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ARTICLE

Highly Promising Discrimination of Various Catecholamine by Ratiometric Fluorescence Probes under Intermolecular Self-Association of Two Sensing Elements

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Two sensing elements based on fluorescence probes have been employed as a promising discriminating sensor (**NB**) of two catecholamines, dopamine (DA) and norepinephrine (NE), acting as a proper guest linker between two self-recognition sensing components. Surprisingly, in the presence of epinephrine (EPI), sensor **NB** containing boronic acid connecting to a napthalamide unit demonstrated a very strong fluorescence enhancement while its large fluorescence quenching was observed in the case of DA and NE. To differentiate the structural similarity of DA and NE, an appropriately designed small fluorescence sensor of **CC** containing crown-ether attached to coumarin showed a complementary recognition to an ammonium ion based catecholamine. The combination of **NB** and **CC** is capable of classification of DA and NE with dual emission bands under PET mechanism. The dual emission ratio (I_{475}/I_{384}) of **NB**-DA-**CC** complex showed the different values from that of **NB**-NE-**CC** complex. Additionally, the PCA analysis using mixed sensors of **NB** and **CC** obviously separated DA and NE better than a single sensing element. This systematic approach is a first report permitting a high potential for identification of DA and NE by ratiometric fluorescence sensors of dual emission by two sensing elements.

Introduction

Catecholamine neurotransmitters, including dopamine (DA), norepinephrine (NE) and epinephrine (EPI), which are a class of biogenic amines and have structural similarity consisting of catechol and amino groups and play an important role as neurotransmitters involving in a variety of central nervous system functions.[1-2] Generally, an unusual catecholamine concentration in body fluids is an essential indicator of patients suffering from Parkinson's disease [3-4] in the clinical diagnosis. Such a critically elevated amount of EPI in the human blood certainly involved the heart failure and the increase in blood pressure.[5] Importantly, a large amount of EPI and NE in blood and urine possibly causes the patients to suffer from tumors of adrenal glands.[6] Specific detection of each catecholamine species is a challenging task for sensing purpose. Detection of specific catecholamine, neurotransmitters, by artificial chemosensors under fluorescence spectroscopy[7-11] which is a versatile technique with high sensitivity, rapid response, and easy performance, is currently of interest subject to pursue. Regarding the optical detection, there are a few reports regarding chemosensor of catecholamine,[12] particularly, containing boronic acid and aldehyde for covalently binding with catechol and amine groups of catecholamine species to form a boronate ester and an iminium ion, respectively.[13] However, most fluorescent chemosensors previously reported the chelation-enhanced fluorescent quenching (CHEQ) effect upon binding with catecholamines.[7] Concerning the discrimination of each catecholamine, many reports regarding fluorescent chemosensors showed the non-specific binding among EPI, DA and NE.[14] None of the fluorescence chemosensors for catecholamine sensing employed the Fluorescence Resonance Energy Transfer (FRET) process as a tool in detection. Interestingly, the factors that give influences on the fluorescent intensity change are environmental condition, probe concentration and instrumental efficiency.[15-16] Ratiometric probe can eliminate most or all vagueness by self-calibration of emission intensities at two wavelengths.[17-21] Chen et al. designed a probe **CouMC** by connecting a coumarin fluorophore and an indolenium block through an ethylene group to address a hybrid fluorophore of coumarin and merocyanine.[22] Addition of HS- to interact at merocyanine moiety in the probe **CouMC** altered the conjugated system and an internal charge transfer (ICT) resulting in a ratiometric sensing behavior by decreasing merocyanine emission band and increasing coumarin emission band. Canary's group[23] reported the ratiometric displacement approach to detect Cu^{2+} by two fluorophore elements which demonstrated the different emission upon the different excitation wavelength. A great deal of works has focused on systematic studies designed to elucidate the optical properties of the sensory molecules for sensing purpose. Our previous work[24] based on FRET-on process for discrimination of catecholamine demonstrated the powerful tool for discrimination of DA and NE from EPI by a FRET-on process induced by an

