3.1. ABSTRACT

With the objective of developing sensitive fluorescence based probes for biomolecules, we have investigated the interactions of a few selected cyclophanes with nucleosides and nucleotides. The addition of ATP or GTP to a solution of the cyclophane 1 in buffer resulted in decrease in its absorbance at 375 nm as compared to the model derivative 4. In contrast, negligible changes were observed with the addition of ADP, AMP, GDP, GMP, adenosine, guanosine and...
phosphate, indicating thereby that the cyclophane 1 undergoes selective interactions only with nucleotide triphosphates with association constants in the order of $10^3$ M$^{-1}$. The cyclophane 1 was further found to undergo efficient interactions with the fluorescence indicator, 8-hydroxy-1,3,6-pyrene trisulfonate (HPTS) resulting in 25\% hypochromicity along with complete fluorescence quenching of HPTS. The subsequent titration of this non-fluorescent [1·HPTS] complex with various nucleosides and nucleotides resulted in the displacement of the indicator, HPTS, leading to the revival of its fluorescence intensity. It was observed that GTP induced the maximum displacement with an overall emission enhancement of ca. 150-fold, whereas ca. 45-fold increase was observed with ATP.

The selectivity towards GTP has been attributed to the presence of a better $\pi$-electron cloud which facilitates effective electronic, $\pi$-stacking and electrostatic interactions inside the cavity of the cyclophane 1. The cyclophane 2, having only one anthracene moiety, behaved similarly, but showed less sensitivity for GTP as compared to 1. In contrast, the cyclophane 3, exhibited efficient interactions with the indicator, HPTS, but was found to be
inefficient as a receptor for nucleotides because of the large cavity size. These results confirm the importance of the cavity size and aromatic surface in the molecular recognition ability of the cyclophanes and demonstrate the potential of the cyclophane 1 as a probe for the detection of GTP and ATP in buffer and bio-fluids.

3.2. INTRODUCTION

Detection of nucleosides and nucleotides has paramount importance as they form the fundamental units of all the life forms. Of all nucleotides, the detection and quantification of adenosine 5'-triphosphate (ATP) and guanosine 5'-triphosphate (GTP) (Chart 3.1) is vital from the view point of clinical diagnosis, taking into account the fact that these nucleoside 5'-triphosphates play important roles in various biological processes. GTP is required for many biological activities in the cell, such as synthesis of DNA, RNA,

\[
\begin{align*}
\text{AMP) } & R_1 = H; & R_2 = NH_2; & n = 1 \\
\text{ADP) } & R_1 = H; & R_2 = NH_2; & n = 2 \\
\text{ATP) } & R_1 = H; & R_2 = NH_2; & n = 3 \\
\text{GMP) } & R_1 = NH_2; & R_2 = OH; & n = 1 \\
\text{GDP) } & R_1 = NH_2; & R_2 = OH; & n = 2 \\
\text{GTP) } & R_1 = NH_2; & R_2 = OH; & n = 3
\end{align*}
\]

Chart 3.1
and proteins, nutrient metabolism, and cell signaling. It is well established that GTP-binding proteins play diverse roles as switches in cell growth, receptor activation, exocytosis, ion channel conductivity and change in cell shape. Moreover, the cascade of reactions initiated through the recognition of GTP by G-proteins regulates the stress factor.

ATP, on the other hand, is known as the biological energy currency and the binding of ATP by proteins is one of the most prominent molecular recognition events in nature. ATP also plays an important role in energy transduction in organisms and controls several metabolic processes, including synthesis of cyclic adenosine monophosphate. The abnormalities in the concentrations of ATP and GTP in the body due to the defect of purine salvage enzymes, particularly, adenosine phosphoribosyl transferase (APRTase) and hypoxanthine phosphoribosyl transferase (HPRTase), result in severe combined immunodeficiency disorder (SCID) and Lesch-Nyhan syndrome (brain gout), respectively.

