Thioflavin-T: A Versatile Optical Probe for Chemo and Biosensing

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Sensing of chemical and biological molecules using extrinsic fluorescence probes has gained a lot of attention in contemporary research due to their high sensitivity and selectivity towards a wide array of analytes. In this review article, we have focused on the photophysical properties of Thioflavin-T and their subsequent modulations in presence of various different classes of analytes which enable sensitive detection of different molecules. The versatility of Thioflavin-T, as a fluorescent probe, is well exhibited by its ability to interact with various molecules which, in turn, act as direct or indirect platforms for detection of both biological and chemical molecules. Through this short review, we aim to encourage other researchers to further explore the interesting photophysical properties of Thioflavin-T for sensing other clinically important biological and chemical analytes.

Keywords: Thioflavin-T; Fluorescence Sensor; Ultrafast Molecular Rotor; Bio-Sensor, Turn On/Turn Off Sensors; Thioflavin-T Aggregates; Thioflavin-T Photophysics

Introduction

Devising sensitive and selective fluorescence sensor platforms for precise detection of chemical and biological analytes holds great relevance in bioanalytical and biomedical research industries (Amdursky et al., 2012; Yao et al., 2014). A plethora of fluorophores have been employed for chemo and biosensing applications in recent times. Owing to the sensitive photophysical response of the fluorescent dyes towards their micro-environment, these photophysical parameters of the dye molecules can easily be tweaked to allow sensing of various analytes (Amdursky et al., 2012; Biju, 2014).

One such cationic dye which has been extensively used as a fluorescent probe for sensing a wide variety of analyte is Thioflavin-T. Thioflavin-T (ThT) belongs to the class of ultrafast molecular rotor dye, which has the inherent ability to undergo the non-radiative ultrafast bond twisting process around single C-C bond (Scheme 1) that makes this molecule practically non-emissive in free state and in solvents of low viscosity (Haidekker et al., 2010; Singh et al., 2010a). However, in a highly viscous media or in a restricted micro-environment, the non-radiative ultrafast bond twisting process is suppressed, as a consequence of which, ThT exhibits fluorescence enhancement by several orders of magnitudes (Singh et al., 2010a; Singh et al., 2010b). This characteristic increase in fluorescence intensity of ThT forms the basis of its sensory mechanism for wide array of analytes like amyloid fibrils, DNA (Murudkar et al., 2012), heparin (Mudliar and Singh, 2016a), metal-ions (Ge et al., 2014; Wang et al., 2011) and so on.

In this short review article, we provide a detailed account of photophysical properties of ThT and its application as a fluorescent sensor. Apart from the routine usage of ThT as an amyloid sensor (Chu et al., 2007; Maezawa et al., 2008; Singh et al., 2015a), it also facilitates sensing of G-quadruplex DNA which are commonly implicated in important cellular functions (Faverie et al., 2014). The ThT-G-quadruplex DNA scaffold acts as a flexible platform for detection of both chemical and biological moieties (Ge et al., 2014; Tong et al., 2013). Very recently, ThT has been also reported to sense an important bioanalyte, Heparin, which is the most widely used blood antiocoagulant in clinical applications (Mudliar and Singh, 2016a). Further, we

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have also provided a brief account of the supramolecular interactions between ThT and macrocyclic host molecules which have been further used for efficient and selective sensing of proteins (Pettiwala and Singh, 2018a) and amino acids (Pettiwala and Singh, 2017). Thus, through this short account, we aim to provide the key sensory applications of Thioflavin-T in chemo and bio-sensing field.

**Photophysics of Thioflavin-T**

To understand the mechanism behind the distinctive fluorescence sensitivity of Thioflavin-T (ThT) towards its local microenvironment, the photophysical properties of ThT have been heavily investigated and debated in the literature. Various hypotheses have been put forward to explain the enormous increase in fluorescence intensity of ThT upon association with amyloid fibrils. One hypothesis which has been highly accepted and supported by both *ab initio* quantum chemical calculations and experimental evidences is that ThT belongs to the class of molecular rotor dyes. The organic dyes which have the ability to undergo an intramolecular twisting motion, in its excited state, are commonly described as molecular rotors (Amdursky *et al.*, 2012; Haidekker *et al.*, 2005; Haidekker *et al.*, 2010; Rettig *et al.*, 1994). By virtue of its molecular rotor property, the benzothiazole moiety of ThT undergoes a very fast rotation relative to dimethyl aminobenzene group in bulk water (Scheme 1). This ultrafast bond twisting process activates an efficient non-radiative decay channel for the molecule which results in a very low emission yield (Amdursky *et al.*, 2012; Voropai *et al.*, 2003). On the other hand, in highly viscous solutions, the ultrafast bond twisting process of ThT is significantly impeded which leads to a drastic enhancement in fluorescence intensity of the dye (Amdursky *et al.*, 2012; Singh *et al.*, 2010a).