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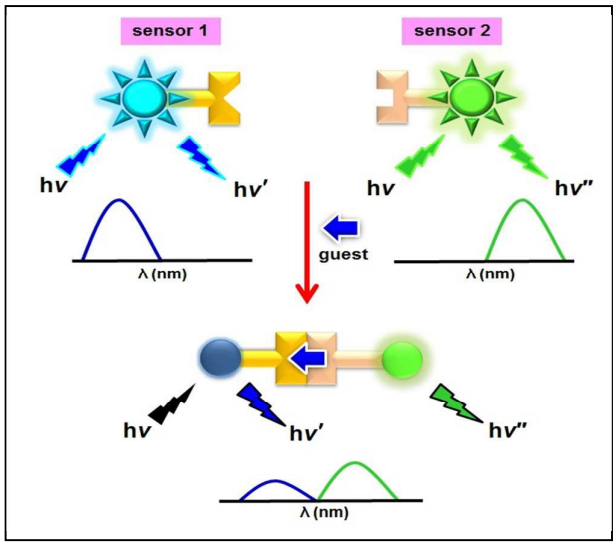
intermolecular assembly of coumarin aldehyde (**CA**) and pyreneboronic acid (**PBA**) with catecholamines as a guest linker. However, this conceptual design showed a well-specific detection of DA and NE. Taking such a definition of well-selective discrimination of the similar structure as the particular challenges for the chemist, the discrimination of similar structure biogenic amines prompts us to develop a more effective sensory system to solve this weak stand point. Deep considering on the structures of DA and NE, NE consists of the additional hydroxyl group addressing on β-position of side chain. As anticipated, this hydroxyl group close to ammonium ion of NE might give a different binding affinity with crown-ether moiety compared to DA without a hydroxyl group on the side chain. Taking on board the idea of intermolecular self-assembly in a spontaneously controllable manner, we have engineered a host-guest complex by suitable two sensing elements which enable to covalently bind with catechol and interact with ammonium unit based catecholamine. Apart from interesting properties of fluorescent sensors, a dual fluorophore giving two different emission responses by the same excitation wavelength gave fascinating sensing properties.[25-30] Our motivation is to design two fluorescence chemosensors: i) NB contains boronic acid connecting to a napthalamide unit for a covalent binding with catechol and as a fluorophore giving the emission band at 381 nm, respectively, ii) CC consists of crown-ether unit connecting to coumarine for non-covalent interaction with ammonium ion based catecholamine and as a fluorophore with emission band at 475 nm, respectively.



Scheme 1 Conceptual hypothesis under dual fluorescence response mechanism

We hypothesized that the suitable catecholamine guest will allow the well-defined linkage between **NB** and **CC** to provide different recognition pattern resulting in the discrimination of DA and NE as the conceptual illustration shown in Scheme 1. It is well-known that most fluorophores were quenched by catecholamine group due to electron rich catechol group. The different quenching patterns of two fluorophores upon binding with an analyte would give a different ratiometric fluorescence according to different binding affinity. This approach can possibly discriminate catecholamine neurotransmitter in biological system. Furthermore, the PCA analysis is utilized for classification of biogenic amine analytes by fluorescence spectral data. Additionally, we also apply this conceptual sensing in human urine samples.

Experimental

General

Nuclear magnetic resonance (NMR) spectra were recorded on Varian Mercury 400 MHz and Bruker 500 MHz nuclear resonance spectrometers (in $CDCl_3$, DMSO-d^6 and D_2O). ESI HRMS spectra were recorded on Varian Cary Eclipse spectrofluorometer.

All materials and solvents were purchased from Aldrich, Fluka, Merck and TCI as standard analytical grade and used without further purification. Commercial grade solvents such as acetone, dichloromethane, methanol and ethanol were purified by distillation before using. Thin-layer chromatography (TLC) was performed on silica and alumina gel plates (Kieselgel 60 F254, 1mm, Merck). Dimethyl sulfoxide as AR grade used in fluorescence measurement was used without further purification. The synthesis of N-(1,8-Naphthaloyl)-3-aminophenylboronic acid (**NB**) was carried out according to the reported procedure. [31]

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Synthesis of sensor CC

The 2-hydroxymethyl-18-crown-6 (0.151g, 0.515 mmol) and triethylamine (0.36 mL, 2.575 mmol) in benzene (15 mL) were stirred at room temperature for 30 min under nitrogen atmosphere. Then, the reaction mixture was added into a solution of coumarine acid chloride (**2**) in benzene (5 mL) by using cannula and refluxed for 24 hours under nitrogen atmosphere. The solvent was removed under reduced pressure. The crude product was purified by column chromatography (Al_2O_3) with 10% EtOH:CH_2Cl_2as eluent to obtain sensor **CC** as a brown oil (0.171 g, 62%). ^{1}H-NMR $CDCl_3$ δ 8.35 (s, 1H, ArH), 7.29 (d, *J* = 8.8 Hz, 1H, ArH), 6.54 (dd, *J* = 3.47 Hz, 1H, ArH), 6.36 (d, *J* = 1.6 Hz, 1H, CCH), 4.39-4.24 (m, 2H, OCH_2), 3.61-3.35 (m, 22H, OCH_2), 3.38 (q, *J* = 7.07 Hz, 4H, CH_2CH_3), 1.19 (t, *J* = 7.1 Hz, 6H, CH_2CH_3). Elemental Analysis: *Calcd*. for $C_{27}H_{39}NO_{10}$: C, 60.32; H, 7.31; N, 2.61, *found*: C, 60.21; H, 7.47; N, 2.42.

