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ARTICLE

A "Turn On" Fluorescent Probe for Heparin and Its Oversulfated Chondroitin Sulfate Contaminant

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Yubin Ding,[a] Leilei Shi[a] and Hui Wei*[a]

Designing "turn on" fluorescent probes for heparin (Hep), a widely used anticoagulant in clinics, is of great importance but remains challenging. By introducing a Hep specific binding peptide AG73 to a typical aggregation induced emission (AIE) fluorogen, tetraphenylethylene (TPE), a sensitive and selective fluorescence "turn on" probe named **TPE-1** for Hep was developed. **TPE-1** was able to detect Hep in a wide pH range of 3-10 without obvious interference from tested anions and biomolecules, especially Hep analogues known as chondroitin sulfate (Chs) and hyaluronic acid (HA). The detection limit of Hep sensing was 3.8 ng/mL, which was far below the clinical demanded concentration of Hep. The probe was applicable to both unfractioned Hep and low molecular weight Hep, the two main heparin products clinically used. Besides, fluorescence of Hep bound **TPE-1** can be turned off via sequential treatment with heparinases. Importantly, this phenomena allows us to develop an enzyme assisted strategy for "turn on" sensing oversulfated chondroitin sulfate (OSCS) with a detection limit of 0.001% (w%), which is a main contaminant in Hep and may cause severe adverse reactions even to death.

Introduction

Heparin (Hep) is a highly sulfated glycosaminoglycan (GAG) with the highest negative charge density of any known biomacromolecules.[1] It can bind antithrombin with a high affinity, and the binding significantly enhances antithrombin's inhibition activity towards thrombin and other coagulation factors.[2] Thus, Hep has been widely used in clinics as both prophylactic and therapeutic agents, especially as an anticoagulant in surgery.[3] The Hep therapeutic dosage should be maintained within a range of 2-8 U/mL for cardiovascular surgery, and a range of 0.2-1.2 U/mL for post-operative and long-term therapy.[4] Overdose of Hep could cause many adverse effects such as hemorrhage, thrombocytopenia, hyperkalemia and osteoporosis.[5] As a result, the dose of Hep must be closely monitored. In clinical laboratories, the dose is determined by activated clotting time assay (ACT) or activated partial thromboplastin time assay (aPTT).[6] These well-established assays, however, are indirect methods, which are time-consuming, costly, and not sufficiently reliable. Therefore, it is urgent to develop efficient and reliable methods for selective and sensitive detection of Hep.

Considerable efforts have been devoted to development of Hep detection methods based on various strategies, such as colorimetric assay, capillary electrophoresis, electrochemical methods, surface enhanced Raman spectroscopy, unbiased sensor array, etc.[7] Recently, particular interests have been focused on the design of small organic molecule based fluorescent probes for sensing various analytes including Hep.[8] These probes have shown distinct advantages of low cost, easy to manipulate and high performance. For example, a 1,3,5-triphenyl-ethynylbenzene based fluorescent probe for Hep sensing was synthesized by functionalizing it with boronic acid and positively-charged ammonium moieties.[9] However, many of the reported methods were based on complicated nano-organic hybrid systems or "turn off" signal response.[9-10] For fluorescent sensing, the "turn on" probes are more reliable than the "turn off" ones, as false results may occur due to the fact that a number of factors other than the target analyte may cause fluorescence quenching.[11] In this regard, the development of sensitive and selective "turn on" fluorescent probes for Hep is still highly desired.

