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Pyrene Excimer-Based Peptidyl Chemosensors for the Sensitive Detection of Low Levels of Heparin in 100% Aqueous Solutions and Serum Samples

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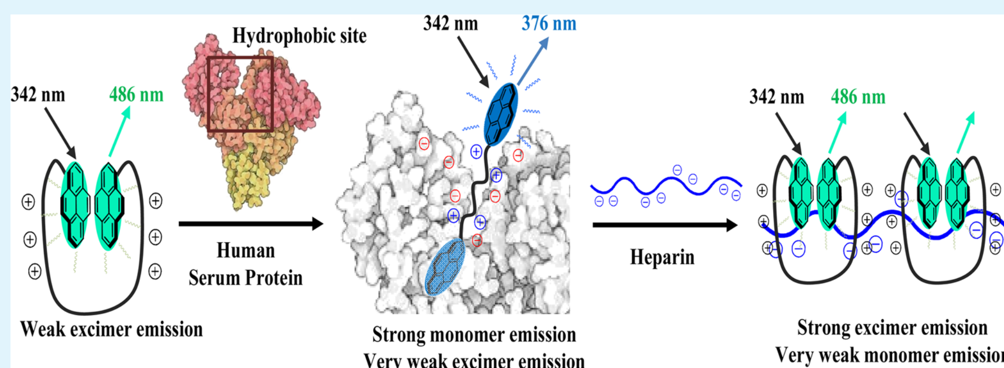
S Supporting Information



ABSTRACT: Fluorescent chemosensors (**1** and **2**, Py−(Arg)$_n$GlyGlyGly(Arg)$_n$Lys(Py)−NH_2, n = 2 and 3) bearing two pyrene (Py) labeled heparin-binding peptides were synthesized for the sensitive ratiometric detection of heparin. The peptidyl chemosensors (**1** and **2**) sensitively detected nanomolar concentrations of heparin in aqueous solutions and in serum samples via a ratiometric response. In 100% aqueous solutions at pH 7.4, both chemosensors exhibited significant excimer emission at 486 nm as well as weak monomer emission in the absence of heparin. Upon the addition of heparin into the solution, excimer emission increased with a blue shift (10 nm) and monomer emission at 376 nm decreased. The chemosensors showed a similar sensitive ratiometric response to heparin independent of the concentration of the chemosensors. The peptidyl chemosensors were applied to the ratiometric detection of heparin over a wide range of pH (1.5−11.5) using the excimer/momomer emission changes. In the presence of serum, **1** and **2** displayed significant monomer emission at 376 nm with relatively weak excimer emission and the addition of heparin induced a significant increase in excimer emission at 480 nm and a concomitant decrease in monomer emission. The enhanced ratiometric response to heparin in the serum sample was due to the interactions between the peptidyl chemosensors and serum albumin in the serum sample. The detection limits of **2** for heparin were less than 1 nM in 100% aqueous solutions and serum samples. The peptidyl chemosensors bearing two heparin-binding sites are a suitable tool for the sensitive ratiometric detection of nanomolar concentrations of heparin in 100% aqueous solutions and serum samples.

KEYWORDS: *fluorescence, chemosensor, ratiometric, heparin, sensor, peptide*

■ INTRODUCTION

Heparin is a highly negatively charged linear polysaccharide with a variable length that consists predominantly (>70%) of trisulfated disaccharide repeating units (Scheme 1).[1] Heparin plays an important role in regulating a range of biological processes, such as cell growth and differentiation, inflammation, immune defense, and metabolism.[2−4] Moreover, it prevents the blood coagulating cascade via an interaction with antithrombin III, a protein inhibitor for thrombin.[1] Clinically, heparin is used to prevent thrombosis during surgery and as an anticoagulant drug to treat thrombotic diseases.[4,5] On the other hand, heparin overdoses cause complications, such as hemorrhages and heparin-induced thrombocytopenia.[6,7] The recommended therapeutic dose of heparin during cardiovascular surgery and in postoperative and long-term care is 2−8 units/mL (17−67 μM) and 0.2−1.2 units/mL (1.7−10 μM), respectively.[8] Therefore, the quantification of heparin in serum is of crucial importance for the regulation of heparin for clinical use.

In recent years, fluorescent chemosensors have attracted considerable attention for the detection of a range of analytes because of the inexpensive instrument, simplicity, and high sensitivity. Although a variety of fluorescent chemosensors for

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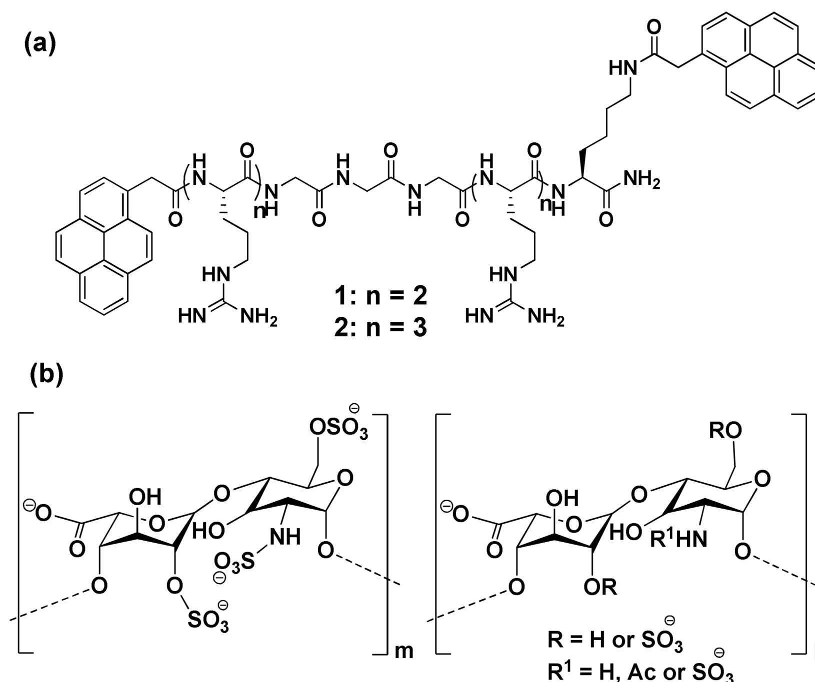
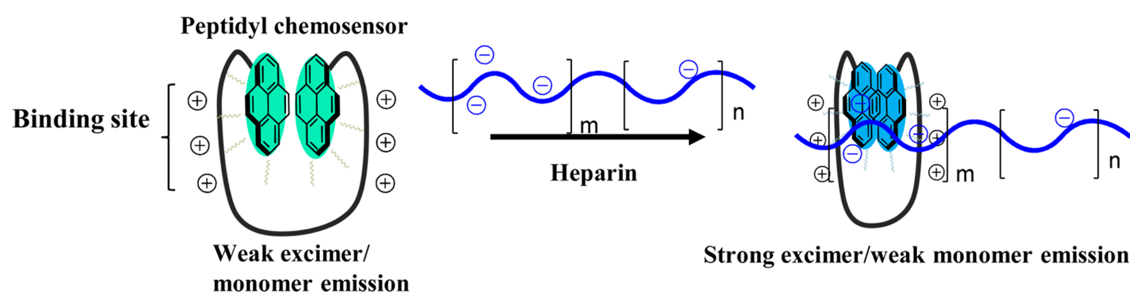
Scheme 1. Structures of (a) Peptidyl Chemosensors and (b) Major and Minor Disaccharide Repeating Units of Heparin