The development of molecular systems capable of recognizing GTP and ATP under physiological pH and biological conditions therefore can have potential applications in basic research as well
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as in medicinal and diagnostic applications. In this context, design of fluorescent probes and methods to distinguish between various nucleotides, in particular GTP and ATP, is gaining importance.\textsuperscript{4,5} Of the reported molecular receptors, most use complementary hydrogen bonding for their recognition. Such molecular recognition in the aqueous medium would be limited due to the competitive hydrogen bonding of the solvent.\textsuperscript{6} Moreover, the sugar moiety of the nucleosides and nucleotides can interfere in such recognition and hence masking of the hydroxyl groups, prior to the recognition event is essential.\textsuperscript{7}

Progress in this area would require new strategies for the complexation under physiological pH conditions and subsequent signaling of the host-guest complex formation. Of the various outputs, the optical methods offer several advantages for studying complexation process and fluorescence based techniques are particularly important for biological applications because of their high sensitivity. In this context, it was of our interest to develop novel cyclophane based systems as probes for nucleosides and nucleotides. These systems are associated with a high degree of structural rigidity and well defined cavity and hence these systems
can encapsulate and stabilize guest molecules through non-covalent interactions.\textsuperscript{8-10} We have selected a few cyclophanes 1-3 (Chart 3.2) and investigated their interactions with various nucleosides and nucleotides under different conditions through photophysical, chiroptical and biophysical techniques. Our results indicate that the cavity size and aromatic surface of the cyclophane play a major role in their biomolecular recognition. Of all the systems, the cyclophane 1 interacts selectively with GTP and ATP and also in presence of other analytes and signals the event through a 'turn on' fluorescence mechanism in buffer and biofluids.

![Chart 3.2](image)
3.3. RESULTS

3.3.1. Interaction with Nucleotides

The synthesis of the cyclophanes 1-3, under investigation as well as the model compound 4, has been achieved as per the procedure described in Chapter 2 of this thesis. To investigate the biomolecular recognition ability, we have monitored the changes in the absorption and emission spectra of these derivatives with the addition of various nucleosides and nucleotides. Addition of adenosine 5'-triphosphate (ATP) to a solution of the cyclophane 1 showed a gradual decrease in the absorbance corresponding to the anthracene chromophore (Figure 3.1). At 0.5 mM of ATP, we

![Graph showing changes in absorption spectra with ATP and adenosine addition](image)

**Figure 3.1.** Changes in the absorption spectra of the cyclophane 1 (11 μM) with the successive addition of (A) ATP and (B) adenosine in phosphate buffer (10 mM, pH 7.4). [Ligand], (a) 0 and (f) 500 μM. Inset shows Benesi-Hildebrand plot for the binding of ATP with 1.
observed 23% hypochromicity in the absorption spectrum of the cyclophane 1, whereas 27% hypochromicity was observed with guanosine 5'-triphosphate (GTP) under identical conditions. Similarly, the addition of ATP to the cyclophanes 2 and 3 resulted in 15% and 17% hypochromicity, respectively (Figure 3.2), whereas negligible changes were observed in the absorption spectrum of the model derivative 4 under identical conditions. The emission spectra of the cyclophanes 1-4, on the other hand, exhibited negligible changes with the addition of both ATP and GTP. Benesi-Hildebrand analysis\textsuperscript{12} of the absorption changes (inset of Figure 3.1A) showed a 1:1 stoichiometry for the complex formed between 1 and ATP with

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure32.png}
\caption{Changes in the absorption spectra of the cyclophanes (A) 2 (21 μM) and (B) 3 (17 μM) with the addition of ATP in 10 mM phosphate buffer and 20% DMSO-water mixture, respectively. [ATP], (a) 0 and (i) 625 μM. Excitation wavelength, 385 nm.}
\end{figure}
a binding constant of $K_{ass} = 4040 \pm 140 \text{ M}^{-1}$ in buffer, while relatively a higher value of $K_{ass} = 4900 \pm 200 \text{ M}^{-1}$ was observed for GTP under identical conditions.

The addition of other guest molecules such as phosphate, pyrophosphate, adenosine, adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), guanosine 5'-monophosphate (GMP) and guanosine 5'-diphosphate (GDP) resulted in negligible changes in the absorption and emission spectra of the cyclophane 1 (Figure 3.1B). Figure 3.3 shows the relative changes in the absorbance of the cyclophanes 1 and 3 as a function of concentration of various ligands. It is evident from Figure 3.3A that the cyclophane 1 shows selectivity towards GTP and ATP, whereas all other ligands have negligible influence on its absorption spectra.

