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PAPER

Study of cavity size and nature of bridging units on recognition of nucleotides by cyclophanes†

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We synthesized a few novel cyclophanes **CP-1** to **CP-4** containing anthracene units linked together through different bridging and spacer groups and have investigated their interactions with various nucleosides and nucleotides. Of these systems, **CP-1** and **CP-3** showed selectivity for 5′-GTP and 5′-ATP as compared to other nucleotides and nucleosides, whereas negligible selectivity was observed with **CP-2** and **CP-4**. Interestingly, **CP-1**, **CP-2** and **CP-3** exhibited significant binding interactions with the fluorescent indicator, 8-hydroxy-1,3,6-pyrene trisulfonate (HPTS), resulting in the formation of non-fluorescent complexes. Titration of these complexes with nucleosides and nucleotides resulted in the displacement of HPTS, leading to the revival of its fluorescence intensity. It was observed that 5′-GTP induced the maximum displacement of HPTS from the complex [**CP-1**·HPTS] with an overall fluorescence enhancement of *ca.* 150-fold, while 5′-ATP induced *ca.* 45-fold. Although the displacement of HPTS from the complexes [**CP-2**·HPTS] and [**CP-3**·HPTS] was found to be similar to that of [**CP-1**·HPTS], these complexes showed lesser selectivity and sensitivity. In contrast, negligible displacement of HPTS was observed from the complex [**CP-4**·HPTS] under similar conditions. These results indicate that **CP-1**, having a well-defined cavity and good electron acceptor (viologen), is capable of forming selective and stable complexes. Though **CP-2** and **CP-3** retain the good electron acceptor (viologen), their reduced aromatic surface and larger cavity, respectively, resulted in lesser sensitivity. In contrast, **CP-4** having a large cavity and a poor acceptor (1,2-bis(pyridin-4-yl)ethene) showed negligible selectivity, thereby indicating the importance of cavity size, bridging unit and aromatic surface on biomolecular recognition properties of cyclophanes.

Introduction

The chemistry of nucleosides and nucleotides has received considerable attention, not only because they constitute the building blocks of nucleic acids, but also because they perform several fundamental life sustaining roles.[1] Deoxynucleotides are found in 0.4–30 μM concentrations in normal cells whereas tumor cells have concentrations up to 11-fold higher.[1b] Of all nucleotides, 5′-GTP is required for many biological activities such as synthesis of DNA, RNA, and proteins; nutrient metabolism; and cell signaling.[2] It is well established that 5′-GTP-binding proteins play a diversity of roles as switches in cell growth, receptor activation, exocytosis, ion channel conductivity and change in cell shape.[3] On the other hand, 5′-ATP is known by the name biological energy currency and the binding of 5′-ATP by proteins is one of the most prominent molecular recognition events in nature.[4] Moreover, 5′-ATP plays important roles in energy transduction in organisms and controls several metabolic processes including the synthesis of cyclic adenosine monophosphate.[5] In this context, the development of molecular systems capable of recognising 5′-GTP and 5′-ATP under physiological pH conditions can have potential applications not only in biology, but also in diagnostics and medicinal chemistry.[6–8]

Recently, we have been interested in cyclophanes as probes for biomolecular recognition.[9,10] By virtue of having a rigid structure with a well defined cavity, cyclophanes can encapsulate and stabilise guest molecules through various non-covalent interactions.[11] Because of the less labour intensive synthesis and ease of functionalisation, cyclophanes have been used as probes not only for simple anions,[12] cations[13] and neutral molecules,[14] but also for biomolecules such as amino acids,[15] proteins,[16] nucleosides,[17] nucleotides[18] and nucleic acids.[19] Even though several cyclophane derivatives have been effectively utilized for host–guest complexation, the design of water soluble cyclophanes that retain the recognition ability in the aqueous medium has been challenging. In addition, the role played by cavity size, bridging units, spacer groups and the aromatic surface in their biomolecular recognition properties, remain less explored.[20] Herein, we report the synthesis

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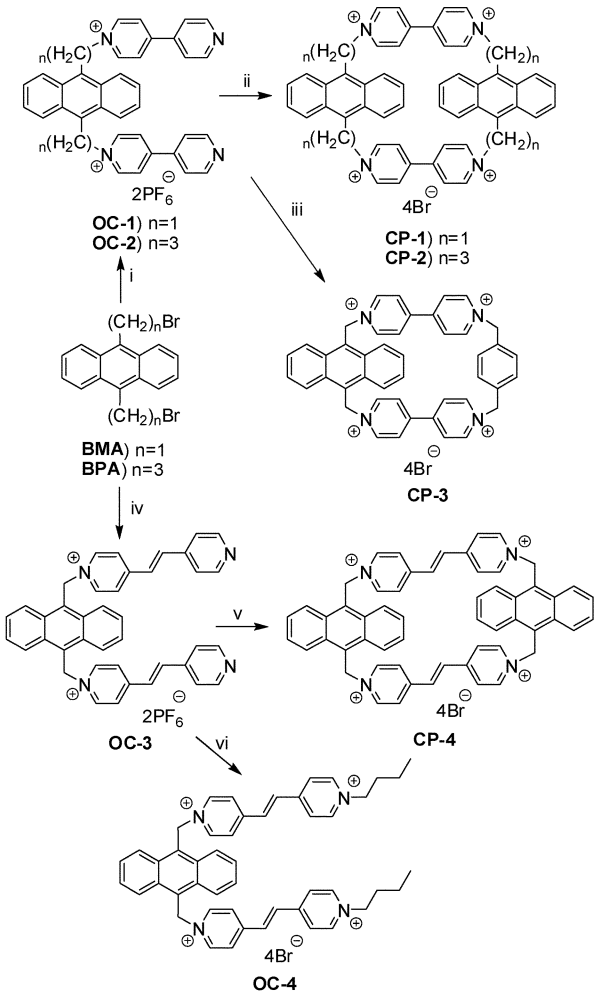
† Electronic supplementary information (ESI) available: Fig. S1–S11 showing changes in the absorption, NMR spectra of **CP-1** and [CP·HPTS] complexes with the addition of HPTS and various nucleotides and ^{1}H NMR spectra of **CP-1**, **CP-2**, **CP-3**, **CP-4** and **OC-4**. See DOI: 10.1039/c0ob00673d

of a series of novel cyclophane derivatives **CP-1**, **CP-2**, **CP-3** and **CP-4**, wherein the nature and size of the cavity have been tuned by varying the bridging units as well as spacer groups. Our investigations indicate that the rigidity of the cavity size, aromatic surface and nature of bridging units dictate the stability of the supramolecular complex and thereby govern the biomolecular recognition properties of cyclophanes.

