Synthesis and studies of phenothiazine based AIE fluorogens

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Received 23 August 2018

Phenothiazine based AIE fluorogens linked to phenyl and tetraphenylethylene groups are reported. The N-butylphenothiazine and N-phenylphenothiazine have been substituted at 3,7-positions with phenyl or tetraphenylethylene (TPE) groups. All the target molecules have been characterized by \textsuperscript{1}H and \textsuperscript{13}C NMR, and mass spectrometry. The UV-Vis absorption, fluorescence and aggregation induced emission (AIE) properties of target molecules have been studied. Typically, the phenyl and TPE substituted phenothiazine molecules exhibit strong absorption band (\(\lambda_{\text{abs}} = 331\) to 358 nm) in THF. Also, weak emission is observed in THF (\(\lambda_{\text{em}} = 405\) to 531 nm) for these compounds; due to AIE phenomenon in water/THF mixture (\(f_w \geq 70\%\)) the emission intensity is drastically enhanced upon aggregation. Bioimaging studies in A549 cells reveal cytoplasmic distribution of the 3,7-diphenylphenothiazine derivatives with \(\geq 90\%\) cell viability.

\textbf{Keywords:} Fluorescence, AIE chromophore, phenothiazine, AIE fluorogens

Tetraphenylethylene (TPE) and hexaphenylsilole (HPS) display unusual phenomena, aggregation-induced emission (AIE)\textsuperscript{1} and aggregation-induced enhanced emission (AIEE)\textsuperscript{2}. These organic molecules have several phenyl rings arranged in propeller shape. In dilute solution, due to fast rotation of the phenyl rings such compounds dissipate the absorbed photon energy through radiationless decay, thus exhibiting very weak fluorescence. However, upon aggregation the restriction of intramolecular motions (RIM) opens up the radiative channel, as a result HPS and TPE molecules become highly emissive in aggregated state\textsuperscript{3}. This phenomenon is known as aggregation-induced emission (AIE) effect, that is opposite of aggregation-caused quenching (ACQ)\textsuperscript{4} effect observed in conventional fluorophores. The intrinsic structural property of being highly emissive in aggregated state, facile synthesis, easy functionalization and good chemical stability of TPE derivatives\textsuperscript{3} make them particularly attractive for various applications in biology and materials. Recent reports suggest that, the coupling of ACQ type fluorophore with TPE molecules can generate novel fluorogens having AIE activity; with improved fluorescence characteristics. This approach has proven to be successful to develop novel AIE fluorogens\textsuperscript{6} for biological applications, where typical ACQ molecules have not been very useful. The “AIE fluorogens” have been employed as biological probes to sense proteins\textsuperscript{7} (mitochondrial monoamine oxidases MAO, Human Serum Albumin, Bovine Insulin), ct-DNA, ds-DNA, G-quadruplex\textsuperscript{8}, cysteine and D-glucose in solution\textsuperscript{9}. Likewise, boron-dipyromethenes containing TPE moieties have been synthesized with red emission in aggregated state and apparently large Stokes shifts\textsuperscript{10,11}. Our group is involved in the design and synthesis of donor-acceptor type BODIPYs\textsuperscript{12-15} and “Boranils”\textsuperscript{16} with large Stokes shifts for biological applications.

In this work, we present the design and synthesis of four novel AIE fluorogens: the N-butyl- and N-phenyl-phenothiazine derivatives containing two phenyl or TPE groups on their 3,7-positions. The absorption and emission behaviors in water/THF (tetrahydrofuran) mixture with varying water content (\(f_w \geq 20\%-80\%\)) were investigated. Also, the application of AIE fluorogens in bioimaging of A549 cells was demonstrated.

\textbf{Results and Discussion}

The target compounds 6-9 were prepared from 10H-phenothiazine (1) in several steps, as shown in Scheme I. The other starting materials like: 10-butyl-10H-phenothiazine (2)\textsuperscript{17}, 10-phenyl-10H-phenothiazine (3)\textsuperscript{17}, 3,7-dibromo-10-butyl-10H-phenothiazine (4)\textsuperscript{18}, 3,7-dibromo-10-phenyl-10H-phenothiazine (5)\textsuperscript{18},
(4-(1,2,2-triphenylvinyl)boronic acid\textsuperscript{19} were synthesized as per the literature reports. Compounds 4 and 5 were coupled with phenylboronic acid in the presence of palladium catalyst and base in a seal tube for 24 h; silica gel column chromatography afforded compounds 6 and 7, respectively in 30-35% yields. By using similar synthetic protocol, compounds 4 and 5 were coupled with (4-(1,2,2-triphenylvinyl)-boronic acid; after silica gel chromatography target compounds 8 and 9 were obtained in 32% and 43% yields respectively. All the target compounds 6-9 were characterized by MALDI-MS, \textsuperscript{1}H and \textsuperscript{13}C NMR spectroscopy. The NMR and mass data of all the compounds are given in supplementary information (SI); the presence of molecular ion peak in the mass spectra confirmed the formation of target compounds.

