Enhanced Two-Photon Absorption and Fluorescence Upconversion in Thioflavin T Micelle-type Aggregates in Glycerol/Water Solution

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Abstract

In this article, we report the systematic characterization of the two-photon absorption of ThT in different mixtures of glycerol/water solution. The relationships of TPA peak position and amplitude revealed a dependence on particle size suggesting that the curious trend observed in TPA with changing glycerol content can be attributed to the presence of micelle-type aggregates. Consequently, the relatively strong TPA cross-section ($\delta_{\text{TPA}} = 300\text{GM}$) obtained in 8.75% glycerol/water solutions could be attributed to the immobilization of dye molecules and the strong coupling of the molecular transition dipoles in micelle-type aggregates. This enhancement of TPA, in addition to the already reported significant fluorescence quantum yield of ThT attached to brain tissue, is expected to boost the application of this compound for *in vitro* and perhaps *in vivo* high resolution multiphoton bioimaging of amyloids in brain tissue.

Introduction

Thioflavin T (ThT) has become a commonly used dye for *in vitro* and *ex vivo* fluorescence imaging of fibrils associated with Alzheimer's and Parkinson's diseases [1-6] since its first study and application completed by Vassar and Culling in 1959 [7]. In their initial work these authors described the dramatic increase and red shift in fluorescence emission of ThT upon selective binding to amyloid fibers. The fluorescence quantum yield (Φ) of ThT increases from nearly zero in aqueous solution to approximately 0.40 in amyloid fibril [8]. The mechanism of this enhancement has been investigated and largely attributed to the restriction of torsional motion between the benzothiazole and aminobenzene rings when the dye binds to the amyloid [9,10]. Several other effects on fluorescence enhancement have also been proposed including morphological differences in fibrils [11], stabilization of charge on the benzothiazole ring [12] and aggregation of the dye molecules [13,14], among others.

The selectivity of the dye to bind to amyloids [14], ease of administration [15] and its non-toxic nature, in addition to its extended π -electron delocalization and intramolecular charge transfer architecture, make ThT an excellent candidate for multiphoton imaging of amyloid fibrils, which could introduce additional advantages. As it is already well-known, two-photon induced fluorescence bioimaging allows for the use of lower excitation energies (longer wavelengths) than for linear processes, making it possible to induce the excitation of the fluorophore in a region where biological tissues are transparent. Consequently, the negligible linear absorption and the minimization of scattering in this spectral region opens a path to greater penetration depth. Additionally, the quadratic dependence of TPA cross section on incident irradiance yields increased spatial resolution [16], and using longer wavelengths in bioimaging reduces

photobleaching of the fluorophore and photodamage to living cells [17]. These advantages have already found a variety of practical applications in the fields of biology [17,18] and medicine [19].

In an effort to expand the application of multiphoton bioimaging in the understanding and diagnosis of Alzheimer's and Parkinson's diseases, herein we report on the systematic experimental analysis of the TPA and fluorescence upconversion of Thioflavin T in glycerol/water solutions. Our results show the potential of ThT as a two-photon fluorescent probe for *in vitro* multiphoton bioimaging of amyloids in brain tissue and may lead to its application *in vivo*.

2. Experimental Methods

Thioflavin T (ThT) (97%) was purchased from AnaSpec and used without any further purification. The linear absorption spectra were measured in aqueous solutions at various concentrations ranging from 9.3 μM to 1 mM and different glycerol content (0-35%), between 190 and 1100 nm on an Agilent 8453 Diode Array UV-Vis single-beam spectrophotometer in a 1 cm quartz cuvette. Contributions from the solvents and the cell were subtracted from the spectra. The fluorescence excitation and emission spectra were measured in the same solutions on an FLS980 spectrofluorimeter (Edinburg Instruments Ltd), using a 1 cm path length spectrofluorimetric quartz cell. The excitation anisotropy spectrum of ThT was obtained upon emission at 420 nm in a 0.1 mM solution in ethylene glycol to inhibit molecular rotational relaxations. The emission intensity was measured through a polarizer oriented parallel ($I_{//}$) and perpendicular ($I_{\perp}$) to the direction of the polarized excitation [20]. Fluorescence quantum yield measurements were made at 25°C *via* the comparative method using lucigenin ($\Phi_f = 0.67$) as a reference [21]. Lucigenin was chosen as a standard since its quantum yield is known and it has a similar excitation wavelength (368 nm) to