![Figure 3.3](image-url)
In contrast, the addition of various nucleosides and nucleotides to the solution of the cyclophane 3 resulted in almost similar hypochromicity in its absorption spectrum, indicating thereby that its utility as a selective receptor is limited (Figure 3.3B).

3.3.2. Nature of Host-Guest Complexation

The complexation between the cyclophane 1 and ATP was further analyzed through cyclic voltammetry and NMR techniques. Figure 3.4 shows the differential pulse voltammograms (DPV) of the cyclophane 1 (0.2 mM) in the aqueous medium, which exhibited two reversible one-electron reduction processes centered at −0.50 and

![Graph showing cyclic voltammetry results](image)

**Figure 3.4.** The differential pulse and square wave voltammograms (inset) of the cyclophane 1 (0.2 mM) in the (——) absence and (----) presence of ATP (1.3 μM) in the aqueous medium.
-0.96 V, characteristic of the viologen moiety. When ATP (1.3 μM) was added, we observed a shift of reduction potentials by 16 and 8 mV, along with a significant decrease in current intensity of 40.04 μA (61%) and 19.33 μA (48%), confirming thereby the formation a stable complex between the cyclophane 1 and ATP. Similarly, in the $^1$H NMR spectrum, the successive addition of ATP to a solution of the cyclophane 1 in D$_2$O resulted in broadening of protons of the methylene group, whereas the protons corresponding to the viologen moiety experienced an up-field shift of $\delta$ 0.03 ppm at 0.35 mM of ATP (Figure 3.5). Based on NMR titration data, the binding constant was determined ($K_{ass} = 4700\pm 200$ M$^{-1}$), which is in good agreement with that obtained through the absorption spectroscopy.

To understand the nature and strength of the complex formed between the cyclophane 1 and ATP, we investigated the effects of

![Figure 3.5](image-url)  
**Figure 3.5.** $^1$H NMR spectra of the cyclophane 1 in D$_2$O in the (a) absence and (b) presence of ATP (0.35 mM).
ionic strength and temperature. As the salt concentration was increased gradually, the changes in the absorption spectrum of 1 induced by ATP were found to be less prominent (Figure 3.6). The values of $K_{ass}$ at different ionic strengths were determined and are found to be 3558, 222 and 137 M$^{-1}$ at 2, 50 and 500 mM of NaCl, respectively. The decrease in $K_{ass}$ values with increase in ionic strength indicates that the viologen unit of 1 is shielded by Na$^+$ ions at higher ionic strength of the buffer from the phosphate groups of ATP,$^{14}$ resulting less significant interactions between the cyclophane 1 and ATP. When the temperature of the complex [1•ATP] was

![Figure 3.6](image-url)

**Figure 3.6.** Relative changes in the absorbance of the cyclophane 1 with the addition of ATP in 10 mM phosphate buffer (pH 7.4) containing (■) 0, (●) 2, (▲) 50 and (▼) 500 mM of NaCl. Inset shows the effect of temperature on the absorbance of complex [1•ATP] from 298 to 343 K.
raised from 293 to 343 K, we observed an increase in the intensity of absorbance corresponding to the cyclophane 1 at 378 nm (inset of Figure 3.6), indicating the dissociation of the complex. However, the complex showed 19% hypochromicity at 343 K, indicating the stability of the complex even at this temperature.

3.3.3. Recognition of Nucleotides Through FID Assay

Eventhough the cyclophane 1 showed selectivity towards GTP and ATP in buffer and signaled the event through changes in the absorption spectroscopy, its utility as a sensitive probe was limited due to its negligible fluorescence yields ($\Phi_F = 0.0007$). By making use of the beneficial non-fluorescent and selective binding properties of the receptor 1, it was of our interest to exploit its potential use as a probe for nucleotides through fluorescent indicator displacement (FID) assay. In this context, we have utilized a highly fluorescent indicator, 8-hydroxy-1,3,6-pyrene trisulfonate (HPTS; $\Phi_F = 0.7$). As described in the first Chapter of this thesis (Section 1.4),$^{15}$ the FID assay involves first the reversible binding of a fluorescence indicator with a receptor followed by a competitive binding of analyte with the receptor resulting in the displacement of
Chapter 3

the fluorescence indicator. Based on this principle, the major requirement is that the affinity between the indicator and the receptor be comparable to that between the analyte and the receptor.