Results and Discussion

Synthesis and photophysical properties of cyclophanes

The synthesis of the cyclophanes **CP-1**, **CP-2**, **CP-3**, **CP-4** and open derivatives **OC-1** to **OC-4** have been achieved as shown in Scheme 1. Reaction of 9,10-bis(bromomethyl)anthracene (**BMA**) with 4,4′-bipyridine in acetonitrile under refluxing conditions gave 88% of **OC-1**, which on reaction with **BMA** or 1,4-bis(bromomethyl)benzene yielded **CP-1** and **CP-3**, respectively, in 26 and 36% yields. 9,10-bis(bromopropyl) anthracene (**BPA**) was treated with 4,4′-bipyridine in dry acetonitrile under refluxing conditions to give the precursor compound **OC-2** in 60% yield. The reaction of **OC-2** with **BPA** under refluxing conditions in dry CH_3CN gave 52% of the cyclophane **CP-2**. Similarly, the reaction of **BMA** with 1,2-bis(pyridin-4-yl)ethene gave the precursor compound **OC-3** in good yields, which on further reaction with **BMA** gave **CP-4** in 22% yield. The reaction of **OC-3** with 1-butyl bromide under refluxing conditions in dry CH_3CN gave **OC-4** in 30% yield. These cyclophanes showed high solubility in the aqueous medium and exhibited absorption maxima at 378–379 nm, corresponding to the anthracene chromophore. However, **CP-4** exhibited an additional absorption maximum at 325 nm owing to the presence of the 1,2-bis(pyridin-4-yl)ethene moiety (Fig. 1). Similarly, the emission spectra of **CP-1**, **CP-2**, **CP-3** and **CP-4** exhibited characteristic anthracene emission maxima at 417, 420, 413 and 431 nm, respectively.



i) (a) 4,4'-Bipyridine, CH_3CN, reflux, (b) aq. NH_4PF_6; ii) (a) **BMA** or **BPA**, CH_3CN, reflux, (b) Bu_4NBr, CH_3CN; iii) 1,4-bis(bromomethyl)benzene, CH_3CN, reflux; iv) (a) 4,4'-bipyridylethylene, CH_3CN, reflux, (b) aq. NH_4PF_6; v) (a) **BMA**, CH_3CN, reflux, (b) Bu_4NBr, CH_3CN; vi) (a) 1-Butyl bromide, CH_3CN, reflux, (b) Bu_4NBr, CH_3CN

Scheme 1 Synthesis of the cyclophanes **CP-1**, **CP-2**, **CP-3**, **CP-4** and open derivatives **OC-1** to **OC-4**

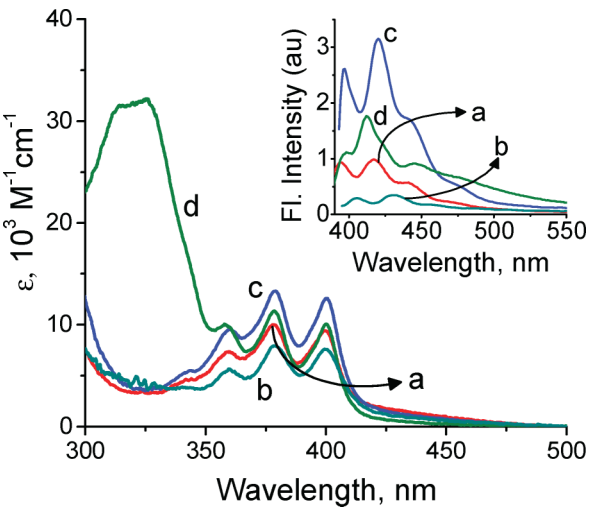


Fig. 1 Absorption and emission (inset) spectra of the cyclophanes (a) **CP-1**, (c) **CP-3** and (d) **CP-4** in phosphate buffer (10 mM, pH 7.4) and (b) **CP-2** in 20% DMSO–water.

Biomolecular recognition properties of cyclophanes

The presence of a well-defined cavity and high solubility in the aqueous medium makes cyclophanes ideal probes for biomolecular recognition.[21] To evaluate their potential as probes, we have monitored the changes in the absorption and emission spectra of these derivatives with the addition of different nucleosides and nucleotides. As shown in Fig. 2, with increasing concentrations of 5′-GTP, we observed a gradual decrease in the absorbance of **CP-1** in phosphate buffer. At 0.5 mM of 5′-GTP, we observed *ca.* 27% hypochromicity in the absorbance of **CP-1**, while 5′-ATP showed *ca.* 23%. Benesi–Hildebrand analysis[22,23] of the absorption changes (inset of Fig. 2) showed 1 : 1 stoichiometry for the complexes of **CP-1** with 5′-GTP and 5′-ATP with binding constants as shown in Table 1. In contrast, negligible changes were observed with the addition of other guest molecules such as adenosine, adenosine 5′-monophosphate (5′-AMP), guanosine 5′-monophosphate (5′-GMP), adenosine 5′-diphosphate (5′-ADP) and guanosine 5′- diphosphate (5′-GDP) (Fig. S1, ESI†). Similarly, negligible changes were observed in the fluorescence spectrum of

Table 1 Association constants (M^{-1}) obtained through Benesi–Hildebrand analysis in buffer

	5′-GTP[a]	5′-ATP[a]	HPTS[b]
CP-1	$4.9 \pm 0.2 \times 10^3$	$4.0 \pm 0.1 \times 10^3$	$4.7 \pm 0.2 \times 10^4$
CP-2	$2.9 \pm 0.2 \times 10^3$	$2.4 \pm 0.2 \times 10^3$	$1.1 \pm 0.1 \times 10^4$
CP-3	$3.9 \pm 0.2 \times 10^3$	$3.4 \pm 0.2 \times 10^3$	$3.7 \pm 0.2 \times 10^4$
CP-4	[c]	[c]	$1.6 \pm 0.2 \times 10^4$

[a] UV-Vis titrations [b] Fluorescence titrations [c] Negligible

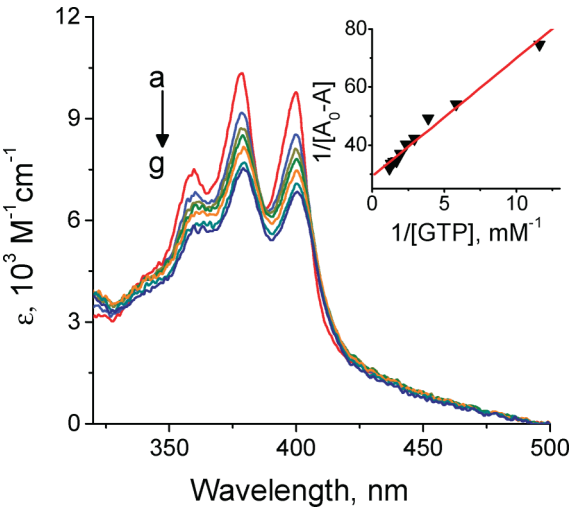


Fig. 2 Changes in the absorption spectrum of **CP-1** (11 μM) with the successive additions of 5′-GTP in phosphate buffer (10 mM; pH 7.4). [5′-GTP], (a) 0 and (g) 500 μM. Inset shows the Benesi–Hildebrand fit of the absorption data.