ThT. Particle sizes were obtained using the Zetasizer nano ZS90, utilizing the He-Ne laser (633 nm) light source, in a solution with concentration of 1 mM and using a 1 cm glass cuvette. The solvents were filtered with syringe filters in order to remove macro-size particles. The glycerol solvent was filtered with a 2 μ m Whatman syringe filter, and water was filtered with a 0.2 μ m Whatman syringe filter before adding the ThT solute. Then, the solution was sonicated until there was no evidence of large particles in solution.

The open aperture Z-scan method [22] was used to perform the nonlinear two-photon absorption (TPA) measurements at room temperature. Samples were prepared in water and glycerol/water mixtures at a concentration of 1 mM. Two-photon excitation was induced with a computer-controlled femtosecond optical parametric amplifier (OPerA Solo) pumped by an amplified laser system from Coherent, Inc. The whole system is capable of generating 90 fs (FWHM) pulses over a wavelength range that spans from 290 nm to 2.6 μ m, with pulse energies of up to 4 mJ and a variable repetition rate ranging from 1 Hz to 1 kHz. Experiments were performed between approximately 450 to 850 nm, with typical input energies ranging from 7 to 45 μ J/pulse at a 50 Hz repetition rate to avoid any contribution from accumulative effects. The pulse width was determined using a single-shot autocorrelator from Coherent, Inc. and a frequency-resolved optical gaiting (FROG) from Swamp Optics LLC.

3. Theoretical and Computational Methods

The molecular structure of ThT was optimized from an initial planar geometry using Density Functional Theory (DFT) [23] in GAUSSIAN 09 [24] using the Becke's three-parameter exchange, Lee, Yang and Parr correlation (B3LYP) hybrid functional [25-27] and the 6-

311++G(d,p) basis set [28,29]. The first 100 excited states and molecular orbitals were performed using Time-Dependent DFT (TD-DFT) [23] with the Coulomb-attenuating method (CAM)-B3LYP exchange correlation functional (XCF) [30] and the same basis set as for the optimization. The CAM-B3LYP XCF was used because it has been proven to be more reliable than B3LYP in molecules with significant intramolecular charge transfer (ICT) [30-32]. Solvent effects for water were considered under the polarizable continuum model (PCM) [33].

The TPA response (degenerate case) for the lowest 20 electronic excited states of the optimized structure *in vacuo* was computed with Dalton 2013 [34], using TD-DFT at the CAM-B3LYP/6-311++G(d,p) level of theory. TPA spectra were calculated using [35,36]

$$\delta_{0f}^{TPA}(\omega) = \frac{4\pi^3\alpha a_0^5}{c} \sum_f (\hbar\omega_f)^2 \delta_{of}^{TPA}(\omega_{0f}) g(2\omega, \omega_{0f}, \Gamma), \quad (1)$$

$$\delta_{0f}^{TPA}(\omega) \approx 1.25273 \times 10^{-2} \times \omega^2 \sum_f g(2\omega, \omega_{0f}, \Gamma) \cdot \delta_{of}^{TPA}(\omega_{0f}) \quad (2)$$

