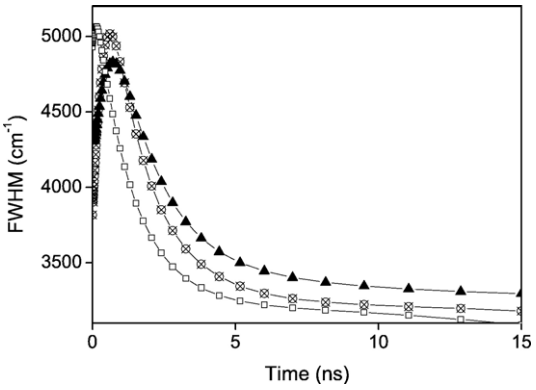

Fig. 4. FWHM of the TRES as a function of time after excitation for Prodan (□), Laurdan (⊗), and Patman (▲) in SPC/Brij (2/1 mol/mol) mixed bilayers. Measurement performed at 25 °C.

3.2. SR in the headgroup region of different simple and mixed bilayers containing phosphatidylcholine and Patman

We have also studied the mobility and the amount of water in the headgroup region of different phosphatidylcholine and phosphatidylcholine/surfactant SUVs by the SR characteristics of Patman. The natural lipid SPC was used in these studies, as well the synthetic DOPC (Fig. 1B). The surfactants Brij 98 and Tween 80 (see Fig. 1A), were mixed with SPC at given molar ratios. To compare the influence of these two surfactants on water structure in the respective headgroup region, vesicles formulated by identical molar SPC/surfactant ratios of 2/1 mol/mol were compared with pure SPC and DOPC bilayers. The latter two differ in the degree of aliphatic chains unsaturation,

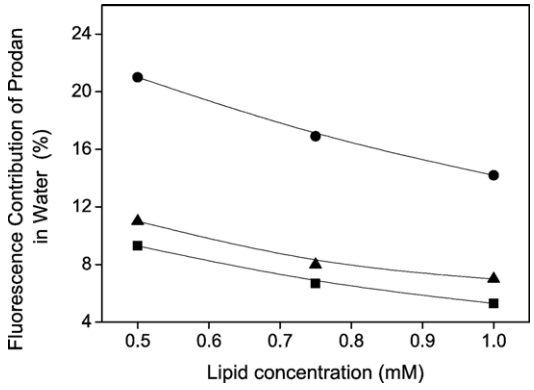

Fig. 5. Fluorescence contribution of Prodan in water to the overall fluorescence signal calculated for the partition coefficient $C_p = 36.2 \times 10^4$ (■), $C_p = 10 \times 10^4$ (●) together with the relative amplitude of the fastest component obtained by three-exponential fitting of the correlation curve $C(t)$ (▲) as a function of lipid concentration. The correlation curve was fitted as described in [23]. The time of the fastest component τ_1 was fixed to the time-resolution limit of the equipment (0.03 ns). The remaining times, i.e. τ_2 and τ_3, were fitted without any constrains yielding the values in the range of 0.3–0.4 ns and 1.7–2.2 ns for all measured lipid concentrations.

SPC having on the average 1.3 and DOPC a single double bond per chain.

The time-dependent Stokes shifts, $\Delta\nu$, and the average solvent relaxation times, τ_r, are not significantly different for DOPC and SPC vesicles as well as for SPC/Brij vesicles, but the tendency is clear (Table 2, Fig. 3). The confinement of water molecules in the headgroup region increases in the sequence DOPC < SPC < SPC/Brij. The mixed bilayer thus appears to be slightly more viscous than the simple SPC or DOPC one. The trend could be due to the less homogeneous chain of SPC as compared to DOPC, leading to larger area per molecule of the latter and to its lower phase transition temperature. This hypothesis is compatible with the observation that the presence of "straight-chain" Brij surfactant slightly reduces the degree of hydration and the water mobility in the headgroup region, as compared to pure SPC. It has been reported that poly-ethyleneglycol (PEG) grafted membranes, which are more expanded [31], are generally less hydrated than the pure lipid bilayers, which has been shown by NMR [32], X-ray, neutron scattering [33], or sound velocity measurements [34]. Additionally, the studies investigating membranes with various relative PEG content show that hydration of headgroup region reaches a minimum at 5–30 mol% of PEG concentration, depending on the method and system used [31,34]. The increase in the degree of hydration at higher PEG concentrations has been attributed to the slight growth in the area per molecule at high PEG content caused by the steric repulsion between the PEG chains attached to phospholipid headgroups [35]. This might be the reason why the dehydration of the herein investigated SPC/Brij mixture does not differ more significantly from that of pure SPC bilayers. Another, and more probable explanation is that PEG on a simple chain surfactant disturbs lipid bilayer differently, i.e. less, than a PEG bound to a dialkenoyl- or diacyl-phospholipid.

