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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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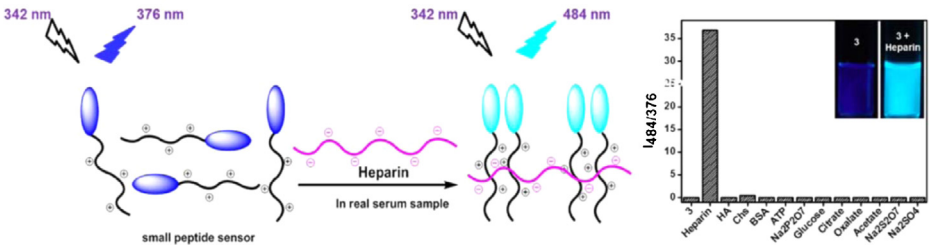
HIGHLIGHTS

- 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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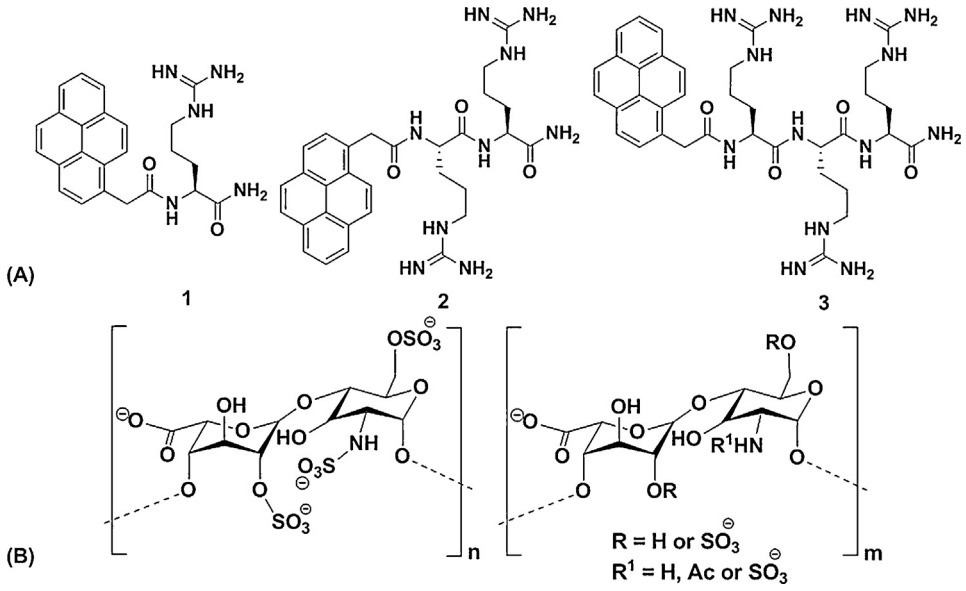

Fig. 1. Structure of (A) **1** (Py–Arg–NH_2), **2** (Py–ArgArg–NH_2), and **3** (Py–ArgArgArg–NH_2), and (B) major and minor disaccharide repeating units of heparin.

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.

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2.2. Instruments

Purification of the synthesized compounds **1**, **2**, and **3** were performed with C-18 semi preparative column on YL 9100HPLC using water (0.1% TFA)/acetonitrile (0.1% TFA) gradient. FT-IR spectra were characterized with VERTEX 80V/Bruker vacuum spectrometer with KBR. ^{1}H NMR and ^{13}C NMR spectra were analyzed using a Varian Unity Inova-400 NMR spectrometer with tetramethylsilane (TMS) as internal standard. LC–MS analyses were carried out with Varian 1200L Quadrupole LC/MS from an Agilent. All UV–visible spectra were measured by UV/visible spectrometer (Lambda 40, PerkinElmer, UK), fluorescence spectra by luminescence spectrometer (LS 55, PerkinElmer, UK).

2.3. Solid phase synthesis: general experimental procedure

The synthesis of Py–Arg–NH_2 (**1**), Py–ArgArg–NH_2 (**2**), Py–ArgArgArg–NH_2 (**3**) were easily and efficiently carried out in solid-phase synthesis with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry [52]. Diisopropylcarbodiimide (DIC) and 1-hydroxylbenzotriazole (HOBt) in situ activation method was used for the coupling reactions. The amino acid, Fmoc–Arg(Pmc)–OH with Fmoc as protecting group (0.3 mmol, 0.3 equiv) was loaded to Rink Amide MBHA resin (0.1 mmol, 0.1 equiv) according to the reported procedure. After deprotection of Fmoc group with 25% piperidine in *N*,*N*-dimethylformamide (DMF) of resin bound Arg the required number of Fmoc–Arg(Pmc)–OH (0.3 mmol, 0.3 equiv) were coupled sequentially (Scheme 1, Supporting information). 1-Pyreneacetic acid (0.3 mmol, 0.3 equiv) was coupled to N-terminal resin bound Arg. After completion of synthesis of target compounds, the resin bound target compounds were washed with DMF and MeOH. Finally, after washing, drying, and the cleavage of **1**, **2**, and **3** from the resin were accomplished with CF_3COOH/1,2-ethanedithiol/thioanisole/water/TIS (86.5/2.5/5/5/1, v/v) at room temperature for 6 h. Following vacuum filtration and removal of TFA with N_2 blow-off, crude product was precipitated from cold ether. The solid precipitate was centrifuged, washed with ether, and lyophilized under vacuum. The crude product was further purified with semi preparative HPLC using water (0.1% TFA)/acetonitrile (0.1% TFA) gradient. The retention times were 38 min for **1**, 36 min for **2**, and 29 min for **3**. The isolated yields of **1**, **2**, and **3** are 75.4, 86.7, and 89.2%, respectively. The products were characterized by using IR, ^{1}H NMR, ^{13}C NMR and ESI-mass spectroscopic data.

2.4. The characterization data of *1*, *2*, and *3*

2.4.1. Compound *1*

Colorless solid, mp 207–208 °C; IR (KBr): 3342 (br s), 3192, 1670, 1541, 1203, 1133 cm^{-1}; ^{1}H NMR (400 MHz, $DMSO_6$) δ 8.43 (br s, 1H) 8.39 (d, J = 8.5 Hz, 1H), 8.28 (d, J = 8.5 Hz, 2H), 8.23 (d, J = 8.4 Hz, 2H), 8.21 (d, J = 8.5 Hz, 2H), 8.06 (*t*, J = 8.4 Hz, 1H), 8.01(d, J = 8.4 Hz, 1H), 7.51 (*t*, J = 8.0 Hz, 2H), 7.44 (br s, 1H), 7.09 (br s, 1H), 4.29–4.21 (m, 3H), 3.09–3.06 (m, 2H), 1.78–1.65 (m, 1H), 1.56–1.41 (m, 3H); ^{13}C NMR (100 MHz, $DMSO_6$) δ 173.5, 170.3, 156.9, 131.2, 130.9, 130.5, 129.9, 129.1, 128.8, 127.4, 127.3, 127.0, 126.3, 125.2, 125.1, 124.9, 124.2, 124.1, 52.2, 40.5, 29.6, 25.3; ESI-mass (m/z): $[M+H]^+$ calculated for $C_{24}H_{26}N_2O_2$: 416.20, observed: 416.20.

