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ORIGINAL CONTRIBUTION

Retinol fluorescence: a simple method to differentiate different bilayer morphologies

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Abstract The fluorescence spectra of retinol obtained in bilayer structures of two different systems with dodecyl tetraethylenglycol ether are shown. A correlation between the fluorescence intensity of retinol and the different topologies of bilayers has been found. We have tested this correlation with the $C_{12}E_4$/benzyl alcohol/water system, and we have also applied this idea to the study of the lamellar phases of the $C_{12}E_4$/PEG/water system. The highest fluorescence intensity of retinol corresponds to unilamellar vesicles, while the lowest is observed for multilamellar vesicles. The kinetic study of the degradation of vitamin A in these media is also related to the different microstructures of the bilayers.

Keywords Lamellar · Vesicle · Retinol · Vitamin A · Surfactant · Dodecyl tetraethylenglycol ether · Bilayer

Introduction

In aqueous surfactant systems [1], the critical micelle concentration (cmc) defines the initial concentration of the aggregation process at which micelles begin to form spontaneously. When more surfactant is added, larger micelles may change from spherical to disc or cylindrical shapes. Commonly, the inter-aggregate interactions are strong enough to promote the macroscopic ordering that can lead to the formation of a sequence of phases by increasing the surfactant concentration. It allows very rich phase behaviour and the formation of different liquid crystals such as hexagonal, cubic or lamellar. Among other structures, microemulsions, gels or a sponge phase can also be formed.

Lamellar structure is very interesting because it can give us a better understanding of biochemically and physiologically related questions due to its structural similarity with cell membranes. On the other hand, the formulation procedures based on polymer–surfactant and alcohol–surfactant lamellar-type mixtures can be used for controlled drug release [2–4], one of their many appealing applications. The lamellar liquid crystal manifests different topologies in which bilayer arrangements constitute the basic block structure of all of them. There exists a structural progression with increasing concentration according to unilamellar vesicles $\left(L_\alpha^+\right)$ → multilamellar vesicles (MLV or $L_{\alpha\mathrm{l}}$) → planar bilayers $(L_{\alpha\mathrm{h}})$ [5]. The $L_{\alpha\mathrm{h}}$ phase connects with the classical lamellar liquid crystalline phase (L_α). Typically, no macroscopic phase separation is observed for these transitions. The microstructure of bilayer-type phases is determined by two factors: the interfacial topology and the degree of connectivity [6]. The presence of a cosurfactant (e.g. aliphatic alcohol, amine or hydrophilic polymers as poly(ethylene oxide)) has been reported extensively to facilitate the formation of vesicles for a variety of different systems [5, 7–17].

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The effect of benzyl alcohol on the formation of bilayer-type microstructures with dodecyl tetraethylenglycol ether ($C_{12}E_4$) has already been studied in detail by our research group [18, 19]. In the phase diagram of this ternary system, a large uniphasic lamellar region was found, which followed the typical bilayer-topological progression. With a further increase in the alcohol concentration, there was an isotropic bicontinuous sponge phase (L_3). However, neither of these techniques showed an abrupt change in some of the properties, which made it difficult to accurately assign the concentration range at which the successive topological changes of the laminar bilayer took place. On the other hand, the addition of charges in the system resulted in the stabilisation of the vesicles in relation to the open bilayers [20] similar to other systems [21].

Diluted aqueous polymer–non-ionic surfactant systems have also been studied previously. PEG (20000)–$C_{12}E_4$ mixtures result in a phase separation, as frequently happens for these kinds of non-ionic systems [22, 23]. However, the phase behaviour results are completely different with the polymer compound PEG (20000), which consists of 2 mol of polyethylene glycol (M_w=7000–9000) joined internally through a homobifunctional aromatic spacer (bisfenol). The increase in polymer hydrophobicity may stabilise the bilayer structures of the non-ionic surfactant. The lamellar region of this system has been inspected with cryo-transmission electron microscopy (TEM), rheological and small-angle X-ray scattering, measurements although the results are not yet clear (unpublished results). Even the direct images were not easy to assign because cryo-TEM pictures of nonionic solutions are rather complicated [24].