Aggregation induced emission (AIE) is a promising strategy for designing "turn on" fluorescent probes, as the AIE fluorogen molecules are almost non-fluorescent in the solution state but become strongly fluorescent after analyte induced aggregation.[12] Tetraphenylethene (TPE) is a widely used fluorogen for designing probes based on AIE mechanism, and it can be facilely functionalized.[13] We envisioned that the conjugation of TPE to a Hep specific binding group could afford a "turn on" probe for Hep with high sensitivity and selectivity. We noticed that the commonly conjugated ammonium moiety showed limited specificity for Hep against its analogues such as chondroitin sulfate (Chs) and hyaluronic acid (HA). Considering

[a] Department of Biomedical Engineering, College of Engineering and Applied Sciences, Nanjing National Laboratory of Microstructures, Nanjing University, Nanjing, Jiangsu, 210093, China. Fax: +86-25-83594648; Tel: +86-25-83593272; E-mail: weihui@nju.edu.cn

† Footnotes relating to the title and/or authors should appear here.
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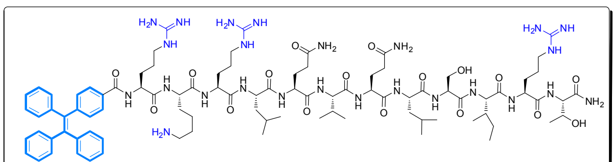
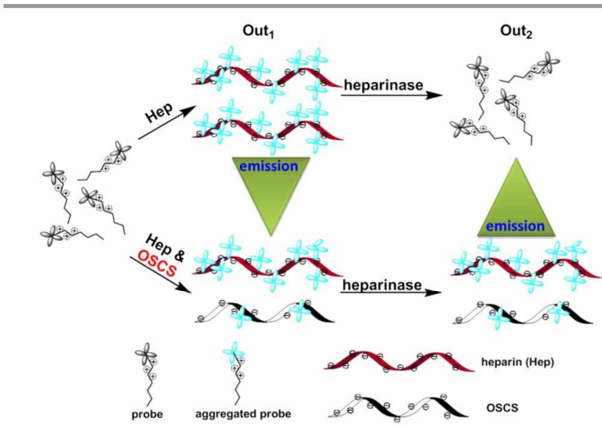
these, a Hep-specific binding peptide, i.e., AG73 (sequence: RKRLQVQLSIRT), was employed to construct the TPE based probe. AG73 is a peptide from the G domain of the laminin α1 chain, which has shown high binding affinity and specificity towards Hep.[14] The probe **TPE-1** was rationally designed by conjugating the TPE fluorogen to the AG73 peptide (Scheme 1). Indeed, the designed **TPE-1** shows excellent fluorescence "turn on" response to Hep with a detection limit of 3.8 ng/mL. More, the Hep detection with **TPE-1** was not interfered by biological abundant anions, biomolecules, and Hep analogues such as Chs and HA in a wide pH range of 3-10.



Scheme 1. Structure of the "signal on" probe **TPE-1**. The probe was constructed by rationally combining the recognition element for Hep (i.e., the AG73 peptide) and the signalling element (i.e., the TPE fluorogen).

On the other hand, oversulfated chondroitin sulfate (OSCS) was reported to be the principal contaminant in Hep, which is a non-natural highly sulfated synthetic GAG.[15] The presence of OSCS in Hep may lead to severe adverse reactions, such as angiodema, hypotension, swelling of the larynx, and even death.[16] Orthogonal methods such as high performance liquid chromatography (HPLC) combined with nuclear magnetic resonance (NMR) and mass spectroscopy are valuable to identify OSCS contaminant in Hep. However, these methods need highly sophisticated instruments and experienced operators. It is thus urgent to develop low cost, easy to operate and highly efficient methods for OSCS detection.[17] Assisted with heparinases treatment, here we also proposed an approach for OSCS detection using **TPE-1** as the probe (Scheme 2). We found that the fluorescence of **TPE-1** can be enhanced by Hep binding and quenched by sequential treatment with heparinases. This fluorescence "off-on-off" process was influenced by OSCS, as it is an inhibitor for heparinases. The OSCS contaminant in Hep thus could be quantified by comparing the output fluorescent signals before and after the enzyme treatment (i.e., the Out_2/Out_1 ratio).



Scheme 2. Fluorescent OSCS detection by **TPE-1** assisted with heparinases treatment. The amount of OSCS present in Hep could be determined by the Out_2/Out_1 ratio.

Results and Discussion

Design, Synthesis and AIE properties of Probe TPE-1.

