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Selective, switchable fluorescent probe for heparin based on aggregation-induced emission



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ABSTRACT

A positively charged tetraphenylethene (TPE) derivative, TPE-4MN, was synthesized as a probe for heparin based on aggregation induced emission. On the addition of 5.0 $\mu\text{g}/\text{mL}$ of heparin, TPE-4MN showed an enhanced emission of about 10-fold. The change in fluorescence at 475 nm was linear over a range of heparin concentrations of 0–1.0 $\mu\text{g}/\text{mL}$ with an $R = 0.99988$ and the limit of detection (LOD) was calculated to be 0.75 $\mu\text{g}/\text{mL}$. The mechanism of the detection was proven to be through an ion pairing interaction. TPE-4MN showed good selectivity for heparin over other types of polysaccharides and could easily distinguish heparin from heparan sulfate, a glycosaminoglycan having a similar structure to that of heparin.

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1. Introduction

Glycosaminoglycans (GAGs) are anionic polysaccharides that play a number of critical physiological and pharmacological roles in human health [1–3]. They are linear polysaccharides, comprised of disaccharide repeating units that contain either sulfo and/or carboxyl groups. The most well studied GAG is heparin, which is widely used as a clinical anticoagulant, critical in the practice of modern medicine [4]. Heparin is considered to be one of the most acidic molecules in nature containing one carboxyl and 2–3 sulfo groups per repeating disaccharide [4]. Because of its clinical relevance and its propensity to be contaminated or adulterated with other difficult to detect molecules [5], there has been an increased interest in developing assays for its detection and analysis [6–12]. Among these assays are

fluorescence assays that rely on cationic dyes [13–19]. One class of fluorescence assays relies on a novel group of fluorogens called aggregation induced emission (AIE) fluorogens.

AIE dyes show no fluorescence (or very low fluorescence) in solution but have strong emission when aggregated or in the solid state, thus, they can be very useful for designing novel bioassays. Tetraphenylethene (TPE), which is easy to synthesize and has a high luminescent efficiency, is currently one of the most widely used AIE fluorogens [20–24]. The introduction of a covalently reactive, malononitrile-functionalized, “thio click moiety” into TPE has been used to construct a biosensor for the selective detection of glutathione in the presence of other thiol-containing compounds [25]. The 4-(diethylamino) TPE derivative (TPE-4N) was used to detect fuming hydrogen chloride vapor and then reversibly regenerated with fuming triethylamine vapor, making it a non-fatiguing solid-state sensor for protonation [26]. AIE molecules can also be used in the field such as metal-organic frameworks [27], micelle transitions in microemulsions [28] and cellular imaging [29].

Recently, there has been interest in preparing AIE probes for the sensitive and selective detection of heparin [30–32]. However, no

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probe has been reported that can distinguish heparin from heparan sulfate (HS), a polysaccharide ubiquitously distributed on the surfaces of animal cells and in the extracellular matrix that mediates various physiologic and pathophysiologic processes. It is very difficult to evaluating the role of heparin and HS ascribed to their similar structure and the sequence of their repeating units. So it is important to find probes that are selective for heparin and HS. Herein, we use an AIE fluorogen, tetra(4-(trimethylamino)phenyl)ethane (TPE-4MN), to study its interaction with heparin and its selectivity for GAG detection (Fig. 1).

2. Materials and methods

2.1. Materials

Trimethylamine, titanium (IV) chloride, zinc dust, 2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid (HEPES), sodium hydroxide, sodium chloride, dextran (DEX), protamine, 4,4'-dihydroxybenzophenone, tetrahydrofuran and 1,2-dibromoethane were purchased from Sigma-Aldrich (St. Louis, MO), ethanol and dichloromethane were purchased from VWR international. Heparin (sodium USP, from porcine intestine), chondroitin sulfate (CS, chondroitin sulfate A, from bovine trachea), hyaluronan (HA, from *Streptococcus zooepidemicus*), dermatan sulfate (DS, from porcine intestine) and HS (from porcine intestine) were purchased from Celsus Laboratories, Inc. (Cincinnati, OH). Low molecular weight heparin (LMWH, enoxaparin sodium, 30 mg/mL) was purchased from Sandoz. ^1H and ^{13}C NMR spectra were measured on a Bruker 600 MHz Spectrometer in D_2O . Mass spectra (MS) were taken on a Thermo Scientific LTQ ORBITRAP XL mass spectrometer operating. UV–Vis absorption spectra were obtained on a Hitachi U2910 UV–Vis Spectrophotometer. FL spectra were recorded on a Spex[®] FluoroLog[®] Tau-3 (Horiba) spectrofluorometer and spectra Max M5.