During the past few years, a remarkable number of spectroscopic investigations, along with high level of quantum chemical calculations have provided proofs to understand the origin of fluorescence enhancement of ThT and its molecular rotor nature. The molecular rotor nature of ThT was first proposed by Voropai *et al.* which was then supported by quantum chemical calculations of ThT dye contributed by Stsiapura and others (Stsiapura *et al.*, 2007; Stsiapura *et al.*, 2008; Stsiapura *et al.*, 2010; Voropai *et al.*, 2003). These calculations led to the proposal that internal rotation around single C-C bond between benzothiazole and dimethyl aminobenzene moiety (Scheme 1) is responsible for non-radiative decay of ThT. Moreover, the formation of charge transfer state, due to the internal rotation between the benzothiazole and dimethyl aminobenzene rings, is favourable in energy. However, the first experimental proof for its molecular rotor nature was provided by ultrafast fluorescence spectroscopic measurements which directly measured the rates for the bond twisting process in water and solvents of varying viscosity (Singh *et al.*, 2010b; Singh *et al.*, 2015a; Srivastava *et al.*, 2010). To further understand the molecular structure and various conformers of ThT, quantum-chemical calculations in ground and excited state of ThT were investigated as a function of dihedral angle of C-C bond (Fig. 1A) (Amdursky *et al.*, 2012; Singh *et al.*, 2010a; Singh *et al.*, 2010b; Stsiapura *et al.*, 2008). These calculations suggested that ThT in ground state $S_0$ has non-planar conformation due to presence of methyl group on nitrogen atom of benzothiazole ring which renders ThT molecule in strictly non-planar conformation. The torsional angle $\varphi$ between benzothiazole and aminobenzene rings was found to be $\sim 37^\circ$ at which potential energy minima was obtained. While, the minimum in potential energy, for ThT, in the excited singlet state ($S_1$), is obtained at $\varphi \sim 90^\circ$ and there is no energy barrier between two states $\varphi \sim 37^\circ$ and $\varphi \sim 90^\circ$. Thus, upon photo-excitation, ThT undergoes ultrafast bond twisting process to move from radiative locally excited (LE) state (quasi minimum state), at dihedral angle $\varphi \sim 37^\circ$, to non-radiative twisted internal charge transfer (TICT) state (global minimum state), at dihedral angle $\varphi \sim 90^\circ$ (Singh *et al.*, 2010a). This transition from LE to TICT state is accompanied by a drop in oscillator strength from initial value of $1.1$ at $\varphi \sim 37^\circ$ to $0.01$ at $\varphi \sim 90^\circ$ (Fig. 1B) (Singh *et al.*, 2010a).
Based on these calculations, a scheme for photophysical processes in the excited state of ThT was proposed. According to this scheme (Fig. 2A), a twisted intramolecular charge transfer state (TICT) is achieved by the ThT molecule, in its excited singlet state, which results in the change of dihedral angle around central single C-C bond from 37° to 90° accompanied by transition from fluorescent LE state to non-fluorescent TICT state. This LE→TICT transition competes with radiative transition from LE state and accounts for significant quenching of ThT in aqueous solution. On the other hand, in highly viscous solvents, the internal rotation between aromatic rings of ThT is blocked and hence the transition from LE→TICT is suppressed, which subsequently leads to high fluorescence yield of ThT (Stsiapura et al., 2007). Further studies, using femtosecond transient-absorption technique, by Stsiapura et al. revealed a modified scheme wherein non-radiative deactivation process proceeds through a conical intersection between TICT(S_1) and S_0 energy levels in concordance with earlier proposed scheme wherein ThT behaves as a molecular rotor (Stsiapura et al., 2010) (Fig. 2B).

In conjunction with quantum chemical calculations, extensive experimental studies were done to understand the effect of solvent viscosity,
temperature, dielectric constant and pressure on photophysical properties of ThT. In 2003, it was first shown that solvent viscosity affects the spectral properties of ThT (Voropai et al., 2003), but only since 2007 a significant progress has been made in this domain. In subsequent studies by Maskevich et al., and Naik et al., it was established that ThT showed viscosity dependent emission with low degree of sensitivity towards solvent polarity (Maskevich et al., 2007; Naik et al., 2009). Moreover, it was shown that the photophysical response of ThT can be modulated by altering the viscosity of solution, such as with acetonitrile-ethylene glycol and glycerol-water solvent mixtures (Stsiapura et al., 2008; Sulatskaya et al., 2012; Sulatskaya et al., 2010). In 2008, Stsiapura et al. demonstrated that decrease in glycerol viscosity induced by heating or varying glycerol-water ratio led to a dramatic reduction in fluorescence yield and decrease of the average decay lifetime of ThT emission (Stsiapura et al., 2008). Huppert et al. studied the non-radiative process of ThT in 1-propanol as a function of temperature, wherein it was found that non-radiative decay rate decreased by three orders of magnitude when temperature was lowered to 88K (Amdursky et al., 2011a). The reduction in non-radiative rate, upon decreasing temperature, was attributed to increase in viscosity by ~2.5 orders of magnitude (Amdursky et al., 2011a). These findings are in concordance with previous works, where it has been described that ThT, in highly viscous solution, exhibits high fluorescence intensity, and hence large reduction in the non-radiative rate processes (Singh et al., 2010b). Similarly, a change in hydrostatic pressure was shown to affect the viscosity and dielectric constant of solution, which in turn, modulates the photophysical properties of ThT (Amdursky et al., 2011b). Photophysical properties of ThT have also been investigated in various confined media like glass matrix (Schirra, 1985), polymer (Raj and Ramaraj, 2001), nano-confined water pool (Singh et al., 2009; Singh et al., 2011b; Singh and Nath, 2012), ionic liquids (Singh et al., 2015b; Singh et al., 2016), micelles (Kumar et al., 2008; Singh and Nath, 2013) and so on. In all these confined media, ThT fluorescence is highly dependent on local microviscosity. Thus, both experimental studies and quantum chemical calculations illustrate that ThT exhibits typical characteristic of molecular rotors described by ultrafast bond twisting process and its photophysics is highly sensitive to the local micro-environment.

