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Characterization of *p*-bis(*O*-methylstyryl)benzene as a lifetime and anisotropy decay standard for two-photon induced fluorescence

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Abstract

We describe the fluorescence spectral properties of *p*-bis(*O*-methylstyryl)benzene (bis-MSB) as a standard for time-resolved measurements of two-photon induced fluorescence. Bis-MSB displays the same single exponential intensity decay in several solvents for one- and two-photon excitation. The anisotropy decay displays the same single correlation time of one- and two-photon excitation. The amplitudes of the anisotropy decay are distinct for one- and two-photon excitation. At some excitation wavelengths the anisotropy amplitude appears to be solely the result of one- and two-photon photoselection, but at shorter wavelengths the anisotropy amplitudes are not related by single constant factor. The absorption range of bis-MSB makes it a suitable standard for one- and two-photon excitation of intrinsic protein fluorescence and extrinsic fluorophores.

Keywords: *p*-Bis(*O*-methylstyryl)benzene; Two-photon induced fluorescence; Fluorescence intensity decay; Protein fluorescence

1. Introduction

Two-photon induced fluorescence (TPIF) has been used to detect simultaneous absorption of two-photons and for determination of the electronic symmetry of the intermediate and excited states [1–5]. More recently, two-photon excitation of fluorescence has become of interest because of the possibility of intrinsic "confocal" excitation in fluorescence microscopy [6–8]. Additionally, TPIF is useful for time-resolved fluorescence because of the possibility for excitation in optically dense media [9] and surprisingly high detection limits [10,11]. Importantly, there may be increased and/or different information content of the two-photon induced anisotropy spectra and/or anisotropy decays [12–16], which may allow the resolution of overlapping states. Such experiments, when combined with studies of two-photon spectroscopy of biomolecules [17–19], may provide new opportunities for spectroscopic studies of macromolecules. Furthermore, two-photon induced fluorescence may find additional applications in fluorescence microscopy. For instance, it is now possible to create contrast in fluorescence microscopy based on the position-dependent fluorescence lifetimes [20–22]. One can imagine TPIF being combined with lifetime and/or anisotropy decay imaging, to provide many novel opportunities for visualization of intracellular structure, dynamics and physiology.

Time-resolved measurements of TPIF, particularly when combined with microscopy, are difficult and prone to error due to the need for intense excitation and the possibility of reflections and stray light. Hence, we felt it is valuable to have standard fluorophores which display known lifetimes and correlation times for OPIF and TPIF. We characterize the one- and two-photon induced intensity and anisotropy decay of bis-MSB in several solvents. Bis-MSB was described previously as a standard for two-photon absorption by Kennedy and Lytle [23] and was chosen for its high two-photon cross-section. Fortunately, the one (540–780 nm) and two-photon absorption spectra (270–350 nm) span the wavelengths of interest for a wide variety of intrinsic and extrinsic probes. In the present report, we describe the lifetimes and correlation times of bis-MSB in several solvents, for one- (OPE) and two-photon excitation (TPE).

2. Experimental methods

Bis-MSB was obtained from Kodak (scintillation grade) and used without further purification. Bis-MSB concentrations were determined using the one-photon extinction coefficient of 56,000 M^{-1} cm^{-1} at 345 nm [24]. For both OPIF and TPIF, the emission was observed through a 420 nm interference filter with approximately 12 nm bandpass. Emission spectra were measured using a monochromator with 2 nm slits yielding a 10 nm bandpass.

For the one-photon measurements, we used 0.5 cm × 0.5 cm cuvettes with excitation and emission near a corner of this smaller cuvette. This cuvette was positioned off center in a 1 cm × 1 cm cuvette holder. For the two-photon measurements, we used 1.0 cm × 0.5 cm cuvettes, with the long axis aligned along the incident light path and with the focal point positioned about 0.5 cm from the surface facing the incident light. The position of the cuvette was adjusted so that the excitation laser beam crossed the solution near the observation window. For both OPE and TPE the concentration of bis-MSB was near 5×10^{-5} M.

