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A facile, sensitive and selective fluorescent probe for heparin based on aggregation-induced emission

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ABSTRACT

A facile, rapidly responsive fluorescence turn-on probe for heparin with high selectivity and sensitivity was reported in this paper. The probe could aggregate on the negatively charged heparin template through electrostatic interactions and then display intense fluorescence due to its aggregation-induced emission (AIE) characteristics. Under optimal condition, the probe showed high selectivity to heparin over chondroitin sulfate(ChS), hyaluronic acid (HA), dextran (DeX) and other substances, with a linear range of 0.2–14 μg/mL, and a detection limit of 57.6 ng/mL. In diluted serum, it also showed good performance.

1. Introduction

Heparin is a highly sulfated glycosaminoglycan with high density of negative charges [1,2]. It is known as an anticoagulant in surgery and treatment of thrombotic diseases due to its quick anticoagulant effect by accelerating the inactivating of thrombin and several other coagulation factors [3]. At high concentrations, however, heparin could induce hemorrhages, thrombocytopenia and hyperkalemia [4,5]. Therefore, it is important to monitor heparin levels in medical applications.

Traditional methods for heparin detection include activated clotting time (ACT), activated partial thromboplastin time (APTT) and anti-factor Xa activity [6–9]. However, these methods are indirect, costly and lack of specificity [10,11]. In order to monitor heparin more effectively, in recent years, many new methods have been developed, such as fluorimetry [12–21], colorimetry [22–26], capillary electrophoresis [27–30], and electrochemical methods [31–34]. Among these methods, fluorescent chemosensors have been demonstrated useful because of their high sensitivity and low cost. However, they still have some drawbacks, such as long time lag [14], short wavelength emission [14], analyte-induced fluorescence quenching [12,14,20], requiring more rigorous testing media [18,21], restricted applicability in real samples [12,13,15,19], and a narrow dynamic response window [12,16]. For example, Anslyn et al. reported a heparin receptor with phenylboronic acids and ammonium groups showed remarkable selectivity and affinity for heparin, but the receptor undergo fluorescence quenching upon the binding of heparin [14]. In contrast to fluorescence quenching, Liu et al. developed a multicolor biosensor for heparin detection based on polymer/heparin complex formation to induce fluorescence resonance energy transfer between the fluorene fragments and the 2,1,3-benzothiadiazole units in the cationic polyfluorene derivative, but its application in complex biological media was not described [15]. Therefore, rapidly responsive fluorescent turn-on sensors that could be applied to quantify heparin in complex biological media with high selectivity and sensitivity are still imperatively demanded.

In 2001 Tang, Zhu and co-workers first reported the phenomenon of aggregation-induced emission (AIE), molecules of which are weakly fluorescent in solution but exhibit intense fluorescence after aggregation [35]. Since then, many AIE active dyes have been described by various research groups, and they have already been successfully used further as fluorescent sensors for metal ions, temperature, pH, and biosensing systems [36–41]. Our group has recently reported a series of salicylaldehyde azine derivatives with AIE characteristics [42]. Because of their long emission wavelength and large stokes shift, they have been applied in the detections of human serum albumin (HSA), bovine serum albumin (BSA) and protamine, as well as applied as optical materials [43–45]. To extend the potential applications of the AIE active salicylaldehyde azine derivatives for biomolecule detection, we developed a new ammonium group functionalized salicylaldehyde azine derivative N,N'-Bis[4-[[3-(trimethylammonio)ethyl]oxy]salicylidene]ethylenediamine bromide (BTASE) with AIE

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characteristics for heparin detection in diluted horse serum through charge–charge interaction in this paper.

2. Experimental

2.1. Apparatus

Fluorescence spectra measurements were recorded on a JASCO FP-6500 spectrofluorimeter (Tokyo, Japan) equipped with a xenon discharge lamp, 1 cm quartz cells. All pH tests were made with a Model pHS-3C pH meter (Shanghai, China). NMR spectra were measured using a JOEL JNM-ECA300 spectrometer operated at 300 MHz. Elemental analyses were carried on a FLASH EA1112 elemental analyzer. All of the measurements were operated at room temperature of 298 K.