2.4.2. Compound *2*

Colorless solid, mp 79–80 °C; IR (KBr): 3375 (br s), 2942, 1668, 1541, 1200, 1134 cm^{-1}; ^{1}H NMR (400 MHz, $DMSO_6$) δ 8.53 (d, J = 8.5 Hz, 1H), 8.38 (d, J = 8.5 Hz, 1H), 8.28 (d, J = 8.4 Hz, 2H), 8.24–8.19 (m, 2H), 8.17 (s, 2H), 8.09–7.98 (m, 3H), 7.66–7.63 (m, 2H), 7.37 (br s, 2H), 7.07 (br s, 3H), 4.34–4.18 (m, 5H), 3.10–3.02 (m, 4H), 1.80–1.40 (m, 8H); ^{13}C NMR (100 MHz, $DMSO_6$) δ 173.2, 171.4, 170.3, 159.1, 158.7, 156.8, 130.9, 130.8, 130.3, 129.9, 128.6, 127.4, 127.2, 126.8, 126.2, 125.1, 124.7, 124.1, 124.0, 123.9, 52.3, 51.9, 40.4, 40.3, 29.3, 29.2, 25.1; ESI-mass (m/z): $[M+H]^+$ calculated for $C_{30}H_{38}N_9O_3$: 572.31, observed: 572.34.

2.4.3. Compound *3*

Colorless solid, mp 170–171 °C; IR (KBr): 3365 (br s), 3191, 1668, 1541, 1202, 1134 cm^{-1}; ^{1}H NMR (400 MHz, $DMSO_6$) δ 8.54 (d, J = 8.3 Hz, 1H), 8.35 (d, J = 8.4 Hz, 1H), 8.27 (d, J = 8.4 Hz, 2H), 8.22 (d, J = 8.4 Hz, 1H), 8.20 (d, J = 8.4 Hz, 1H), 8.10–8.05 (m, 2H), 7.99 (d, J = 8.3 Hz, 1H), 7.91 (d, J = 8.4 Hz, 1H), 7.57 (*t*, J = 8.0 Hz, 2H), 7.49 (*t*, J = 8.0 Hz, 1H), 7.42 (br s, 2H), 7.09 (br s, 4H), 4.36–4.27 (m, 5H), 3.18–3.03 (m, 6H), 1.50–1.38 (m, 6H), 1.32–1.21 (m, 6H); ^{13}C NMR (100 MHz, $DMSO_6$) δ 173.5, 171.8, 171.3, 170.7, 159.2, 158.6, 156.9, 156.8, 130.9, 130.8, 130.5, 129.9, 129.1, 128.8, 127.5, 127.4, 127.0, 126.4, 125.3, 125.1, 124.9, 124.2, 124.1, 124.0, 52.6, 52.4, 52.2, 40.6, 40.4, 29.2, 29.1, 25.2, 25.1, 25.0; ESI-mass (m/z): $[M+H]^+$ calculated for $C_{36}H_{50}N_{13}O_4$: 728.40, observed: 728.36.

2.5. General fluorescence measurements

A stock solutions of **1** (1.38×10^{-3} M), **2** (1.14×10^{-3} M), and **3** (1.26×10^{-3} M) were prepared in distilled water and stored in a cold and dark place. The concentration of **1**, **2**, and **3** were confirmed by UV absorbance at 342 nm for pyrene group. The molar extension coefficient (ε) of **1**, **2**, and **3** is 16,000 cm^{-1} M^{-1}. These stock solutions were used for all spectrofluorometric experiments after appropriate dilution. The fluorescence experiments were carried out using the above referred solution after maintaining the pH of the solution to 7.4 using 10 mM HEPES buffer solution. Fluorescence emission spectrum of a sample in a 10 mm path length quartz cuvette was measured in 10 mM HEPES buffer solution at pH 7.4 using a PerkinElmer luminescence spectrophotometer (model LS 55). Emission spectra (365–600 nm) of **1**, **2**, and **3** in the presence of several anions (sodium citrate, sodium oxalate, sodium acetate, $Na_2P_2O_7$, $Na_2S_2O_7$, Na_2SO_4, adenosine triphoshphate (ATP), glucose, bovine serum albumin (BSA), chondroitin 4-sulfate (ChS), and hyaluronic acid (HA) and heparin were measured by excitation with 342 nm. The slit size for excitation and emission were 15 and 2.5 nm, respectively.

2.6. Preparation of biological relevant sample containing human serum

After collection of the whole blood from young male adult (age group between 27 and 35 years), the blood was allowed to clot for 1 h at room temperature. After removing the clot part by centrifuging at 1500 rpm for 15 min, the obtained serum was incubated at 4 °C. The stock serum solution was used for all spectrofluorometric experiments after appropriate dilution. We used sample solution containing 2% serum by dilution of the serum with aqueous (10 mM HEPES, pH 7.4 or 150 mM NaCl, 2 mM KCl and 10 mM HEPES) solutions.

2.7. Quantum yield measurement

As anthracene (Φ, 0.27) was used as a standard, quantum yields of the peptide-based sensors were obtained [53]. The absorbance was recorded in aqueous buffered solutions containing 25% CH_3CN in 10 mm cell. The fluorescence spectrums of the solutions were recorded with the excitation wavelength of 342 nm and the relative fluorescence was determined by weighing the area beneath the corrected fluorescence emission spectrum. Finally, the quantum yield of the peptide-based sensors was calculated as follows [54].