CP-1 with the addition of all nucleotides and nucleosides under similar conditions (Fig. S2, ESI†).

The selective complexation of **CP-1** with 5′-GTP and 5′-ATP was further confirmed through NMR and electrochemical measurements. For example, the successive additions of 5′-ATP to a solution of **CP-1** in D_2O resulted in broadening of protons of the methylene group in the ^1H NMR spectrum, whereas the protons corresponding to the viologen moiety experienced an upfield shift of δ 0.03 ppm in the presence of 5′-ATP (Fig. S3, ESI†). The differential pulse voltammograms (DPV) of **CP-1** in the aqueous medium exhibited two reversible one-electron reduction processes centred at –0.5 and –0.96 V, characteristic of the viologen moiety. When 5′-ATP 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 (61%) and 19.33 μA (48%), thereby confirming the formation of a stable complex.

Subsequently, we have investigated the interactions of the cyclophanes **CP-2**, **CP-3** and **CP-4** with various nucleosides and nucleotides. The cyclophane **CP-2** showed less selective interactions with all the nucleosides and nucleotides. However, **CP-3**, in which one of the anthracene moieties was replaced with a phenyl unit, exhibited *ca.* 17% and 15% hypochromicity in its absorption spectrum with the addition of 5′-GTP and 5′-ATP, respectively, whereas negligible changes were observed in the presence of other nucleotides. In contrast, **CP-4**, having a large cavity, exhibited less significant interactions with all the nucleotides and nucleosides (Fig. S1, ESI† and Table 1).

Interaction of cyclophanes with fluorescence indicator, HPTS

To improve the sensitivity of the signaling event as well as to understand the role of the cavity size in the complexation process, fluorescence indicator displacement (FID) assay[24] was employed. For this, highly fluorescent ($\Phi_F = 0.7$) and water soluble 8-hydroxy-1,3,6-pyrene trisulfonate (HPTS) was chosen as the indicator. The successive additions of **CP-1** to a solution of HPTS in buffer resulted in a regular decrease in the absorbance and decrease in the fluorescence intensity of HPTS. The addition of *ca.* one equivalent of **CP-1** (6.3 μM) resulted in 25% hypochromicity in the absorption spectrum along with complete fluorescence quenching (*ca.* 99%) of the HPTS (7 μM) (Fig. 3 and Fig. S4, ESI†). We observed similar changes in the fluorescence properties of HPTS upon the addition of **CP-2** and **CP-3**. The addition of one equivalent of these cyclophanes (6.6 μM) resulted in significant hypochromicity in the absorption spectrum and complete quenching in the fluorescence of HPTS (insets of Fig. 3 and 4). In contrast, **CP-4** showed less significant changes under similar conditions. For example, upon the addition of one equivalent of **CP-4** (3.1 μM), we observed *ca.* 8% hypochromicity in the absorption spectrum and 39% quenching in the fluorescence intensity of HPTS (3.3 μM). However, upon increasing the concentration of **CP-4** to 7.2 equivalents (23.8 μM), we observed complete quenching of the fluorescence of HPTS (Fig. S5, ESI†). Benesi–Hildebrand analysis of the fluorescence titration data gave 1 : 1 stoichiometry for the complexation between HPTS and various cyclophanes (for K_{ass} values, refer Table 1).

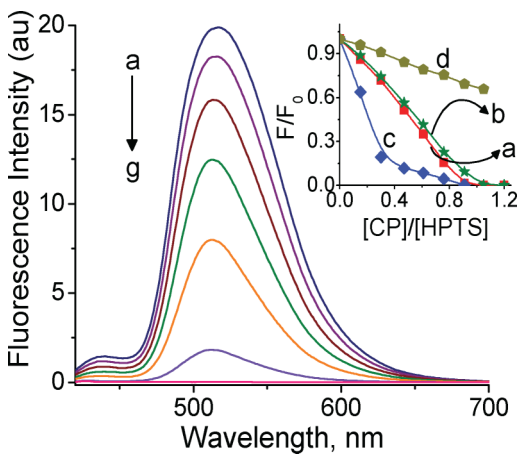


Fig. 3 Changes in the fluorescence spectrum of HPTS (7 μM) with gradual addition of **CP-1** in phosphate buffer (pH 7.4). [**CP-1**], (a) 0 and (g) 6.3 μM. Inset shows the relative quenching in the fluorescence of HPTS by (a) **CP-1**, (b) **CP-2**, (c) **CP-3** and (d) **CP-4**. Excitation wavelength, 364 nm.

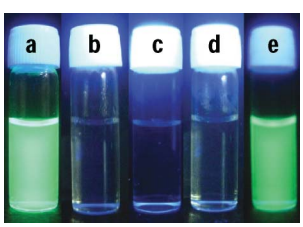


Fig. 4 Visual fluorescence of (a) HPTS (16 μM) alone and HPTS in presence of equimolar concentrations of (b) **CP-1**, (c) **CP-2**, (d) **CP-3** and (e) **CP-4**. Excitation wavelength 360 nm.

The interaction between HPTS and the various cyclophanes was subsequently analyzed through picosecond time-resolved fluorescence analysis (Fig. 5). HPTS alone exhibited a mono-exponential decay with an excited state lifetime of 5.3 ± 0.1 ns in buffer. The addition of one equivalent of **CP-1** (6.3 μM) resulted in a bi-exponential decay with lifetimes of 0.22 ± 0.01 (70%) and 6.2 ± 0.1 ns (30%), indicating significant interaction between these molecules. Similar changes were observed in the lifetime of HPTS in the presence of one equivalent of either **CP-2** (4.3 μM) or **CP-3** (4.3 μM). For example, we observed lifetimes of 2.67 ± 0.2 (24%), 9.59 ± 0.5 (72%) and 0.27 ns (4%) in the presence of **CP-2** (Fig. S6, ESI†). However, upon the addition of one equivalent of **CP-4** (3.1 μM), less significant changes in the lifetime of HPTS (3.3 μM) were observed. Nevertheless, upon increasing the concentration of **CP-4** to 7.2 equivalents (23.8 μM), a tri-exponential decay with lifetimes of 1.41 (20%), 7.21 ± 0.3 (30%) and 0.2 ns (50%) were observed (inset of Fig. 5).

