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A series of triphenylamine-based two-photon absorbing materials with AIE property for biological imaging

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A specific series of D-π-A (1A-3A) and D-π-D (1B-3B) structural chromophores with various electron donors and π-conjugated bridges were designed, synthesized, and fully characterized. Their crystal structures were determined by single crystal X-ray diffraction analysis. The one/two-photon absorption properties of the chromophores have been successfully tuned by using different electron donors and π-bridges. Interestingly, it was found that chromophore 3B shows quite weakly fluorescent in pure DMSO, while a significant AIE (aggregation-induced emission) effect is observed in water/DMSO (v/v 90%) mixtures with a sharp increase in fluorescence intensity about 11 times. Furthermore, chromophore 3B shows strong two-photon excited fluorescence (TPEF) and large 2PA action cross section (394 GM). The results of live-cell imaging experiments show that 3B can be effectively used as a bio-imaging probe in two-photon fluorescence microscopy towards HepG2 cells in vitro. The TPEF images of 3B not only exhibit cellular cytosol uptake but also exhibit actin regulatory protein uptake which are different from OPEF images.

Introduction

Organic molecules with large two-photon absorption (2PA) draw great interest in the field of material science due to their various applications including optical limiting,1–3 up-converted lasing,4 3D optical data storage,5 micro-fabrication,6 bio-imaging,7 and photodynamic therapy.8 With the significant potential applications, a large amount of compounds with large 2PA cross sections (σ) and good processabilities have been developed. The molecular design strategies include symmetrical donor-acceptor-donor (D-A-D), donor-π-bridge-acceptor (D-π-A), and donor-π-bridge-donor (D-π-D) structure.9–16 These studies revealed that the intramolecular charge transfer (ICT) from the donor groups to the π-bridge can enhance the 2PA cross sections. Other factors such as strength of the donor and acceptor, the character of the conjugated bridge, the planarity of the chromophores, and the dimensionality of the charge-transfer network17 can also enhance the 2PA cross section. For bio-photonic applications, a good 2PA labeling agent with a high 2PA cross section and large 2PA action cross section (Φσ, where Φ is the fluorescence quantum yield) is highly desired. It is necessary that 2PA chromophores are water soluble or dispersable and remain highly fluorescent in aqueous media. However, highly efficient 2PA fluorescence has suffered the defect of low fluorescence efficiency in aqueous media because most 2PA molecules are hydrophobic and their fluorescence quantum yields are considerably reduced in water, by self-aggregation, which generally leads to fluorescence quenching.18 To overcome this limitation, a special molecular design for a 2PA chromophore is required not only to ensure a large two-photon activity, but more importantly, to overcome fluorescence quenching at high concentration or in biological aqueous environment, which is generally observed for common organic fluorophores. Recently, some novel 2PA chromophores were synthesized both with large two-photon activity and high fluorescence quantum yields in aggregation. Prasad and co-workers had synthesized a chromophore with aggregation-enhanced fluorescence and two-photon absorption in nano-aggregates due to the hindering of molecular internal rotation.19 Tang discovered luminogenic molecules and polymers of AIE and 2PA based on tetraphenylethene.20 Recently, our group have reported several series of compounds based on isophorone21 or triphenylamine22 with good AIE property. Triphenylamine has been widely used in opto- and electro-active materials for the good electron-donating and transporting capabilities, as well as their special propeller starburst molecular structure.23, 24 Recently, 2PA materials with triphenylamine as the electron donor have aroused great interest and become the focus of intensive research in this field.25, 26 Ethyoxyl was also attached to the triphenylamine group to enhance its electron-donating ability and improve solubility of the compounds. Moreover, cyano-substituted compounds show good optical and electrical properties due to their high electron affinities. Molecules including an electron-withdrawing cyano group on the central π-bridge exhibit strong fluorescence and higher 2PA cross section value.27, 28 Some cyano-substituted compounds have been reported to show unique enhanced emission rather than a fluorescence quenching in the solid state,29, 30 which enlightened us to design the novel functional molecules with both AIE and 2PA properties. In this present, we report...
synthesis and optical properties (linear and nonlinear) of the six novel type D-π-A (1A-3A) and D-π-D (1B-3B) chromophores (Scheme 1) based on cyanosubstituted triphenylamine with derivatives. The correlation between molecular structures and spectral properties are discussed by quantum-chemical calculation and X-ray crystallography. Interestingly, a simple modification on π-bridge of chromophore 3B results in good AIE characteristic together with fascinating 2PA properties compared with other related chromophores, suggesting the suitability of 3B for high-contrast bio-imaging application.

