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Photophysical properties of the phenoxazin dyes resazurin and resorufin in soybean lecithin microemulsions

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ABSTRACT

Photophysical properties of resazurin and resorufin were investigated in a homogeneous solution of binary solvent mixtures (isooctane/1-propanol) and in soybean lecithin microemulsions. Resorufin does not show any important changes in the position of either absorption or fluorescence bands when lecithin is added to the mixture isooctane-1-propanol; when microemulsions are formed by the addition of water, a new band appears in the absorption and fluorescence spectra of the dye with an isosbestic or isoemissive point. In contrast, resazurin presents a clear dependence in the absorption and emission spectra with the concentration of lecithin in the isooctane-1-propanol mixture and with the water content. Triplet state properties were also investigated. The results are discussed in terms of the localization of the dyes in the microheterogeneous systems.

1. Introduction

Synthetic dyes have been frequently employed to characterise organised systems [1], in particular reverse micelles [2]. Owing to electrostatic and/or hydrophobic interactions, dyes can be incorporated within the less polar region of the reverse micellar system and significant changes occur in their absorption and emission properties [3]. In previous work, we reported the photophysics and photochemical behaviour of the phenoxazin-3-one dyes resazurin (RZ) and resorufin (RF) Scheme 1, in both aqueous [4–6] as well as direct and reverse micellar solutions [7]. Resazurin is a heterocyclic *N*-oxide dye that is often used to study biological material [8,9]. The "resazurin reduction test" has been used for about 50 years to monitor bacterial and yeast contamination of milk, and also for assessing semen quality. RZ is blue and scarcely fluorescent, and in biological tests it is reduced to RF (pink and highly fluorescent), which is further reduced to hydroresorufin (uncoloured and nonfluorescent). Recently, the dye has gained popularity as a very simple and versatile way of measuring cell proliferation and cytotoxicity [10]. Most of these applications are based on the oxygen atom transfer reaction with RZ as donor. In this way, RZ can be reduced to RF by a thermal reaction using organic compounds or enzymes as catalysts [11–14], or by a photochemical reaction [15,16]. In the latter, the irradiation of RZ in the presence of amines leads to deoxygenation of the *N*-oxide group giving RF. This photoreaction is highly dependent on the amine structure and is efficient only in the presence of tertiary aliphatic amines [4].

The photophysical properties of RF are strongly dependent on the environment and its strong fluorescence has resulted in RF frequent use as a probe for studying solute–solvent interactions. Thus RF has been used to study rotational diffusion of dye molecules [17] in water and DMSO solutions and as a probe for studying rotational dynamics of dyes bound to cyclodextrins [18]. However, the influence of self-organized systems on their spectroscopy and dynamics has been scarcely explored.

A number of surfactants are known to form water-in-oil reverse micelles with microdroplet-like aggregates. Among these are phospholipids and, in particular, lecithins. Lecithin (Lec) is the generic name for a group of fatty substances occurring in animal and plant tissues, composed mainly of phospholipids (e.g., phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol). Lecithin is sometimes used as a synonym for pure phosphatidylcholine, a phospholipid that is the major component of its phosphatide fraction. In aqueous solution phospholipids can form liposomes, bilayer sheets, micelles, or lamellar structures, depending on hydration and temperature [19]. In the case of reverse micelle formation, a third component, an aliphatic alcohol

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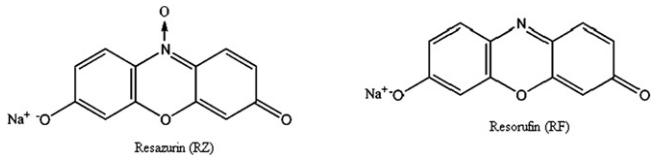

Scheme 1.