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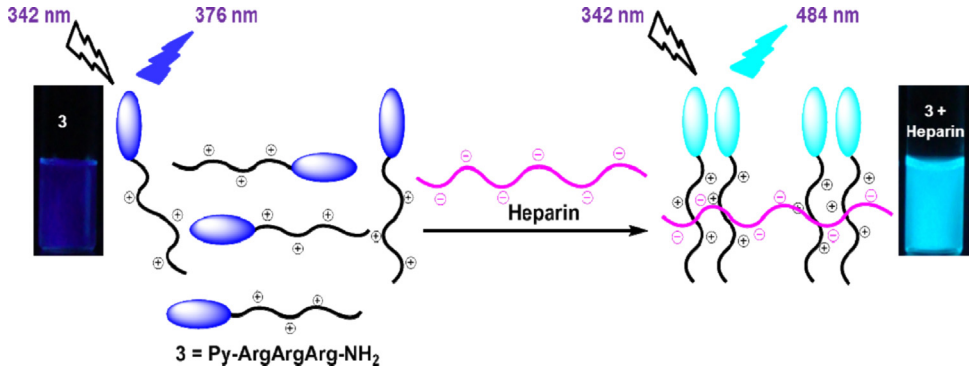

Scheme 1. Schematic illustration of ratiometric detection of heparin detection by short peptide sensors.

$$Q\ (\text{unknown}) = Q\ (\text{standard})\frac{F\ (\text{unknown})}{F\ (\text{standard})} \times \frac{A\ (\text{standard})}{A\ (\text{unknown})}$$

where F is the relative fluorescence and A is the absorbance of the solutions at the exciting wavelength.

2.8. Detection limits of the peptide-based sensor for heparin

The detection limit was calculated based on a fluorescence titration. To determine the S/N ratio, the emission intensity of free peptide sensor was measured ten times and the standard deviation of the blank measurements was determined. Three separate measurements of the emission intensity were measured in the presence of heparin, and the mean intensity was plotted as a concentration of heparin to determine the slope. The detection limit was calculated using the following equation:

$$\text{Detection limit} = 3\sigma/m,$$

where σ is the standard deviation of the intensity of free sensor, and m is the slope between the intensity at 376 nm vs concentration [55].

3. Result and discussion

3.1. Design and solid phase synthesis of peptide-based sensors for heparin

A pyrene fluorophore was incorporated into the peptide consisting of arginine amino acid(s) because the pyrene fluorophore has interesting photophysical properties such as high fluorescence quantum yield, chemical stability, and dual fluorescence emissions (monomer and excimer) depending on the distance between two pyrene fluorophores [56,57]. We reasoned that when the peptide-based sensor may interact with the sulfated disaccharide repeating unit of the heparin, two pyrene fluorophores of the peptide on heparin may come closer to each other, resulting in the decrease of the pyrene monomer at 376 nm as well as the increase of excimer emissions around 480–490 nm (ratiometric response), as depicted as in Scheme 1. Three pyrene (Py) labeled peptide-based sensors, (Py–Arg–NH_2, **1**; Py–ArgArg–NH_2, **2**; Py–ArgArgArg–NH_2, **3**) were easily synthesized in solid phase synthesis using Fmoc chemistry with isolated yield of 75.4, 86.7, and 89.2%, respectively as shown in Fig. 1 [52]. The detailed experimental procedure for the synthesis and characterization of **1**, **2**, and **3** are described in Section 2 (Figs. S1–S6, Supporting information).

3.2. Fluorescence emission optimization studies with heparin

As the peptide-based sensors showed a good solubility in aqueous solutions, fluorescence experiments of the peptide-based sensors carried out in 100% aqueous solution without organic co-solvent. The stock solutions of **1**, **2**, and **3** were prepared in distilled water and stored in a cold and dark place. UV–visible absorption spectra of **1**, **2**, and **3** exhibited a typical pyrene absorption band at 343 nm in aqueous buffered (10 mM HEPES) solutions at pH 7.4.

The fluorescent emission response of **1**, **2**, and **3** to heparin were investigated in aqueous buffered solution (10 mM HEPES, pH 7.4). The fluorescent emission intensity changes of **1** (10 μM) to heparin was measured (Fig. S7, Supporting information). Upon gradual addition of heparin to the solution, there is no change of excimer emission at 480–490 nm and only a decrease of monomer emission intensity at 376 nm was observed. The monomer emission intensity of **1** (10 μM) was plotted as a function of the concentration of heparin. A complete change in the monomer emission intensity required about 650 nM (0.065 equiv) of heparin.

As shown in Fig. 2a, the fluorescence emission spectra of **2** (10 μM) in aqueous buffered solution (10 mM HEPES, pH 7.4)

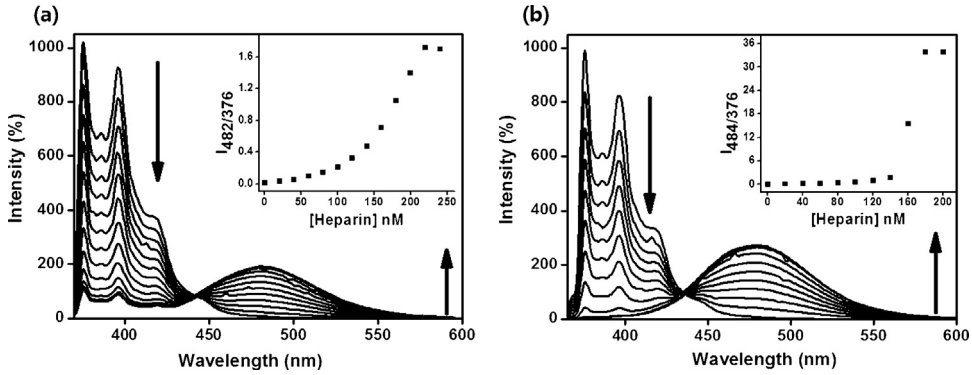

Fig. 2. Fluorescence spectra of (a) **2** (10 μM) upon gradual addition of heparin (0, 0.002, 0.004, 0.006, 0.022 and 0.024 equiv); (b) **3** (10 μM) upon gradual addition of heparin (0, 0.002, 0.004, 0.006, 0.020 and 0.022 equiv) in aqueous (10 mM HEPES, pH 7.4) solutions (λ_{ex} = 342 nm, slit = 15/2.5 nm).

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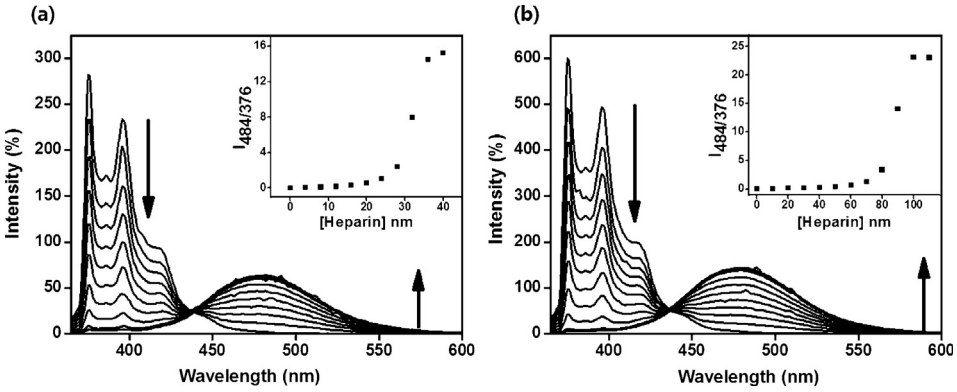