**Experimental Section**

**General procedures**

All chemicals were commercially available and used as obtained. The solvents were purified by conventional methods before used. IR spectra were recorded with a Nicolet FT-IR NEXUS 870 spectrometer (KBr discs) in the 4000-400 cm⁻¹ region. H and 13C NMR spectra were recorded on a 400 and 100 MHz NMR instrument using (CD3)2SO or (CD3)2CO as solvent. Chemical shifts were reported in parts per million (ppm) relative to internal TMS (0 ppm) and coupling constants in Hz. Splitting patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m). MALDI-TOF mass spectra were recorded on a time-of-flight (TOF) mass spectrometer using a 337 nm nitrogen laser with alpha-cyano-4-hydroxycinnamic acid as matrix.

**Optical measurements**

The one-photon absorption (OPA) spectra were obtained on a UV-265 spectrophotometer. The one-photon excited fluorescence (OPEF) spectra measurements were performed using a Hitachi F-7000 fluorescence spectrophotometer. OPA and OPEF of all chromophores were measured in five organic solvents of different polarities with the concentration of 1.0 × 10⁻³ mol L⁻¹. The quartz cuvettes used are of 1 cm path length. The fluorescence quantum yields (Φ) were determined by using coumarin 307 as the reference according to the literature method. Quantum yields were corrected as follows:

\[ \Phi = \Phi_{ref} \frac{A \eta^2 D_s}{A \eta^2 D_r} \]

Where the s and r indices designate the sample and reference samples, respectively, A is the absorbance at λexc, η is the average refractive index of the appropriate solution, and D is the integrated area under the corrected emission spectrum.

For time-resolved fluorescence measurements, the fluorescence signals were collimated and focused onto the entrance slit of a monochromator with the output plane equipped with a photomultiplier tube (HORIBA HuoroMax-4P). The decays were analyzed by ‘least-squares’. The quality of the exponential fits was evaluated by the goodness of fit (χ²).

Two-photon absorption (2PA) cross sections (σ) of the samples were obtained by two-photon excited fluorescence (TPEF) method at femtosecond laser pulse and Ti: sapphire system (680-1080 nm, 80 MHz, 140 fs, Chameleon II) as the light source. The sample was dissolved in benzene solvent at a concentration of 1.0 × 10⁻³ mol L⁻¹. The intensities of TPEF spectra of the reference and the sample were determined at their excitation wavelength. Thus, σ of samples was determined by the following Eq,

\[ \sigma = \sigma_{ref} \frac{F_{ref} \cdot \Phi_{ref} \cdot C_{ref} \cdot n_{ref}}{F \cdot \Phi \cdot C \cdot n} \]

Where the ref subscripts stand for the reference molecule (here fluorescein in the aqueous NaOH solution (1 mol L⁻¹) at concentration of 1.0 × 10⁻³ mol L⁻¹ was used as reference). σ is the 2PA cross-section value, c is the concentration of the solution, Φ is the refractive index of the solution, F is the TPEF integral intensities of the solution emitted at the exciting wavelength, and Φ is the fluorescence quantum yield. The σref value of reference was taken from the literature.

**Computational studies**

Density functional theory (DFT) calculations on all the compounds were carried out in vacuo for a better understanding of the charge transfer state. Optimizations were carried out with B3LYP/LANL2DZ without any symmetry restraint, and the time-dependent density functional theory (TD-DFT) B3LYP/LANL2DZ calculations were performed on the optimized structure. All calculations, including optimizations and TD-DFT, were performed with the G03 software. Geometry optimization of the singlet ground state and the TD-DFT calculation of the lowest 25 singlet-singlet excitation energies were calculated with a basis set composed of 6-31 G* for C H N O atoms were downloaded from the EMSL basis set library.

**X-ray structural determinations**

Single crystals of the compounds used in X-ray determination were obtained by slow evaporation of their mother liquors. X-ray diffraction data of 1A-3A and 3B were collected on a Bruker Smart 100 CCD area detector diffractometer. Both of the radiation sources were Mo Ka (λ = 0.71073 Å). Empirical absorption correction was applied to the data. The structures were solved by direct methods and refined by full-matrix least-squares methods on F². All the nonhydrogen atoms were located form the trial structure and then refined anisotropically with SHELXTL using the full matrix least-squares procedure. The hydrogen atom positions were geometrically idealized and generated in idealized positions and fixed displacement parameters. Cambridge Crystallographic Data Centre (CCDC) as supplementary publication numbers CCDC 1A-854148 (see ref. 39), 2A-1006493, 3A-1006514, 3B-1006515.