Scheme 2. Proposed Binding Mode of Peptidyl Chemosensors with Heparin



monitoring heparin have been reported,[9−25] most showed turn-on responses to heparin with a change in the single emission intensity, and a few satisfied the nanomolar sensitivity for heparin in mixed aqueous−organic solvent.[9−20] On the other hand, the turn-on response was not ideal because the emission intensity can be affected by environmental effects, such as pH, polarity of the media, and temperature. Therefore, the ratiometric response using two different emission bands was recommended for practical application because the ratio between two emission intensities could correct for the environmental effects.[10,26,27] Recently, a few ratiometric fluorescent chemosensors for heparin based on the mechanism of fluorescence resonance energy transfer (FRET), internal charge transfer (ICT), or pyrene monomer/excimer have been reported.[21−25] Some ratiometric fluorescent chemosensors were suitable for the detection of low concentrations of heparin in aqueous solutions, but a few of them were tested for the ratiometric detection of heparin in serum samples. In addition, few ratiometric chemosensors were suitable for the detection of nanomolar concentrations of heparin in serum samples and most ratiometric sensors showed a response to heparin dependent on the concentration of the chemosensors because heparin as a biopolymer consisting of disaccharide repeating units, as shown in Scheme 1.[23−25] Therefore, the synthesis of new ratiometric chemosensors for detecting nanomolar concentrations of heparin in aqueous solutions as well as in serum samples independent of the concentration of the chemosensors is still a challenge.

Over the past few years, several fluorescent peptide-based chemosensors for the detection of metal ions and small biological active molecules have been reported because peptides have high biological compatibility and high solubility in aqueous solutions.[14,28−38] Peptide is expected to be a promising receptor for various kinds of analytes because peptides, unlike proteins, cannot be denatured through exposure to UV light, high or low pH, and organic solvents and have potent binding affinities for specific target molecules, such as metal ions, biologically active small molecules, and biopolymers. On the other hand, there are few reports on peptide-based sensors for various analytes. Recently, a fluorescent peptidyl chemosensor for heparin based on the 12mer peptide (RKRLQVQLSIRT) was reported to mimic the heparin-binding primary structure of the G domain of the laminin α1 chain.[24,39] The peptidyl chemosensor showed a sensitive ratiometric response to nanomolar concentrations of heparin in aqueous solutions and biologically relevant samples containing serum. On the other hand, the ratiometric response to heparin is strongly dependent on the concentration of the chemosensor itself because heparin is a biopolymer consisting of the disaccharide repeating unit for the binding site of the chemosensor.

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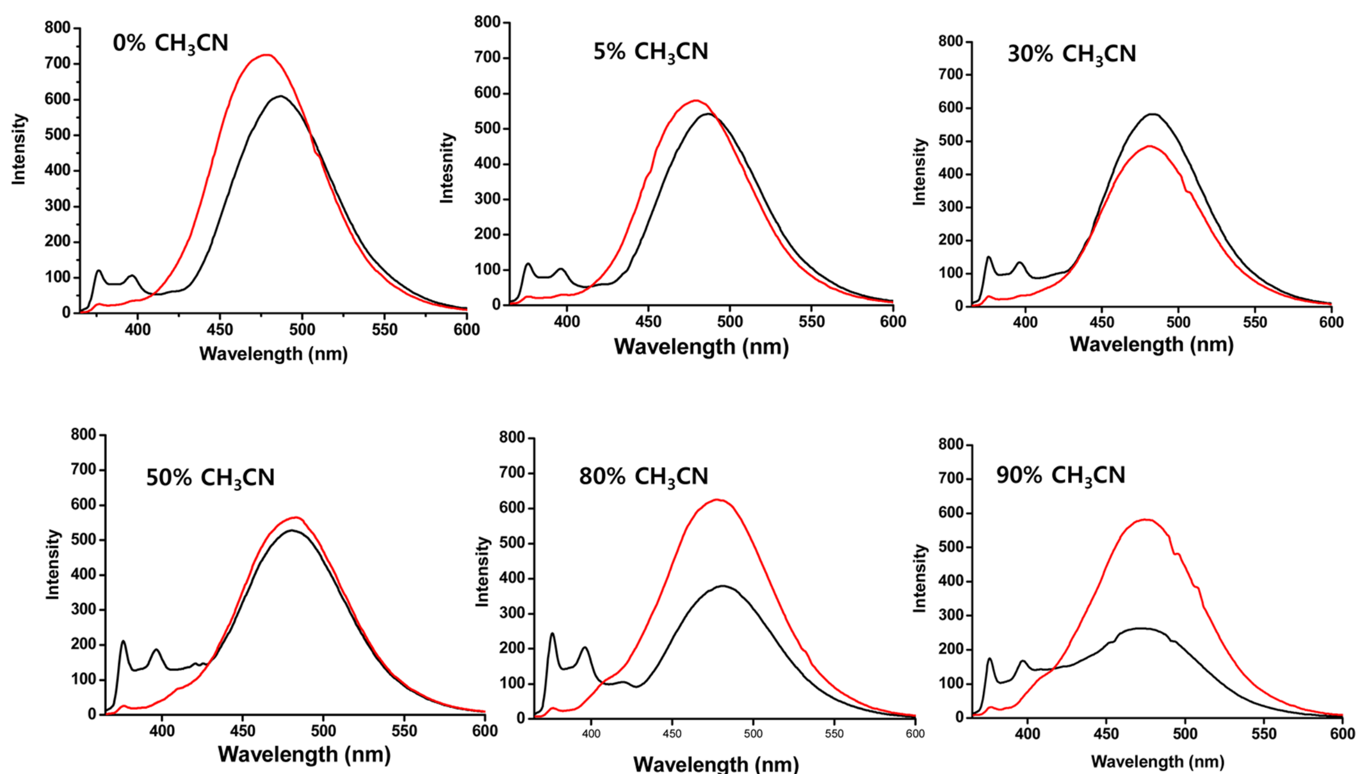

Figure 1. Fluorescence spectrum of **1** (1 μM) in the presence or absence of heparin (50 nM) in an aqueous solution (10 mM HEPES, pH 7.4) containing different percentages of CH_3CN, λ_{ex} = 342 nm. The red line indicates the presence of heparin, and the black line indicates the absence of heparin.