All-*trans* retinol is a hydrophobic vitamin A compound that exerts a potent influence on maintaining the health of the skin and surface tissues. Moreover, retinol protects the skin against skin ageing by neutralising unstable oxygen molecules (free radical). In spite of a wide range of biological and pharmacological effects, the therapeutic and cosmetic uses of this vitamin are still limited due to its poor chemical stability when exposed to air, water or light [25]. The serious problem of the instability of retinoids, and in particular retinol, has generated a large number of studies to find chemical media that can decrease the degradation of these compounds. In recent years, surfactant-organised media have been tested in order to solve the problem of the instability of retinoids. Complex self-assembly structures like liposome or vesicle, micelle, reversed direct or reverse micelle and microemulsion have potential industrial applications in cosmetics, pharmaceuticals, food processing, oil-recovery, etc [26, 27].

There have been studies of the photoisomerization of several retinoids (retinol, retinal and retinoic acid) in the presence of β-cyclodextrins [28] and alkyl-γ-cyclodextrins [29]. It has been demonstrated that the formation of inclusion complexes between retinoids and cyclodextrins protects retinoids from the process of degradation. Results obtained in the studies of retinol stability in the presence of micelles of sodium dodecylsulfate, hexadecyltrimethylammonium bromide, polyoxyethylenelaurylether (Brij-35) and *iso*-octylphenylpolyoxyethylenether (Triton X-100), have been compared. In this case, the degradation of the vitamin is faster than in the presence of cyclodextrins.

With respect to vitamin A, using liposomes to encapsulate retinol and other retinoids can increase stability and reduce toxicity. Moreover, these systems reduce blood viscosity and cause less lysis of red blood cells than retinoid compounds not encapsulated in liposomes [30, 31]. Recently, the newly emerging area of encapsulation using inorganic particles prepared in O/W/O emulsions [32, 33] and lipid particles [34, 35] have shown great potential.

Surfactant media have been used in fluorescence analysis for many years [36, 37]. Surfactants change the absorption and emission wavelengths of the spectral bands of retinol and improve the sensitivity of spectroscopic measures due to the solubilization of the vitamin in an aqueous medium in which it is insoluble in the absence of a surfactant [38–40].

In this paper, we focus on the development of an easy and low-cost technical application (fluorescence measurements) with the advantage of characterising the microstructure of bilayer structures. We present the fluorescence spectra of retinol in lamellar phases obtained for two systems containing dodecyl tetraethylenglycol ether. We have analysed the fluorescence intensity as a function of the different topologies of bilayers. We used our previous knowledge of the microstructural characterization of the $C_{12}E_4$/benzyl alcohol/water system to discover whether fluorescence spectroscopy was a useful tool for differentiating the different topologies of bilayers. We have also applied this technique to the study of the bilayer structures of the $C_{12}E_4$/polyethylene glycol (PEG)/water system. Finally, we have studied the variation in the fluorescence intensities of retinol as a function of time in order to obtain the kinetic study of vitamin A degradation in these media.

Experimental

Reagents

All the reagents used in this work were of analytical grade. The standard used was all-*trans*-retinol (synthetic, crystalline, ≥95%) from Sigma-Aldrich. Ultrapure water was obtained from a Milli-Q system (Millipore). The dodecyl tetraethylenglycol ether ($C_{12}E_4$; ≥98% gas chromatography (GC)) surfactant was from Fluka. Benzyl alcohol was from Merck, and the polyethylene glycol compound, mol. wt.

15,000–20,000 (PEG 20000), which consists of 2 mol of polyethylene glycol (M_w=7,000–9,000) joined internally through a homobifunctional aromatic spacer (bisfenol), was from Sigma.

The solutions were prepared by weighing the appropriate quantities of the surfactant, alcohol or polymer and by adding water in a graduate tube to a total volume of 5 mL. The tubes were left to stand in a thermostatic bath (Julabo ED-19) at 30 °C (±0.03 °C).

For the $C_{12}E_4$/benzyl alcohol/water system, we have carried out two series of experiments: at a fixed surfactant concentration of 50 and 150 mM, varying the amount of benzyl alcohol in both cases.

For the $C_{12}E_4$/PEG/water system, we have prepared samples in two ways: at a fixed surfactant concentration of 50 mM, varying PEG contents, and at a fixed PEG concentration of 8 g L^{-1}, varying surfactant contents.

Stock solutions of retinol were prepared by dissolving the appropriate quantity of this standard in methanol. These solutions were stored in dark flasks at −18 °C. The mixtures containing retinol were prepared by diluting very low volumes of stock solutions of retinol in the measurement system. The final retinol concentration of every sample was equal to 0.5 mg L^{-1}.