**Thioflavin-T: Amyloid Fibril Sensor**

In 1959, ThT was first described as a potent amyloid fibril marker. Since then, ThT has emerged as a gold standard probe for detection of amyloid fibrils (Grenning et al., 2007b; LeVine, 1993; Rodríguez-Rodríguez et al., 2009; Sabate and Saura, 2007). The distinct increment in fluorescence intensity of ThT, on interaction with amyloid fibrils or amyloid like fibrils, makes it the most convenient diagnostic probe for detection of amyloid fibrils. Apart from acetylcholinesterase (Harel et al., 2008) and serum albumins (Sen et al., 2009), ThT does not associate with globular proteins in native state or amorphous aggregates of proteins, thus making it highly specific probe for amyloid fibrils (LeVine, 1993; Vetri et al., 2007). Amyloid fibrils are insoluble, disordered proteinaceous aggregates, rich in β-sheet structure, which are associated with various neurodegenerative disorders like Parkinson’s disease, Alzheimer’s disease and transmissible spongiform encephalopathies etc. (Biancalana and Koide, 2010; Chiti and Dobson, 2006; Gestwicki et al., 2004; Singh et al., 2015a). Thus, sensing of amyloid fibrils and tracing the fibrillation process becomes important from medical point of view. The applicability of ThT is not only limited to simple sensing of amyloid fibrils, but has also been extended to elucidate the mechanism of fibril formation and to study the structure of fibrils.

Although it is well established fact that ThT is an excellent tool for sensing amyloid fibrils, the mechanism of its binding to amyloid fibrils, and the reason behind observed changes in its photophysical properties are still ambiguous. The observed fluorescence enhancement of ThT on interaction with amyloid fibrils has been previously attributed to formation of excimers (Groenning et al., 2007a), micelles (Khurana et al., 2005), dimers or aggregates (Groenning et al., 2007b) of ThT in the fibrillar medium. However, these propositions stand weak as the optical microscopy results have demonstrated that ThT interacts with amyloid fibrils in the monomeric form (Kitts and Bout, 2009). More importantly, the spectral properties of ThT aggregation, reported in a number of recent works, has clearly rejected the theory of ThT dimerization or aggregation in the fibrillar medium (Mudliar and Singh, 2016b; Singh et al.,
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Thus, the mechanism behind fluorescence enhancement of ThT, upon incorporation into amyloid fibrils, is the restriction of ultrafast bond twisting process around single C-C bond in the excited state of ThT, which is strongly supported by experimental evidences as described earlier in the article.

Another aspect of ThT-amyloid fibril interaction, which is highly investigated, is the binding mode of ThT to amyloid fibrils. ThT specifically recognizes cross-β structures in amyloid fibrils, which form specific arrangement of side chains referred to as cross-strand ladders (Biancalana and Koide, 2010; Groenning et al., 2007a; Noel et al., 2013). These cross-strand ladders contain repetitive side-chain interactions running across β-strands in β-sheets which are parallel to the long axis of the fibril (Biancalana and Koide, 2010; Ivanova et al., 2009; Nelson and Eisenberg, 2006). Krebs et al., using polarized fluorescent microscopy, proposed that ThT binds along surface side-chain grooves which run parallel to long-axis of β-sheet that also consist of cross-strand ladders (Fig. 3). This binding model of ThT to amyloid fibrils is referred to as Channel binding model (Biancalana and Koide, 2010; Krebs et al., 2005). Thus, ThT interacts with a common structural feature of amyloid fibrils irrespective of protein type, be it Alzheimer’s related fibrils (Maezawa et al., 2008) or prion associated amyloid fibrils (Sabate et al., 2008) or fibrils of generic protein like BSA (Mudliar et al., 2016). Importantly, ThT has been routinely employed in in vitro assays and kinetic studies of amyloid fibril formation, which not only gives insights into the molecular mechanism behind amyloid fibrillation process, but also aids in identifying potential inhibitors of protein fibrillation process. Interestingly, very recently, using ultrafast fluorescence spectroscopy, it has been shown that apart from the channel binding mode, a large fraction of fibril bound ThT, also occupies surface binding sites on the fibril (Singh et al., 2015a). Interestingly, these surface bound ThT molecules do not contribute to the characteristic emission enhancement observed for ThT in the fibrillar medium (Singh et al., 2015a).

Although ThT displays diverse applicability in association of amyloid fibrils, but it cannot be employed for in vivo imaging due to its positive charge which makes it hydrophilic and prevents it from entering blood brain barrier (Klunk et al., 2001). Additionally, the unaltered spectral position of ThT in association with amyloid fibrils further limits its applications in imaging studies (Maskevich et al., 2007; Mora et al., 2016; Singh et al., 2010a). Thus, recently researchers have focused on developing neutral derivatives of ThT which displays prominent spectral changes on association with amyloid fibrils for the efficient detection of amyloid fibrils. One such work has been demonstrated, wherein a neutral derivative of ThT was synthesized 2-[2’-Me,4’-(dimethylamino)phenyl] benzothiazole (2Me-DABT) and its interaction with insulin fibrils was studied using time-resolved and steady-state fluorescence techniques (Mora et al., 2016). Upon addition of insulin fibrils to aqueous solution of 2Me-DABT, a significant increase in emission intensity was observed accompanied by a distinct hypsochromic shift in emission spectra. Such a large shift in emission spectra of 2Me-DABT, upon interaction with insulin fibrils, suggests its potential use as ratiometric sensor for amyloid fibrils, and it
also provides a distinctive advantage in imaging applications due to relatively low background signal (Mora et al., 2016). Thus, ThT continues to be the most extensively utilized probe molecule for sensing amyloid fibrils with minor alterations, in order to find better and efficient methods to detect amyloid fibrils.