As shown in Scheme 1, the molecular structure of **TPE-1** consists of two parts, both of which play important roles in Hep sensing. The TPE moiety is a typical AIE fluorogen, which emits strong fluorescence only after aggregation and acts as the signal reporting group in **TPE-1**. While the AG73 peptide with a sequence of RKRLQVQLSIRT was reported to show high affinity towards Hep,[14a] and this moiety is incorporated as the Hep recognition group. **TPE-1** was synthesized via solid phase peptide synthesis with an acceptable yield of 33%, and a high purity of 99.7% (See ESI for more details, Figs. S1-S4†). The AIE properties of **TPE-1** were then evaluated. As shown in Fig. 1, the fluorescence of free **TPE-1** was enhanced by only 114% even in 100% HEPES compared with 80% HEPES, which was attributed to its high solubility in water. On the other hand, TPE still retained its excellent AIE property after bioconjugation, as indicated by the gradual fluorescence increase with the increase of HEPES buffer fractions. Interestingly, there was an obvious inflection point at 95% HEPES buffer. When the fraction of HEPES buffer in the mixture was higher than 95%, **TPE-1** was sharply turned on and emitted stronger fluorescence. Based on these results, a mixture of HEPES buffer/DMSO solution with a volume ratio of 95:5 was chosen for Hep sensing.

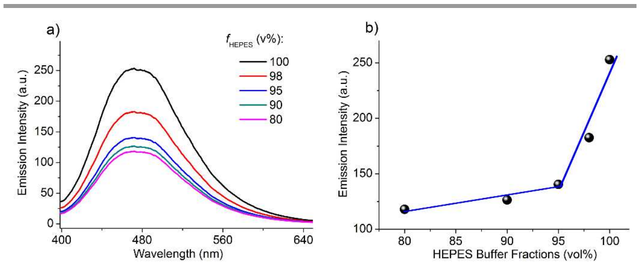


Fig. 1 AIE properties of **TPE-1**. a) Fluorescence spectra of **TPE-1** (10 μM) in HEPES buffer/DMSO mixed solutions with different HEPES buffer fractions. b) Plot of the fluorescence intensity at 470 nm *versus* the HEPES buffer fraction. λ_{ex} = 340 nm.

Sensitive and selective fluorescence "turn on" detection of Hep.

As mentioned above, no fluorescence can be observed by the naked eye when **TPE-1** was dissolved in a 95% HEPES buffer solution. However, vivid blusih green fluorescence "turn on" was observed immediately after addition of Hep to 10 μM **TPE-1** solution (Fig. 2). Fluorescence titration experiments were carried out to investigate the sensing performance of **TPE-1** towards Hep. In the absence of Hep, **TPE-1** showed a very weak fluorescence. With increasing amount of added Hep, the fluorescence intensity of the peak at around 470 nm was enhanced gradually. 5.8 μg/mL of Hep was needed to saturate the fluorescence enhancement by a factor of about 31 (Fig. 2a). Analysis of the titration data reveals that the emission intensity increased linearly with the increasing concentrations of Hep in a dynamic range of 0.32-5.5 μg/mL, and the corresonding correlation coefficient R was 0.9995. The calculated detection limit of Hep based on 3σ/s was 35 ng/mL, where σ was the standard deviation of 10 blank measurements and s was the slope of the emission intensity as a function of the Hep concentration (Fig. 2b). The detection limit can be further lowered by adjusting the amount of **TPE-1** used. When the concentration of **TPE-1** used was 1 μM, the detection limit towards Hep was as low as 3.8 ng/mL, which was much lower than the clinical demanded concentration of Hep (Fig. S5†). And the corresonding dynamic range was 0.064-0.32 μg/mL when the concentration of **TPE-1** was 1 μM.