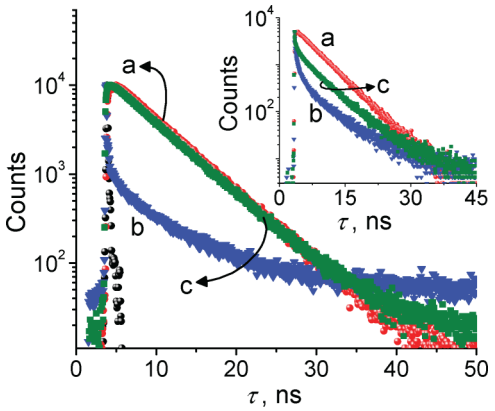


Fig. 5 Fluorescence decay profiles of HPTS (7 μM) (a) alone, (b) and (c) in the presence of **CP-1** (6.3 μM) with the addition of GTP (1.6 mM). Inset shows decay profiles HPTS (7 μM) (a) alone, (b) and (c) in the presence of **CP-1** (6.3 μM) and **CP-4** (24 μM). Excitation wavelength, 375 nm.

The changes in the optical properties of HPTS in the presence of different cyclophanes indicate the formation of stable complexes. The nature and strength of the complex formed between HPTS and these cyclophanes could be assessed by investigating the effect of temperature on the fluorescence of HPTS. It is expected that at higher temperatures these complexes would be disassembled leading to an enhancement in the emission corresponding to HPTS. When the temperature at which the equimolar complexes formed between HPTS and **CP-1**, **CP-2** and **CP-3** was raised from 298 to 358 K, we observed a regular increase in the emission intensity of HPTS, indicating a gradual dissociation of the complex at these temperatures (Fig. 6 and Fig. S7, ESI†). In contrast, when the temperature of a mixture of HPTS and **CP-4** was raised from 298 to 358 K, only negligible changes were observed in the emission spectra (inset of Fig. 6).

To understand the mode of interaction between HPTS and **CP-4**, we monitored the binding event at different salt concentrations (Fig. S8, ESI†). We observed *ca.* 15% decrease in the fluorescence quenching of HPTS, with the addition of **CP-4** in the presence of 1 M NaCl, indicating that electrostatic interactions together with outside stacking plays a role in the interaction between HPTS and **CP-4**. Further, to understand the mechanism of binding of **CP-4**,

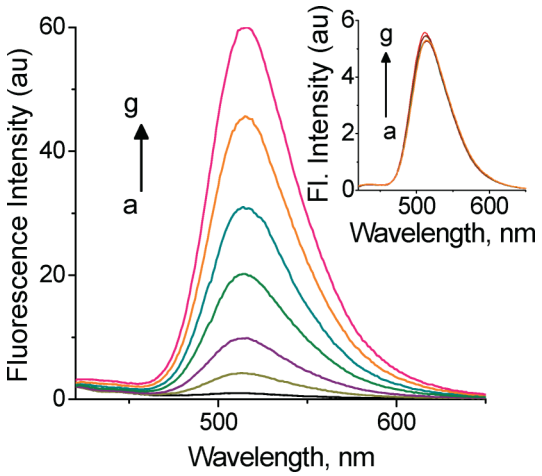


Fig. 6 Effect of temperature on the emission spectra of the complex [**CP-1**·HPTS] in phosphate buffer (10 mM, pH 7.4) and (inset) a mixture of **CP-4** and HPTS. (a) 298 to (g) 358 K. Excitation wavelength, 364 nm.

control experiments were carried out using the model open system **OC-4**. We observed significant quenching of the fluorescence intensity of HPTS with the addition of **OC-4** (Fig. S9, ESI†). The observation of similar results with **CP-4** and its corresponding open system, **OC-4** indicates that the presence of cavities is not essential for their interaction with HPTS. The temperature and ionic strength dependent titrations of HPTS with **CP-4** and also the results with **OC-4**, demonstrate that the interaction between HPTS and **CP-4** is not through inclusion complexation, but rather, could be attributed to electrostatic and/or outside stacking interactions, while **CP-1**, **CP-2** and **CP-3** form inclusion complexes with HPTS.

Selective recognition of 5′-ATP and 5′-GTP through displacement of HPTS

The competitive binding ability of the fluorescence indicator HPTS with various cyclophanes was effectively employed to develop a fluorescence indicator displacement (FID) assay for the detection of various nucleotides and nucleosides in the aqueous medium. Fig. 7 shows regular release of HPTS from the complex [**CP-1**·HPTS] by the gradual addition of 5′-GTP. The successive additions resulted in a regular enhancement in the fluorescence intensity of HPTS at 512 nm. In buffer, *ca.* 150-fold enhancement was observed at 1.6 mM of 5′-GTP, which led to the visual detection of 5′-GTP through "turn on" fluorescence intensity (inset of Fig. 7). Such a large excess of 5′-GTP was necessitated due to the strong binding of HPTS to **CP-1**. In contrast, addition of adenosine, 5′-AMP, 5′-GMP, 5′-ADP, 5′-GDP, cytidine 5′-triphosphate (5′-CTP) and uridine 5′-triphosphate (5′-UTP) showed negligible changes, whereas *ca.* 45-fold enhancement in intensity was observed with 5′-ATP (Fig. 8). The displacement of HPTS from the complex [**CP-1**·HPTS] by 5′-GTP was also reflected in the time-resolved fluorescence analysis. Upon the addition of 1.6 mM of 5′-GTP, we observed complete revival of the emission decay profile with lifetimes of 5.4 ± 0.1 (98%) and 9.6 ± 0.3 ns (2%) (Fig. 5). The observation of the species with a lifetime of 5.4 ns, clearly shows the presence of the displaced (free) HPTS.

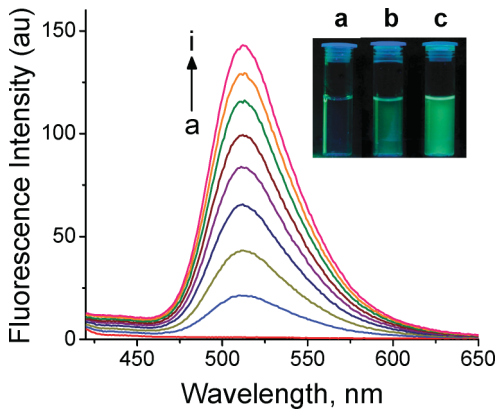


Fig. 7 Fluorescence indicator displacement (FID) from the complex [**CP-1**·HPTS] by 5′-GTP in 10 mM phosphate buffer (pH 7.4). [5′-GTP] (a) 0 and (i) 1.6 mM. Excitation wavelength, 364 nm. Inset shows the visual fluorescence of (a) the complex [**CP-1**·HPTS] and [**CP-1**·HPTS] in the presence of (b) 5′-ATP and (c) 5′-GTP in buffer.

