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Rapid Report

Singlet oxygen production and fluorescence yields of merocyanine 540: a comparative study in solution and model membrane systems

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Singlet oxygen and fluorescence quantum yields of merocyanine 540 were measured in solution (methanol, ethanol, *n*-heptanol) and in model membrane systems (cationic micelles, unilamellar dimyristoyl- and dipalmitoylphosphatidylcholine vesicles). Both singlet oxygen quantum yields and fluorescence quantum yields increase with increasing viscosity/rigidity of the surrounding medium: the yield of singlet oxygen production (24°C) goes from 0.002 in methanol to 0.04 in dipalmitoylphosphatidylcholine vesicles, and fluorescence yields (25°C) change from 0.14 to 0.61 in the same media. The data are consistent with previous findings that photoisomerization is in direct competition with intersystem crossing and radiative relaxation. Therefore, a singlet oxygen yield close to the maximum value of 0.11 can only be achieved after both photoisomerization and internal conversion are prevented by a highly viscous environment.

Merocyanine 540 (MC540), an anionic tetramethine cyanine dye, has the ability to selectively sensitize the photoinactivation of leukemia cells, lymphoma cells, and enveloped pathogenic viruses [1–3]. At present, MC540 is used in phase I clinical trials for the extracorporeal purging of autologous bone marrow and in preclinical evaluations as a sterilizing agent for blood and blood products [4,5]. In spite of these important future medical applications, the mechanism of photodynamic action is poorly understood and remains to be elucidated. A recent structure-bioactivity study with herpes simplex Type I virus using MC540 and analogues revealed a correlation between in vitro photoinactivation capabilities and the production of singlet molecular oxygen ($^1O_2$; $^1\Delta_g$) [6]. This result is in support of previous findings that the photoinactivation of neoplastic cells requires the presence of molecular oxygen and is enhanced in deuterated media [7,8]. The MC540 sensitized production of $^1O_2$ has already been studied in methanol by two groups [9,10]. The results, however, are contradictory and differ at least by a factor of ten. Singlet oxygen generation has also recently been investigated in dimyristoylphosphatidylcholine (DMPC) liposomes, but the methods used allow only an estimation of an upper and lower limit of the singlet oxygen quantum yield (Φ_Δ) [11,12]. Due to the absence of accurate data, this study investigates the generation of $^1O_2$ in different solvents and model membrane systems (micelles, liposomes), and provides quantitative data. In addition, comparing these data with fluorescence quantum yields (Φ_f) gives insight into the factor(s) governing the MC540-sensitized generation of $^1O_2$ in vitro and in vivo.

The studies in homogeneous media were performed in ethanol, methanol, and *n*-heptanol. As model membrane systems, cationic dodecyltrimethylammonium bromide (DTAB) micelles, DMPC- and dipalmitoylphosphatidylcholine-(DPPC) liposomes were used. Unilamellar liposomes with a diameter of 60 to 80 nm were prepared by the injection method described by Kremer et al. [13] using a 30 mM potassium phosphate buffer (pH = 7.0 at 20°C). The injected ethanolic solution already contained the dye and the $^1O_2$-scavenger 1,3-diphenylisobenzofuran (DPBF). For the preparation of micelles, appropriate amounts of dye and DPBF in an ethanolic solution were placed into a volumetric

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPBF, 1,3-diphenylisobenzofuran; DTAB, dodecyltrimethylammonium bromide; MC540, merocyanine 540; $^1O_2$, singlet molecular oxygen; Φ_Δ, singlet oxygen quantum yield; Φ_f, fluorescence quantum yield.

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flask, ethanol was removed by a stream of nitrogen and a 0.1 M DTAB surfactant solution was added. Solubilization was completed after stirring for 1–2 h.