2.2. Reagents

All reagents and solvents used in this paper were of analytical grade without further purification. Heparin sodium salt of 150 U/mg and hyaluronic acid were purchased from Beijing Kehaijunzhou Biotechnology Development Center (Beijing, China). Chondroitin sulfate and dextran were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Human serum albumin was purchased from Beijing Biodee Biotechnology Co. Ltd. (Beijing, China). Other reagents were purchased from Alfa Aesar Co. (Tianjin, China). Deionized water was used throughout the experiment. Tris–HCl solutions were prepared by adding HCl/NaOH to reach proper pH. The 1 mM stock solution of the dye reagent was prepared by dissolving the compound in dimethyl sulfoxide.

2.3. Analytical procedure

For heparin detection, to a 5.0 mL flask with 3.88 mL buffer (10 mM Tris–HCl at pH 7.0) containing different amount of heparin, 0.12 mL of the BTASE stock solution was added to each flask by a pippet to obtain solutions of 30 μM BTASE. After well mixed, the solution was allowed to stand at room temperature for 2 min. Then 2.0 mL of the solution was transferred to a 1 cm cell for fluorescence measurement by excitation/emission at 391/530 nm.

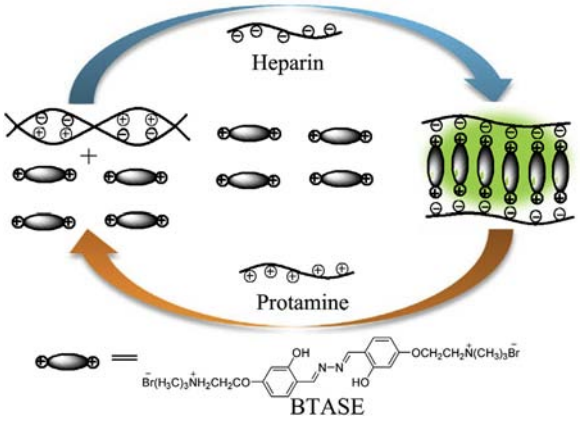
Heparin detection in horse serum was carried out as follows. Horse serum was diluted in buffer (10 mM Tris–HCl at pH 7.0) to produce a 1% serum sample. Then 0.12 mL of the BTASE stock solution was added to a 5.0 mL flask with 3.88 mL serum sample containing different amount of heparin. After well mixed, the solution was allowed to stand at room temperature for 2 min. Then 2.0 mL of the solution was transferred to a 1 cm cell for fluorescence measurement by excitation/emission at 391/530 nm.

3. Results and discussion

3.1. The fluorescence turn-on detection of heparin

As shown in Scheme 1, positively charged tertiary amines was introduced into BTASE to improve its water solubility. BTASE displayed very weak fluorescence in aqueous solution. Upon addition of heparin, BTASE could aggregate on the negatively charged heparin template due to electrostatic interactions between the positively charged tertiary amines in BTASE and the negatively charged sulfate and carboxylate groups in heparin, and thus display intense AIE fluorescence.

In 10 mM Tris–HCl at pH 7.0, 30 μM BTASE displayed very weak fluorescence at 530 nm, while the fluorescence increased in the presence of heparin. Nearly 40-fold fluorescence enhancement



Scheme 1. The design principal of the fluorescence turn-on detection of heparin based on AIE characteristics of BTASE.

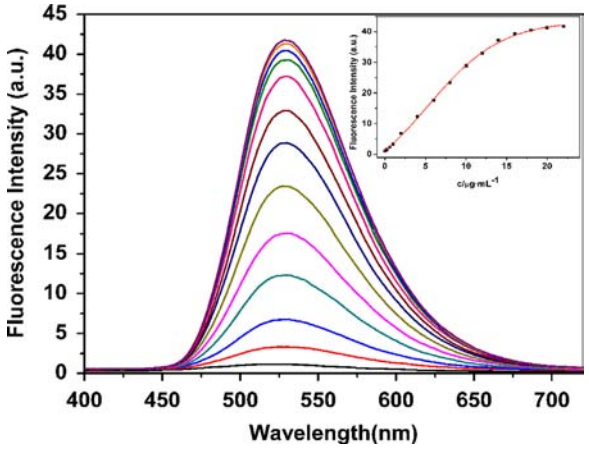


Fig. 1. Fluorescence spectra of BTASE (30 μM in 10 mM Tris–HCl buffer solution, pH=7.0) in the presence of different amounts of heparin (from 0 to 22 μg/mL), λ_{ex}=391 nm.