Complexation studies by fluorescence spectrophotometry

Typically, a stock solution of 1 x 10^{-4} M of sensor **NB** in DMSO was prepared. All of biogenic amines (DA, NE, EPI, TY, Glu, Lys, His and Hist) were prepared at concentration of 2 x 10^{-3} M in 0.01 M. phosphate buffer, pH 7.4. Fluorescence spectra were recorded from 350-800 nm at ambient temperature. For fluorescence titration, the solution of guests was added directly to 2.00 mL of 1 x 10^{-5} M of sensor **NB** in a 1-cm quartz cuvette by a micropipette and the portion of the mixture solution was stirred for 5 min prior to the measurement.

Principle component analysis (PCA) method

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For PCA analysis, the solution of guests (100 equiv.) were added to the solution of sensor **NB** (1 x 10^{-5} M) and stirred for 5 min. Then, 0.1 mL of 15-crown-5 was added to the solution mixture and stirred for 10 min. The solution of sensor **CC** (5 x 10^{-5} M) was added in the same 1-cm quartz cuvette by a micropipette and stirred for 5 min. The fluorescence spectra were monitored after each addition. The complexation was repeatedly detected for 5 times for each guest. The evaluation of PCA was calculated from the responsive fluorescence spectra in the range of 350 to 800 nm using MATLAB 7.11 (version R2011a) program.

Complexation study of sensor NB with EPI in human urine sample

A stock solution of 1 x 10^{-4} M of sensor **NB** was prepared in DMSO. The stock solution of 2 x 10^{-3} M of EPI analyte was prepared in 0.01 M phosphate buffer pH 7.4 and the synthetic urine was prepared in milli-Q water as listed in Table S1 in the ESI. For calibration curve, the synthetic urine was spiked into the 1 x 10^{-5} M of sensor NB solution by the 100-fold diluted solution of urine. The EPI solution was added to the mixture solution as various concentrations ($10^{-7}\mu$M) and stirred for 5 min. The fluorescence spectra were recorded from 350-800 nm at the excitation of 340 nm. Therefore, a calibration curve was built from the plot of $I-I_0$ at the emission wavelength of 490 nm versus concentration of EPI.

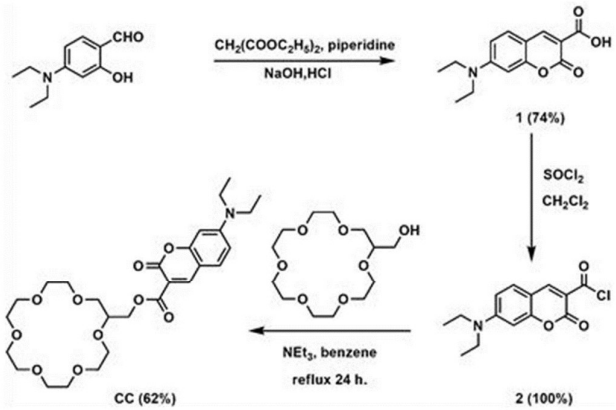
Then, the 100-fold diluted solution of urine samples was spiked into the 1 x 10^{-5} M of sensor NB solution. The 40 µM of EPI solution was added to the mixture solution and stirred for 5 min. The manipulation was repeated for three times. The fluorescence spectra were recorded from 350-800 nm at the excitation of 340 nm. After that, the found amount and percent recovery were calculated from the calibration curve.

Results and discussion

Molecular sensors were designed and synthesized for sensitive and selective detection of catecholamines through dual fluorescence responses under one excitation wavelength. The structure of catecholamines consists of two functional groups, catechol and amino group. Therefore, the designed sensor should contain the binding sites for the selective binding with each part of catecholamine guests.

Synthesis of Sensor CC

The sensor **NB** was prepared in a single-step reaction by a nucleophilic substitution reaction between 3-aminophenylboronic acid hemisulfate and 1,8-naphthalene dicarboxylic acid anhydride in 56% yield. Sensor **CC** was obtained by a coupling reaction of **2** and 2-hydroxymethyl-18-crown-6 by nucleophilic substitution using triethylamine as base (as shown in Scheme 2). From the NMR spectrum of **CC**, a singlet proton of coumarin showed the upfield shift from 8.68 to 8.35 ppm and other aromatic protons presented the upfield shift possibly caused by the electron donating of methylene bridge as shown in Fig S8 in the ESI. In addition, the ^{13}C-NMR spectrum showed ester group signals at 163.97 and 158.54 ppm. HR-ESI mass spectrum showed the intense peak of m/z at 560.251 corresponding to the structure of **CC**. (Fig. S10 in the ESI).