Equipment

The fluorescence spectra of the different mixtures were obtained in a luminescence spectrophotometer Perkin-Elmer model LS-50B, equipped with a 1-cm quartz cell light pass thermostatised with a bath Thermomix BU at 30 °C. The acquisition and data treatments were carried out with the Perkin-Elmer Flwin Lab software. Statistical analysis was accomplished with the STATGRAPHICS® Plus 5.1 software.

Methodology

Study of the fluorescence intensity of retinol in the systems being studied

One week after their preparation, the fluorescence spectra of the $C_{12}E_4$/benzyl alcohol/water systems were obtained from 350 to 600 nm emission wavelength under excitation at 330 nm. The excitation and emission slit widths were 2.5 and 5 nm, respectively.

The fluorescence spectra of the different mixtures were recorded again immediately after the addition of the appropriate aliquot of the retinol stock solution in order to obtain a final concentration of 0.5 mg L^{-1}. The region of the fluorescence spectra selected for the study of the structures formed in the different systems was that corresponding to the characteristic band of retinol: from 420 nm to approximately 550 nm. This study, as has been described, was also carried out on the $C_{12}E_4$/PEG/water system.

All the systems studied ($C_{12}E_4$/benzyl alcohol/water and $C_{12}E_4$/PEG/water), with and without retinol added, has a common peak at 396 nm approximately in the emission spectrum when exciting at 330 nm. In order to compare all the fluorescence spectra, a normalisation process was accomplished. With this purpose, the highest value of the fluorescence intensity in a given spectrum at wavelength 396 nm was set as "1", and the source data of the other emission spectra were multiplied by a factor to match this fluorescence intensity value at the selected wavelength. In a second step, the normalised fluorescence values were plotted as a function of the emission wavelength.

The fluorescence spectra of retinol have a lower intensity than other fluorescent molecules, and its spectra have significant noise as can be observed in Figs. 1, 2, 4 and 5. However, the variation of retinol fluorescence intensity is very sensitive to medium changes. Thus, the retinol molecule can be analysed with very good correlations between the fluorescence signal and its concentration. In addition, surfactants have been used in retinol determinations [38–40], and it was found that the kind of surfactant and its concentration had an enormous effect on the fluorescence signal. The medium is a determinant in the fluorescence signal of retinol.

Study of retinol stability in different media

We have carried out a kinetic study of vitamin A degradation in different solvents: alcohols (butanol and benzyl alcohol) and in a water solution of PEG (8 g L^{-1}). The fluorescence intensity was registered as a function of time. The results obtained in this study have been interpreted in terms of a first-order reaction kinetic:

$$dC/dt = -kC$$

where C is the vitamin concentration at any time, t is the time and k the reaction rate constant. Integrating and letting $C=C_0$ at $t=0$ gives:

$$\ln C/C_0 = -kt$$

The first-order rate equation was tested and the constant evaluated using a graphical procedure. The fluorescence intensity is linearly dependent on the concentration of probe (vitamin) in such a way that:

$$C/C_0 = (I - I_\infty)/(I - I_0)$$

where I_∞ is the fluorescence intensity after infinite time, and I_0 is the fluorescence intensity at $t=0$. A plot of