The successive additions of the receptor 1 to a solution of HPTS in buffer resulted in a regular decrease in the absorbance and quenching of the fluorescence intensity of HPTS centered at 512 nm (Figure 3.7). At ca. 6.25 \( \mu \text{M} \) of the cyclophane 1, we observed 25% hypochromicity in the absorption spectrum of HPTS along with the quantitative fluorescence quenching (99%). In contrast, the addition

![Figure 3.7](image)

**Figure 3.7.** Changes in the (A) absorption and (B) fluorescence spectra of HPTS (7 \( \mu \text{M} \)) with addition of the cyclophane 1 in phosphate buffer (pH 7.4). Inset of (B) shows the corresponding fluorescence changes with the addition of the model compound 4 in buffer (pH 7.4). [1 or 4], (a) 0 and (g) 6.25 \( \mu \text{M} \). Excitation wavelength, 364 nm.
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of the model compound 4 resulted in only negligible changes in the absorption and fluorescence properties as shown in the inset of Figure 3.7B. Similarly, we have carried out the titrations of the cyclophanes 2 and 3 with HPTS under similar conditions (Figure 3.8). While the addition of the cyclophane 2 resulted in significant hypochromicity in the absorption spectrum and complete quenching of the fluorescence of HPTS, the cyclophane 3 showed less significant changes.

![Fluorescence spectra](image)

**Figure 3.8.** Changes in the fluorescence spectra of HPTS with the gradual addition of the cyclophanes (A) 2 in buffer (10 mM phosphate, pH 7.4) and (B) 3 in 20% DMSO-water. [2 or 3], (a) 0 and (f) 11.3 μM. Excitation wavelength, 364 nm.

The changes in the optical properties of HPTS in the presence of the cyclophanes 1-3 are indicative of the formation of a stable complex. Benesi-Hildebrand analysis of the emission data gave a 1:1
stoichiometry for the complex [1·HPTS], 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 higher value of $K_{ass} = 6.56 \pm 0.3 \times 10^4$ M$^{-1}$ was obtained in the aqueous medium. The complexation between the cyclophane 1 and HPTS was further analyzed by picosecond time-resolved fluorescence analysis and NMR techniques. HPTS alone exhibited a single exponential fluorescence decay with a lifetime of 5.3 ns (Figure 3.9)$^{16}$ whereas a biexponential decay with lifetimes of 215 ps (70%) and 6.2 ns (30%) was observed in the presence of the cyclophane 1. Similarly, the successive additions of the cyclophane 1 to a solution of HPTS in

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure3.9}
\caption{Fluorescence decay profiles of (a) HPTS, (b) complex [1·HPTS] and (c) complex [1·HPTS] in the presence of GTP.}
\end{figure}
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Figure 3.10. $^1$H NMR spectra in D$_2$O of (a) HPTS alone, (b) complex [1·HPTS] and (c) [1·HPTS] in presence of GTP.

D$_2$O resulted in broadening of the peaks corresponding to H$_4$, H$_5$, H$_9$ and H$_{10}$ protons of HPTS in its $^1$H NMR spectrum (Figure 3.10).

To understand the nature and strength of the complex formed between the cyclophanes 1-3 and HPTS, we have investigated the effects of ionic strength and temperature on the complexation process (Figure 3.11). For example, the quenching of emission of HPTS by the cyclophane 1 was found to be less prominent as we increase in ionic strength of the buffer. We obtained a lower value of $K_{ass} = 1.9 \times 10^4$ M$^{-1}$ at higher ionic strengths (500 mM), indicating thereby that the viologen units of the cyclophane 1 are shielded from the sulfonate groups of HPTS by Na$^+$ ions, resulting in less favorable interactions between the cyclophane 1 and HPTS. Moreover, when the temperature of the complex [1·HPTS] was raised from 293 to 358 K, we observed a regular increase in the
Figure 3.11. (A) The relative fluorescence quenching of HPTS by the cyclophane 1 in 10 mM phosphate buffer (pH 7.4) containing (■) 0, (■) 2, (▲) 50 and (▼) 500 mM NaCl. (B) Effect of temperature on the emission spectra of complex [1·HPTS]. (a) 293 and (f) 358 K. Excitation wavelength, 364 nm.

emission intensity of HPTS, indicating a gradual dissociation of the complex at these temperatures.

