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We report self-assembly and photophysical properties of a new pyridothiazole based aggregation-induced-emission enhancement (AIEE) luminogen 4-(5-methoxy-thiazolo[4,5-b]pyridin-2-yl)benzoic acid (PTC1) and its application for the sensitive detection and monitoring of amyloid fibrillation. The self-assembling properties of the new AIEE probe are extensively studied by AFM and it was noted that as aggregation increases there is enhancement of fluorescence. The fluorescence of PTC1 is quenched upon addition of cupric (Cu^{2+}) ions while the fluorescence is regenerated in the presence of amyloid fibers. AFM studies reveal that PTC1 self associate/aggregate to hairy micelle structures which gets disrupted on the addition of Cu^{2+} and again reassembles in the presence of amyloid fibers. Hence, the fluorescence quenching and regeneration may be attributed to disaggregation and AIE respectively. Further, a comparative analysis of the performance of PTC1 is done with the conventional ThT which confirms it to be a more sensitive probe for the detection of amyloid both in the presence and absence of Cu^{2+} . Of note, a very simple, facile and cost-effective methodology for the detection of amyloid fibres is presented, wherein fluorescence quenching/enhancement can be visualized under UV without the use of sophisticated instrumentation techniques. To the best of our knowledge and literature survey, this is first report wherein the self-assembling properties of AIEE probe is studied extensively via microscopy and the photophysical properties compared w.r.t to the morphological transformations. The AIEE probe has been designed using an unusual pyridothiazole scaffold unlike the commonly used archetypal AIE scaffolds based on tetraphenylethene (TPE) and hexaphenylsilole (HPS) and hence, the work also has implications in designing new generation AIEE dyes based on novel scaffold reported.

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Synthesis and self-assembly studies of a new AIEE probe and its application in sensing amyloid fibrillation

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Abstract: We report self-assembly and photophysical properties of a new pyridothiazole based aggregation-induced-emission enhancement (AIEE) luminogen 4-(5-methoxy-thiazolo[4,5-b]pyridin-2-yl)benzoic acid (**PTC1**) and its application for the sensitive detection and monitoring of amyloid fibrillation. The self-assembling properties of the new AIEE probe are extensively studied by AFM and it was noted that as aggregation increases there is enhancement of fluorescence. The fluorescence of **PTC1** is quenched upon addition of cupric (Cu^{2+}) ions while the fluorescence is regenerated in the presence of amyloid fibers. AFM studies reveal that **PTC1** self associate/aggregate to hairy micelle structures which gets disrupted on the addition of Cu^{2+} and again reassembles in the presence of amyloid fibers. Hence, the fluorescence quenching and regeneration may be attributed to disaggregation and AIE respectively. Further, a comparative analysis of the performance of **PTC1** is done with the conventional ThT which confirms it to be a more sensitive probe for the detection of amyloid both in the presence and absence of Cu^{2+} . Of note, a very simple, facile and cost-effective methodology for the detection of amyloid fibres is presented, wherein fluorescence quenching/enhancement can be visualized under UV without the use of sophisticated instrumentation techniques. To the best of our knowledge and literature survey, this is first report wherein the self-assembling properties of AIEE probe is studied extensively via microscopy and the photophysical properties compared w.r.t to the morphological transformations. The AIEE probe has been designed using an unusual pyridothiazole scaffold

unlike the commonly used archetypal AIE scaffolds based on tetraphenylethene (TPE) and hexaphenylsilole (HPS) and hence, the work also has implications in designing new generation AIEE dyes based on novel scaffold reported.

Keywords: Amyloid sensing; Aggregation Induced Emission Enhancement (AIEE); Atomic force microscopy; Fluorescence Turn OFF/ON; Molecular docking and simulation

Introduction

A major challenge in the Alzheimer's disease (AD) is its timely diagnosis.¹⁻² Main hallmarks of this disease is the aggregation of protein due to misfolding. Amyloid β ($A\beta$) aggregates have been proposed as the most feasible biomarker for the diagnosis of AD as the neuronal plaques are mainly formed by the aggregation of $A\beta_{42}$ peptide which is known to induce a range of cytotoxicities.^{3,4} One of the major obstacles in the diagnosis and detection of AD is the lack of effective methodologies that could sense amyloid aggregates timely and selectively. The results of amyloid binding assays known so far in literature are not infallible and often produce false positives/negatives.⁵⁻⁷ Thioflavin T is the most commonly used dye for sensing amyloid fiber however its application is also limited by weak fluorescence and sensitivity.^{8,9} These drawbacks of the conventional fluorophores may be rectified by new class of molecules exhibiting aggregation induced emission (AIE) or Aggregation induced emission enhancement (AIEE).¹⁰⁻¹² The main difference between AIE and AIEE probe is AIE probe remain non fluorescent in molecular dissolved state while AIEE probe is fluorescent under dissolved state too and its fluorescence

increases as aggregation increases.¹² AIE/AIEE probes have many advantages over conventional fluorophore since it remains unaffected by quenching which occurs at a higher concentration or prolonged incubation.¹³ In conventional fluorophores, aggregation causes quenching (ACQ) at higher concentrations, however, in AIE/AIEE probes, aggregation leads to remarkable enhancement of fluorescence along with exceptionally good signal-to-noise ratio.¹⁴ The most common and typical examples of AIE probes are tetraphenylethene (TPE)¹⁵ and hexaphenylsilole (HPS)¹⁶, like propeller or rotor molecules AIE arises in these probes by the restricted twisting or rotation caused by aggregation which leads to stabilization of locally excited (LE) state.¹⁷ Several literature reports have demonstrated the excellent application of the use of AIE/AIEE probes in biosensing, for the detection and monitoring of plethora of disease biomarkers and as theranostic tool.¹⁸⁻²⁴ Recent reports also suggest that AIE probes could detect amyloids with high sensitivity as compared to conventional ThT due to their excellent quantum yield and resistance to quenching.²⁵⁻²⁷ Herein, we report the facile synthesis and photophysical properties of the new pyridothiazole construct 4-(5-methoxythiazolo[4,5-b]pyridin-2-yl)benzoic acid (**PTC1**) which exhibits aggregation-induced emission enhancement (AIEE) properties and its application as a sensor for sensitive detection of amyloid fibers. The fluorescence of the new probe is quenched in the presence of Cu²⁺ while a significant recovery in fluorescence (turn-ON) can be visualized on the addition of amyloid fibers. **PTC1** has a tendency to self-associate and aggregation leads to photoluminescence. When Cu²⁺ is added the aggregates are disrupted and hence the fluorescence is quenched while upon addition of amyloid re-aggregation occurs which leads to the