Single photon excitation from 282 to 310 nm was accomplished using the frequency-doubled output of a rhodamine 6G (R6G) dye laser, and from 350–375 nm using the frequency-doubled output of a pyridine2 dye laser. Two-photon excitation from 564 to 620 nm and 700–750 nm was accomplished with the fundamental output of these dye lasers, respectively. The dye lasers were synchronously pumped with the 514 nm output of a mode-locked argon ion laser, and cavity-dumped at 3.795 MHz. Signal from the solvents alone were less than 0.5% for both one- and two-photon excitation. The excitation beam was focused by using a 5 cm focal length lens. A similar lens was used for the collection of the fluorescence. Steady state anisotropy spectra were obtained in the usual L-format manner [25,26] by measuring the intensity of the parallel and perpendicular components of the emission, with correction for the G-factor which was close to unity.

Frequency-domain intensity and anisotropy decays were obtained on the GHz instrument describe previously [27,28]. Intensity decays ($I(t)$) were fit to the multi-exponential model using

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i), \qquad (1)$$

where α_i are the amplitudes associated with the decay time τ_i [29,30]. The differential phase and modulated anisotropy data were fit to the multi-correlation time anisotropy decay ($r(t)$) model

$$r(t) = \sum_j r_{0j} \exp(-t/\theta_j), \qquad (2)$$

where r_{0j} is the portion of the total anisotropy ($r_0 = \Sigma r_{0j}$) which decays with a correlation time θ_j [31]. The expressions relating the time-resolved equations to the frequency-domain data are summarized in [12].

3. Results

3.1. Emission spectra

The emission spectra of bis-MSB in cyclohexane are shown in Fig. 1. The emission depends in linearity on intensity for one-photon excitation at 290 nm (○), and quadradically on intensity for two-photon excitation at 580 nm (●). Identical emission spectra were observed for one- (— —

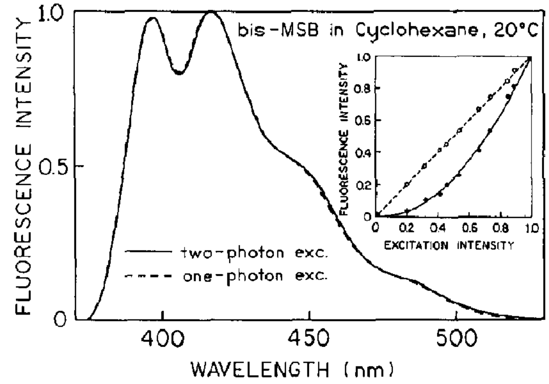

Fig. 1. Emission spectra of p-bis(O-methylstyryl)benzene (bis-MSB) in cyclohexane at 20°C, resulting from one- (— — —) and two- (———) photon excitation, at 290 and 580 nm, respectively. The insert shows the dependence of the emission intensity on the excitation intensity at 290 (○) and 580 (●) nm.

—) and two-photon (———) excitation in cyclohexane (CH), and in propylene glycol (PG) and ethanol (not shown). The emission intensity was stable with continuous illumination and bis-MSB did not appear to display significant photodecomposition or bleaching.

3.2. Fluorescence lifetimes

Frequency-domain intensity decay data for bis-MSB in cyclohexane are shown in Fig. 2. The frequency-response for OPE and TPE are essentially identical. The intensity decay was found to be a single exponential in cyclohexane (Fig. 2), ethanol and propylene glycol (Table 1). Essentially identical lifetimes were observed for both one- and two-photon excitation (Table 1). Examination of Table 1 reveals that the lifetimes for two-photon excitation are smaller to a small extent (about 50 ps) then for one-photon excitation. At present we are uncertain as to the origin of this minor effect.

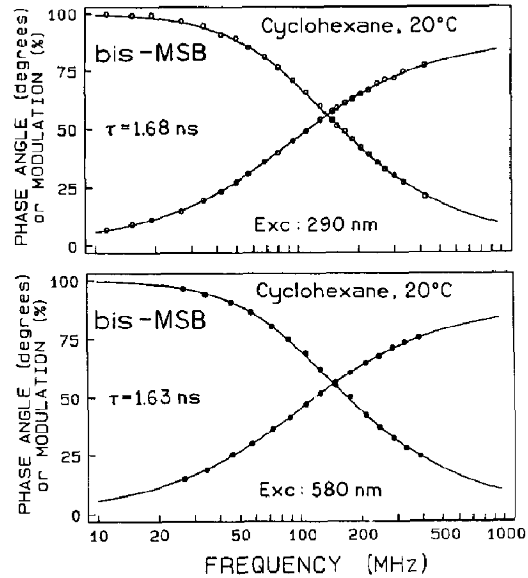

Fig. 2. Frequency-domain intensity data for bis-MSB in cyclohexane at 20°C, obtained using one- (top) or two-photon (bottom) excitation. The solid lines show the best single-exponential fits to the data.