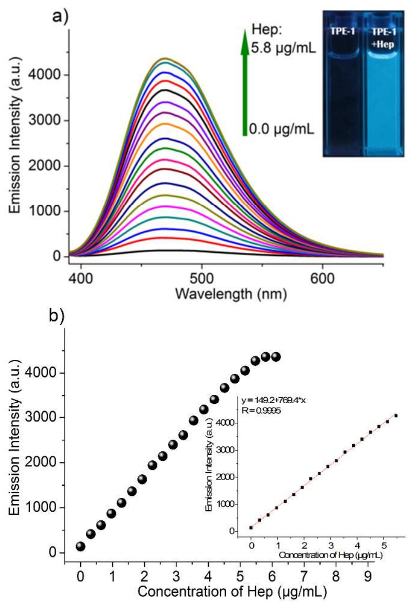


Fig. 2 Hep detection with **TPE-1**. a) Fluorescence titration profile of **TPE-1** (10 μM) with increasing amount of Hep in 10 mM pH 7.4 HEPES/DMSO (95:5, v:v). λ_{ex} = 340 nm. Inset: a photograph showing the fluorescence "turn on" behavior of **TPE-1** after addition of 5.8 μg/mL Hep. b) Corresponding calibration curve of Hep. Inset: linear response curve of Hep.

We also noticed that there are mainly two forms of Hep used in clinics, and the one tested above was unfractionated Hep (UFH) from hog intestine. Thus we also tested the Hep sensing property of **TPE-1** towards the other form of Hep, i.e., the low molecular weight Hep (LMWH). The **TPE-1** probe also exhibited good dynamic range and sensing sensitivity towards the two LMWH tested (Dalteparin and Enoxaparin), indicating the general capability of **TPE-1** for Hep detection (Figs. S6 and S7†).

Hep detection is easily interferenced by its GAG analogues, especially Chs and HA. To obtain good selectivity, highly specific recognition group is demanded. With the Hep binding peptide AG73 incorporated as the recognition group, **TPE-1** showed high selectivity to Hep over tested anions, Hep analogues and important biomolecules. As illustrated in Figs. 3a and 3b, only the addition of Hep enhanced the fluorescence intensity by about 31-fold, while the addition of 0.05 mg/mL BSA induced a fluorescence enhancement by about only 3-fold. The addition of glucose (10 μM), ATP (10 μM), PO_4^{3-} (10 μM), DNA (2 μM) and RNA (2 μM) did not produce distinguished fluorescence enhancement. It is worth pointing out that tested Hep analogues including as high as 10 μM of Chs and HA also did not significantly influence the selective detection of Hep using **TPE-1** as the probe (Fig. 3c).

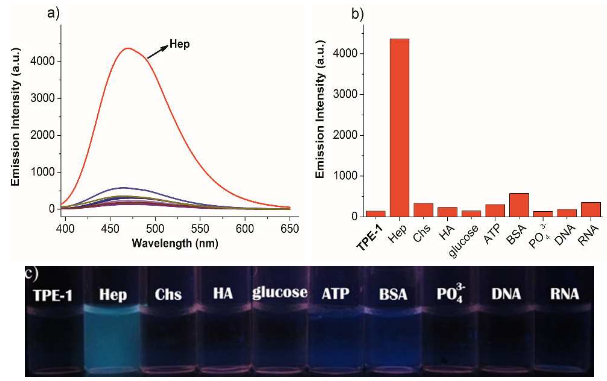


Fig. 3 Selective detection of Hep with **TPE-1**. a) Fluorescence spectra of **TPE-1** (10 μM) upon addition of various anions and biomolecules in 10 mM pH 7.4 HEPES/DMSO (95:5, v:v). λ_{ex} = 340 nm. b) Corresponding fluorescence intensity of **TPE-1** at 470 nm. c) A photograph showing the fluorescence change of **TPE-1** (10 μM) upon addition of various anions and biomolecules in 10 mM pH 7.4 HEPES/DMSO (95:5, v:v) under a portable UV lamp irradiation.

High stability of TPE-1 probe.