regeneration of fluorescence (Fig.1). Notably, the fluorescence turn-OFF/ON can be visualized with the naked eye under UV chamber without the use of sophisticated instrumentation hence a very simple, rapid and cost-effective methodology for the detection of amyloid is presented.

Figure 1

Experimental Section

Materials and Chemicals: All the starting materials for **PTC1** synthesis were obtained from commercial suppliers and used as received. 2-Amino-6-methoxypyridine and Benzoyl isothiocyanate were purchased from Combi-Blocks, USA. Acetone, THF, Lithium bromide, Lithium hydroxide, ethyl acetate, hexane, N, N dimethyl formamide (DMF), sodium hydroxide, sodium bicarbonate, sodium sulphate was purchased from Sisco Research Laboratories (SRL), India. Sodium hydroxide, Bromine (Br₂), Tert-Butyl nitrite, 4-Bromo phenyl acetate, Cu(I)Br, Palladium(II)Acetate and Xantphos was from Spectrochem, India. Acetic acid and Toluene were from Finar, India while HCl was from Merck. Moisture sensitive reactions were performed under an atmosphere of dry Nitrogen. All the solvents used for reactions were distilled prior to use. The water used for all microscopy and spectroscopy measurement was ultra pure water obtained from Millipore Direct-Q® 5 UV Water Purification System. A β (1-42) fragment, A β (1-40) fragment, diphenylalanine (FF), were purchased from Sigma (USA). Thioflavin T (ThT) was purchased from sigma and further recrystallized prior to use. Column chromatography was carried out on silica gel (200–300 mesh). The R_f was recorded in Analytical TLC Silica Gel 60F₂₅₄ purchased from Merck (Germany). The melting point of various intermediates and **PTC1** was recorded in Visual Melting Range Apparatus (MR-VIS) provided by LABINDIA. ¹H NMR (400 MHz) and ¹³C NMR (100 MHz) spectra were recorded on Avance III 400 NMR spectrometer instrument.

Proton chemical shifts are reported in parts per million. HPLC was done using Waters E2695 machine. HPLC was performed using ammonium bicarbonate buffer (ABC) 27 minutes run time and water: ACN as mobile phase in C-18 Column. LC-MS was obtained on an Waters 2690 LC-MS instrument. The method used was Ammonium bicarbonate Buffer (ABC); 7 minutes run time and water: ACN as mobile phase in C-18 Column. UV-visible spectra were recorded on a LABINDIA UV-Vis Spectrophotometer 3000⁺ with 10 mm quartz cell at 25 °C. Fluorescence spectra were recorded on RF-5301PC Spectrofluorophotometer (Shimadzu) with a 1 mm quartz cell at 25 °C. Fluorescence lifetime was measured by single photon counting method using a commercial setup from Jobin-Yvon. The samples were excited at appropriate wavelength using a nano-LED. Stock solution of PTC1 were prepared by making 20mM PTC1 solution in DMSO. Subsequently, 1mM stock solution was prepared by dissolving 50μL of **PTC1** in 1mL water.

Synthesis of PTC1: The pyridothiazole construct (**PTC1**) was synthesized by using the standard synthetic methodology in 6 steps starting from 2-amino-6-methoxy pyridine (See ESI for more detail). To introduce a thiazole moiety, first, the amino group of 2-amino-6-methoxy pyridine was coupled with benzyl isothiocyanate followed by hydrolysis leading to the formation of a thiourea derivative of 6-methoxy pyridine. Next intramolecular cyclization was carried out in the presence of lithium bromide and bromine by using acetic acid as a solvent to yield a fused thiazole ring. In the next step, deamination was carried out via previously reported methodology which is necessary for further C-C coupling. Further, C-H activation was done using palladium acetate and Xantphos and C-C coupling were done with 4-bromophenyl acetate which upon subsequent hydrolysis yielded **PTC1** (ESI).

Fluorescence spectroscopy of PTC1: The Excitation and Emission spectra of **PTC1** were recorded at 10 μ M concentration in deionized water. The emission spectra of **PTC1** was recorded by giving excitation wavelength as 340 nm and emission was recorded in range 350-600 nm keeping slit width 3. Similarly, excitation spectra were recorded in range 250-600 nm using emission wavelength of 418 nm.

Fluorescence spectroscopy measurements of the interaction of PTC1 with Cu^{2+} : 10 μ L of **PTC1 (1mM)** was added to 990 μ L DI water and fluorescence of **PTC1** alone was recorded at 10 μ M concentration. **PTC1** was added always fresh before the assay to prevent unwanted aggregation. To assess **PTC1** interaction with Cu^{2+} increasing concentration of $\text{Cu}(\text{NO}_3)_2 \cdot 2.5\text{H}_2\text{O}$ was added from the stock solution (100mM) from 1 μ L-20 μ L (100 μ M – 2 mM). The concentration of **PTC1** was kept fixed at 10 μ M.

Preparation of stock solution of $\text{A}\beta_{42}$ fibrils: A fresh stock solution of concentrated $\text{A}\beta_{42}$ peptide was prepared by dissolving 1 mg of the lyophilized peptide into 440 μ L of anhydrous DMSO. Then, a working solution of $\text{A}\beta_{42}$ was prepared for a fibrillation study by diluting 125 μ L of peptide solution in a 5 mL buffer solution at pH 2.0 by adding HCl. Additionally; the solution had NaCl (137 mM) and KCl (2.7 mM). The peptide solution was then incubated at 37 $^\circ\text{C}$ for 15 days to achieve complete fibrillation.