2.2. Synthesis of tetra(4-(trimethylamino)phenyl)ethane (TPE-4MN)

This AIE probe was prepared according to previously published experimental procedures [33]. A light yellow solid was obtained in 28.7% yield. ^1H NMR (800 MHz, D_2O , δ): 7.03–6.96 (d, 8H), 6.78–6.71 (d, 8H), 4.40–4.32 (t, 8H), 3.75–3.67 (t, 8H), 3.14 (s, 36H). ^{13}C NMR (150 MHz, D_2O , δ): 158.93, 142.34, 141.00, 135.84, 117.24, 68.38, 65.02, 57.17 (see Supplementary Information, Fig. S1).

2.3. Preparation of HEPES buffers

HEPES (0.595 g) was dissolved in distilled water in a 500 mL volumetric flask and 1.0 M NaOH was added to adjust the pH of

solutions to 5.0, 7.4, 9.0 and 11.0. The volume of each solution was brought to 500 mL by adding distilled water to afford solutions of 5 mM HEPES buffers at various pH values.

2.4. Preparation solutions in HEPES buffer for fluorescence determination

GAGs (5.0 mg of either heparin, HS, CS, HA and DEX) were made up with HEPES buffer in a 25 mL volumetric flask. An aliquot of the resultant stock solution (0.2 $\mu\text{g}/\text{mL}$) was diluted with HEPES buffer in another volumetric flask to obtain a sample solution with concentrations of between 0.02 $\mu\text{g}/\text{mL}$ and 0.002 $\mu\text{g}/\text{mL}$.

TPE-4MN (1.482 mg) was added into 100 mL of HEPES buffer to obtain a 20 μM solution. An aliquot (50 μL) of the stock solution of heparin (0.02 $\mu\text{g}/\text{mL}$), 50 μL of HEPES buffer and 100 μL of the solution of TPE-4MN (20 μM) were added into a 96 well Costar blk/clrbtm. After shaking for a few seconds, a solution of TPE-4MN with 5.0 $\mu\text{g}/\text{mL}$ of heparin was obtained. The solution was allowed to stand for 1 min before its fluorescence spectrum was measured ($\lambda_{\text{ex}} = 315 \text{ nm}$, $\lambda_{\text{em}} = 475 \text{ nm}$).

A solution of TPE-4MN (10 μM) with 1.0 $\mu\text{g}/\text{mL}$ of heparin in the HEPES buffer was prepared by adding 100 μL of the stock solution of heparin (0.002 $\mu\text{g}/\text{mL}$) into 100 μL of a stock solution of TPE-4MN (20 μM) in the HEPES buffer.

Similarly, the concentrations of the solutions of other GAGs (0.2 $\mu\text{g}/\text{mL}$, 0.02 $\mu\text{g}/\text{mL}$ or 0.002 $\mu\text{g}/\text{mL}$) were adjusted by adding an appropriate amount of HEPES buffer to a volumetric flask and brought to volume with HEPES buffer.

2.5. Preparation of solution of TPE-4MN and heparin containing interfering substances

Solutions of heparin with different concentrations (0.002–0.2 $\mu\text{g}/\text{mL}$) of heparin and solutions of CS, HA, HS and DEX (0.2 $\mu\text{g}/\text{mL}$) were prepared in HEPES buffer. An aliquot (100 μL) of the solution of TPE-4MN (20 μM) in the HEPES buffer was added into a 96-well costar black/clear bottom plates, followed by the addition of 5 μL of a solution of CS (0.2 $\mu\text{g}/\text{mL}$), 50 μL of a solution of heparin (0.02 $\mu\text{g}/\text{mL}$) and 45 μL of HEPES buffer. After shaking for a few seconds, a solution of TPE-4MN (10 μM) with 5.0 $\mu\text{g}/\text{mL}$ of heparin and 5.0 $\mu\text{g}/\text{mL}$ of CS in the HEPES buffer was obtained. The solution was allowed to stand for 1 min at room temperature before its fluorescence spectrum ($\lambda_{\text{ex}} = 315 \text{ nm}$, $\lambda_{\text{em}} = 475 \text{ nm}$) was measured. A solution of TPE-4MN and heparin containing other polysaccharides, HA, HS or DEX, were similarly prepared and their fluorescence determined.

3. Results and discussion

The TPE-4MN probe was chemically synthesized based on literature methods [33] and its structure was confirmed by NMR and MS analysis (see Supplementary Information, Figs. S1 and S2). A number of GAGs and related polysaccharides were used in the current study to examine the utility of TPE-4MN as a probe to undergo AIE. The mechanism of the detection we proposed was based on the interaction between positive and negative charges. The charge density (negatively charged groups/disaccharide) of the polysaccharides studied were heparin (–3.5) > CS (–2) > HS (–1.5) > HA (–1) > DEX (0). HS is a polysaccharide has similar structure but has a much lower charge density to heparin and DEX is a linear (de-branched) polysaccharide consisting of α -1,6 glucose and has no charged functional groups.