was observed when the concentration of heparin reached 22 μg/mL (Fig. 1), which should be attributed to the aggregation of BTASE on the negatively charged heparin template through electrostatic interactions. The formation of aggregates of BTASE-heparin aggregates was directly observed using TEM (transmission electron microscope): particle size of micrometre sizes were detected in the solution of BTASE containing 16 μg/mL heparin; while no particle was observed in the solution of BTASE without heparin (Fig. S1).

3.2. Optimization of the analytical condition

3.2.1. Effect of BTASE concentration

In 10 mM Tris–HCl at pH 7.0 containing 16 μg/mL heparin and 30–40 μM BTASE, a maximum fluorescence intensity could be obtained (Fig. 2). The excessive BTASE (> 40 μM) were well dispersed in solution and displayed almost no fluorescence, but could absorb the excitation light and induce inner filter effects, which led to fluorescence decrease of BTASE and heparin aggregates [46]. Thus, 30 μM BTASE was used for the following tests.

3.2.2. Effect of pH

The effect of pH on fluorescence intensity is shown in Fig. 3. In 10 mM Tris–HCl with 30 μM BTASE and 16 μg/mL heparin, the

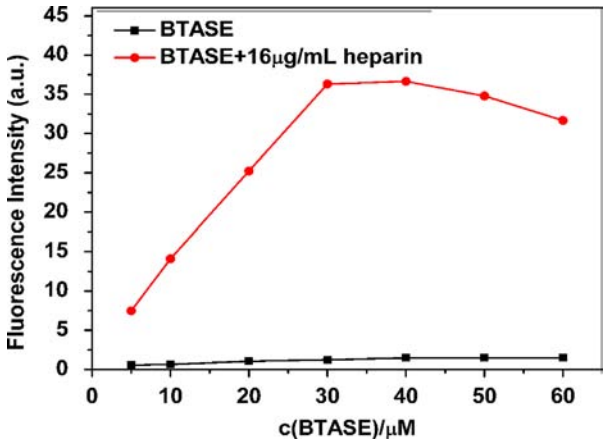


Fig. 2. The effect of BTASE concentration on the fluorescence intensity, in 10 mM Tris–HCl buffer solution at pH 7.0 with 16 μg/mL heparin, λ_{ex} =391 nm, λ_{em} =530 nm.

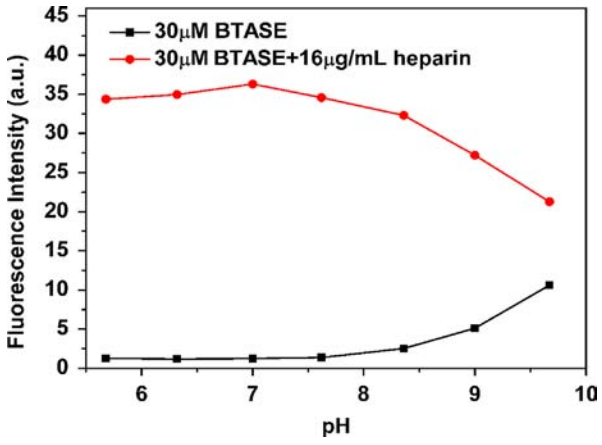


Fig. 3. The effect of pH on the fluorescence intensity, in 10 mM Tris–HCl buffer solution with 30 μM BTASE and 16 μg/mL heparin, λ_{ex}=391 nm, λ_{em}=530 nm.

fluorescence intensity reached its maximum within the range of pH 5.7–7.6, after which it started to decrease because the hydroxyl groups of BTASE underwent deprotonation at high pH, leading to the increase of repulsion between the probe and heparin molecules and weakening the binding. Therefore, pH 7.0 was chosen for this work.