Fluorescence spectroscopy analysis of PTC1 assay with Phe-Phe (FF) and FF:Tannic acid: 50 μ L of **PTC1** was taken from 1mM stock solution and 20 μ L $\text{Cu}(\text{NO}_3)_2 \cdot 2.5\text{H}_2\text{O}$ (100mM) was added. Subsequently 400 μ L FF (1mM) and 400 μ L FF with Tannic acid (1mM:1mM) were added. The final volume of the solutions was adjusted to 1000 μ L with DI water. The fluorescence spectra were recorded by diluting the sample 5 times.

PTC1 interaction with amyloid fibrils (0.5 μ M) in absence of Cu^{2+} : A stock solution of PTC1 (1 mM) was prepared in deionised water, and 5 μ L of this solution was mixed with 10 μ L of $\text{A}\beta_{42}$ peptide (15 days aged solution) and solution was adjusted to final volume upto 500 μ L by Tris buffer pH 7.4. The fluorescence was measured under 340 nm excitation.

PTC1 interaction with amyloid fibrils (0.5 μ M) in presence of Cu^{2+} : A stock solution of PTC1 (1 mM) was prepared in deionised water, and 5 μ L of this solution was mixed with 10 μ L of $\text{A}\beta_{42}$ peptide (15 day aged solution) and 2.5 μ L $\text{Cu}(\text{NO}_3)_2 \cdot 5\text{H}_2\text{O}$ (100 mM stock) solution was adjusted to final volume of 500 μ L by Tris buffer pH 7.4. The fluorescence was measured under 340 nm excitation.

Sequential regeneration of fluorescence of PTC1- Cu^{2+} complex by amyloid $\text{A}\beta_{42}$: A stock solution of PTC1 (1 mM) was prepared in deionised water, and 5 μ L of this solution was mixed with 10-150 μ L of $\text{A}\beta_{42}$ peptide (15 day aged solution) and 5 μ L $\text{Cu}(\text{NO}_3)_2 \cdot 5\text{H}_2\text{O}$ (100 mM stock) solution was adjusted to final volume of 500 μ L by 20 mM Tris buffer pH 7.4. The fluorescence was measured under 340 nm excitation.

2.8. Fluorescence spectroscopy analysis of PTC1 interaction with heat induced amyloid in BSA in presence of Cu^{2+} : 50 mg/mL BSA was dissolved in DI water and heated at 75°C and 85°C for 10 minutes. The sample of BSA were kept in eppendorf thermomixer at 950 rpm shaking. Subsequently to 980 μ L BSA, 10 μ L of PTC1 (from stock: 1mM) and 5 μ L Cu^{2+} (from 100 mM $\text{Cu}(\text{NO}_3)_2 \cdot 2.5\text{H}_2\text{O}$) was added.

Monitoring of amyloid fibrillation by PTC1 assay in presence of Cu^{2+} : PTC1 binding assay was done by collecting aged $\text{A}\beta$ peptide solution- incubated for different time periods- and

mixing them with **PTC1**, Cu^{2+} and 20 mM Tris buffer pH 7.4. 50 μL of A β peptide/fibril solution (aged for 1, 2, 3, 5, 7, 10, 12 and 15 days) was added with 2.5 μL $\text{Cu}(\text{NO}_3)_2 \cdot 5\text{H}_2\text{O}$ and 10 μL **PTC1**. The volume this solution was adjusted to 500 μL by Tris buffer pH 7.4. The sample was incubated for 5 mins at room temperature and the fluorescence was measured under 340 nm excitation.

ThT binding assays: In the case of the Thioflavin T probe, a stock solution of ThT (400 μM) was prepared in a 20 mM Tris buffer at pH 7.4, and 50 μL of this solution was mixed with 5 μL of A β peptide solution (aged for 15 days) and solution was adjusted to 500 μL by Tris buffer pH 7.4.. After 5 min of incubation, the fluorescence of ThT was measured under 450 nm excitation. For studies in the presence of Cu^{2+} , 2.5 μL $\text{Cu}(\text{NO}_3)_2 \cdot 5\text{H}_2\text{O}$ (100 mM stock) solution was added additionally. For monitoring amyloid fibrillation, 50 μL of A β peptide/fibril solution (aged for 1, 2, 3, 5, 7, 10, 12 and 15 days) was mixed with 50 μL of ThT stock solution in presence or absence of 5 μL $\text{Cu}(\text{NO}_3)_2 \cdot 5\text{H}_2\text{O}$.

Fluorescence life time measurements: Fluorescence lifetime was measured by single photon counting method using a commercial setup from Jobin-Yvon. For lifetime measurement, excitation of samples is 340 nm by Nano-LED and emission is recorded at 420 nm.

Atomic Force Microscopy (AFM): Neat and co-incubated solutions of **PTC1**, **PTC1** with Cu^{2+} and **PTC1** with amyloidogenic peptides were imaged with an atomic force microscope. The samples were placed on freshly cleaved muscovite mica surfaces followed by imaging with an atomic force microscope (INNOVA, ICON analytical equipment, Bruker, operating under the acoustic AC mode (AAC or tapping mode), with the aid of a cantilever (NSC 12(c) from MikroMasch, Silicon Nitride Tip) by NanoDrive™ version 8 software. The force constant was 2.0 N/m, while the resonant frequency was ~ 276 kHz. The images were taken in the air at room

temperature, with the scan speed of 1.5 lines/sec. The data analysis was done using of nanoscope analysis software. The sample-loaded substrates were dried at dust-free space under a 40W lamp for 30 minutes followed by high vacuum drying and subsequently examined under AFM.