is employed in order to stabilize the microemulsion [20]. The alcohol content has great influence on the type of microemulsion formed. At low alcohol content, microemulsions are rich in oil, at intermediate alcohol content they are rich in both solvents, and at high alcohol content they are rich in water [21]. The higher water solubilisation achieved by using 1-propanol as compared to the other alcohols tested, is probably due to the fact that 1-propanol is partitioned within the different microphases of emulsions, i.e., dispersed aqueous phase, continuous oil phase and lecithin membrane [22]. The applications of microemulsions are numerous, and they find uses as microreactors for enzymatic reactions, synthesis of monodisperse polymers, and in many cases as simplified models of biological membranes. Since many of these applications involved one or more reactants, knowledge of the microscopic distribution of substrates is very important. Three water types have been proposed in reverse micelles based on their proximity to the surfactant interface, i.e., water strongly bound to the surfactant polar heads, water bound to the interface and free bulklike water molecules [23]. As the water content increases, the characteristics of the reverse micelle water approach those of bulk water. Also the amount of water solubilised affects the micellar shape, radius and the structure of the polar interface. Therefore, reverse micelles are commonly described in terms of w, the ratio of water to surfactant molarities $w = \frac{[H_2O]}{[surf]}$.

In spite of the biological applications of these dyes, few studies on the effect of organized media on their properties have been carried out. In this work we present a detailed study on the effect upon the spectroscopic and photophysical properties of resazurin and resorufin of lecithin water-in-oil microemulsions prepared with isooctane as the bulk organic phase and 1-propanol as co-surfactant. Absorption and fluorescence emission spectra and lifetimes were determined, as well as triplet state properties. The results are discussed in terms of the distribution of the dye molecules in the different pseudophases of the system. It was established that the effect of the microemulsion on the photophysics of the dyes is markedly dependent on the presence or absence of the *N*-oxide function.

2. Experimental

2.1. Materials

Soybean Lecithin (Epikuron 200) (Lec) was obtained from Lucas Meyer and used without further purification. It has a distribution of fatty acids with a major contribution of C18:2. Lec concentrations were estimated using 770 for the average molecular weight [24]. Resazurin (RZ) (dye content > 91%) and resorufin (RF) (dye content > 95%) were from Sigma and used without further purification. Their photophysical properties coincide with those previously reported [16]. All the organic solvents (1-propanol and isooctane) were from Sintorgan (HPLC grade) and used as received. Water was purified through a Millipore Milli-Q system. Lecithin microemulsions containing the dyes were prepared by adding a small amount of the dyes dissolved in 1-propanol to a 0.05 M of soybean lecithin in isooctane-10% 1-propanol solution. The water to lecithin molar ratio w ($w = [H_2O]/[\text{lecithin}]$) was varied by adding water with pH adjusted at 8.5. The mixture was shaken for a few minutes until a visually clear solution was obtained. The final concentration of the dyes was in the order of 5×10^{-6} M.

2.2. Measurements

Absorption spectra were determined on a Hewlett Packard 6453E diode array spectrophotometer. Static fluorescence determinations were carried out at room temperature in air equilibrated solutions with a Spex Fluoromax spectrofluorometer. Fluorescence quantum yields were determined from the area under the corrected spectrum, relative to that of cresyl violet in methanol as a standard [25]. Fluorescence lifetime measurements were performed with an OB 900 Edinburgh Instruments fluorometer using the time-correlated-single-photon-counting (TCSPC) technique. All measurements were carried out at 25 ± 1 °C.

Transient absorption measurements were made using a laser flash photolysis equipment as previously described [26]. Measurements were performed in samples subjected to continuous bubbling with high purity argon. Quantum yields of triplet species (Φ_T) were determined using zinc tetraphenylporphyrin (ZnTPP) triplet state as a reference actinometer. Values of 7.3×10^4 M^{-1} cm^{-1} and 0.83 were used for the absorption coefficient and quantum yield of ZnTPP triplet state, respectively [27]. The molar absorption coefficients of triplet dyes were determined by the ground state depletion technique [28]. For both dyes, the negative absorption of the difference transient spectra matches the ground-state band. This is consistent with the lack of photoproduct formation under our conditions of laser experiments, and it permits the application of the ground state depletion method to determine the molar absorption coefficients of the triplet state.

The diameter of the reverse micelles was determined by dynamic light scattering (DLS), Malvern 4700 goniometer and 7132 correlator with an argon-ion laser operating at 488 nm. All measurements were made at a scattering angle of 90° at a temperature of 25 °C. Temperature was controlled by the built-in thermostatted system of the instrument. The measurements were analyzed by triplicate and CONTIN analysis to obtain the size distribution of the particles.

