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ARTICLE TYPE

An unique fluorescence biosensor for selective detection of tryptophan and histidine†

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A novel photoinduced electron transfer (PET) based substituted calix[4]arene fluroionophore synthesized has been used for the selective recognition of tryptophan (L-Trp.) and histidine (L-His.) by emission spectra. The detection limit of synthesized receptor was found to be 0.00826 nM for L-Trp. and 0.00158 nM for L-His. Moreover, this probe has been applied for recognition L-Trp. and L-His. from blood serum.

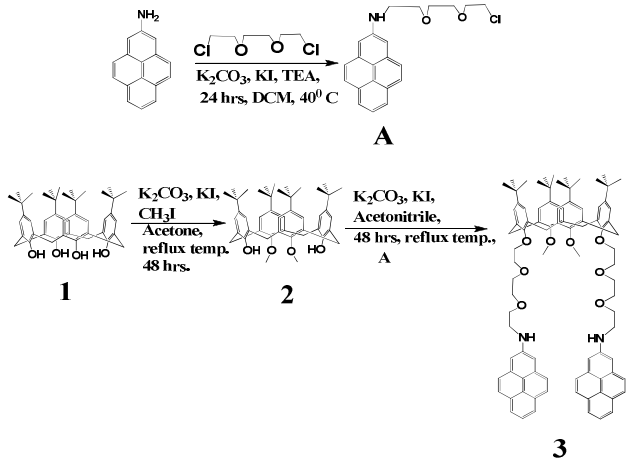
Research on molecular recognition of amino compounds, such as biogenic amines, amino acids, peptides, proteins, and carbohydrate like essential substrates in biological processes, by synthetic receptors is an issue of great concern from both a supramolecular chemistry and analytical application point of view[1]. Among naturally occurring amino acids L-Trp. is the most fluorescent, the indole ring of L-Trp. have recently been the subject of much investigation due to the functional and structural prominence of such interactions in chemistry and biology[2]. Recently, it was found that the reduced nutritional state of patients with chronic kidney disease could be attributed to the deficiency of L-His. L-His. rich proteins are found to play many important roles in humans and their abnormal level could indicate a variety of diseases[3]. Therefore, selective detection of L-His. and L-Trp. in biological fluids have become a significant objective and a number of methods have been developed for this purpose.

The leading issue in the design of any active chemosensor is the association of a selective molecular recognition event with a physical signal highly sensitive to its occurrence. Changes in both the absorption and emission of light can be employed as signals provided appropriate chromophores or fluorophores are available, and two important classes of sensors are those of the optical and fluorimetric types. Fluorescecne technique is commonly considered superior than other electrochemical methods[4-11] because of its sensitivity, selectivity, response time and in situ monitoring ability. This route of detection embroils, a fluorophore module is the site of both photonic transactions of excitation and emission. A receptor module is liable for guest complexation and decomplexation. A spacer module holds the fluorophore and receptor close to, but separate from, each other. The design of the fluoroionophore is crucial to this technique and requires a high number of aromatic fluorophores in close proximity to create van der Waals contact and π- π stacking. Under these conditions, electronic excitation of one ring can cause an enhanced interaction with its neighbour, leading to what is termed as an excited-state dimer or an excimer for a fluoroionophore. In recent years, digital colour tone for fluorescence sensing: a direct comparison of intensity, ratiometric and hue based quantification is demanding area of detection.[12-13] Extensive research has been done during last decade on highly π – π delocalized planar systems such as pyrene, quinoline, coumarine, antharcene and dansyl chloride which have been used for this purpose[14].