3.2.3. *Effect of reaction time*

The results of time-dependent fluorescence measurement of the mixture containing 30 μM BTASE and 16 μg/mL heparin in 10 mM Tris–HCl at pH 7.0 is displayed in Fig. 4. Aggregation of BTASE to heparin was very fast and could be finished in 100 s. A time of 2 min was adopted in following experiments to ensure the reaction of BTASE with heparin was fully completed.

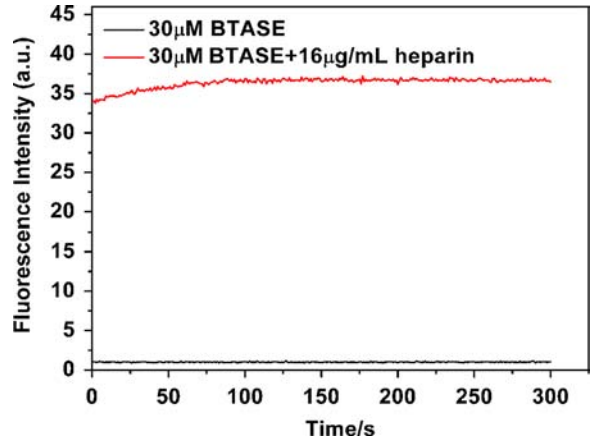


Fig. 4. The effect of reaction time on the fluorescence intensity, in 10 mM Tris–HCl buffer solution at pH 7.0 with 30 μM BTASE and 16 μg/mL heparin, λ_{ex}=391 nm, λ_{em}=530 nm.

3.2.4. *Selectivity over other polysaccharides*

To investigate selectivity, other three polysaccharides, chondroitin sulfate (ChS), hyaluronic acid (HA) and dextran (DeX) were also tested with BTASE. As shown in Fig. 5, there was almost no response of BTASE to HA and DeX, and ChS displayed much weaker fluorescence enhancement compared with heparin. The high selectivity of BTASE to heparin was because ChS, DeX and HA contained low charge density and could not bind to BTASE efficiently to form significant aggregations of BTASE.

3.2.5. *Interference of foreign substances*

The interference of some foreign substances to 16 μg/mL heparin (within 5% error) was tested under the optimum condition. As shown in Table 1, most of the foreign substances displayed no significant influence on heparin detection. The major interferences were Fe^{3+}, Co^{2+} and Cu^{2+}, which might strongly bind with BTASE, and their interferences can be eliminated by adding metal ion masking agents such as EDTA.

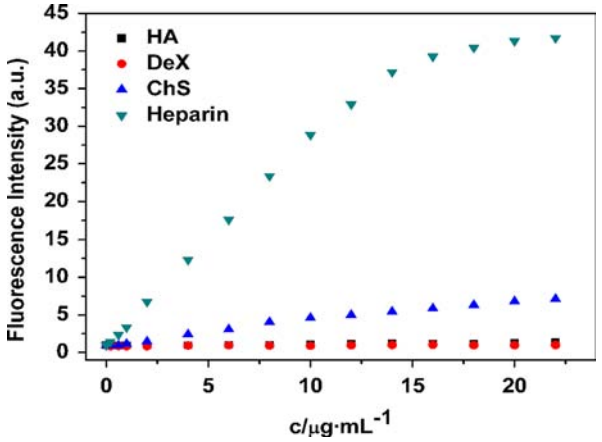


Fig. 5. The fluorescence intensity of BTASE (30 μM in 10 mM Tris–HCl buffer solution, pH=7.0) in the presence of different amounts of HA, DeX, ChS and heparin, λ_{ex}=391 nm, λ_{em}=530 nm.