Here c is the speed of light in vacuum, a_0 is the Bohr's radius, α is the fine structure constant, $E = \hbar\omega$ is the photon energy (half of the transition energy for the degenerate case), $\bar{\delta}_{0f}^{TPA}(\omega_{0f})$ is the orientational averaged two-photon probability for the degenerate case, and ω is the excitation frequency. A normalized Lorentzian lineshape function ($g(2\omega, \omega_{0f}, \Gamma)$) was employed to broaden electronic transitions (Eq. (3)),

$$g(2\omega, \omega_{0f}, \Gamma) = \frac{1}{\pi} \frac{\Gamma_{gf}/2}{(\omega_{gf} - 2\omega)^2 + (\Gamma_{gf}/2)^2}, \quad (3)$$

where Γ is the FWHM linewidth for all transitions, in this specific case, 0.12 eV to best fit experimental spectra. The TPA calculated spectra are in Göppert-Mayer units (GM), i.e. $10^{-50} \text{cm}^4 \cdot \text{s} \cdot \text{molec}^{-1} \cdot \text{photon}^{-1}$ when atomic units are used for the elements of eq. (1) and (2).

4. Results and Discussion

Figure 1 displays the normalized excitation, absorption and emission spectra of an aqueous solution of ThT at a concentration of 0.625 mM. It also shows the excitation anisotropy (ThT/ethylene glycol) and the calculated one-photon absorption (OPA) spectrum. These results are in agreement with the linear optical properties of ThT described in the literature [9], where the excitation, absorption and emission maxima are located at 340, 410 and 460 nm, respectively. In the fluorescence anisotropy spectrum one can identify three different spectral regions corresponding to $S_0 \rightarrow S_1$, $S_0 \rightarrow S_2$ and $S_0 \rightarrow S_n$ at 350-370 nm, 300-320 nm and 260-290 nm, respectively. According to this outcome one could anticipate a strong TPA in the 300-320 nm range, a region that corresponds to a transition between states of equal parity ($S_0 \rightarrow S_2$). Although ThT is not a centrosymmetric molecule, and it is known that selection rules are more relaxed in such a scenario, considering parity restriction is still essential.

Interesting to note is the poor overlap between excitation and absorption. Chen and co-workers attribute this effect to a degradation of pure ThT into three kinds of derivatives (dThT-1, dThT-2, and dThT-3) after storage or by UV irradiation [37]. Specifically, dThT-1 is responsible for the emission observed at approximately 450 nm when excited at 340 nm. Emission is not observed in pure ThT, dThT-2, and dThT-3 unless the molecules are bound to amyloid fibrils [37]. This suggests an additional advantage for using ThT as a TPA biomarker; since UV light is not used for excitation, one would only expect to see fluorescence from bound molecules.

Next, in order to truly assess the potential application of ThT for two-photon bio-imaging, we measured the full TPA spectrum in a 1 mM aqueous ThT solution. In Figure 2 we present the experimental and theoretical TPA spectra along with the associated transitions. First, it is important to point out the remarkable spectral match between theory and experiment. The theoretical spectrum was slightly red-shifted (+30 nm) to obtain the best theoretical-experimental overlap (this is common practice in theoretical-experimental works [31,38-41]). Interesting to highlight is the fact that most of the TPA spectrum, within the regions of highest measureable TPA cross section, is determined by the transitions to the first and fourth excited states. Second, two of the three prominent bands observed in the theoretical spectrum (760 nm and 540 nm) can be distinguished in the experimental spectrum. However, because of the occurrence of linear absorption below 480 nm the third theoretical band centered at 410 nm cannot be accessed experimentally. The presence of the band at approximately 540 nm (equivalent to a 270 nm excitation by one-photon) was anticipated based on excitation anisotropy (see Figure 1). Finally, the presence of the two relatively important TPA bands is ascribed to the significant intramolecular charge transfer (ICT) associated with transitions to the first and fourth excited states. It is well-known that a large charge transfer within a molecule can be associated with a large electric transition dipole moment, which favors TPA absorption [42]. Our hypothesis can be corroborated by a close inspection of the molecular orbitals involved in the dominant transitions, those to the first and fourth excited states (Figure 3). While the former mainly involves a transition between HOMO and LUMO, the latter is dominated by the following two transitions: HOMO-3 $\rightarrow$ LUMO (21%) and HOMO $\rightarrow$ LUMO +2 (55%). In both cases, one can observe a relatively strong ICT. This effect is more pronounced between HOMO-3 and LUMO where a significant charge transfer

occurs from the benzothiazole to the aminobenzene ring, and between HOMO and LUMO +2 where the charge transfer looks significant but in the opposite direction within the molecule.