In the present study, new ratiometric peptidyl chemosensors bearing two heparin-binding sites were designed for the following reasons. Cardin et al. reported that the peptide sequence corresponding to XBBBXXBX (where B is a basic and X a nonbasic amino acid) was proposed to be common in heparin-binding peptides.[40] The role of the pattern and spacing of basic amino acids in the heparin-binding peptides were reported.[41,42] The peptide space (20 Å) between the positively charged amino acids of the peptides played an important role in heparin binding, irrespective of the α-helix or β-sheet structures of the peptides. As shown in Scheme 2, if one peptidyl chemosensor bearing two heparin-binding sites can bind to one trisulfated disaccharide in the repeating disaccharide units, the ratiometric response to heparin will be quite sensitive to the concentration of heparin and less dependent on the chemosensor concentration.

In the present work, fluorescent peptidyl chemosensors consisting of two pyrene labeled heparin-binding peptides and one Gly-rich peptide as the space between two heparin-binding peptides were synthesized (Scheme 1). Two pyrene (Py) fluorophores were incorporated into the peptide (**1**, Py−ArgArgGlyGlyGlyArgArgLys(Py)−NH_2; **2**, Py−ArgArgArgGlyGlyGlyArgArgArgLys(Py)−NH_2) because the pyrene fluorophore exhibits interesting photophysical properties, such as a high fluorescence quantum yield, chemical stability, and dual fluorescence emissions (monomer emission at 376 nm and excimer emissions at 486 nm) depending on the distance between the two pyrenes.[36,43] The reasoning was that if the peptidyl chemosensors containing two heparin-binding sites can interact with the same or close disaccharide unit of the heparin (Scheme 2), the two pyrene fluorophores of the peptide may come closer or farther away from each other, resulting in a change in the pyrene excimer emissions and monomer emissions depending on the proximity between the two pyrene fluorophores.

In addition, if the pyrene excimer/monomer emission changes induced by heparin occur via intramolecular excimer formation rather than intermolecular excimer formation, the chemosensors will show a more sensitive ratiometric response to heparin that is less dependent on the concentration of the chemosensor itself. As expected, the chemosensors (**1** and **2**) containing two heparin-binding sites exhibited a sensitive ratiometric response to heparin in a 100% aqueous solution as well as in the serum sample by the increasing excimer emission and concomitant decrease in monomer emission. Interestingly, the change in the intensity ratio (I_{486}/I_{376}) of the peptidyl chemosensors by heparin was independent of the concentration of the chemosensor. Moreover, the change in I_{486}/I_{376} by heparin in the serum sample was much greater than that measured in a 100% aqueous solution.

RESULTS AND DISCUSSION

Fluorescence Emission Spectra of the Chemosensors in Different Solvent Systems. The peptidyl chemosensors were synthesized easily in solid-phase synthesis in high yield (86% for **1** and 89% for **2**) using Fmoc chemistry (Scheme S1, Supporting Information).[44]The detailed experimental procedure for the synthesis and characterization of **1** and **2** are described in the Experimental Section (Figures S1−S9, Supporting Information). Stock solutions of **1** (1.42×10^{-3} M) and **2** (1.20×10^{-3} M) were prepared in 100% aqueous solution and stored in a cold and dark place. The UV−visible absorption spectra of **1** and **2** exhibited a typical pyrene absorption band at 343 nm in a 100% aqueous solution at pH

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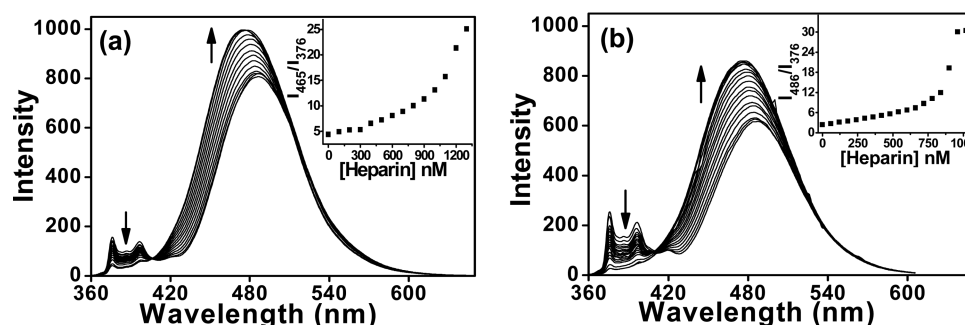

Figure 2. Fluorescence spectra of (a) **1** (30 μM) and (b) **2** (30 μM) upon the gradual addition of heparin in 100% aqueous solutions (10 mM HEPES, pH 7.4), λ_{ex}= 353 nm, slit = 15/2.5 nm.

7.4 (Figures S8 and S9, Supporting Information). Therefore, the effects of the organic solvent on the fluorescence response of the chemosensors to heparin were investigated systematically.

The fluorescence response of **1** to heparin was measured in aqueous solutions (10 mM HEPES, pH 7.4) containing different percentages (0−90%, v/v) of acetonitrile (Figure 1 and Figure S10, Supporting Information). As shown in Figure 1, significant excimer emission at 486 nm was observed, even in the absence of heparin in a 100% aqueous solution at pH 7.4. This indicates that two hydrophobic pyrene fluorophores of the peptide may come closer to each other in a hydrophilic environment, resulting in significant intramolecular excimer emission at 486 nm and weak monomer emission at 376 nm. As the percentage of acetonitrile ranged from 0 to 5% in aqueous solutions (10 mM HEPES at pH 7.4), the addition of heparin induced an increase in excimer emission with a blue shift (~10 nm) and a considerable decrease in monomer emission at 376 nm.

As the percentage of acetonitrile ranged from 10 to 40% in aqueous solution (10 mM HEPES at pH 7.4), the addition of heparin induced a significant decrease in monomer emission and a slight decrease in excimer emissions. This suggests that the excimer emission may be quenched by the negatively charged groups of heparin under this solvent condition. As the percentage of acetonitrile ranged from 50 to 90% in aqueous solution (10 mM HEPES at pH 7.4), the addition of heparin induced a significant increase in excimer emissions and a significant decrease in monomer emission. The enhancement of excimer emission at 486 nm by heparin increased with increasing percentage of acetonitrile (50−90%) in solution. In these solvent systems, two pyrene fluorophores of the peptide may be far away compared to that in a 100% aqueous solution because this solvent system provides a hydrophobic environment for the two hydrophobic pyrene fluorophores. Therefore, the peptide exhibited weaker excimer emission than that measured in a 100% aqueous solution. Upon the addition of heparin, two heparin-binding sites of the peptide interacted with the disaccharide repeating unit of heparin and the two pyrene fluorophores may come closer to each other, resulting in enhanced excimer emission and a concomitant decrease in monomer emission.