Photophysical Studies

The UV-Vis absorption studies of compounds 6-9 were carried out in tetrahydrofuran (THF) and the data are presented in Table I. The comparison of absorption spectra of compounds 6-9 is shown in Figure 1. In solution compounds 6-9 exhibited a broad absorption band having absorption maxima between 331-348 nm; and the absorption coefficients corresponding to this band were in the range of 78,000-86,000 M\textsuperscript{-1}cm\textsuperscript{-1}. This band can be attributed to the $\pi\rightarrow\pi^*$ transitions within the molecules. As compared to the absorption of the previously reported parent N-butyl-phenothiazine compound ($\lambda_{abs} = 310$ nm\textsuperscript{20} the phenyl and TPE substituted compounds 6-9 exhibited significant red shifts (21-38 nm) in their absorption spectra. The significant bathochromic
shifts in the absorption bands are attributed to the electron rich aromatic substituents attached to the 3, 7-positions of the phenothiazine core in these compounds. The enhanced electronic communication between the phenothiazine core and the 3,7-diphenyl or 3,7-diTPE substituents causes red shifts with higher molar absorptivity of the phenothiazine derivatives.

Fluorescence studies of compounds (6-9) were carried out in THF and the emission quantum yields were calculated. Emission data are presented in Table I and comparative emission spectra are shown in Figure 2. The compounds 6-9 exhibited moderate emission in the range of 400–650 nm, in dilute concentrations. In general, the emission maxima of the major emission band in compounds 6-9 were observed between 430 to 492 nm. The compounds 6 and 8 displayed considerably red shifted emission (16-42 nm) w.r.t. the parent N-butyl-phenothiazine compound ($\lambda_{em} = 450$ nm)$^{20}$. On the other hand 3, 7-diTPE substituted compound 9 showed 20 nm blue

<table>
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<th>Compd</th>
<th>$\lambda_{ah}$ (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\lambda_{em}$ (nm)</th>
<th>Stokes shift (cm$^{-1}$)</th>
<th>$\Phi_f$</th>
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<td>332</td>
<td>78000</td>
<td>492, 531(sh)</td>
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<td>7</td>
<td>334</td>
<td>82000</td>
<td>406(sh), 454</td>
<td>7914</td>
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<tr>
<td>8</td>
<td>331</td>
<td>86000</td>
<td>466</td>
<td>8752</td>
<td>0.191</td>
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<tr>
<td>9</td>
<td>348</td>
<td>81000</td>
<td>405(sh), 430</td>
<td>5818</td>
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</table>

Table I — Absorption and emission data of compound 6-9 in tetrahydrofuran; $\lambda_{ex} = 330$ nm, quinine sulphate was used as reference ($\Phi_f = 0.546$ in 1N H$_2$SO$_4$).

Figure 1 — Comparison of absorption spectra of compounds 6-9 in THF.

Figure 2 — Comparison of emission spectra of compounds 6-9 in THF ($\lambda_{ex} = 330$ nm).
shift in emission as compared to the parent \textit{N}-butylphenothiazine compound. Furthermore, large Stokes shifts of the order of 4677 to 8752 cm\(^{-1}\) were observed for compounds 6-9 in dilute THF solutions; the calculated emission quantum yields were in the range of 0.012 to 0.191. The substantial red shifts in emission maxima and large Stokes shifts observed for these compounds 6-8, can be ascribed to the substituents present on the 3,7-positions of phenothiazine core. Typically, in polar solvent, the molecules turn from the locally excited state (LE) to twisted intramolecular charge transfer state (TICT) by intramolecular rotations. This result into TICT emission peak at longer wavelength when compared to LE emission, hence large Stokes shift\(^{21}\). In compounds 6-9, the TICT emission peak was further enhanced due to AIE effect.

