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A pyrene-based fluorescent sensor for ratiometric detection of heparin and its complex with heparin for reversed ratiometric detection of protamine in aqueous solution

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ABSTRACT

An imidazolium-modified pyrene derivative, **IPy**, was used for ratiometric detection of heparin, and its complex with heparin was used for reversed ratiometric detection of protamine in both aqueous solution and serum samples. The cationic fluorescent probe could interact with anionic heparin via electrostatic interaction to bring about blue-to-green fluorescence changes as monomer emission significantly decreases and excimer increases. The binary combination of **IPy** and heparin could be further used for green-to-blue detection of protamine since heparin prefers to bind to protamine instead of the probe due to its stronger affinity with protamine. The cationic probe shows high sensitivity to heparin with a low detection limit of 8.5 nM (153 ng/mL) and its combination with heparin displays high sensitivity to protamine with a detection limit as low as 15.4 nM (107.8 ng/mL) according to the 3σ IUPAC criteria. Moreover, both sensing processes are fast and can be performed in serum solutions, indicating possibility for practical applications.

1. Introduction

Heparin is a linear polysaccharide with abundant of sulfate and carboxylate groups, and therefore has the highest negative charge density among any known biological macromolecules [1,2]. Heparin plays an important role in regulating various biological processes, such as cell growth and differentiation, inflammation, and blood coagulation [3,4]. More importantly, heparin has been widely used in clinical applications such as preventing thrombosis during surgery and as an anticoagulant drug to treat thrombotic diseases [5]. However, overdose of heparin can induce some complications such as hemorrhages and heparin-induced thrombocytopenia [3]. The suggested therapeutic dose of heparin is 2–8 U/mL (17–67 μM) during cardiovascular surgery and 0.2–1.2 U/mL (1.7–10 μM) for postoperative and long-term care, respectively [6,7]. Protamine, as a well-known heparin antidote, is a highly cationic protein (pI = 13.8) with high content of basic arginine residues. The anticoagulant effect of heparin can be reversed by protamine due to their combination through electrostatic interaction [8]. Thus, protamine is often used for treatment of heparin overdose. However, the over-use of protamine could also induce adverse effect such as hypotension and idiosyncratic fatal cardiac arrest [9]. Therefore, the detection and quantification of both heparin and protamine are of crucial significance for clinical procedures.

Among various methods including colorimetric sensor [10,11], electrochemical sensor [4], and surface-enhanced Raman sensor [12], fluorescent sensors have drawn extensive attention because of their advantages in terms of high sensitivity, high selectivity, easy operation and real time detection [13]. Up to now, a great amount of fluorescent sensors have been developed for detecting heparin and protamine. These sensors exhibit either turn-off responses [2,14–16] or turn-on [17–19] or ratiometric responses [3,5,20,21] to the presence of the target analyte. The turn-off and turn-on sensors rely on fluorescence intensity variation at one wavelength and are easily affected by instrumental and environmental conditions. By comparison, ratiometric fluorescent sensors are advantageous because they usually depend on the intensity ratio at two emission wavelengths, which can help avoiding the above-mentioned interferences [22].

Most of the above mentioned fluorescent sensors detect only one target, either heparin or protamine. Very recently, fluorescent sensors for recognizing both heparin and protamine are attracting attention since they are more convenient and multi-functional. Zhang and coworkers used gold nanoparticles coupled with fluorescent emitting materials such as silicon quantum dots [23], fluorescein [24], and upconversion nanoparticles [25] for detecting both protamine and heparin. These gold nanoparticle-attached fluorescent materials are fluorescence inert due to the quenching effect of gold surface, and show turn-on responses to protamine as protamine interacts with gold nanoparticles and de-attaches fluorescent materials from gold surface, and then show turn-off responses to heparin because heparin has stronger affinity with protamine that results in binding fluorescent materials

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with gold nanoparticles again. John et al. reported using folic acid capped gold nanoparticles for detecting both protamine and heparin based on aggregation and de-aggregation of gold nanoparticles induced by the added analyte [26]. Su et al. developed a fluorescein-labeled DNA probe for turn-off sensing protamine and turn-on sensing heparin [8]. Although these sensors show some interesting sensing behaviors, they have some drawbacks like long time of incubation [23–25], or low sensitivity [9]. Moreover, they are all based on single signal of fluorescence variation at one wavelength. Fluorescent ratiometric sensors for detecting both heparin and protamine have never been reported, to our knowledge.

In the present study, we reported using a cationic pyrene-based fluorophore for ratiometric detection of both heparin and protamine in total aqueous solution. Pyrene-based fluorophores have been widely used to develop ratiometric sensors as they can emit monomer and excimer emission [27–29], and more recently used for ratiometric detection of various biomolecules such as G-quadruplex structures of G-rich DNA [30], human breast cancer cell [31], and proteins [32]. Their fluorescence emission is highly dependent on the conformation or aggregation of pyrene derivatives and very sensitive to the microenvironment. Although a variety of pyrene derivatives were reported for ratiometric sensing heparin [3,5,21,33,34], they were not used to detect protamine or both targets. The present imidazolium-modified pyrene derivative (**IPy**, Scheme 1) exhibits only monomer emission in aqueous buffer solution, and shows blue-to-green emission change upon addition of heparin as monomer emission significantly decreases accompanied by excimer emission increasing. The binary combination of **IPy** and heparin displays green-to-blue changes to the addition of protamine as witnessed by recovered monomer emission and decreased excimer emission. Moreover, both sensing processes show high sensitivity and can be performed in serum solutions.