The change in intensity ratio (I_{486}/I_{376}) by heparin was analyzed in aqueous buffered solutions containing different percentages of acetonitrile. As the percentage of CH_3CN was increased from 40 to 90%, the change in intensity ratio by heparin increased from 8.83-fold to 12.08-fold. The intensity ratio change (12.7-fold) by heparin was the greatest in the aqueous solution (10 mM HEPES at pH 7.4) containing 80% CH_3CN. Although the intensity ratio change by heparin was not great in a 100% aqueous solution, the addition of heparin induced a unique blue shift in the maximum excimer emission at 486 nm. Therefore, a 100% aqueous solution and an aqueous solution containing 80% acetonitrile were chosen as the solvent condition for further studies.

As shown in Figure 2, a fluorescent titration of the peptidyl chemosensors (**1** and **2**) with heparin was carried out. The peptidyl chemosensor **1** (30 μM) itself displayed a strong excimer emission band at 486 nm and weak monomer emissions at 386 and 397 nm in a 100% aqueous solution. The gradual addition of heparin to a solution of **1** resulted in a significant decrease in the pyrene monomer emission intensities at 386 and 397 nm with a concomitant increase in pyrene excimer emission with a blue shift from 486 to 476 nm (Figure 2a). This suggests that the binding of **1** with heparin induced an increase in the intramolecular interactions between the two pyrene fluorophores. The intensity ratio (I_{465}/I_{376}) increased gradually from 4.41 to 25.08 (ca. 5.7-fold enhancement) with increasing heparin concentration from 0 to 1300 nM. Approximately 1300 nM (0.043 equiv) of heparin was necessary for the complete change in the emission intensity of **1**. Similarly, **2** also showed a sensitive ratiometric response to heparin via a decrease in monomer emissions and a concomitant increase in excimer emission with a blue shift, as shown in Figure 2b. I_{486}/I_{376} between the excimer and monomer emission increased from 2.43 to 30.4 (ca. 12.5-fold). Approximately 1000 nM (0.034 equiv) of heparin was required for saturation of the intensity ratio change. Peptidyl chemosensor **2** showed a more sensitive ratiometric response to heparin than **1** because **2** required smaller amounts of heparin for a change in the saturation of the intensity ratio and showed more enhanced excimer emission and a change in intensity ratio.

The increase in pyrene−pyrene excimer emission by heparin was confirmed further by UV−visible spectroscopy (Figure S11, Supporting Information). In the UV−visible absorption titration of **1** and **2** with heparin, a significant decrease in absorbance at 350 nm was observed in the presence of increasing concentrations of heparin. A complete change in the absorbance required approximately 1200 nM (0.0040 equiv) of heparin for **1** and 1140 nM (0.0038 equiv) for **2**, which is consistent with the saturation concentration of heparin measured in the fluorescent titration experiments. The decrease in the absorbance bands of the chemosensors at 350 nm by heparin indicated that the dimerization of two pyrene

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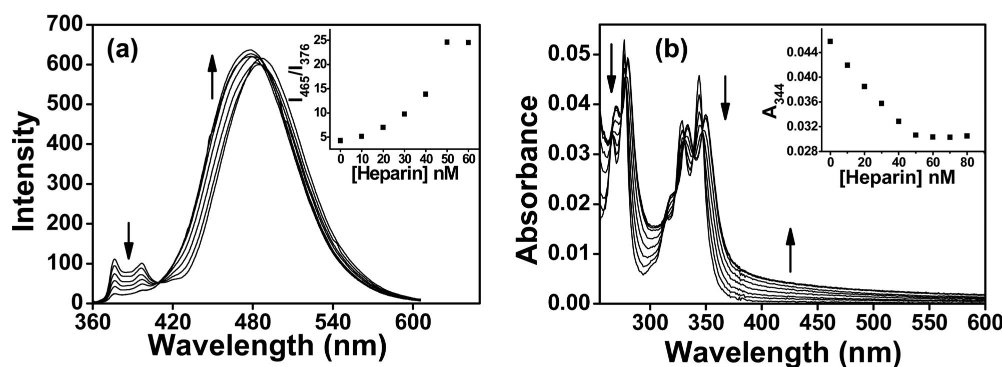

Figure 3. (a) Fluorescence spectra of **1** (1 μM) upon the gradual addition of heparin (λ_{ex}= 353 nm). (b) UV−visible absorption spectra of **1** (1 μM) upon the gradual addition of heparin in aqueous (10 mM HEPES, pH 7.4) solutions.

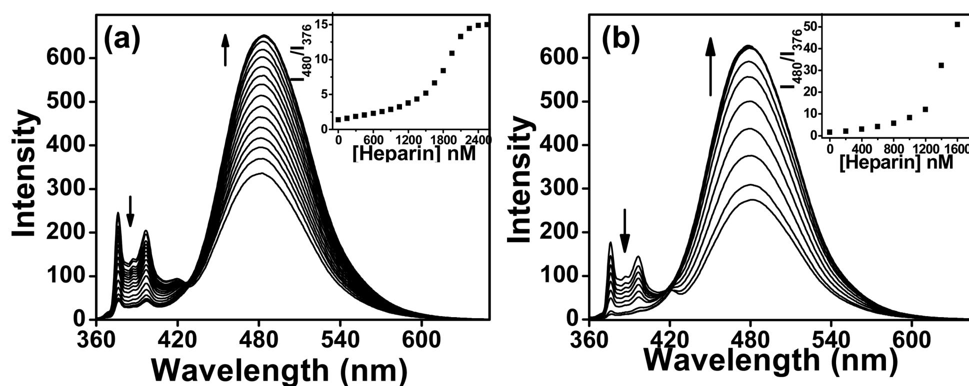

Figure 4. Fluorescence spectra of (a) **1** (30 μM) and (b) **2** (30 μM) upon the gradual addition of heparin in aqueous solutions (10 mM HEPES, pH 7.4) containing 80% CH_3CN, λ_{ex}= 353 nm, slit = 15/2.5 nm.

fluorophores of the peptidyl chemosensor occurred after binding of the peptidyl chemosensor with heparin.[36,43]

Because the chemosensors contained two pyrene labeled heparin-binding sites, it was expected that pyrene excimer emission occurred via intramolecular excimer formation rather than intermolecular excimer formation. In general, the intramolecular excimer is less dependent on the concentration, whereas intermolecular excimer formation is dependent on the concentration. Therefore, the fluorescent titration of low concentrations of the peptidyl chemosensor (1 μM) with heparin was also carried out (Figure 3). Although the concentration of the peptide was low, excimer emission was observed in the absence of heparin in the aqueous solutions. This suggests that two pyrene fluorophores of the peptide came into close contact, resulting in intramolecular excimer formation. Upon the addition of heparin, the maximum intensity of excimer emission increased with a blue shift and monomer emission decreased. I_{465}/I_{376} increased gradually from 4.26 to 24.4 (ca. 5.7-fold enhancement) as the concentration of heparin was increased from 0 to 50 nM (Figure 3a). The intensity ratio change of **1** induced by heparin appeared to be less dependent on the concentration of the peptide, which strongly supports the enhanced pyrene excimer emission occurring mainly via intramolecular excimer formation.