Scheme 2 Synthesis pathway of sensor CC

Complexation studies of sensor NB with various guests by fluorescence spectrophotometry

The sensor **NB** contains boronic acid moiety as a binding unit for condensation reaction with catechol group of analyte and napthalimide moiety as a fluorophore. The selectivity of sensor NB was examined in biological system (1:9 v/v of DMSO:phosphate buffer at 0.01 M, pH 7.4) toward 100 equiv. of various biogenic amines including dopamine (DA), norepinephrine (NE), epinephrine (EPI), tyramine (TY), L-glutamic acid (Glu), L-lysine (Lys), histidine (His) and histamine (Hist).The chemosensor **NB** showed the characteristic emission band at 384 nm. As similar to previous reports, most of chemosensors upon complexing with catecholamine underwent the PET process since electron transfer from phenyl based catecholamine to napthalimide moiety causes the fluorescence quenching. Likewise, sensor **NB** in the presence of DA and NE showed the fluorescence quenching. On the contrary, sensor **NB** covalently bound with EPI surprisingly exhibits a strong fluorescence with a concomitant of a large red shift from 384 nm to 490 nm as shown in Fig. 1. This finding suggests that after complexation, the combination of donating ability of the secondary amine based EPI and a poor acceptability of boronic ester in **NB**-EPI adduct extremely enhances the totally orientation of dipole moment to acceptor part of napthalimide resulting in the predominant ICT state (Intramolecular Charge Transfer) as shown in Fig. 1. In the case of DA and NE, the PET process is more dominative than the ICT process because the complex of sensor **NB** with DA and NE gives a slight influence on their dipole moments. On the other hand, other analytes without the catechol group including TY, Glu, Lys, His and Hist could not induce the fluorescence changes implying that boronic ester did not form on sensor **NB**.

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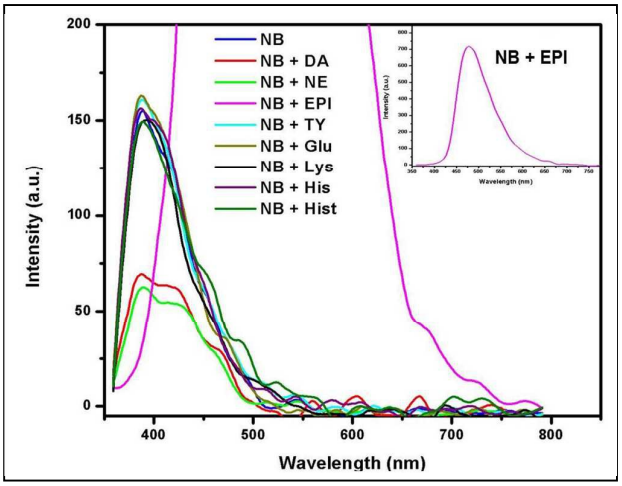

Figure 1. Fluorescence spectra of sensor **NB** (1 x 10^{-5} M) in 1:9 v/v of DMSO:phosphate buffer at 0.01 M, pH 7.4) in the presence of various guests at 100 equivalent and inset shows the fluorescence spectrum of sensor **NB** and EPI.

Regarding to the fluorescence changes of sensor **NB** in the presence of catecholamines (DA, NE and EPI), it is indicative of the selective binding between sensor **NB** and catecholamines. To evaluate the binding mode between sensor **NB** and catecholmines, the stoichiometry of complexes was measured by the Job's method using fluorescence technique showing the stoichiometry of 1:1 binding mode for sensor **NB** with DA, NE and EPI (as shown in Fig. S12 in the ESI)

Binding Properties of sensor NB with DA, NE and EPI by fluorescence spectrophotometric titration technique

To verify the binding affinity of sensor **NB**, fluorescence titration of sensor **NB** was carried out in phosphate buffer solution pH 7.4.

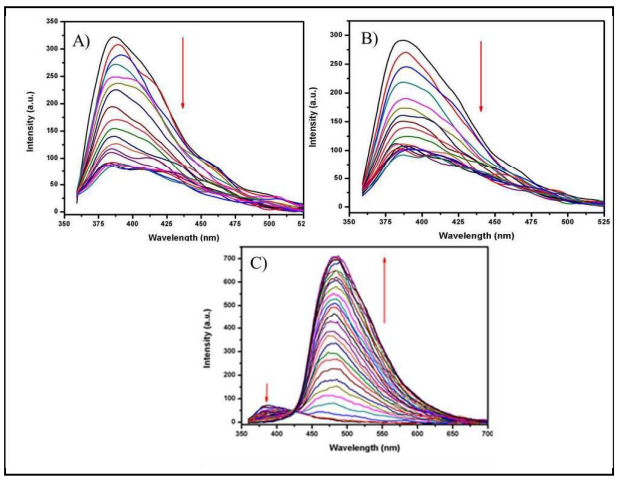

Figure 2 The fluorescence titration spectra of sensor **NB** (1 x 10^{-5} M) upon gradual addition of (A) DA, (B) NE and (C) EPI at 0-80 equiv. in 1:9 v/v DMSO: phosphate buffer at 0.01 M, pH 7.4 (λ_{ex} = 340 nm)