Molecular docking studies: The molecular docking was performed using the AutoDockVina²⁸ and the necessary input files were prepared using the AutoDock 4.2.²⁹ For docking the PDB:2BEG was chosen as the receptor consisting of 5 A β ₄₂ peptides from the Protein Data Bank³⁰ The TT molecule was taken from PubChem (ID: 16953). The TT and **PTC1** were converted to sdf format and minimized using mmff94 force field using Steepest Descent method for 1000 steps. The atomic charges of **PTC1** were assigned using antechamber with AM1-BCC method. The rotatable bonds were kept by default rotatable and no change was made. A grid box centered on the protein was defined using the dimension of 40 X 40 X 40 using XYZ coordinates (-7.593, 0.520, -8.532) and 0.375 Å grid step. The grid size and dimension was chosen in such a way that plausible amino acids are covered in the probable site which is proposed in earlier studies.³¹ The same coordinates for A β ₄₂ peptides were used for both ThT and **PTC1**. Vina uses iterated local search global optimizer algorithm for docking calculations. All the parameters for vina run were set as default except the exhaustiveness for the search was set to 8.

Molecular Dynamics Simulations (MDS): To access the binding stability of **PTC1** in comparison to ThT with the A β ₄₂ and for validation of molecular docking, the best docking pose was selected as initial conformation for 5 ns molecular dynamics simulations (MDS) as employed earlier.³² A total of three simulations system were generated: one for apo-protein (A β ₄₂), A β ₄₂ in complex with ThT (A β ₄₂-ThT) as a reference compound and A β ₄₂ in complex with **PTC1**

(A β ₄₂_PTC1). For all the MDS, the structure for A β ₄₂ was taken from the Protein Data Bank (PDB:2BEG).³⁴ MDS was carried out in GROMACS 2018 version³⁵ using GROMOS96-43a1 force-field. For inhibitor topology and parameter generation, PRODRG tool was used.³⁶ All the MDS system were solvated using with Simple Point Charge (SPC) water model in dodecahedron box maintaining a distance of 1 nm from all the directions of protein and periodic boundary. As the net charge of A β ₄₂ is -5, an equal number of Na⁺ counterions were added for neutralization of systems. In order to remove steric clashes and bad contacts, all the MDS systems were subjected to energy minimization using the steepest descent algorithm to generate maximum force below 1000 kJ.mol⁻¹ nm⁻¹ (50,000 steps max.). After energy minimization, the systems were equilibrated with position restraint simulation of 1 ns carried out under NVT (the constant Number of particles, Volume and Temperature) and NPT (the constant Number of particles, Pressure and Temperature) conditions. Briefly, the first equilibration was carried at a constant volume for 100 ps at a constant temperature of 300 K using a modified Berendsen thermostat algorithm. Subsequently, the second equilibration was carried out for 100 ps at a constant pressure of 1 bar maintained by Parrinello-Rahman barostat (Parrinello, 1981). Finally, all the MDS were subjected to 5 ns production dynamics saving the coordinates at every 2 fs time-step with all the other parameters similar as described above. Finally, the trajectories were visualized by VMD³⁷ and Chimera.³⁸ The root mean square deviation (RMSD) and Radius of gyration (Rg) were calculated from final trajectories using rms, gyrate and GRACE (xmgrace) was used for generating and visualization of plots ([http:// plasma-gate.weizmann.ac.il/Grace](http://plasma-gate.weizmann.ac.il/Grace)). The binding

energy of A β ₄₂ with **PTC1** and ThT complex was predicted by free energy calculations. The widely used approach for calculations of free energy binding is Poisson–Boltzmann or generalized Born and surface area continuum solvation (MM-PBSA and MM-GBSA) methods.³⁹ The g_mmpbsa tool with all the default parameters⁴⁰ was used for molecular mechanics potential energy (electrostatic + van der waals interactions) and free energy solvation (polar + non-polar solvation energies) calculations. The binding free energy was calculated for an entire trajectory of 5 ns, with a total of 50 frames with the time frame of 100 ps in order to encompass different conformational states of the complexes.

Results and Discussion

The new pyridothiazole construct (**PTC1**) was synthesized by using standard organic synthesis protocols in 6 steps from 2-amino-6-methoxy pyridine (ESI[†]) followed by the structural characterization. The synthetic procedure for preparing **PTC1** is illustrated in scheme 1. To design **PTC1** the basic skeleton of the molecular rotor ThT was retained, but herein we replaced the benzothiazole moiety by methoxy substituted pyridothiazole group, a well-known metal chelator. Previously, many charge transfer based fluorescence switch systems were synthesized using pyridothiazole moiety by varying its electron releasing and withdrawing group, hence this modification was made.⁴¹ The insertion of electron withdrawing group i.e. –COOH group in

PTC1 instead of electron donating group (N, N-dimethylamino) of ThT was done to ensure different photophysical properties of **PTC1**. It was envisaged that an electron donating methoxy group on pyridothiazole and electron withdrawing group on another side of the rotor will cause restricted rotation and push **PTC1** towards locally excited (LE) state rather than twisted intramolecular charge transfer (TICT) which exist in ThT. Interestingly, it could be deciphered by TLC spots while synthesis, that **PTC1** exhibits good fluorescence under UV chamber.