Fig. 3. Fluorescence spectra of (a) **3** (2 μM) and (b) **3** (5 μM) upon gradual addition of heparin in aqueous buffered solutions (10 mM HEPES, pH 7.4) (λ_{ex} = 342 nm, slit = 15/2.5 nm).

displayed strong emission intensities at 376 and 400 nm, corresponding to the typical pyrene monomeric emission band. This indicates that the peptide-based sensor fully dissolved well without aggregates in aqueous solutions. The gradual addition of heparin to the solution of **2** resulted in the significant decrease of the pyrene monomer emission intensities at 376 and 400 nm and concomitant increase of the pyrene excimer emission at 482 nm with a clear iso emission point at 442 nm. The intensity ratio (I_{482}/I_{376}) gradually increased from 0.0075 to 1.714 (ca. 228.6-fold enhancement) as the concentration of heparin increased from 0 to 220 nM (Fig. 2a, inset). A complete change of the ratiometric response required ~220 nM (0.022 equiv) of heparin. Similarly, **3** also showed a sensitive ratiometric response to heparin by decrease of monomer emissions and concomitant increase of the pyrene excimer, as shown in Fig. 2b. The intensity ratio (I_{484}/I_{376}) between excimer and monomer emission increased by ca. 5947-fold (from 0.0057 to 33.84). About 180 nM (0.018 equiv) of heparin was required for the saturation of the intensity ratio change (Fig. 2b, inset). Peptide-based sensor **3** showed more sensitive ratiometric response to heparin than **2** because **3** required lesser amount of heparin for the saturation of the intensity ratio and showed a more enhanced excimer emission and intensity ratio.

UV–visible absorption spectrum of **2** or **3** (10 μM) exhibited a hypochromic shift upon addition of heparin. A complete change of the absorbance required about 220 nM of heparin for **2** and 180 nM for **3** (Fig. S8, Supporting information). This is consistent well with the saturation point measured in fluorescent

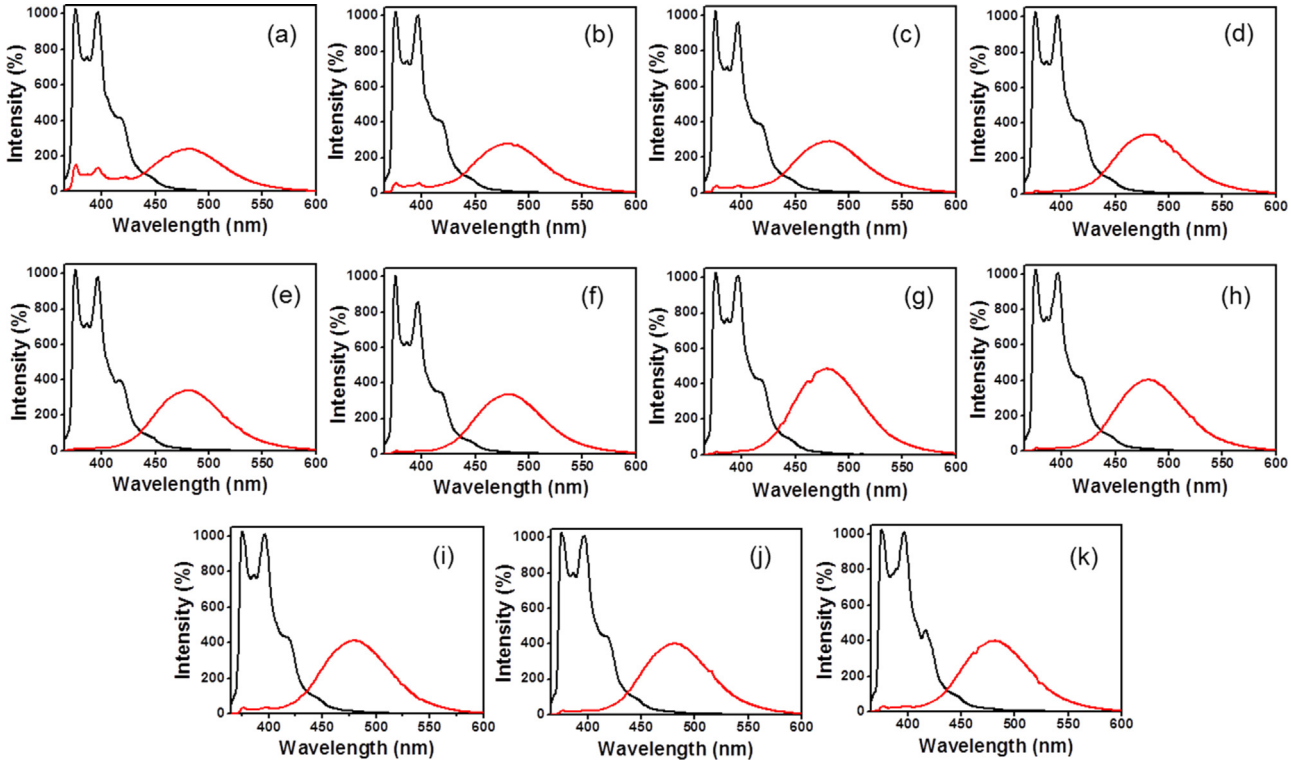

Fig. 4. Fluorescent ratiometric response of **3** (10 μM) to heparin (0.025 equiv) at different pH (a) 1.5, (b) 2.5, (c) 3.5, (d) 4.5, (e) 5.5, (f) 6.5, (g) 7.5, (h) 8.5, (i) 9.5, (j) 10.5, and (k) 11.5 solutions (λ_{ex} = 342 nm, slit = 15/2.5 nm).

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titration with heparin. The decrease of the absorbance band at 343 nm with a considerable red shift indicated that intermolecular dimerization between two pyrene moieties occurred [56,57].

We investigated whether the peptide-based sensor showed a ratiometric response to heparin depending on the concentration of the sensor because heparin as a biopolymer consisted of the repeating sulfated disaccharide units. As shown in Fig. 3, we chose the most sensitive sensor, **3** and tested the fluorescent response depending on the concentration of the sensor. Interestingly, the ratiometric response to heparin was observed independent of the concentration; however the saturation concentration and the enhancement of the excimer emission showed the dependence of the concentration of **3**. When the sensor concentration was 2 μM, the intensity ratio (I_{484}/I_{376}) increased from 0.0057 to 15.2 (ca. 2666-fold enhancement) as the concentration of heparin increased. About 40 nM of heparin was necessary for the saturation of the intensity ratio change (Fig. 3a, inset). In the case of 5 μM concentration, the intensity ratio (I_{484}/I_{376}) increased from 0.0055 to 23.06 (ca. 4192.7-fold) by gradually increasing concentration of heparin and complete change in the intensity ratio required about 100 nM of heparin (Fig. 3b, inset). As shown in Fig. 2, at the concentration of 10 μM, the intensity ratio (I_{484}/I_{376}) gradually increased from 0.0057 to 33.84 (ca. 5947-fold) as the concentration of heparin increased. About 180 nM (0.018 equiv) of heparin was required for the saturation of the intensity ratio change.