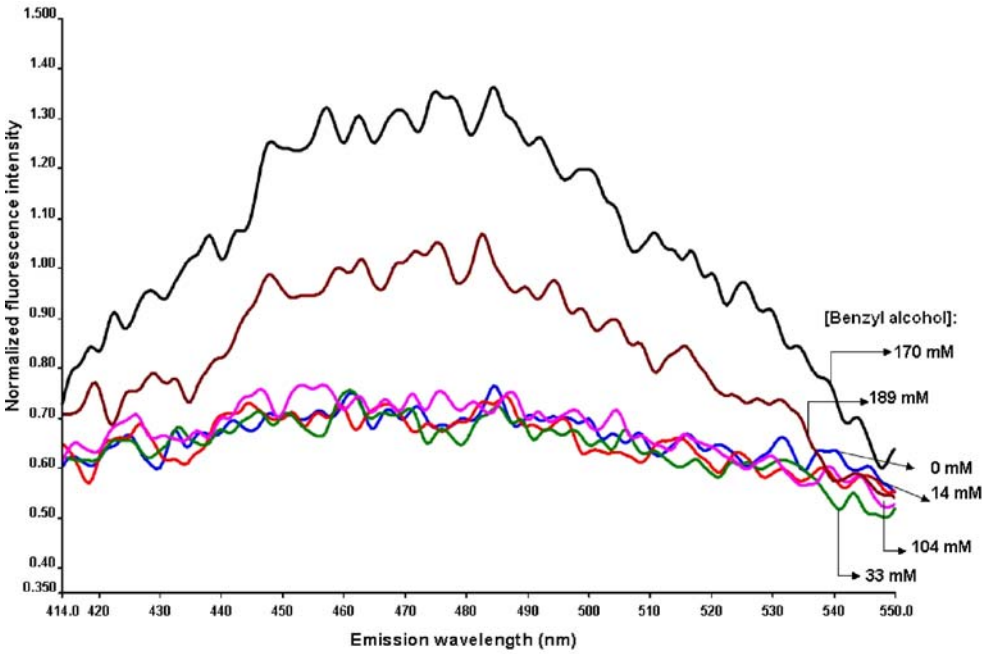

Fig. 1 Retinol fluorescence spectra obtained for the $C_{12}E_4$/benzyl alcohol/water system, at a fixed $C_{12}E_4$ concentration of 150 mM and benzyl alcohol concentrations varying from 0 mM to 189 mM. ($\lambda_{exc.}$=330 nm)

$\ln(I - I_\infty)$ versus t gives a straight line for a first-order rate equation. The rate constant is the slope of this plot.

Results and discussion

Study of the fluorescence intensity of retinol in the $C_{12}E_4$/benzyl alcohol/water system

We have carried out experiments at two fixed surfactant concentrations of 50 and 150 mM by varying benzyl alcohol contents. The results obtained for some of the different systems prepared containing 150 mM $C_{12}E_4$ and different concentrations of benzyl alcohol and at 0.5 mg L^{-1} retinol are shown in Fig. 1.

According to these results, the fluorescence intensity is not dependent on an alcohol content below 100 mM, while at higher alcohol concentrations, the intensity increases. If we compare fluorescence results with the phase diagram of the system [18], we find there is a good correlation between these data and the different subregions of lamellar phase, $L_{\alpha l}$, $L_{\alpha l} + L_{\alpha h}$, and $L_{\alpha h}$. In all the samples corresponding to $L_{\alpha l}$ phase, the fluorescence intensity of retinol is almost undetected. At higher amounts of alcohol, when open bilayers exist ($L_{\alpha h}$), the fluorescence spectra of retinol appear again. This sequence of phases can be statistically confirmed from the

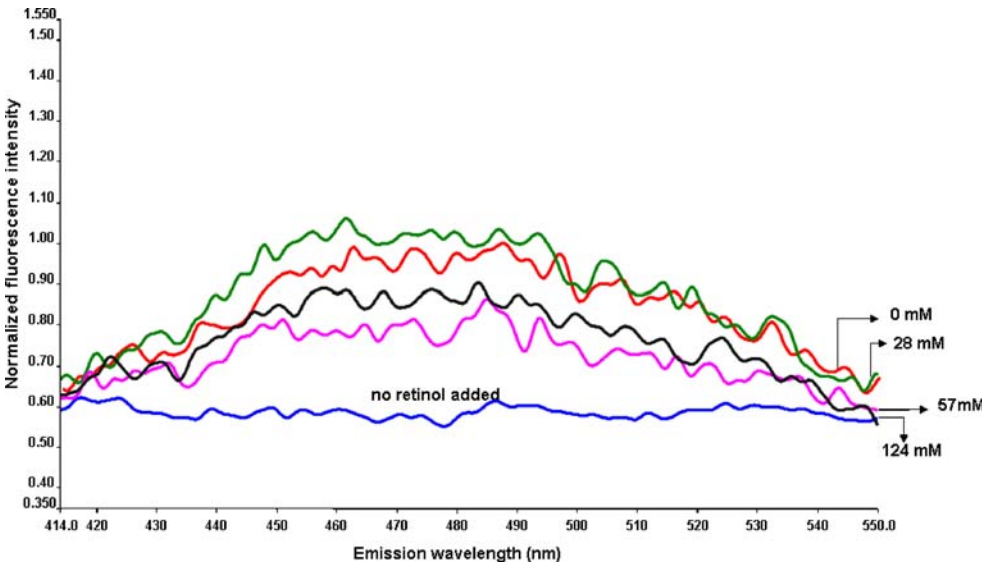

Fig. 2 Retinol fluorescence spectra obtained for the $C_{12}E_4$/benzyl alcohol/water system, at a fixed $C_{12}E_4$ concentration of 50 mM and benzyl alcohol concentrations varying from 0 mM to 124 mM. ($\lambda_{exc.}$=330 nm)

significant differences between fluorescence retinol intensities at 95% confidence level by means of the analysis of variance.

