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Organized media effect on the photochemical deoxygenation of resazurin in the presence of triethanolamine

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ABSTRACT

The photodeoxygenation of the synthetic dye resazurin in the presence of triethanolamine was investigated in water, CTAC and SDS direct micelles, AOT and BHDC reverse micelles, and soybean lecithin microemulsions (LEC). In all cases the only product observed was the deoxygenated dye resorufin. Triplet and reaction quantum yields were determined in all media. The photoreaction proceeds more efficiently in the microheterogenous systems with positive interface, CTAC and BHDC, while the lower yield is observed in AOT, SDS and LEC. The initial step in the mechanism is the interaction of the triplet state of the dye with the amine, and the effect of the interface is interpreted by a decrease of the recombination rate of the radicals formed in the initial electron transfer step. Negative and zwitterionic interfaces have no effect on the quantum yield.

1. Introduction

In the last years there has been an increasing interest in the study of different photoprocesses occurring in various organized microheterogeneous environments like micelles, reverse micelles, cyclodextrins, liposomes, proteins, etc., because of their numerous applications [1]. Nanometer-sized cavities and cages can be generated in these systems which can serve as "nanoreactors" in which photophysical and/or photochemical reactions can take place, differing significantly from analogous reactions in a homogeneous solution [2]. Synthetic dyes have been frequently employed to characterise organized systems. Owing to electrostatic and/or hydrophobic interactions, organic dyes can be incorporated to the less polar regions of the system, and significant changes occur in their absorption and emission properties and in their photochemical behaviour. [3,4]

Resazurin (RZ, Scheme 1) is a heterocyclic N-oxide dye that is often used to study biological materials [5–8]. The 'resazurin reduction test' has been employed for more than 50 years to monitor bacterial and yeast contamination of milk [9], for assessing semen quality [5,10] and for measuring cell proliferation and cytotoxicity [11]. Resazurin, also known as Alamar Blue, is blue and scarce fluorescent, and when used in biological tests it is reduced to resorufin (RF) (pink and highly fluorescent) which is further reduced to hydroresorufin (uncoloured and nonfluorescent). It is still not known how this reduction occurs, intracellularly via enzyme activity or in the medium as a chemical reaction, although the reduced fluorescent form of Alamar Blue was found in the cytoplasm of living cells and nucleus of dead cells. Most of these applications are based on the oxygen atom transfer reaction with the dye as donor. In this way RZ is reduced to RF, which can be used as a target fluorescent probe. The process may be induced by a thermal reaction using organic compounds or enzymes as catalysts [7,12,13] or by light [14].

We have demonstrated that irradiation of RZ at 600 nm in the presence of aliphatic amines leads to deoxygenation of the N-oxide group giving RF [15]. The detailed study of these photoreactions was of interest in our group, in particular the interactions of the excited states of both dyes RZ and RF with amines [16] and aminoacids [17]. It was proved that the photodeoxygenation of RZ occurs from the triplet state of the dye and the efficiency is highly dependent on the amine structure. It is effective only in the presence of tertiary aliphatic amines. This structural effect was ascribed to the redox potential of the amines. The mechanism most likely involves, as an initial step, and electron transfer reaction from the amine to the triplet of the dye [16]. The redox potential of aliphatic amines increases from tertiary to secondary to primary amines [18], making the reaction highly inefficient for primary and secondary amines. This effect of the amine structure on the photoreduction of N-oxides heterocycles was also reported many years ago by Pietra et al. [19] in a study of the photodeoxygenation of 2-nitrophenaxine-10- oxide, where it was found that the reaction occurs quite efficiently in the presence of triethylamine and is much less efficient for other amines. Free radicals are produced during the

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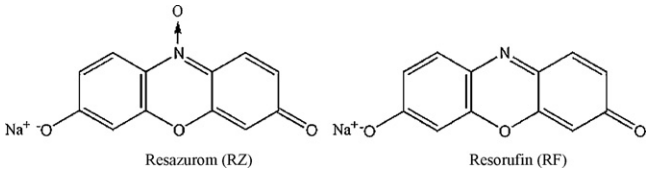


Scheme 1.

photoreduction by amines, making the system RZ-tertiary amine an efficient photoinitiator for vinyl polymerization [20]. Photoreduction was also observed in the presence of aliphatic aminoacids. Although aminoacids quench both, the excited singlet and triplet of RZ, the photoreduction takes place only with aminoacids containing the –SH group [17].