The fluorescence of TPE-4MN increases when its molecular motion is restricted and this is associated with its AIE properties. Hence, we speculated that the binding of the positively charged TPE-4MN

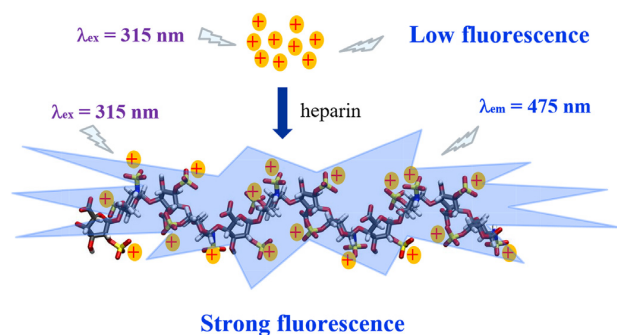


Fig. 1. The scheme for the proposed mechanism of the interaction between TPE-4MN and heparin.

(+4/molecule) to negatively charged GAGs would restrict the motion of TPE-4MN through its aggregation or stacking on the GAG chain. When TPE-4MN was added into a solution containing heparin we expected an ion-pairing interaction to occur, resulting in a change of the fluorescence intensity as depicted in the illustration shown in Fig. 1. And when TPE-4MN was added into a solution containing other less negative charged GAGs, we expected to observe differences in the fluorescence spectra compared to that of heparin.

The AIE properties of TPE-4MN were first examined. The most common method used to test the AIE of a dye molecule is to measure its fluorescence in a mixed solvent/non-solvent system with different fractions of non-solvent. Aggregates gradually form when sufficient non-solvent is introduced into the solution so that fluorescence increases with increasing fractions of a non-solvent. Since TPE-4MN dissolves well in most of the common solvents, it was difficult to find a non-solvent to promote its aggregation. Thus, we tested the AIE property of TPE-4MN by decreasing the temperature of TPE-4MN in a glycerol/water mixture containing up to 99% of glycerol. At higher temperatures, the molecule undergoes dynamic intramolecular rotations, which non-radiatively annihilate its excited state, reducing the luminescence of TPE-4MN. In contrast, at lower temperatures in highly viscous 99% of glycerol, intramolecular rotations are greatly restricted, blocking non-radiative pathways and favoring the radiative pathways, greatly increasing the luminescence of TPE-4MN. A solution of TPE-4MN showed a very low fluorescence with an emission wavelength at ~460 nm at 30 °C that increased when the temperature was decreased to 0 °C (Fig. 2). When the temperature was decreased from 30 °C to -50 °C, the fluorescence of the molecule increased over 3-fold and showed a blueshift from 460 nm to 430 nm, indicating the restricted conformation of the molecule under low

temperatures. The results obtained for TPE-4MN were typical of those of an AIE fluorogen.

The conditions were next selected. Detection at 25 °C in 5 mM HEPES buffer at a pH of 7.4 gave the best signal to noise ratio (Fig. S3). The fluorescence of TPE-4MN solution containing heparin was found to be stable for up to 60 min (Fig. S4), which is important in developing a fluorescence assay. Next, increasing amounts of heparin was added to TPE-4MN to examine the effect of this polyanion on AIE (Fig. 3). TPE-4MN itself almost gave no emission owing to its good solubility in HEPES buffer and its fluorescence at ~475 nm gradually increased as a function of heparin concentration reaching a maximum at 5.0 µg/mL, increasing in fluorescence intensity by ~10-fold. This results from the interaction between the positively-charged TPE-4MN and the negatively-charged heparin chain, resulting in restricted intramolecular rotation of TPE-4MN and increased fluorescence emission. The relation between the fluorescence intensity at 475 nm and heparin concentration was next examined. The results show that the change in fluorescence was linear over a range of heparin concentrations of 0–1.0 µg/mL with an $R = 0.99988$ (Fig. 3B). The LOD was calculated to be 0.75 µg/mL, which is a typical value for these reported fluorescence probes [34–36].