Table 1

Fluorescence intensity decay analysis of bis-MSB

Solvent 20°C	One-photon excitation			Two-photon excitation		
	λ_{ex} (nm)	τ (ns) [a]	χ^2_R	λ_{ex} (nm)	τ (ns) [a]	χ^2_R
Cyclohexane	290	1.68	1.5	580	1.63	1.3
Ethanol	290	1.63	1.6	580	1.63	1.9
	365	1.58	0.9	730 [b]	1.56	1.7
Propylene glycol	290	1.51	1.2	580	1.46	1.6
	283	1.51	1.3	566	1.46	1.7
	305	1.50	1.1	610	1.45	1.6

[a] Standard deviations of the decay times from the least squares analyses [12,32] were smaller than 0.005 ns. The values of χ^2_R were calculated using 0.2° uncertainty in the measured phase angles and 0.005 uncertainty in the measured modulation values.

[b] All measurements used scattered light as the reference, except for 730 nm excitation where DMSS (4-diethylamine-ω-methylsulfonyl-*trans*-styrene) was used with a reference lifetime of 34 ps [36].

3.3. Anisotropy decays

The steady state and time-resolved anisotropy behavior of bis-MSB make it a suitable standard for one- and two-photon experiments. Bis-MSB displays a single rotational correlation time in CH, PG and ethanol, and the same correlation time for one- and two-photon induced fluorescence (Table 2). This allows direct comparison of the measured correlation times for a variety of experimental configurations. For instance, the one-photon correlation time measured in a standard cuvette can serve as the reference for two-photon excitation in a fluorescence microscope. The fact that the elongated bis-MSB molecule displays a single correlation time, and the same correlation time for OPIF and TPIF, implies that the one- and two-photon transitions are parallel and probably aligned along the long axis of the molecule and only rotational motions which displace this axis contribute to the anisotropy decay.

The amplitudes of the bis-MSB anisotropy decays are somewhat more complex, but in fact, provide opportunities for comparing one- and two-photon experiments. The amplitudes for one- and two-photon excitation (r_0^1 and r_0^2, respectively) were recovered from the frequency-domain data (Fig. 3). In all cases the differential phase angles and modulated anisotropies were larger for TPE than for OPE (Fig. 3), but the shapes of the frequency responses were similar, as were the frequencies at which the differential phase angles displayed the maximal values. For co-linear absorption and emission transition measurements, for both OPE and TPE, one expects $r_0^2/r_0^1 = 10/7 = 1.43$ [12]. Ratios larger than 1.43 were found for bis-MSB in cyclohexane,

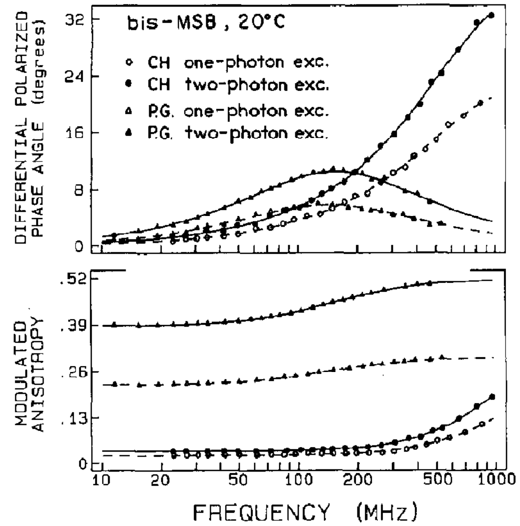

Fig. 3. Frequency-domain anisotropy decays data for bis-MSB in cyclohexane (○, ●) and propylene glycol (△, ▲) at 20°C, resulting from one- (○, △) and two-photon (●, ▲) excitation.