The photophysics of RZ and RF in homogeneous aqueous solution, in direct and reverse micellar solutions and in LEC microemulsions was previously investigated by us [21,22]. The absorption and emission characteristics of the dyes are strongly affected by the medium and the results depend on the charge and type of the interface. In addition, the absorption and fluorescence properties of these dyes are dependent on pH. In particular RF has been used as a probe molecule to study the reorientation of solvent molecules, and has shown interesting spectroscopic properties in protic solvents that strongly depend on temperature, viscosity and structure of the solvent [23].

Due to importance of the use of RZ in biological materials and its photoreduction by biological substrates, it is of interest to investigate the effect of organized media on the photoprocesses of the dye. Here we present a mechanistic study of the photoreduction of RZ by triethanolamine (TEOA) in microheterogeneous systems, comprising direct and reverse micelles and lecithin microemulsions. Quantum yields were determined and the results are discussed in terms of the effect of the medium on the photophysics of the dye and the localization of the amine in the different regions of the organized systems.

2. Experimental

2.1. Materials

The dyes resazurin (RZ), resorufin (RF) and methylene blue (MB) were from Sigma and were used as supplied. Sodium 1,4-bis (2-ethylhexyl) sulfosuccinate (AOT) from Sigma was dried under vacuum over P_2O_5. The surfactant benzylhexadecyldimethylammonium chloride (BHDC) from Fluka was twice recrystallized from ethyl acetate and dried under vacuum. The amines triethanolamine (TEOA) (Aldrich) was purified by vacuum distillation before use. Cetyltrimethylammonium chloride (CTAC) (Kodak) and SDS (Aldrich) were purified by recrystallization. Soybean Lecithin (LEC, Epikuron 200) was obtained from Lucas Meyer and was used without further purification. It has a distribution of fatty acids with a major contribution of $C_{18:2}$. LEC concentrations were estimated using 770 for the average molecular weight [24]. 9,10-Dimethylanthracene (DMA) was from Aldrich and it was also used without further purification. All the orbanic solvents: methanol, benzene, 1-propanol, isooctane and heptane (Sintorgan HPLC grade) were used as received. Water was purified through a Millipore Milli-Q system. The pH was adjusted at pH = 8.5 by the incorporation of a concentrated NaOH solution.

2.2. Measurements

Reverse micelles solutions were prepared with AOT 0.2 M in n-heptane and BHDC 0.1 M in benzene. LEC microemulsions containing the dyes were prepared by the addition of a small amount of the dyes dissolved in propanol to a 0.05 M of soybean lecithin in isooctane-10% 1-propanol solution.

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 $20 \pm 1\,^{\circ}C$.

Transient absorption measurements were made using a laser flash photolysis equipment previously described [26]. Measurements were performed in samples subjected to a 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 $7.3 \times 10^4\,M^{-1}\,cm^{-1}$ 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 matched the ground-state band. This is consistent with the lack of photoproduct formation under our conditions of laser experiments, and allows the application of the ground state depletion method to determine the molar absorption coefficients of the triplet state.

