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A Supramolecular ON−OFF−ON Fluorescence Assay for Selective Recognition of GTP

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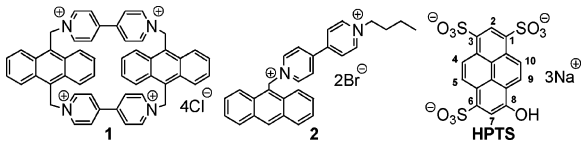
Detection of nucleosides and nucleotides has paramount importance as they form the fundamental units of all the life forms.[1,2] Of all the nucleosides and nucleotides, the recognition of ATP and GTP is vital[3,4] since ATP plays an important role in energy transduction in organisms and controls several metabolic processes,[5] whereas GTP is involved in RNA synthesis, citric acid cycle, and acts as an energy source for protein synthesis.[6] Most known receptors for these nucleotides use complementary hydrogen bonding, but such a recognition in the aqueous medium would be limited due to the interference from hydroxyl groups of the sugar moiety and competitive hydrogen bonding of the solvent.[7,8] Progress in this area would require new strategies for the selective recognition and subsequent signaling of the event under physiological pH conditions.

We recently reported a novel cyclophane receptor **1**, which selectively complexes with ATP in buffer and signals the event through changes in absorption spectroscopy.[9] However, the utility of **1** as a sensitive receptor is limited due to its negligible fluorescence yields ($\Phi_F = 0.0007$). Herein, we report a highly sensitive fluorescence assay for GTP, which makes use of the beneficial nonfluorescent and selective binding properties of the receptor **1**, highly fluorescent properties of the indicator, 8-hydroxy-1,3,6-pyrene trisulfonate (HPTS; $\Phi_F = 0.7$), and competitive assay,[10] pioneered by Anslyn and co-workers.[11] The uniqueness of our system is that it differentiates between ATP and GTP involving synergistic effects of electronic, π-stacking, and electrostatic interactions inside the cavity and recognizes micromolar quantities of GTP in buffer and biological fluids through a visual "turn on" fluorescence mechanism.



The successive additions of the receptor **1** to a solution of indicator, HPTS, in buffer resulted in a regular decrease in the absorbance (Figure S1, Supporting Information) and fluorescence quenching of HPTS centered at 512 nm (Figure 1A).[12] At ca. 6.25 μM of **1**, nearly complete fluorescence quenching (99%) along with 25% hypochromicity in the absorption spectrum of HPTS was observed. In contrast, the addition of the model compound **2** showed negligible changes in the absorption and fluorescence properties of HPTS (inset of Figure 1A). The Benesi−Hildebrand analysis of the emission data gave a 1:1 stoichiometry for the complex [**1**·HPTS] (inset of Figure 1B), with an association constant (K_{ass}) of $(4.66 \pm 0.2) \times 10^4$ M^{-1} and change in free energy of −27 kJ mol^{-1} in buffer, while a relatively higher value of K_{ass} ($(6.56 \pm 0.3) \times 10^4$ M^{-1}) was obtained in aqueous medium.

The complex formation between **1** and HPTS was analyzed by picosecond time-resolved fluorescence analysis, cyclic voltammetry (CV), and NMR techniques. HPTS alone exhibited a single exponential emission decay with a lifetime of 5.3 ns (Figure 1B),[13]

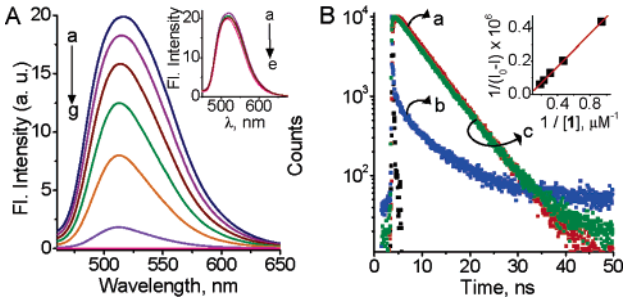


Figure 1. (A) Changes in fluorescence spectra of HPTS (7 μM) with gradual addition of **1** and **2** (inset) in phosphate buffer (pH 7.4): [**1** or **2**] = 0−6.25 μM; $\lambda_{ex} = 364$ nm. (B) Fluorescence decay profiles of (a) HPTS, (b) complex [**1**·HPTS], and (c) complex [**1**·HPTS] in the presence of GTP. Inset shows Benesi−Hildebrand fit for emission quenching of HPTS by **1**.