**Thioflavin-T: Nucleic Acid Sensor**

Nucleic acids (DNA/RNA) are one of the most crucial biomolecules present in living system, which monitor essential cellular process. The inevitable importance of nucleic acids and their non-canonical structures in functioning of living system has led to the development of various strategies to detect them (Cosnier and Mailley, 2008; Wu and Guo, 2014). Considering the significance of mutated DNA in fatal diseases like cancer, sensing of both natural DNA and disease related DNA has been targeted by researchers (Cosnier and Mailley, 2008). Some of the sensitive and efficient platforms developed for detection of nucleic acids are Polymerase Chain Reaction (PCR) (Schaerli et al., 2009; Tassone et al., 2008), DNA microarrays (G. Ventimiglia and S. Petralia, 2013; Sassolas et al., 2008), rolling circle amplification (RCA) (Ji et al., 2012), blotting methods (Glenn and Andreou, 2013; Huang et al., 2010), hybridization chain reaction (HCR) (Guo et al., 2016) and so on. Although these conventional techniques are commonly used, but they involve complex procedures, thus, limiting their utility in wider scenarios. Apart from these conventional techniques, extrinsic fluorescent probes have been highly investigated for their interaction with nucleic acids. ThT turns out to be a very efficient and facile probe molecule, among others, for detection of nucleic acid, owing to its unique fluorescence light up attribute on interacting with nucleic acids.

ThT has been identified as an amyloid fibril sensor for last 50 years, but now, it has recently been in focus for its interaction with nucleic acids/DNA (Ilanchelian and Ramaraj, 2004; Liu et al., 2013). ThT, upon interacting with DNA, displays a dramatic fluorescence enhancement, compared to other DNA binding dyes like ethidium bromide and 4’6-diaminodino-2 phenylindole (Murudkar et al., 2012). The increase in fluorescence intensity of ThT, on association with DNA, is attributed to restriction of its intramolecular torsional relaxation. On binding to DNA, the ultrafast bond twisting process around central C-C bond in ThT, is suppressed and as a result the non-radiative deactivation channel is set off and fluorescence is switched on (Ilanchelian and Ramaraj, 2004; Murudkar et al., 2012). This remarkable increase in fluorescence intensity of ThT on binding to DNA led to tremendous research activity in constructing sensor platforms based on ThT for detection of different types of DNA.

Few studies, involving ThT and DNA, concentrate on the binding mechanism of ThT to DNA. One such study to probe the interaction between ThT and DNA was reported using steady-state and femtosecond time-resolved transient emission techniques (Murudkar et al., 2014). The alteration in spectral properties of ThT, on interaction with DNA, were found to be in concordance with earlier studies (Liu et al., 2013). However, the binding mode of ThT to DNA was found to be electrostatic interaction and intercalation, as indicated by salt, viscosity and resonance energy transfer studies (Murudkar et al., 2014). In another study, a combination of absorption and emission titrations under different conditions (salt content, temperature), fluorescence quenching, T-jump relaxation methods and viscosity experiments were employed to understand the mechanistic aspects of ThT-DNA interaction (Biancardi et al., 2014). The results pointed out that ThT in its monomeric form, undergoes intercalates between DNA base pairs. In addition to intercalation and electrostatic interaction as binding modes of ThT to DNA, it was observed that under ThT excess condition, ThT dimer bind to the DNA grooves as shown by QM/MM MD simulation (Biancardi et al., 2014). In 2017, the same group further explored the binding interaction between ThT and DNA using molecular dynamics and quantum chemical modelling (Biancardi et al., 2017).

ThT-DNA interaction has been exploited in various formats to construct sensors for secondary structural change in DNA or DNA structures implicated in disease conditions. One such study is employing ThT as sensor for studying premelting structural changes in DNA, wherein ThT was found to be sensitive to structural changes induced by temperature, in pre-melting regime, which remains undetected by other spectroscopic techniques like Circular Dichroism spectroscopy (Murudkar et al., 2012). Another interesting work, wherein ThT acts
as a fluorescent probe to understand the interaction between imidazolium based ionic liquids and calf thymus DNA was reported in 2012 (Singh et al., 2012). The addition of ionic liquid to DNA bound ThT leads to a decrease in fluorescence intensity, indicating a displacement of ThT from DNA surface, owing to strong interaction between DNA and ionic liquids. This proposition was further supported by femtosecond fluorescence up conversion studies (Singh et al., 2012). Furthermore, ThT was employed to probe the interaction between DNA and ionic liquids of varying hydrophobicity which revealed that hydrophobicity of ionic liquids, in addition to electrostatic interaction, plays an important role in ionic liquid-DNA interaction (Singh et al., 2012).

In 2013 Liu et al. demonstrated that ThT was able to selectively recognize ds DNA containing cavity structures such as gap site, mismatch site or abasic site (Liu et al., 2013). These are commonly implicated in mutagenic DNA and carcinogenic lesions. Selective recognition of these cavity structures by ThT was illustrated by increase in fluorescence enhancement as compared to full matched DNA which showed comparatively negligible fluorescence (Fig. 4). This increase in fluorescence of ThT on associating with ds DNA cavity structures originated from restriction of its non-radiative torsional relaxation of single C-C bond (Liu et al., 2013). This ability of ThT to selectively recognize DNA cavity structures presents it as a potential platform for development of efficient and practical DNA sensors.

Natural self-assembled DNA/RNA structures such as G-quadruplexes and i-motif are commonly observed in the promoter regions of various human genes including proto-oncogene (RET) (Guo et al., 2007) and retinoblastoma (Rb) gene (Xu and Sugiyama, 2006; Zhou et al., 2010). Rapid and convenient sensors for detection of these natural self-assembled DNA/RNA is highly important from the perspective of medical diagnostics. In 2015, ThT was employed as fluorescence sensor for detection of i-motif in retinoblastoma (Rb) and RET proto-oncogene using CD spectroscopy and fluorescence studies at different pH values. It was found that ThT acted as an efficient sensor to probe the conformational changes in i-motifs by the virtue of its high degree of sensitivity towards changes in its local microenvironment (Lee et al., 2015).