The surfactant Tween 80 lowers headgroup polarity much more markedly (Table 2, Fig. 3). This implies higher viscosity in the micro-surrounding of Patman. We assume that the branching of the PEG chains on Tween 80 molecules enables more efficient contact with the bilayer headgroup region. Moreover, as the PEG chains are shorter on Tween than on Brij molecules they are packed and alter the area per molecule differently. Additionally, the sugar moiety in Tween 80 may affect bilayer hydration per se [36,37]. All this translates into different headgroup hydration in Brij or Tween containing aggregates.

The tested surfactant effect on interfacial microviscosity may also be explained by the steric repulsion between the PEG chains, which can affect the mobility of lipid headgroups or whole molecules in the bilayer. The mobility of bound water close to Patman is much reduced in the Tween containing membranes, and less so in the Brij containing membranes.

The observed differences between Tween and Brij containing bilayers, thus, may be due to the different chemical structure of the polar parts of these detergents which, in turn, results in different interfacial PEG packing. While the polar part of Brij 98 only little interferes with the headgroup organization, the more bulky polar head of Tween 80 seems to expose more hydrophobic residues on the bilayer and thus leads to a less hydrated and more rigid water structure around the binding site of the tested dye (Fig. 1B). Please note that Patman is giving microscopic information on the amount of (bound) water and its mobility at the level of the ester-groups within the bilayer. The detected dehydration at that level induced mainly by Tween does not allow any conclusion on the amount of water bound to the hygroscopic ethylenglycol groups, which are supposed to be located directly at the lipid water interface. Thus, in our opinion a Tween induced macroscopic increase in the water uptake [38] does not contradict our Patman results.

3.3. Aggregates with different surfactant phospholipid, Brij 98/ SPC, ratios compared by SR of Patman

Increasing relative amount of the surfactant Brij 98 in phospholipid vesicle suspension, ultimatively, leads to complete solubilisation of the phospholipid bilayers into the form of mixed lipid micelles. SPC/Brij 98 vesicles (2/1 mol/mol) as used in our study had an average diameter of 100 nm. The related formulation with an 8-times higher surfactant content (SPC/Brij 98 = 1/4 mol/mol) contained 10 times smaller 10 nm small aggregates. The SR probed by Patman in the former kind of aggregates corresponds to processes at nanosecond time-scale (see Table 2 and Fig. 3). Only 65% of the SR in SPC/Brij 98 (1/4 mol/mol) mixtures is captured by the time resolution of 30 ps (see Fig. 6). Similar to SR investigations on different micelles reported in the literature [39,40], a large part of the SR in the latter case occurs on an "ultrafast" time-scale, indicating contribution of the "bulk" water to the SR probed in the polar part of micellar structures. The SR probed by Patman in pure Brij 98 micelles yields a qualitatively similar SR (see Fig. 6). The observed quantitative difference diminishes gradually with decreasing relative phospholipid concentration.