Singlet oxygen quantum yields were measured using DPBF as a chemical $^1O_2$-scavenger. In all media, rose bengal was used as a reference, since Φ_Δ values are published [14–16], and MC540 and rose bengal have overlapping absorption spectra. In homogeneous solution, Φ_Δ was additionally determined without the use of a reference according to Eqn. 1 [17,18],

$$\frac{1}{\Phi_{-\mathrm{DPBF}}} = \frac{1}{\Phi_\Delta}\left(1 + \frac{k_d}{k_r} \cdot \frac{1}{[\mathrm{DPBF}]}\right) \qquad (1)$$

where $1/\Phi_{-\mathrm{DPBF}}$ represents the quantum yield of DPBF consumption, k_d the natural decay of $^1O_2$ to its ground state, and k_r the rate constant for the chemical quenching of $^1O_2$ by DPBF (physical quenching can be omitted as demonstrated by Merkel and Kearns [19]). The singlet oxygen quantum yield can be calculated from the intercept obtained by plotting $1/\Phi_{-\mathrm{DPBF}}$ versus 1/[DPBF] according to Eqn. 1. This latter method requires actinometry for the determination of yields of DPBF consumption, which was performed with Actinochrome 475/610 according to literature [20]. Both Φ_Δ determination methods gave identical results. As previously shown, the method described by Eqn. 1 cannot be used in model membrane systems, since high local concentrations of DPBF solubilized inside the hydrophobic phase cause a chain reaction leading to erroneous results [17,18]. The steady-state irradiation experiments were made with 2 ml air saturated samples, which were placed in 1 cm quartz cuvettes and irradiated through a GG475 cut-off filter, a 552 ± 10 nm narrow band interference filter and various calibrated neutral density filters using a 150 W xenon lamp as light source. During the irradiation procedure, the samples were stirred and kept at 24 ± 1°C. The consumption of DPBF was followed by absorption spectroscopy at 410 nm (homogeneous media) or 418 nm (microheterogeneous media) and was always < 15%. For each Φ_Δ determination and each method three to five runs were carried out, and concentrations of MC540 and rose bengal were varied (for details see Table I). In DTAB micelles, varying the dye concentrations leads always to a single occupancy of the micelle. Singlet oxygen quantum yields were always independent of the dye concentrations used. In liposomes, measurements were also performed with dye and DPBF locally separated by solubilizing dye and scavenger in separate liposomes. Separation and cosolubilization of the substrates gave the same results indicating the absence of interactions between DPBF and MC540 at these high local concentrations.

Fluorescence quantum yields were obtained from corrected fluorescence spectra and with cresyl violet perchlorate as a reference (λ_{exc} = 560 nm, Φ_f = 0.54 in methanol [21]). Reference and sample had absorbances around 0.05 and were matched at the excitation wavelength. In the case of liposomes, the fluorescence of liposomes without any dye was measured and the stray light was found to be < 1%. The temperature was controlled to ± 1 C°.

TABLE I

Singlet oxygen production, fluorescence yields and adsorption maxima of merocyanine 540 in different media

Singlet oxygen quantum yields Φ_Δ were measured at 24 ± 1°C. In homogenous solution, values include data of both determination methods: (1) using Eqn. 1 without a reference, (2) with rose bengal as reference. Values in micelles and liposomes were determined with method 2 only. Dye concentrations varied between 2 and 15 μM in solution, and between 3 and 10 μM in model membrane systems (lipid dye ratios: from 150 to 550). Fluorescence yields Φ_f were measured after excitation at 520 nm and 560 nm using cresyl violet perchlorate as a reference. The fluorescence yields shown were obtained at 25 ± 1°C. Values are means ± S.D.

Medium	Φ_Δ ($\times 10^3$)	Φ_f	λ_{max} (nm)
Methanol	2.2 ± 0.2	0.14 ± 0.01	555
Ethanol	3.4 ± 0.5	0.15 ± 0.02	559
n-Heptanol	5.6 ± 0.4	0.40 ± 0.04	566
DTAB-micelles	6.6 ± 0.8	0.33 ± 0.06	564
DMPC-liposomes	15.8 ± 2.8	0.48 ± 0.05	567
DPPC-liposomes	37.2 ± 6.2	0.61 ± 0.10	567

In homogeneous solution, an increase in Φ_Δ by a factor of 3 was found on going from methanol to *n*-heptanol (Table I). This increase is paralleled by a 14-fold increase in solvent viscosity (viscosities at 20°C: methanol 0.60 cp; ethanol 1.20 cp; *n*-heptanol 8.53 cp). Still, as Table I shows, Φ_Δ's are extremely low and have values well below 0.01 meaning that less than 1% of the absorbed light energy is used for $^1O_2$-generation. The fluorescence quantum yields follow the same pattern: changing the solvent from methanol to *n*-heptanol raises Φ_f from 0.14 to 0.40. In addition, Φ_f's in all solvents used decrease with increasing temperature; e.g., Φ_f in methanol has a value of 0.20 ± 0.01 at 5°C and is reduced to 0.11 ± 0.01 at 35°C.