3. Results and discussion

3.1. Microemulsion characterisation

Avramoities et al. [29], based on results of luminescence quenching of $Ru(bpy)_3^{2+}$ by $Fe(CN)_6^{3-}$ in lecithin–alcohol w/o microemulsions, concluded that there is a threshold for water content in order to obtain reverse micelles. According to their results only for $w > 20$ pure reverse micellar structures exist. However, when we varied the amount of water as measured by $w = [H_2O]/[\text{Lec}]$, in the range $1 < w < 30$, we found that our results of dynamic light scattering (DLS) measurements could be analyzed with a model of spherical reverse micelles. In all cases the distribution obtained showed poly-dispersivity between 0.2–0.7. The mean hydrodynamic radius was ~1.0 nm at $w = 1$ and 3.0 nm at $w = 30$. A linear dependence of the radius with w was obtained over the explored range, indicating the presence of spherical non interacting aggregates. Similar values were reported by Aliotta et al. [30] from a small-angle neutron scattering study of lecithin with cyclohexane as the organic phase and by Shinoda et al. [21] when the microemulsion is formed with 1-propanol as co-surfactant.

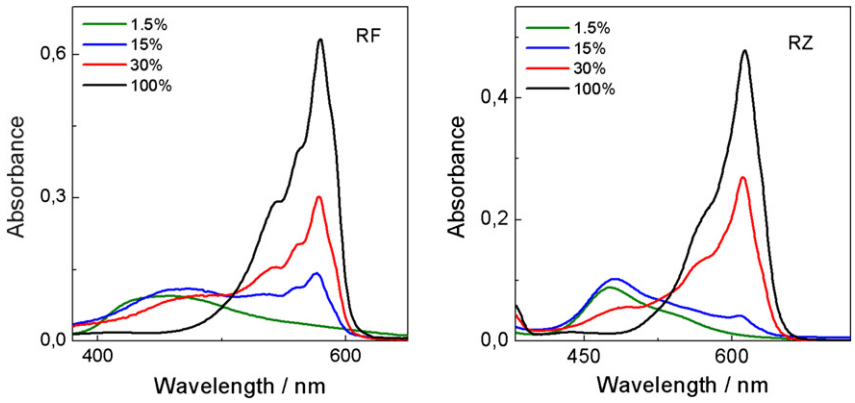

Fig. 1. Visible absorption spectra of RF and RZ in isooctane-1-propanol mixtures as a function of the percentage of 1-propanol.

3.2. Absorption spectra

The absorption spectra of the dyes are solvent dependent, in polar solvents the maxima are in the region 570–580 nm and 600–610 nm for RF and RZ respectively. This absorption may be ascribed to a delocalised $\pi\pi^*$ transition of the heterocyclic ring. On the other hand, in non polar solvents both dyes present an unstructured band in the region of 450 nm which can be assigned to the undissociated ion-pair form. The formation of this ion-pair may remove the resonance delocalisation and the red band disappears. In Fig. 1 the spectra are shown in 1-propanol-isooctane mixtures as a function of the percentage of 1-propanol. An isosbestic point should be expected if only two species were involved. However, there are at least two factors for explaining the absence of the isosbestic point. First, as stated above, the band corresponding to the delocalised absorption moves with the solvent polarity; second, the broad band at 450 nm may correspond to more than one species, including small neutral aggregates of the dye molecules in an associated form. It is known that RF forms ion-pairs with monovalent cations, especially in non aqueous media [31], hence, it may be expected that a strong interaction of the cation with one of the carbonyl oxygen atoms may disturb the charge delocalisation on the heterocyclic ring and shift the absorption maxima towards the blue.

The effect of lecithin concentration on the absorption spectra is demonstrated in Fig. 2. For RF the spectrum remains practically unchanged in the range 0–0.1 M of lecithin, with a band centered at 470 nm. On the other hand, for RZ a significant effect is observed. The absorption intensity is augmented, and structured bands appear. The apparent structure is most probably due to the combination of the spectrum of the dissociated dye and the ion-pair form. These two species are present over the concentration range of lecithin. It is interesting to note how the effect of the phospholipids on the absorption spectra differ from one dye to the other, and this is due only to the presence of the N–O bond in RZ. This difference may be interpreted in terms of the stability of the ion-pair. In the case of RF the interaction with the zwitterionic heads of the surfactant is not strong enough to dissociate the tight ion-pair, and the spectrum is practically unaffected by the presence of the phospholipid. In the case of RZ the presence of the N–O bond reduces the electron density in the ring, the ion-pair becomes less tightly bound and dissociation may be induced by the bipolar heads of phospholipid molecules.