**Cell image**

HepG2 cells were seeded in 6 well plates at a density of 2 × 10⁴ cells per well and grown for 96 h. For live cell imaging cell cultures were incubated with the complexes (10% PBS: 90% cell media) at concentration of 40 μM and maintained at 37 °C in an atmosphere of 5% CO₂ and 95% air for incubation times ranging.
tetrazolium bromide (MTT) assay was performed. HepG2 cells were trypsinized and plated to ~70% confluence in 96-well plates 24 h before treatment. Prior to the treatment of compounds, the DMEM was removed and replaced with fresh DMEM, and aliquots of the compound stock solutions (500 µM DMSO) were added to obtain final concentrations of 0, 10, 20, 40, 60, and 80 µM. The treated cells were incubated for 24 h at 37 ºC and under 5% CO2. Subsequently, the cells were treated with 5 µg/mL MTT (40 µL/well) and incubated for an additional 4 h (37 ºC, 5% CO2). Then, DMEM was removed, the formazan crystals were dissolved in DMSO (150 µL/well), and the absorbance at 490 nm was recorded. The cell viability (%) was calculated according to the following equation: cell viability (%) = OD490(sample)/OD490(control) × 100, where OD490(sample) represents the optical density of the wells treated with various concentration of the compounds and OD490(control) represents that of the wells treated with DMEM + 10% FCS. Three independent trials were conducted, and the averages and standard deviations are reported. The reported percent cell survival values are relative to untreated control cells.

**Synthesis**

The chromophores 1A and 1B were prepared as literature. N-(4-(4-nitrostyryl)phenyl)-4-ethoxy-N-(4-ethoxyphenyl)benzamine (2A). diethyl(4-nitrophenyl)methyphosphonate (M1) (2.73 g, 10 mmol), t-BuOK (2.24 g, 20 mmol), 4-(bis(4-ethoxyphenyl)amino)benzaldehyde (M2) (3.61 g, 10 mmol), and a modicum of 18-crown-6 were placed into a dry mortar. The mixture milled vigorously for about 20 min, after completion of the reaction (monitored by TLC), the mixture was dissolved in 150 mL CH2Cl2 and washed with DI-water, the organic layer was separated and dried over MgSO4, filtered, and concentrated to afford a yellow solid. Purified by silica gel column chromatography using petroleum ether (b.p. 60–90 ºC)/ethyl acetate (10:1 by volume) to get 0.55 g yellow powder. Yield: 54%. 1H-NMR (400 MHz, (CD3)2SO) δ (ppm): 7.31 (d, J = 8.8 Hz, 2H), 7.21 (d, J = 8.4 Hz, 2H), 6.97 (d, J = 8.8 Hz, 4H), 6.88 (t, 5H), 6.82 (d, J = 13.2 Hz, 1H), 6.73 (d, J = 8.8 Hz, 2H), 6.54 (d, J = 8.4 Hz, 2H), 5.22 (s, 2H), 3.99 (q, J = 6.9 Hz, 4H), 1.32 (t, J = 6.8 Hz, 6H). 13C-NMR (100 MHz, (CD3)2SO) δ (ppm): 154.83, 148.24, 146.92, 140.03, 137.05, 131.43, 129.07, 126.99, 125.05, 124.72, 119.79, 116.27, 64.24, 15.16. MALDI-TOF Calcd for C30H28N2O5, 505.20; Found, 505.28.

N-(4-(4-aminophenyl)phenyl)-4-ethoxy-N-(4-ethoxyphenyl)benzamine hydrochloride (2B). 2A (0.96 g, 2 mmol) was dissolved in ethanol (25 mL) and added into a round-bottom flask equipped with a magnetic stirrer then heated to 80 ºC. 0.06 g Pd/C catalyst was added into the preceding reaction system and a solution of ethanol (25 mL) contained 85% hydrazine hydrate (5 mL) was added dropwise for 0.5 h. After the reaction completed, the solvent was removed under reduced pressure. The mixture was washed with DI-water and extracted with ethyl acetate. The organic phase was dried over MgSO4, filtered, and concentrated to afford a light red oil. Purified by silica gel column chromatography using petroleum ether (b.p. 60–90 ºC)/ethyl acetate (10:1 by volume) to get 0.55 g yellow powder. Yield: 54%. 1H-NMR (400 MHz, (CD3)2SO) δ (ppm): 7.79 (d, J = 8.8 Hz, 4H), 7.73 (d, J = 8.4 Hz, 4H), 6.83 (d, J = 8.8 Hz, 2H), 4.04 (q, J = 6.8 Hz, 4H), 1.37 (t, J = 6.8 Hz, 6H). 13C-NMR (100 MHz, (CD3)2SO) δ (ppm): 157.88, 152.95, 148.03, 146.17, 142.68, 139.56, 132.55, 129.07, 126.99, 125.05, 124.72, 119.79, 116.27, 64.24, 15.16. MALDI-TOF Calcd for C30H28N2O5, 505.28; Found, 505.28.
115.55, 113.93, 106.16, 63.20, 14.64. MALDI-TOF Calcd for C$_{31}$H$_{29}$N$_3$O$_2$, 475.23; Found, 475.30.

