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# A facile four-armed AIE fluorescent sensor for heparin and protamine

Yuxia Gao[a,b,1], Kailun Wei[c,1], Jianzuo Li[c], Ying Li[c,*], Jun Hu[a,*]

[a] Beijing Advanced Innovation Center for Soft Matter Science and Engineering, Beijing University of Chemical Technology, Beijing, 100029, China
[b] Department of Applied Chemistry, College of Science, China Agricultural University, Beijing, 100193, China
[c] College of Chemistry and Material Science, Shandong Agricultural University, Tai'an, 271018, China

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ABSTRACT

In this research a four-armed amino-tailored tetraphenylethene **TPE-NH$_2$** was synthesized, and it could detect heparin (Hep) quantitatively, primarily promoted by the electrostatic interactions between its positively charged amino groups and the sulfates, carboxylates of heparin. On account of the aggregation-induced emission (AIE) nature of tetraphenylethene backbone, the aggregation restricted the rotation of C—C single bond through rigidifying the conformation of **TPE-NH$_2$**, thus making it display intense fluorescence with a broad working range (≤2.0 μg/mL) and a low detection limit (down to 35.89 ng/mL) for heparin. Meanwhile, the **TPE-NH$_2$**/Hep complex can be further disaggregated by protamine due to the stronger affinity between heparin and protamine, consequently leading to the highly sensitive detection of protamine. What's more, the low toxicity of **TPE-NH$_2$** makes it a potential functional sensor for detecting heparin in the matrix of human serum.

## 1. Introduction

Heparin (Hep), a linear sulfated glycosaminoglycan, owns the highest negative charge density among the biological macromolecules [1]. It has been used as anticoagulant in surgery and treatment of thrombotic diseases over 80 years, due to it can bind antithrombin with a great affinity and enhance the inhibition of antithrombin significantly [2]. But the overdose of heparin induces serious side effects, like hemorrhages, hyperkalemia, and thrombocytopenia [3,4]. As a well-known heparin antidote, protamine is a positively charged protein (pI = 13.8) and often employed to reverse the anticoagulant effect of heparin through the electrostatic interactions [5]. Similarly, the overuse of protamine could also induce many adverse effects including hypotension and idiosyncratic fatal cardiac arrest [6]. Therefore, it is of great significance to detect and quantify heparin and protamine in medical applications.

Traditional detection methods mainly include activated clotting time (ACT), anti-factor Xa activity, and activated partial thromboplastin time (APTT) [7–9], which are either inaccurate, expensive, or lack of specificity [10–12]. Currently, various strategies have been developed to monitor heparin and protamine effectively, such as capillary electrophoresis [13], surface enhanced Raman spectroscopy [14], electrochemical methods [15,16], colorimetric assay [17–19] and fluorescent methods [20–22]. Among them, fluorescent methods have drawn extensive attention on account of the advantages in terms of high sensitivity and selectivity, easy operation, and real time detection [23,24]. For example, Schrader group developed a chromophore-tethered polymeric sensor which could measure heparin in serum with "turn off" response, due to the formation of cyclic esters with aminoethanol and glycol sulfate moieties [25]; Liu and co-workers have described $CuInS_2$ QDs for detecting heparin with a limit of 12.46 nM, promoted by the electrostatic interactions between amino groups on $CuInS_2$ and sulphates in heparin [26]. While these conventional fluorescent chemosensors tend to form aggregates in aqueous solutions, where aggregation-caused quenching (ACQ) effect may induce strong interference during the detection process. Thus, rapidly responsive fluorescent sensors that can quantify heparin and protamine in biological media without ACQ effect are still imperatively demanded.