Similarly, when the emission of the complex [2·HPTS] was recorded as a function of temperature, we observed a regular increase in the emission intensity of HPTS at higher temperatures due to the dissociation of the complex. In contrast, when the temperature of a mixture of the cyclophane 3 and HPTS was raised from 293 to 358 K, we observed negligible changes in the emission spectrum (Figure 3.12). The negligible effect of temperature on the emission spectrum of a mixture of 3 and HPTS strongly suggests that 3 is unable to form a stable complex with HPTS.
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Figure 3.12. Effect of temperature on the emission spectra of (A) the complex [2.HPTS] and (B) a mixture of the cyclophane 3 and HPTS. (a) 293 to (g) 358 K. Excitation wavelength, 364 nm.

The beneficial competitiveness of the assay is demonstrated by comparing the efficiency of fluorescence indicator displacement (FID) by various nucleotides and nucleosides. Figure 3.13 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, which led to the visual detection of GTP through "turn on" fluorescence intensity. 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 (Figure 3.14). Similarly, the utility of the cyclophanes 2
Figure 3.13. Fluorescence indicator displacement (FID) from the complex [1-HPTS] by GTP in buffer. [GTP], (a) 0 and (i) 1.6 mM. Excitation wavelength, 364 nm.

and 3 as receptors for nucleotides was investigated through FID assay. Figure 3.15 shows the changes in the emission spectra of a mixture of the cyclophanes 2 or 3 and HPTS with the addition of GTP. The successive additions of GTP to a solution of [2.HPTS] resulted in regular enhancement in the fluorescence of HPTS at 512 nm. At ca. 1.6 mM of GTP, we observed a net fluorescence enhancement of ca. 73-fold as compared to ca. 150-fold enhancement observed with the cyclophane 1. The titrations carried out with the cyclophane 3, on the other hand, resulted in contrasting results as compared to the cyclophanes 1 and 2. When GTP was successively added to a solution of a mixture of HPTS and
the cyclophane 3, we observed negligible displacement of HPTS and showed non-negligible quenching in the fluorescence intensity of HPTS (Figure 3.15B).

To further demonstrate the selectivity of the FID assay, it was tested in the presence of other nucleotides and also in biological fluids. Even in the presence of various nucleotides, the complex [1·HPTS] exhibited selectivity towards GTP. Similarly, in biological fluids, we observed ca. 140-fold enhancement in fluorescence intensity, which is comparable to the results obtained in the buffer medium (Figure 3.14). 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 [1·HPTS], we observed a biexponential decay having lifetimes 5.4 ns (98%) and 9.6 ns (2%) (Figure 3.9). The former lifetime has been attributed to the free HPTS in solution. Similarly, $^1$H NMR spectrum of free HPTS was almost completely revived when GTP was added, thereby confirming the quantitative displacement of the indicator, HPTS from the complex (spectrum 'c' in Figure 3.10).

Figure 3.15. (A) FID from the complex [2·HPTS] by GTP in phosphate buffer (10 mM, pH 7.4). Inset shows the displacement of HPTS from [1·HPTS] and [2·HPTS] by GTP. (B) Changes in the emission spectrum of a mixture of 3 and HPTS with the addition of GTP. [GTP], (a) 0 and (c) 1.6 mM. Excitation wavelength, 364 nm.

3.4. DISCUSSION

The optimized geometries obtained through B3LYP level theoretical calculations\textsuperscript{17} revealed interplanar distances of 10.4, 9.9
and 13.9 Å between the two aromatic units for the cyclophanes 1-3, respectively (Figure 3.16). In comparison to the cyclophane 1, the replacement of one of the anthracene moieties by a phenyl unit in 2 results in a cavity having a lesser aromatic surface. On the other hand, cyclophane 3 having three methylene groups has a cavity with large dimensions. These variations in the cavity size could be correlated directly to the behavior of these derivatives towards various guest molecules like nucleotides and HPTS.

Figure 3.16. Optimized geometries of the cyclophanes 1 – 3.

