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**Simple and fast detection of homocysteine by cucurbit[7]uril fluorescent probe
based on competitive strategy**

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Abstract

This research put forward a simple and rapid fluorescence probe method based on competition strategy to detect homocysteine. The host-guest interaction behavior between cucurbit[7]uril and homocysteine was further studied via UV-vis spectroscopy, Fourier transform infrared spectroscopy, molecular modeling, and ¹H NMR spectroscopy in aqueous solution. The binding constant of cucurbit[7]uril-homocysteine measured by ¹H NMR titration is $2.40 \times 10^3 \text{ L mol}^{-1}$. Under the optimal detection conditions, the fluorescence intensity of the cucurbit[7]uril-berberine complex declined linearly with increasing concentration of homocysteine in the range from 5 to 200 μM , and the limit of detection was 1.76 μM . This established fluorescent probe method exhibits high selectivity for coexisting interfering substances. Meanwhile, it was also successfully employed to detect homocysteine in serum samples with the recovery rate of 85.1% to 102.0%. This supramolecular fluorescent probe based on competitive strategy provides a simple and fast method for the determination of homocysteine in practical samples.

Keywords: Supramolecular probe · Host-guest interaction · Competitive strategy ·

Homocysteine detection

Introduction

Homocysteine (Hcy), as a reactive sulfhydryl-comprising amino acid, is an important intermediate creation during the metabolism of methionine and cysteine, which can be released into the blood [1, 2]. The plasma of a normal adult can contain 5-15 μM homocysteine [3, 4]. Moreover, hyperhomocysteinemia (HHcy) is characterized by the irregular increase of homocysteine concentration in plasma. HHcy is classified into mild (16-30 μM), modest (31-100 μM) and severe (>100 μM) according to elevated concentrations of total Hcy [2]. Abnormal Hcy levels can cause a variety of diseases, such as cardiovascular diseases, neurological diseases, osteoporosis, inflammatory bowel disease, and complications with pregnancy [5, 6].

So far, a great number of methods have been reported for detecting Hcy, including radioenzyme immunoassay (RIA) [7], high performance liquid chromatography (HPLC) [8-13], electrochemistry [2, 14], capillary electrophoresis (CE) [15, 16], colorimetry [1, 17-20], liquid chromatography-mass spectrometry (LC-MS) [21-23], etc. [24]. However, radioenzyme immunoassay utilizes radioactive adenosine, which can cause pollution and require expensive antibody costs. The instrumental analysis has some inevitable limitations such as tedious sample pretreatment, complicated chemical modification, precise instruments, long analysis time and low sensitivity [25]. Compared with the above analytical methods, the fluorescence probe method has attracted extensive concern owing to its rapidness, simplicity, high sensitivity, good stability, and low cost. Therefore, it is extremely indispensable to develop a convenient, fast, sensitive and stable fluorescence probe method for the determination of Hcy content in practical applications.

Cucurbit[n]urils (CB[n], $n = 5-8, 10$) are the macrocyclic molecular containers, which are consisted of n glycoluril monomers linked with $2n$ methylene groups. Symmetrical CB[n] hosts have hydrophobic cavities and restrictive polar uridylcarbonyl portals [26, 27]. CB[n] and its derivatives can combine with various guest molecules through hydrogen bonding, hydrophobic interaction, and ion-dipole interaction [26, 28, 29]. Guest molecules are encapsulated in the cavity of CB[n] via specific host-guest interactions. Supramolecular complex usually alters the photophysical and photochemical properties of the contained guest molecules [29, 30]. Owing to its excellent solubility in aqueous solutions and the appropriate cavity size, CB[7] has received special attention in fluorescence analysis in recent years. In

addition, CB[7] possesses the extraordinary competency to constitute host-guest complexes with organic compound and cations [28, 31].

In this study, a fluorescent probe method based on the competitive strategy has been developed to detect Hcy in practical samples. The host-guest interaction mechanism and binding constant between the CB[7] and Hcy have also been investigated. On the basis of host-guest recognition and concentration advantage, Hcy can occupy the hydrophobic cavity of CB[7] by competing with berberine hydrochloride (BER), resulting in a significant change in the fluorescence spectrum. The proposed method can achieve simple, rapid and highly selective determination of Hcy without expensive equipment and complicated operations.