Calixarenes, with their unique three-dimensional exterior, are one of the superlative known host molecules along with cyclodextrins, cucurbiturils, cryptands, and crown ethers. Calix[4] arene based chemosensors have engrossed a great deal of consideration due to their ability to visually sense analytes with high sensitivity as well as fast response time[15]. The mechanism of fluorescence involved in the calix[4]arene system is mainly PET[16], Förster (Fluorescence) resonance energy transfer (FRET)[17], photoinduced charge transfer (PCT)[18] and intramolecular charge transfer (ICT)[19] used for molecular recognition. Recently, we have reported an ICT[20], PET[21] and PET with ICT[22] fluoroionophores for selective detection and determination various cations and anions. These results prompted us to design novel dual ion sensing fluroionophore linked with pyrene for selective detection of biomolecules such as L-Trp. and L-His.

Herein we propose, a simple, sensitive and selective method, with a low detection limit and fast response time by using synthesized 5,11,17,23 tetra-tert-butyl 26, 28 dimethoxy 25, 27 diamino pyrene 2-yl calix[4]arene **(TDPC)** receptor. This fluoroionophore has been applied for selective recognition of L-Trp. and L-His. in presence of other amino acids. For the first time, we have synthesized TDPC ligand to provide rigidity as well as flexibility for molecular sensing. There has been no report yet on TDPC which is used for dual recognition of L-Trp. and L-His. via photoinduced electron transfer (**PET**) fluorescence mechanism.

Pyrene is well established and extensively studied fluorophore for transition metal detection as well as anions[23]. The fluorescence spectra of the compound were recorded in acetonitrile in presence of 100-fold excess of various amino acids which were prepared in water and slight amount of concentrated HCl. We evaluated the interaction of ligand with L-Trp. and L-His. in the presence of other amino acids. To explore sensing ability of our fluoroionophore, we added 1×10^{-6} M solutions of L-Trp. and L-His. into 1×10^{-6} M solution to the fluoroionophore (TDPC) 1×10^{-6} M. We observed that L-Trp. enhances the fluorescence intensity and L-His. quenches the fluorescence intensity via photoinduced electron transfer from free receptor to guest molecule. Fluorescent indicating via the PET approach is distinguished by its intrinsically supramolecular nature since different components perform each one (or more) of the required functions. This also means that guest-binding properties of the components allow the quantitative prediction of the signalling parameters of the systems



Scheme: 1 Synthetic route for 5, 11, 17, 23-tetra t-butyl-26, 28-dimethoxy 25, 27 diamino pyrene 2-yl calix[4]arene (**TDPC**)

have natural "all or none" switch ability: guest-induced "off-on" and "on-off" fluorescence probes are both designable.

quantitatively through the fluorescence emission intensity change. Spectroscopic properties of TDPC was examined in mixed aqueous organic medium (acetonitrile/aqueous phosphate buffer (8:2, v/v; pH=7.2). Before addition of L-Trp., ligand has intensity of 17,100 at 445 nm and 471 nm wavelength. After gradual addition of L-Trp. into the ligand, we observed significant increase in the fluorescence intensity due to hydrogen bonding of L-Trp. with ligand. Additionally L-Trp. has good fluorescence property, when it comes in contact with π-rich pyrene molecule it enhances fluorescence intensity via PET mechanism. Likewise in the case of L-His., we observed quenching phenomenon in the fluoresce intensity due to hydrogen bonding between oxygen atom of ligand with L-His. molecule. From these emission studies, we have calculated the limit of detection (LOD) for the synthesized probe and it was found to be 0.00826 nM for L-Trp. and 0.00158 nM for L-His. (**Fig. 1A and B**). Stern-Volmer plots are worthwhile for appreciative the mechanism of emission quenching[22] and hence were utilized to probe the nature of the quenching process in the complexation of L-His. with ligand TDPC. From the data, dynamic or static quenching processes can be determined by plotting relative emission intensities (I_0/I) against quencher concentration [Q]. Expressed by the following equation (1), the slope of the plotted line yields Ksv(the static quenching constant).