Another important self-assembled noncanonical structure formed by DNA/RNA is G-quadruplex. G-quadruplexes have gained widespread attention due to their potential applications in both in vitro and in vivo studies. These structures are often identified by the presence of signature G-tetrads formed by hydrogen bonding between four guanine residues which are further stabilized by Hoogsteen hydrogen bonding (Gellert et al., 1962; Phan et al., 2007). G-quadruplexes are involved in various biological functions like chromosome stability by blocking cellular polymerases and helicases, gene regulation, serving intermediates in recombination and telomerase activity. Sequences with potential to form G-quadruplex (G4) in human genome account for about 40% of all genes of which human telomeric regions contribute majorly. Apart from human telomeric regions, these sequences are encountered in the promoter regions of oncogenes like C-KIT, H-RAS, K-RAS, C-MYC, and also in untranslated regions of mRNAs, indicating their inevitable significance in proper functioning of biological systems and hence their precise detection is crucial (Faverie et al., 2014). There are various techniques which detect G4 structures like nuclear
magnetic resonance (NMR), melting temperature determination (Mergny and Lacroix, 2009; Mergny et al., 2005), isothermal difference spectra and circular dichroism (CD). However, these techniques are time-consuming and require special expertise. Thus, there is a need for rapid and facile screening method for detection of G4 structure. It has been observed that, ThT becomes fluorescent in presence of G4 structures and hence acts as a sensitive label free probe for detecting G4 structures (Faverie et al., 2014; Gabelica et al., 2013) (Fig. 5).

ThT has been found to selectively induce and stabilize G4 structures (Mohanty et al., 2013). In 2013, the dual role of ThT was reported in inducing G4 structures in 22AG human telomeric DNA, and sensing the same through its fluorescence switch on mechanism attributed to the restriction of its ultrafast molecular rotor property (Mohanty et al., 2013). More importantly, it was noted that ThT, on interaction with other DNA forms (ss or duplex DNA and calf thymus DNA), resulted in relatively low emission enhancement, thus indicating the high specificity of ThT towards quadruplexes compared to other DNA forms. Additionally, a comparative study with thiazole orange (TO) interaction with G4, and other DNA forms, was performed wherein it was observed that TO showed similar fluorescence enhancement for all forms of DNA, indicating the lack of specificity towards G4 structures. Thus, ThT acts as a highly specific fluorescence sensor for G4 structures (Mohanty et al., 2013).

The increase in fluorescence of ThT, on associating with G4, is attributed to intercalation, groove binding and end stacking of ThT molecules which restricts the non-radiative ultrafast bond twisting process (Faverie et al., 2014; Mohanty et al., 2013). To further generalize and extend the application of ThT as fluorescent probe for detection of G4, a detailed screening of G4 forming sequence was carried out. It was distinctly noted that ThT displayed a drastic increase in emission intensity on association with G4 forming sequences, while in the case of control duplexes and single strands, relatively very less enhancement in emission intensity was observed for ThT (Fig. 6). Using a plate reader format hundreds of oligonucleotides with potential to form G4 structure were analysed (Faverie et al., 2014).

Fig. 5: Schematic representation of fluorescence turn-on of ThT in G-quadruplex (Adapted from Gabelica et al. 2013 with permission Copyright (2013) American Chemical Society)

![Fig. 5: Schematic representation of fluorescence turn-on of ThT in G-quadruplex](https://example.com)

Fig. 6: Each point corresponds to fluorescence enhancement in the presence of a different oligonucleotide. The change in fluorescence emission is plotted for DNA and RNA quadruplexes on the left (in blue) and non-G-quadruplex structures on the right. Green dots correspond tootrinucleotides, purple dot to parallel-duplex, brown dot to the triplex and red to other nonquadruplex-forming sequences (Adapted from Faverie et al., 2014 with permission from Oxford University Press)
Very recently, ThT has been demonstrated as an efficient fluorescent sensor for detection of RNA G-quadruplexes using fluorescence, absorption and emission lifetime studies (Fig. 7). ThT showcased a drastic fluorescence enhancement on interacting with RNA G-quadruplexes compared with other RNA forms (Xu et al., 2018b). This differential fluorescence response of ThT generated for G4 and non-G4 RNA structures renders ThT a simple and efficient sensor for RNA G-quadruplexes, in addition to DNA G-quadruplexes. The interesting and selective fluorescence properties showcased by ThT on associating with DNA G4 structures has allowed to devise facile and label-free fluorescence sensor platforms for detection for metal ions and biomolecules, which will be discussed in detail in the next section.

In 2014, Ge et al. reported a Hg$^{2+}$ ions sensor platform based on ThT-DNA G-quadruplex framework. In this work, the interaction between ThT, Hg$^{2+}$ ions and oligonucleotide sequences rich in guanosine and thymine loops was studied. Hg$^{2+}$ ions are known to specifically interact with Thymine (T) rich oligonucleotides, thus, as speculated, Hg$^{2+}$ ions interacted with the oligonucleotide, and blocked the process of G-quadruplex formation, which is observed in absence of Hg$^{2+}$ ions (Ge et al., 2014). This blocking of G-quadruplex formation was reflected by decrease in fluorescence intensity of ThT which is normally high in the absence of Hg$^{2+}$ ions due to formation of G4 structures. This sensor platform enabled sensitive and selective detection of Hg$^{2+}$ ions with limit of detection (LOD) of 5 nM (Ge et al., 2014).

Based on the similar principle of Hg$^{2+}$ ions specific interaction with T-rich oligonucleotide sequences to form thymine-thymine mismatched base pair with Hg$^{2+}$ ions (T-Hg$^{2+}$-T), a turn on fluorescent sensor for detection of Hg$^{2+}$ ions was designed (Ono et al., 2011; Xu et al., 2018a). The sensors based on ThT-G4, reviewed so far, involve fluorescence quenching as signal output, which, in general, suffers from low sensitivity and is not a desirable feature. In this particular work, a turn on fluorescent sensor based on the strategy of proximity-dependent G-quadruplexes was devised, wherein in the absence of Hg$^{2+}$ ions the 2 strands of G-rich DNA failed to form G4 structure resulting in negligible fluorescence intensity of ThT (Xu et al., 2018a). On addition of Hg$^{2+}$ions, a remarkable increase in ThT fluorescence was observed. This increase in fluorescence was attributed to formation of G4 assisted by Hg$^{2+}$ ions which bridged the 2 strands of DNA by forming T-Hg$^{2+}$-T complexes. The LOD was found to be 10 nM, and importantly, this sensor also enabled detection of Hg$^{2+}$ ions in fetal calf serum (Xu et al., 2018a). Also, the ThT-G4 format has been utilised for selective recognition of K$^+$ ions with LOD of 1 mM. The sensor showed minimum specificity towards other tested metal ions (Liu et al., 2014).