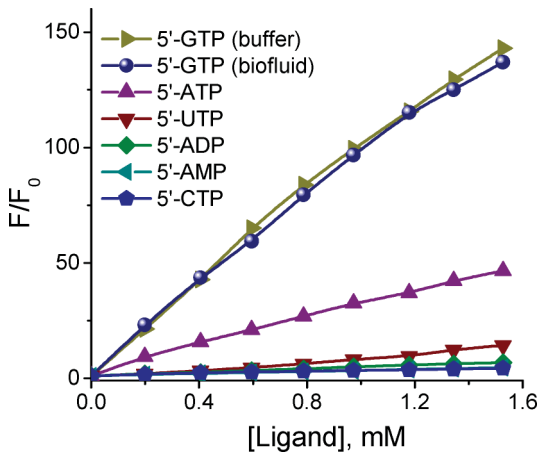


Fig. 8 Concentration dependent relative FID efficiency from the complex [**CP-1**·HPTS] by various nucleotides.

Furthermore, the selectivity of the assay was tested in the presence of 5′-ATP and other nucleotides and also in biological fluids. Results indicate that this assay is highly sensitive towards 5′-GTP even in the presence of various nucleotides. Similarly, in biological fluids, we observed *ca.* 140-fold enhancement in the fluorescence intensity with the addition of 5′-GTP, which is comparable to that obtained in buffer.

Subsequently, we have investigated the utility of the cyclophanes **CP-2**, **CP-3** and **CP-4** as receptors for FID assay. The successive additions of 5′-GTP to [**CP-2**·HPTS] and [**CP-3**·HPTS] resulted in regular enhancement in the fluorescence of HPTS (Fig. S10, ESI†). These observations are similar to those obtained with the complex [**CP-1**·HPTS]. However, a notable difference between these cyclophanes is the extent to which HPTS was displaced from these complexes by the addition of 5′-GTP. As shown in Fig. 9, the addition of 5′-GTP to the solutions of the complexes [**CP-2**·HPTS] and [**CP-3**·HPTS] resulted in fluorescence enhancement, but the extent of enhancement was found to be *ca.* 70- and 73-fold, respectively. In contrast, negligible displacement of HPTS was observed in the case of the cyclophane **CP-4** under these conditions (Fig. S11, ESI†) indicating that **CP-4** forms a non-

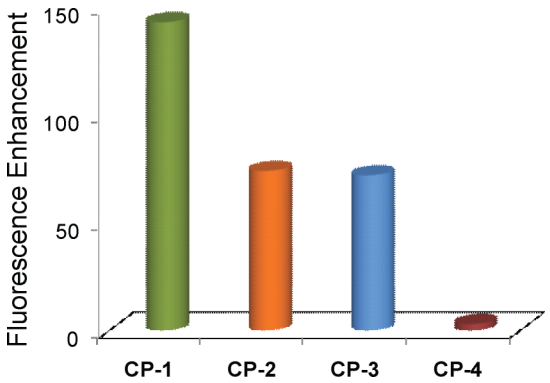


Fig. 9 Relative enhancement in fluorescence intensity observed by the displacement of HPTS from various complexes [**CP**·HPTS] with the addition of 5′-GTP.

encapsulated static complex with HPTS involving electrostatic interactions. These results demonstrate that the cyclophanes **CP-1**, **CP-2** and **CP-3** form non-fluorescent HPTS encapsulated complexes, from which HPTS can be selectively and competitively displaced with 5′-GTP and 5′-ATP, but with different efficiencies, depending on the stability of the complexes.

Evaluation of the binding thermodynamics through ITC

Isothermal titration calorimetry (ITC) is a valuable tool to study the thermodynamics of ligand/nucleotide binding, providing not only the binding constants (K_{ass}), but also important data on the relative significance of enthalpic *versus* entropic factors associated with the binding interactions.[25] Evaluation of these data provides a rational understanding of the encapsulation of the nucleotides within the cyclophane. For example, Fig. 10 shows the ITC curve for the interaction between the indicator HPTS and **CP-1**. As can be seen, the enthalpy change associated with each injection was exothermic, with a net heat release of $-13.3 \pm 0.1 \times 10^4$ J mol^{-1}. The net entropy change was -332 J mol^{-1}deg^{-1}, indicating that this process is enthalpically driven.

Table 2 summarises the thermodynamic parameters obtained from the ITC titrations for the interaction of **CP-1** and **CP-4** with HPTS and 5′-GTP. The entropy change indicates that the guest molecule becomes spatially confined in the cyclophane cavity after encapsulation, thus bringing out ordering in the system. Compared to **CP-1**, **CP-4** shows an increase in its entropy change, which indicates that the nature of the complex formed is different from that of **CP-1**. Further, these results support outside stacking and electrostatic interactions as the main interactions involved in the complexation.

Table 2 Thermodynamic parameters obtained for the various cyclophanes with HPTS and nucleotides[a]

	K_{ass} M^{-1}, 10^4	ΔH J mol^{-1}, 10^4	ΔS J mol^{-1}deg^{-1}	ΔG^b J mol^{-1}, 10^4
CP-1+ HPTS	44.0 ± 0.2	−13.3 ± 0.1	−332	−12 ± 0.1
CP-1+ 5′-GTP	87.5 ± 1.1	−3.1 ± 0.04	13.2	−3.1 ± 0.01
CP-4+ HPTS	25.5 ± 0.1	−5.1 ± 0.1	−67.5	−4.9 ± 0.01
CP-4+5′-GTP	17.7 ± 0.4	−2.8 ± 0.7	6.91	−2.8 ± 0.02

[a] Average of more than three independent experiments. [b] Calculated using the equation $\Delta G = \Delta H - T\Delta S$.