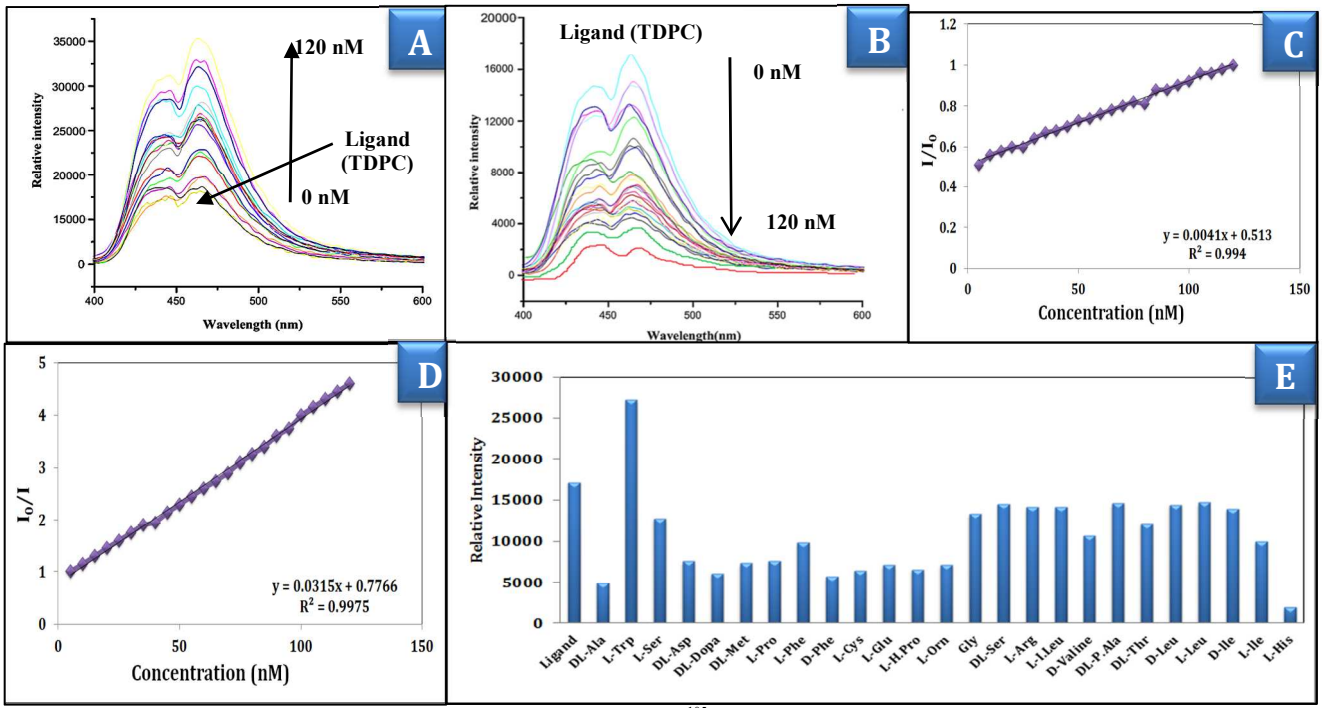


Fig. 1 (A and B) Emission spectra of increasing concentration of L-Trp (0, 5, 10,….120 nM) and L-His (0, 5, 10,….120 nM) with TDPC (1×10^{-8} M) (C and D) Linearity curve of TDPC (1×10^{-8} M) with L-Trp (0-120 nM) and Stern-Volmer plot with L-His(0-120 nM) (E) Selectivity plot of TDPC (1×10^{-6} M) with amino acids(DL-Alanine, L-Tryptophan, L-Serine, DL-Aspartic acid, DL-DOPA, DL-Methonine, L-Proline, L-Phenyl alanine, D-Phenyl alanine, L-Cystine, L-Glutamic acid, L-Hydroxyproline, Glycine, DL-Serine, L-Arginine, L-Isoleucine, D- Valine, DL-Phenyl alanine, DL-Threonine, D-Leucine, L-Leucine, D-lle, L-lle, L-Histidine (1×10^{-6} M).(λex= 380 nm and λem = 440 nm)

$$I_0/I = 1 + K_{sv}[Q] \text{ …….. (1)}$$

To explore the sensitivity of our fluorescent probe, we evaluated the probe sensitivity by optimizing different concentrations of L-Trp. and L-His. from 5 nM to 120 nM with ligand and estimated