The emission intensity changes of compounds 6-9 due to aggregation in aqueous media were also investigated by fluorescence spectroscopy, all four compounds showed the anticipated AIE phenomenon in solution. The emission spectra of AIE fluorogens 6-9 are shown in Figure 3 and Figure 4. Upon decreasing the solvation by increasing the water content in THF solutions, gradual rise in emission intensity was observed for all the compounds (Figure 5). The AIE fluorogens 6-8 showed moderate fluorescence in pure THF solution; upon increasing the water content of the solutions (\(f_w \geq 20-60\%\)) steady rise in the fluorescence intensity was observed. In the case of compound 9 having two bulky TPE groups, the initial emission was very weak in pure THF solution; and drastic rise in the emission intensity with the increase in water content (\(f_w \geq 20-60\%\)) was observed.

![Figure 3 — Emission spectra of (a) compound 6 and (b) compound 7 in water/THF mixtures with different water volume fractions (\(f_w\)); \(\lambda_{ex} = 330\) nm.](image)

![Figure 4 — Emission spectra of (a) compound 8 and (b) compound 9 in water/THF mixtures with different water volume fractions (\(f_w\)); \(\lambda_{ex} = 330\) nm.](image)
observed. Compounds 6, 7 and 9 showed drastic augmentation in fluorescence in water/THF mixture ($f_w \approx 80\%$); whereas for compound 8 maximum enhancement in emission was observed up to 60% water/THF mixture (Figure 5). Typically, for compounds 6 (2.5 fold) and 7 (5 fold) enhancement in fluorescence was observed in 80% water/THF mixture at 492 and 454 nm, respectively. For compound 8, emission intensity at 466 nm was enhanced by 1.5 fold in 60% water/THF mixture. For compound 9, fluorescence intensity was increased by 36 fold with significant red shift (53 nm) in emission peak as compared to its emission in pure THF. The increase in the emission intensity upon aggregation could be ascribed to the hydrophobic effect and the restricted intramolecular rotations of phenyl rings in these compounds 6-9. The photographs given in Figure 6 evidently show emissive nature of AIE fluorogens upon aggregation in water/THF mixture ($f_w \geq 60-80\%$) under UV illumination. The clustering of hydrophobic amorphous aggregates of 6-9 was further confirmed by the SEM images shown in Figure 7. Furthermore, the aggregated structures are quite stable under ambient conditions as these photographs are taken one day after sample preparation.

**Biological Studies**

The biocompatibility and the usage of the phenothiazine based drugs are well reported in literature. Phenothiazine derivatives have been tested for anti-microbial activities against protozoa and parasites. In order to test potential biological applications of our compounds, cytotoxicity of 6 and 7 was evaluated using the alamar blue assay after exposure of A549 cells to a concentration range of 0–50 $\mu$M for 48 h. The results showed that after 48 h of

![Figure 5](image-url) — Plot of emission intensities of compound 6-9 versus compositions of aqueous mixtures; ($\lambda_{ex} = 330$ nm), concentration used was $10^{-4}$ M.

![Figure 6](image-url) — Digital photographs of compounds 6,7 and 9 in 80% water/THF mixture and compound 8 in 60% water/THF mixture (a) under white light (b) under UV light($\lambda_{ex} = 365$ nm).
treatment of 6, at all concentrations nearly 90% cells were viable, showing no significant cell death. The difference between the cell proliferation rate of all the concentrations and control was evaluated as to be non-significant at 95% confidence intervals.

The cell viability data of 7 had shown similar biocompatibility with around 95% cell viability at all the concentrations. These data clearly indicated excellent biocompatibility of compounds 6 and 7 for biological applications (Figure 8). The compounds 6 and 7 were internalized into the cells and fluorescence imaging showed the internalization of both the compounds in A549 cells (Figure 9). The bright fluorescence and overlaid images displayed intracellular localization of fluorescence signals in A549 cells, indicating a cytoplasmic distribution and good cell membrane permeability of compounds 6 and 7.

Experimental Section

Materials and physical measurements

Unless otherwise mentioned, all the reagents and solvents were purchased from Aldrich, Acros Organics or Merck and used without further purification. Silica gel (60–120 and 230- 400 mesh size) was used for column chromatography. The NMR spectra of compounds were recorded with Bruker Avance III 500 MHz NMR spectrometer. The MALDI-MS data for all the compounds were recorded with Bruker Daltonics Ultraflx Xtreme MALDI-TOF instrument. Absorption spectra were recorded with JASCO V-750 spectrophotometer. Fluorescence emission measurements were recorded

Figure 7 — SEM images of self-organised compounds 6, 7, 9 in 80% water/THF mixture and compound 8 in 60% water/THF mixture.