The continuous photolysis experiments were carried out with a 150 W Xe lamp coupled to a grating monochromator (Photon Technology International). Irradiation wavelength was 620 nm in all cases. It was chosen with the compromise of overlapping the absorption spectrum of the sample with that of the actinometer and to avoid absorption by the photoproduct. RZ solutions were deoxygenated by argon bubbling. The singlet oxygen mediated photooxidation of dimethyl anthracene (DMA), sensitized by methylene blue (MB) was used as actinometer. Air equilibrated methanolic solutions of MB, with matched absorbances for RZ solutions in the different media, were irradiated at 620 nm in the presence of DMA. The consumption of DMA was measured by the absorption at 397 nm. Quantum yields for the photoreduction of RZ, Φ_R, were determined relative to the photobleaching of DMA in methanol sensitized by MB, Φ_{DMA}, according to:

$$\frac{S_{(RZ)}}{S_{(DMA)}} = \frac{\Phi_R}{\Phi_{DMA}} \quad (1)$$

where $S_{(RZ)}$ and $S_{(DMA)}$ are the initial slopes of the plots of [DMA] and [RZ] vs. time respectively. S_{RZ} was measured at the absorption maximum of RZ in each medium. The quantum yield for DMA photobleaching was calculated by

$$\Phi_{DMA} = \Phi_{\Delta} \frac{k_r[DMA]_0}{(k_r + k_Q)[DMA]_0 + k_d} \quad (2)$$

where $[DMA]_0$ is the initial concentration of DMA (1.2×10^{-4} M), Φ_Δ is the singlet oxygen quantum yield of MB (0.50) [29], k_r and k_Q are the reactive and physical quenching rate constant for the interaction of DMA with singlet oxygen, 6.3×10^7 and $5 \times 10^6\,M^{-1}\,s^{-1}$ respectively, in methanol [30]. k_d is the unimolecular decay rate constant of singlet oxygen in methanol ($1 \times 10^5\,s^{-1}$) [30]. In this way $\Phi_{DMA} = 0.036$ which is of the same order than Φ_R.

3. Results and discussion

In air equilibrated solutions RZ is stable upon irradiation, even in the presence of amines. When oxygen free solutions of RZ are irradiated in its visible band in the presence of tertiary aliphatic amines, a photobleaching of the dye takes place with the concomitant

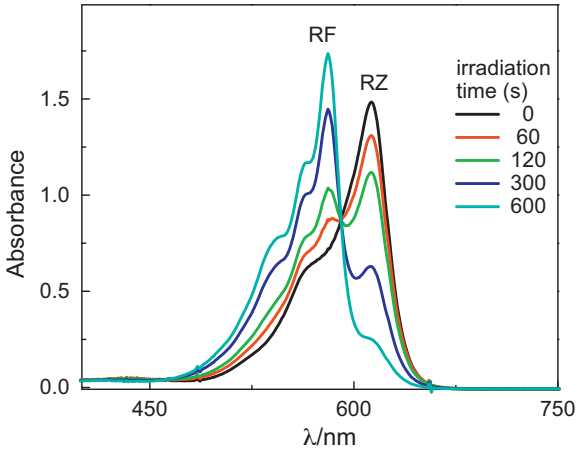


Fig. 1. Photolysis of RZ in CTAC direct micelles. CTAC 0.025 M, TEOA = 5.5×10^{-4} M, irradiation at 620 nm.

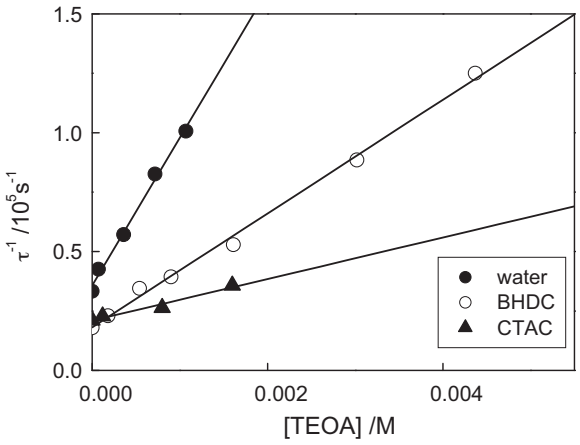


Fig. 2. Kinetics of triplet quenching of RZ by TEOA in water (•), BHDC (○) and CTAC (▲).