Scheme 1

The photophysical properties of **PTC1** were assessed by first taking its UV visible absorption spectra (Fig. S1, ESI) which revealed the absorption maximum at around 350 nm. Further, the fluorescence spectra of **PTC1** reveal excitation maximum at 337 nm and emission maximum at around 420 nm (Fig.2A). Interestingly, the fluorescence of **PTC1** can be visualized with the naked eye under UV Chamber and intense blue emission could be assessed in a vial containing **PTC1** while ThT did not reveal any such fluorescence (Fig. S2, ESI). The emission spectra of **PTC1** indeed revealed that the fluorescence of the new probe is exceptionally good with high signal to noise ratio. Since the structure of **PTC1** was analogous to ThT, a relatively low quantum yield fluorescence probe, it was surprising that **PTC1** revealed such exceptionally high

fluorescence (Fig 2A). Hence, we were motivated to study the aggregation properties of **PTC1** probe since it's reported that aggregation of the molecule leads to the restricted intramolecular rotation(RIR) and yield high fluorescence.⁴²⁻⁴⁴ Accordingly, AFM studies were pursued to investigate the self-assembly of **PTC1**. The AFM studies of **PTC1** revealed that it assembles into cylindrical hairy micelles or supermicelles like structures with small fibers protruding from sides of micelle (Fig.2B). Such micelle morphology also indicated that **PTC1** will have a tendency to self-aggregate further and assembled into febrile structures. Prolonged aggregation studies indeed revealed the formation of mature fibers (5 days, Fig.2C) which get converted into twisted fibers like morphology when incubated for 15 days (Fig.2D). Hence, it was evident that the aggregation of **PTC1** might play a crucial role in its exceptional fluorescence. In addition, **PTC1** also revealed other characteristic features of AIEE probes i.e. a continuous increase in emission intensity could be assessed with respect to time and concentration. The fluorescence of the conventional fluorophores, unlike AIEgen, quenches with prolonged incubation periods and concentration due to aggregation. The aggregation of **PTC1** may be mainly attributed to pi-pi stacking interactions along with hydrogen bonding as in other AIE probes.⁴⁵⁻⁴⁷

Figure 2

Further, a solvent dependent study was done to understand the effect of solvent polarity in aggregation of **PTC1**. For these studies **PTC1** aggregation studies were done in a mixture of tetrahydrofuran (THF) and water. While water is polar in nature, THF is a non polar solvent. Hence this binary mixture is ideal for studying self-assembly of amphiphilic molecules and it was expected that the addition of THF will further induce aggregation of **PTC1**.⁴⁴ Interestingly, as the ratio of water increased in THF/water mixture, there was increase in fluorescence. AFM micrograph of 1:9 water:THF revealed micellar structures with fibers protruding outside while AFM micrograph of 9:1 water:THF revealed dense fibrillar tape-like aggregates. From the AFM micrograph and the corresponding fluorescence, it could be surmised that the aggregation of **PTC1** was effected by the solvent polarity and the fluorescence increases with aggregation (Fig. 3). Hence, **PTC1** clearly exhibits aggregation-induced-emission characteristics.

Figure 3

Since **PTC1** also has pyridine moiety which acts as an effective metal chelator, we were also curious to investigate the effect of the interaction of metal ions with **PTC1** and visualize its effect on the **PTC1** fluorescence property. Metal ions are also reported to be very effective fluorescence quenchers.⁴⁸ Since the basic idea of designing **PTC1** was to assess its application in amyloid sensing, we wanted to first assess the interaction of **PTC1** with metals like Cu and Zn as they are known to have a high affinity for amyloids and many metal-assisted amyloid fibrillation are

reported with these metal ions.⁴⁹ As expected, the fluorescence of **PTC1** was quenched by both Cu^{2+} and Zn^{2+} (Fig. S3, ESI). However, quenching was more effective in the case of Cu^{2+} hence, the fluorescence emission spectra were recorded using varying concentrations of Cu^{2+} only (Fig. 4A). AFM micrograph of **PTC1** in the presence of Cu^{2+} revealed that the aggregates/assembly of **PTC1** got disrupted on the addition of Cu^{2+} (Fig. 4B). Prolonged incubation studies of **PTC1** were also done with Cu^{2+} (till 15 days) and notably, the fluorescence signal of the fluorophore **PTC1** remains quenched and also no regeneration of aggregates could be assessed (data not shown). Hence the AFM and fluorescence studies of **PTC1** with Cu^{2+} further implicated aggregation plays an important role, in the fluorescence of **PTC1** and quenching may be attributed to disaggregation caused by Cu^{2+} . The observed quenching of fluorescence intensity after the addition of Cu^{2+} ions may be due to photo-induced electron transfer (PET) process and static quenching mechanism, indicating the formation of a complex between Cu^{2+} and **PTC1**.⁵⁰⁻⁵³

The quantitative effect of fluorescence quenching was also determined by plotting Stern-Volmer plot (Fig. S4, ESI). The obtained values of the Stern–Volmer constant for **PTC1** in presence of Cu^{2+} was found to be $1860.7 \times 10^{-6} \text{ M}^{-1}$ respectively. The plot between used Cu^{2+} ions concentration and τ_0/τ furnished straight line which hints that the nature of quenching in the present case is static, i.e. Cu(II) ions directly formed complex with the **PTC1** molecular structure (Fig.S4B, ESI).⁵¹⁻⁵³

Since there was a structural similarity between **PTC1** and ThT, it was also expected that **PTC1** should have an affinity for binding amyloid fibers. Hence, we were motivated to assess the application of **PTC1** as an amyloid sensor and investigate, if the fluorescence of **PTC1** which was quenched by Cu^{2+} could be regenerated by the addition of $\text{A}\beta_{42}$ fibers. $\text{A}\beta_{42}$ mature fibrils were formed by previously reported methodology (ESI).²⁷ As $\text{A}\beta_{42}$ fibers were added in increasing concentration to the solution of Cu(II) coordinated **PTC1**, a surprising recovery of the quenched fluorescence intensity could be assessed (Fig. 4C). To investigate whether the aggregation properties of **PTC1** are also regenerated on the addition of $\text{A}\beta_{42}$, AFM micrograph was recorded. Interestingly AFM revealed re-assembled **PTC1** and the micelles of **PTC1** could be seen binding to amyloid fibers (Fig. 4D). It may be interpreted that due to the high affinity of **PTC1** to $\text{A}\beta_{42}$ fibers, **PTC1** cling to amyloid fibers followed by the self-association and leading to regeneration of fluorescence. Notably, **PTC1** binding assays were also effective for detection of other amyloid model like Phe-Phe which is used as reductionist model for $\text{A}\beta_{42}$ ²⁰ (Fig. S9a, ESI).