3.3. *Analytical figures of merit*

According to the above optimized condition, the calibration curve for the determination of heparin by BTASE was constructed (Fig. S2). The relationship between fluorescence intensity of BTASE at 530 nm and concentration of heparin in μg/mL was as $I_{530}=0.75+2.76\times$ [heparin], the linear range was 0.2–14 μg/mL (0.03–2.1 U/mL) with a correlation coefficient of $R^2=0.999$ ($n=10$). The detection limit based on IUPAC ($C_{DL}=3\,S_b/m$) [47] was 57.6 ng/mL (8.64×10^{-3} U/mL) according to 10 blank measurements, which is comparable to or even lower than those of several other reported fluorescent sensors for heparin [12,15,18,19,21]. This detection range is suitable for heparin monitoring during postoperative and long-term care (0.2–1.2 U/mL) [15].

Table 1
Interference of foreign substances.[a]

Substance	concentration (μM)	Response ratio[b] (%)
Na^+, K^+	2000	< −5.0
NH_4^+	500	< −4.1
Zn^{2+}, Ag^+, Al^{3+}, Hg^{2+}, Mn^{2+}, Ca^{2+}, Ba^{2+}	20	< −5.0
Cd^{2+}, Mg^{2+}	10	< −4.5
Co^{2+}	1	−1.9
Fe^{3+}	0.5	−4.8
Cu^{2+}	0.1	−3.2
L-Histidine, L-Threonine, L-Phenylalanine, L-Proline, L-Cyseine, L-Lysine, L-Aspartic acid, L-Glutamic acid	1000	< −5.0
ATP(Adenosine triphosphate), ADP(Adenosine diphosphate), AMP(Adenosine monophosphate), ppi(Pyrophosphoric acid)	200	< −4.7
Adenosine	500	−4.7
Glucose	1000	2.0
BSA(Albumin from bovine serum)	20	−3.8

[a] Conditions: 30 μM BTASE, 16 μg/mL (1 μM) heparin, 10 mM Tris–HCl buffer solution at pH=7.0, 2 min reaction at room temperature. Excitation/emission was performed at 391/530 nm.
[b] response ratio=$(I-I_0)/I_0$, where I_0 and I denote fluorescence intensity before and after addition of a foreign substance to the solution.

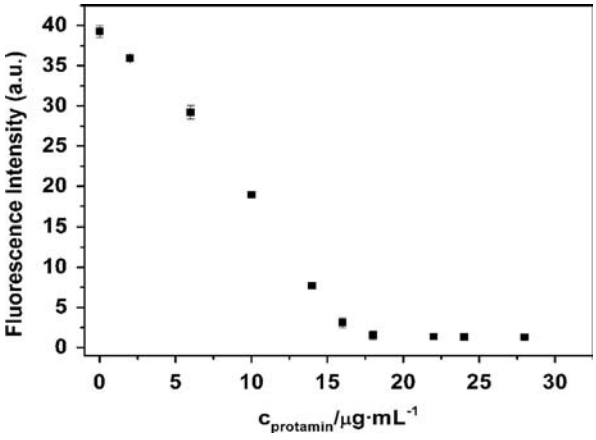


Fig. 6. The fluorescence intensity of 30 μM BTASE with 16 μg/mL heparin (in 10 mM Tris–HCl buffer solution, pH=7.0) in the presence of different amounts of protamine (from 0 to 28 μg/mL), λ_{ex}=391 nm.

3.4. Competitive binding with protamine

Protamine is a highly cationic protein, and it could strongly bind with heparin through electrostatic interactions [1]. In medicine, it is used in the treatment of heparin overdose. Therefore, we studied the effect of protamine on the AIE fluorescence of BTASE-heparin complex. As shown in Fig. 6, the fluorescence of BTASE-heparin decreases gradually upon the addition of increasing amounts of protamine, most likely because the strong binding between protamine and heparin weakens the aggregation of BTASE. Therefore, BTASE could also be a potentially useful fluorescence probe to study the interaction between heparin and certain proteins [1].