Experimental

Materials and reagents

The homocysteine (Hcy) was obtained from Shanghai Hanhong Technology Co., Ltd. Berberine hydrochloride (BER) was purchased from Nanjing Dulai Biotechnology Co., Ltd. Cucurbit[7]uril (CB[7]) was acquired from Shanghai Nafu Biotechnology Co., Ltd. All other chemicals were analytical reagent grade, and double-distilled water was worked through the whole of the process.

Apparatus

Fluorescence spectra (FL) were recorded using a RF-5301PC fluorescence spectrophotometer (Shimadzu, Japan). Fourier-transform infrared spectroscopy (FT-IR) was acquired by a FT-IR 8400s Fourier-transform infrared spectrophotometer (Shimadzu, Japan). Nuclear magnetic resonance spectroscopy (^1H NMR) was performed on a Bruker Avance-500 (500 MHz, Bruker, Switzerland). Ultraviolet-visible (UV-vis) absorption spectra were recorded through a UV-1800 UV-vis spectrophotometer (Shimadzu, Japan). All optical records were measured under stable ambient conditions.

Spectra measurement procedure

The fluorescence measurement was performed at an excitation wavelength of 343 nm in the absence or presence of Hcy solution. 2 mL CB[7]-BER (3 μM : 2 μM) standard stock solution was set in a 4 mL EP tube. Subsequently, a given concentration of Hcy aqueous solution was added in sequence. Afterwards the mixture was shaken fully for 15 minutes at room temperature. Finally, the fluorescence change values ($F/F_0 = F_{\text{CB[7]}\cdot\text{BER}+\text{Hcy}}/F_{\text{CB[7]}\cdot\text{BER}}$) were calculated.

Examination of spiked bovine serum

The fluorescent probe based on CB[7] was applied to determine Hcy in actual serum samples. Disparate concentrations of Hcy were appropriately subjoined to the diluted bovine serum samples, and then the spectroscopic method described in Section 2.3 was performed.

Results and discussion

Characterization of CB[7]-Hcy inclusion complex

UV-vis spectroscopy

First, UV-vis technology was utilized to explore the formation of the CB[7]-Hcy system in aqueous solution. The trial consequence was shown in Fig. S1, BER displayed absorption peaks at 228 nm, 263 nm and 345 nm [31]. A weak hypochromicity was observed after CB[7] was annexed to the BER solution. However, an enhancement in absorption spectrum was observed after addition of Hcy into the CB[7]-BER supramolecular system. This should be attributed to the occupation of the CB[7] hydrophobic cavity by Hcy, giving rise to some BER molecules being expelled.

FT-IR spectroscopy

The FT-IR spectrum was also analyzed to explore the host-guest interaction between CB[7] and Hcy (Fig. S2). CB[7] has polar carbonyl groups at its two portals and its important representative absorption peak emerges at 1740 cm^{-1} . Hcy has sulfhydryl, methylene and C-S groups, and its special absorption peaks are 2633 cm^{-1} , 2940 cm^{-1} , and 545 cm^{-1} , respectively. Compared with the FT-IR spectrum of CB[7], the carbonyl characteristic peak of CB[7]-Hcy was blue-shifted to 1732 cm^{-1} . The reason should be the electrostatic interaction between the amino group of Hcy and the carbonyl group of CB[7] portals. At the same time, the hydrogen of the amino group forms a hydrogen bond with the oxygen of the carbonyl group. In addition, it was observed that both the methylene and the sulfhydryl absorption peaks disappeared, while the C-S characteristic peak weakened in the CB[7]-Hcy system. This indicated that the methylene and sulfhydryl groups of Hcy may have entered the hydrophobic cavity of CB[7].

Molecular simulation

Then, the host-guest recognition between CB[7] and Hcy was explored via theoretical calculations. The CB[7]-Hcy molecular model was optimized at the B3LYP/6-31G(d) level of density functional theory adopting the Gaussian program (Fig. 1). The outcomes verified the partial inclusion of Hcy in the hydrophobic cavity

of CB[7]. In the energy-minimized structure ($-13057221.14 \text{ kJ mol}^{-1}$), the hydrophobic side chain of the Hcy is embedded in the CB[7] macrocyclic molecule cavity, and the amino nitrogen moiety is located in the vicinage of the host carbonyl portal.