At a fixed amount of surfactant equal to 50 mM (Fig. 2), we observe a decrease in the fluorescence intensity above 30 mM of alcohol. This abrupt change in the fluorescence intensity agrees with the phase behaviour previously described for this system. In fact, this concentration corresponds to the transition from unilamellar vesicles (L_{α}^{+}) to multilamellar vesicles $(L_{\alpha l})$. At higher alcohol contents, the intensity hardly varies at all where multilamellar vesicles exist.

In order to compare all the results as a whole, we have plotted the fluorescence intensity of retinol at its optimum wavelengths in all the systems ($\lambda_{exc.}$=330 nm; λ_{em}= 474 nm) as a function of the alcohol content (Fig. 3a, b). According to this figure, we can observe that the lowest values correspond to multilamellar vesicles phase $(L_{\alpha l})$, while the highest values correspond to open bilayer phase $(L_{\alpha h})$. In fact, the vesicle structure is such that the polar head groups of the surfactant are exposed to the aqueous phase on both sides of the bilayer. The hydrocarbon chains align themselves in the inner core forming a bilayer. Localization of the probe can be restricted to one of several regions: in the inner hydrophobic surfactant region, close to the head group region, or in the inner or outer aqueous phase. The chemical structure of retinol induces us to think that it is located in the bilayer and not in the inner or outer aqueous phase. The hydroxyl group in the structure of the vitamin can favour its localization between the surfactant chains, with this group located between the head groups of surfactant. In vesicle structures, the probe can be entrapped in the vesicles. Our previous study, by small-angle neutron scattering [19], of this system shows that the unilamellar vesicle region (L_{α}^{+}) is composed of vesicles with an average radius of about 100 nm, while the MLV phase $(L_{\alpha l})$ has around 13 shells. So in this multilamellar vesicle region, the lower intensity values are observed. On the other hand, when the bilayers build up stacked flat bilayers, the probe located between the surfactant chains makes it possible to reach higher intensity values. Thus, the highest intensity values are reached in vesicles with only one shell. Sponge phase (L_3) is built up from the bicontinuous structure of open bilayers. The retinol fluorescence in the L_3 phase is very similar to that in the $L_{\alpha h}$ phase.

Study of the fluorescence intensity of retinol in the $C_{12}E_4$/PEG/water system

Two different types of experiments were carried out. One of them was performed at a fixed surfactant concentration equal to 50 mM, and the other was performed at a fixed amount of polymer of 8 g L^{-1} (Fig. 4a, b)

Fluorescence intensity as a function of surfactant content can be observed in Fig. 4a. The quenching of fluorescence increases when surfactant concentration increases at a fixed amount of polymer equal to 8 g L^{-1}. On the other hand, fluorescence intensity is not sensitive to polymer concentration at 50 mM of surfactant.

If we assume that the effect of surfactant concentration on fluorescence intensity is due to the different microstructures of bilayers, we can conclude that the polymer does not affect its structure. This is reasonable because the polymer is water soluble, and it can be considered as a cosolvent. In this way, the reduction in intensity by the increase in the surfactant content of the system is, as we have already seen in the absence of a polymer, due to the transition from unilamellar to multilamellar vesicles. The relation between intensities and the sequence of phases can be better observed in Fig. 5. The linear reduction in fluorescence intensity is due to the increase in the number and size of vesicle shells. The fluorescence of retinol is lower when it is entrapped in larger multilamellar vesicles.