Figure 9 — Fluorescence images of 6 and 7 (2 µM) in live A549 cells. The upper panel shows 6 and the lower panel shows 7 internalized A549 cells. (a and d) bright field images, (b and e) fluorescence images, (c and f) merged images.

Figure 8 — Cell viability test of 6 and 7 in A549 Cells (concentration range was 20 to 50 µm). The values showing above the bars indicate the P values between the control and each concentration.
with JASCO spectrofluorometer FP-8300. The relative fluorescence quantum yields (Φf) were calculated by using Quinine sulphate as the reference compound (Φf = 0.546 in 1N H2SO4), λex = 330 nm. To study the particle morphology of compounds, samples were examined under Scanning Electron Microscopy (SEM). The aqueous suspension was drop casted on to the silica wafer, dried overnight and mounted on an aluminium stub. The samples were then coated with platinum prior to the analysis and analyzed using SEM (JSM7600F, JEOL).

Alamar blue assay
Lung adenocarcinoma (A549) cells were procured from NCCS, India. Resazurin was purchased from Sigma Aldrich, India. The A549 cells were grown in a RPMI 1640 medium supplemented with 10% FBS (foetal bovine serum) at 37°C and 5% CO2. For the Alamar blue assay, the cells were seeded in 96well plates as 8 x 103 cells per well. After 24 h, the compound mixed culture medium (20 to 50 µM) was added to the wells and incubated for 48 h. The media was replaced with fresh media containing 10% v/v of the alamar blue solution and incubated at 37°C in the dark for 1 h. The media was taken out and the change in the colour had been evaluated using a multi-plate reader by recording the absorbance at 350 nm to evaluate the cell viability. All the experiments were performed in triplicate.

Cell culture
Bioimaging of compounds 6 and 7 were performed in A549 cells. Cultured cells were cropped and seeded on 14 mm coverslips. A fluorescent probe supplemented in culture media (2 mL, final concentration: 2 µM) was added to the cell culture well. The samples were incubated at 37°C for 30 min. Then, culture media were removed and the cells were washed three times with PBS and coverslips were placed on glass slides for imaging. Fluorescence imaging was carried out with a ZEISSAxio Scope A1 Fluorescence Microscope.

Synthesis

**Compound 6**: In a clean dry seal tube compound 4 (0.10 g, 0.24 mmol), phenyl boronic acid (0.07 g, 0.56 mmol) and Pd(PPh3)4 (12.80 mg, 0.01 mmol) were dissolved in dry toluene (1.50 mL) under nitrogen atmosphere. Then ethanol (0.75 mL) and aqueous 2 M potassium carbonate (1.00 mL) was added in the reaction. The mixture was stirred at 85°C for 24 h. The reaction mixture was extracted with dichloromethane and dried over anhyd. Na2SO4. The solvent was evaporated using rotary evaporator under reduced pressure. The desired product was purified by silica gel column chromatography using 3% dichloromethane/n-hexane. Removal of solvent produced fluorescent green solid. Yield 30%. 1H NMR (DMSO-d6, 500 MHz): δ 7.65 (d, J = 7.5 Hz, 4H), 7.52 (d, J = 8.5 Hz, 2H), 7.45 (m, 6H), 7.33 (t, J = 7 Hz, 2H), 7.12 (d, J = 8.5 Hz, 2H), 3.95 (t, J = 7 Hz, 2H), 1.73 (m, 2H). 4.15 (m, 2H), 0.92 (t, J = 7.5 Hz, 3H); 13C NMR (DMSO-d6, 125 MHz): δ 144.25, 139.36, 134.86, 129.36, 127.53, 126.50, 126.34, 125.46, 124.22, 116.45, 46.75, 28.84, 19.91, 14.12; MALDI-MS: C19H22NS+ [M]+: Calcd m/z 407.17. Found m/z 407.14.