LMWH was also examined with TPE-4MN to test the influence of molecular weight on detection and the results are shown in Fig. 4. With the addition of LMWH, fluorescence increased gradually reaching about a 4-fold enhancement at 5.0 µg/mL. This 4-fold increase in fluorescence was considerably lower than the 10-fold enhancement observed with heparin. These results were predictable because LMWH has chains that are only about one-third the size of heparins chains. As a result, the motion of TPE-4MN was not as restricted, and lower increases in fluorescence were observed. Thus, the detection of heparin by TPE-4MN also depends on the

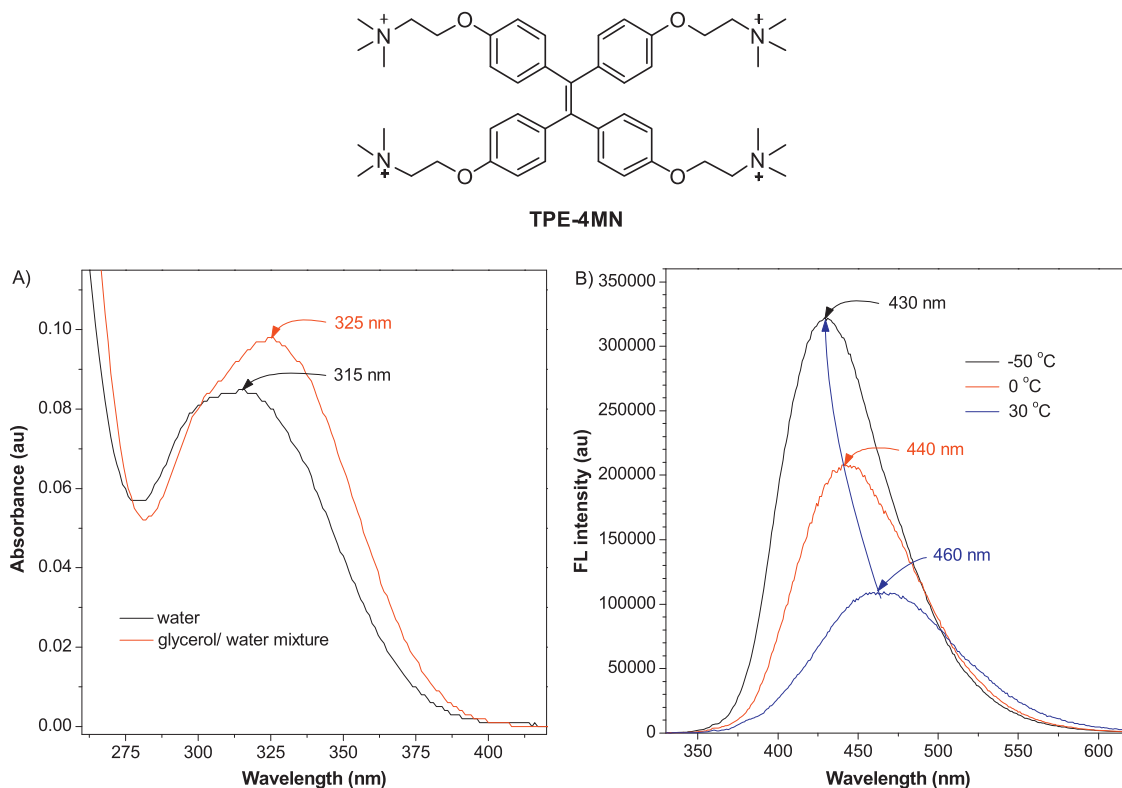


Fig. 2. The structure of the fluorogen (TPE-4MN) and its fluorescence properties with added glycerol. (A) UV–Vis spectra of TPE-4MN in water and glycerol/water mixture with 99% glycerol. (B) Fluorescence spectra of TPE-4MN in glycerol/water mixture with 99% of glycerol at different temperatures. λ_{ex} : 325 nm; concentration: 10 µM.