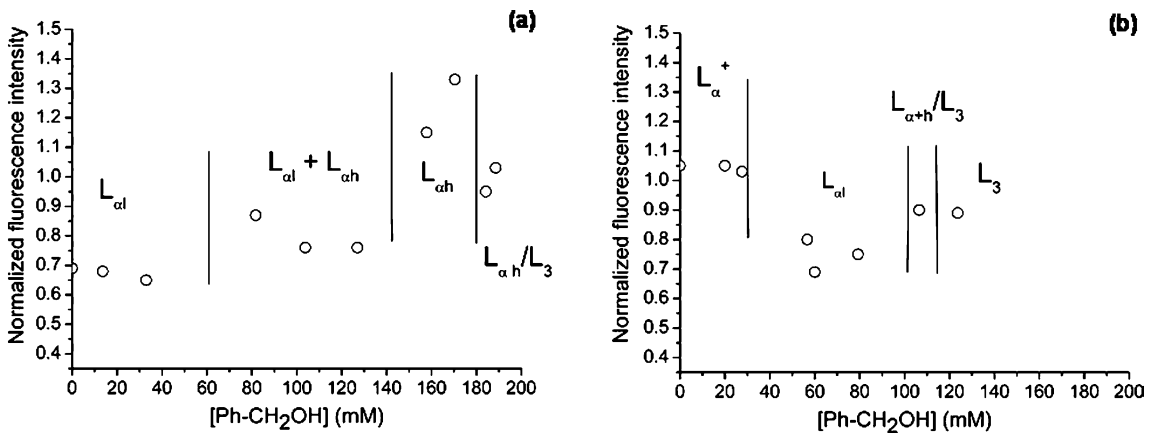

Fig. 3 Correspondence between the normalised fluorescence intensities of the retinol added to the $C_{12}E_4$/benzyl alcohol/water system and the different regions of its diagram phase. ($\lambda_{exc.}$=330 nm and $\lambda_{em.}$=474 nm): **a** fixed $C_{12}E_4$ concentration of 150 mM and **b** fixed $C_{12}E_4$ concentration of 50 mM