The UV−visible absorption spectrum of **1** (1 μM) displayed a hypochromic shift upon the addition of heparin (Figure 3b). A complete change in the absorbance required approximately 50 nM of heparin (Figure 3b, inset). The decrease in the absorbance band at 344 nm with a considerable red shift indicated that intramolecular dimerization of the pyrene fluorophores of the peptide occurred after the peptide was bound to heparin.[36,43]

This study further investigated whether the peptidyl chemosensors might operate well to detect heparin in a mixed aqueous−organic solvent system. As shown in Figure 4, **1** and **2** also exhibited a sensitive ratiometric response to heparin in aqueous solutions at pH 7.4 containing 80% CH_3CN. Upon the addition of heparin, the maximum intensity of excimer emission at 486 nm increased without a blue shift, and the monomer emission decreased. I_{480}/I_{376} of **1** increased gradually from 2 to 15 as the concentration of heparin was increased from 0 to 2500 nM. I_{465}/I_{376} of **2** increased from 0.2 to 50 as the concentration of heparin was increased from 0 to 1800 nM. A complete change in the intensity ratio required approximately 2500 nM (0.0083 equiv) and 1800 nM (0.0060 equiv) of heparin for **1** and **2**, respectively

The UV−visible absorption spectra of **1** or **2** displayed a hypochromic shift in the presence of heparin in an aqueous solution containing 80% CH_3CN (Figure S12, Supporting Information). Upon the addition of heparin, a significant decrease in the absorbance band at 343 nm corresponding to pyrene was observed. This suggested that the dimerization of two pyrene fluorophores readily occurred, even in mixed aqueous−organic solvent via the strong π−π stacking interactions between the two pyrene moieties of the peptide bound to heparin.[36,43]

pH Effect on the Fluorescent Response of the Chemosensors to Heparin. To examine the effects of pH on the response, fluorescent responses of the chemosensors to

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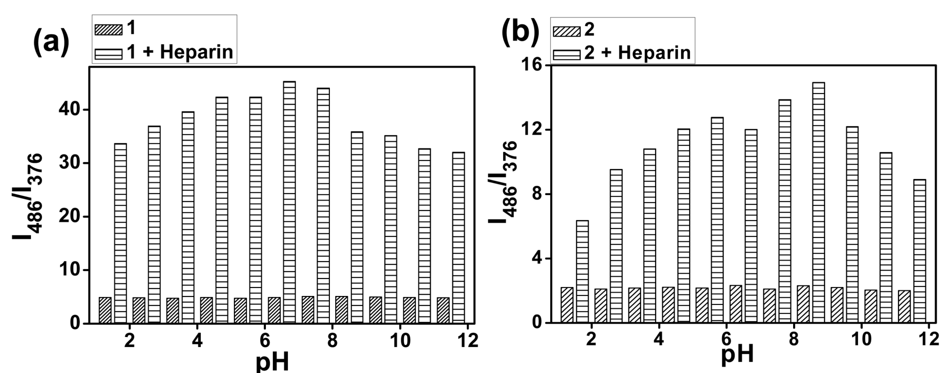

Figure 5. pH effect on the intensity ratio (I_{486}/I_{376}) of (a) **1** (30 μM) and (b) **2** (30 μM) in the absence and presence of heparin (1500 nM), λ_{ex}= 342 nm, slit = 15/2.5 nm.

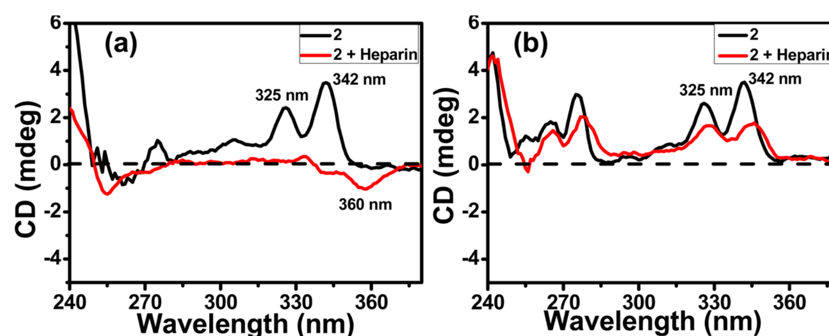

Figure 6. Induced CD spectra of **2** (30 μM) in the presence of heparin (2 μM) (a) in 100% aqueous solutions (10 mM HEPES, pH 7.4) and (b) in a mixed aqueous−organic solution containing 80% CH_3CN (10 mM HEPES, pH 7.4).

heparin were measured under a range of pH conditions (Figure 5). Interestingly, **1** showed a ratiometric response to heparin in a wide range of pH (1.5−11.5; Figure 5a). On the other hand, I_{486}/I_{376} by heparin showed some dependence on the pH. In acidic pH (1.5−4.5), a slight decrease in the ratiometric response was observed. This might be due to the decrease in electrostatic interactions between heparin and **1**, by protonation of the carboxylate groups of heparin under acidic pH. The largest intensity ratio change induced by heparin was observed at neutral pH, whereas in basic pH (>10.5), the change in I_{486}/I_{376} by heparin decreased slightly with increasing pH. Peptide-based sensor **2**, like **1**, showed a similar ratiometric response behavior to heparin depending on pH (Figure 5b). Interestingly, both peptide-based sensors showed ratiometric responses to heparin over the wide range of pH (1.5−11.5), and the change in intensity ratio by heparin was the greatest at a physiological pH.