In addition to metal ion sensing, ThT-G4 platform has also been explored for detection of various biomolecules. In one such work, a label-free turn on fluorescent sensor for detection of biothiols was constructed on ThT-G4 framework (Tong et al., 2013). The sensing approach is as follows: In the absence of oligonucleotide sequence ARGO100, ThT displays a weak fluorescence, however, upon addition of ARGO100, a remarkable increase in emission intensity is observed due to formation of G4 structure. Upon addition of Hg$^{2+}$ ions, ThT-G4 complex is disrupted due to specific interaction between Hg$^{2+}$ ions and thymine residues present in oligonucleotide. On
introducing biothiols (cysteine and glutathione), Hg$^{2+}$ ions form stronger interaction with biothiols (Hg–S) as a result of which the G-quadruplex structure is restored and fluorescence enhancement is obtained. This sensor platform provides a sensitive mode for detection of cysteine and glutathione in the range of 2x10$^{-8}$-2.5x10$^{-6}$ M and 3x10$^{-8}$-2x10$^{-6}$ M with a LOD values 8.4 nM and 13.9 nM respectively (Tong et al., 2013). Based on a similar concept, detection method for Ag$^{+}$ ions and biothiols was developed using G-quadruplexes as a label free platform (Yang et al., 2016). This approach exploits the coordination of Ag$^{+}$ ions with guanine, which does not allow the G-quadruplexes to form and leads quenched emission from ThT. However, when biothiols are added to this solution, Ag$^{+}$ ions are released owing to stronger coordination of Ag$^{+}$ ions and GSH leading to enhanced emission from ThT (Fig. 8). This method shows a good linear response for GSH in the range of 50-3600 nM with a LOD value of 16 nM.

Liu et al. developed a Forster resonance energy transfer (FRET) based label free fluorescent sensor for detection of thrombin (Liu et al., 2015). The sensor system comprised of a conjugated polymer (CP) as energy donor while ThT as energy acceptor. In the absence of thrombin, ThT induced the transition from aptamer to G4 structure and resulted in fluorescence turn on signal. Due to electrostatic interactions between anionic G4 structure and cationic CP, distance between the donor and the acceptor shortened and hence a high FRET signal was achieved. While in the presence of thrombin, aptamer forms a G4/thrombin complex first, followed by binding of ThT. Due to steric hindrance of thrombin, a long distance between the donor and acceptor exists, and hence a weak FRET signal is observed. Importantly, this sensor system allows rapid and sensitive detection of thrombin not only in aqueous solution but also in serum samples and hence presenting its potential utility in practical applications (Liu et al., 2015).

A label free and enzyme free biosensor for detection of liver cancer related short gene (MXR7) was devised based on target recycling, and ThT induced G4 formation in human serum (Li et al., 2015). The detection system consists of ThT and two hairpin DNA which contain domains showing affinity towards MXR7 and G-rich regions. The interaction between these domains, G-rich sequences and MXR7 results in formation of G-quadruplex which ultimately binds to ThT resulting in fluorescence enhancement. This sensor system facilitates sensitive detection of MXR7 in the range of 0 fM to 350 fM with LOD value of 10 fM (Li et al., 2015).

Another sensor system based on ThT-G4 complexation was devised for detection of important biomolecule streptavidin. The sensor system consisted of target molecule streptavidin, ThT and G-rich biotinylated ssDNA (Bai et al., 2017). It was noted that in the presence of ThT, G-rich biotinylated ssDNA was transformed to form G-quadruplex DNA structure, which on association with ThT, resulted in high emission yield. In the absence of streptavidin, ThT-G-quadruplex DNA complex was digested by exonucleases resulting in release of ThT which exhibits weak fluorescence intensity. However, on addition of streptavidin the digestion of ThT-G-quadruplex DNA by exonuclease was prevented and thus the high emission yield was attained. The range of detection for streptavidin was found to be 0.05-2500 ng/ml with low LOD value of 0.02 ng/ml. Additionally the sensor also gave satisfactory results in 5% human serum (Bai et al., 2017).

Thioflavin-T Interaction with Supramolecular Host: Sensor for Various Chemical and Biomolecules
The modulation in photophysical properties of ThT is observed when placed in confined spaces like amyloid fibrils, water nanopools of reverse micelle, micelles, DNA, etc. One such confined space which have been highly studied for their interaction with ThT, and has been further propagated as a sensory platform for various biomolecules, are supramolecular host. One such class of supramolecular host is cyclodextrin. The interaction of ThT with cyclodextrins has been studied in detail using steady-state and femtosecond resolved emission measurements (Singh et al., 2011a). It has been shown that the inclusion of ThT by β-cyclodextrin cavity leads to a sizeable increase in emission intensity consistent with the molecular rotor picture of ThT (Singh et al., 2011a). While β-CD leads to a sizeable complexation with ThT, α-CD, owing to its smaller cavity size, fails to form any complex with ThT, (Singh et al., 2011a) and contrastingly, γ-CD is able to form both 1:1 complex, (Murudkar et al., 2015) and 1:2 complex, (Singh et al., 2015a) where two ThT molecules are included, displaying an excimer like emission for ThT. Interestingly, sulfobutyl ether derivative of β-CD (SBE_7-β-CD) significantly improves the complexation of ThT, suggesting crucial role of extended hydrophobic cavities in determining the binding strength of ThT with this class of host molecules (Singh et al., 2015c). The 1:1 complex of γ-CD with ThT has been projected as a sensor for hydrocarbon chains, where significant emission enhancement is observed for ThT-γ-CD complex in the presence of hydrocarbon chains of surfactant molecules (Murudkar et al., 2015). This emission enhancement has been ascribed to the formation of ternary complex. ThT-γ-CD-hydrocarbon, which provides a rigid packing of the free space in the cyclodextrin cavity, leading to significant restriction to the torsional relaxation of ThT and thus yielding increase in emission intensity (Murudkar et al., 2015).