Table 2

Anisotropy decay analyses of bis-MSB fluorescence

Solvent 20°C	One-photon excitation				Two-photon excitation				
	λ_{ex} (nm)	r_0	θ (ns)	χ_R^2	λ_{ex} (nm)	r_0	θ (ns)	χ_R^2	r_0^2/r_0^1
Cyclohexane	290	0.304 (0.004) [a]	0.130 (0.002)	1.4	580	0.509 (0.004)	0.121 (0.001)	1.5	1.67
Ethanol	290	0.301 (0.002)	0.129 (0.001)	1.0	580	0.517 (0.003)	0.125 (0.001)	1.9	1.72
	365	0.373 (0.002)	0.117 (0.001)	2.0	730	0.537 (0.002)	0.114 (0.001)	2.5	1.44
Propylene glycol	290	0.299 (0.001)	4.41 (0.03)	1.0	580	0.516 (0.001)	4.40 (0.01)	1.2	1.73
	283	0.262 (0.001)	4.45 (0.04)	1.2	566	0.508 (0.001)	4.49 (0.02)	2.5	1.94
	305	0.342 (0.001)	4.39 (0.02)	1.1	610	0.522 (0.001)	4.45 (0.02)	2.3	1.53

[a] Standard deviations from the least squares analyses [12,32]. Experimental uncertainties of the phase angles and modulation ratio were assumed the same as in Table 1.

propylene glycol and ethanol (Table 2). However, this effect was found to depend on the excitation wavelength, with the expected ratio of 1.43 being observed for excitation above 310(620) nm. This caused us to investigate the steady state anisotropy spectra of bis-MSB in the absence of rotational diffusion (Fig. 4). At wavelength above 310 nm, the steady state anisotropies display a ratio near 1.43 (Fig. 4). At shorter wavelengths below 310 nm, the anisotropy of bis-MSB decreases for one-photon excitation, but remains high for two-photon excitation. Apparently, a second transition contributes to the one-photon absorbance at 300 nm, but the two-photon absorbance from 280(560) to 370(740) is due to a single transition. This result seems to be consistent with the one- and two-photon absorption spectra of bis-MSB, reproduced from [23] as the insert in Fig. 4. We verified that these wavelength-dependent anisotropy amplitudes were in fact present in the time-resolved anisotropy decays. Frequency-domain anisotropy data for bis-MSB in the same propylene glycol solvent are

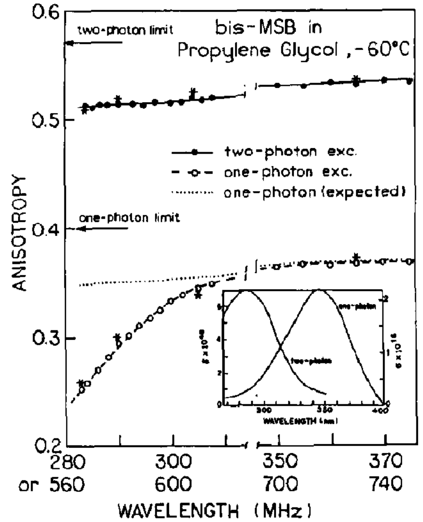

Fig. 4. Steady state anisotropy spectrum of bis-MSB in propylene glycol at −60°C measured with one- (○) and two-photon (●) excitation. The dotted line (....) is the one-photon excitation anisotropy spectrum predicted for co-linear transition moments from the two-photon anisotropy spectrum. The asterisks (∗) are anisotropies at $t = 0$, obtained from time-resolved measurements. The insert shows the one- and two-photon absorption spectra reproduced from [23].