Figure4

It is well known that tannic acid is a very effective amyloid inhibitor and it effectively breaks the amyloid fibrils. The TEM image of amyloid with tannic acid clearly reveals complete break down of amyloid fibrils. Hence we added tannic acid to **PTC1**-amyloid regenerated system.

Addition of tannic acid indeed induced fluorescence quenching suggesting strongly that PTC1 bind to amyloid fibrils and show more enhancement with fibers than monomer. Hence the study clearly revealed utility of PTC1 for monitoring amyloid fibrillation process. We will be studying utility of PTC1 for screening amyloid inhibitors in greater detail in our future work. Next, to assess the selectivity of PTC1 binding assay, for amyloid, we took native bovine serum albumin (BSA) as negative control. Native BSA protein does not have beta sheet however, beta sheets can be thermally induced in BSA if it is heated upto 85°C..³³ Figure 4A reveals the fluorescence spectra of PTC1-Cu (II) complex with native BSA and heat treated BSA. It may be noted that the addition of native BSA did not induced any recovery of PTC1 fluorescence, however addition of heat treated BSA caused partial recovery. Hence, the study implicates that since BSA acquired β -sheet structures on heating, PTC1 was now able to bind to BSA and hence fluorescence *turn-ON* could be assessed. The experiment also show that fluorescence is regenerated only if BSA form fibrils and not with native BSA.

Figure5

Further, comparative analysis of the performance of ThT and **PTC1** for the detection of amyloid was also performed in the presence of amyloid. Interestingly, the fluorescence of **PTC1** was enhanced on the addition of 0.5 micromole of A β_{42} (Fig. 6A). ThT, on the other hand, reported no

fluorescence enhancement at such a low concentration of amyloid. Hence, **PTC1** clearly revealed a more sensitive detection of amyloid fibrillation assisted by metal ions wherein ThT show false negative. The quantum yield of **PTC1** both in presence and absence of amyloid was also exceptionally high as compared to ThT (Fig. 5B). Moreover, **PTC1** binding assays were more sensitive than ThT assay even for the detection of other amyloid models- FF (Phe-Phe, dipeptide)- which is used as reductionist model for $A\beta_{42}$ ⁵⁶ and $A\beta_{40}$ (Fig S7, ESI).

Figure 6

Since $A\beta_{42}$ monomer transforms from oligomer to protofibril stage followed by fibril formation in a time-dependent fashion,⁵⁷ we also performed a time-dependent fluorescence recovery assay with $A\beta_{42}$ incubated at different time intervals. The fluorescence which was quenched in fresh condition by cupric ions was gradually recovered during the course of 15 days, implicating that **PTC1** binding affinity for $A\beta$ increases as it gradually changes from oligomer to fibril stage. A conventional ThT binding assay was also done under similar conditions in presence of Cu^{2+} and addition of amyloid aged at different time intervals. Surprisingly there was no enhancement in fluorescence of ThT in presence of Cu^{2+} . The fluorescence enhancement which was expected to occur on the binding of ThT to amyloid fibers could not be seen in the presence of Cu^{2+} . Hence a false negative result was obtained via ThT binding assay in presence of Cu^{2+} . As compared to ThT, **PTC1** binding assay showed significant recovery of fluorescence and it could be surmised

that **PTC1** is a more effective probe for sensing amyloid as compared to ThT. Even in the absence of Cu^{2+} ions, the fluorescence intensity of ThT binding assay was very less as compared to **PTC1**. The experiment henceforth clearly revealed superior detection properties of **PTC1** binding assay as compared to ThT binding assay and confirmed that **PTC1** is a better alternative probe to ThT for detection of amyloid fibrillation (Fig. 5C). The fluorescence lifetime of **PTC1** was also very high (3.9 ns, Fig. 5D) as compared to that reported for ThT (1ps).⁸⁵ Hence, **PTC1** clearly exhibits superior photophysical properties as compared to ThT and it is potentially a better probe. The photophysical properties of **PTC1** are summarized in (Table 1, ESI).

Additionally, to assess the binding and interaction of **PTC1** with $\text{A}\beta_{42}$, theoretically, we performed docking studies of **PTC1** and ThT with amyloid fibrils obtained from the protein database (PDB code: 2BEG). The results suggested that **PTC1** can only be docked to the flanks of the fibril i.e. particularly to the entry cleft site and not into the core sites. However, our results also suggest that **PTC1** is able to dock more tightly in the entry flanking region compared to ThT as shown in figure 6(A). Like the fact that aromatic amino acids are very crucial to the interaction of ThT to $\text{A}\beta_{42}$, we found similar results that in both the cases -ThT and **PTC1**-, Phe19 plays an important role as assessed by figure 6 (B,C). The docking empirical score for the **PTC1** was found to be -6.6 kcal/mol which is higher than that of ThT (-6.2 kcal/mol).

