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SHORT COMMUNICATION

The Contribution of Raman Scattering to the Fluorescence of the Polyene Antibiotic Amphotericin B

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Abstract Fluorescence has recently been applied to the analysis of the molecular organization state of the polyene antibiotic amphotericin B (AmB) in solution or in lipid membranes. The polyene chain of AmB monomer gives rise to two fluorescence emissions; $S_1(2^1A_g) \rightarrow S_0(1^1A_g)$ between 500 and 700 nm, $S_2(1^1B_u) \rightarrow S_0(1^1A_g)$ between 400 and 500 nm. However, Raman scattering might interfere with the $S_2 \rightarrow S_0$ emission fluorescence due to the weak fluorescence quantum yield and close proximity to the exciting lines. In fact, we show here that a change in the excitation wavelength results in a shift of three emission bands, an effect which excludes their assignment to fluorescence. These bands originate from the water Raman at 3382 $cm^{-1}$and AmB resonance Raman at 1556 and 1153 cm^{-1}. As a consequence, some former conclusions on the molecular organization state of AmB should be reconsidered.

Keywords Fluorescence · Raman · Amphotericin B · Polyene antibiotic · Fungizone

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Introduction

Fluorescence has recently been applied to the analysis of the molecular organization state of the polyene antibiotic amphotericin B (AmB) in aqueous solution or in lipid membranes [1–8]. In the particular case of AmB monomers, their heptaene chain gives rise to strong absorption spectra ($S_0(1^1Ag) \rightarrow S_2(1^1B_u)$), presenting a vibronic structure around 400 nm (408, 384 and 364 nm), and two weak fluorescence emissions, $S_1(2^1A_g) \rightarrow S_0(1^1A_g)$ between 500 and 700 nm, $S_2(1^1B_u) \rightarrow S_0(1^1A_g)$ between 400 and 500 nm. Therefore, the emission from $S_2(1^1B_u)$ is close to the absorption, and the fluorescence quantum yield of AmB being low (0,0006 in 2-propanol-water and excitation at 408 nm)(1), interference of its Raman scattering must be considered. AmB is known to give rise to two intense resonance Raman peaks (C = C streching and a mixture of C-C and C = C stretching with C-H bending) at 1556 and 1153 cm^{-1} (9). The Raman spectrum of water can also complicate interpretation of the fluorescence spectrum of AmB. Indeed, self-screening resulting from the high absorption and low quantum yield, strongly limit both the range of concentrations over which AmB can be studied and the intensity of the AmB fluorescence spectra obtained. Front-face geometry could solve this limitation but has not yet been used.

Interference of Raman spectra can be easily demonstrated: i.e., by changing the excitation wavelength, fluorescence spectra remain at the same wavelength, whereas Raman peaks shift.

Experimental

Generic AmB (Pharma-Tek® AmB [PTAmB], Pharmatek Laboratories, Inc.; San Diego, CA) was obtained as a

lyophilized powder containing AmB, sodium deoxycholate and sodium phosphate, respectively, in milligram ratios of 50:41:20.2. High purity AmB (AmBHP) was prepared from generic AmB by semi-preparative high-pressure liquid chromatography (HPLC) as described previously [2]. In brief, a semi-preparative HPLC was performed by applying aliquots of Pharma-Tek AmB reconstituted in HPLC grade water to a 10 mm (I.D.)×250 mm 5 micron AquaC18TM column (Phenomenex U.S.A., Torrance, CA). Minimal apparent purity of the purified AmB (AmBHP), based on absorbance at 405 nm, was 98%. Fungizone is a commercial micellar dispersion of AmB in deoxycholate. DMSO and SDS were purchased from Sigma France.

For studies of AmBHP in water alkalinized to pH 12, the procedure of Gagos et al. [12] was applied. For studies on Fungizone in the presence of detergent, 0.5 M SDS prepared in Milli-Q water was added to concentrated aqueous Fungizone suspensions so that SDS:AmB was 200:1. The mixtures were adjusted to the desired concentrations.

Absorbance spectra of aqueous suspensions of AmBHP in 0.1 or 1 cm path length quartz cuvettes were recorded on a Cary Varian 3E UV-visible spectrophotometer. Fluorescence spectra were recorded on a spectrofluorimeter Aminco Bowman series 2. Fluorescence of diluted samples presenting a low absorbance, were recorded in a 1 cm quartz cell. For higher concentrations, samples were held in a quartz dual path length cuvette (0.25 and 1 cm). The orientation of the cuvette was such as to minimize the inner filter effects [4].

The spectrofluorimeter operated at a 4 nm band width and a 1 nm step size. Under some experimental conditions, self-screening (inner filter) effects had to be taken into consideration and a correction factor to be applied to fluorescence intensities [15].