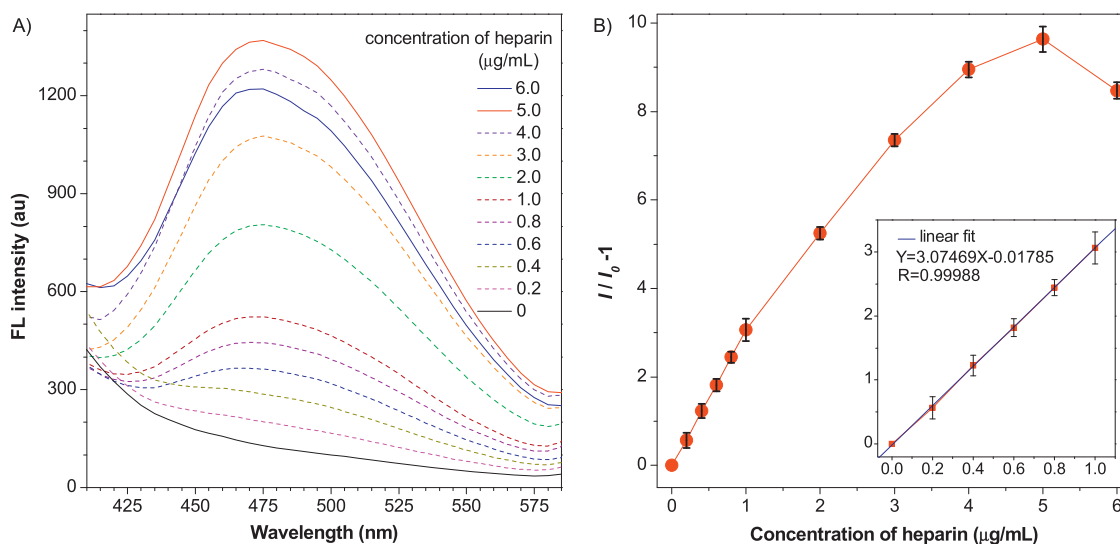


Fig. 3. (A) Fluorescence spectra of TPE-4MN in HEPES buffer (pH = 7.4, 5 mM) with different concentrations of heparin. (B) Plot of the fluorescence intensity of TPE-4MN in HEPES buffer (pH = 7.4, 5 mM) with different concentrations of heparin. Insert: The result of linear fit of plot. λ_{ex} : 315 nm; concentration of heparin: 0–6.0 $\mu\text{g/mL}$; concentration of TPE-4MN: 10 μM .

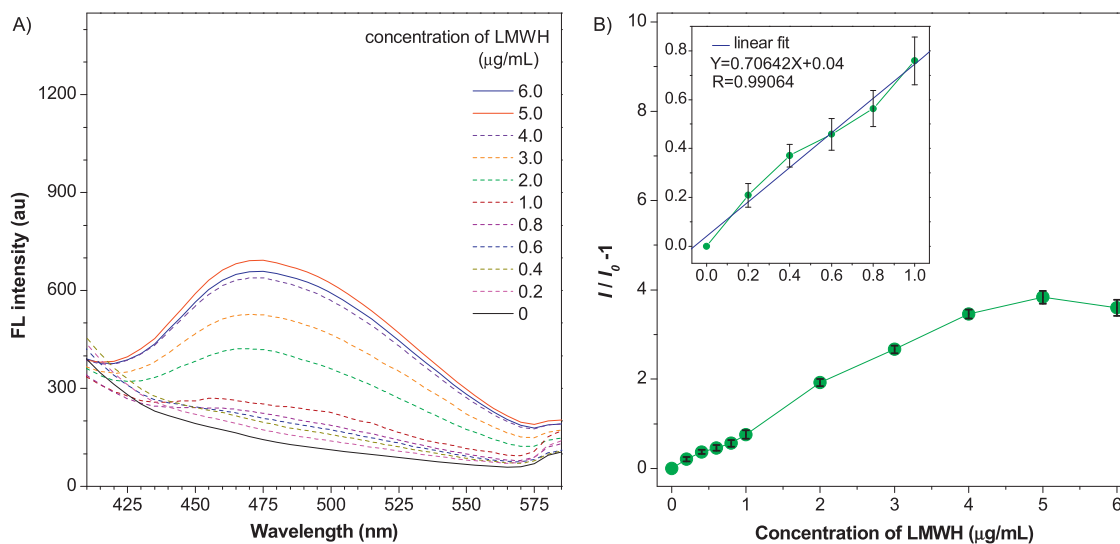


Fig. 4. (A) Fluorescence spectra of TPE-4MN in HEPES buffer (pH = 7.4, 5 mM) with different concentrations of LMWH. (B) Plot of the fluorescence intensity of TPE-4MN in HEPES buffer (pH = 7.4, 5 mM) with different concentrations of LMWH. Insert: The result of linear fit of plot. λ_{ex} : 315 nm; concentration of low molecular weight heparin: 0–6.0 $\mu\text{g/mL}$; concentration of TPE-4MN: 10 μM .

molecular weight of heparin. Heparin with higher molecular weight gave higher signals suggesting that TPE-4MN may also be useful in determining the molecular weight of heparins.

The fluorescence of the uncharged TPE-4OBr synthetic precursor was next used to compare with the positively charged TPE-4MN under identical conditions to confirm the mechanism of the interaction involves ion-pairing. TPE-4OBr is not charged in HEPES buffer (pH = 7.4, 5 mM) with 2% of THF, so it should have no interaction with heparin and show no change in fluorescence. In contrast, TPE-4MN is positively charged in HEPES buffer containing 2% of THF so it should be responsive to heparin. As expected, TPE-4MN showed a 5-fold increase in emission when heparin was added, while TPE-4OBr showed strong fluorescence in the mixture with 98% of HEPES buffer because its AIE properties but the

addition of up to 5.0 $\mu\text{g/mL}$ heparin resulted in almost no change in fluorescence (Fig. 5). Considering the similar structure between TPE-4OBr and TPE-4MN, these results confirm that the positive charge in the fluorogen is necessary and it plays an important role in the fluorogen's interaction with heparin.