Fig. 1 Energy-minimized structure of the CB[7]-Hcy complex in the ground state. Color code for Hcy and CB[7]: oxygen, red; nitrogen, blue; carbon, gray; hydrogen, white; sulfur, yellow

^1H NMR spectroscopy

The generation of the CB[7]-Hcy inclusion complex can be further affirmed by ^1H NMR spectroscopy (Fig. 2). The consequences obtained are consistent with the results of complex reported in the literature [32]. Compared with the proton signal of the unbound Hcy molecule, the resonance of protons H_γ and H_β of the bound Hcy in the ^1H NMR spectrum of the CB[7]-Hcy system underwent a evident up-field shift, indicating that the hydrophobic cavity of the CB[7] has a shielding effect on the $-\text{CH}_2\text{CH}_2-$ of Hcy molecule. Simultaneously, the chemical shift of the resonance hydrogen in the CB[7] molecule also varied because of the inclusion of Hcy. In short, CB[7] can combine with Hcy to compose host-guest inclusion complexes due to the synergistic hydrophobic effect, hydrogen bonding, ion-dipole interaction, and good size and morphology matching.

Fig. 2 ^1H NMR spectra (500 MHz) of CB[7], Hcy and CB[7]-Hcy complex

Apparent binding constant and stoichiometry

The Hcy has no fluorescence in aqueous solution, and there was no obvious change in its UV-vis absorption and fluorescence emission spectra after the addition of CB[7]. Thus, NMR titration and fluorescence spectroscopy was employed to measure the association constant of CB[7]-Hcy and CB[7]-BER inclusion complex, respectively [33, 34].

For the 1:1 host-guest inclusion complex (H-G), the fluorescence intensity of the guest (G) was enhanced with the additional non-fluorescent host (H). The fluorescence intensity depends on the concentration of host [28, 35], and the relationship can be expressed by the following equation:

$$F/F_0 = 1 + (F_\infty/F_0 - 1) \frac{[H]K}{(1 + [H]K)} \quad (1)$$

Where F represents the fluorescence intensity measured by adding different concentrations of H , F_0 represents the fluorescence intensity of G in the absence of H , F_∞ is the fluorescence intensity when G is completely complexed, and K is the equilibrium binding constant for the 1:1 complexation.

$$K = \frac{[H-G]}{[H][G]} \quad (3)$$

The 1:1 complexation (and, thence, the applicability of Eq. (1)) can be proved from the double reciprocal Benesi-Hildebrand graph of $1/(F-F_0)$ versus $1/[H]$. The graph of the 1:1 complex is linear, while the graph of higher-order complexes is nonlinear.

The equilibrium reaction between CB[7] and BER is as follows:

The equilibrium binding constant is expressed as follows:

$$K_{\text{CB[7]-BER}} = \frac{[\text{CB[7]-BER}]}{[\text{CB[7]}][\text{BER}]} \quad (5)$$

Fig. 3a illustrated the augmentation of BER fluorescence as a function of the addition of CB[7]. The solid line demonstrated the best fit of the data to Eq. (1) adopting the nonlinear least squares method. Three such tests were carried out, producing the average value of $K_{\text{CB[7]-BER}} = 1.34 \times 10^5 \text{ L mol}^{-1}$, which is consistent with the corresponding literature result ($1.77 \times 10^5 \text{ L mol}^{-1}$) [36]. At the same time, a successive variation Job's plot (Fig. S3) also proved that the CB[7]-BER inclusion complex was constituted with a 1:1 stoichiometry.

The binding affinity between CB[7] and Hcy was determined by utilizing ^1H NMR chemical shift changes of the proton hydrogen resonances after the addition of CB[7] supramolecular host. The NMR version of the Benesi-Hildebrand equation is expressed as follows [33]:

$$1/\Delta\delta = 1/(K_a\Delta\delta_{\text{max}}[\text{H}_0]) + 1/\Delta\delta_{\text{max}} \quad (6)$$

where $\Delta\delta$ represents the measured change in chemical shift after adding the host referenced to that of the free guest molecule, $\Delta\delta_{\text{max}}$ represents the discrepancy in chemical shifts between that observed in the guest and in the host-guest inclusion complex, and $[\text{H}_0]$ is the known host concentration.

The production of a 1:1 CB[7]-Hcy host-guest system can be confirmed from the graph of $1/\Delta\delta$ versus $1/[\text{H}_0]$ (Fig. 3b). Simultaneously, the calculated binding constant of $K_{\text{CB[7]-Hcy}}$ is $2.40 \times 10^3 \text{ L mol}^{-1}$, which is close to the data $(1.09 \pm 0.147) \times 10^3 \text{ L mol}^{-1}$ calculated by isothermal titration calorimetry [32].