If the evolution of I_0/I plots, according to the concentration of quencher, is linear for the whole range of quencher concentrations, fluorescence quenching can be attributed either to being purely

dynamic, or purely static, the latter mechanism being due to the formation of a ground-state non-fluorescent complex. In contrast, if the ratio I_0/I are not linear and show an upward curve at higher quencher concentrations, the fluorescence quenching mechanism can be attributed to the presence of simultaneous dynamic and static quenching. In our case, typical linear plots for L-His. with TDPC was observed which indicate that fluorescence quenching is purely static because of a non-fluorescence ground state complex between L-His.[23] with TDPC ligand. The calibration curve shows good linearity with correlation coefficient of 0.994 for L-Trp and 0.997 for L-His (**Fig. 1C and D**). Our experimental result evidently shows sensitivity and selectivity towards L-Trp. and L-His.

To know about selectivity of our fluoroionophore in presence of other amino acids, we have carried out emission titration in presence of other amino acids which is displayed in **Fig.S1 (ESI†).** The results indicated that other amino acids did not produce noticeable effects on the emission spectra as compared to L-His. and L-Trp. It may be because L-His. and L-Trp. make stronger hydrogen bonding than any other amino acids which lead to change in fluorescence intensities which has been shown in **Fig. 1E.**

Binding constants for fluoroionophore was determined by using emission titration data following the previously reported articles[24]. The titration experiment was carried out following the method described in the experimental section **(ESI †).** The plots log [(F_0 - F)/(F-Fœ)] vs.log[M] for selected compounds are shown in **Fig. S2–S3 (ESI†)**.The observed binding constant from fluorescence spectra for tryptophan is 10.21×10^8 and for histidine 12.13×10^8. We have also calculated binding constant for DL-Ala and L-Ser. for major interferent during this analysis. **Fig. S4–S5 (ESI†)**.

To support the results obtained from the fluorescence studies, similar titrations were carried out even by absorption spectroscopy. The absorption spectral studies were carried out for the titration of L-His. and L-Trp. with the ligand in acetonitrile. With addition of 35 nM of L-Trp. and 30 nM of L-His., the absorbance at 327nm and 357 nm increase dramatically and new bands at 366 nm , 415 nm and 381 nm, 438 nm upturn which show a bathochromic shift (λ_{max}) as shown in **Fig. S6 and S7 (ESI†),** while other amino acids exhibit no significant change in the absorption spectra. The shifting of bands shows the interaction of L-Trp. with the oxygen atom of fluoroionophore and also hydrogen bonding between them. We also performed UV-titration by varying the concentration of L-Trp. (10 nM –35 nM) and L-His. (10 nM – 30 nM) to study absorption changes **(Fig. S8 and S9, ESI†).**

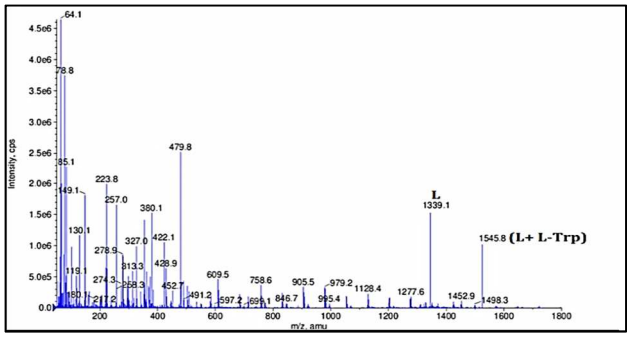


Fig.2 ESI mass spectrum showing the isotopic peak pattern of a molecular ion peak for the 1:1 complex formed between TDPC with L-Trp.