**Compound 7**: In a clean dry seal tube compound 5 (0.050 g, 0.14 mmol), phenyl boronic acid (0.04 g, 0.32 mmol) and Pd(PPh3)4 (0.007 g, 0.006 mmol) were dissolved in dry toluene (0.75 mL) under nitrogen atmosphere. Then ethanol (0.40 mL) and aqueous 2 M potassium carbonate (1.00 mL) was injected in the reaction. The mixture was stirred at 85°C for 24 h. The reaction mixture was extracted with dichloromethane and dried over anhyd. Na2SO4. The solvent was evaporated using rotary evaporator under reduced pressure. The desired product was purified by silica gel column chromatography using 5% dichloromethane/n-hexane. Removal of solvent produced as fluorescent green solid. Yield 35%. 1H NMR (CDCl3, 500 MHz): δ 7.61 (d, J = 7.5 Hz, 5H), 7.42 (d, J = 7.5 Hz, 8H), 7.32 (m, 8H); 13C NMR (CDCl3, 500 MHz): δ 141.59, 140.28, 139.05, 138.79, 136.96, 134.42, 130.26, 129.70, 129.10, 128.87, 128.07, 128.00, 127.74, 126.47, 126.24, 125.72, 125.34, 124.51, 124.16, 116.91, 115.29; MALDI-MS: C19H22NS+ [M]+: Calcd m/z 427.13. Found m/z 427.14.

**Compound 8**: In a clean dry seal tube compound 4 (0.10 g, 0.24 mmol), compound B (0.22 g, 0.56 mmol) and Pd(PPh3)4 (0.013 mg, 0.00 mmol) were dissolved in dry toluene (1.50 mL) under inert atmosphere. Then ethanol (0.75 mL) and aqueous 2 M potassium carbonate (1.00 mL) was added in the reaction. The mixture was stirred at 85°C for 24 h. The reaction mixture was extracted with dichloromethane and dried over anhyd. Na2SO4. The solvent was evaporated using rotary evaporator under reduced pressure. The desired product was purified by silica gel column
chromatography using 20% dichloromethane/n-hexane. Removal of solvent produced as fluorescent yellow solid. Yield 32%. \(^1\)H NMR (DMSO-\(d_6\), 500 MHz): 6.743 (m, 8H), 7.15 (m, 18H), 7.02 (m, 18H), 3.91 (t, \(J = 7\) Hz, 2H), 1.68 (m, 2H), 1.42 (m, 2H), 0.89 (t, \(J = 7.5\) Hz, 3H); \(^3\)C NMR (DMSO-\(d_6\), 125 MHz): \(\delta\) 144.09, 143.71, 143.67, 142.36, 141.12, 140.62, 137.07, 133.98, 131.73, 131.20, 131.12, 128.39, 128.32, 128.26, 127.10, 127.04, 126.96, 126.04, 125.64, 125.09, 116.40, 46.73, 28.76, 19.84, 14.10; MALDI-MS: C\(_{68}\)H\(_{53}\)NS\(^+\) [M\(^+\)]: Calcd m/z 915.38. Found m/z 915.33.

**Compound 9:** In a clean seal tube compound 5 (0.05 g, 0.14 mmol), compound B (0.12 g, 0.56 mmol) and Pd(Ph\(_3\))\(_2\)(0.007 g, 0.006 mmol) were dissolved in dry toluene (1.50 mL) under inert atmosphere. Then ethanol (0.75 mL) and aqueous 2 M potassium carbonate (1.00 mL) was injected in the reaction. The mixture was stirred at 85°C for 24 h. The reaction mixture was extracted with dichloromethane and water/THF mixture. The rise in emission intensity was significant upon aggregation in water/THF mixture. All the compounds showed bright green fluorescence. The Stokes shifts (~8600 cm\(^{-1}\)) of phenothiazine based AIE fluorogens make them useful for potential applications as theranostic agents in biology.

**Supplementary Information**
Characterization data related to this article is provided in supplementary information available in the journal web-site.

**Conclusions**
In summary, the synthesis and photophysical studies of phenothiazine based fluorogens is reported. The substitution of phenyl or tetraphenylethylene groups on the 3,7-positions of the phenothiazine, resulted in the significant red shifts in their absorption and emission maxima. All the compounds showed moderate fluorescence in dilute solutions and enhanced emission upon aggregation in water/THF mixture. The rise in emission intensity was significant in all the compounds upon aggregation. The bioimaging studies in live A549 cells revealed cytoplasmic distribution of phenothiazine-diphenyl compounds with bright green fluorescence. The cytotoxicity data indicated non-toxic nature of the compounds with 90-95% cell viability. Also, the large Stokes shifts (~8600 cm\(^{-1}\)) of phenothiazine based AIE fluorogens make them useful for potential applications as theranostic agents in biology.

**Acknowledgements**
Financial support from SERB (EMR/2015/000779) Govt. of India is greatly acknowledged. NM and VP thank IIT Gandhinagar for fellowship and infrastructural support.

**References**