A similar host-guest complex of ThT with another supramolecular host, cucurbit[7]uril (CB7), has been employed as sensor for fluoride anion, when suitable amount of Ca^{2+} was added to the stoichiometrically mixed CB7 and ThT (CB7:ThT = 2:1). This ternary supramolecular nanocapsule, Ca^{2+}-CB7-ThT displayed excellent selectivity towards fluoride anions in complete aqueous solution. Importantly, the LOD of the fluoride ion, achieved using this system was 68 times lower than the maximum limit defined by Environmental Protection Agency (EPA) which suggests the prospect of this fluoride sensor in real application (Zhu et al., 2014).

Recently, the interaction of ThT with another supramolecular host, sulphated β-CD (SCD), has been probed (Mudliar and Singh, 2016b). Interestingly, it was observed that this heavily negatively charged host molecule, SCD, induced the formation of emissive H-aggregates of ThT instead of forming a conventional 1:1 inclusion complex with ThT. The formation of emissive H aggregates of ThT, upon interaction with SCD, was attributed to suppression of non-radiative torsional relaxation of ThT, which, in turn, activated the fluorescence channel, and hence ThT-SCD exhibited significant fluorescence enhancement as compared to ThT in bulk water. Most importantly, a distinctive enhanced red-shifted emission band at 545 nm, in contrast to weakly emissive monomeric band at 490 nm band was observed. Appearance of this new emission band at 545 nm, along with other changes in spectral properties of ThT, upon interaction with SCD, strengthened the hypothesis that SCD induced the formation of highly emissive ThT H-aggregates (Mudliar and Singh, 2016b).

In 2017, this ThT-SCD supramolecular dye aggregate template was explored to devise sensors for important biomolecules-arginine and lysine (Pettiwala and Singh, 2017). This sensor system was based on the principle that basic amino acids such as arginine and lysine would interact electrostatically with the anionic SCD which, may result in dissociation of ThT aggregates from SCD. This dissociation of ThT-SCD assembly will, in turn, bring about modulations in monomer-aggregate equilibrium allowing ratiometric detection of arginine and lysine (Fig. 9). This proposition was well supported by absorbance, fluorescence CD spectroscopy and emission life time studies (Pettiwala and Singh, 2017).

In fluorescence measurements, it was evident that gradual addition of arginine to ThT-SCD complex led to a corresponding decrease in fluorescence intensity of the complex. This decrease in fluorescence intensity can be accounted to the disassembly of ThT H-aggregates from SCD surface as a result of electrostatic interaction and hydrogen bonding between arginine and SCD. Since the free ThT is weakly emissive in water, the dissociation of
ThT H-aggregates from the surface of SCD towards monomeric form of ThT resulted in decrease of emission intensity. The shift in the population of ThT-SCD aggregate form (highly emissive) to ThT monomer form (weakly emissive) on addition of arginine, allows ratiometric detection of arginine by monitoring the ratio of emission intensities at 545 nm (aggregate form) and 490 nm (monomeric form), thus yielding a quantitative estimation of arginine (Pettiwala and Singh, 2017).

In absorption studies, addition of arginine to ThT-SCD complex resulted in a bathochromic shift of absorption spectra from 406 nm (ThT aggregate form) to 413 nm (ThT monomer). This red-shift in absorption spectra indicated the dissociation of ThT aggregates from SCD surface, and release of ThT monomer in bulk water. The excited-state lifetime results were in conjunction with fluorescence and absorbance studies wherein it was observed that addition of arginine in ThT-SCD results in faster excited-state lifetime, thus indicating the disruption of ThT-SCD complex and release of ThT monomer in solution (Pettiwala and Singh, 2017).

Similar changes in photophysical properties of ThT-SCD complex was observed on addition of lysine as both arginine and lysine contain positively charged side chains, hence their mode of interaction remains identical with ThT-SCD complex, based on electrostatic interactions and H-bonding. However, arginine shows marginally better response to supramolecular-dye aggregate platform than lysine. Moreover, it was demonstrated that arginine exhibits multi-wavelength distinct recognition pattern which differentiates it from lysine, using the current sensor system. More importantly, this sensor system based on ThT-SCD complex was able to detect arginine and lysine in biologically complex media like serum (Pettiwala and Singh, 2017).

Very recently, the ThT-SCD supramolecular dye aggregate assembly was further utilized to design a sensitive and discriminative platform for detection of two different classes of proteins (Pettiwala and Singh, 2018a). The extreme sensitivity of the photophysical properties of ThT towards its local microenvironment and previous reports of interaction between protein and cyclodextrins prompted us to investigate the ThT-SCD supramolecular assembly as sensory system towards protein detection. The ThT-SCD system displayed a differential fluorescence response on interaction with proteins i.e., a turn-on signal towards non-metalloproteins and turn-off signal towards metalloproteins (Fig. 10). Crucially, the sensor system also enabled discrimination within same class of proteins using Principal component analysis (PCA) of the fluorescence patterns (Pettiwala and Singh, 2018a).