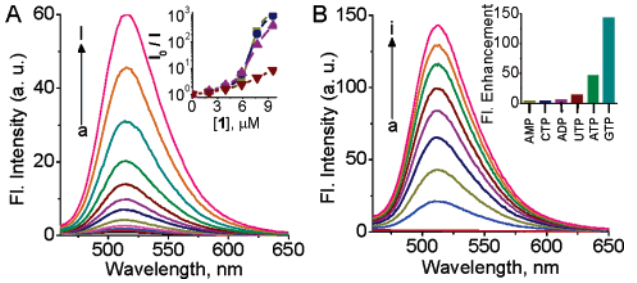


Figure 2. (A) Effect of temperature on the emission spectra of complex [**1**·HPTS]: (a) 293 to (l) 348 K. Inset shows the relative fluorescence quenching of HPTS by **1** at different salt concentrations. [NaCl] (■) 0, (●) 2, (▲) 50, (▼) 500 mM. (B) Fluorescence indicator displacement (FID) from the complex [**1**·HPTS] by GTP in buffer: [GTP] = 0−1.6 mM. Inset shows the relative FID efficiency of various analytes at 1.6 mM.

whereas a biexponential decay with lifetimes of 215 ps (70%) and 6.2 ns (30%) was observed in the presence of the receptor **1**. In CV, we observed a peak at 0.45 V, corresponding to one-electron oxidation of HPTS,[14] while a significant decrease in current intensity (67%) was observed in the presence of **1** (Figure S2). The successive additions of **1** to a solution of HPTS in D_2O resulted in broadening of the peaks corresponding to H_4, H_5, H_9, and H_{10} protons of HPTS. These results confirm the formation of a stable complex between HPTS and the receptor **1**.

To understand the nature and strength of the complex formed between the receptor **1** and HPTS, we investigated the effects of temperature and ionic strength. When temperature of the complex [**1**·HPTS] was raised from 293 to 348 K, we observed a regular increase in the emission intensity of HPTS, indicating a gradual dissociation of the complex (Figure 2A) at these temperatures. As shown in the inset of Figure 2A, the emission quenching of HPTS by **1** becomes less and less prominent with an increase in ionic strength (Figures S3−S6). The lower value of $K_{ass} = 1.9 \times 10^4$ M^{-1} obtained at higher ionic strengths (500 mM) indicates that the viologen units of **1** are shielded from the sulfonate groups of HPTS by Na^+ ions, thereby decreasing the interactions between **1** and HPTS.

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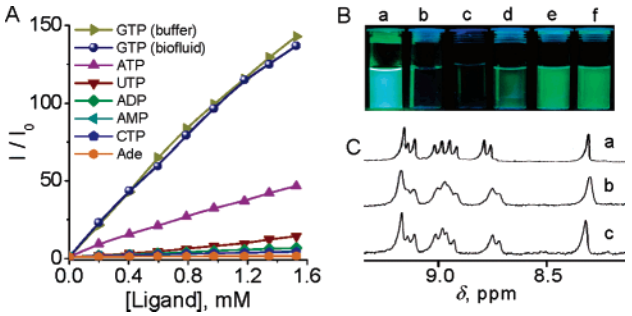

Figure 3. (A) Concentration-dependent relative FID efficiency by various analytes. (B) Visual fluorescence of (a) HPTS alone, (b) complex [**1**·HPTS], and (c−f) complex [**1**·HPTS] in the presence of CTP, ATP, GTP (buffer), and GTP (biofluid). (C) ^{1}H NMR spectra in D_2O of (a) HPTS alone, (b) complex [**1**·HPTS], and (c) [**1**·HPTS] in the presence of GTP.

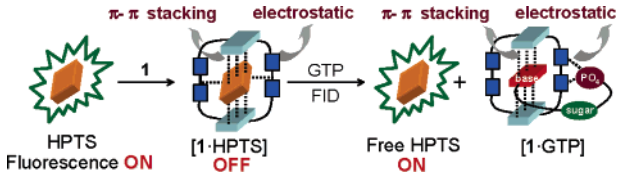

Figure 4. Schematic representation of GTP recognition through FID.