Results

Fluorescence of Monomeric AmBHP: AmB in Alkalinized (pH 12) Water

AmB is soluble in water alkalinized to pH 12, and it is only present as a monomer in this solvent [7, 10, 11]. In Fig. 1 are presented spectra obtained between 400 and 700 nm with excitations at 408 nm (maximum of absorption) and 390 nm. It appears that AmB spectra are clearly divided in two parts. Above 500 nm, peaks were observed at 502, 523, 562 and 602 nm. These four bands can be rationalized in terms of the $S_1 \rightarrow S_0$ fluorescence [13, 14], and the spectra did not depend on the excitation wavelength. Below 500 nm, the spectra have been proposed to correspond to the $S_2 \rightarrow S_0$ transition [4, 14]. However, as illustrated, they

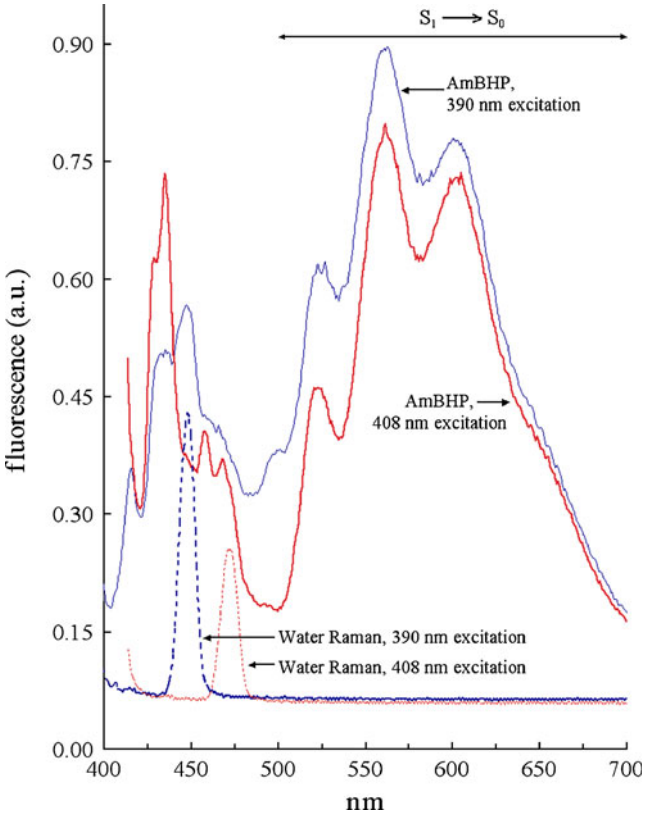

Fig. 1 Influence of excitation wavelength on AmBHP emission spectra and water Raman. AmBHP was dissolved in water (pH 12) at a final concentration of 6 μM. Emission was recorded between 400 and 700 nm with excitation set at either 390 nm or and 408 nm. Water Raman was recorded under the same conditions. No correction factors were applied

depended on the wavelength of excitation. In each spectrum, a strong peak at 3382 cm^{-1} of the excitation (emissions at 449 and 473 nm, respectively, for excitations at 390 and 408 nm) could be assigned to the solvent since it coincided with the O-H stretching Raman peak of water at 3382 cm^{-1}. As could be expected, the intensity of this peak remained constant with increasing AmB concentrations.

Emission spectra of AmB between 400 and 500 nm for excitations wavelengths of 390, 408, 415 and 420 nm, after subtraction of the water Raman bands and correction for self-screening (15), are presented in Fig. 2. Four or five band were observed, depending on the excitation wavelength. If the $S_2 \rightarrow S_0$ emission spectrum is assumed to be the mirror image of the $S_0 \rightarrow S_2$ absorption (408, 384, 364 nm), emission peaks would be expected at approximately 435 and 464 nm. As a matter of fact, such emission peaks were observed with an excitation at 390 nm (431-466 nm and 461-466 nm).

Another peak was at 1556 cm^{-1} of the excitation (emissions at 415, 435, 443 and 449 nm, respectively, for excitations at 390, 408, 415 and 420 nm). The implication