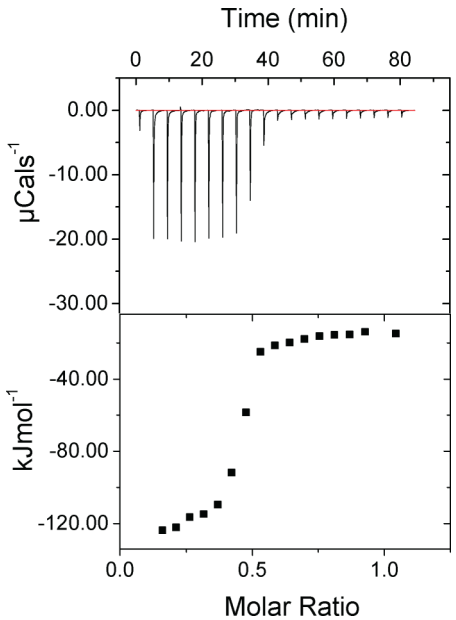


Fig. 10 ITC titration of **CP-1** with HPTS in 10 mM phosphate buffer, pH 7.4. (A) Differential power recorded in the experiment; (B) Integration of areas under peaks corresponding to the amount of heat released upon the addition of **CP-1** to HPTS as a function of the molar ratio ([**CP-1**]/[HPTS]).

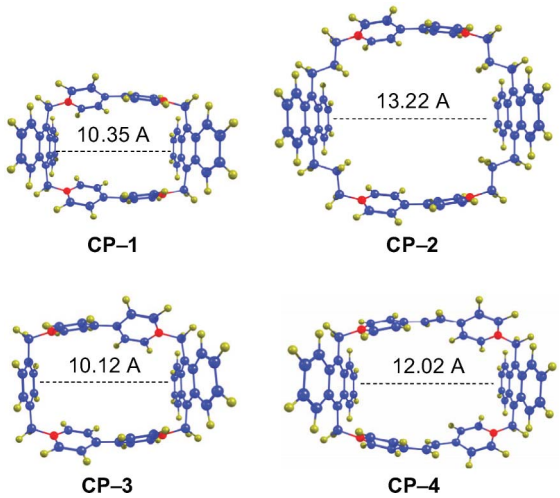


Fig. 11 Optimized geometries of **CP-1**, **CP-2**, **CP-3** and **CP-4**.

In order to gain further information on the cyclophane binding behaviour, we have studied the binding thermodynamics for the association of 5′-GTP with the various cyclophanes. **CP-1** exhibits a binding constant of K_{ass} = 87.5 ± 1.1 × 10^4 M^{-1} with 5′-GTP. The thermodynamic parameters calculated using ITC technique provides a quantitative evaluation of the different non-covalent interactions including the electrostatic interactions.[25b] The lower Δ*G* value of the **CP-1**·5′-GTP complex, in contrast to **CP-1**·HPTS complex having Δ*G* value of −12 ± 0.1 × 10^4 J mol^{-1}, indicates that although 5′-GTP can effectively displace HPTS from the cyclophane cavity, it can only revive <50% of the fluorescence of HPTS. The entropy change in the interaction between 5′-GTP and **CP-1** can be attributed to the loss of freedom upon encapsulation of the guest molecule in the cavity. The **CP-4**·5′-GTP complex shows a free energy change (Δ*G* = −2.8 ± 0.02 × 10^4 J mol^{-1}), which should essentially indicate an efficient displacement of HPTS from the **CP-4**·HPTS complex. However, the lack of a rigid cavity and the outside stacking of HPTS precludes the displacement of HPTS from the complex. The various non-covalent forces like π–π stacking, hydrophobic, electrostatic and Van der Waals interactions, constitute the enthalpic contribution of the binding process, while the changes in solvation, translational and conformational entropy contribute to the total entropy of the system. All the cyclophane systems show a negative value of Δ*H* indicating that non-covalent forces play a major role in the recognition process, which in turn substantiates the importance of the aromatic moiety, bridging unit and the cavity size on the biomolecular recognition.

Role of cavity size on biomolecular recognition

Fig. 11 shows the optimized geometries[26] which reveals an interplanar distance of 10.35, 13.22, 10.12 and 12.02 Å between the two aromatic units for the cyclophanes **CP-1**, **CP-2**, **CP-3** and **CP-4**, respectively. Based on the experimental evidence, the selective binding of **CP-1** to nucleotides such as 5′-GTP and 5′-ATP could be attributed to the synergistic effects of π–π stacking in combination with electrostatic interactions inside the cavity[27] The presence of the cavity in **CP-1** and three phosphate groups in nucleotides are essential for the selective recognition and for the formation of a stable 1 : 1 inclusion complex.

The interactions of **CP-2** and **CP-3** with HPTS resulted in similar observations, albeit with varying sensitivities. The mechanism of the quenching of fluorescence of HPTS by these cyclophanes has been attributed to an efficient electron transfer process from the excited state of HPTS to the viologen moiety on the basis of experimental evidence and the theoretically calculated favourable change in free energy (Δ*G* = −1.72 eV). In contrast, the quenching of fluorescence of HPTS by **CP-4** was less significant which has been attributed to the poorer encapsulating affinity and less favourable change in free energy (Δ*G* = −1.67 eV). In the competitive displacement assay, the fluorescent indicator, HPTS is successfully displaced by nucleotides and nucleosides from the complex [**CP-1**·HPTS]. Interestingly, 5′-GTP induced the maximum displacement resulting in a fluorescence enhancement of *ca.* 150-fold.

By virtue of having extended aromatic surface and more cationic charges when compared to other nucleotides, 5′-GTP and 5′-ATP exhibited better complexing ability with **CP-1** through the synergistic effects of electronic, π–π stacking and electrostatic interactions in the cavity. The results obtained with **CP-2**, consisting of a flexible and large cavity is indeed interesting, although not completely unexpected and provides crucial information that confirms the role played by the cavity size. The less significant selectivity exhibited by **CP-2** indicates its inability to form stable complexes which reiterates the importance of efficient packing between the host and the guest. The replacement of one of the anthracene moieties by a phenyl unit in **CP-3** reduces its aromatic surface which hinders π–π stacking interactions. This eventually leads to less efficient complexation which obviously results in less significant enhancement in FID (*ca.* 70-fold). The inability of **CP-4** to undergo selective encapsulation with any of the guest molecules

could be attributed to the presence of a larger cavity as well as to the poor acceptor strength of the bridging unit.

Conclusions

In summary, we have synthesized a series of novel cyclophane derivatives containing anthracene and/or phenyl moieties bridged together through different bridging units. The cavity size and flexibility in these systems could be varied depending on the bridging and spacer units. The interaction of **CP-1** with 5′-GTP and 5′-ATP resulted in the formation of a stable 1 : 1 inclusion complex, as demonstrated by steady-state and time-resolved absorption and fluorescence, NMR, electrochemical and isothermal calorimteric techniques. We have also demonstrated a highly sensitive and selective fluorescence indicator displacement assay for the recognition of 5′-GTP using the fluorescence indicator, HPTS. The biomolecular recognition properties of **CP-2** and **CP-3** were similar to that of **CP-1**, albeit with less sensitivity due to their larger cavity size and lesser aromatic surface, respectively. On the other hand, **CP-4** showed less significant binding affinity towards various guest molecules due to the presence of a larger cavity as well as its reduced electron acceptor strength. Thus, each of these molecules serves to explain one of the aspects of the recognition event.