The beneficial competitiveness of the assay is demonstrated by comparing the efficiency of fluorescence indicator displacement (FID) by various nucleotides and nucleosides. Figure 2B shows regular release of HPTS from the complex [**1**·HPTS] by gradual addition of GTP. The successive additions resulted in a regular enhancement in fluorescence intensity corresponding to HPTS at 512 nm. In buffer, ca. 150-fold enhancement was observed at 1.6 mM of GTP (Figure 3A), which led to the visual detection of GTP through a "turn on" fluorescence, as shown in Figure 3B. In contrast, addition of adenosine, AMP, ADP, CTP, and UTP showed negligible changes, whereas ca. 45- and 50-fold enhancement was observed with ATP and ITP, respectively.

The selectivity of the assay was tested in the presence of ATP and other nucleotides and also in biological fluids. Results indicate that this assay is highly sensitive toward GTP even in the presence of various nucleotides. In biological fluids, we observed ca. 140-fold enhancement in fluorescence intensity, which is comparable to the results obtained in the buffer medium (Figures S7−S15). The displacement of HPTS from the complex [**1**·HPTS] was confirmed by time-resolved fluorescence and NMR techniques. When GTP was added to the complex, we observed a biexponential decay having lifetimes of 5.4 ns (98%) and 9.6 ns (2%). The former decay is attributed to the free HPTS in solution. Similarly, ^{1}H NMR spectrum of free HPTS was almost completely revived (Figure 3C) when GTP was added, thereby confirming the quantitative displacement of indicator, HPTS, from the complex.

A schematic representation of the supramolecular ON−OFF−ON fluorescence assay for GTP recognition is shown in Figure 4. The indicator, HPTS, undergoes an efficient complexation with the receptor **1** resulting in complete quenching of its fluorescence. The mechanism of the quenching is due to the electron transfer from the excited state of HPTS to the viologen moiety on the basis of experimental evidence and the theoretically calculated favorable change in free energy ($\Delta G = -1.7$ eV).[15] The driving force for such a complexation is attributed to the synergistic effects of π-stacking and electrostatic interactions inside the cavity,[16] as confirmed by the sigmoidal nature of the relative fluorescence quenching curves obtained at different ionic strengths and the effects of temperature and Debye−Huckel ionic strength function of the medium on K_{ass} and negative results obtained with the model system **2**.

In the competitive displacement assay, the fluorescent indicator, HPTS, from the complex [**1**·HPTS] is successfully displaced by nucleotides and nucleosides. Interestingly, GTP induced the maximum displacement resulting in fluorescence enhancement of ca. 150-fold. The time-resolved fluorescence analysis and revival of the original ^{1}H NMR spectrum of HPTS confirm the quantitative displacement of HPTS from the complex by GTP. The competitive displacement of the indicator by various analytes is found to be in the order GTP (buffer) ≈ GTP (biofluid) ≫ ITP ≈ ATP > UTP > CTP ≈ ADP ≈ AMP ≈ Ade. By virtue of having a better π-electron cloud and low ionization potential when compared to other nucleotides, GTP unusually exhibits better complexing ability with the receptor **1** through synergistic effects of electronic, π-stacking, and electrostatic interactions inside the cavity.

In conclusion, we demonstrated a highly sensitive and selective fluorescence assay for GTP through beneficial properties of the receptor **1** and the fluorescence indicator, HPTS. The uniqueness of this assay is that it successfully discriminates GTP from ATP and other nucleotides and nucleosides through an ON−OFF−ON fluorescence mechanism with a visual change in fluorescence intensity. Studies are in progress to evaluate the selectivity of the receptor **1** toward other biologically important analytes and through FID utilizing different indicators.

Acknowledgment. We dedicate this work to Professor Gary B. Schuster on his 60th birthday, and thank CSIR and DST for the financial support. This is Contribution No. PPD-219 from RRLT.

Supporting Information Available: Details of synthesis, calculations, processing of biological fluids, and Figures S1−S15 showing photophysical properties of HPTS, the receptor **1**, and the complex [**1**·HPTS] in the presence of various analytes. This material is available free of charge via the Internet at http://pubs.acs.org.

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