In conclusion, SR becomes much faster in micelles in comparison with the mixed lipid/surfactant vesicles. Thus, the SR method is suitable for detecting the micelle formation, as has been also concluded by others for the DPPC/octylglucoside system [41]. Once the micelles are formed, however, the additional presence of lipid molecules does not alter micellar hydration as observed with SR, and the PEG chains have the determining effect on micelle-associated water organization. Viard and colleagues came to a similar conclusion

Table 2
Characteristics of the solvent relaxation process probed by Patman in DOPC, SPC, SPC/Brij (2/1 mol/mol) and SPC/Tween (2/1 mol/mol) SUVs

	$\Delta\nu$ (cm^{-1}) [a]	τ_r (ns) [b]	Observed SR (%) [c]
DOPC	3 450±50	1.02±0.03	91
SPC	3 450±50	1.08±0.03	93
SPC/Brij	3 400±50	1.16±0.03	95
SPC/Tween	3 250±50	1.44±0.03	95

[a] $\Delta\nu$ ($\Delta\nu = \nu(0) - \nu(\infty)$); $\nu(0)$ was determined by time-zero spectrum estimation [24].

[b] $\langle\tau_r\rangle \equiv \int_0^\infty C(t)\mathrm{d}t$

[c] Obtained by comparison of the $\Delta\nu$ values determined by using the $\nu(0)$ values from the time-zero spectrum estimation [24] with those obtained exclusively by TRES reconstruction.

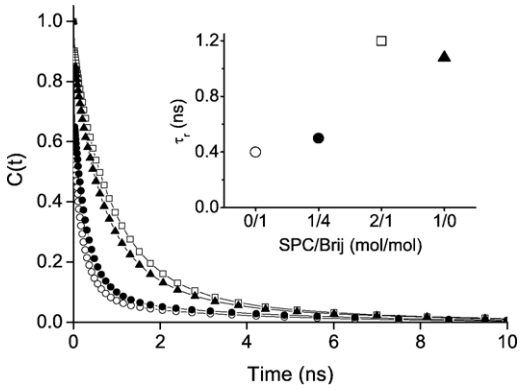

Fig. 6. Correlation functions $C(t)$ and resulting relaxation times, τ_r, for Patman in SPC/surfactant aggregates representing micellar structures (SPC/Brij (0/1 mol/mol) (○), SPC/Brij (1/4 mol/mol) (●)), and vesicles (SPC/Brij (2/1 mol/mol) (□), SPC/Brij (1/0 mol/mol) (▲)). Measurements performed at 25 °C. The incorporated, smaller figure plots the same relaxation time data versus SPC/surfactant ratio.

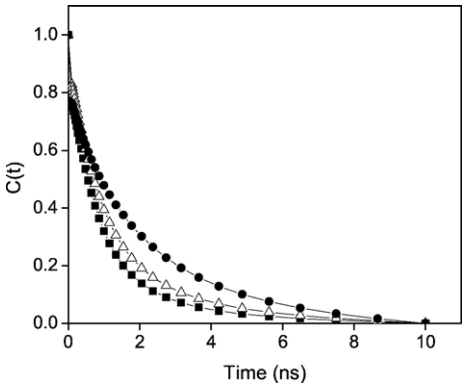

Fig. 7. Correlation functions $C(t)$ for Patman and the effect of melittin binding: POPC alone (■), POPC+melittin (△), POPC/DOPS(1/1 mol/mol)+melittin (●). Comment: Correlation curves for vesicles containing POPC/DOPS are similar to those obtained for POPC alone and they are not shown for higher readability of the plot.

for the dielectric relaxation process in the micelles containing phospholipid–octylglucoside mixtures [41].