Theoretical estimation of changes in quantities such as electron density and dipole moments could provide some insight into the origin of the diverse behavior of both dyes. Density functional theory calculations (B3LYP at the 6-311G* level) were carried out for both dyes. Solvation by water molecules was taken into account using an IFE-PCM model. More significant than examining the result for the individual dyes is to note the differences. The most significant change occurs in the dipole moment. The calculated dipole moments are 5.70 and 4.16 D for RF and RZ respectively. Since the direction of the dipole moment is across the molecular plane, and the distance is shorter in the case of RF, it implies higher fractional charges for this structure. This may explain the differences in the ion pair stabilities of the dyes.

When water is added to Lec dissolved in isooctane-10% 1-propanol, reverse micelles are formed, and further changes in the

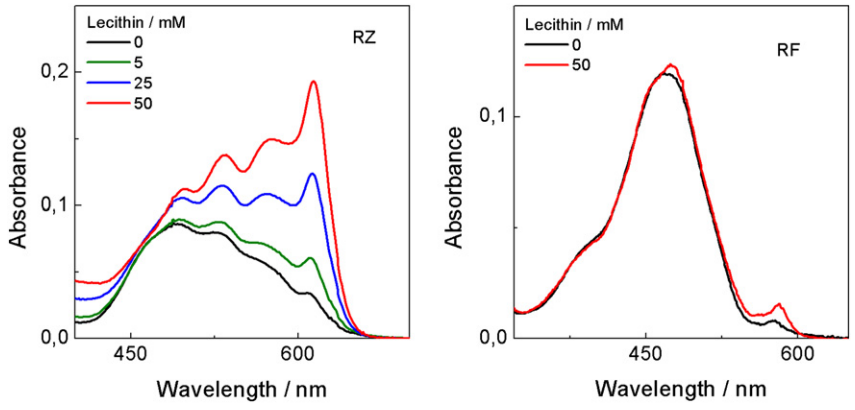

Fig. 2. Absorption spectra in isooctane-10% 1-propanol as function of lecithin concentration.

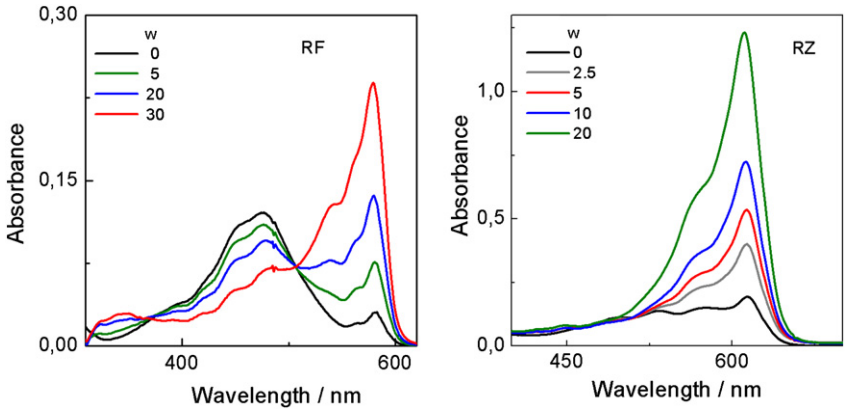

Fig. 3. Absorption spectra in lecithin microemulsions formed with [Lec] = 0.05 M in isooctane-1 propanol 10% as a function of the water content.

absorption spectra are apparent, Fig. 3. In both cases, starting with the band in the region of 450 nm (isooctane – 10% 1-propanol) with increasing w a new band appears in the red region, and at high water content the spectrum resembles that in a polar solvent like an alcohol. At $w = 30$ the maxima are located at 580 nm for RF and 612 nm for RZ, these values are different from those in water (572 and 602 nm, respectively) and similar to those observed in normal cetyltrimethylammonium chloride (CTAC) micelles [7] or in 1-propanol (Fig. 1). Accordingly, it can be concluded that even at high values of w the dyes remain in a region of less polarity than water, probably the interface of the reverse micelles.