Results and discussion

Structural Studies

The synthetic routes of the targeted chromophores (1A-3A and 1B-3B) are presented in Scheme 1. The molecule structures are changed from DE$_\pi$EA to DE$_\pi$ED by restoring NO$_2$ to NH$_2$. These six chromophores and their intermediates were prepared via the Knoevenagel and Witting-Ehorner reactions in high yield. All the chromophores are soluble in common organic solvents, such as benzene, ethyl acetate, ethanol, acetonitrile, and N,N-dimethylformamide (DMF). They were fully characterized by $^1$H and $^{13}$C-NMR, mass spectrometry, and elemental analysis. The structures of 1A-3A and 3B were also characterized by single crystal X-ray diffraction analysis.

The crystal structure descriptions of 1A-3A and 3B

The crystal structures of the chromophores are shown in Fig. 1-3 and Fig. S1-S3. The crystal data collection and refinement parameters are listed in Table S1. The selected bond distances and angles are given in Table S2. The structure of chromophore 1A has been reported previously, here is listed for the comparison.

The ORTEP diagram of chromophore 2A with atom numbering scheme has been depicted in Fig. 1a. As shown in this figure, the central nitrogen (N2) and its three bonded carbon atoms are in distorted pyramidal geometry of the bond angles in the range of 117.2-120.6$^\circ$. The dihedral angle between the two benzene rings P1 and P2 is 4.37$^\circ$, which is much smaller than that of 1A (29.59$^\circ$), revealing that the planarity of 2A is much better than that of 1A by introducing ethyoxyl chain to the triphenylamine moiety. Furthermore, the linkage between the rings is quite conjugated with bond lengths of 1.498(6) Å (C23-C22), 1.280(5) Å (C22-C21) and 1.465(5) Å (C21-C20). The bond-length alternation (BLA, the difference between the average lengths of carbon-carbon single and double bonds) across the $\pi$-bridge of 2A is 0.20 Å, which is much smaller than that of 1A (1.27Å). These structural features suggest that chromophore 2A possesses a higher delocalization within the molecule and more charge-transfer features of the ground state in the solid state compared with those of 1A.

Fig. 1 (a) ORTEP diagram of 2A, Hydrogen atoms are omitted for clarity. (b) the side elevation of 2A

Fig. 2 (a) ORTEP diagram of 3A, Hydrogen atoms are omitted for clarity. (b) the side elevation of 3A

The unit cell of chromophore 3A contains two molecules as shown in Fig. 2a, while the unit cell of chromophore 3B contains one molecule (Fig. 3a). Similar to 2A, around the central nitrogen, three phenyl ring planes are arranged in a pyramidal geometry. The dihedral angles between P1 and P2 (13.58$^\circ$ for 3A and 12.63$^\circ$ for 3B) are greater than that of 2A. Obviously, the cyano group plays an important role in twisting the main structure in comparison with 2A. However, a direct comparison of the linkage between phenyl rings clearly indicates that the conjugation degree of the chromophore 3A and 3B seems to be extended by the cyano group. Noticing that the value of BLA across the $\pi$-bridge (0.11 Å for 3A and 0.12 Å for 3B) are smaller than that of 2A (0.20 Å). It is crucial that the BLA has been established as a useful parameter in relation to NLO response. What’s more, cyano group also influences their stack structure. In the case of chromophore 1A and 2A, the molecules are fixed into centrosymmetric antiparallel dimers by N-O···H hydrogen bond into a one-dimensional chain...
along axis \(a\) (Fig. S1c and Fig. S2). Differently from them, chromophore 3A and 3B are fixed into centrosymmetric antiparallel dimers by weak C-N···H stacking interactions into a one-dimensional chain along axis \(b\) (Fig. S3 and Fig. 3c). Meanwhile, from further inspection of the crystal structure of 3B, the 2D layer structure is formed through, however, multiple C-H···π hydrogen bonds with distance of 2.861 Å between the hydrogen atom in one molecule and the π electron cloud of planar phenyl ring in the other molecule (Fig. 3d). This is a common feature of AIE active molecules.\(^{41,42}\) Beside C-H···π interaction, there also exists C-H···N, N-H···O hydrogen bonds within the aggregate structure. The various inter-molecular interactions help rigidify the conformation and lock the intramolecular rotations of the phenyl rings. As a result, the excited-state energy consumed by intramolecular rotation is greatly reduced, thus enabling the molecule to emit intensely in the solid state.

Hence, the structural characters of 3B, such as distorted molecular plane, steric hindrance of cyano segment in case of π-π stack, and the smaller value of BLA make the chromophore highly promising candidate for AIE and two-photon absorption materials.