During the investigation of using **TPE-1** as the fluorescent "turn on" probe for Hep, we found that **TPE-1** was very stable. Its stock solution can be stored at room temperature for more than 2 months without obvious decomposition. We then tested the photostability of **TPE-1** after binding with Hep. After addition of 5.8 μg/mL Hep to 10 μM **TPE-1**, the fluorescence intensity of the obtained solution was monitored every 20 min. Note, during the monitoring, the solution was under continuous UV lamp irradiation. It was found that there was negligible decrease in the emission intensity of **TPE-1** throughout 4 hours of UV light irrdiation, indicating that **TPE-1** showed high photostability after binding to Hep (Fig. 4a).

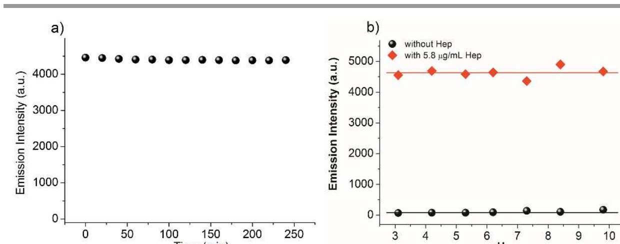


Fig. 4 High stability of **TPE-1**. a) Photostability of **TPE-1** upon Hep binding under continuous UV light irradiation. b) Fluorescence emission intensity of **TPE-1** (10 μM, black dots) and **TPE-1** + 5.8 μg/mL of Hep (red squares) over a pH range of 3-10. λ_{ex} = 340 nm.

For a fluorescent probe, its sensing performance under differnt pH should also be evaluated. Since the pK_a value of the guanidyl group in arginine residue is about 12.4 and the pK_a of the ω-NH_3^+ group in lysine residue is about 10.5, we envisioned that the guanidyl group in arginine residues and ω-NH_3^+ group in lysine residue of the peptide AG73 would remain positively charged within a wide pH range lower than 10. Accordingly, the electrostatic interactions between the positively-charged peptide in **TPE-1** and the negatively-charged Hep would not be affected in such a wide pH range. By changing the pH values from 3 to 10, we measured the emission intensity of probe **TPE-1** (10 μM) in the absence and presence of 5.8 μg/mL Hep. As shown in Fig. 4b, the emission properties of **TPE-1** and its Hep binding complex were not affected in the tested pH range from 3-10. This indicates that Hep sensing using **TPE-1** as the probe fully meets broad pH requirements.

OSCS detection assisted with heparinases treatment.

After establishing the sensitive and selective method for Hep sensing in a wide pH range with **TPE-1** probe, we then proposed an approach for OSCS detection assisted with heparinases treatment (Scheme 2). Since the fluorescence of **TPE-1** was turned on when it was assembled on Hep specifically, we envisioned that the cleavage of Hep by heparinases would disassemble **TPE-1** from the cleaved Hep fragments and thus turn off the AIE fluorescence. To test our hypothesis, cocktail solution of heparinases I, II and III was prepared and added into a solution containing 10 μM **TPE-1** and 5.8 μg/mL Hep, and then incubated at 37 °C for fluorescence measurements. It clearly showed that the fluorescence intensity decreased sharply to 55% of the initial intensity after heparinases treatment (Fig. S8†). This result indicates that indeed the enhanced fluorescence of **TPE-1** after Hep binding could be turned off by heparinases treatment.

Then the influence of OSCS on **TPE-1** fluorescence before and after heparinases treatment was investigated. When **TPE-1** in 10 mM pH 7.4 HEPES/DMSO (95:5, v:v) was titrated with OSCS alone, the AIE fluorescence "turn on" of **TPE-1** was observed (Fig. S9†). However, only 8-fold fluorescence enhancement was observed after addition of 6.6 μg/mL OSCS, while addition of 5.8 μg/mL of Hep enhanced the fluorescence by ~31-fold. This indicates that OSCS can also bind to **TPE-1** and turn on its fluorescence, but to a much less degree than Hep. We then studied the inhibition effect of OSCS on heparinases activity. Even after 4 hours of enzyme cocktail treatment, the fluorescence intensity of Hep with 1% OSCS was still stronger than that of Hep alone (Fig. S10†). Thus, the presence of OSCS indeed inhibited the activity of heparinases.