Inspired by the above, herein, in this research we reported a four-armed fluorescent "turn on" sensor **TPE-NH$_2$** for heparin by making use of aggregation-induced emission (AIE) effect (Fig. 1) [27,28], molecules of which exhibited weak fluorescence in solution but intense fluorescence upon aggregation. In this four-armed sensor, the hydrophobic tetraphenylethene (TPE) moiety, a widely used AIE fluorophore, was linked to four positively charged amino groups by the flexible alkyl chains. The results showed that **TPE-NH$_2$** could detect heparin quantitatively through the electrostatic interactions with a low detection limit of 35.89 ng/mL in a working range (≤2.0 μg/mL). Moreover, the

* Correponding authors.
*E-mail addresses:* ly8049@sdau.edu.cn (Y. Li), jhu@mail.buct.edu.cn (J. Hu).
[1] These authors contributed equally to this work.

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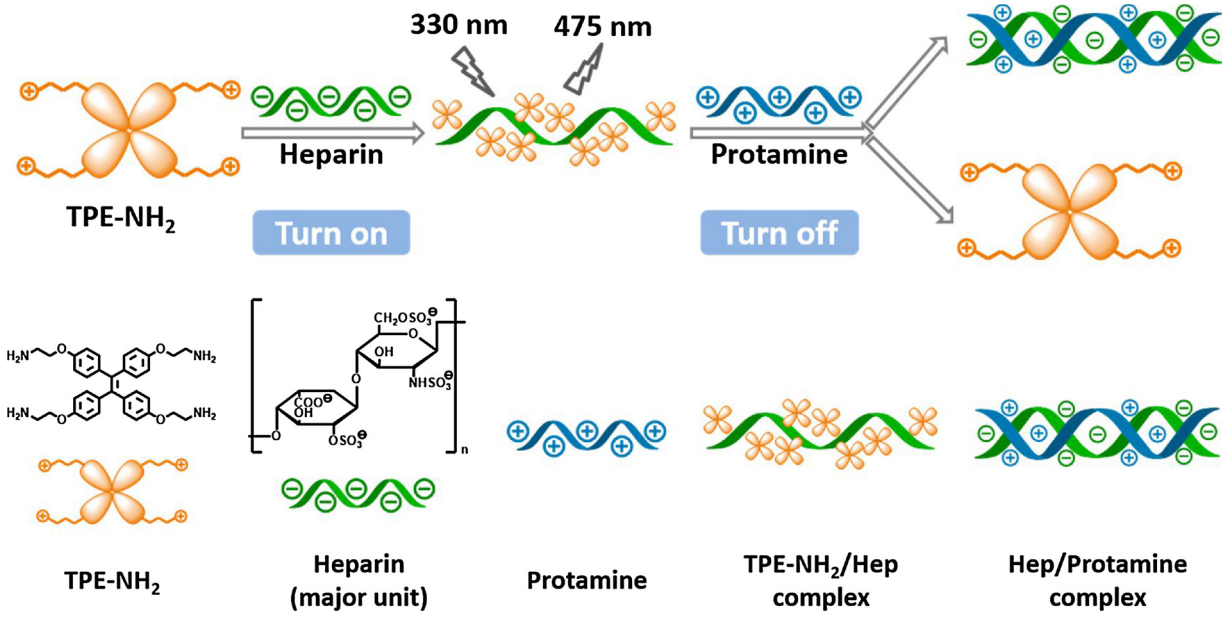


**Fig. 1.** Schematic representation of the detection process of the four-armed sensor **TPE-$NH_2$**.

**TPE-$NH_2$**/Hep complex could further measure protamine effectively due to the greater affinity between heparin and protamine (Fig. 1). This work may provide a simple approach for designing "turn on" probe, which can be beneficial to monitor the dose of heparin and protamine, respectively.

## 2. Experimental

### 2.1. Materials and apparatus

Heparin sodium salt, protamine sulfate, hyaluronic acid (HA), 4,4′-dimethoxybenzophenone, borontribromide, titanium tetrachloride, sodium citrate, sodium phosphate and glucose were purchased from Energy Chemical. 1,2-Dibromoethane, triphenylphosphine, bovine serum albumin (BSA) and calcium hydride were provided by Aladdin. Human serum was provided by Tai'an Hospital of Traditional Chinese Medicine. Tris(hydroxy-methyl)aminomethane hydrochloride (Tris – HCl), sodium azide, and other common chemicals were from local commercial suppliers. AR grade solvents were distilled before use.