Absorption properties The absorption spectra of chromophores are shown in Fig. S4. Two major absorption bands can be observed: the band at about 305 nm is attributed to π-π* transition of the triphenylamine moiety, which remains almost unchanged. While the other band locates at about 380-460 nm with stronger intensity, is assigned as intramolecular charge-transfer (ICT) absorption band. This band shows regular red-shift from chromophore 1A(B) to 3A(B), as shown in Fig. 4. This is attributed to the introduction of ethoxyl and cyano groups which increase the electron-donating strength of the end group and extend the conjugation length of the system. Additionally, the introduction of cyano group also leads to the disappearance of short wavelength band of 3A(B).

Quantum Chemical Calculations TD-DFT computational studies were performed to establish the electronic structure of the chromophores and aid in assignment of optical transitions. The energy and composition of ICT are listed in Table 1 and the spatial plots of selected TD-DFT frontier molecular orbitals are shown in Fig. 5 and Fig. S5. The HOMOs (H) of all chromophores are localized on main conjugated framework between the triphenylamine moiety and aniline (or nitrobenzene), and the LUMOs (L) are mainly located on the styrylbenzene (or nitro-styrylbenzene) units.

As shown in Fig. 5, two transitions of 1B and 2B resemble those observed in the experimental linear absorption spectra. The low-energy band originating from H→L+1 transition is assigned to intramolecular charge transfer (ICT) transition (aniline→triphenylamine moiety), while the high-energy band is assigned in five different solvents (\(c = 1 \times 10^{-5}\) mol L\(^{-1}\)) are collected in Table S3.
to H→L+2 (1B) or H→L+5 (2B) transition (ICT) and mixed with the π-π* transition of the triphenylamine. In the case of 3B, the transition at ca. 396 nm originating from H→L+1 transition is assigned as the ICT transition (triphenylamine→π-bridge).

Additionally, the energy gap between the H and L of 3B (0.62 eV) is much smaller than that of 1B (3.44 eV) and 2B (3.33 eV), which is caused by the introduction of cyano group in the π-bridge, which may make the electron transfer easily in conjugated systems. This may be the condition for 3B bearing stronger two-photon absorbing active. It is obvious that the optical properties of the chromophores strongly depend on the length of the π-bridge and the nature of peripheral substituting donor groups relying on the theoretical calculation. The results of 1A-3A are similar to that of 1B-3B with a satisfying agreement with experimental results.

Table 1 Selected experimental and computed optical data for chromophores 1A-3A and 1B-3B

<table>
<thead>
<tr>
<th>compd</th>
<th>Transitions(S.No.)</th>
<th>OP (Tc)a</th>
<th>E (eV)b</th>
<th>Cal.λmax (nm)c</th>
<th>Obs.λmax (nm)d</th>
<th>f</th>
<th>Character</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>1 102(H-1)→104(L)</td>
<td>3.2869</td>
<td>377.2</td>
<td>426.3</td>
<td>0.7868</td>
<td>π→π*ICT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 103(H)→108(L+4)</td>
<td>4.0296</td>
<td>307.7</td>
<td>303.4</td>
<td>0.2042</td>
<td>π→π*ICT</td>
<td></td>
</tr>
<tr>
<td>2A</td>
<td>1 126(H-1)→128(L)</td>
<td>2.7525</td>
<td>450.4</td>
<td>446.7</td>
<td>0.6268</td>
<td>π→π*ICT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 127(H)→132(L+4)</td>
<td>4.0483</td>
<td>306.3</td>
<td>304.5</td>
<td>0.2063</td>
<td>π→π*ICT</td>
<td></td>
</tr>
<tr>
<td>3A</td>
<td>1 133(H)→135(L+1)</td>
<td>2.6702</td>
<td>464.3</td>
<td>463.6</td>
<td>0.5896</td>
<td>π→π*ICT</td>
<td></td>
</tr>
<tr>
<td>1B</td>
<td>1 96(H)→100(L+3)</td>
<td>4.1425</td>
<td>299.3</td>
<td>302.1</td>
<td>0.0405</td>
<td>π→π*ICT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 96(H)→98(L+1)</td>
<td>3.6687</td>
<td>337.9</td>
<td>352.4</td>
<td>0.0331</td>
<td>π→π*ICT</td>
<td></td>
</tr>
<tr>
<td>2B</td>
<td>1 120(H)→122(L+1)</td>
<td>3.4673</td>
<td>357.6</td>
<td>372.2</td>
<td>0.0294</td>
<td>π→π*ICT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 120(H)→125(L+4)</td>
<td>4.0808</td>
<td>303.8</td>
<td>303.4</td>
<td>0.0428</td>
<td>π→π*ICT</td>
<td></td>
</tr>
<tr>
<td>3B</td>
<td>1 125(H)→127(L+1)</td>
<td>3.1295</td>
<td>396.2</td>
<td>405.5</td>
<td>0.0221</td>
<td>π→π*ICT</td>
<td></td>
</tr>
</tbody>
</table>

a: Orbitals involved in the excitations, b: Transition coefficients, c: Excitation energies (eV), d: Calculate peak position of the longest absorption band, e: Observed peak position of the longest absorption band, f: Oscillator Strengths.