appearance of the deoxygenated product RF. After completeness of the photoreaction the final absorbance of RF at its maximum is ca. 20% higher than the initial absorbance of RZ. This factor corresponds to the differences in molar absorption coefficients of the dyes. This implies that the photoreaction is a 100% conversion of RZ to RF, Eq. (3). This is further confirmed by the presence of an isosbestic point in the spectrum as exemplified in Fig. 1 for the reaction in CTAC micelles.

$$\mathrm{RZ} \xrightarrow[\text{amine}]{h\nu} \mathrm{RF} \qquad (3)$$

The reaction is only effective in the presence of tertiary amines; irradiation of RZ in the presence or primary or secondary aliphatic amines or aromatic amines does not lead to RF [16].

The effect of different organized media on the photoreaction was investigated. The reaction was carried out in water at pH 8.5, direct SDS and CTAC micelles, reverse micelles of AOT/heptane and BHDC/benzene and LEC microemulsions. In all cases the same photoreaction was observed with the presence of a neat isosbestic point in the spectrum, indicating that the reaction is unaltered by the presence of the organization. The photoreaction was followed by the decrease of RZ absorbance in the maximum, around 600–620 nm in all systems.

Triplet and photoreaction quantum yields were determined as discussed in the experimental section and are collected in Table 1. As can be seen in the table, the photoreaction quantum yields vary by one order of magnitude, being the highest in CTAC and BHDC.

Since the excited singlet lifetime of RZ is too short, around 0.7 ns in water and 1 ns in CTAC micelles [21], at TEOA concentrations used practically there is not interaction of the amine with the dye in its excited singlet. Therefore, the most likely mechanism for the photodeoxygenation is via the triplet state. In order to confirm this, two kinds of experiments were carried out. First, the kinetics of triplet quenching was investigated, and second, the effect of TEOA concentration on the quantum yield was analyzed.

Table 1
Photophysical and photochemical parameters for RZ in different media.

Medium	Φ_T	τ_T/μs	$k_d/10^4$ s^{-1}	$k_T/10^7$ M^{-1} s^{-1}	Φ[a]	η
H_2O pH 8.5	0.08	35	2.9	6.7	0.033	0.49
SDS 0.02 M	0.08	30	3.4	5.7	0.028	0.5
CTAC 0.025 M	0.12	57	1.8	0.7	0.14	1.75
AOT 0.2 M w = 20	0.08	58	1.7	3.0	0.013	0.19
BHDC 0.1 M w = 20	0.11	52	1.9	2.4	0.12	1
LEC 0.05 M w = 30	0.22	3.1	32	18	0.035	0.22

[a] Photoreaction quantum yield at [TEOA] = 0.005 M.

Triplet quenching rate constants, k_T, were determined from triplet lifetime determinations by laser flash photolysis according to Eq. (4)

$$\tau^{-1} = \tau_o^{-1} + k_T\,[\mathrm{TEOA}] \qquad (4)$$

where τ_o and τ stand for the triplet lifetime, measured by laser flash photolysis at 825 nm, in the absence and the presence of TEOA, respectively. The effect of the organized medium on the triplet quenching is exemplified in Fig. 2. The values of k_T are also collected in Table 1. It must be noted that k_T is evaluated with the analytical concentration of TEOA and not with the local concentration in the pseudophase of the organized system.

The photoreaction mechanism can be written as (Scheme 2)

