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Fluorescent peptide-based sensors for the ratiometric detection of nanomolar concentration of heparin in aqueous solutions and in serum

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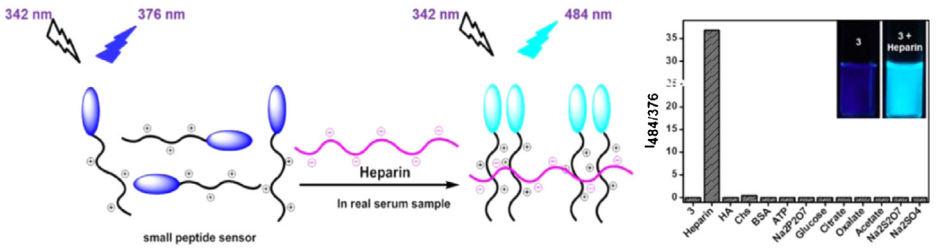
HIGHLIGHTS

- Readily synthesized in high yields (yield > 75%) using solid-phase synthesis.
- Ratiometric responses to heparin in 100% aqueous solution.
- Detection of nanomolar concentration of heparin in real biological samples.
- The detection limit for heparin in real biological sample was 205 pM.

GRAPHICAL ABSTRACT



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ABSTRACT

New fluorescent peptide-based sensors (**1**–**3**) for monitoring heparin in serum sample were synthesized using short peptides (1∼3mer) as a receptor. The peptide-based sensors (**2** and **3**) showed a sensitive ratiometric response to heparin both in aqueous buffered solution (10 mM HEPES, pH 7.4) and in 2% human serum sample by increase of excimer emission of pyrene at 480 nm and concomitant decrease of monomer emission of pyrene at 376 nm, whereas the peptide-based sensor **1** showed a turn off response only by decrease of monomer emission at 376 nm. **2** and **3** exhibited excellent selectivity toward heparin among various anions and competitors of heparin including chondroitin 4-sulfate (ChS) and hyaluronic acid (HA). Peptide-based sensor **3** showed a more sensitive response to heparin than **2**. The detection limit of **3** was determined as 36 pM (R^2 = 0.998) for heparin in aqueous solution and 204 pM (R^2 = 0.999) for heparin in aqueous solutions containing 2% human serum. The peptide-based sensors, **2** and **3** provided a practical and potential tool for the detection and quantification of heparin in real biological samples.

1. Introduction

Heparin is a highly negatively charged linear polysaccharide (glycosaminoglycan (GAG) family member), with a variable length that consists predominantly (>70%) of trisulfated disaccharide repeating units (Fig. 1) [1–3]. Heparin plays a crucial role in the regulation of various biological processes such as cell growth, cell differentiation, inflammation, immune defense, lipid transport, and metabolism [4–7]. Moreover, it prevents the blood coagulating cascade through interaction with antithrombin III, a protein inhibitor for thrombin [2]. Thus, heparin as an anticoagulant drug has been used to prevent thrombosis during surgery and to treat thrombotic diseases [2,4,8]. The therapeutic recommended dosage of heparin is 2–8 U mL^{-1} (17–67 μM) during cardiovascular surgery and 0.2–1.2 U mL^{-1} (1.7–10 μM) in postoperative long-term care. However, an overdose of heparin has caused some

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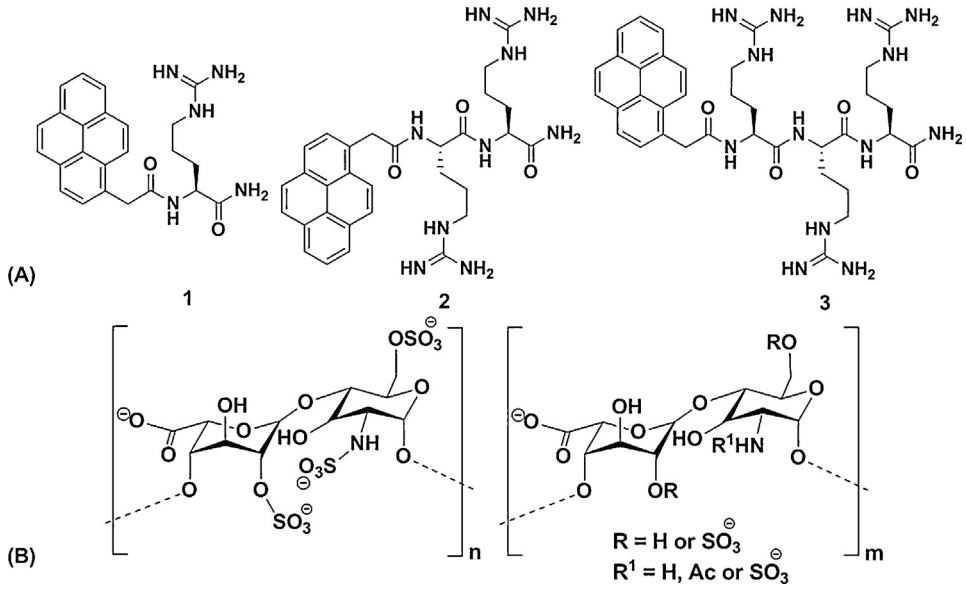