**One-photon exited fluorescence (OPEF)**

Representative one-photon induced emission spectra of 2B in different solvents are shown in Fig. 6, the others are included in the supporting information (Fig. S6). These chromophores (especially 1B-3B) show regular red-shift as the solvent polarity increase, indicating that this charge separation increases in the excited state, resulting in a larger dipole moment than that in the ground state, which explains the sensitivity of the emission spectra of these chromophores to solvent polarity. It is noticed that these chromophores exhibit much remarkable bathochromic shift in ethanol compared to other solvents, indicating that hydrogen bond interactions may occur between the chromophores and ethanol.

As shown in Fig. 7a, chromophores 1A-3A and 1B-3B in benzene all show obvious bathochromic shift from 1A(B) to 3A(B), respectively. They are all in agreement with the order of the extension of the π-system: 4-ethoxy-N-(4-ethoxy-phenyl)-N-phenyl aniline > triphenylamine. Meanwhile, noting that 1B-3B exhibit much stronger fluorescence intensity compared with that of 1A-3A by changing from nitro into amino. Additionally, it can be seen from Fig. 7b that the fluorescence wavelength of 3B is evidently red-shift by 2B and the intensity becomes much weaker, which is due to the introduction of cyano group.

Meanwhile, the time-resolved fluorescence measurements were performed and the detailed data of the fluorescence decay curves are listed in Table S3. The experimental errors are estimated to be ±11% from sample concentrations and instruments. The lifetimes of 1B-3B in the solution were obtained by monitoring at the monomer emission. The decay behaviors of 1B-3B were double-exponential manner obtained by monitoring at the monomer emission. As shown in Table S3, the weighted mean lifetime of 3B (0.97 ns) in ethyl acetate is longer than that of 1B (0.51 ns) and 2B (0.73 ns). This may be attributed to the larger delocalization, leading to a larger molecular stabilization effect for the excited state.

Additionally, the fluorescence lifetime of 3B in ethyl acetate was calculated by multiplying the corresponding quantum yield on natural lifetime, which could be easily calculated from the known Strickler-Berg equation (eqn. 1). A calculated fluorescence lifetime of 1.02 ns was obtained, which is in
excellent agreement with the experimental value (0.97 ns) and further validate the experimental time-resolved technique.

\[
\frac{1}{\tau_0} = 2.88 \times 10^{-9} n^2 \int \frac{I(\tilde{\nu}) d\tilde{\nu}}{\tilde{\nu}^2} \int \frac{g(\tilde{\nu}) d\tilde{\nu}}{\tilde{\nu}}
\]

(1)

in which \( n \) is the refractive index, \( I \) is the fluorescence emission, \( s \) \( \varepsilon \) is the extinction coefficient, and \( \tilde{\nu} \) is the wavenumber. The natural radiative lifetime \( \tau_0 \) and the fluorescence lifetime \( \tau \) are related through the quantum yield \( \Phi \) by

\[
\Phi = \frac{\tau}{\tau_0}
\]

(2)

Except for the tunable fluorescence and increased FL lifetime, the exciton-plasmon interaction and energy transfer of \( 3B \) also results in dramatically optimization on NLO activity, such as 2PA and TPEF.

![Image 1](https://example.com/image1.png)

**Fig. 6** The one-photon excited fluorescence spectra of \( 2B \) in different solvents

![Image 2](https://example.com/image2.png)