$^1H$ and $^{13}C$ NMR spectra were recorded on JEOL JNM-ECA 400 spectrometer in $CDCl_3$ or $d_6$-DMSO at 25 °C. Electrospray ionization mass (ESI-MS) was analyzed on Bruker ESQUIRE-LC spectrometer in positive mode. Fourier transform infrared (FTIR) spectra were recorded on Nicolet 360 spectrophotometer by incorporating samples in KBr pellets. UV–vis spectra were recorded on NANODROP 2000C spectrophotometer. Fluorescence measurements were carried out on Fluorescence Spectrometer of Agilent Cary Eclipse. Fluorescent decay curves were performed on a FLSP-920 spectrophotometer (Edinburgh Instruments) with a 375 nm laser. Zeta potential was recorded by Malvern Zetasizer (Nano-ZS).

### 2.2. Analytical procedure

#### 2.2.1. Heparin detection

20 μL TPE-$NH_2$ stock solution (1 mM) was added to 2 mL Tris – HCl buffer (10 mM, pH 6.0) containing different amount of heparin (0–20 mg/mL). After incubating at room temperature for 0.5 h, fluorescence measurements were performed with excitation at 330 nm. The detection limit was calculated according to the formula "detection limit = 3 × σ/S", where σ is the standard deviation of blank samples and S is the slope of calibration line.

Heparin detection in human serum. Before the measurement, the standard curve was obtained as follows: initially different amounts of heparin (1 mg/mL, 0–4.0 μL) were mixed with 20 μL TPE-$NH_2$ solution (1 mM), and then the mixtures were diluted to 2.0 mL by 1% human serum, which was prepared in Tris – HCl buffer (10 mM, pH 6.0). The samples were incubated under ambient temperature for 0.5 h, and fluorescence spectra were conducted with excitation at 330 nm.

For the recovery measurement, different amounts of heparin (1 mg/mL, 0.6–3.0 μL) were mixed with TPE-$NH_2$ (1 μM, 20 μL), and diluted to 2.0 mL by 1% human serum samples. After that, their fluorescence were investigated and the recovery of heparin were calculated. Three replicates were performed, and the equation of recovery was as follows:

$$\textit{Recovery} = \frac{\textit{Founded}}{\textit{Added}} \times 100\%$$

#### 2.2.2. Protamine detection

20 μL Tris – HCl solution of TPE-$NH_2$ (1 mM) was first added to a heparin aqueous (1 mg/mL, 10 μL), and then different amounts of protamine was added. After diluting to 2.0 mL by Tris – HCl buffer, the mixture was incubated for 30 min, and fluorescence measurements were performed with excitation at 330 nm.

#### 2.2.3. Cytotoxicity measurement

Michigan Cancer Foundation-7 (MCF-7) cells were provided by Norman Bethune Health Science Center of Jilin University and grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) in humidified atmosphere of 5% $CO_2$ at 37 °C. Cells were seeded in 96-well plates and cultured for 24 h with a density of $1 \times 10^4$ cells per well. After incubation, the previous media was replaced by fresh DMEM containing TPE-$NH_2$ at certain concentrations and incubated for 24 h. Subsequently, 10 μL CellTiter-Blue™ reagent was added to each well and incubated for another 3 h to perform the cell viability assay. The fluorescence intensity was measured at 560/590 nm ($E_x/E_m$) using a BioTek Synergy H1 microplate reader.

### 2.3. Synthesis of the four-armed AIE sensor TPE-$NH_2$

As shown in Scheme 1, boron tribromide (12.90 g, 51.50 mmol) was added dropwise to a solution of 4,4'-dimethoxybenzophenone (5.00 g, 20.60 mmol) in dry dichloromethane (DCM, 20 mL) in an ice bath, and