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A 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 fluoroionophore has been used for the selective recognition of tryptophan (L-Trp) and histidine (L-His) by emission spectroscopy. The detection limit of the 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 the recognition of 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 has 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 has 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. The fluorescence technique is commonly considered superior to other electrochemical methods[4–11] because of its sensitivity, selectivity, response time and *in situ* monitoring ability. In this detection technique, 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 the receptor close together, 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, the digital colour tone for fluorescence sensing: a direct comparison of intensity, ratiometric and hue based quantification is a demanding area of detection.[12,13] Extensive research has been done during the last decade on highly π–π delocalized planar systems such as pyrene, quinoline, coumarine, anthracene 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 ICT,[20] PET (ref. 21) and PET with ICT (ref. 22) fluoroionophores for selective detection and determination of various cations and anions. These results

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prompted us to design novel dual ion sensing fluoroionophores 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 a synthesized 5,11,17,23-tetra-*tert*-butyl-26,28-dimethoxy-25,27-diamino pyrene-2-yl-calix[4]arene (TDPC) receptor (Scheme 1). This fluoroionophore has been applied for selective recognition of L-Trp and L-His in the presence of other amino acids. For the first time, we have synthesized a 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* the photoinduced electron transfer (PET) fluorescence mechanism.

Pyrene is a well established and extensively studied fluorophore for detection of transition metals as well as anions.[23] The fluorescence spectra of the compound were recorded in acetonitrile in the presence of 100-fold excess of various amino acids which were prepared in water and a small amount of concentrated HCl. We evaluated the interaction of the ligand with L-Trp and L-His in the presence of other amino acids. To explore the sensing ability of our fluoroionophore, we added 1×10^{-6} M solutions of L-Trp and L-His into a 1×10^{-6} M solution of the fluoroionophore (TDPC). We observed that L-Trp enhances the fluorescence intensity and L-His quenches the fluorescence intensity *via* photoinduced electron transfer from the free receptor to the guest molecule. Fluorescent indication *via* the PET approach is distinguished by its intrinsically supramolecular nature since different components perform one (or more) of the required functions. This also means that guest-binding properties of the components allow the quantitative prediction of the signaling parameters of the systems (Scheme 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 the ligand and estimated quantitatively through the fluorescence emission intensity change. Spectroscopic properties of TDPC were examined in a mixed aqueous organic medium (acetonitrile–aqueous phosphate buffer) (8 : 2, v/v; pH = 7.2). Before addition of L-Trp, the ligand has an intensity of 17 100 at 445 nm and 471 nm wavelength. After gradual addition of L-Trp into the ligand, we observed a significant increase in the fluorescence intensity due to hydrogen bonding of L-Trp with the ligand. Additionally L-Trp has good fluorescence properties, when it comes in contact with a π-rich pyrene molecule it enhances fluorescence intensity *via* the PET mechanism. Likewise in the case of L-His, we observed a quenching phenomenon in the fluoresce intensity due to hydrogen bonding between the oxygen atom of the ligand and the 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).

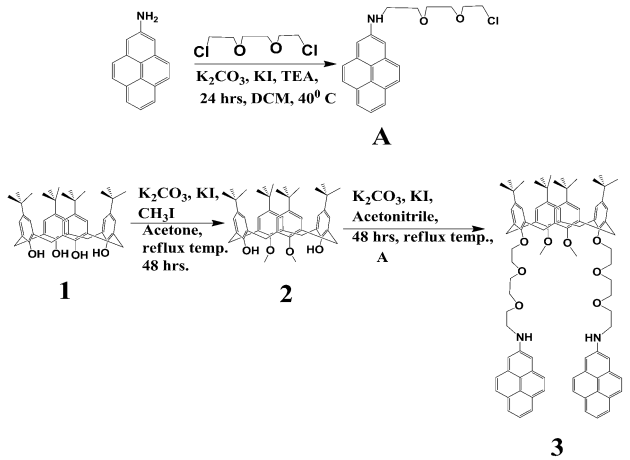
Stern–Volmer plots are worthwhile for appreciating the mechanism of emission quenching[22] and hence were utilized to probe the nature of the quenching process in the complexation of L-His with ligand TDPC. From the data, dynamic or static quenching processes can be determined by plotting relative emission intensities (I_0/I) against quencher concentration [Q]. As expressed by eqn (1), the slope of the plotted line yields K_{sv} (the static quenching constant).

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

If the evolution of I_0/I plots, according to the concentration of the 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 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 were observed which indicate that fluorescence quenching is purely static because of a non-fluorescence ground state complex between L-His (ref. 23) and the TDPC ligand. The calibration curve shows good linearity with a 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 the selectivity of our fluoroionophore in the presence of other amino acids, we have carried out emission titration in the 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 form stronger hydrogen bonds than any other amino acids which leads to a change in fluorescence intensities which has been shown in Fig. 1E.