3.3. Fluorescence emission

The effect on the fluorescence emission spectra of the addition of 1-propanol to solutions of the dyes in isooctane was studied first. To this end, excitation was performed at 500 nm for RF and 520 nm for RZ since at these wavelengths the absorption presents the smaller dependence on the alcohol concentration, as can be seen in Fig. 1. The spectra at different percentage of the alcohol are presented in Fig. 4.

In the absence of the alcohol the dyes are practically non fluorescent. With increasing alcohol content a fluorescence spectrum, similar to that in 1-propanol, evolves. This implies that the species responsible for the absorption at 450 nm are barely fluorescent and that the observed emission arises from excitation of the blue tail of the absorption band corresponding to molecules preferentially solvated by alcohol molecules.

Fluorescence quantum yields were also determined and are shown in the inset (Fig. 4). It can be seen that at ca. 50% 1-propanol there are no further changes and the fluorescence emission is similar to that in pure 1-propanol. This behavior confirms a preferential solvation of the dye by the alcohol, even at low volume fraction. This preferential solvation is further corroborated by the fluorescence lifetime determinations. At low alcohol content the lifetimes are very close to those in 100% 1-propanol. The fluorescence parameters of both dyes under different conditions are collected in Table 1.

In the absence of added water, $w = 0$, RF presents an absorption maximum at 470 nm, while for RZ it is ca. 520 nm. When RF is excited at 470 nm the fluorescence emission spectrum presents two bands, one as shoulder ca. 550 nm and the other, with a maximum at ca. 590 nm, Fig. 5. The latter is similar to that in 100% 1-propanol while the former can be ascribed to the undissociated dye in a less polar region. It must be noted that the relative intensity of the two bands depends upon the excitation wavelength. Similarly, for RZ, excitation at 520 nm also produces two bands, one at 590 nm as a shoulder, and the other at 640 nm.

The addition of water causes a noticeable effect on the emission of the dyes, Fig. 5. In the case of RF the fluorescence intensity at 590 nm increases, while that at 550 nm decreases with the increase of *w*. An isoemissive point can be observed at 570 nm which can be correlated with that in the absorption spectrum of RF (Fig. 3).

The presence of the isosbestic and isoemissive points in the case of RF when the water content increases, Figs. 3 and 5 respectively,

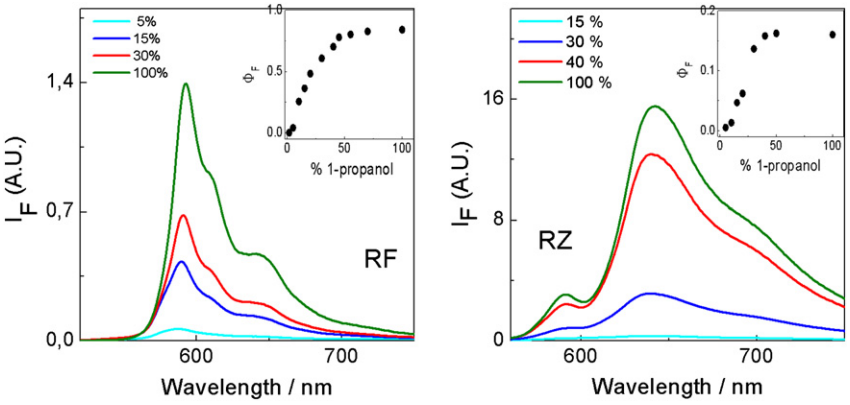

Fig. 4. Fluorescence spectra of RF and RZ in isooctane as a function of 1-propanol percentage; $\lambda_{exc} = 500$ nm (RF) and 520 nm (RZ). Inset: Fluorescence quantum yield as a function of 1-propanol percentage.

Table 1
Photophysical Properties of RF and RZ in homogenous solvents and lecithin microemulsion.