In model membrane systems, both Φ_Δ and Φ_f increase further and reach maximum values in DPPC-liposomes with a Φ_Δ = 0.04 and a Φ_f = 0.61. The results are summarized in Table I. The shift of the absorption maxima towards longer wavelengths (Table I) in connection with the known hypsochromic solvatochromism of the dye [22], is a clear indication that MC540 has been incorporated into the organized assemblies and does not reside in the water bulk phase. By comparison, the absorption maximum of MC540 in pure water is 533 nm [23]. In addition, Φ_f's show the same trend in temperature dependence as in homogeneous solution.

The results in model membrane systems and in homogeneous solution are consistent. The changes in Φ_Δ and Φ_f in combination with the observed temperature dependence of Φ_f's are in agreement with previous findings that cyanine dyes and also MC540 undergo photoisomerization from the first excited singlet state [24]. In homogeneous solution, an increase of the solvent viscosity obviously reduces photoisomerization and most likely also internal conversion. Therefore, in a more viscous medium more of the absorbed light is dissipated by a radiative relaxation mechanism (fluorescence) and via intersystem crossing resulting in an increase in Φ_f and in triplet-sensitized $^1O_2$. The same effect takes place in model membrane systems. Due to the incorporation of MC540 into the organized assemblies, the mobility of the dye is decreased, and consequently photoisomerization and internal conversion are again hindered. The data in cationic DTAB micelles indicate, that MC540 experiences less restrictions in this medium than in liposomes. This is easy to understand taking into account that micelles form smaller and less structured entities than liposomes. Although both DMPC- and DPPC-liposomes have the same size (= same curvature of the bilayer membrane) and the same bilayer structure, they have very different phase transition temperatures. DMPC-liposomes undergo a phase transition at 23°C, whereas DPPC-liposomes change from a gel- to a liquid-like state at 41°C [25]. Since Φ_Δ's were measured at 24°C, the higher Φ_Δ in DPPC-liposomes reflects the higher rigidity of the bilayer membrane at this temperature.

The results from this study clearly show that the production of $^1O_2$ sensitized by MC540 is dependent on the viscosity or rigidity of the solubilization site. Consequently, the production of toxic $^1O_2$ will only reach a maximum, if the surrounding medium is able to cause total inhibition of both photoisomerization and internal conversion. By taking the rate constants for the different deactivation processes of the excited singlet state into consideration, the highest achievable Φ_Δ can be estimated. With a radiative rate constant of $4 \cdot 10^8\ s^{-1}$ calculated from absorption data and the emission maximum according to the Strickler-Berg relationship [26] and a rate constant for intersystem crossing of $5 \cdot 10^7\ s^{-1}$ [24], the maximum upper limit of triplet yield and therefore also Φ_Δ is estimated to be 0.11. Although this maximum Φ_Δ value is 55-times higher than the actual Φ_Δ in methanol, and still three times larger than Φ_Δ in DPPC-liposomes, MC540 remains even under optimum conditions a very modest producer of $^1O_2$. These results clearly suggest that MC540 has to be altered chemically in order to increase $^1O_2$-production and consequently also photoinactivation efficiencies in vivo. From this study it is also evident, that the first alteration should change the rate constant of intersystem crossing so that it is larger or at least of the same order of magnitude as the radiative rate constant. As a next and less important step, introduction of bulky groups might be considered to inhibit photoisomerization. However, as shown in this work, photoisomerization and internal conversion in vivo are obviously not lowering $^1O_2$-production significantly due to the highly structured localization site of the photosensitizer.

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