Experimental Section

General techniques

The equipment and procedure for spectral recordings are described in our earlier publications.[28] Solvents and reagents were purified and dried by usual methods prior to use. Doubly distilled water was used in all the studies. All experiments were carried out at room temperature (25 ± 1 °C), unless otherwise mentioned. All melting points 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 or 500 MHz Bruker advanced DPX spectrometer. Mass spectra were recorded either on a JEOL AX503 (HRMS) or Shimadzu Biotech Axima CFRplus instrument equipped with a nitrogen laser in the linear mode (2,5-dihydroxybenzoicacid (DHB) as matrix) mass spectrometers. The electronic absorption spectra were recorded on a Shimadzu UV-Vis spectrophotometer. Fluorescence spectra were recorded on a SPEX–Fluorolog F112X spectrometer. Fluorescence lifetimes were measured using a IBH picosecond time correlated single photon counting system. The fluorescence decay profiles were deconvoluted using IBH datastation software V2.1 and minimizing the χ^2 values of the fit to 1 ± 0.1. Isothermal titration calorimetric titrations were carried out using a Microcal iTC$_{200}$ microcalorimeter (Microcal, Northampton, MA). All of the data were calculated with Origin ITC data-analysis software using one set of binding site model.

Calculation of association constants (K_{ass})

All the analyte solutions were prepared in buffer and the binding affinities were calculated using Benesi–Hildebrand eqn (1),

$$\frac{1}{A_f - A_{ob}} = \frac{1}{A_f - A_{fc}} + \frac{1}{K(A_f - A_{fc})[\text{Ligand}]} \quad (1)$$

where, K is the equilibrium constant, A_f is the 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.

Calculation of change in free energy (ΔG_{ET})

The change in free energy (ΔG_{ET}) for the photoinduced electron transfer reaction was evaluated according to the simplified Rehm–Weller eqn (2),[29]

$$\Delta G_{ET} = E_{ox} - E_{red} - E_{(0,0)} \quad (2)$$

where, $E_{(0,0)}$ is the singlet excitation energy in eV, E_{ox} is the oxidation potential of the donor and E_{red} is the reduction potential of the acceptor. For calculation, the oxidation potential of anthracene and HPTS were taken as 1.9 and 0.42 V,[30,31] respectively and the reduction potential of viologen and 1,2-bis(4-pyridinium)ethene were taken as –0.45 and –0.50 V,[32,33] respectively. Singlet state energy of anthracene and HPTS were 3.18 and 2.59 eV,[30,31] respectively. The change in free energy for the electron transfer from the singlet excited state of anthracene to viologen and 1,2-bis(4-pyridinium)ethene were found to be –0.83 and –0.78 eV, respectively. Similarly, the change in free energy value for the electron transfer from the excited state of HPTS to viologen and 1,2-bis(4-pyridinium)ethene were found to be –1.72 and –1.67 eV, respectively.

Processing of biofluid

Fresh blood samples were treated immediately with EDTA and centrifuged at 3000 rpm for 5 min and the supernatant was collected.[10b] This supernatant was divided into two portions. One of the portions was subjected to deproteinization by stirring with 20% trichloroacetic acid followed by centrifugation at 3000 rpm for 5 min and used for further studies after diluting 1000 times with water. The other portion was used as such after dilution with water. The pH of all blood samples used for the experiments was maintained at 7.4.

Materials

Anthracene, paraformaldehyde, sodium, diethylmalonate, and *p*-xylene were obtained locally and used as received. Lithium aluminium hydride, 1,2-bis(pyridin-4-yl)ethene and 4,4′-bipyridine as well as all nucleosides, nucleotides and the fluorescence indicator, 8-hydroxy-1,3,6-pyrene trisulfonate (HPTS), were purchased from Sigma–Aldrich and used as received. All experiments were carried out in 10 mM phosphate buffer (pH 7.4) containing 2 mM NaCl at room temperature (25 ± 1 °C), unless otherwise mentioned. Synthesis of 9,10-bis(bromomethyl)anthracene (**BMA**), mp >300 °C (lit. mp >300 °C),[34] 9,10-anthracene-bis(3′-propanol), mp 172–173 °C (lit. mp 174–175 °C)[35] **OC-1**, mp >300 °C (lit. mp >300 °C)[10] and **CP-1**, mp >300 °C (lit. mp >300 °C)[10] was achieved by reported procedures. Petroleum ether used was fraction with boiling range 60–80 °C.

Preparation of 9,10-bis(bromopropyl)anthracene (**BPA**): A mixture of 9,10-anthracene-bis-(3′-propanol) (0.34 mmol) and aqueous HBr (5 mL) was refluxed at 120 °C for 2 h. Upon

cooling to room temperature, 10 mL dichloromethane was added to the reaction mixture. After neutralising with aqueous sodium bicarbonate solution, the organic layer was separated, dried over anhydrous Na_2SO_4 and the solvent was evaporated to give the product which was purified by column chromatography over silica gel. Elution of the column with petroleum ether yielded 74% of **BPA**, mp 149–150 °C, ^{1}H NMR (300 MHz, $CDCl_3$, TMS) δ 8.24–8.27 (4H, m), 7.43–7.47 (4H, m), 3.71 (4H, t), 3.54 (4H, t), 2.25–2.34 (4H, m); ^{13}C NMR (75 MHz, $CDCl_3$, TMS) δ 132.2, 129.5, 125.3, 124.9, 34.0, 33.8, 26.5; HRMS (FAB) m/z calcd for $C_{20}H_{20}Br_2$ 420.1808, found 420.1938.