3.5. Application in real sample analysis

The proposed method was also applied to the analysis of heparin in a horse serum sample. Considering horse serum contains proteins such as albumin, globulin and fibronectin, 100-fold diluted horse serum was selected for sample treatment to avoid the possible interference caused by these biomolecules. In 100-fold diluted horse serum containing 30 μM BTASE, the fluorescence intensity of BTASE increased linearly with the addition of heparin (Fig. S3), very similar to that in buffer solution (Fig. S2). The relationship between the fluorescence intensity of BTASE at 530 nm and the concentration of heparin in μg/mL was as $I_{530}=1.31+2.37\times$[heparin], and the linear range was found as 0.2–14 μg/mL (0.03–2.1 U/mL) with a correlation coefficient of $R^2=0.992$ (n=10). Recovery experiments were carried out by adding 4.5 μg/mL and 9.0 μg/mL heparin to 100-fold diluted serum samples, and shown in Table 2. The results showed that the proposed method was satisfactory and effective in heparin detection in diluted serums.

Table 2
The results of real sample measurements.[a]

Sample	Heparin added /μg·mL^{-1}	heparin found /μg·mL^{-1}	Recovery (%)	R.S.D.[b] (%)
1% Diluted horse serum	4.50	4.58	102	3.6
	9.00	9.04	101	1.4

[a] Conditions: 30 μM BTASE, 10 mM Tris–HCl buffer solution at pH=7.0, 2 min reaction at room temperature. Excitation/emission was performed at 391/530 nm.
[b] $n=3$.

3.6. Comparing with other methods for heparin detection

This method was compared with other reported fluorescent sensors for heparin (Table 3). Although some methods exhibit a wider linear range [13–15,18], they still have some limitations, such as long time lag [14], short wavelength emission [14], analyte-induced fluorescence quenching [14], requiring more rigorous testing media [18], and restricted applicability in real samples [13,15]. Our newly developed fluorescent sensor based on salicylaldehyde azine showed a number of attractive analytical features such as high sensitivity, good selectivity, simple operation process, short analysis time and good reproducibility.

4. Conclusion

In summary, we have described a new AIE fluorescence turn-on probe for heparin detection with high sensitivity and selectivity to heparin through electrostatic interaction. With a short response time of 2 min, the proposed method had a detection limit of 57.6 ng/mL heparin with a linear range of 0.2–14 μg/mL in 10 mM Tris–HCl at pH 7.0, and could be used for heparin detection in diluted serums.

Table 3
Comparison of different fluorescent sensors for heparin detection.

Sensor	Work mode	Linear range	Detection limit	Test media	Applications	Reference
tripodal boronic acid	Turn off	0–9 U/mL	Not given	HEPES(10 mM, pH=7.4, containing 19% serum)	19% serum	[14]
chromophore-tethered copolymer	Turn off	0.075–0.55 U/mL	Not given	HEPES (25 mM, pH=7.12)	Not available	[12]
Poly(fluorine-alt-benzothiadiazole)derivative	Turn on	0–8 U/mL	Not given	PBS (2 mM, pH=7.4)	Not available	[13]
Poly(fluorine-alt-benzothiadiazole)derivative	Turn on	0–5 U/mL	3.3×10^{-3} U/mL	PBS (2 mM, pH=7.4)	Not available	[15]
Butterfly-shaped conjugated oligoelectrolyte and graphene oxide	Turn on	0–1.76 U/mL	0.046 U/mL	PBS (10 mM, pH=7.4)	Not available	[19]
Quinolinium labled peptide	Turn on	0–0.4 U/mL	Not given	HEPES (10 mM, pH=7.4)	10% bovine serum	[16]
Benzimidazolium dyes	Turn on	0.012–1.2 U/mL	Not given	HEPES (10 mM, pH=7.4, containing 1% DMSO)	20% human plasma	[17]
Quinine derivative	Turn on	0.2–4 U/mL	0.075 U/mL	HEPES (10 mM, pH=7.4, C2H5OH/H2O=1:3)	2% bovine serum	[18]
Pyrene derivative	Ratiometric	0.6–3.6 U/mL	0.019 U/mL	HEPES (10 mM, pH=7.4, C2H5OH/H2O=15/85)	5% Fetal bovine serum	[21]
Salicylaldehyde azine derivatives	Turn on	0.03–2.1 U/mL	8.64×10^{-3} U/mL	Tris–HCl (10 mM, pH=7.0)	1% horse serum	Present work

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.09.055.

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