Protamine, a clinical antidote for heparin, was used to further examine the nature of the interaction between TPE-4MN and heparin. Protamine binds electrostatically to heparin's anionic chain influencing its fluorescence with TPE-4MN. The addition of protamine decreases the assay signal linearly until it is completely quenched at protamine concentration of 10 $\mu\text{g/mL}$ (Fig. S5). Thus, protamine can competitively reverse the interaction between TPE-4MN and heparin. DS, a GAG with iduronic acid residues similar to those found in heparin, was also tested to see if the structure of the

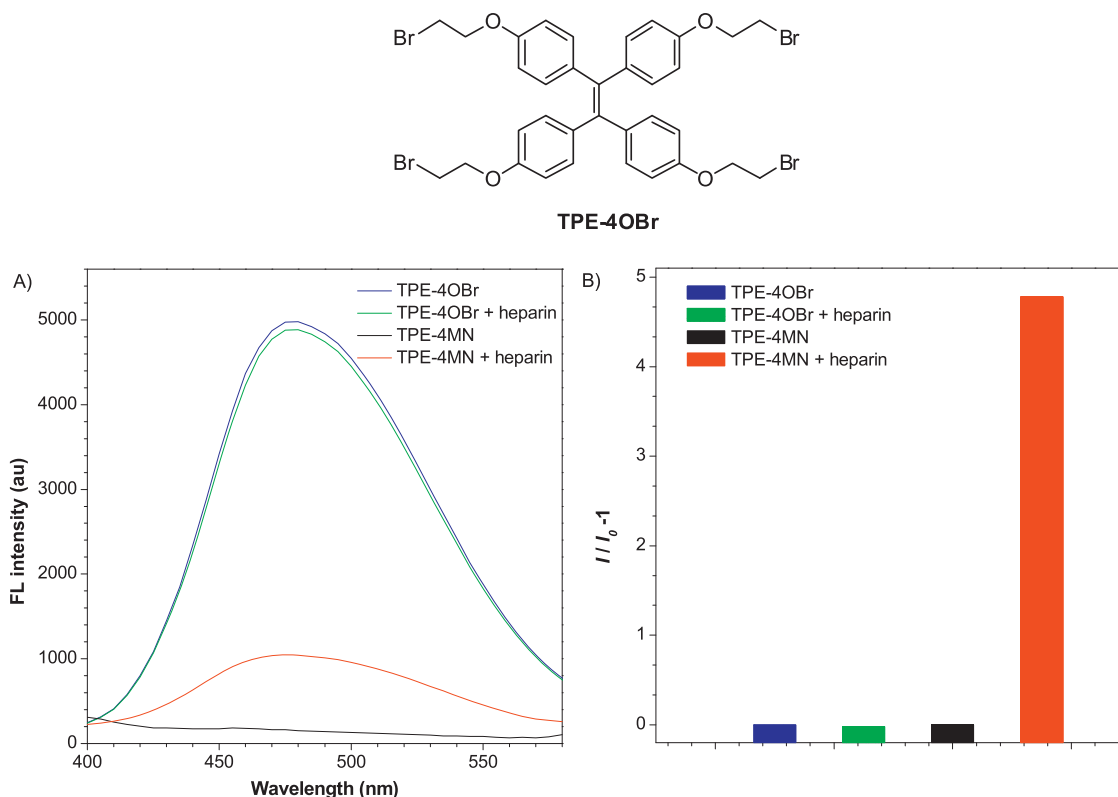


Fig. 5. (A) Fluorescence spectra of TPE-4OBr and TPE-4MN in HEPES buffer (pH = 7.4, 5 mM) with 2% of THF with heparin. (B) Column of the fluorescence intensity of TPE-4OBr and TPE-4MN in HEPES buffer (pH = 7.4, 5 mM) with 2% of THF with heparin. λ_{ex} : 315 nm; concentration of heparin: 5 $\mu\text{g}/\text{mL}$; concentration of TPE-4OBr and TPE-4MN: 10 μM .

repeating unit of GAGs influenced fluorescence detection. The mixture of DS and TPE-4MN shows much lower fluorescence at 475 nm than that observed for heparin (Fig. S6). DS has a similar negative charge density as CS and shows similar fluorescence properties with TPE-4MN. While it would also be interesting to examine how domains of charge patterns within GAGs influence TPE-4MN fluorescence, GAG samples with such defined patterns of charges are not currently available.