Fig. 3 a: Fluorescence alteration of the 2 μM BER solution as a function of the addition of CB[7] aqueous solution; b: ^1H NMR chemical shift alteration of the Hcy solution as a function of the addition of CB[7] aqueous solution. The inset displayed the linear double reciprocal plot indicating 1:1 inclusion complex. $C_{CB[7]} = 3 \mu\text{M}$, $C_{BER} = 2 \mu\text{M}$, $\lambda_{ex}/\lambda_{em} = 343/500 \text{ nm}$

Response mechanism of the fluorescent probe

Due to the isoquinoline and substituted benzene rings in BER are not in the same plane, it prevents the formation of a conjugated system. As a result, BER has feeble fluorescence emission in aqueous solution. When CB[7] was added into the BER solution, the isoquinoline ring part of BER entered the CB[7] cavity, arousing the electrostatic attraction between the heterocyclic nitrogen of BER and the carbonyl oxygen of the macrocycle. Eventually, the formed complex system leads to the increasing fluorescence of the guest molecule. In addition, the extent of freedom of movement of the BER molecules is reduced, thereby reducing the possibility of radiationless transition [33, 37]. When Hcy was mixed with the CB[7]-BER system, Hcy and BER contended to occupy the hydrophobic cavity of CB[7]. The electrostatic ion-dipole interaction and hydrophobic interaction between the amine group of Hcy and the carbonyl group of CB[7] portal promote the formation of complexation [27]. The photochemical performance of BER extremely depends on its local microenvironment. Hence, the addition of Hcy makes BER lose the protection of the CB[7] cavity, causing a reduction in the fluorescence intensity of BER (Scheme 1).

Scheme 1 Competitive recognition of CB[7] to Hcy against BER

Optimization of experimental conditions

Firstly, the influence of changing the BER and CB[7] concentrations on the fluorescence intensity of the supramolecular system was studied. CB[7] is spectroscopically inert in aqueous solution [37]. The fluorescence intensity of the CB[7]-BER complex increased obviously in the range from 1 to 10 μM (Fig. S4). In this study, it is extremely important to choose an appropriate BER concentration because it acts as a signal probe. If the concentration of BER is very low, the sensitivity of the probe will also be low. In turn, very high concentration may be detrimental to obtain an optimal detection limit for the analyte. Thus, 2 μM was chosen as the optimal BER concentration. Furthermore, the BER aqueous solution itself can only emit weak fluorescence (Fig. S5), but the results showed that the fluorescence intensity was gradually enhanced and then tended to balance after the addition of 3 μM CB[7] (Fig. S6). Therefore, 3 μM was chosen as the optimal CB[7] concentration.

The effect of pH on $F/F_{\text{pH}=7}$ was investigated. When the sample pH was maintained in the range of 5 to 10, the fluorescence variation of the system was relatively stable (Fig. S7). However, too high or too low pH was not conducive to the steady change of the fluorescence intensity of the reaction system. Thus, the pH

would not be adjusted in subsequent tests.

The impact of temperature on F/F_0 was also researched. As shown in Fig. S8, the formed complexes were stable below 30 °C. F/F_0 weakened greatly when the ambient temperature rose from 30 °C to 50 °C in view of the dissociation of the inclusion complexes at higher temperatures. Relying on the above events, all succeeding determinations were carried out at room temperature.

In addition, the outcomes confirmed that F/F_0 reached a minimum quickly within 8 min after the addition of Hcy and kept constant for several hours (Fig S9). Hence, incubation for 8 min at room temperature was selected as a standard measurement condition.

Analytical performance

Fig. 4 displayed that the fluorescence intensity of the formed CB[7]-BER complex gradually weakened with the increasing concentration of Hcy. Under the optimized test conditions, the standard calibration graph of F/F_0 versus Hcy concentration showed a good linear relationship ranging from 5 to 200 μM . The linear regression equation was $F/F_0 = -0.0003C_{\text{Hcy}} + 0.8783$ ($R^2 = 0.9999$) with the detection limit (LOD) of 1.76 μM ($\text{LOD} = 3\sigma/S$, σ : standard deviation, S : slope). Compared with other reported detection methods (Table 1), this established fluorescence method has advantages of fast detection time, satisfactory sensitivity, a wider linear range as well as without sample pretreatment.