Figure 6

Further, the Molecular Dynamics Simulations (MDS) were also carried out to study the conformational stability and prediction of binding free energy in complex formed by $A\beta_{42}$ -**PTC1** and $A\beta_{42}$ -ThT. In the present study, we analyzed the RMSD, gyrate as well as the binding free energy of the complexes and compared with apo-protein's MDS. In order to assess the dynamic stability of $A\beta_{42}$, $A\beta_{42}$ -ThT, $A\beta_{42}$ -**PTC1**, RMSD values for C_{α} backbone were calculated for the entire 5 ns simulations. RMSD is a means to measure the structural variation between the C_{α} back-bone from its initial conformation to its final position during the entire simulation trajectory. Smaller RMSD values indicate higher stability of the simulation. The RMSD values are shown in the plot of RMSD (nm) vs. Time (ns) in figure 7A. The average RMSD values with S.D. for $A\beta_{42}$, $A\beta_{42}$ -ThT, $A\beta_{42}$ - **PTC1** were 0.29 (0.040), 0.30 (0.034) and 0.22 (0.024) nm respectively. The RMSD analysis revealed that the $A\beta_{42}$ - **PTC1** was much more stable in comparison to the apo and $A\beta_{42}$ -ThT reference MDS. The radius of gyration (R_g) is a measure of how compact is the protein fold during the course of the simulation. The stable values of R_g indicate a stable fold during the MDS and unfolded state will have varying R_g values over the time. The R_g values are shown in the plot of R_g (nm) vs. Time (ns) in figure 7B. The average R_g values with S.D. for $A\beta_{42}$, $A\beta_{42}$ -ThT, and $A\beta_{42}$ -**PTC1** was 1.37 (± 0.025), 1.39 (± 0.015) and 1.40 (± 0.009) nm respectively. From the R_g graph, it is clearly evident that the apo-protein ($A\beta_{42}$) has varying R_g values as compared to the $A\beta_{42}$ -ThT and $A\beta_{42}$ -**PTC1**. These stable R_g values over the time indicate that there is stable complex formation maintaining the fold and interestingly, for $A\beta_{42}$ -**PTC1** the R_g

values have the least deviation indicating a better complex formation. The binding free energy (ΔG), for both the complexes, was calculated using the MM-PBSA method for entire 5 ns trajectories. The (ΔG) is an estimation of the non-bonded interaction energies and a total of 50 frames at every 100ps were taken for the calculations. The estimated value of (ΔG) calculated for $A\beta_{42}$ -ThT and $A\beta_{42}$ -**PTC1** were -165.982 ± 3.743 and $-125.675 \pm 1.626 \text{ KJ.mol}^{-1}$ respectively. Details of energy contribution from each component are shown in figure 7C. It is a well-known fact that the ThT binds to diverse fibrils irrespective of the primary sequence of them which strongly indicates that the ThT recognizes a common structural feature amongst the fibrils.⁵⁸ Earlier reports have hypothesized that the amyloid interactions by ThT are a result of hydrophobic interactions at the surface of the cross- β architecture of fibrils. It is known that the ThT do not bind uniformly to the β -sheet surface but binds at shallow groove channels formed by aromatic residues (for instance, a Val-Phe cross-strand motif and contacts with the adjacent Leu ladder). The primary reason for ThT binding is because the aromatic residues present a large hydrophobic surface and the capacity to π -stack with the dye.⁵⁹⁻⁶¹ Similar results were obtained in our MDS for both the dyes ThT and **PTC1**. MDS in case of **PTC1** reveals, the molecule was almost in a similar location with hydrophobic interaction with the aromatic residues (Phe) and interaction with Leu and Val (Figure 7D) as identified earlier with ThT.²⁶ The binding free energy estimation for each component (Fig. 7C) also confirmed the increase in Van der Waal interactions in case of **PTC1** compared to ThT. Overall, our MDS results indicated that the **PTC1** have much more stable interaction (though the binding constant of is less than $A\beta_{42}$ -ThT)

with the A β ₄₂ compared to ThT with similar binding free energy estimates. From computational studies it was also evident that, though the binding constant of A β ₄₂-**PTC1** was less than A β ₄₂-ThT, the binding was more stable and the affinity of **PTC1** for amyloid was stronger due to which **PTC1** could also potentially displace ThT (used in excess) and the fluorescence could be recovered with the same intensity even in presence of competing ThT probe (Fig. S8, ESI)

Figure7

Since the simulations suggested that **PTC1** has an affinity for binding to amyloid fiber, we also performed a binding assay of **PTC1** to amyloid to get an experimental proof for **PTC1**-amyloid binding. To quantify the intermolecular interactions between **PTC1** and amyloid, the variation in the fluorescence intensity with fibril concentration is fitted with the following modified Benesi–Hildebrand equation for 1: 1 complexation.⁶²

$$f_i = \frac{f_i^o + f_i^\infty K_b [Amyloid]}{1 + K_b [Amyloid]}$$

Where f_i is the observed fluorescence intensity, f_i^o & f_i^∞ are the fluorescence intensities of free and entirely bound **PTC1** with amyloid, respectively. K_b is the binding constant. f_i^∞ & K_b were estimated by fitting the fluorescence data using the above equation. A good correlation ($R^2 = 0.89$) between the fitted and fluorescence experimental data plainly assist the single mode of binding between **PTC1** and amyloid fibrils. The estimated binding constant from the plot fitting was found to be $6.63 \times 10^5 \text{ M}^{-1}$, indicating good affinity of **PTC1** towards the amyloid fibrils (ESI). The binding constant of **PTC1** was found to be much better than that of pico green ($2.6 \times 10^4 \text{ M}^{-1}$) and comparable to that of ThT (Fig. 8),⁶³ Earlier, it has been reported, that PG is a better probe than ThT since it revealed higher fluorescence enhancement and sensitivity on binding with amyloid. It was further explained that although, PG had weaker binding constant than ThT, only 10% of bound ThT caused fluorescence enhancement while in the case of PG, most of the PG molecules which are bound to amyloid show fluorescence enhancement and hence better sensitivity.⁶⁴ Similar observation can be noted for **PTC1**, Though, **PTC1** may have weaker association constant with amyloid than ThT, the binding of **PTC1** to amyloid is more stable and hence it produced more fluorescence enhancement. owing to its high quantum yield which in turn lead to higher signal to noise ratio as compared to ThT.