Induced CD Spectra of the Peptidyl Chemosensors in the Presence of Heparin. Induced circular dichroism (CD) spectroscopy was used to examine the interactions between the pyrene fluorophore of the peptide and heparin. Achiral chromophores, such as pyrene and anthracene, displayed induced CD upon binding to the biomolecules with chiral centers, such as DNA, cyclodextrin, and heparin.[45−47] On the other hand, the induced CD was observed by interactions between the peptides and achiral chromophores. Surprisingly, **2** showed significant induced CD even in the absence of heparin in 100% aqueous solution and mixed aqueous−organic solvent systems (Figure 6). The positive ellipticity observed at 325 and 342 nm, corresponding to the absorbance of pyrene, suggests that the pyrene fluorophores are surrounded by the peptide part in both solvent systems. Upon the addition of heparin to the 100% aqueous solution, the positive ellipticity at 342 and 325 nm was changed to a negative ellipticity at 340 and 360 nm. The inverse, decrease, and red shift of the CD bands in the presence of heparin suggest that, after peptidyl chemosensor **2** was bound to heparin, the surrounding environment for the pyrene fluorophores was changed significantly and the pyrene fluorophores were dimerized. The split CD band corresponding to 240 and 360 nm suggests that the two pyrenes in the peptide bound on heparin interacted mainly via the long and short axis interactions.[48]

Upon the addition of heparin into the mixed aqueous−organic solution (v/v, 20:80) containing **2**, a decrease and red shift of the CD bands (325 and 342 nm) corresponding to the absorbance of pyrene were observed. This suggests that after the peptidyl chemosensor was bound to heparin, the two pyrenes of the peptide may be close to each other and become dimerized. The CD spectrum of **1** was also measured in the presence or absence of heparin (Supporting Information Figure S13). The induced CD spectra of **1** in the presence and absence of heparin were similar to those of **2** in the presence and absence of heparin. This indicates that in the absence of heparin, the peptide moiety of **1** surrounded the pyrene fluorophore, and after **1** bound to heparin, the surrounding environment for the pyrene fluorophores was changed significantly and the pyrene fluorophores were dimerized.

Selectivity Study of the Chemosensors for Heparin. This study next investigated the fluorescence response of **1** and **2** 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

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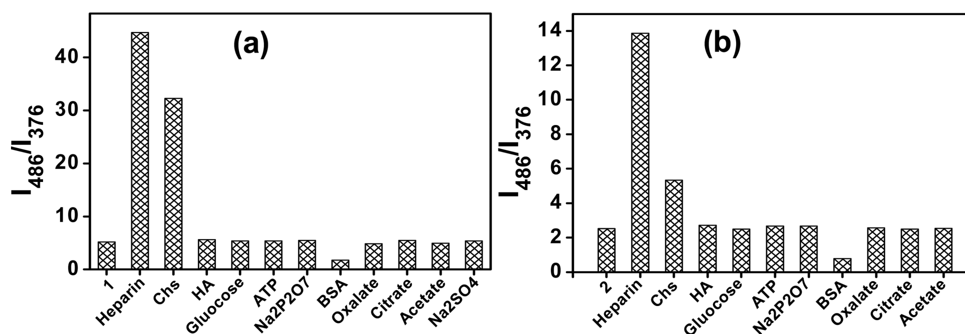

Figure 7. Fluorescence intensity ratio (I_{486}/I_{376}) of (a) **1** (30 μM) and (b) **2** (30 μM) in the presence of heparin or other competitors (1500 nM), such as chondroitin-4-sulfate (ChS), hyaluronic acid (HA), and bovine serum albumin (BSA), in aqueous (10 mM HEPES, pH 7.4) solutions, λ_{ex}= 342 nm, slit = 15/2.5 nm.

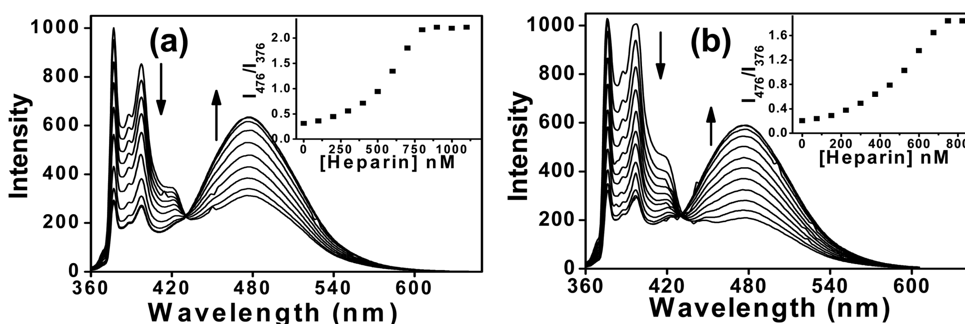

Figure 8. Fluorescence spectra of (a) **1** (30 μM) and (b) **2** (30 μM) upon gradual addition of increasing concentrations of heparin in aqueous solutions (150 mM NaCl, 2 mM KCl, and 10 mM HEPES, pH 7.4) containing 5% human serum, λ_{ex}= 353 nm, slit = 15/2.5 nm.

shown in Figure 7a, I_{486}/I_{376} increased significantly from 5.2 to 44.7 (8.6-fold) in the presence of heparin, whereas the intensity ratio was not changed considerably by other anions, such as ATP, $Na_2P_2O_7$, sodium oxalate, sodium acetate, sodium citrate, $Na_2S_2O_7$, and Na_2SO_4, except for ChS and BSA. I_{486}/I_{376} increased from 5.2 to 32.3 (6.2-fold) in the presence of ChS, which consisted of repeating disaccharide units containing at least one or two sulfate groups. This suggests that electrostatic interactions between the peptidyl chemosensors and heparin might play an important role in the ratiometric response because the major disaccharide repeating unit of heparin included three sulfated groups, whereas the major disaccharide repeating unit of ChS contained at least one sulfated group and one carboxyl group. The most abundant blood protein, bovine serum albumin (BSA), caused a decrease in I_{486}/I_{376} from 5.22 to 1.51. Highly charged chemosensors with an aliphatic chain for heparin were reported to show a considerable response to BSA because highly negatively charged amino acids were located predominantly in the surface of BSA, and BSA has several binding sites for hydrophobic molecules, such as fatty acid and steroids.[25,49−53] As shown in Figure 7b, **2** exhibited high selectivity toward heparin over other biological competitors, such as ChS and HA in an aqueous buffered solution at pH 7.4. I_{486}/I_{376} increased significantly by 5.5-fold in the presence of heparin, whereas I_{486}/I_{376} increased 2.1-fold in the presence of ChS. The selectivity study indicated that **2** showed relatively higher selectivity for heparin than **1**.

Ratiometric Detection for Heparin in Serum Sample. The practical applications of the peptidyl chemosensors for the detection of heparin in the presence of human blood serum were investigated. As shown in Figure 8, the peptidyl chemosensors showed a sensitive ratiometric response to heparin in aqueous solutions containing 5% human serum. Approximately 7.1-fold enhancement of I_{486}/I_{376} of **1** was measured by the addition of 1000 nM heparin. Interestingly, the change in I_{476}/I_{376} by heparin in the human serum sample was much larger than that (ca. 5.7-fold enhancement) measured in a 100% aqueous solution. Upon the addition of an increasing concentration (0−1000 nM) of heparin in the serum sample, I_{476}/I_{376} of **2** increased approximately 9.2-fold (from 0.20 to 1.84; Figure 8b). The saturation concentration (1000 nM) of heparin in the serum sample was similar to that measured in the 100% aqueous solution; however, the intensity ratio change of **2** by heparin in the serum sample was much larger than that measured in the 100% aqueous solution.