Based on the mechanism demonstrated above, TPE-4MN was used to interact with HS to determine if there was different performance compared with heparin. Because of their similar structure and repeating units, it can be difficult to distinguish heparin from HS. Their major difference is that they have very different charge densities [37], making an ion-pairing interaction with TPE-4MN a potentially useful approach. The fluorescence spectra of TPE-4MN mixed with different concentrations of HS are shown in Fig. 6 (blue line (in the web version)). No increase in fluorescence at 475 nm was observed even after the addition of 5.0 $\mu\text{g}/\text{mL}$ of HS (Fig. 6, blue bar (in the web version)), which is quite a different from that obtained using heparin (Fig. 3). Heparin's -3.5 charges/disaccharide allows it to strongly interact with TPE-4MN and restrict intramolecular motion, leading to a large increase in fluorescence. In contrast, HS has a much lower charge density, <-1.5 charges/disaccharide, allowing only weak interactions with TPE-4MN giving no change in the fluorescence. The data demonstrate that TPE-4MN is a good probe to distinguish heparin and HS.

Next we examined a variety of polysaccharides, CS, HS and DEX, to better understand the selectivity of TPE-4MN on different polysaccharides. The fluorescence was again tested under the

same conditions. On the addition of different polysaccharides, TPE-4MN showed the greatest AIE response to heparin with much weaker response to CS and almost no response to HA and DEX (Fig. 6). These results are consistent with the different charge densities of these polysaccharides. CS has a -2 charge/disaccharide, promoting a much weaker interaction with TPE-4MN than heparin, and resulted in a much weaker influence on TPE-4MN fluorescence. The other polysaccharides examined with less than a -2 charge/disaccharide apparently an insufficient charge density to result in a fluorescence change in the $+4$ charge/molecule TPE-4MN. At a GAG concentration of 5.0 $\mu\text{g}/\text{mL}$, the fluorescence of the different GAG/TPE-4MN solutions was quite different. Heparin shows a 10-fold higher response than the other polysaccharides (Fig. 6B), thus, making the selective detection of heparin possible using TPE-4MN. The fluorescence of a mixture containing 5.0 $\mu\text{g}/\text{mL}$ of these other polysaccharides with a range of heparin concentrations was measured to further test the selectivity of TPE-4MN on heparin. The fluorescence of these solutions increases with the addition of heparin, indicating that the detection of heparin by TPE-4MN is not seriously influenced by other polysaccharides (Figs. S7–S10). The upper ionic strength limits of this assay were also measured, as this is important considering the potential uses of TPE-4MN. The fluorescence at ~ 475 nm decreased gradually with the addition of NaCl (Fig. S11). The fluorescence was completely quenched at a NaCl concentration of 5 mg/mL. As expected, the addition of salts reduces the ion pairing interaction between the heparin/dye complex.

Finally, we examined a semi-synthetic GAG, OSGS with a charge density of -4 /disaccharide. OSGS is a known adulterant of heparin [5]. As with heparin, the fluorescence of TPE-4MN

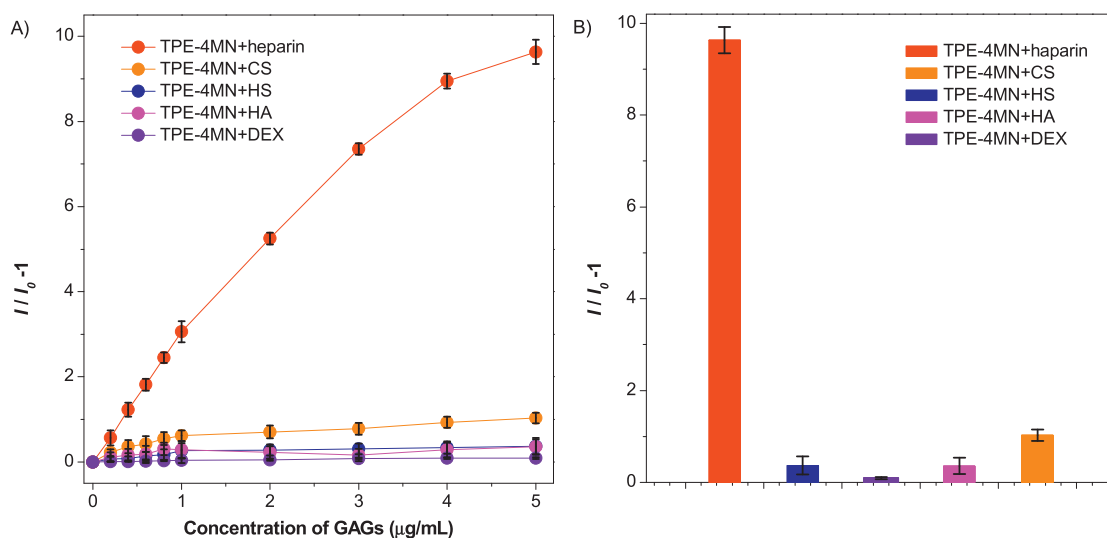


Fig. 6. (A) Plot of the fluorescence intensity of TPE-4MN in HEPES buffer (pH = 7.4, 5 mM) with different GAGs. (B) Column of the fluorescence intensity of TPE-4MN in HEPES buffer (pH = 7.4, 5 mM) with different GAGs. λ_{ex} : 315 nm; concentration of GAGs: 0–6.0 $\mu\text{g/mL}$; concentration of TPE-4MN: 10 μM .