Based on the above interesting phenomena, a heparinases assisted strategy for detection of OSCS contaminant in Hep was developed using **TPE-1** as follows (Scheme 2). The fluorescence intensities before and after enzyme treatment were defined as Out_1 and Out_2, respectively. The ratio of Out_2/Out_1 was employed as the output signal for measuring different contents of OSCS in Hep. Different amounts of OSCS were spiked into Hep (w%: 0, 0.001, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 50, 70, 90, 100), and then added to the solution of **TPE-1** for fluorescence measurements, giving Out_1. Then, these solutions were treated with enzyme cocktail for 4 h, and the fluorescence intensities were recorded as Out_2. The values of Out_2/Out_1 as a function of OSCS amounts were plotted in Fig. 5a. It is obvious that with increasing OSCS amounts, the value of Out_2/Out_1 increased accordingly. Thus the amounts of OSCS contaminant in Hep can be quantified by **TPE-1** with heparinases treatment in a signal "turn on" manner. The detection limit for OSCS was found to be 0.001% (w%), since the presence of 0.001% (w%) of OSCS induced vivid change in Out_2/Out_1 (Fig. S11†). Besides, the value of Out_2/Out_1 shows a linear relationship to % (w/w) of OSCS in Hep in a range of 1%-70%, indicating that the presence of OSCS can be detected reliably with **TPE-1** (Fig. 5b).

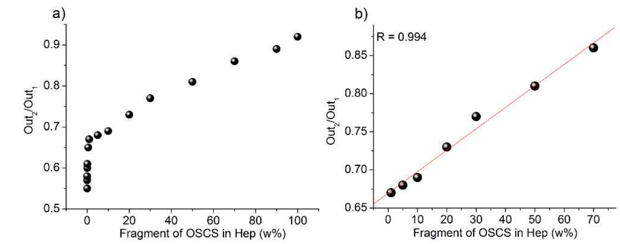


Fig. 5 Heparinases assisted detection of OSCS contaminant in Hep with **TPE-1**. a) Out_2/Out_1 as a function of amounts (w%) of OSCS in Hep. b) Linear response curve of OSCS in Hep. Out_1: fluorescence intensity measured upon addition of Hep (OSCS) to **TPE-1**; Out_2: fluorescence intensity measured after 4 hours of enzyme treatment of aforementioned solution.

Conclusions

The conjugation of TPE, a typical AIE fluorogen, to heparin-binding AG73 peptide afforded a fluorescence "turn on" probe **TPE-1** for Hep. The fluorescence "turn on" behavior of **TPE-1** was ascribed to the aggregation induced emission upon Hep binding. **TPE-1** was sensitive towards Hep in a wide pH range with a detection limit of 3.8 ng/mL, and showed high selectivity towards Hep over tested possible competitors, including various anions, biomolecules and especially Hep analogues such as Chs and HA. More, the **TPE-1** probe was applicable to sensing both UFH and LMWH. When further assisted with heparinases treatment, the OSCS contaminant in Hep was successfully determined with **TPE-1**. Compared with existing detection methods, **TPE-1** shows excellent sensitivity and selectivity towards Hep and OSCS (Tables S1 and S2†).[18] In general, in this work we designed and constructed a fluorescent probe **TPE-1** for efficient detection of both Hep and its OSCS contaminant in a reliable signal "turn on" manner. This work provides an ideal approach for designing "turn on" probes. It can be envisioned that more wide targets will be detected in the future when other specific biorecognition elements such as aptamers are conjugated.

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Graphics for Contents

A "Turn On" Fluorescent Probe for Heparin and Its Oversulfated Chondroitin Sulfate Contaminant

Yubin Ding,[a] Leilei Shi,[a] and Hui Wei*[a]

The conjugation of tetraphenylethene and heparin binding peptide afforded a fluorescence "turn on" probe for reliable determinaton of heparin and its contaminant oversulfated chondroitin sulfate.