As the concentration of **3** increased, the intensity ratio (I_{484}/I_{376}) at the saturation concentration increased and the saturation concentration of the intensity ratio also increased. Considering the significant enhancement of the excimer emission, 10 μM was used for further experiments such as pH titration and selectivity study.

3.3. The pH Effect on the fluorescent response of *3* to heparin

To investigate the working pH of the peptide-based sensor, we measured the fluorescent response of **3** to heparin in various pH (1.5–11.5). Interestingly, the ratiometric response to heparin was observed in the wide range of pH (3.5–11.5). However, the ratiometric response induced by heparin showed the dependence on pH (Fig. 4). In acidic pH, the maximum intensity ratio change (I_{484}/I_{376}) induced by heparin decreased as the pH decreased. This suggests that the carboxylate groups of heparin play a critical role in the binding with the peptide sensors because the protonation of the carboxylate groups of heparin in acidic pH might induce the decrease of the electrostatic interactions between heparin and the positively charged peptide sensor. The largest intensity ratio change induced by heparin was observed at pH 7.5. As pH increased over 8, the intensity ratio (I_{484}/I_{376}) induced by heparin slightly decreased. However, **3** showed a considerable ratiometric response to heparin at pH 11.5. Considering the pK_a value (12.5) of the guanidine group of arginine, the slightly decrease of the intensity ratio induced by heparin at pH 11.5 was maybe due to the deprotonation of the guanidine groups of **3** at high pH.

The peptide-based sensor, **2** like **3** also showed a similar ratiometric response behavior to heparin depending on pH (Fig. 5). However, the maximum intensity ratio change (I_{484}/I_{376}) induced by heparin considerably decreased as pH decreased. For example, at acidic pH (1.5∼3.5), upon the addition of heparin did not completely decrease of the monomer emission intensities and slightly induced the excimer emission. This result clearly indicates that the carboxylate groups of heparin play a critical role in the binding with the peptide sensor **2**. At the basic pH (11.5), the monomer emission band at 370 nm and 405 nm did not completely decrease in the presence of heparin. This suggests that charge–charge interactions between **2** and heparin seemed to be

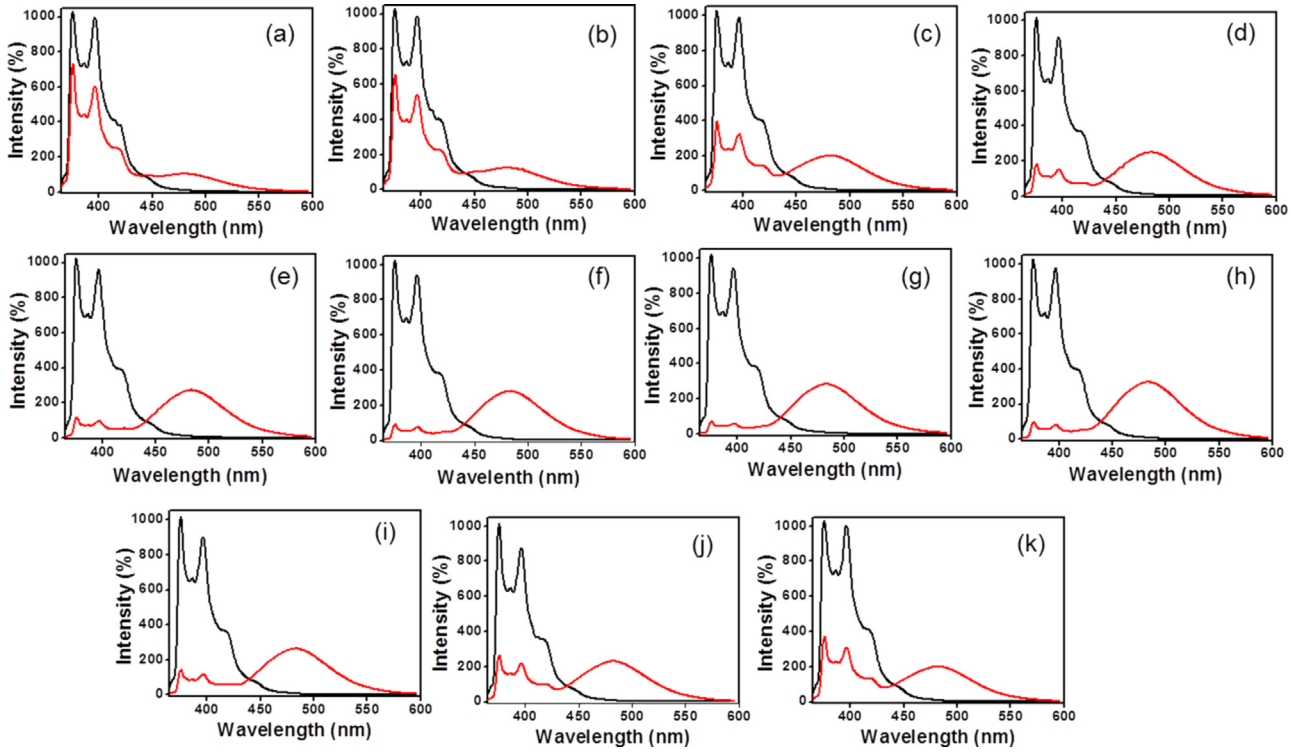

Fig. 5. Fluorescent ratiometric response of **2** (10 μM) to heparin (0.025 equiv) at different pH (a) 1.5, (b) 2.5, (c) 3.5, (d) 4.5, (e) 5.5, (f) 6.5, (g) 7.5, (h) 8.5, (i) 9.5, (j) 10.5, and (k) 11.5 solutions (λ_{ex} = 342 nm, slit = 15/2.5 nm).

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important for the binding and ratiometric response. Interestingly, both **2** and **3** showed ratiometric responses to heparin in the wide range of pH (1.5–11.5), however, **3** showed a much sensitive ratiometric response to heparin than **2** at acidic and basic pH.