Fig. 1. Structure of (A) **1** (Py–Arg–NH_2), **2** (Py–ArgArg–NH_2), and **3** (Py–ArgArgArg–NH_2), and (B) major and minor disaccharide repeating units of heparin.

complications such as hemorrhages and thrombocytopenia [9–11]. Considering the therapeutic dosage of heparin and 10∼20 fold dilution of serum for the preparation of the biologically relevant samples, selective and sensitive quantification of nanomolar concentration of heparin in the biologically relevant samples are of clinical importance.

Up to now, there are several assays to monitor heparin, including the activated clotting time assay (ACT), activated partial thromboplastin time assay (aPTT), chromogenic antifactor Xa, potentiometric assays, and electrochemical assay [12–22]. However, these methods based on indirect measurements are not sufficiently reliable for the accurate measurement of heparin because of low specificity and interferences by charged species or biological competitors of heparin in serum. Thus, it is much desirable for the development of new methods for monitoring heparin with high accuracy and reliability.

In recent years, fluorescence has received great attention for the monitoring of various analytes due to inexpensive instrument, simple, rapid, and high sensitivity. A variety of fluorescent sensors for heparin based on organic compounds, polymers, and biomolecules has been reported [23–37]. However, most of them showed turn-off or turn-on responses to heparin in aqueous solutions or in mixed aqueous–organic solutions, whereas some of them showed ratiometric responses to heparin. Turn-on response type was more preferred because turn-off response could not be differentiated with the false signal induced by the precipitation of sensors or the decrease of the absorbance by impurities. However, enhanced emission intensity induced by sample could be affected by environmental effects such as pH, polarity of the media, photo-bleaching, and temperature [38–39]. Thus, ratiometric response using two different emission bands was more ideal than the turn-on response type in practical application because the ratio between two emission intensities could correct the concentration of sample as well as the environmental effects [38,39]. Up to now, a few of fluorescent chemosensors and biosensors showed ratiometric responses to heparin in aqueous solutions and some of them are tested for the suitable detection of heparin in real biological samples including serum or plasma [23,26,33,35]. However, almost all did not satisfy the sensitivity for the nanomolar concentration of heparin in real biologically relevant samples. Thus, there is a highly challenging for the development of new ratiometric fluorescent sensors for detecting nanomolar concentration of heparin in aqueous solutions as well as in real biologically relevant samples.

In recent years, peptide has been frequently used as the receptor for fluorescent biosensors and chemosensors because of their potent binding affinities for specific analytes, biological compatibility, and high solubility in aqueous solutions [40–51]. Recently, we reported a peptide-based sensor for heparin based on 12mer peptide to mimic the heparin-binding sequence (RKRLQVQLSIRT) of the G domain of the laminin α1 chain, which showed a sensitive ratiometric response to heparin in aqueous solution and in biologically relevant samples [26]. Considering the primary structures of the heparin binding peptides including protamine and our peptide-based sensor, almost all heparin binding peptides commonly shared arginine rich sequences as a critical binding site for heparin. In the present study, we synthesized fluorescent sensors for heparin based on peptides (1∼3mer) consisting of arginine amino acid(s) and investigated the relationship between the primary structure of the peptides and the sensitivity, selectivity, and ratiometric response to heparin in aqueous solution and biologically relevant samples. Furthermore, we investigated and discussed about the binding mode of heparin with the peptide-based sensors consisting of the simplified amino acid sequences.

2. Experimental

2.1. Reagents

Rink amide MBHA, Fmoc–Arg(Pmc–OH, *N*,*N*-diisopropylcarbodimide (DIC), and 1-hydroxybenzotriazole (HOBt) resin were purchased from Advanced ChemTech. Other reagents for solid phase synthesis including 1-pyreneacetic acid, trifluoroacetic acid (TFA), 1,2-ethane dithiol (EDT), thioanisole, *N*,*N*-dimethylformamide (DMF), triisopropylsilane (TIS), piperidine, phenylsilane and $Pd(PPh_3)_4$ were purchased from Aldrich. The heparin sodium salt from porcine intestinal mucosa, chondroitin 4-sulfate sodium salt (ChS) from the bovine trachea, and hyaluronic acid sodium salt (HA) from *Streptococcus equinus* were purchased from Sigma–Aldrich.