Preparation of 1,1′-(3,3′-(anthracene-9,10-diyl)bis(propane-3,1-diyl))di-4,4′-bipyridin-1-ium bishexafluorophosphate (**OC-2**): To a solution of 4,4′-bipyridine (1.19 mmol) in 5 mL dry acetonitrile was added **BPA** (0.24 mmol) in 5 mL dry acetonitrile over 30 min. The reaction mixture was then refluxed for 12 h and after cooling to room temperature the precipitated product was filtered and washed with dry CH_3CN. The residue was then dissolved in water, filtered and an aqueous solution of NH_4PF_6 was added to precipitate the product. The product was filtered and recyrstallised from acetone and dried in vacuum to yield 60% of **OC-2**, mp >250 °C, ^{1}H NMR (300 MHz, CD_3COCD_3, TMS) δ 9.38–9.40 (4H, d, J = 6 Hz), 8.64–8.66 (4H, d, J = 6 Hz), 8.95–8.96 (4H, m), 8.45–8.49 (4H, m), 8.15–8.17 (4H, m); ^{13}C NMR (75 MHz, CD_3COCD_3, TMS) δ 149.1, 145.6, 132.1, 130.1, 129.5, 126.1, 125.9, 125.7, 125.1, 123.0, 61.3, 32.5, 24.4. HRMS (FAB) m/z calcd for $C_{40}H_{36}N_4PF_6$ 717.71, found 717.74 $[M–PF_6]^+$

Synthesis of the cyclophane **CP-2**: To a solution of **OC-2** (0.058 mmol) in 5 mL dry acetonitrile, **BPA** (0.058 mmol) in dry acetonitrile (10 mL) was added slowly in 20 min. The reaction mixture was stirred and refluxed for 3 days and after cooling to room temperature, the precipitate obtained was filtered. It was then washed with dry acetonitrile, recrystallised from methanol and dried under vacuum to yield 52% of **CP-2**, mp. > 300 °C, ^{1}H NMR (300 MHz, [D_6]DMSO, TMS) δ 7.61–9.52 (32H, m), 5.06 (8H, s), 3.72 (8H, s), 2.41 (8H, s); ^{13}C NMR (75 MHz, [D_6]DMSO, TMS) δ 148.5, 145.9, 132.0, 129.0, 126.6, 125.6, 125.1, 60.8, 32.4, 24.3; MALDI–TOF MS m/z calcd for $C_{60}H_{56}N_4Br_4$ 1152.73, found 1152.80 $[M]^+$.

Synthesis of the cyclophane **CP-3**. A solution of the compound **OC-1** (1.2 mmol) and 1,4-bis(bromomethyl) benzene (0.8 mmol) in dry CH_3CN (80 mL) was heated under reflux at 80 °C for 24 h. The reaction mixture was cooled to room temperature to give a precipitate which was filtered and washed with dry CH_3CN (10 mL). The precipitate was recrystallised from a 1 : 1 mixture of water and methanol to yield 36% of the cyclophane **CP-3**, mp >300 °C; ^{1}H NMR (500 MHz, [D_6]DMSO, TMS) δ 6.02 (4H, s), 7.23–7.26 (4H, d), 7.72–7.78 (8H, m), 8.58–8.78 (12H, m), 9.18–9.21 (4H, t), 9.59 (4H, s); ^{13}C NMR (75 MHz, D_2O) δ 63.2, 70.9, 121.2, 123.2, 125.0, 126.2, 127.2, 130.4, 134.4, 135.8, 138.6, 139.1, 140.3, 141.3; HRMS (FAB) m/z Calcd for $C_{44}H_{36}N_4Br_2$ 780.5903. Found: 780.6964 $[M–2Br]^+$.

Synthesis of (*E*)-1,1′-(anthracene-9,10-diylbis (methylene))bis-(4-((*E*)-2-(pyridin-4-yl)vinyl)pyridinium) bishexafluorophosphate (**OC-3**): A solution of **BMA** (20 mmol) and (*E*)-1,2-di(pyridin-4-yl)ethene (33 mmol) in dry acetonitrile (150 mL) was refluxed for 8h. The reaction mixture was then cooled to room temperature, filtered, washed with acetonitrile and dried under vacuum. It was then dissolved in water and an aqueous saturated solution of ammonium hexaflurophosphate was added to precipitate the product **OC-3**, which was filtered and washed with water. The precipitate was purified by recrystallisation from acetone to yield 63% of **OC-3**, mp 238–240 °C, ^{1}H NMR (500 MHz, CD_3CN, TMS) δ 6.82 (4H, s), 7.60–7.78 (10H, m), 7.93–7.94 (2H, d), 8.05–8.07 (4H, d), 8.19 (4H, s), 8.37–8.39 (4H, t), 8.56–8.58 (4H, d), 8.66 (4H, s), ^{13}C NMR (125 MHz, CD_3CN, TMS) δ 55.7, 122.7, 123.2, 124.0, 125.2, 128.3, 129.0, 131.4, 137.1, 143.5, 144.9, 147.3, 152.6; HRMS (FAB) m/z calcd for $C_{40}H_{32}N_4P_2F_{12}$ 858.6372, found 858.6358 $[M]^+$.

Synthesis of the cyclophane **CP-4**: A solution of **BMA** (0.27 mmol) and **OC-3** (0.27 mmol) in dry acetonitrile (30 mL) was refluxed for 24 h. The reaction mixture was cooled to room temperature, filtered, washed with hot acetonitrile and dried under vacuum. The precipitate was then purified by recrystalliation from methanol to yield 22% of **CP-4**, mp >300 °C; ^{1}H NMR (500 MHz, [D_6]DMSO, TMS) δ 6.98 (8H, s), 7.82–7.83 (12H, m), 8.17–8.18 (8H, d), 8.39–8.46 (8H, t), 8.73–8.77 (8H, d); ^{13}C NMR (125 MHz, CD_3CN, TMS) δ 56.0, 123.9, 125.3, 125.9, 128.3, 131.4, 133.7, 143.8, 151.3; MALDI-TOF MS m/z calcd for $C_{56}H_{44}N_4Br_4$ 1092.59, found 1092.69 $[M]^+$.

Synthesis of the 4,4′-(1*E*,1′*E*)-2,2′-(1,1′-(anthracene-9,10-diylbis(methylene))bis(pyridinium-4,1-diyl))bis(ethene-2,1-diyl)-bis(1-butylpyridinium) bromide (**OC-4**): A solution of 1-butyl bromide (0.27 mmol) and **OC-3** (0.27 mmol) in dry acetonitrile (30 mL) was refluxed for 24 h. The reaction mixture was cooled to room temperature, filtered, washed with hot acetonitrile and dried under vacuum. The precipitate was then purified by recrystalliation from methanol to yield 30% of **OC-4**, mp >300 °C; ^{1}H NMR (500 MHz, D_2O) δ 0.55–0.58 (6H, t), 0.97–1.02 (4H, m), 1.59–1.64 (4H, m), 4.21 (4H, s), 6.56–8.62 (4H, s), 7.42–7.44 (8H, m), 7.78–7.82 (8H, m), 8.03–8.05 (4H, m), 8.38–8.4 (4H, d), 8.43–8.44 (4H, d); ^{13}C NMR (125 MHz, D_2O) δ 12.6, 18.7, 32.5, 55.9, 61.3, 123.8, 125.3, 125.7, 125.9, 128.6, 131.3, 133.3, 134.0, 143.8, 144.4, 150.8, 151.6; HRMS (FAB) m/z calcd for $C_{48}H_{50}N_4$ 682.40, found 681.8 $[M–2Br]^+$.

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