The fluorescence intensity at 384 nm of the complexation of sensor **NB** toward DA, NE and EPI gradually decreased upon the increment of catecholamine guests as illustrated by Fig. 2(A), (B) and (C), respectively. Interestingly, the new emission band at 490 nm of complexation **NB**-EPI was significantly developed. Fluorescence titration data were analyzed by using non-linear regression plot (Fig. S13) to provide the log K_s values of complex **NB** with DA, NE and EPI of 4.13, 4.17 and 4.01, respectively. All guests employed the catechol group to covalently bind with boronic acid based **NB**. It can be reasonably explained that the ammonium ion on DA and NE preferred to promote the condensation reaction of boronic acid and catechol group producing a negative boronate ester. It is reliably rationalized that the log K_s values of sensor **NB** with DA and NE is slightly higher than the sensor **NB** with EPI.

It is particularly important to extensively investigate the visual detection of EPI observing from a large fluorescence change and strong fluorescence intensity. To verify the selective detection of **NB** towards various analytes by naked-eyes sensing, the solution of **NB** in the presence of various analytes was exposed to 356 nm UV-visible light. The fluorescence color changes of sensor **NB** toward analytes were displayed in Fig. 3. The solution of sensor **NB** in the presence of DA or NE showed a very low brightness of luminescence. On the other hand, the solution of **NB**-EPI adduct exhibited a highly green luminescence. Among other guests including TY, Glu, Lys, His and Hist, sensor **NB** solution still remained unchanged.

Figure 3 Fluorescence responses of (A) sensor **NB** (1 x 10^{-5} M) in 1:9 v/v DMSO:phosphate buffer at 0.01 M, pH 7.4) in the presence of the various guests 100 equiv. and Fluorescence images (B) of (a) sensor **NB** and (b) The filter paper dipped in sensor **NB** solution, and the word "EPI" with EPI solution was exposed to the UV light.

Furthermore, to develop the EPI sensor on the solid support, the filter paper was dipped in 5 x 10^{-5} M of sensor **NB** solution. Then, this filter paper was painted the word "EPI" using a paintbrush dipped in EPI solution. The prepared filter paper exposed to 356 nm UV light, showed a strong green luminescence in word "EPI" as illustrated in Fig. 3B. As a result of fluorescence changes on solid support, sensor **NB** offers an excellently promising detection of EPI

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on the paper base. The results also implied that the sensor **NB** can bind with EPI yielding the **NB**-EPI adducts on the solid support.

Determination of detection limit of sensor NB with DA, NE and EPI by fluorescence spectrophotometry

The detection limits of sensor **NB** toward DA, NE and EPI measured in a range of 11.9-95.2 μM, 13.0-130.0 μM and 5.0-95.0 μM were 7.71, 8.50 and 1.54 μM, respectively. As consistent with a large fluorescence change of **NB**-EPI complex, sensor **NB** can serve as an effective EPI sensing in a very low concentration compared to DA and NE. (Fig S15 in the ESI)

Complexation studies of sensors NB and CC with various guests by fluorescence spectrophotometry technique

Although sensor **NB** offers considerable promises as EPI selective fluorescence probes, our further purpose is to discriminate the similar structure of DA and NE. Therefore, we developed ratiometric fluorescence probes using two fluorescence sensory elements (**NB** and **CC**) containing the different binding sites and employed intermolecular self-assembled complex to give different emission bands upon the same excitation wavelength. We hypothesized that the adaptive self-assembled complexation of boronic acid based **NB** and crown-ether based **CC** by an induced fit of a proper guest linker, DA or NE, would perform the different recognition patterns as a consequence of the different fluorescence responses of dual fluorophores. Notably, naphthalimidefluorophore containing boronic acid and crown-ether coumarin exhibit the different emission bands at 384 and 475 nm, respectively, upon excitation at 340 nm. Since the structures of DA and NE are different in the side chain, we expect that the different ratio of dual fluorescent responses after complexation could be obtained.

Sodium cation in phosphate buffer solution could interfere the recognition affinity of ammonium ion of guest with crown-ether based **CC**. To overcome this problem, the competitive receptor for Na^+ ions was utilized. From the previous report, the binding constant of 15-crown-5 with Na^+ ion is much higher than that of 15-crown-5 with ammonium ion.[32] For all manipulation, the complexation study of **CC** system in buffer solution will add the 15-crown-5 to preferentially bind with Na^+ ions.