**Fig. 7** The one-photon excited fluorescence spectra of \( 1A-3A, 1B-3B \) (a) and \( 2B-3B \) (b) in benzene

### Aggregation-Induced Enhanced Emission

To investigate the AIE attribute of \( 3B \), different amounts of water (a poor solvent for \( 3B \)) were added into the pure DMSO solution by defining the water fractions \( (f_w) \) of 0-99% and then monitored the photoluminescence (PL) change with the excitation wavelength of 503 nm, respectively. Fig. 8a shows that the emission of \( 3B \) is dramatically weakened with a gradual addition of water into the DMSO and the emission band is bathochromically shifted when \( f_w \leq 50\% \). The light emission is invigorated from \( f_w \approx 60\% \) and reaches its maximum value at 90% water content, which is 11-fold higher than that in pure DMSO solution. Meanwhile, the emission maximum is gradually red-shifted to 556 nm when \( f_w \) reaches 99%. It is presumable that molecule of \( 3B \) may cluster together to form random, amorphous aggregates in the mixture with low water fractions (0-50%). When the water fraction becomes higher, the chromophore may agglomerate in an ordered fashion to form crystal-like aggregates. This phenomenon is also probably caused by the change of solvent polarity with addition of water at low water fractions, then the water can interact with solute molecules immediately, which would weaken the emission gradually. As can be seen in the inset of Fig. 8a, after reaching a maximum intensity at 90% water content, the PL intensity decreases with increasing water content. This phenomenon was often observed in some compounds with AIE properties, but the reasons remain unclear. There are two possible explanations for this phenomenon: (1) After the aggregation, only the molecules on the surface of the nano-particles emit light and contribute to the fluorescent intensity upon excitation, leading to a decrease in fluorescent intensity. However, the restriction of intramolecular rotation in the aggregation state can enhance light emission. The net outcome of these antagonistic processes depends on which process plays a predominant role in affecting the fluorescent behavior of the aggregated molecules. (2) When water is added, the solute molecules can aggregate into two kinds of nano-particle suspensions: crystal particles and amorphous particles. The former one results in an enhancement in the PL intensity, while the latter leads to a reduction in intensity.

The absorption spectra of \( 3B \) in the DMSO/water mixtures (c = 1 × 10^{-3} mol L^{-1}) are shown in Fig. 8b. The spectral profiles are significantly changed when \( f_w > 60\% \), respectively. The intensity of absorption peaks of the compound \( 3B \) positioned at 400 nm gradually decrease with the increasing water content, while the new peak located at about 490 nm emerges afterward, indicating the formation of nanoscopic aggregates of \( 3B \). The representative scanning electron microscopy (SEM) image in Fig. 9a shows the formation of nano-aggregates of \( 3B \) by the addition of the 90% fraction of water, the image indicates that the AIE dots are well dispersed and have a needle-like with a mean size of about 200 nm. Fig. 9b, c show fluorescence images of \( 3B \) in the DMSO solution, nanoparticle suspension (90% water content) and powder under UV light.

To quantitatively evaluate the AIE effect of \( 3B \), the quantum efficiency of the solid powder was measured and the value is 7.13%, measured by using an integrating sphere, which are higher than that of \( 3B \) in the solution (Φ < 0.4%) and manifest its AIE feature.
Figs. 8-10 illustrate the TPEF properties and applications of chromophores 2B and 3B. The TPEF spectra reveal a significant red-shift of the OPEF in ethyl acetate due to reabsorption effects. The addition of water to DMSO/water mixtures causes a red-shift of the PL peak intensity, with 52 and 23 nm shifts for 2B and 3B, respectively. Two-photon excited fluorescence (TPEF) spectra at different excitation wavelengths show a two-photon absorption (2PA) cross section that increases with input laser power, indicating a two-photon process. The 2PA cross sections suggest a possible two-photon behavior due to the AIE property. The enhanced 2PA cross section in ethylene bond systems indicates an effective approach for increasing the 2PA cross section of the molecule.

The 2PA cross sections (σ) of chromophores 2B and 3B were measured within the wavelength range of 680-1050 nm in the water/DMSO mixture with 90% water fraction. The TPEF spectra data of 2B and 3B are described in Table S3, which were measured in ethyl acetate (c = 1 × 10^{-3} mol L^{-1}). The TPEF spectra of chromophores 2B and 3B in ethyl acetate pumped by femtosecond laser pulse at 300 mw at different excitation wavelengths are presented in Fig. S7 (left). Fig. S7 (right) shows logarithmic plots of the fluorescence integral versus pumped power with slopes of 1.93 and 1.97, indicating a two-photon excitation mechanism. No linear absorption was observed in the range from 680-1080 nm, the emission excited by 700 nm laser wavelength can be attributed to the TPEF mechanism. The TPEF bands located at 500 nm for 2B and 550 nm for 3B, which are evidently red-shifted by 52 and 23 nm compared with those of OPEF in ethyl acetate due to the reabsorption effect. In addition, a red-shift of 3B compared with 2B is observed, revealing that the nature of π-bridge plays an important role in TPEF. It is reasonable that the cyano group substituted on the ethylenic bond increases the electron density of the π-bridge (shown in DFT calculations), favoring the ICT between the core and the two end groups.