	RF				RZ			
	water pH = 8.5	1-propanol	isooctane − 10% 1-prop	Lec 0.05 M *w* = 30	water pH = 8.5	1-propanol	isooctane − 10% 1-prop	Lec 0.05 M *w* = 30
Absorption λ_{max} (nm)	572	579	470	580	602	612	490	612
Fluorescence λ_{max} (nm)	583	592	588	590	634	642	642	644
Φ_F	0.41[a]	0.84	0.25	0.57	0.11	0.16	0.01	0.21
τ (ns)	2.90	4.90	4.30	4.83	0.65	1.2	0.50	1.25
Φ_T	0.04	0.005	n.d	0.01	0.08	0.14	n.d	0.22

[a] In Ref. [4] Φ_F was erroneously given as 0.71. The present value is the correct one.

suggests a partition of the dye between two pseudophases of the microemulsion, one of lower polarity, most likely the interfacial region between the phospholipid polar head and the organic phase, and the other of higher polarity close to the polar heads in proximity of the water pool.

Similar changes are observed for RZ upon the addition of water. However, in this case there are not clear isosbestic or isoemissive points in the spectra. This may be due to more than two locations for the dye, or a gradual change in the microenvironment as *w* increases. What is clear is that the dye moves toward a more polar region upon increasing the water content as evidenced by the enlargement of the emission intensity. However, the spectral properties do not reach those in pure water either for RF or RZ. The emission maxima are red-shifted by ca. 8 nm with respect to water. Therefore, it may be concluded that the dyes remain in the interfacial region, close to the zwitterionic heads of the phospholipids molecules even at the highest *w*.

Another experimental quantity which provides information about the microenvironment sensed by the dye molecules is the fluorescence lifetime. These data appear in Table 1. Both in the homogeneous solvents and in Lec solutions, the data were fitted to monoexponential decays. In Lec solutions, the fitting did not improve when a biexponential function was used. Since the spectral evidence suggests more than one population of the dye molecules, the observed decays may correspond to an average value of two or more lifetimes that are sufficiently close so that they cannot be distinguished by a multiexponential analysis. In effect, when a lifetime distribution model is used for the fitting of the decay in the microemulsion, the resulting distribution is broader than that observed in pure solvents. What is observed is that the average lifetime in the microemulsion is longer than that in water for both dyes, and similar to the value in 1-propanol. Fluorescence quantum yields follow a similar trend to the fluorescence lifetime.

3.4. Triplet state properties

The triplet state properties of both dyes were investigated by laser flash photolysis. In Fig. 6 the transient absorption spectra of RZ in water and Lec reverse micelles are shown. The spectra can be assigned to the triplet state of the dyes by comparison with those in homogeneous solvents [7]. The coarse spectral characteristics of RF are similar to those in water. RF present a characteristic red band around 700 nm with a shoulder at 640 nm, a strong negative band (not shown) due to the bleaching of the ground state, and a broad band centred at 400 nm. The most important feature is the reduced absorption intensity in the microemulsions. This effect was also observed in CTAC direct micelles and benzylhexadecyldimethylammonium chloride (BHDC) reverse micelles [7]; however, in these cases the band near 400 nm was red shifted in the micellar systems. This effect was ascribed to a reduced intersystem crossing in the organized media, accompanied by an increase in the fluorescence emission.

On the other hand, for RZ the spectrum is also similar to that in water but the absorption intensity is higher in the Lec microemulsions. The *T*–*T* spectrum in this case presents a strong band around 400 nm. The decrease in intensity at ca. 390 nm is due to a ground state absorption band in this zone.

The changes in absorption intensity might be due to differences in the intersystem crossing quantum yields or in the molar absorption coefficients. In order to unravel the origin of the effect, triplet state quantum yields were determined. The procedure is discussed in our previous publications [32]. Briefly, the product $\Phi_T\epsilon_T$, where Φ_T is the triplet quantum yield and ϵ_T is the molar absorption coefficient of the triplet state at the working wavelength, was measured by laser flash photolysis with ZnTPP (zinc tetraphenyl porphyrin) as the actinometer in benzene. The triplet parameters are collected in Table 1. It can be seen that the differences in absorption intensities are mainly due to changes in