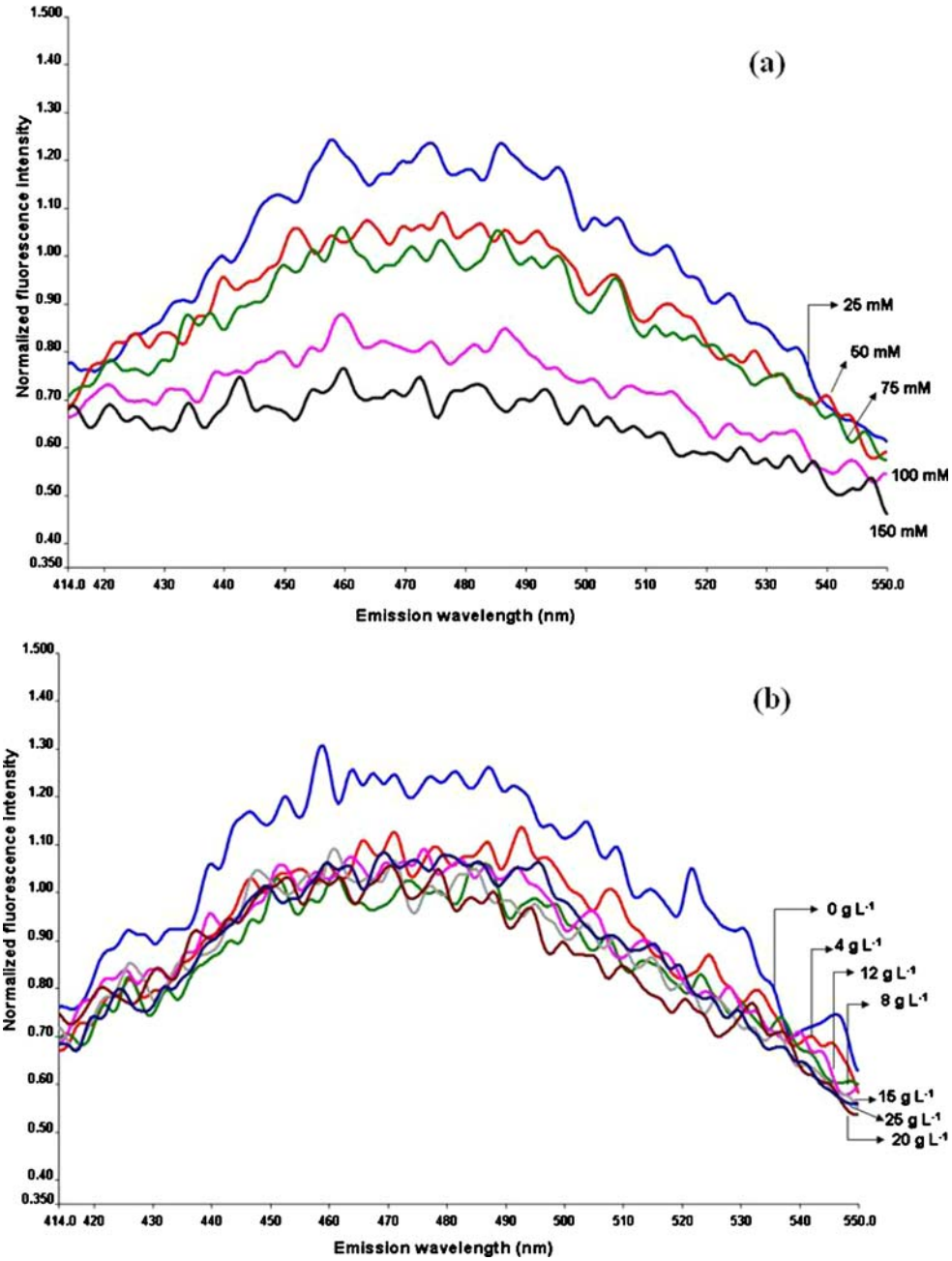

Fig. 4 Retinol fluorescence spectra obtained for the $C_{12}E_4$/PEG/water system at: **a** fixed PEG concentration (8 g L^{-1}) and variable $C_{12}E_4$ concentrations and **b** fixed $C_{12}E_4$ (50 mM) and different PEG concentrations. ($\lambda_{exc.}$=330 nm)

Study of retinol stability in different media

On the other hand, it is already known that liposomes stabilise vitamin A compounds [30]. We have investigated whether a correlation exists between the different microstructures of lamellar phases and the stabilisation of retinol in these media. We aimed to investigate whether stabilisation of vitamin A could be achieved by the microstructure effect instead of a kinetic mechanism determination.

First of all, we studied retinol degradation in alcohol (butanol and benzyl alcohol) solutions and in the aqueous solution of PEG (8 g L^{-1}) in order to evaluate the solvent effect. This kinetic analysis appears in Fig. 6. The data fit a first-order rate constant with a half-life time, given by ln2/k, of 19 min for benzyl alcohol, 79 min for butanol and 311 min for PEG in water. The kinetic data show a solvent effect on kinetic constant. The stability of retinol is increased by increasing the solvent polarity.

Retinol stability has been studied in the lamellar phases of both surfactant systems. We have found that the stability of retinol in the unilamellar vesicles of $C_{12}E_4$ (50 mM) is similar to that in the aqueous solution of PEG (8 g L^{-1}) with a slightly higher half-life time. But the addition of low amounts of alcohol sharply increases retinol stability to a half-life time of 17 days. When more alcohol is added and the vesicles grow in size to become multilamellar vesicles,

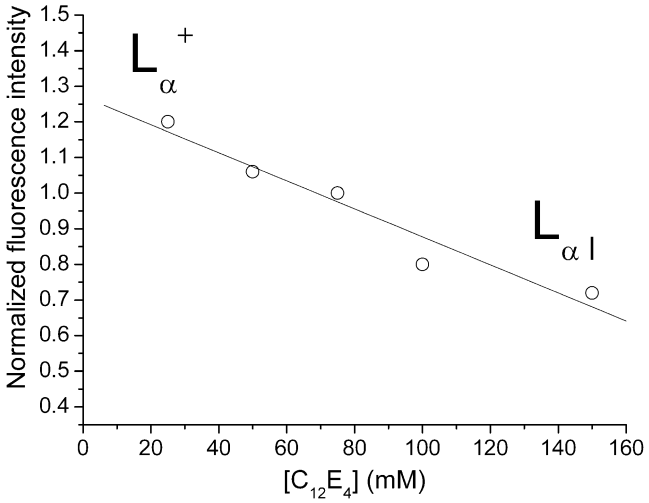

Fig. 5 Variation of normalised fluorescence intensity values of retinol added to the $C_{12}E_4$/PEG/water system as a function of surfactant content. ($\lambda_{exc.}$=330 nm)

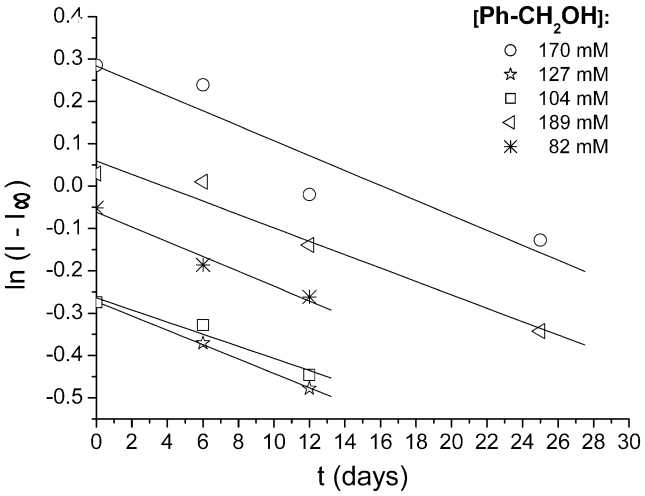

Fig. 7 Kinetic study of retinol stabilisation in the $C_{12}E_4$/benzyl alcohol/water system at a constant $C_{12}E_4$ concentration (150 mM) and different alcohol contents

the stability of retinol becomes lower with half-life times of less than 12 days.