Mass spectra of ligand were recorded in acetonitrile upon addition of an excess amount of L-Trp. and L-His. where the spectrum shows molecular ion peak m/z at 1339.1 and in the presence of L-Trp. and L-His., it exhibits the formation of 1:1 complex by showing peak at 1545.8 (**Fig.2**) and 1495.1 respectively **(Fig. S10, ESI†)** which will give assurance of binding of amino acids with ligand. The stoichiometry of the complex formed (1:1) was also derived based on Job's plot**. (Fig.S11-S12, ESI†)**.

^{1}H NMR investigation was performed to get insight the binding mechanism and also to find out effect of L-Trp. and L-His. on ^{1}H NMR. We have exposed here comparison spectra of ligand in presence of L-Trp. and L-His. We perceived shifting of –NH peak to δ = 8.41 ppm from δ = 8.59 ppm for L-Trp. suggesting hydrogen bonding between L-Trp. with ligand which has been displayed in **Fig. S13 (ESI †).**

For L-His., due to strong hydrogen bonding with ligand, the –NH peak at δ = 8.59 disappears upon addition of 10 fold excess L-His. into the ligand which has been presented in **Fig. 3**. We have also perceived that the fluorescence sensor gives the maximum enhancement and quenching at pH 7. The plot of pH study is given in the **Fig. S14-S15 (ESI †).**

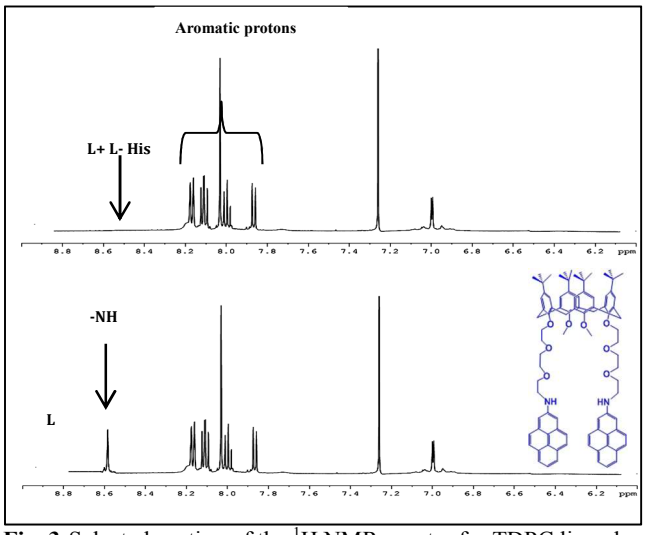


Fig. 3 Selected portion of the ^{1}H NMR spectra for TDPC ligand and recorded in $CDCl_3$ upon addition of 10 equivalent amount of L-His.

To assess whether our ligand can be of any use in the recognition of amino acids by naked eye detection, ligand was titrated with various amino acids in acetonitrile by maintaining a 1: 1 mole concentration and observing the corresponding colour changes. The yellow colour ligand was observed to be almost dark brown upon addition of L-His and no significant colour change was observed upon addition of other amino acids. **(Fig S16, ESI†)**.