Although the initial measurements of the TPA spectrum on ThT in aqueous solutions seem promising, a maximum cross section of 60 GM at 540 nm is still low for practical applications in nonlinear bioimaging. However, considering the restrictive environment available for ThT in amyloid plaques one could, in principle, anticipate a larger TPA cross-section in addition to the greater fluorescence quantum yield for attached dye molecules. While the latter has already been proven by Turoverov *et al* [8], TPA has never been attempted under these conditions.

In order to prove this point, we started by measuring the quantum yield of ThT in glycerol/water solutions of varying composition. As shown in Figure SI-1, the quantum yield of ThT increased linearly from 0.006 in water to 0.068 in a 35% glycerol aqueous solution. This result is not totally unanticipated considering previous results [8] and the fact that as the glycerol content is increased, the viscosity of the mixture increases, restricting the degree of freedom of ThT in solution. Next, we measured the TPA spectra of ThT in similar glycerol/water solutions of varying composition. Figure 4 displays the TPA spectra of ThT in these solutions. At first, one cannot identify any obvious dependence of the TPA cross-section with the content of glycerol in the solvent mixture. It is clear that the TPA cross section does, indeed, change with glycerol content, but in a not linear fashion. Interesting to highlight, is the fact that the TPA cross section is the highest in a solution with 8.75% glycerol. Additionally, the peak position corresponding to the second transition also varies with glycerol content. This seems to indicate that TPA also presents a dependence with viscosity.

In 2014, Manners and co-authors presented multicompartment micelles in which the emission of each segment was controlled to produce any color in the visible range [43]. Additionally, Krishnamoorthy *et al* reported that ThT preferred to reside inside anionic micelles resulting in an enhancement in the fluorescence emission by more than an order of magnitude [44]. Furthermore, Singh and co-workers showed that the ThT forms micelles in aqueous solutions due to the combination of polar and hydrophobic regions within the molecule [14]. These findings, in combination with the observed enhancement of the TPA cross section, led us to reconsider our first hypothesis based solely on viscosity and to contemplate the possibility of formation of aggregates which could indeed affect both properties. In order to examine this possibility, we measured particle size using dynamic light scattering (DLS) in the solutions used to obtain the TPA spectra. In fact, in the aqueous solution, particles with diameter of approximately 620 nm were detected, confirming the presence of micellar-type aggregates. An interesting relationship between micelle size and glycerol content is reported in Figure 5. The smallest particle size was measured in the 8.75% glycerol solution, which corresponds to the solution exhibiting the highest TPA cross section. This outcome suggests that the curvature of the micelles formed in this specific solution induce an optimal intermolecular distance for a strong coupling of the molecular transition dipole moments due to the dihedral angle afforded (approximately 37°) within each molecule that is optimal for ICT [10]. Also, important to note is the similar particle size for solutions with 5 and 12 % glycerol content as well as those with 17.5 and 35%. Both, the amplitude and peak positions of the most intense measureable TPA bands are comparable within these sets of glycerol/water solutions (see Figure 6). The average micelle size for the 5% and 12% glycerol aqueous solutions were 355 and 320 nm, respectively. Their peak positions were 580 nm and 590 nm, correspondingly and the cross sections of 63 and 68 GM, respectively. This relationship is not

clear for the 17.5% and 35% set since the two peaks are not clearly distinguishable in the spectrum of the 35% glycerol solution. In an effort to clarify this point we attempted to separate the contributions of the different bands upon deconvolution. We were able to establish that the peak position and amplitude of the bands of the 35% solution are more noticeably comparable to that of the 17.5% band (the deconvoluted spectrum is presented in Figure SI-2). Interesting to highlight is the fact that for both solutions the average particle size was approximately 420 nm, the peak position 560 nm, and the TPA cross-sections were 51 and 66 GM for the 17.5 and 35% solutions, respectively. These results further support the notion that micelle formation is responsible for the change in TPA spectrum and that different glycerol/water ratios cause micelle formation with different sizes, thus affecting the corresponding dihedral angles and intermolecular distances between molecules. Since the ICT and intermolecular coupling of ThT molecules in these solutions (17.5% and 35% glycerol/water solutions) is similar, the TPA peak position and amplitude are also similar.