Thus, the precise and tuneable interactions of ThT with supramolecular host have facilitated researchers to design sensor platforms for important biomolecules, which in near future can be used in practical applications.

**Thioflavin-T: Sensor for Heparin**

Heparin is the most widely used anticoagulant, administered to a patient undergoing surgical operations as well as for post-operational maintenance.
However, an overdose of Heparin may lead to certain post-operative complications. This makes it inevitable to monitor the levels of Heparin in post-operative care. Recently, the spectroscopic investigation of the direct interaction between ThT and clinically important biomolecule, Heparin, has been reported which yielded unprecedented changes in the spectral features of ThT (Mudliar and Singh, 2016a). Accrediting to high negative charge density on the surface of Heparin, ThT molecules were made to form aggregate with a head-to-head arrangement i.e., H-aggregate. Generally, H-aggregates are poorly emissive in nature, however, ThT forms highly emissive H-aggregates on Heparin, which enables a turn-on sensing mechanism for Heparin. In fluorescence titration of ThT with Heparin, a characteristic enhanced emission is noted at a large red shifted wavelength of 560 nm in contrast to a very weak emission band at 490 nm. This largely shifted enhanced emission band centred at 560 nm is quite different compared to the usually observed enhanced emission band at 490 nm for ThT when in association with amyloid fibrils or G-quadruplex DNA. The 560 nm band is attributed to ThT aggregates formed on Heparin surface, and this proposition is further supported by evidence from absorption, excitation, CD spectroscopy and time-resolved fluorescence experiments. Both fluorescence and absorption measurement allowed detection of Heparin within range of 0-15 µM with LOD value 18 nM and 26 nM respectively enabling a dual mode of detection for Heparin. Furthermore, this sensor system demonstrated excellent selectivity towards Heparin compared to its structural analogues and contaminants namely Hyaluronic acid and Chondroitin sulphate. Also, this sensor system detected Heparin in biological complex media like fetal bovine serum and human serum-Heparin mixtures, thus exhibiting its practical utility. Further, ThT H-aggregates formed on the surface of Heparin were able to monitor the interaction of heparin with protamine which is its only clinically approved antidote. On addition of protamine to ThT-Heparin solution a decrease in fluorescence at 560 nm was observed (Fig. 11). This decrease in fluorescence intensity was accounted for disassembly of ThT aggregates from Heparin surface, as a result of electrostatic interaction between positively charged protamine and negatively charged Heparin. Thus, ThT, by the virtue of changes in its photophysical properties, was able to sense Heparin and the same platform was used to probe interaction of protamine with Heparin (Mudliar and Singh, 2016a).

Based on similar concept of dissociation of ThT H aggregates from Heparin surface upon interaction with basic amino acids, a sensor for arginine and lysine was devised (Pettiwala and Singh, 2018b). The strong electrostatic interactions and hydrogen bonding between Heparin and basic amino acids, lead to changes in photophysical properties of ThT, which enabled ratiometric detection of arginine and lysine (Fig. 12). Moreover, this sensory ensemble allowed dual mode of detection for basic amino acids that is both fluorimetry and colorimetry, with LOD values as low as 1µM. Thus, this sensing scheme based on ThT provides a facile and selective platform for detection of basic amino acids (Pettiwala and Singh, 2018b).

![Fig. 11: Schematic representation of Heparin Induced ThT aggregates and its Dissociation upon heparin "Protamine Interaction (Adapted from Mudliar and Singh 2016a with permission from American Chemical Society)](image-url)
ThT has also shown to act as sensor for few enzymes (Ma et al., 2016), microRNA (Fan et al., 2017) and aptamers (Wang et al., 2016) based on the common mechanism of modulation of its fluorescence property on interacting with different target analytes. Although these sensors have certain limitations, however they might act as potential sensory systems for real-time applications in biomedical and clinical areas.

At the end, we would like to add some general remarks on the usage of this versatile probe molecule with respect to the practical aspects in sensing applications. Since ThT belongs to the class of molecular rotors, whose photophysical properties are quite sensitive to the temperature, thus, the performance of ThT based sensors are prone to be affected by the increase in temperature of the environment. However, the photostability of ThT is quite good, and moreover, ThT is also stable in a wide pH range of 2 to 9 at room temperature, which assures the reproducibility of ThT based sensors. Additionally, ThT is available commercially at a very economical price which avoids the time-consuming and tedious synthetic efforts, generally required for the synthesis of a sensor probe, and thus this can largely impact the usage of this probe in real-life applications. These useful attributes of ThT makes it a very popular probe for sensing applications which is evidenced by a large number of literature reports on its sensing applications in recent past.

**Conclusion**

In this review article, we attempted to cover all the recent works which outline the application of Thioflavin-T as fluorescent sensor for detection of various target analytes. All of the aforementioned chemo and biosensing application of ThT are based on either turn-on or turn-off fluorescent mechanism of dye, which arises because of alterations in its photophysical properties in response to the changes in the micro-environment of the dye. The initial section of the review article is dedicated to the photophysics of ThT followed by the common sensory application of ThT to detect amyloid fibrils. Next, we have discussed the detailed interactions of ThT with various forms of DNA including G-quadruplex DNA and supramolecular hosts which act as sensor platforms for metal-ions, amino acids, biothiols, Thrombin and so on. Finally, we have outlined the interaction of Thioflavin-T with Heparin, where ThT aggregates, instead of ThT monomers works as sensing ensemble. These sensor platforms based on Thioflavin-T are highly sensitive, simple and cost-effective compared to other conventional methods employed for detection.

Fig. 12: Schematic representation of arginine/lysine induced dissociation of ThT aggregates from Heparin surface (Adapted from Pettiwala and Singh 2018b with permission from Elsevier)
of clinically relevant molecules. Through this short review of Thioflavin-T sensory applications, we hope to stimulate new ideas and constructs for detection of other biologically important molecules.

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