$$\mathrm{RZ} \xrightarrow{h\nu} {}^{S}\mathrm{RZ}^*$$

$${}^{S}\mathrm{RZ}^* \longrightarrow {}^{T}\mathrm{RZ}^*$$

$${}^{S}\mathrm{RZ}^* \longrightarrow \mathrm{RZ}$$

$${}^{T}\mathrm{RZ}^* \xrightarrow{k_d} \mathrm{RZ}$$

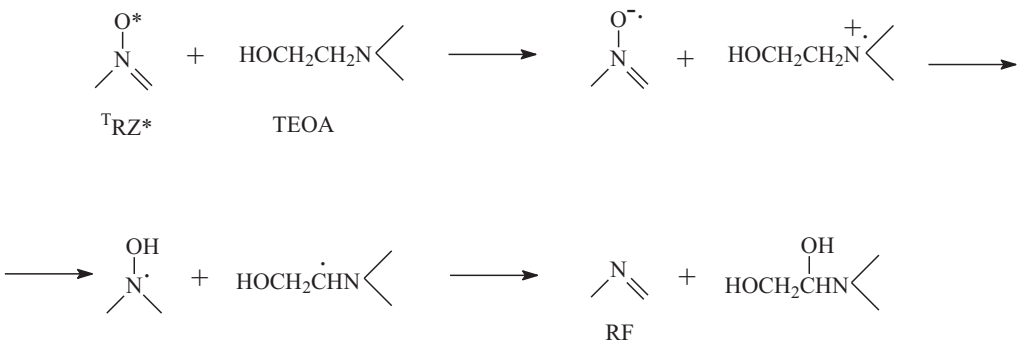
$${}^{T}\mathrm{RZ}^* + \mathrm{TEOA} \xrightarrow{k_T} \mathrm{RZ}^{-\bullet} + \mathrm{TEOA}^{+\bullet}$$

$$\mathrm{RZ}^{-\bullet} + \mathrm{TEOA}^{+\bullet} \xrightarrow{k_b} \mathrm{RZ} + \mathrm{TEOA}$$

$$\mathrm{RZ}^{-\bullet} + \mathrm{TEOA}^{+\bullet} \xrightarrow{k_R} \mathrm{RF} + \text{other products}$$

Scheme 2.

In the mechanism RZ and RF stand for the monoanionic form of the dyes, therefore, the species formed in the electron transfer quenching of the triplet state is the dianionic radical of the dye. The last step is not intended to represent an elementary reaction, but a series of reactions leading to the final product. An open question is which species is responsible for accepting the oxygen atom from RZ. A detailed mechanism was proposed for the photochemical deoxygenation of 2-nitrophenazine N-oxide in the presence of amines by Pietra et al. [19] consisting in the initial electron transfer



Scheme 3.

to the excited state of the dye followed by proton transfer. In the present case the steps depicted by Scheme 3 may be operating.

Accordingly, k_R should be a complex function of the rate constants of the elementary steps in Scheme 3

From the mechanism in Scheme 2 the reaction quantum yield can be written as

$$\Phi_R = \Phi_T \frac{k_T\,[TEOA]}{k_T\,[TEOA] + k_d}\eta \quad (5)$$

where Φ_T is the triplet quantum yield (unaffected by the presence of TEOA), k_d is the unimolecular rate constant for the triplet decay and $\eta = k_R/(k_R + k_b)$ is the efficiency of the process leading to the deoxygenated product RF.

Photoreaction quantum yields as a function of TEOA concentration are shown in Figs. 3 and 4.

The solid lines were calculated with Eq. (5) with the values Φ_T given in Table 1 and k_d and k_T determined by laser flash photolysis. The efficiency η was the only adjustable parameter and its value is also shown in the table.

From the analysis of the quantum yield dependence on TEOA concentration it can be concluded that the photoreaction involves exclusively the triplet state of the dye. The quantum yield increases with the amine concentration reaching a plateau.

The results show that the main factor controlling the efficiency of the reaction is the sign of the electric charge of the polar heads in the interface. This is clearly evidence by comparison of the values in the table for AOT and BHDC reverse micelles. In both cases the triplet properties, quantum yield, lifetime, and quenching rate constant, are of the same order. However, the photoreaction quantum yield is one order of magnitude lower in AOT (negative interface) than in BHDC (positive interface). Similarly, in direct micelles the higher efficiency is observed for CTAC.