2. Experimental methods

2.1. Materials and instruments

The pyrene derivative (**IPy**) was synthesized and characterized according to our previously reported method [35]. Heparin sodium salt (from hog intestine) was purchased from TCI. N-(2-hydroxyethyl) piperazine-N′-ethanesulfonic acid (HEPES), chondroitin sulfate sodium salt, adenosine 5′-triphosphate disodium salt hydrate (ATP, ≥99%), cytidine 5′-triphosphate disodium salt (CTP, ≥95%), uridine 5′-triphosphate trisodium salt hydrate (UTP, ≥96%), L-arginine (99%), L-lysine (99%), L-histidine (99%) and all proteins including protamine (from salmon), bovine serum albumin (≥98%), lysozyme (from chicken egg, ≥90%), trypsin (from bovine pancreas), pepsin (from porcine gastric mucosa), β-lactoglobulin (from bovine milk, ≥90%), cytochrome c (from bovine heart, ≥95%), ovalbumin egg (from chicken egg white, ≥ll p, hyaluronic acid sodium salt, and poly-L-lysine hydrobromide were purchased from Sigma-Aldrich. HIV-1 TAT peptide (Sequence: YGRKKRRQRRR) was obtained from Chinese Peptide Company. Fetal bovine serum was purchased from Zhejiang Tianhang Biotechnology Co. Ltd. All chemicals were used as received.

Steady-state fluorescence spectra were measured on a single photon-counting fluorescence spectrometer (FS5, Edinburgh Instruments) with xenon light (150 W) as the excitation source, and the excitation and emission slit widths were set at 2.1 and 0.3 nm, respectively. All samples were excited at 346 nm. Time-resolved fluorescence decays were measured on a time-resolved single photon-counting fluorescence spectrometer (FS920, Edinburgh Instruments) with 343.4 nm laser as the excitation source. UV–vis absorption spectra were recorded on a spectrophotometer (U3900, Hitachi Instrument).

2.2. Preparation of samples

The aqueous stock solution of HEPES (100 mM) was first prepared by dissolving solid HEPES in neat water and using NaOH (1.0 M) to regulate pH to 7.4. HEPES buffer solution (10 mM, pH 7.4) was prepared by diluting the stock HEPES (100 mM) solution with water. The stock solutions of heparin (2.5×10^{-5} M and 2.5×10^{-4} M), the stock solutions of all anions and proteins (2.5×10^{-4} M) were prepared in 10 mM HEPES buffer (pH 7.4) solution and stored at 0–4 °C. The aqueous stock solution of **IPy** (2.5×10^{-4} M) was prepared in neat water. Biological samples were prepared using fetal bovine serum by diluting it with HEPES solution to the corresponding percentage in volume. All aqueous solutions were prepared from Milli-Q water (18.2 MΩ cm at 25 °C).

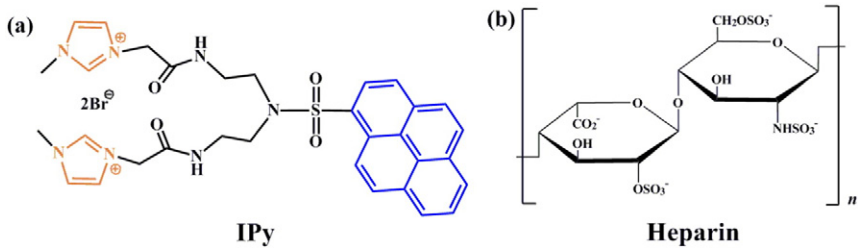
2.3. Heparin detection in aqueous buffer and serum solutions

For detection in buffer solution, the testing solution containing **IPy** (1, 5, 10, 20, or 50 μM) was first prepared by diluting the aqueous stock solution of **IPy** with HEPES buffer solution (10 mM, pH 7.4). Then, 2.5 mL of testing solution was put in a quartz cell and the corresponding emission spectra were scanned. Next, heparin stock solution was added dropwise into the quartz cell and stirred by capillary tube. Then the emission spectra were scanned right away to record heparin-induced variation. The added volume of heparin stock solution is <4%, and for most experiments <0.4% of the volume of the testing solution.

When tested in serum solution, the serum solution containing certain percentage of serum (0.2%, 5% and 10%) was first prepared by dilution of the serum into HEPES buffer solution (10 mM, pH 7.4), and then the testing solution containing **IPy** (10 μM) was prepared by adding the stock solution of **IPy** (100 μL, 2.5×10^{-4} M) into the corresponding serum solutions (2.4 mL). The fluorescence assay of sensing heparin was similar to that measured in buffer solution.

2.4. Protamine detection in aqueous buffer and serum solutions

The fluorescence assay of detecting protamine was quite similar to that of detecting heparin. The big difference was the preparation of the testing solution. For detection in buffer solution, the testing solution



Scheme 1. Structures of **IPy** (a) and major unit of heparin (b) used in this study.