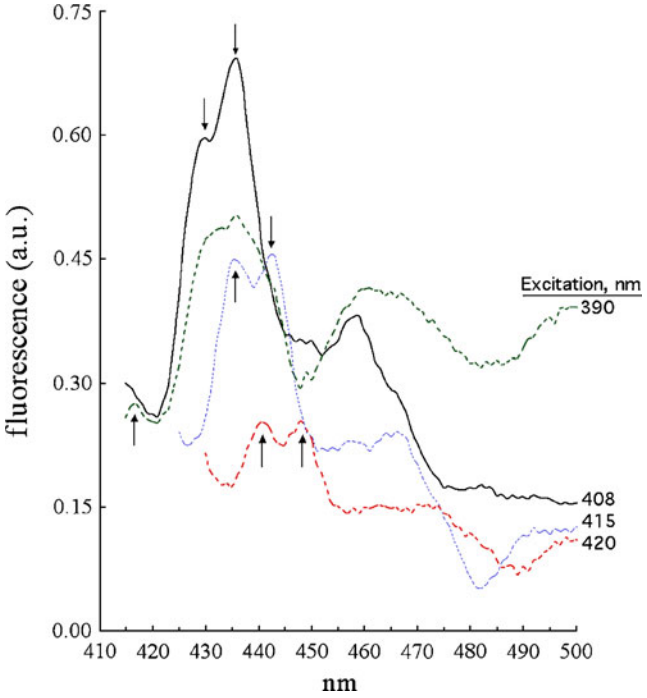

Fig. 2 Influence of excitation wavelength on emission spectra of AmBHP after correction for water Raman. AmBHP was dissolved in water (pH 12) to a final concentration of 10 μM. Spectra of AmB or water (pH 12) were recorded between 400 and 500 nm at excitations of 390, 408, 415 and 420 nm. Samples were held in the quartz dual path length cuvette (A_{408}=0.34) The Raman spectrum of water was subsequently subtracted from the spectrum of AmB obtained at the corresponding excitation. The arrows indicate the theoretical position of the AmB resonance Raman peaks

of these findings is interference by Raman scattering and more precisely to the well-known intense resonance Raman band of AmB (C = C stretching vibration) [9, 16]. Similarly, another peak was observed at 1153 cm^{-1} of the excitation (emission at 431, 435, 441 nm for excitations at 408, 415, 420 nm), and it corresponds to the resonance Raman involving a mixture of C-C and C = C stretching with C-H bending vibrations [9].

Our data differ from those of Gruszecki et al. [8]. In our experiments, at 3 μM AmB and 390 nm excitation, the Raman band of water at 449 nm was twice the AmB fluorescence at 435 nm, whereas it was smaller in their experiment. Although spectra were similar for concentrations of AmB above 10 μM, data presented herein argues strongly for the assignment of the 415 nm line to resonance Raman and not to fluorescence. Furthermore, the life time measurements obtained by excitation at 405 nm and measurement at 430 nm [8] are compatible with an assignment to Raman bands: a single decay component characterized by $t \leq 10$ ps was observed. It is well known that Raman lifetimes are typically in the scale of picoseconds or less [17].

Fluorescence of AmBHP in Water at pH 7

In contrast to the situation in water at pH 12, AmB at concentrations greater than 1 μM is no longer only in a monomeric form in water at pH 7. Self-associated species are also present, and the absorption spectra of these polymeric forms are shifted to wavelengths below 360 nm [18]. The amount of monomeric AmB, for a given total AmB concentration, is lower in comparison to the amount at pH 12, as evidenced by the decrease of absorption in the region (around 400 nm) attributed to monomers [7]. When spectra of aqueous solutions of AmB of pH 7 and pH 12 were compared, neither the increased fluorescence nor the change of emission wavelengths described by Gruszecki et al. [8] for 3.6 μM AmB was observed. The fluorescence emission they described at 448 nm is presumably dominated by the water Raman emission (theoretically at 449 nm). Taking into account the expected decrease of fluorescence as noted here, it is not necessary to consider a non-rigorously parallel orientation of the dipole transition of neighbouring transitions in self-associated species, as did these authors.

Fluorescence of Fungizone at pH 7 in the Presence or Absence of SDS

Stoodley et al. [4] have presented fluorescence emission spectra of 50 μM Fungizone in the presence and absence of SDS, i.e. in its monomeric or self-associated form, respectively.

Proceeding by the same approach as above, i.e. wavelength excitation changes, it appeared that the C = C stretching resonance Raman of AmB brings a strong contribution to the peak described by Stoodley at 437 nm (data not shown). Furthermore, in the particular case of Fungizone in the absence of SDS, elastic scattering background should also be taken into account. Therefore, their interpretation of the change in the ratio of "fluorescence" intensity from S2 and S1 energy levels with increasing surfactant concentration, is to be taken with caution.

Conclusion

The conjunction of the weak quantum yield of the $S_2(1^1B_u) \rightarrow S_0(1^1A_g)$ emission between 400 and 500 nm and close proximity to the exciting lines, with strong absorption, self-screening, water Raman and AmB resonance Raman scattering (and elastic scattering) renders the study of this fluorescence unreliable to obtain information on the organization of AmB in water or in lipid membranes. The present data indicate that only the fluorescence of the $S_1(2^1A_g) \rightarrow$

$S_0(1^1A_g)$ transition of monomeric AmB, observed between 500 and 700 nm, is reliable. Unfortunately, for this transition, the quantum yield is also low (0.0006) [1], which limits its usefulness as a tool for the study of AmB/membrane interaction. Light scattering by the biological material makes the fluorescence of AmB, at concentrations needed to reliably study its mechanism of action and avoid self-screening effects, difficult to accurately discern.

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