It was generally accepted that heparin interacted well with the positively charged peptides [58–60]. However, it was not clear how many charged amino acids were required for the heparin binding. Cardin et al. reported that heparin binding peptides commonly shared the primary sequence of XBBBXXBX, where B is a basic and X is a non-basic amino acid [58–60]. Fromm et al. [60] investigated the role of the pattern and spacing of basic amino acids in the heparin binding peptides containing ArgArgGly$_m$ArgArg and ArgArgArgGly$_m$ArgArgArg (m = 2 or 3 glycine) sequences. The previous results indicated that multiple charged amino acids with the space of non-basic amino acid(s) in the peptide might be necessary for the heparin binding. However, the result employed in this study showed that mono, di, and triarginine(s) without the space of non-basic amino acid(s) were enough for the binding with heparin. Even though the peptide sensor **1** based on one arginine residue did not show a ratiometric response to heparin, the decrease of the monomer emission intensity of **1** by heparin indicated that the peptide sensor **1** might bind with heparin and the fluorescence of **1** was decreased by quenching effects of the negatively charged groups of heparin. Considering the data of fluorescence titration of **2**, two arginine amino acids in the binding site of the peptide sensor provided enough binding affinity for the interaction with heparin and a ratiometric response with an enhancement of the excimer emission. Multiple arginine amino acids in **2** and **3** might provide more potent electrostatic interactions with the peptide sensor and heparin and enough length for the interaction between the two pyrene fluorophores of the peptides for excimer emission. As shown in the data of pH titrations, the maximum intensity ratio change (I_{484}/I_{376}) of **2** induced by heparin considerably decreased at acidic pH (1.5 and 2.5) in which the electrostatic interactions between **2** and heparin was weakened at acidic pH. This result confirmed that electrostatic interactions between **2** and heparin play a critical role in binding and ratiometric response to heparin. At the same acidic pH, the **3** still maintained larger intensity ratio change (I_{484}/I_{376}) induced by heparin than **2**. This suggests that the peptide-based sensor (**3**) had more potent binding affinity for heparin than **2**, which is well consistent with the concentration of heparin for the saturation of intensity ratio change, as shown in Fig. 2. The binding mode of the **3** with heparin was proposed based on the results of fluorescent and UV–visible titration experiments, as shown in Scheme 1. After the binding of the peptide sensor with heparin, the two pyrene fluorophores of the peptides on the heparin came closer to each other and dimerization occurred by help of intermolecular π–π stacking interactions, resulting in the increase of excimer emission and a decrease of monomer emission.

3.4. Fluorescent selectivity of **3** to heparin

We investigated the fluorescence response of **3** to various anions and biological competitors of heparin such as sodium pyrophosphate, sodium oxalate, sodium citrate, sodium thiosulfate, sodium sulfate, sodium acetate, glucose, adenosine triphosphate (ATP), bovine serum albumin (BSA), chondroitin 4-sulfate (ChS) and hyaluronic acid (HA), as shown in Fig. 6. The intensity ratio (I_{484}/I_{376}) increased significantly from 0.0058 to 33.38 in the presence of heparin while the intensity ratio was not considerably changed in the presence of other anions such as ATP, $Na_2P_2O_7$, sodium oxalate, sodium acetate, sodium citrate, $Na_2S_2O_7$, and Na_2SO_4. The intensity ratio slightly increased from 0.0058 to 0.40 in the presence of chondroitin 4-sulfate maybe due to the presence of negatively charged sulfated groups of chondroitin 4-sulfate. Unexpectedly, **3** did not show any ratiometric response to bovine serum albumin (BSA), the most abundant blood protein. In the previous reports about chemosensors for heparin, the selectivity of the chemosensors between heparin and BSA was not well investigated. According to the recent report [37], highly positively charged chemosensors for heparin showed some response to BSA because highly negatively charged amino acids were predominately located in the surface of BSA. Overall results revealed that **3** showed excellent selectivity for heparin among various anions and biological competitors of heparin in aqueous buffered solution at pH 7.4. As shown in Fig. 6, peptide-based sensor, **2** also exhibited high selectivity toward heparin among various anions and biological competitors of heparin in aqueous buffered solution at pH 7.4.

Fig. 7 represents a visible emission change of **3** (10 μM) in the presence of various anions and heparin (0.025 equiv) under UV light (λ_{em} = 365 nm) of UV lamp. The solution of **3** exhibited a blue color in the absence or presence of the other competitors, whereas the solution of **3** in the presence of heparin displayed an intense cyan color in aqueous buffered solution (10 mM HEPES, pH 7.4). Interestingly, **3** displayed a less intense cyan color in the presence of heparin in aqueous buffered solution (10 mM HEPES, pH 7.4) containing 2% serum.

3.5. Interference effect of other anions on the detection ability of **3**

The interference effect of other anions on the detection ability of **3** for heparin was investigated, as shown in Fig. S9b

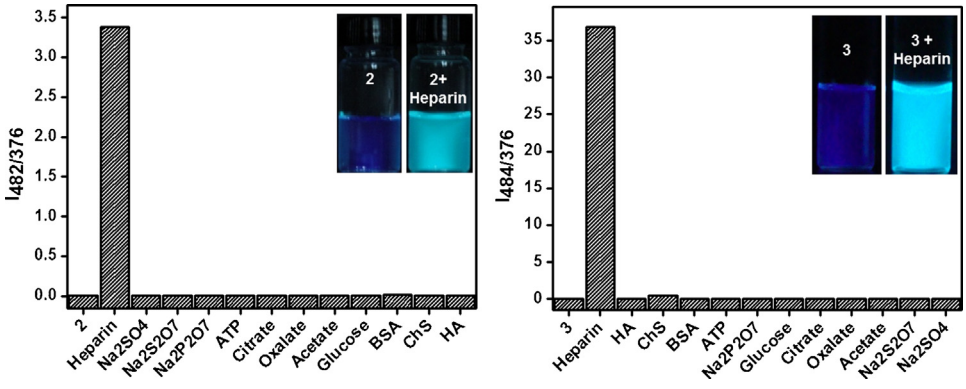

Fig. 6. Fluorescence spectra of (a) **2** (10 μM), and (b) **3** (10 μM) in the presence of anions (0.5 equiv) heparin (0.5 equiv), and biological competitors (0.5 equiv) of heparin such as hyaluronic acid (HA) chondroitin 4-sulfate (ChS), bovine serum albumin (BSA) in aqueous buffered solutions (10 mM HEPES, pH 7.4) (λ_{ex} = 342 nm, slit = 15/2.5 nm).