Figure8

Conclusions

In summary, we report a facile synthesis of novel pyridothiazole based fluorophore, **PTC1** which conceptually has a similar structure with ThT but exceptionally high quantum yield and fluorescence lifetime. Fluorescence of **PTC1** was attributed to its aggregation propensity which resulted in AIE. Due to AIE the QY and signal to noise ratio of **PTC1** were very high and no quenching of fluorescence occurred over prolonged time. **PTC1** could effectively detect amyloid fibers in presence of metal ions like Cu and Zn wherein ThT gives a false negative result. The application of **PTC1** as an amyloid sensor through fluorescence turn-OFF-ON has been illustrated using different amyloid models. Fluorescence of **PTC1** is quenched in presence of Cu^{2+} (turn-OFF), while the addition of A β aggregates caused recovery of fluorescence (turn-ON). The fluorescence OFF/ON switch can be assessed with the naked eye under UV chamber too. Hence, **PTC1** provides a very economical tool to assess the presence of amyloid fibers in solution without the aid of sophisticated instrumentation techniques. The method can potentially be very useful for the detection of amyloids in vitro and may pave the way for an effective diagnostic tool for early-stage detection of A β aggregates which are the biomarker of Alzheimer's

disease. Future endeavors will include a more rigorous study of **PTC1** as labeling agent and amyloid imaging dye and understanding the mechanism of its aggregation in more detail. The free acid group presents in **PTC1** offer a facile modification site in its structure and many new generation AIE dyes could be designed using this as scaffold. The efforts will also be pursued to tune **PTC1** absorption properties towards visible and near-infrared region for its wider applicability as amyloid sensing dye in vivo.

ASSOCIATED CONTENT

The supporting information of this manuscript is available and contains detailed synthesis of **PTC1**, additional fluorescence with other amyloid models, AFM images, NMR, LCMS and HPLC data.

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Conflict of Interest

The authors declare no competing financial interest.

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Figures and Scheme

Figure 1. Depicts *Top*: the basic skeleton of the designed new pyridothiazole based construct (**PTC1**) and its energy minimized structure; along with the structure of Thioflavin T from which the structure of **PTC1** is derived. *Bottom*: Schematic representation of **PTC1** binding assay for detection of amyloid. **PTC1** shows good fluorescence which is quenched on the addition of Cu^{2+} and regenerated on the addition of amyloid. AFM and fluorescence studies reveal that the

fluorescence enhancement occurs by the aggregation while the quenching occurs by the disaggregation. Representative vial images of **PTC1** (100 μ M) show blue emission under long UV in the chamber (365 nm); Fluorescence is quenched by the addition of Cu^{2+} and regenerated by the addition of amyloid.

Scheme-1: Synthesis of the new molecule **PTC1**. i) benzoyl isothiocyanate, acetone, rt for 2-3 h; ii) aqueous NaOH, THF, rt, 3h; iii) acetic acid Br_2 , LiBr, 60 $^\circ\text{C}$, 3-4h; iv) *tert*-Butyl nitrite, in DMF 70 $^\circ\text{C}$, 3h; v) 4-bromophenyl acetate Cs_2CO_3 , palladium acetate, Cu(I)Br , Xantphos, toluene, 120 $^\circ\text{C}$ 16 h; vi) Lithium hydroxide, THF, rt, 4 h.

Figure 2. (A) Excitation and Emission spectra of **PTC1**; AFM micrograph of **PTC1** 10 μM . (B) **PTC1** (fresh); (C) **PTC1** (5 days); (D) **PTC1** twisted fibers (15 days), the inset shows magnified images of **PTC1** twisted fibers.

Figure 3: (A) Fluorescence spectra of **PTC1** in THF:Water mixture; AFM micrograph of **PTC1** 10 μM in (B & C) 2D and corresponding 3D image of **PTC1** in 90% water in THF; (D) 2D AFM image of **PTC1** in 10% water in THF; (E & F) High resolution 2D and corresponding 3D image of **PTC1** in 10% water in THF.

Figure 4: (A) Quenching of **PTC1** fluorescence by addition of Cu^{2+} metal ions and (B) corresponding AFM micrograph showed the disaggregation of **PTC1** assembly; (C) Recovery of

fluorescence in the presence of Amyloidogenic fibers when added to **PTC1**-Cu(II) complex solution and (D) corresponding AFM micrograph showed binding of **PTC1** with amyloids.

Figure 5: (A) PTC1 assay with FF and FF+Tannic acid (amyloid inhibitor), (inset) shows vials of FF and FF with Tannic acid; (B) SEM image of FF and (C) SEM image with FF+amyloid inhibitor Tannic acid.

Figure 5: (A) Fluorescence spectra of **PTC1** and ThT in presence of 0.5 micromole amyloid; (B) Quantum yield of **PTC1** and ThT in presence and absence of amyloid; (C): A comparative analysis of **PTC1** (square) and ThT (triangle) in presence of Cu $^{2+}$ (1mM) and 5 micromole A β_{42} aged for 1, 2, 3, 5, 7, 10, 12 and 15 days. ThT (circle) binding assay was also preformed without Cu $^{2+}$; (D) Fluorescence lifetime of **PTC1**.

Figure 6: The binding mode of **PTC1** and ThT in the entry site of the five Aβ₄₂ fibrils. (A) The Aβ₄₂ fibril is shown in cartoon mode, the binding site residues in stick mode and **PTC1** (blue) and ThT (brown) in stick mode; (B) 2D diagram for ThT interaction with Aβ₄₂ residues; (C) 2D diagram for **PTC1** interaction with Aβ₄₂ residues.

Figure7. Molecular Dynamics analysis of $A\beta_{42}$, $A\beta_{42}$ -ThT, and $A\beta_{42}$ -PTC1. **(A)** RMSD of the C_{α} backbone atoms. **(B)** Computed R_g of C_{α} backbone. **(C)** Representative contributions of each energy component in the total binding free energy for $A\beta_{42}$ -ThT, and $A\beta_{42}$ -PTC1 complexes calculated for entire trajectory at 100 ps interval. **(D)** MDS snapshot of $A\beta_{42}$ -PTC1 complex simulations indicating the molecular interactions labeled with Phe, Val, and Leu residues.

Figure 8. (a) Fluorescence titration of molecule **PTC1** in aqueous solution in the presence of different concentrations of 15 days aged A β 42 fibrils (0–20 μ M); (b) The variation of the area under fluorescence spectra with the amyloid concentration and the black solid line represents the best fitting by the equation.

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