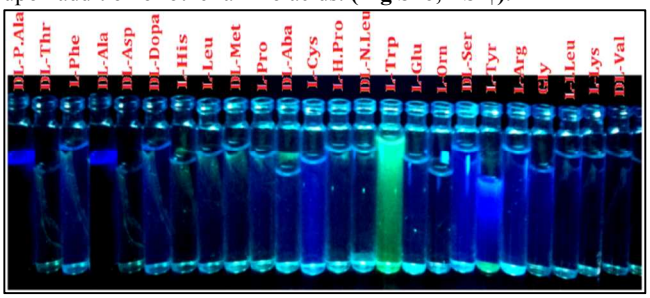


Fig.4 Visual colour changes obtained upon addition of TDPC with various amino acids in 365 nm of UV in the dark

The sensing property of ligand for L-Trp. has been supported by observing the fluorescent colour change visually in the presence of different amino acids under an incident light of 365 nm and ligand was found to give deep yellow fluorescence only in the case of L-Trp. in **Fig. 4**. Further, this study was carried out in the presence of other amino acids added to an initial solution possessing a 1: 2 ligand to L-Trp. ratio and we found no changes in the deep yellow fluorescence suggesting the stability of complex. This investigation suggests the ability of a fluoroionophore for sensing L-Trp. in the presence of other competitive amino acids which is also supported by doing emission titration with TDPC ligand and L-Trp complex with other amino acids in **Fig.S17 (ESI†)**. The schematic representation of amino acid is displayed in **Fig.5** and proposed binding mechanism through hydrogen bonding is presented in **Fig. S18 (ESI †).**

We have also optimized our general procedure for detection of L-Trp. and L-His. The fluorescence of TDPC ligand was quenched by the addition of L-His. and then recovered by sequential addition of L-Trp. We investigated the factors influencing the fluorescence of the system. The fluorescence of TDPC decreased quickly in the presence of L-His, and reached stability around 45 seconds. The recovery of the TDPC- L-His was accomplished in 45 seconds. Therefore, 45 second was chosen for further experiments. **Fig. S19-S20 (ESI †).**

This PET probe is applied for the analysis of L-Trp. and L-His. in blood serum. The standard addition method was applied to evaluate the validity of the proposed sensor. The preparation of serum samples and analytical results for the blood samples are shown in **Table S1-S2 (ESI†).** The result obtained with excellent recovery of spiked L-Trp. and L-His. ranged from 101 to 105%, illustrating the validity of the developed technique.

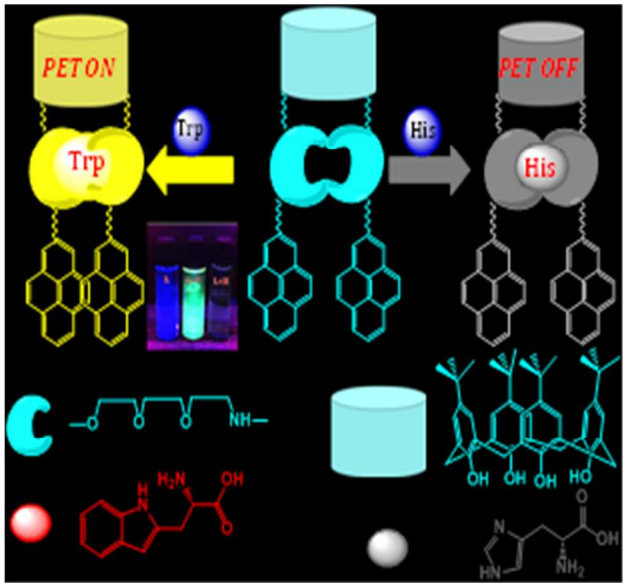


Fig.4. Schematic design of TDPC PET probe with L-Trp and L-His.

In conclusion, we have reported for the first time highly selective and sensitive dual biomolecule PET fluorescence probe for L-Trp and L-His. Proposed fluorescence probe has lower sensing limit as well as high selectivity towards L-Trp (0.00826 nM) and L-His (0.00158 nM). Furthermore, the present system has been applied for blood serum sample for selective detection of L-Trp and L-His with 101 to 105% recovery. This highly sensitive, selective, easy and cost-effective fluorometric method will provide great interest for routine analysis of L-Trp and L-His.

Acknowledgements

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† **Electronic Supplementary Information (ESI)** contains materails and metods, Synthesis procedure, UV-spectra,real sample analysis result table, ESI-MASS spectra, ^{1}H NMR spectra, selectivity plot by emission spectra, and pH study graph.