The 2PA cross sections (σ) of chromophores 2B and 3B were measured in the wide wavelength range from 680-1050 nm. Fig. 10 (left) indicates that in the measured region, the 2PA cross-section σ value of chromophore 3B is much enhanced compared with 2B. Furthermore, two-photon action cross-sections (Φσ) are shown in Fig. 10 (right), the maximum value is 527 GM for 2B and 394 GM for 3B, respectively. Noticing that though the OPEF intensity of 3B is much lower than that of 2B, the two-photon action absorption cross section of 3B is only slightly smaller than that of 2B. On account of the extended π-system and enhanced intra-molecular charge transfer (ICT) from the triphenylamine to the cyano group, the result proves that the strategy of increasing the ICT effect is an effective approach for enhancing the 2PA cross section of the molecule. Therefore, the value of the 2PA cross section to the functional material can be modulated by simply modify on π-bridge. Significantly, by comparing 3B with 2B, it not only shows larger σ and Φσ value, but also exhibits higher PL intensity at 90% water content due to AIE property, which spurred us further to explore its potential application in biological imaging.

Two-Photon Microscopy Biological Imaging Application of 3B

Considering the comprehensive merits of the chromophores, we singled out 3B for biological imaging application research. To analyze the potential of 3B as an antitumor agent, its cytotoxicity was measured toward the human hepatoma cancer cells (HepG2). The tested chromophore was dissolved in DMSO and then serially diluted in complete culture medium. It is noteworthy that the live cells were stained with 3B, the cells were active for 24 h during the incubation time. The results clearly indicate that HepG2 cells incubated with concentration of 10 μm of 3B remained 90% viable after 24 h of feeding time, demonstrating the superior biocompatibility of 3B.

To evaluate the internalisation of 3B in living cells, one and two-photon fluorescence microscopy micrographs were obtained from HepG2 incubated with 3B, owing to its relatively lower toxicity toward live cells and two-photon behavior together with AIE property. A bright-field image (Fig. 11a) of each cell was
taken immediately prior to the imaging. To minimize the side effects of organic solvent toward live cells, 3B was dissolved in DMSO at high concentration and diluted with phosphate buffer solution to a working concentration. OPEF images (Fig. 11b) of each live cell were successfully taken and clearly display the cytoplasm structure. The TPEF images of 3B (Fig. 11c) exhibit similar cellular cytosol uptake at the excitation wavelength of 700 nm. It shows the remarkable stability of the fluorescence from cellular cytosol as we switch one photon excitation to two-photon excitation. It’s worth noting that the TPEF images of 3B not only exhibit cellular cytosol uptake but also exhibit actin regulatory protein staining. As most of currently commercial available cellular actin probes are membrane impermeable, highly toxic and require pre-fixation, therefore, 3B might be able to have potential as more safety fluorescence probe for cellular cytosol and actin in two-photon excitation range.\textsuperscript{50} Our preliminary confocal microscopy studies reveal that 3B with function as a luminescent cellular cytosol probe for HepG2 cells being successfully taken up by live cells and clearly emerged from cellular cytoplasm (Fig. 11d, Fig. 11e). We presume that the luminescence from punctuate bright dots outside the nuclei region is because of the aggregation of the complex inside the lysosome. This result demonstrates that the observed lysosome uptake may be due to the presence of the intact 3B.

Fig.11  (a) Bright-field image of HepG2 cells. (b) One-photon image of HepG2 cells incubated with 20 mM 3B after 30 min of incubation, washed by PBS buffer. $\lambda_{ex} = 416$ nm (emission wavelength from 536 to 556 nm). (c) Two-photon image of HepG2 cells incubated with 20 mM 3B after 30 min of incubation, washed by PBS buffer. $\lambda_{ex} = 690$ nm (emission wavelength from 540 nm to 560 nm). (d) The overlay of a-c. (e) The overlay of b-c. Scale bars represent 20 $\mu$m.

**Conclusion**

A series of triphenylamine derivatives with D-$\pi$-A (1A-3A) or D-$\pi$-D (1B-3B) configuration have been synthesised by convenient method. Their photophysical properties can be tuned by simple modification of electron donor and $\pi$-bridge groups, as correlate both experimentally and theoretically. Interestingly, the introduction of cyano-group in chromophore 3B results in lower OPEF intensity but stronger intensity of TPEF than that of corresponding 2B. Significantly, 3B emits intensely upon aggregated formation in water/DMSO mixture, demonstrates a typical AIE feature. Therefore the simple modification on $\pi$-bridge of chromophore 3B resulting excellent 2PA and AIE properties which pave the way for its biophotonic application. Our initial investigation demonstrates that 3B is successfully applied to a two-photon fluorescent probe for labeling the cytoplasm and actin regulatory protein in live cells. Finally, it can be said that the research results open a new way to search for applicable new TPA material.

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**Notes and references**

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Graphical abstract

One- and two-photon fluorescence properties of six chromophores were successfully tuned by different electron donors and π-bridges, the simple structure modify of 3B resulting in a significant AIE effect and large 2PA action cross section, being promising in bioimaging applications.