Finally, the results displayed in Figure 6 revealed an inverse relationship between micelle size and peak amplitude, and a blue shift of the peak associated with the $S_0 \rightarrow S_2$ transition as micelle size become larger. These effects are attributed to differences in the dihedral angle between the benzothiazole and aminobenzene ring when micelles form in different aqueous solution with varied glycerol content. As a result, a change in ICT takes place as predicted theoretically in reference [10]. In a purely aqueous solution, dihedral angles would not be restricted while in more viscous solutions, the larger size of the ThT micelles are more likely to contain molecules with varying dihedral angles. Based on our results, we may consider that ThT molecules form micelles in 8.75% glycerol/water solution immobilized nearest the 37° dihedral angle, the optimum dihedral angle for strong ICT [10].

5. Conclusion

We have shown, for the first time to our knowledge, the full theoretical and experimental TPA spectra of ThT in pure aqueous solution and in glycerol/water solutions of different compositions. We demonstrated that while viscosity enhances the fluorescence quantum yield of ThT in solution, the formation of relatively small micelle-type aggregates could be responsible for the observed enhanced TPA in glycerol/water solutions. This is due to the strong coupling of the molecular transition dipoles of ThT molecules in that arrangement. The combination of the relatively high TPA cross-section ($\delta^{\text{TPA}} = 300 \text{ GM}$) at 540 nm in an 8.75% glycerol/water solution and the already reported fluorescence quantum yield (0.4) of ThT bound to amyloid plaques, [8] may lead to potential application of ThT as a two-photon biomarker for *in vitro*, and perhaps, *in vivo* multiphoton bioimaging of amyloids in brain tissue.

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Figure Captions:

Figure 1. Normalized excitation (purple dash-dotted line), one-photon absorption (blue dashed line), and emission (green dotted line) spectra of ThT in water. Theoretical one-photon absorption (blue solid line) and excitation anisotropy (red solid line) for ThT in ethylene glycol. The theoretical spectrum was calculated using TD-DFT/CAM-B3LYP/6-311++G** in water using PCM for the first 100 electronic excited states of the optimized structure. The Lorentzian convolution was obtained using a linewidth of 0.35 eV (FWHM) to best fit the experimental spectrum. The inset exhibits the molecular structure of ThT.

Figure 2. Theoretical and experimental TPA of ThT. Experimental TPA was obtained in a 1 mM aqueous solution. The theoretical spectrum was computed for the first 20 electronic excited states with TD-DFT/CAM-B3LYP/6-311++G** *in vacuo* using Dalton 2013 and was spectrally shifted (+30 nm). The Lorentzian convolution was obtained using a linewidth of 0.12 eV (FWHM) to best fit the experimental spectrum. Transitions 1 and 4 are indicated as the main contributors to the measurable bands around 760 nm and 540 nm. The inset presents a closer view of the TPA spectrum from 460 to 820 nm.

Figure 3. Molecular orbitals (MOs) of ThT modeled using GAUSSIAN 09 at the CAM-B3LYP/6-311++G** level of theory in water. Arrows represent the first (95% HOMO → LUMO) and fourth (21% HOMO -3 → LUMO and 55% HOMO → LUMO +2) two-photon excited states at their corresponding maximum absorption.

Figure 4. Two-photon absorption spectra of ThT (δ_{TPA}) in varying glycerol content. TPA was measured using the open-aperture z-scan method with an amplified femtosecond laser (Coherent, Inc.).

Figure 5. Average particle size as a function of glycerol content.

Figure 6. TPA peak position (a) and cross section (b) as a function of micelle size.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

TOC