Fig. 4 Fluorescence spectra of the CB[7]-BER system in the existence of dissimilar concentrations of Hcy (from a to j: 0, 1, 5, 10, 50, 80, 100, 200, 500, and 1000 μM). $C_{\text{CB}[7]} = 3 \mu\text{M}$, $C_{\text{BER}} = 2 \mu\text{M}$, $\lambda_{\text{ex}} = 343 \text{ nm}$. Inset displays the calibration curve of F/F_0 versus Hcy concentration

Table 1 Comparison with other reported technologies for the detection of Hcy

Detection technique	Sample pretreatment	Detection time (min)	Linear range (μM)	LOD (μM)	Appliction	Ref.
Colorimetric probe	/	10	2.5-30	2.09	/	[1]
Differential pulse voltammetry	Hcy-binding-aptamer was grafted onto the surface of Au nanoparticles/carbon electrode	60	0.05-20	0.01	Human blood serum and urine samples	[2]
HPLC-UV	Precolumn derivatization	10	0.7-10	0.2	Pig brain homogenates	[8]
Capillary zone electrophoresis	Reduction, derivatization and capillary electrophoresis separation	11.9	2-20	0.7	Human plasma samples	[15]
Fluorescence probe	Modified carbon dots	15	5-50	3.5	Human plasma samples	[38]
Fluorescent probe	Not needed	8	5-200	1.76	Bovine serum	This work

Effect of interfering substances

The influence of some common interferents on the determination of Hcy was investigated before applying the proposed method to actual sample analysis (Fig. 5).

The results demonstrated that ions (Na^+ , K^+ , Ca^{2+} , HCO_3^- , CO_3^{2-}), amino acids, except high-concentration phenylalanine (alanine, cysteine, valine, methionine, arginine, histidine, lysine, tryptophan), peptides (glutathione) and glucose in serum cannot significantly interfere with the determination of Hcy. Although high concentration of phenylalanine can interfere with the detection of Hcy by the CB[7]-BER probe, the normal concentration of phenylalanine in adult serum is low about μM level, which has little influence in actual serum detection applications. Accordingly, fluorescent probes based on the CB[7] host have good selectivity for the detection of Hcy.

Fig. 5 Influence of interfering substances on the detection of Hcy. The concentration of interferents and Hcy are both 1 mM

Analytical application

In order to verify the practicability of this method, the standard addition method was used to determine Hcy in the real bovine serum samples. From Table 2, the recoveries of examined samples spiked with three different concentrations of Hcy were in the range from 85.1% to 102.0%, and the related RSDs were lower than 5%. The above analysis results suggested that the fluorescent probe method is effective and reliable, with satisfactory accuracy and precision.

Table 2 Determination of Hcy in spiked serum

Sample	Added (μM)	Detected (μM) ^a	Recovery (%)	RSD (%)
Bovine serum	40	40.8 $\pm$ 1.1	102.0	2.8
	100	98.5 $\pm$ 1.6	98.5	1.6

	160	136.2 ± 6.3	85.1	4.6
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^a Mean ± standard deviation (n = 3)

Conclusions

This work reported a new method for detecting Hcy by means of the host-guest competitive interaction of CB[7] with Hcy and BER. It was identified that the formation of CB[7]-BER inclusion complexes strongly enhanced the fluorescence emission of BER because of their powerful coplanarity and rigidity. Because of the host-guest interaction and concentration advantage between CB[7] and Hcy, the BER in the hydrophobic cavity of CB[7] can be substituted by Hcy, leading to reduce the fluorescence emission of CB[7]-BER complex. This fluorescence probe method based on the competitive strategy was successfully applied to determine Hcy in biological serum, which exhibits good sensitivity and selectivity. The interaction mechanism between the CB[7] host and the analyte Hcy was also proved that the hydrophobic alkyl chain of Hcy located inside the host through a series of characterization techniques. In conclusion, this supramolecular fluorescent probe provides a promising way for the simple and fast determination of Hcy in clinic.

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Author Contributions

Chenrui Jiang: Method establishment, Data analysis, Writing - original draft. Deli Xiao: Supervision, Writing - reviewing & editing. Ping Yang: Software, Theoretical calculation. Wenyao Tao: Literature investigation. Zhaorui Song: Method establishment, Data analysis. Hua He: Supervision, Funding acquisition, Writing - reviewing & editing.

Data Availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval We declare that we have not violated any ethical responsibilities. No

Human Participants and/or Animals were used in this research.

Consent to Participate This is not applicable as we have not used any human participants.

Consent for Publication This is not applicable as we have not used any human participants.

Conflict of Interest The authors declare that they have no conflict of interest to the publication of this article.

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