To verify the selectivity of dual sensory system, the complexation of sensors **NB** and **CC** toward 100 equiv. of various guests including DA, NE, EPI, TY, Glu, Lys, His and Hist was evaluated in 1:9 v/v DMSO:phosphate buffer at 0.01 M, pH 7.4). In Fig. 4(a), the observation of the fluorescence changes of mixed sensors **NB** and **CC** upon adding DA, NE, and EPI signified a well-specific recognition of dual sensory system towards catecholamines. Other biogenic amines with the two sensory system showed a small change of both emission bands indicating non-fitted complexation for both sensing elements.

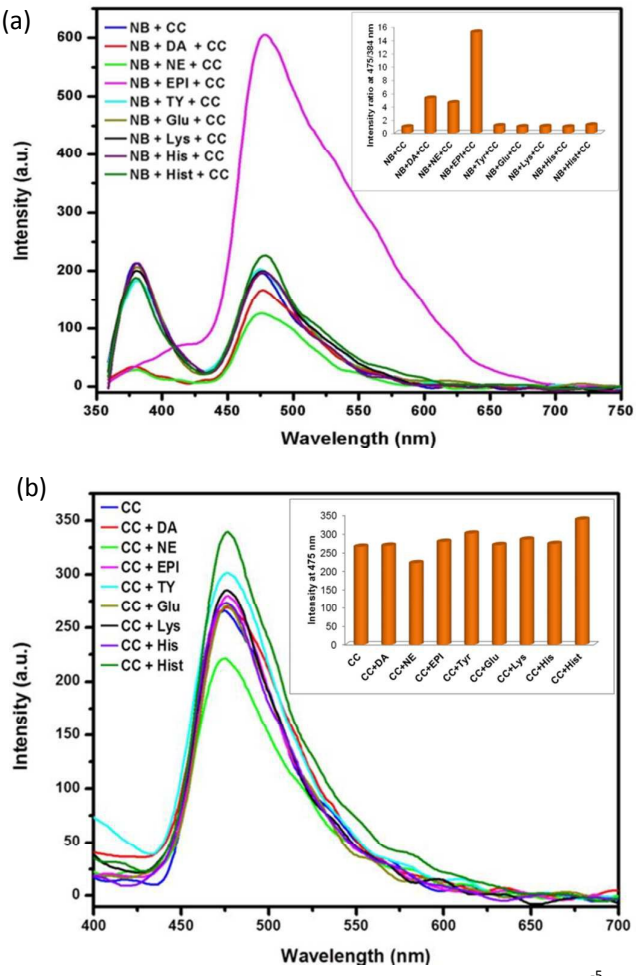

Figure 4. Fluorescence spectra of (a) mixing sensors **NB** (1 x 10^{-5} M) and **CC** (5 x 10^{-5} M) (b) sensor **CC** (5 x 10^{-5} M), in the presence of various guests 100 equiv. in 1:9, v/v DMSO: phosphate buffer (0.01 M, pH 7.4) with 0.1 mL 15-crown-5 (λ_{ex} = 340 nm)

Upon the addition of DA or NE, the fluorescence intensity of both sensors was quenched possibly caused by PET process from phenyl donor group to napthalimide and coumarin acceptor group as shown in Fig. 4. Compared to the emission band at 475 nm of sensor **CC** with various analytes, the emission band at 384 nm of complex **NB** with DA along with NE showed a larger quenching while other guests did not effect to the spectral change. This suggested that napthalimide obtained a stronger effect of energy transfer from guests than coumarin in the case of DA and NE. The emission band at 475 nm of **CC** for NE exhibited a larger decrease (I_0-I = 100 a.u.) than that for DA (shown in green and red lines, respectively, in Fig. 4(a)). It should be noted that the spectra of sensor **CC** with guests showed a large quenching only with NE with (I_0-I = 40 a.u.) and no change of emission band for **CC** and DA (shown in green and red lines, respectively, in Fig. 4(b)). This implies that NE could bind more strongly with sensors **NB** and **CC** than DA because the hydroxyl group of NE may undergo the complementary hydrogen bonding interactions with crown-ether

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based **CC**. For better understanding of the binding behaviors, structures of the adduct of **NB**⊂NE⊂**CC** have been calculated by using density function theory (DFT) at the B3LYP/6-31+G(d) level. Figure 5 exhibited the complementary hydrogen bonding (HB1 and HB2) between OH based NE and oxygen based napthlimide with hydrogen based crown-coumarine, respectively, and electrostatic force of $-NH_3^+$ and negative charge of boronate ester. This evidence clearly supports a strong binding of mixed sensors **NB** and **CC** with NE resulting in a larger fluorescence quenching at 475 nm compared to single sensing element of **CC**. Definitely, the mixing of sensors **NB** and **CC** enables to enhance the binding affinity of **CC** with DA and NE.