Based on our experimental evidence, the binding of ATP or GTP to the cyclophanes 1-3 is a result of π-π stacking in combination with electrostatic interactions inside the cavity as shown in Figure 3.17. The attraction between the phosphate groups of nucleotides and the viologen moiety of the cyclophane 1 involving multiple electrostatic interactions result in the formation of a tight complex,
which is further stabilized by π-π stacking of the nucleobase inside the cavity. The presence of a cavity in the molecular system 1 as well as three phosphate groups in ATP or GTP are very essential for the selective recognition and for the formation of a stable 1:1 complex, as evidenced from the negative results obtained with the model system 4 and with various other guest molecules. Evidence for this comes from the fact that the cyclophane 1 exhibits non-negligible interactions with ADP, containing two phosphate groups, while no binding interactions were observed with AMP, adenosine and phosphate.

This mode of complexation was further confirmed by making use of the Debye-Huckel ionic strength function of the medium on
the $K_{ass}$ values and the thermodynamic parameters such as $\Delta H^\circ$ and $\Delta S^\circ$ (-11.15 kJmol$^{-1}$ and -37.41 JK$^{-1}$mol$^{-1}$), obtained using Van't Hoff's plot$^{20}$ (Figures 3.18-3.19). Thermodynamic parameters obtained are consistent with the expected non-classical hydrophobic interactions usually observed in the case of the cyclophane systems.$^{10a}$ As a consequence of the complex formation between 1 and ATP, (i) current intensity decreases as observed in the differential pulse and square wave voltammograms,$^{21}$ (ii) shielding of protons of the viologen moiety occurs due to the interactions with the phosphate groups, (iii) broadening of signals corresponding to the methylene

![Graph](image)

**Figure 3.18.** Effect of Debye-Huckel ionic strength function of the medium on the association constants for the complex [1·ATP] in 10 mM phosphate buffer (pH 7.4) under various salt concentrations. [NaCl] (a) 0, (b) 2, (c) 50 and (d) 500 mM.
protons is observed because of $\pi$-stacking of the aromatic part of ATP, and (iv) decrease in entropy ($\Delta S^\circ$) is observed due to the formation of an ordered complex through non-classical hydrophobic interactions.

As observed with ATP and GTP, the fluorescence indicator, HPTS undergoes efficient complexation with the receptor 1 resulting in complete quenching of its fluorescence. The mechanism of the quenching is due to photoinduced electron transfer process from the excited state of HPTS to the viologen moiety as characterized through experimental evidence and the theoretically
calculated favorable change in free energy \( \Delta G = -1.7 \text{ eV} \). The driving force for such a complexation is attributed to the synergistic effects of \( \pi \)-stacking and electrostatic interactions inside the cavity. This was confirmed by the sigmoidal nature of the relative fluorescence quenching curves obtained at different ionic strengths and the effect of temperature and negative results obtained with the model system 4.

A schematic representation of the selective recognition of GTP by [1.HPTS] and [2.HPTS] is shown in Figure 3.20. In the competitive displacement assay, the fluorescent indicator, HPTS, is successfully displaced from the complex [1·HPTS] by nucleotides and nucleosides. Interestingly, GTP induced maximum displacement resulting in net fluorescence enhancement of ca. 150-fold leading to visual changes in fluorescence (Figure 3.21). The time-resolved

![Figure 3.20](image-url)  
**Figure 3.20.** Schematic representation of GTP recognition through fluorescence indicator displacement assay (FID).
fluorescence analysis and revival of the original $^1$H NMR spectrum of HPTS confirms the quantitative displacement of HPTS from the complex by GTP. The competitive displacement of the indicator, HPTS by various analytes is found to be in the order GTP (buffer) $\approx$ GTP (biofluid) $>$ ITP $\approx$ ATP $>$ UTP $>$ CTP $\approx$ ADP $\approx$ AMP $\approx$ Ade. By virtue of having a better $\pi$-electron cloud and low ionization potential, GTP unusually exhibits better complexing ability with the receptor 1 through synergistic effects of electronic, $\pi$-stacking and electrostatic interactions inside the cavity.

![Figure 3.21](image)

**Figure 3.21.** Visual observation of fluorescence intensity of (a) HPTS alone, (b) complex [1·HPTS] and (c) to (f) [1·HPTS] in presence of CTP, ATP, GTP (buffer) and GTP (bio-fluid), respectively.