This effect of the interface can be the result of the combination of two factors. One is the localization of the dye and the amine in the organized system. TEOA is a polar molecule capable of hydrogen bonding to water molecules, the octanol–water partition coefficient is 0.1 [31], therefore, it will be located preferentially in the aqueous phase or in the hydrated region of the interfaces. In CTAC direct micelles the red shift of the absorption and emission of RZ with respect to pure water indicates that the dye is localized in a less polar environment. Since the dye bears a negative charge it is most likely localized in the positive micellar interface [21]. A similar scenario is expected in BHDC reverse micelles. On the other hand, in SDS direct micelles the dye is practically excluded from the micellar pseudo phase, while in AOT it is preferentially located in the water pool [21]. If the higher efficiency in the organized systems with positive interface should be due only to a high local concentration of the amine, a considerable increase in the triplet quenching rate constant should be noticeable in these cases. Since this is not the case, the higher reaction quantum yield must be due to the interface effect on the events following the initial quenching reaction. The first effect to be considered is on the separation yield of the initial reactive species, most likely the semireduced form of the dye and the radical cation derived from the amine. The positive charge may expulse the latter to the aqueous phase avoiding the back recombination, k_b in the mechanism, and increasing η. Secondly, the charged interface may affect the secondary reactions leading to the final products, in particular the fact that the negatively charged product RF remains bound to the interface may protect it from a back oxidation. In LEC microemulsions the zwitterrionic interface has not effect and the quantum yield is similar to that in water.

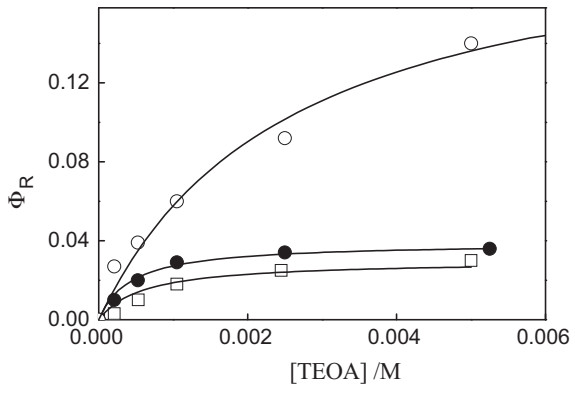


Fig. 3. Photoreaction quantum yield as a function of TEOA concentration in CTAC (○), water pH 8.5 (●) and SDS (□).

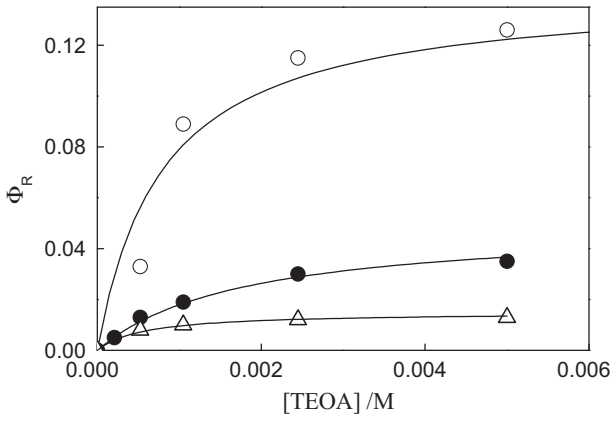


Fig. 4. Photoreaction quantum yield as a function of TEOA concentration in BHDC (○), LEC (●) and AOT (△).

It can be seen in the table that the efficiency in CTAC is higher than unity. We think that this is probably due to the experimental uncertainties arising from an underestimation of the triplet yield and an over evaluation of the reaction quantum yield. Nevertheless, an extra source of RF, not considered in the mechanism, might be a process originating in the singlet excited state of RZ. We disregard this route because at the TEOA concentrations used there is not singlet quenching in homogeneous media [16], and as discussed above, the similar triplet quenching rate constants in micellar and homogeneous media indicates that there is not a high local concentration in the vicinity of the dye.

In summary, the photodeoxygenation yield of RZ is greatly affected by the interface in the organized systems. It is higher when the interface is positive and it is interpreted by a decrease of the recombination rate of the radicals formed in the initial electron transfer step. Negative and zwitterionic interfaces have no effect on the quantum yield.

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