In the serum sample, free peptidyl chemosensors **1** and **2** showed strong monomer emission with relatively weak excimer emission, and upon the addition of increasing concentrations of heparin, a significant decrease in monomer emission and a concomitant increase in excimer emission at 486 nm were observed. Surprisingly, the ratiometric responses of the peptidyl chemosensors to heparin were greater than those of the peptidyl chemosensors measured in 100% aqueous solutions. This may be due to the interactions between the peptidyl chemosensors and serum albumin in the serum sample. Serum albumin is the most abundant (∼50%) in human blood plasma protein. Serum albumin plays an important role in the transport of fatty acids, steroid hormones, and hydrophobic drugs through the bloodstream because BSA has hydrophobic binding sites for fatty acids, steroids, and hydrophobic drug.[49−53] Therefore, this study examined whether serum albumin plays an important role in the fluorescence response of the peptide sensors to heparin in the presence of serum. Because BSA was highly homologous (76%) to human serum albumin, the fluorescent spectra of the peptide sensors were measured in the presence of BSA. The fluorescent spectrum of the peptidyl

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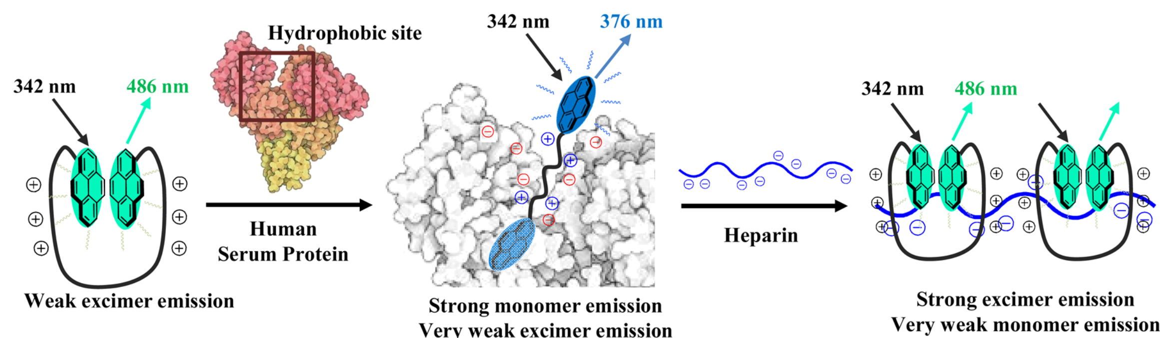
Scheme 3. Proposed Binding Mode of Peptidyl Chemosensors with Heparin in Serum



chemosensors was first measured in the presence of BSA in aqueous solutions (Figure S14, Supporting Information). Both peptides exhibited significant monomer emission and weak excimer emission in the presence of BSA. When the peptide was bound to BSA, the two hydrophobic pyrene fluorophores of the peptide might bind to each hydrophobic binding site and were far away, resulting in significant monomer emission and weak eximer emission. Upon the addition of heparin to the solution containing the peptide sensor and BSA, the increase in excimer emission at 486 nm and the concomitant decrease in monomer emissions at 386 and 397 nm were observed. This suggests that heparin removed the peptide sensor from the complex between the peptide and BSA and formed a new complex with the peptide sensors and heparin, resulting in an increase in excimer emission and a decrease in monomer emission.

As shown in Scheme 2, the binding mode of the peptide sensor with heparin could be proposed based on the results of the fluorescent measurements and CD spectrum. In a 100% aqueous solution, two hydrophobic pyrene fluorophores of the peptide came closer and partially dimerized in a hydrophilic environment, resulting in considerable pyrene excimer emissions and relatively weak monomer emissions. The induced CD showed that the two pyrene fluorophore moieties were surrounded by the positively charged peptide part in aqueous solutions. After the peptide was bound to heparin mainly by electrostatic interactions between the positively charged arginine amino acid moieties of the peptide and the negatively charged group in the disaccharide unit of heparin, the two pyrene fluorophores of the peptide might come closer more than the unbound form, resulting in enhanced pyrene excimer emission with a blue shift in excimer emission. According to the induced CD spectra, the environment of the pyrene fluorophores of the peptide bound to heparin was somewhat different from that of the pyrene fluorophores of the peptide in aqueous solutions.

In the serum sample, the peptidyl chemosensors bound to serum proteins, such as BSA (Scheme 3). Since BSA has several binding sites for hydrophobic molecules, such as fatty acid and steroid, the two hydrophobic pyrene fluorophores of the peptide might bind to different hydrophobic binding sites of BSA, resulting in significant monomer emission and weak excimer emission. The addition of heparin removed the chemosensors from the complex between the chemosensor and BSA mainly via strong charge−charge interactions and formed a new complex with the peptidyl chemosensor, resulting in an increase in excimer emission and a decrease in monomer emission.

Figure 9 represents a visible emission change in **2** in the presence of various anions and heparin under UV light (λ_{em}= 365 nm) with a UV lamp. In the absence of heparin, the solution of **2** showed a blue-green color in aqueous solution and displayed a cyan−blue color in the presence of 5% serum. Compound **2** displayed a cyan-blue color in the absence of heparin or in the presence of other competitors in the serum sample, whereas **2** displayed a cyan color in the presence of heparin in the serum sample.

Figure 9. Visible emission color change in **2** (30 μM) in aqueous solutions containing 5% serum (10 mM HEPES, pH 7.4), in the presence of HA, heparin, glucose, BSA, ATP, ChS, and $Na_2P_2O_7$ under UV light (λ_{em} = 365 nm). The concentration of heparin and other competitors was 1500 nM, respectively.

Detection Limits of the Chemosensors for Heparin in Serum Samples. As the peptidyl chemosensors displayed a sensitive ratiometric response to heparin in a pure aqueous buffered solution and in aqueous buffered solution containing the serum sample, the detection limits of **1** and **2** were measured. The change in the fluorescence intensity at 376 was in proportion to the heparin concentration (Figures 2 and 3). Therefore, the sensitivity was measured based on the linear relationships between the changes in the monomer emission intensity at 376 nm and the concentration of heparin (Supporting Information Figures S15 and S16). The detection limit of **1** was determined to be 1.0 nM (R^2 = 0.998) for heparin in a 100% aqueous solution at pH 7.4 and 1.2 nM (R^2 = 0.998) in aqueous solutions containing 80% CH_3CN at pH 7.4. The detection limit of **2** was determined to be 763 pM (R^2 = 0.996) for heparin in a 100% aqueous solution at pH 7.4 and 963 pM (R^2 = 0.998) for heparin in aqueous solutions containing 80% CH_3CN at pH 7.4, respectively.