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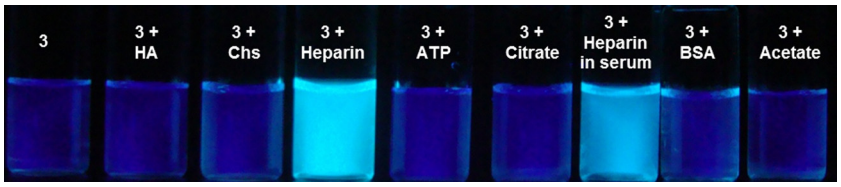

Fig. 7. A visible emission color change of **3** (10 μM) in the presence of various anions and heparin under UV light (λ_{em} = 365 nm) in aqueous buffered solution (10 mM HEPES at pH 7.4) and serum sample (v/v, 2%). HA, ChS, heparin, BSA, and ATP (0.025 equiv) were utilized, whereas sodium citrate and sodium acetate (0.5 equiv) were used. (For interpretation of the references to color in the text, the reader is referred to the web version of this article.)

(Supporting information). The ratiometric response to heparin was not considerably affected in the presence of high concentration of anions such as ATP, $Na_2P_2O_7$, $Na_2S_2O_7$, Na_2SO_4, sodium citrate, sodium oxalate, sodium acetate, and glucose. Furthermore, the ratiometric response (I_{484}/I_{376}) by heparin was not changed even in the presence of HA, structurally similar polysaccharide to heparin. Even though **3** did not show response to ChS and BSA, respectively, a relatively low intensity ratio (I_{484}/I_{376}) change by heparin was observed in the presence of ChS and BSA. **2** also showed ratiometric response to heparin without interference of anions and HA, as shown in Fig. S9a (Supporting information). However, the intensity ratio change of **2** by heparin considerably decreased in the presence of ChS and BSA.

Peptide-based sensor **3** displayed an excellent selective and sensitive ratiometric response to heparin among the possible competitors in aqueous buffered solutions at pH 7.4. For the practical application, we investigated whether the **3** successfully detected heparin by ratiometric response in real biological media containing serum. The real biological media containing 2% and 5% human serum was prepared in aqueous buffered solution at pH 7.4, respectively. By considering the osmotic pressure of red blood cell [61], we examined the fluorescence response of **3** to heparin in aqueous buffered solutions with high salts (150 mM NaCl, 2 mM KCl and 10 mM HEPES) containing 2% and 5% human serum. Fig. 8 showed fluorescence response of **3** to heparin in biologically relevant sample containing 2% human serum. **3** still showed a sensitive ratiometric response to heparin. The gradual addition of heparin induced a considerable decrease of the monomeric emission intensity at 376 nm and a concomitant increase of excimer emission at 480 nm. Both emission intensities were completely changed in the presence of ~180 nM (0.018 equiv) of heparin. Even though the intensity ratio (I_{480}/I_{376}) change by heparin in biologically relevant sample containing 2% serum was much decreased than that measured in aqueous buffered solution, the peptide sensor **3** showed a ratiometric response to heparin in real biologically relevant sample containing serum and the saturation concentration (0.018 equiv, 180 nM) of heparin measured in biologically relevant sample was similar to that measured in the aqueous buffer solution (Fig. 2b). This indicates that the sensitivity of **3** was not considerably decreased in the biologically relevant sample. Moreover, we tested the fluorescent response of **3** to heparin in the real biological sample containing higher amount (5%) of human serum. Peptide-based sensor **3** still showed a highly sensitive ratiometric response to heparin in this condition containing 5% human serum (Fig. 8). The intensity ratio (I_{480}/I_{376}) change to heparin increased gradually depending on the concentration of heparin. About 180 nM (0.018 equiv) of the heparin was required for the saturation of the intensity ratio change, which confirmed that the high sensitivity of **3** for heparin was maintained in real biological samples containing 2% and 5% serum.

The peptide-based sensor, **2** also showed a sensitive ratiometric response to heparin in aqueous buffered solution containing 2% human serum (Fig. S10, Supporting information). Upon the addition of increasing concentration of heparin, a significant decrease of monomer emission intensity at 376 nm and concomitant increase of excimer emission at 476 nm were observed. The complete change in the intensity ratio (I_{476}/I_{376}) of **2** required about 200 nM of heparin in biologically relevant sample containing human serum. This indicated that the **2** and **3** showed a considerable ratiometric response to heparin even in the biologically relevant samples containing human serum and the high sensitivity of the **2** and **3** was not considerably changed even in real biological samples in comparisons with that measured in aqueous buffered solution. The quantum yield (ϕ_F) of peptide-based sensors **1**, **2**, and **3** were determined in the absence and presence of heparin with reference to the anthracene, as shown in Table 1.

3.6. Detection limits for heparin in biological samples containing serum

The detection limits of the peptide-based sensors (**2** and **3**) for heparin was investigated in aqueous buffered solution (10 mM HEPES, pH 7.4) and in biologically relevant sample containing

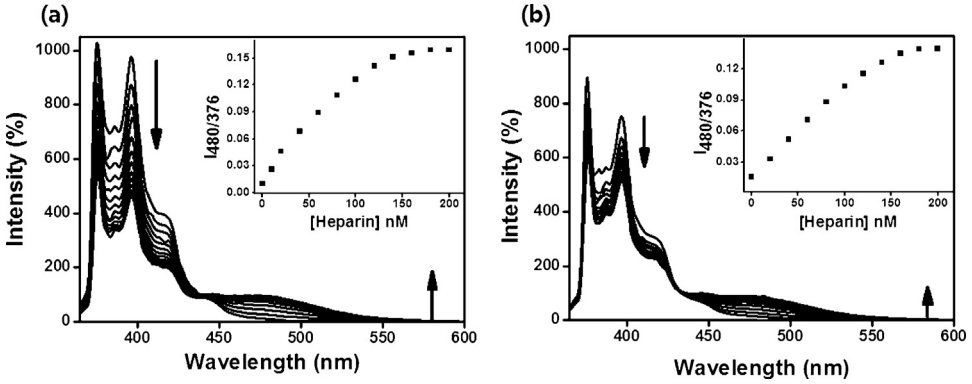

Fig. 8. Fluorescence spectra of **3** (10 μM) upon gradual addition of heparin in aqueous buffered solutions at pH 7.4 (150 mM NaCl, 2 mM KCl and 10 mM HEPES) containing (a) 2% human serum, and (b) 5% human serum (λ_{ex} = 342 nm, slit = 15/2.5 nm).

Table 1
Fluorescence quantum yield (ϕ_F) of **1**,**2**, and **3**.