Binding constants for fluoroionophores were 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 $\log[(F_0 - F)/(F - F_\infty)]$ *vs.* log[M] for selected compounds are shown in Fig. S2 and S3 (ESI†).The observed binding constant from fluorescence spectra for tryptophan is 10.21×10^8 and for



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

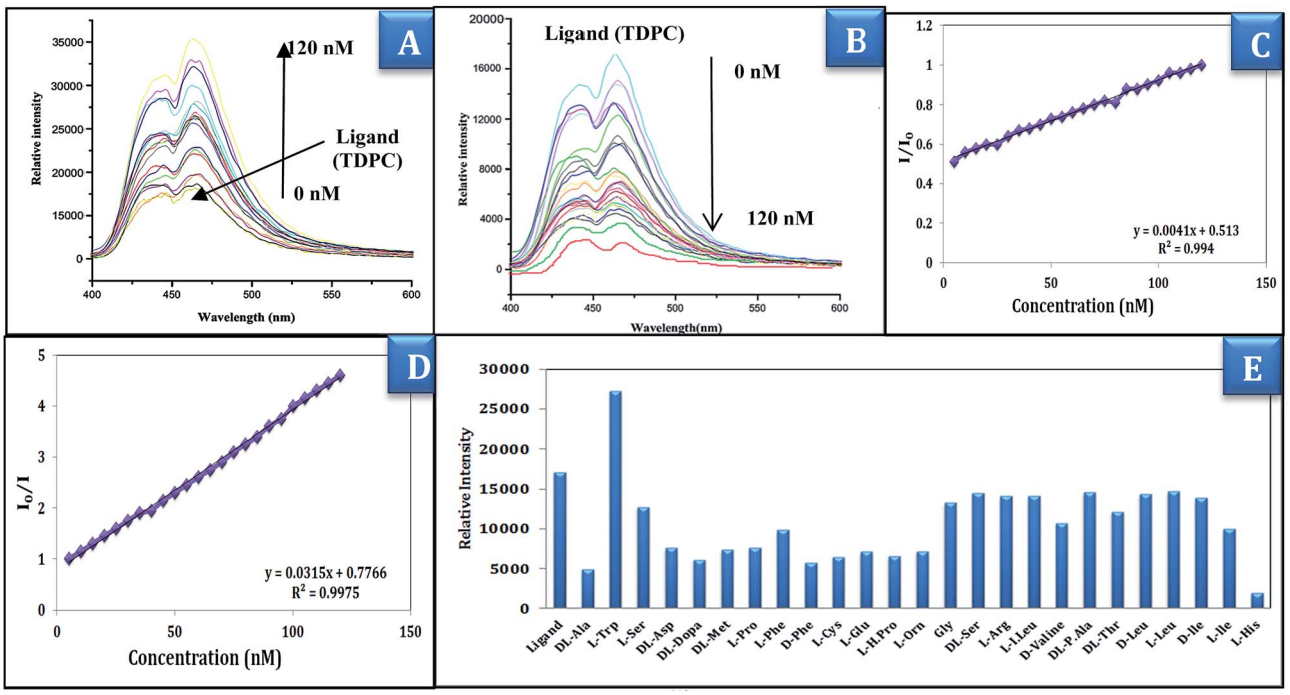


Fig. 1 (A and B) Emission spectra of increasing concentration of L-Trp (0, 5, 10,…120 nM) and L-His (0, 5, 10,…120 nM) with TDPC (1×10^{-8} M), (C and D) linearity curve of TDPC (1×10^{-8} M) with L-Trp (0–120 nM) and Stern–Volmer plot with L-His(0–120 nM), and (E) selectivity plot of TDPC (1×10^{-6} M) with amino acids (DL-alanine, L-tryptophan, L-serine, DL-aspartic acid, DL-DOPA, DL-methionine, 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, and L-histidine) (1×10^{-6} M). ($\lambda_{ex} = 380$ nm and $\lambda_{em} = 440$ nm).

histidine is 12.13×10^8. We have also calculated the binding constant for DL-Ala and L-Ser. For major interferent analysis see Fig. S4 and 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 the addition of 35 nM of L-Trp and 30 nM of L-His, the absorbance at 327 nm and 357 nm increases dramatically and new bands at 366 nm, 415 nm, 381 nm, and 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 the fluoroionophore and also hydrogen bonding between them. We also performed UV-titration by varying the concentration of L-Trp (10–35 nM) and L-His (10–30 nM) to study absorption changes (Fig. S8 and S9, ESI†).

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

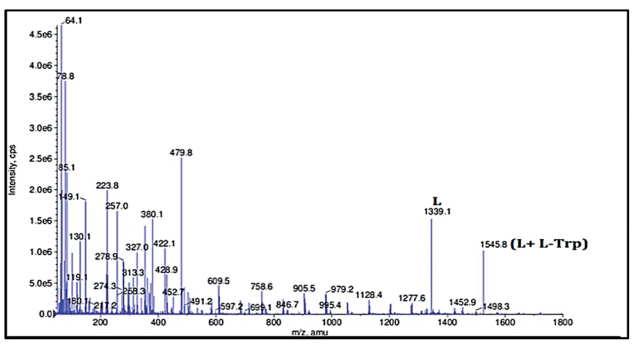


Fig. 2 ESI mass spectrum showing the isotopic peak pattern of a molecular ion peak for the 1 : 1 complex formed between TDPC and L-Trp.