Similarly, the very good linearity between the intensity changes as a function of the heparin concentration were obtained in real biological media containing 5% human serum (Supporting Information Figure S17). Compounds **1** and **2** have detection limits of 1.7 nM (R^2 = 0.998) and 903 pM (R^2 = 0.999), respectively, for heparin in the serum sample. The detection limits of the peptidyl chemosensors for heparin in aqueous solutions were slightly lower than those measured in the serum sample. This may be due to the nonspecific interactions between the peptidyl chemosensors and various components in human serum.[18]

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On the other hand, the decrease in the detection limits for heparin in the serum sample was not considerable. Considering the dilution factor for the serum sample (5% serum) and normal therapeutic recommended dosage of heparin (2−8 U/mL; 17−67 μM) during cardiovascular surgery and (0.2−1.2 U/mL; 1.7−10 μM) in postoperative and long-term care,[8] the detection limits of **1** and **2** for heparin in the serum sample were sensitive enough for therapeutic use.

■ CONCLUSIONS

Fluorescent peptidyl chemosensors **1** and **2** were synthesized for the monitoring of heparin in serum via a ratiometric response. Both chemosensors exhibited a sensitive ratiometric response to heparin in aqueous buffered solutions and in a human serum sample by decreasing the monomer emission with a concomitant increase in intramolecular excimer emission. Compounds **1** and **2** were applicable for the monitoring of heparin over a wide range of pH (1.5 to 11.5). The detection limits of **1** and **2** for heparin were determined to be 1.7 nM (R^2 = 0.998) and 903 pM (R^2 = 0.999) in a 5% serum sample solution, respectively. The detection limits and the ratiometric detected concentration of heparin in the serum sample solution were much lower than the clinical demanded concentration of heparin in serum. Peptidyl chemosensors bearing two heparin-binding sites provided a potential and practical tool for the detection of low levels of heparin in the serum samples.

■ EXPERIMENTAL SECTION

Reagents. Rink Amide MBHA, Fmoc−Lys(alloc)−OH, Fmoc−Arg(Pmc)−OH, Fmoc−Gly−OH, *N*,*N*-diisopropylcarbodiimide (DIC), and 1-hydroxybenzotriazole (HOBt) resin were purchased from Advanced ChemTech. The 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.

General Experimental Procedure. *Solid-Phase Synthesis.* Py−ArgArgGlyGlyGlyArgArgLys(Py)−NH_2, **1**, and Py−ArgArgArgGly-GlyGly−ArgArgArgLys(Py)−NH_2, **2**, were synthesized efficiently in solid-phase synthesis with 9-fluorenyl methoxycarbonyl (Fmoc) chemistry (Scheme 1).[44] In situ activation method using DIC and HOBt was used for the coupling reactions. First, Fmoc−Lys(alloc)−OH (0.3 mmol, 0.3 equiv) was loaded to Rink Amide MBHA resin (0.1 mmol, 0.1 equiv). The deprotection of the Fmoc group was achieved by a treatment of 20% piperidine in DMF. The deprotection of the alloc group of the lysine side chain was achieved with phenylsilane (0.3 mmol, 0.3 equiv) and $Pd(PPh_3)_4$ (0.02 mmol, 0.02 equiv) in dichloromethane. The cleavage of the peptide from resin was accomplished with CF_3COOH/1,2-ethanedithiol/thioanisole/water/TIS (TFA/EDT/thioanisole/TIS/H_2O, 86.5:2.5:5:5:1, v/v) at room temperature for 6 h. The crude product was purified further with semipreparative HPLC using a water (0.1% TFA)/acetonitrile (0.1% TFA) gradient. The isolated yields were 86% and 89% for **1** and for **2**, respectively. The high purity (>97%) of the peptides was characterized by HPLC with an analytical column. The peptides were also characterized by UV, IR, and mass spectroscopies.

Characterization Data of 1. Compound 1. Colorless solid, mp 133−134 °C. IR (KBr): 3370 (br s), 2928, 1672, 1318, 1205, 1122 cm^{-1}. MS-FAB (*m*/*z*). $[M]^+$ calculated for $C_{72}H_{93}N_{22}O_{10}$: 1425.7445. Observed: 1425.7445.

Compound 2. Colorless solid, mp 105−106 °C. IR (KBr): 3375 (br s), 2942, 1668, 1314, 1200, 1134 cm^{-1}. ESI-MS (*m*/*z*). $[M + H]^+$ calculated for $C_{84}H_{116}N_{30}O_{12}$: 1737.94. Observed: 1736.87.

General Fluorescence Measurements. Stock solutions of **1** (1.42×10^{-3} M) and **2** (1.20×10^{-3} M), (1.26×10^{-3} M) were prepared in distilled water and stored in a cold and dark place. The concentrations of **1** and **2** were confirmed by UV absorbance at 342 nm for the pyrene group. The stock solution was used for all fluorescent experiments after the appropriate dilution. The fluorescence emission spectrum of a sample in a 10 mm path length quartz cuvette was measured in a 10 mM HEPES buffer solution at pH 7.4 using a PerkinElmer luminescence spectrophotometer (model LS 55). The emission spectra (365−600 nm) of the sample were measured by excitation with 342 or 353 nm. The detection limit was calculated based on a fluorescence titration.[54] The emission intensity at 376 nm of the chemosensor was measured 10 times, and the standard deviation of the blank measurements was determined. Three separate measurements of the emission intensity at 376 nm were performed in the presence of heparin, and the mean intensity was plotted versus the concentration of heparin to determine the sensitivity. The detection limit was calculated using the following equation: detection limit = $3\sigma/m$, where σ is the standard deviation of the intensity of free chemosensor and m is the sensitivity. Aqueous buffered solutions at different pH were prepared in distilled water using the following chemicals. 2-Morpholinoethanesulfonic acid (MES) was used to prepare the buffered solutions with pH ranging from 1.5 to 5.5. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was used for the buffered solutions at pH ranging from 7.5 to 8.5. *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) was used for the buffered solution at pH ranging from 9.5 to 11.5.

Human Serum Preparation Method. After collecting the whole blood from young male adults (age group between 27 and 35 years), the blood was allowed to clot for 1 h at room temperature. After removing the clot by centrifuging at 1500 rpm for 15 min, the obtained serum was incubated at 4 °C. The serum stock solution was used for the experiments after the appropriate dilution. The sample solution containing 5% serum by dilution of the serum stock solution with aqueous (10 mM HEPES, 150 mM NaCl, 2 mM KCl, pH 7.4) solutions was used.

■ ASSOCIATED CONTENT

Supporting Information

Information on the synthesis and corresponding characterization data for compounds **1** and **2**, HPLC, mass spectra, UV visible absorption spectra, induced CD spectra, and fluorescence spectra for the detection limit. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b01932.

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The authors declare no competing financial interest.

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