S. no.	Peptide-based sensor	Quantum yield (ϕ_F)[a,b,c]
1	**1**	0.19[a]
2	**1** + heparin	0.07[a]
3	**2**	0.20[a], and 0.19[b]
4	**2** + heparin	0.18[a], and 0.24[b]
5	**3**	0.16[a], 0.21[b], and 0.18[c]
6	**3** + heparin	0.27[a], 0.24[b], and 0.23[c]

[a] In aqueous buffered solutions.
[b] In 2% serum containing aqueous buffered solutions.
[c] In 5% serum containing aqueous buffered solutions.

human serums because the **2** and **3** displayed a sensitive and selective ratiometric response to heparin. Upon the addition of increasing concentrations of heparin in the aqueous buffered solutions containing 0%, 2%, and 5% human serum, the maximum intensity at 376 nm was changed more sensitively in proportion to the concentration of heparin than the excimer emission at 480 nm. As shown in Fig. S11 (Supporting information), the change of the fluorescence intensity at 376 nm was well proportioned to the concentration of heparin ranging from 0 to 900 pM in aqueous solution. The detection limit of **3** was calculated as 36 pM ($R^2 = 0.998$) for heparin in an aqueous solution based on the linear relationships between the monomer emission intensity change and concentration of heparin using $3\sigma/m$, where σ is the standard deviation of the blank measurements and m is the slope of the intensity ratio as a function of heparin concentration. Similarly, a very good linearity between intensity changes as the function of heparin concentration (from 0 to 2400 pM) were obtained in real biological media containing 2% and 5% human serum, respectively (Fig. S12). The detection limit of **3** was determined as 204 pM ($R^2 = 0.999$) for heparin in real biological media containing 2% human serum and 299 pM ($R^2 = 0.997$) in real biological media containing 5% human serum.

The sensitivity of **2** for heparin was also measured based on the change of the monomer emission intensity in aqueous solution and in real biological media containing 2% serum. The detection limit of **2** was calculated as 232 pM ($R^2 = 0.998$) and 468 pM ($R^2 = 0.997$) for heparin in aqueous solution and in real biological media containing 2% serum, respectively (Fig. S13, Supporting information). The detection limit and linearity range of peptide-based sensors (**2** and **3**) were compared with other reported fluorescent chemosenors, as shown in Table 2. The detection limits of **2** and **3** for heparin in real biological media were higher than those measured in aqueous buffered solutions. This is maybe due to the nonspecific binding of the peptide-based sensor with some components in human serum such as serum albumin [37]. However, the decrease of the detection limits for heparin in biologically relevant samples was not very significant and the detection limit of **2** or **3** for heparin in aqueous solution containing human serum were much lower than therapeutic recommended dosage of heparin (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 and long-term care [9]. Considering the low detection limits of the **2** and **3** for heparin and the dilution factors for the serum sample preparation, the peptide-based sensors employed in this study might be suitable for the clinical use of detecting heparin. Table 3 lists the concentrations of heparin in various biological samples [62–65].

Table 3
The concentrations of heparin in various biological samples.

Species	Origin	Quantity of heparin	Ref.
Human	Plasma	1–2.4 mg L^{-1}	[62]
	Serum	1.2-1.8 mg L^{-1}	[62]
	Lungs	108 U mg^{-1}/gram of tissue[a]	[63]
	Mastocytoma	71 U mg^{-1}/gram of tissue[a]	[63]
	Liver	28 U/100 g of tissue[a]	[64]
Horse	Plasma	0.24 U mL^{-1}[a]	[65]
	Serum	0.2–4.0 U mL^{-1}[a]	
Rabbit	Liver	16–28 U/100 g of tissue[a]	[64]
	Lung	12–16 U/100 g of tissue[a]	
	Kidney	36–54 U/100 g of tissue[a]	
Dog	Liver	760–1800 U/100 g of tissue[a]	[64]
	Lung	440–500 U/100 g of tissue[a]	
	Kidney	200–520 U/100 g of tissue[a]	
Ox	Lung	1600–2100 U/100 g of tissue[a]	[64]

[a] 100 U = 1 mg.

4. Conclusions

Fluorescent peptide-based sensors (**2** and **3**) for heparin were synthesized using short peptides (1∼3mer) as a receptor. The **2** and **3** showed a sensitive ratiometric response to heparin in aqueous buffered solution by decreasing monomer emission at 376 nm and

Table 2
Comparison of the peptide-based sensors, **2** and **3** with reported fluorescent chemosenors.

Structure of the chemosenor	Aqueous buffered solutions		Aqueous buffered solutions containing serum[a,b,c]		Ref.
	Detection limit	Linearity range	Detection limit	Linearity range	
Silacyclopentadiene derivative	23 nM	0–11 μM	–	–	[23]
Functional ruthenium polypyridyl complex	0.38 nM	21–77 nM	0.68 nM[a]	35–98 nM[a]	[24]
Polyadenosine–coralyne complex	4 nM	0–1000 nM	–	–	[25]
Py12mer	3 pM	0–50 pM	34 pM[c]	0–250 pM[c]	[26]
$CuInS_2$ quantum dots	12.46 nM	0.05–15 μM	–	–	[27]
Salicylaldehyde azine derivative	57.6 ng mL^{-1}	0.2–14 μg mL^{-1}	–	–	[28]
Cationic conjugated polyfluorene derivative	30 nM	0–0.7 μM	–	–	[33]
Quinine derivative	30 nM	0.8–16 μM	–	–	[34]
Pyrene derivative	0.157 μM	5–30 μM	0.068 μM[c]	0–22.5 μM[c]	[35]
Conjugated Oligoelectrolyte/Graphene	0.046 U mL^{-1}	0–1.76 U mL^{-1}	–	–	[36]
Tetraphenylethene (TPE)	20 nM	0–15 μM	–	–	[37]
Peptide-based sensor **2**	232 pM	0–3500 pM	468 pM[b]	0–3500 pM[b]	Present
Peptide-based sensor **3**	36 pM	0–900 pM	204 pM[b]	0–2400 pM[b]	Present
			299 pM[c]	0–2400 pM[b]	

[a] 1% serum.
[b] 2% serum.
[c] 5% serum.

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concomitantly increasing excimer emission at 480 nm. The **2** and **3** displayed excellent selectivity for heparin among various anions and biological competitors of heparin in aqueous buffered solution. The detection limit of **2** and **3** for heparin was determined as 232 pM ($R^2 = 0.998$) and 36 pM ($R^2 = 0.998$) in aqueous solutions, respectively. The detection limit of **2** and **3** for heparin was determined as 468 pM ($R^2 = 0.997$) and 204 pM ($R^2 = 0.999$) in biologically relevant samples containing 2% human serum. The detection limits of the **2** and **3** were much lower than the clinical recommended concentration of heparin in serum. The ratiometric sensor based on di and tripeptide consisting of arginine amino acids showed enough sensitivity and selectivity for the detection and quantification of heparin in real biological samples containing human serum.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2015.03.001.

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