The kinetic study at a constant amount of surfactant equal to 150 mM is plotted in Fig. 7. It was not possible to accurately determine the half-life times for every sample, but a correlation between this kinetic parameter and microstructures was clearly demonstrated. The kinetic constant is almost invariable with an alcohol content higher than 80 mM. The half-life time of the reaction is around 24 days in every sample where open and stacked bilayers exist. While at a lower alcohol content when multilamellar vesicles exist, the stability of retinol is lower with a half-life shorter than 12 days.

We could explain these data if the inhibition of the degradation is connected with the immobilisation and protection effect of vitamin A in the bilayers, as it has been already published to explain the stabilisation in lipid particles [35]. It is well known that encapsulation offers lasting stability of drug. Multiple emulsions [33] have been prepared for encapsulating retinol. We must bear in mind that retinol is an amphiphilic molecule. There is a partition between bilayer and water phases, and we postulate this partition coefficient depends on the bilayer topology, so that the partition is higher for open bilayers than for closed bilayers. Do not forget the solutions without retinol were prepared at least 1 week before these were measured, while retinol was added at the moment the fluorescence was measured. It is likely that retinol is mainly solubilized in the outer shell of the vesicles during the measured time, and the volume fraction of outer bilayer is much higher in unilamellar vesicles than in multilamellar vesicles. The more retinol is solubilised in bilayers, the protection effect of bilayers against degradation is stronger. In his manner, the higher stability of vitamin A corresponds to closed bilayers and to unilamellar vesicles.

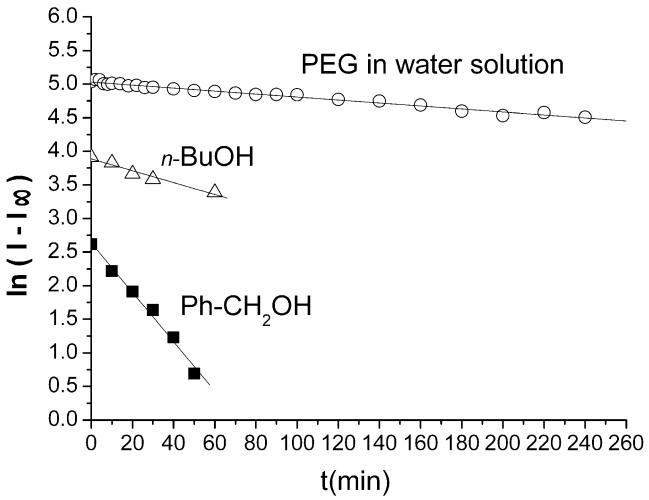

Fig. 6 Kinetic study of retinol stabilisation in three different media: an aqueous solution of 8 g L^{-1} PEG, *n*-butanol and benzyl alcohol

The stability of retinol in vesicles of $C_{12}E_4$ in the presence of PEG is slightly improved. The half-life time increases by more than 1 day. But the effect is much less pronounced than for the benzyl alcohol system. As we have already mentioned, PEG acts as cosolvent, and retinol solubility in PEG solutions is higher than in water.

Summary

We have studied the effect of the different topologies of lamellar phases on the fluorescence of retinol. The fluorescence intensity of retinol was very sensitive to the different topologies of lamellar phases. The previously proposed sequence of phases for the diluted $C_{12}E_4$/benzyl alcohol/

water system has been corroborated by the fluorescence measurements. The fluorescence intensity of retinol entrapped in multilamellar vesicles is almost undetected, whereas the fluorescence intensity of retinol is higher in unilamellar vesicles and in open-stacked bilayers. From fluorescence measurements in the $C_{12}E_4$/PEG/water system, we deduced that the polymer behaves as a cosolvent. Moreover, the retinol solubilized in vesicles degraded significantly more slowly than the free retinol in alcohol solutions.

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