3.4. The use of SR-technique to investigate protein binding to lipid vesicles

Various methods, such as the steady-state fluorescence [42], indicate that the protein IFN alfa-2b interacts with lipid vesicles composed of phosphatidylcholines at high protein/vesicle ratio (e.g. of 3000/1). To elucidate the nature of the underlying "protein–membrane interaction" we applied the solvent relaxation method to such system. For the purpose, the headgroup dye Patman was chosen owing to its attractive location in the lipid organization [9,10,12,14] and its invariance upon at least some protein binding [10]. To check the chromophore suitability, we also assessed the binding of the antimicrobial peptide melittin to a phospholipid membrane using the probe Patman. The mechanism of melittin binding to a lipid bilayer appears to be understood [20–22]. This amphipathic and positively charged peptide binds to neutral membranes in the interfacial region. The SR of Patman in pure, neutral POPC bilayers is nearly unaffected by the peptide binding, except for a small, yet noticeable increase in the relaxation times (Table 3, Fig. 7). When the bilayer is negatively charged, the peptide is attracted stronger to its surface due to ionic interaction and then inserts itself more deeply into the bilayer (see Table 3, results obtained for POPC/DOPS, Fig. 7). Addition of melittin to a charged bilayer, for example, substantially doubles the relaxation times, τ_r, from 0.9 ns to 1.8 ns. The binding of protein IFN alfa-2b to pure SPC vesicles, in contrast, does not change much the degree of headgroup hydration. The protein only minimally decreases the SR dynamics by 5% (see Table 4), inducing an even smaller effect than the peripheral binding of melittin to neutral bilayers. We conclude that the interaction of protein IFN alfa-2b with neutral soy-phosphatidylcholine bilayers is only peripheral. IFN alfa-2b therefore does not affect the degree of bilayer hydration and has only a minor influence on the mobility of the hydrated glycerol region in the tested phospholipid bilayer.

Table 3
Parameters obtained by SR for POPC and POPC/DOPS (1/1 mol/mol) vesicles without[a] and with peptide[b] probed by Patman

	$\Delta\nu$ (cm^{-1})	τ_r (ns)	Observed SR (%)
POPC	3400±50	1.01±0.05	84
POPC+melittin	3400±50	1.29±0.03	90
POPC/DOPS	3450±50	0.93±0.05	70
POPC/DOPS+melittin	3350±50	1.82±0.05	87

[a] Total lipid concentration was 0.2 mM.
[b] Final peptide concentration was 8 μM.

4. Conclusions

In the present work, we have investigated the solvation dynamics of the headgroup region in mixed lipid/surfactant vesicles. First, we demonstrate that a high lipid/surfactant concentrations ratio (2:1 mol/mol) does not preclude the use of dyes Prodan, Laurdan, and Patman for probing solvent relaxation in the headgroup region of the bilayer. These dyes have similar relative locations in surfactant containing and pure phosphatidylcholine bilayers and the gained SR parameters are in either cases of the same order of magnitude. Secondly, we show that the mixed vesicles containing Brij 98 exhibit nearly identical solvent relaxation behavior to that observed in the pure phospholipid bilayer. In contrast, the presence of surfactant Tween 80, containing shorter branched PEG chains, apparently leads to dehydration of the headgroup region, and to the decreased mobility of the hydrated functional groups. It should be stressed that this conclusion can only be drawn for the case

Table 4
Characteristics of the solvent relaxation process probed by Patman in DOPC and SPC vesicles, and SPC vesicles in the presence of protein IFN alfa-2b

	$\Delta\nu$ (cm^{-1})	τ_r (ns)	Observed SR (%)
DOPC	3450±50	1.02±0.03	91
SPC	3450±50	1.08±0.03	93
SPC+IFN	3450±50	1.17±0.03	98

that the addition of the detergent does not change the localization of the Patman's chromophore. We have shown that factors like variation of the lipid acylchains [43], ethanol addition [44], or membrane curvature [27] do not affect the position of the chromophore of Patman. Moreover, the FWHM do not change significantly due to addition of Tween 80 or Brij 98, arguing against a possible relocalisation of Patman. Though we are thus confident that the addition of the neutral detergents does not affect the position of that chromophore, we certainly cannot fully rule out this possibility based on the presented data. An alternative explanation for the observed trends would be that the incorporation of surfactants into phosphatidylcholine bilayers allows the chromophore of Patman better access to the deeper bilayer parts. Thirdly, the process of micellization, resulting from relative surfactant/lipid concentration ratio increase, is accompanied by an accelerated SR kinetics. The SR behavior is controlled solely by the surfactant molecules, once the micelles have formed. Finally, we tried to measure interferon alfa-2b penetration into SPC bilayer using solvation dynamics. The results of our measurements in comparison with the data obtained for melittin leads to the conclusion that the binding of the former protein to uncharged mixed surfactant/phosphatidylcholine bilayers is purely peripheral.

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