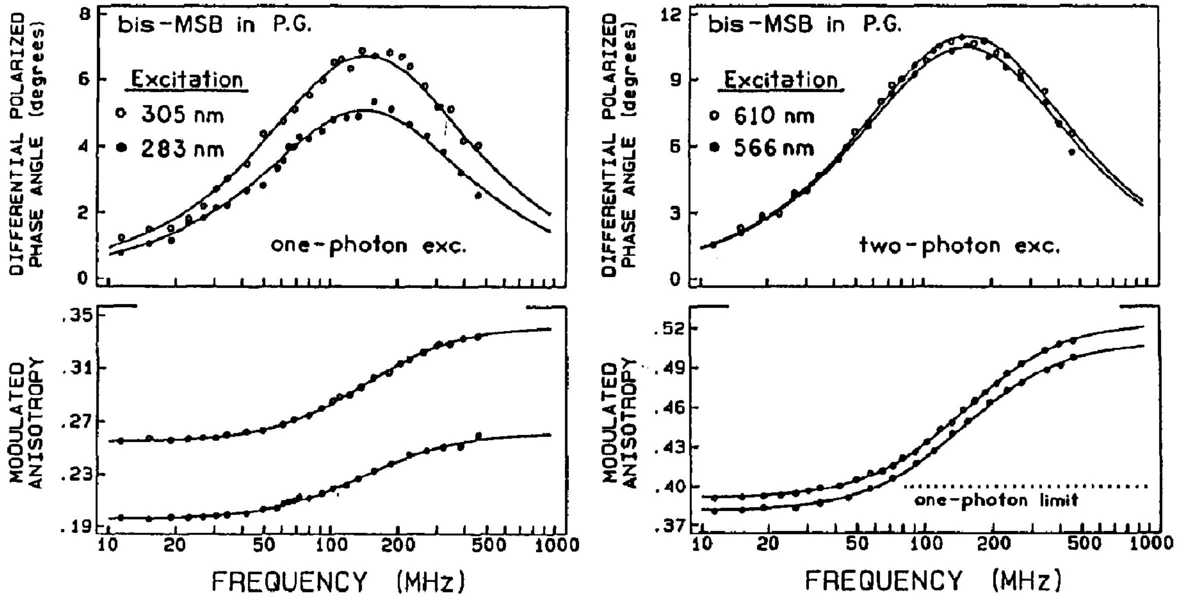

Fig. 5. Frequency-domain anisotropy decays data for bis-MSB in propylene glycol at 20°C, resulting from one- (left) and two-photon (right) excitations.

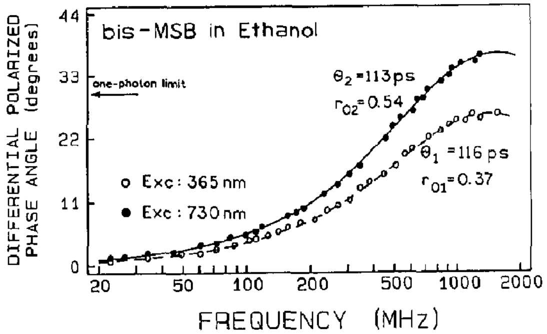

Fig. 6. Differential polarized phase angles for bis-MSB in ethanol at 20°C, obtained for one- (○) and two-photon (●) excitation.

shown in Fig. 5 for one- and two-photon excitation. For TPE, the frequency-domain anisotropy data are nearly the same for excitation at 566 and 610 nm (right), which indicate that the time-zero anisotropies are nearly the same. In contrast, the frequency-domain anisotropy data for OPE at 283 and 305 nm are distinct (left), and the difference is due to a difference in the time-zero anisotropies. In all cases, the time-zero anisotropy values recovered from the frequency-domain data (∗) were in agreement with the steady-state anisotropies observed at low temperatures (Fig. 4).

And finally, we present frequency-domain anisotropy data, which in isotropic media can only result from TPE. Differential phase angles of bis-MSB in ethanol are shown in Fig. 6. We demonstrated elsewhere [13], that the maximum possible phase difference between linearity polarized components of the emission is 30° for OPE. For bis-MSB in ethanol, differential phase angles as large as 36° were observed (Fig. 6).

4. Discussion

The availability of a two-photon lifetime and anisotropy standard should be useful in future two-photon experiments. For instance, most phototubes have decreased or no sensitivity above 600 nm. Consequently, it is difficult to perform time- or frequency-domain measurements with TPE cause of the inability to measure a light scattering reference. Bis-MSB can be used to convert the red wavelength excitation to visible emission with known decay time characteristics. Lifetime measurements may then be done using the reference corrections described for the time- [33] and frequency-domain [34]. Additionally, the one- and two-photon anisotropy values can be used to determine and/or correct for the effect of wide aperture microscope objectives on the measured anisotropy values [35]. And finally, we note that it is probable that fluorescent molecules will display unusual anisotropy spectra and other properties for TPE, as has been observed for indole [14]. Hence, it will be valuable to have a two-photon reference molecule to exclude instrumental effects or other artifacts in the two-photon measurements.

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