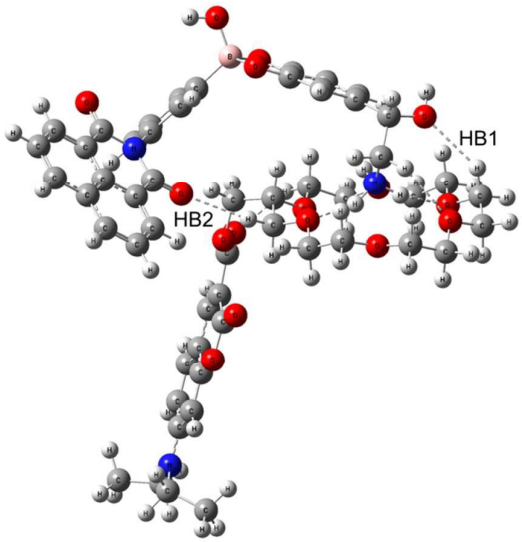

Figure 5.DFT/B3LYP/6-31–G(d) optimized structures of the induced fits of **NB**-guest-**CC** complex.

Owing to a well-classified determination, this approach offers very promising features for a discriminate-sensing purpose for DA and NE. These indicated that DA and NE act as a proper guest linker between **NB** and **CC.**

As advantage of different fluorescence responses upon complexation, the self-coordination between sensors **NB** and **CC** with the bridging analytes of DA or NE induced the different ratiometric fluorescence intensity of coumarin and napthalimide ($I_{coumarin}/I_{napthalimide}$) as shown in Fig. 4a (inset). As a result, the ratio of dual emission of complex **NB**-DA-**CC** of 4.84 showed a slight larger than that observed for complex **NB**-NE-**CC** (R = 4.28). Fantastically, the ratiometric fluorescence changes of each complex still allowed the constant ratio upon the different concentration of sensors and guest as well as the different condition in the presence of excess amount of guest.

Principle component analysis (PCA) method for analysis of complexation[33]

Furthermore, to easily identify various biogenic amines by using the different recognition patterns in terms of the different fluorescent responses upon complexation, PCA method was reliably utilized to reduce the dimensionality of data set for easier interpretation.

Due to the highly selective binding affinity of sensor **NB** with EPI as previous mentioned result, we have examined the clustering discrimination of other biogenic amines under excluding EPI.

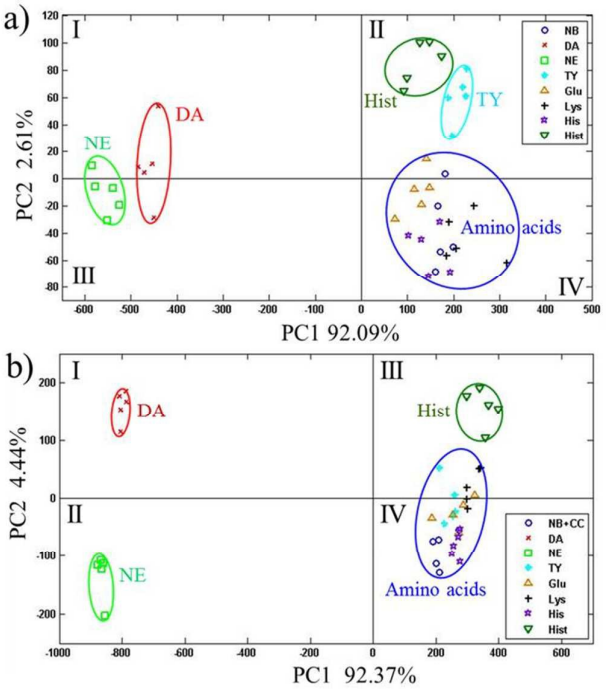

Figure 6. PCA score plots of (A) sensor NB and (B) sensors NB and CC upon addition of various guests (100 equiv.) in 1:9 v/v DMSO: phosphate buffer at 0.01 M, pH 7.4) and excluding fluorescence data of EPI for determining of PCA analysis

Two dimensional score plot for two principal components (PC1 and PC2) represents 94.70% variance of the classification of biogenic amines as shown in Fig 6. The appearance of catecholamine clusters including DA and NE on the border line between quadrant I and quadrant III was separated from other biogenic amines while, the cluster of Hist and TY appeared in quadrant II. This implied that the sensor NB can bind slightly with Hist and TY. Cross-validation LDA of this model showed 77.5% accuracy of discrimination for 7 biogenic amines.