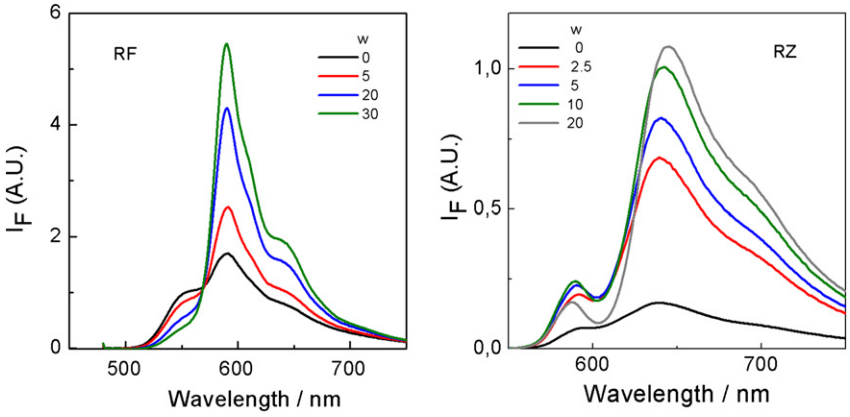

Fig. 5. Fluorescence spectra of RF (λ_{exc} = 470 nm) and RZ (λ_{exc} = 520 nm) 8 × 10^{-6} M in microemulsions of lecithin 0.05 M at 25 °C as a function of *w*.

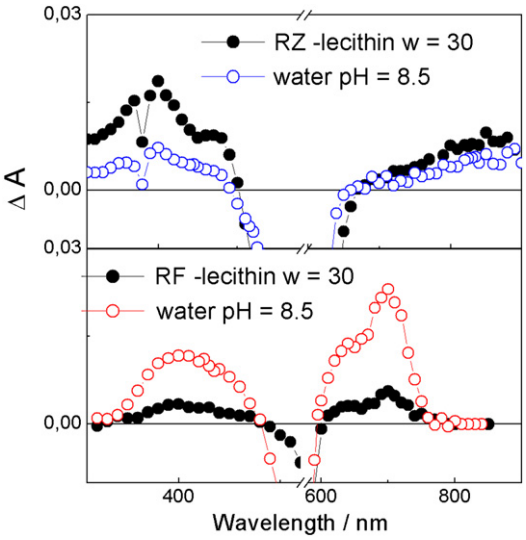

Fig. 6. Transient absorption spectra of RZ and RF in water pH 8.5 in microemulsions of lecithin 0.05 M at $w = 30$ at 2 μs after the laser pulse.

intersystem crossing quantum yields and not in the absorption coefficients. Opposed to what is observed for the ground state absorption and fluorescence emission, the *T*–*T* absorption spectrum presents only minor changes in the shape and position of the bands in the presence of lecithin. On the other hand, large effects are apparent in the triplet yield, and what is most interesting, is the fact that the yield increases in the microemulsion in the case of RZ, while it decreases for RF.

4. Conclusions

The emission and absorption spectra reveal that in the reverse micelles of Lec at high water content, the dyes sense a microenvironment of polarity similar to 1-propanol and do not migrate to the interior of the water pool. The dyes are bound to the interface by hydrophobic or electrostatic forces and penetrate close to the bulk organic phase at low *w* and afterwards change to a more polar medium when w increases. However, even at the higher water content investigated, the photophysical properties do not show evidence of an aqueous environment. The dyes bear a delocalized negative charge and the polar heads are zweitterionic in character. It seems that the interaction with the positive group of the polar head is stronger than the eventual repulsion with the phosphate negative charge.

The presence of the N–O bond in RZ produces some important changes with respect to RF, especially at low water content. The latter equilibrium between two species is evidenced by the presence of an isosbestic point in the absorption spectrum and an isoemissive point in the fluorescence spectrum as the water content in the microemulsion increases. This is not the case for RZ, here the absorption and fluorescence spectra clearly sense a microenvironment of higher polarity at high w but there is no indication of an equilibrium, and most likely several species and/or locations are involved. For RF the two species in equilibrium are proposed to be an ion-pair in the less polar regions of the systems and the dissociated dye in a high polarity medium. The difference between the dyes might be traced to higher fractional charges in RF and consequently, a tighter ion pair for this dye.

It can be seen form Table 1 that for both dyes the fluorescence quantum yield in Lec microemulsions is higher than in water and this effect is concomitant with an increase in the fluorescence lifetime. On the other hand the triplet yield is lower for the highly fluorescent dye RF, and it decreases from water to the microemulsion. Alternatively the triplet yield for RZ is higher in the organized system. The cause of this opposite effect it is not clear, but it may be related to the much higher fluorescence quantum yield of RF. This increment in triplet quantum yield for RZ may be important with regard to the possible uses of the dye as a sensitizer in biological systems.

Acknowledgments

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