^{1}H NMR investigation was performed to get an insight into the binding mechanism and also to find out the effect of L-Trp and L-His on ^{1}H NMR. We have exposed here comparison spectra of the ligand in the presence of L-Trp and L-His. We perceived shifting of the –NH peak to $\delta = 8.41$ ppm from $\delta = 8.59$ ppm for L-Trp suggesting hydrogen bonding between L-Trp and the ligand which has been displayed in Fig. S13 (ESI†). For L-His, due to strong hydrogen bonding with the ligand, the –NH peak at $\delta = 8.59$ 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 the pH study is shown in Fig. S14 and S15 (ESI†).

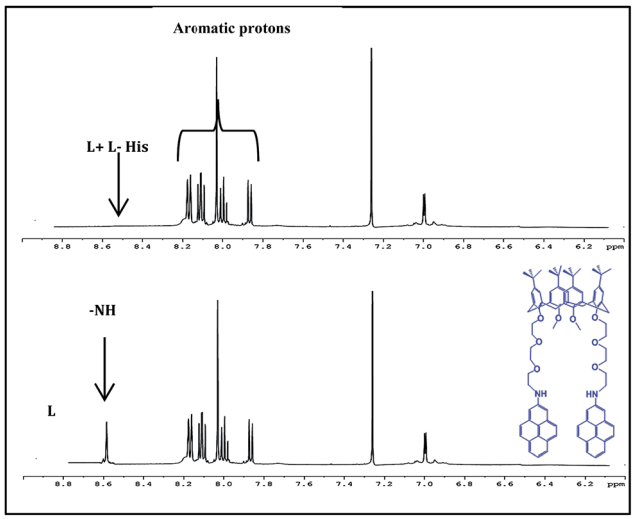


Fig. 3 Selected portion of the ^{1}H NMR spectra for the TDPC ligand and recorded in $CDCl_3$ upon addition of a 10 equivalent amount of L-His.

To assess whether our ligand can be of any use in the recognition of amino acids by naked eye detection, the 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†).

The sensing property of the 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 the ligand was found to give deep yellow fluorescence only in the case of L-Trp as shown 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 the 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 performing emission titration of the TDPC ligand and the L-Trp complex with other amino acids as shown in Fig. S17 (ESI†). The schematic representation of amino acids is displayed in Fig. 5 and the proposed binding mechanism through hydrogen bonding is presented in Fig. S18 (ESI†).

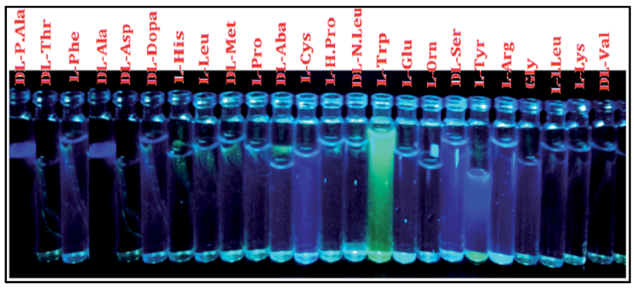


Fig. 4 Visual colour changes obtained upon addition of TDPC with various amino acids in 365 nm of UV in the dark.

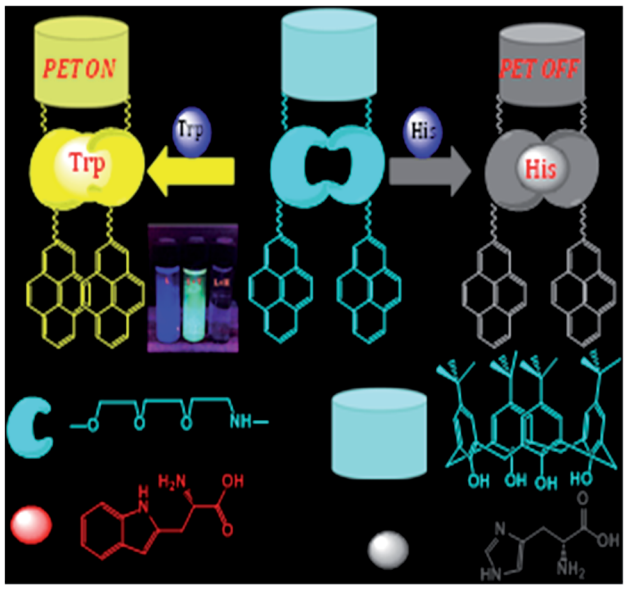


Fig. 5 Schematic design of the TDPC PET probe with L-Trp and L-His.

We have also optimized our general procedure for detection of L-Trp and L-His. The fluorescence of the 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 seconds was chosen for further experiments. Fig. S19 and 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 Tables S1 and S2 (ESI†). The results obtained with excellent recovery of spiked L-Trp and L-His ranged from 101 to 105%, illustrating the validity of the developed technique.

In conclusion, we have reported for the first time a highly selective and sensitive dual biomolecule PET fluorescence probe for L-Trp and L-His. The proposed fluorescence probe has a 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 to blood serum samples 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.

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