solution increased with the addition of OSCS (Fig. 7A) demonstrating that TPE-4MN can strongly interact with OSCS to undergo AIE. At an OSCS concentration of 5.0 $\mu\text{g/mL}$, the fluorescence increased about 14.5-fold (Fig. 7B) affording an LOD of 0.67 $\mu\text{g/mL}$. Thus, it appeared that heparin (−3.5 charges/disaccharide) and OSCS (−4.5 charges/disaccharide) interact similarly with TPE-4MN. Future studies are currently underway to study TPE derivatives having different numbers of positive charge groups as well as functional groups capable of hydrogen bonding and complexation. The fine-tuning of these functional groups to match selected disaccharide repeating structures in different GAGs may be useful in designing a class of fluorescent probes for the sensitive and selective detection of these biologically and pharmacologically important biopolymers.

4. Conclusions

In summary, a probe for heparin, TPE-4MN, has been prepared and its aggregation induced emission property has been confirmed by measuring its fluorescence in glycerol/water mixture containing up to 99% of glycerol under different temperatures. With the addition of heparin, TPE-4MN shows an increasing in fluorescence with a wavelength at around 475 nm and the increasing fold reaches ~10 times when the concentration of heparin is up to 5 $\mu\text{g/mL}$. The change in fluorescence at 475 nm is linear over a range of heparin concentrations of 0–1.0 $\mu\text{g/mL}$ with an $R = 0.99988$ and the LOD was calculated to be 0.75 $\mu\text{g/mL}$. The mechanism of the detection has been demonstrated to be the interaction between the positive charge from TPE-4MN and the negative charge from

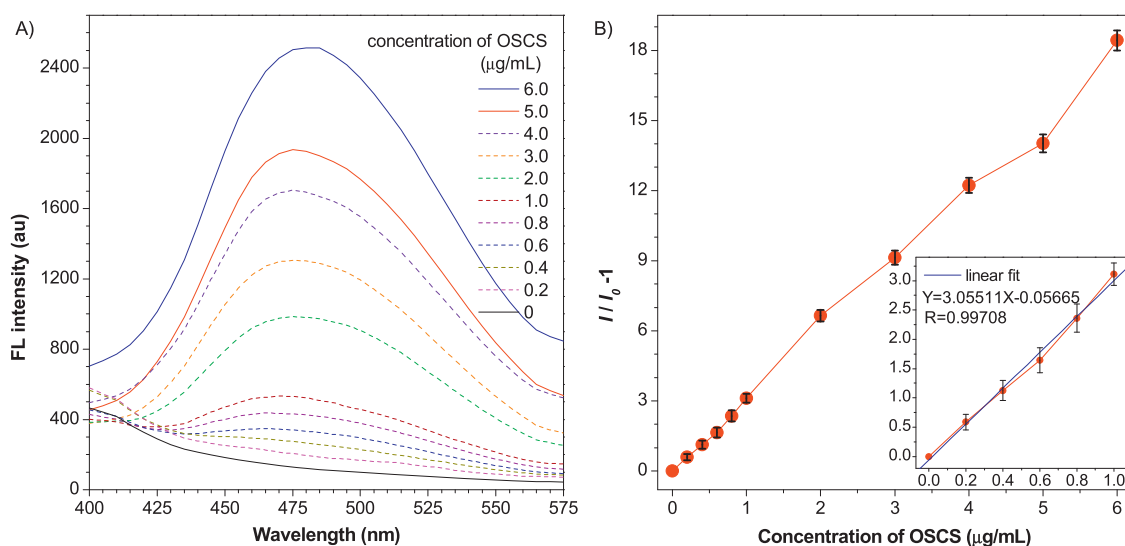


Fig. 7. (A) Fluorescence spectra of TPE-4MN in HEPES buffer (pH = 7.4, 5 mM) with different concentrations of OSCS. (B) Plot of the fluorescence intensity of TPE-4MN in HEPES buffer (pH = 7.4, 5 mM) with different concentrations of heparin. Insert: The result of linear fit of plot. λ_{ex} : 315 nm; concentration of OSCS: 0–6.0 $\mu\text{g/mL}$; concentration of TPE-4MN: 10 μM .

heparin. Based on this mechanism, TPE-4MN shows a significant difference while interact with heparin and HS, indicate that it is a useful probe to distinguish these two similar polysaccharides. Further study shows that TPE-4MN has good selectivity to heparin and can distinguish heparin from other common polysaccharides such as CS, HA and DEX.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ab.2016.09.007>.

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