The behavior of the cyclophane 2 towards various nucleotides and HPTS is comparable to the results obtained with the cyclophane 1, but with lesser sensitivity. In this case, ca. 73-fold fluorescence enhancement was observed upon addition of GTP to the complex [2·HPTS]. Based on our experimental evidence, the observation of
relatively lesser sensitivity for 2 in comparison to 1 can be attributed to the presence of a cavity with lesser aromatic surface. The cyclophane 2 with lesser aromatic surface in the cavity undergoes not so strong π stacking and hydrophobic interactions as compared to the cyclophane 1. Because of these weak interactions, a less favorable complex between 2 and GTP will be formed and as a result lesser displacement of HPTS occurs from [2.HPTS] by GTP.

The contrasting results obtained with the cyclophane 3, containing a flexible spacer group with a larger cavity, are indeed interesting, although not completely unexpected. Moreover, it helps to confirm the role played by cavity size in the molecular recognition process.\textsuperscript{23} The negligible interactions exhibited by the cyclophane 3 with various guest molecules such as nucleotides and nucleosides indicate that the cyclophane 3 is unable to form a stable complex with these analytes. Even though the fluorescence quenching of HPTS by the cyclophane 3 is an indicative of complexation, the investigation of the effect of temperature on their interaction shows that the quenching may be attributed to an outside stacking mechanism involving electrostatic interactions between the viologen moiety of the cyclophane 3 and HPTS.\textsuperscript{22,24}
The investigation of the effect of temperature on fluorescence of a mixture of the cyclophane 3 and HPTS substantiates our hypothesis. Moreover, if a complex is formed between the cyclophane 3 and HPTS, the addition of GTP should, in principle, result in the displacement of HPTS leading to an enhancement in the emission of HPTS. In contrast, we observed non-negligible quenching of the fluorescence of HPTS with the addition of GTP. This observation confirms the fact that the cyclophane 3 is unable to form a stable complex with the fluorescent indicator, HPTS. The inability of the cyclophane 3 in selectively recognizing any of the guest molecules could be attributed to the presence of a larger cavity, which in turn is not favorable for the formation of a tightly packed complex involving various non-covalent interactions such as \( \pi \)-stacking and hydrophobic interactions.

3.5. CONCLUSIONS

In summary, we have investigated the biomolecular recognition properties of a series of selected novel cyclophane derivatives containing anthracene and viologen moieties wherein the cavity size was varied by changing the spacer groups as well as
the aromatic moiety. The cyclophane 1 acts as a selective probe for GTP and ATP through absorption spectroscopy. Subsequently, a highly sensitive and selective fluorescence assay was developed for GTP through the beneficial properties of the cyclophane 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.

The behavior of the cyclophane 2 towards various guest molecules is similar to that of the cyclophane 1, but with lesser sensitivity, i.e. ca. 73-fold fluorescence enhancement was observed as against ca. 150-fold enhancement with the cyclophane 1 towards GTP through FID. On the other hand, the utility of the cyclophane 3 having a larger cavity as a receptor for nucleotides, nucleosides and HPTS is limited due to its larger cavity which leads to less favorable interactions with the guest molecules. These results are important in understanding the role of cavity size and aromatic surface in the biomolecular recognition and in the design of efficient receptors based on cyclophanes for nucleosides and nucleotides.
3.6. EXPERIMENTAL SECTION

3.6.1. General Techniques

The equipment and procedures for melting point determination and spectral recordings have been described elsewhere. All melting points are uncorrected and were determined on a Mel-Temp II melting point apparatus. An Elico pH meter was used for pH measurements. $^1$H and $^{13}$C NMR spectra were measured on a 300 MHz or 500 MHz Bruker advanced DPX spectrometer. HRMS were recorded on a JEOL mass spectrometer. The electronic absorption spectra were recorded on a Shimadzu UV-VIS-NIR spectrophotometer. Fluorescence spectra were recorded on a SPEX-Fluorolog F112X spectrofluorimeter. The fluorescence quantum yields were determined by using optically matched solutions. Quinine sulphate ($\Phi_f = 0.54$) in 0.1 N H$_2$SO$_4$ was used as the standard. The quantum yields of fluorescence were calculated using the equation 3.1, where, $A_s$ and $A_u$ are the absorbance of