An unique fluorescence biosensor for selective detection of tryptophan and histidine †

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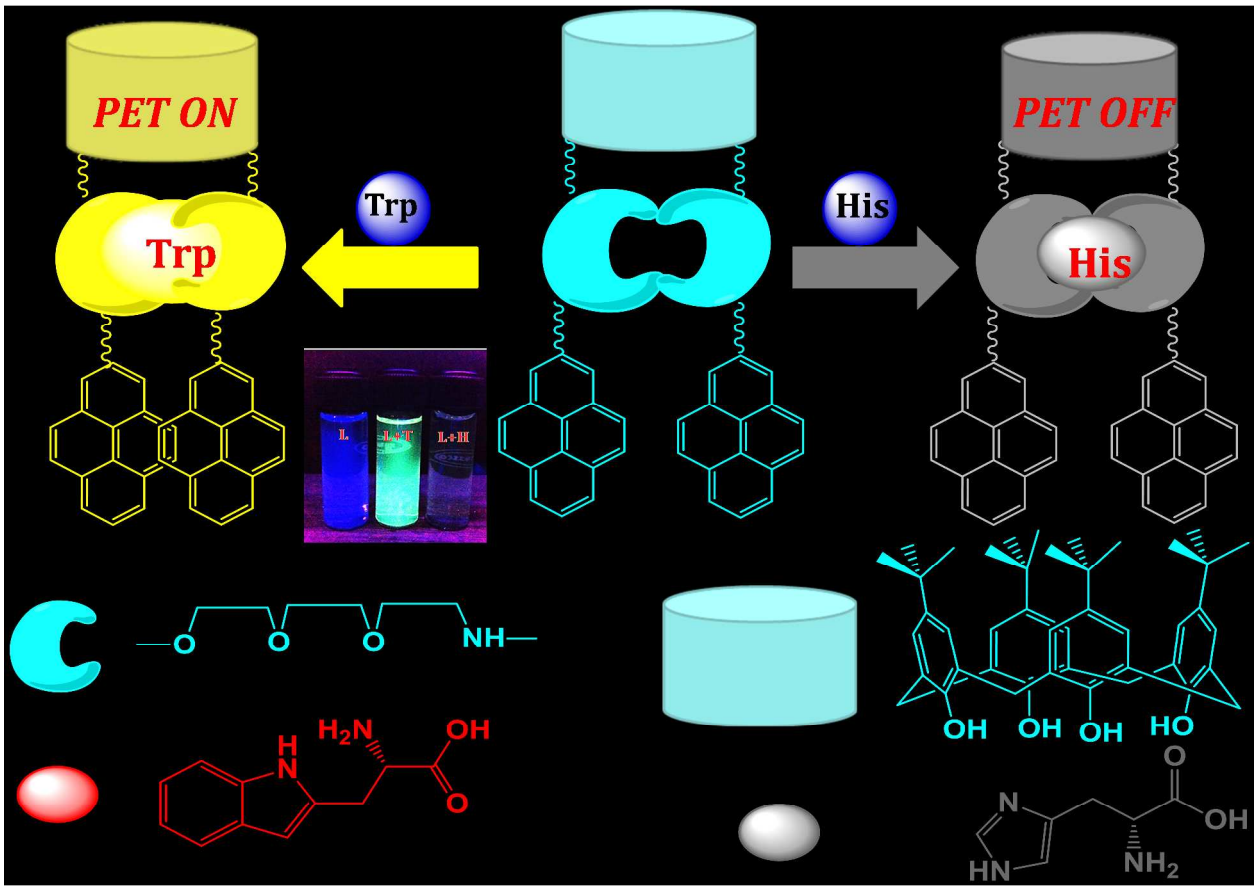
Graphical Abstract



Abstract:

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