In our further purposes to separate the similar structures of DA and NE, the fluorescence spectra of mixing sensors **NB** and **CC** toward biogenic amines were classified by using PCA analysis. Two dimensional score plot for PC1 and PC2 representing 96.81% variance was illustrated in Fig. 6b. This PCA model demonstrated that two sensing elements of sensors **NB** and **CC** could clearly differentiate the similar molecules of DA and NE in quadrant I and quadrant III, respectively. Hence, both sensors give a high performance for discrimination of DA and NE while, Hist cluster appeared in quadrant II closed to the cluster of sensors and other biogenic amines. Moreover, cross-validation LDA exhibited 77.5% accuracy of discrimination for all 7 biogenic amines. The PCA score

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plot of two sensing elements of **NB** and **CC** definitely gave a higher discrimination of DA and NE than a single sensing element of **NB**.

Complexation study of sensor NB with EPI in human urine sample

To develop fluorescence sensor **NB** in the analytical application, we applied the detection of EPI in real biological system of human urine samples. A linear range of 10-70 μM was obtained by plotting the intensity of fluorescence at 490 nm versus the spiked various concentration of EPI in the synthetic urine as indicated in Fig. S19 in the ESI. The spiked urine samples were prepared by the 100-fold diluted solution of urine by standard addition method. The % recovery for EPI at 40 μM of the spiked solution is in the range of 100.14 to 101.06% which are the acceptable recovery in sensing application as displayed in Table 1. Therefore, the fluorescence sensor **NB** can serve as an effective sensing of EPI in the analytical application of real samples.

Table 1 Results for the determination of EPI in human urine samples

samples	spiked (μM)	found (μM)	recovery[a] (%)
human urine-1	40	40.05± 1.09	100.14± 2.74
human urine-2	40	40.42± 1.74	101.06± 4.34

[a] mean ±std, n = 3

Furthermore, the discrimination of catecholamine in urine samples was carried out by PCA method as show in Fig 7. Two dimensional score plot for two principal components (PC1 and PC2) represents 96.74% variance of the classification of biogenic amines. The clusters of DA and NE appeared in quadrant I and quadrant II, respectively, which was remarkably separated from other biogenic amines. This PCA plot of urine samples is consistency with that in Fig 6. It satisfied that this dual emission system of mixed sensors **NB** and **CC** highlights an effective discrimination of DA and NE in clinical diagnosis.

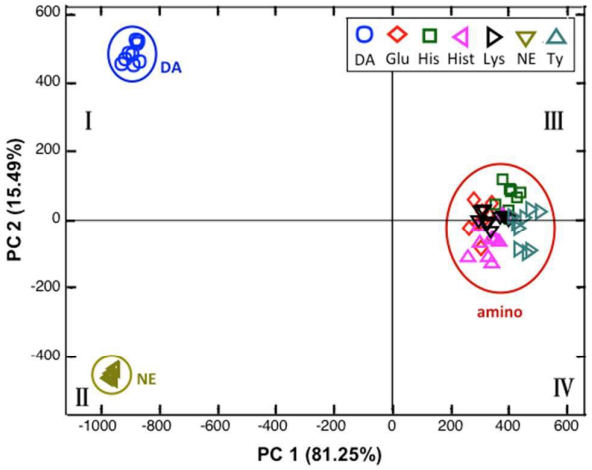

Figure 8. PCA score plots of mixed **NB** and **CC** upon addition of various guests (100 equiv.) in urine samples and excluding fluorescence data of EPI for determining of PCA analysis

Many techniques have been devoted for determination of DA shown in Table S2. These methods provide ultrahigh sensitive approach. It can be seen from Table S2 that most of the detection limits obtained by fluorescence, electrochemistry and colorimetric method are lower than micromolar range. Although the detection limit of our sensory system is not better than that found in the previous reports, the normal amount of DA in human serum should be in the range of 10^{-7}-10^{-5} M.[34] For outstanding results of our sensory system, an effective discrimination of DA and NE are definitely remarkable and fantastic which is a rare report focusing in this important approach due to quite similar structure of guest. This aspect is still challenge for chemists.

Conclusions

An effective discrimination of EPI, DA and NE has been achieved by the development of ratiometric fluorescence of two sensing elements of **NB** and **CC**. EPI was clearly discriminate from other biogenic amines with a strong emission band at 490 nm. Ratiometric fluorescence sensors **NB** to **CC** displayed the emission ratio (I_{490}/I_{384}) of **NB**-DA-**CC** and **NB**-NE-**CC** complexes as 4.84 and 4.28, respectively, at the excess amount of guest. We have also successfully reported the obviously different recognition pattern of two sensors **NB** and **CC** toward catecholamines by PCA method. For analytical application, chemosensor **NB** offers a good result for detection of EPI in human urine samples. This is the first example of the significant discrimination of DA and NE by intermolecular self-assembled recognition of two sensors. This potential research highlights the promising concept of intermolecular ratiometric fluorescence sensors using two sensing elements.

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Notes and references

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