$$F_u = \frac{A_s F_u n_s^2}{A_u F_s n_u^2} F_s$$  \(3.1\)
standard and unknown, respectively. $F_u$ and $F_s$ are the areas of fluorescence peaks of the unknown and standard and $n_s$ and $n_u$ are the refractive indices of the standard and unknown solvents, respectively. $\Phi_s$ and $\Phi_u$ are the fluorescence quantum yields of the standard and unknown. Fluorescence lifetimes were measured using a IBH Picosecond single photon counting system. The fluorescence decay profiles were deconvoluted using IBH data station software V2.1, fitted with monoexponential decay and minimizing the $\chi^2$ values of the fit to $1 \pm 0.1$.

3.6.2. Materials

All nucleosides and nucleotides and the fluorescence indicator, HPTS, were purchased from Sigma-Aldrich and used as received. The synthesis of the cyclophane derivatives 1 - 3 and the model derivative 4 used in the present study was achieved as described in Chapter 2 of the present thesis. Doubly distilled water was used for all the experiments. All experiments were carried out in 10 mM phosphate buffer (pH 7.4) containing 2 mM NaCl at room temperature ($25 \pm 1 ^\circ C$), unless otherwise mentioned.
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3.6.2. Measurement of GTP in biofluids

Fresh blood samples collected from healthy people were immediately treated with EDTA and centrifuged at 3000 rpm for 5 min. The supernatant was collected and divided into two portions. One of the portions was subjected to deproteinization by stirring with 20% trichloroacetic acid for 20 min. followed by centrifugation at 3000 rpm for 5 min. The supernatant was used for further studies after diluting 1000 times with water. The other portion (not subjected to deproteinization) was used as such after dilution with water. The pH of all blood samples used for all the experiments was maintained at 7.4.

3.6.3. Calculation of association constants \((K_{ass})\)

Nucleotides, nucleosides and other analyte solutions were prepared in distilled water. The binding affinities of the cyclophanes and model derivatives were calculated using Benesi-Hildebrand equation 3.2, where, \(K\) is the equilibrium constant, \(A_f\) is the

\[
\frac{1}{A_f - A_{ob}} = \frac{1}{A_f - A_{f_{c}}} + \frac{1}{K(A_f - A_{f_{c}})[\text{Ligand}]} \tag{3.2}
\]
absorbance of free host, \( A_{ob} \) is the observed absorbance in the presence of various ligands and \( A_{fc} \) is the absorbance at saturation. The linear dependence of \( 1/(A_f - A_{ob}) \) on the reciprocal of the ligand concentration indicates the formation of a 1:1 molecular complex between ligands and the host.

### 3.6.4. Calculation of change in free energy (\( \Delta G_{ET} \))

The change in free energy (\( \Delta G_{ET} \)) for the photoinduced electron transfer reaction was evaluated according to Rehm-Weller equation 3, where, \( E_{(0,0)} \) is the singlet excitation energy in eV, \( w_p \) is

\[
\Delta G_{ET} = E_{ox} - E_{red} - w_p - E_{(0,0)} \quad (3.3)
\]

the work term which was taken as -0.056 eV in water,\(^{28} \) \( E_{ox} \) is the oxidation potential of the donor and \( E_{red} \) is the reduction potential of the acceptor. The oxidation potential of anthracene (1.9 eV), reduction potential of viologen (-0.45 eV) and singlet state energy of anthracene (3.18 eV) were used for calculations.\(^{5} \) The change in free energy value for the electron transfer from the singlet excited state of anthracene to the viologen was found to be -0.77 eV, which predicts a facile quenching of the anthracene fluorescence by the viologen moiety through an electron transfer mechanism.
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The oxidation potential of HPTS (0.42 eV),
29 reduction potential of the viologen moiety (-0.45 eV) and singlet state energy of HPTS (2.59 eV) were used for calculations. The change in free energy value for the electron transfer from the excited state of HPTS to the viologen moiety was found to be -1.72 eV, which predicts a facile quenching of the fluorescence of HPTS by the viologen moiety through an electron transfer mechanism.

3.7. REFERENCES


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