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A supercharged fluorescent protein based FRET sensing platform for detection of heparin contamination†

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Development of biosensors for the detection of oversulfated chondroitin sulfate (OSCS), the key contaminant in heparin (Hep), is a greatly demanding but challenging task. In this work, a FRET system for the detection of OSCS was successfully constructed by using supercharged green fluorescent protein (ScGFP) as the energy donor and dye labelled Hep (Hep-RF1) as the energy acceptor. With heparinase treatment, Hep-RF1 was hydrolyzed into small fragments, resulting in quenching of the FRET signal. On the other hand, since OSCS is an inhibitor for heparinase, the presence of OSCS would enable the effective FRET from ScGFP to Hep-RF1 even after heparinase treatment. With this ScGFP based FRET sensing platform, as low as 0.001% (w/w) OSCS in Hep has been successfully detected.

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Introduction

Heparin (Hep), a highly sulfated glycosaminoglycan (GAG), has been widely used in clinics, especially as an anticoagulant in surgery.^{1–3} One of the most important issues in using Hep is the drug purity, which is directly associated with the Hep drug safety.³ During 2007–2008, the global Hep supply chain was contaminated and led to severe adverse reactions such as angiodema, hypotension, and even patient death. Later investigations established that the principal contaminant in Hep was oversulfated chondroitin sulfate (OSCS), a non-natural highly sulfated synthetic GAG.^{4,5} The detection of OSCS in Hep, however, was very challenging due to their similar structures (Scheme 1). Therefore, high performance liquid chromatography (HPLC) was coupled with nuclear magnetic resonance (NMR) and mass spectroscopy (MS) to distinguish OSCS from Hep.⁴ However, these methods required highly sophisticated instruments, experienced operators and complicated data analysis skills. Thus the development of low cost, easy to perform and reliable methods for OSCS detection is still greatly needed.

Optical probes are ideal tools for the detection of various target analytes such as ions, bioactive small molecules, and biomacromolecules.^{6–12} For this kind of sensing technology, the detection of an analyte is accomplished by reading a light signal as the output, which is straightforward and easy to operate.^{13–18} The development of optical probes for OSCS detection has attracted particular attentions recently.^{19–23} For example, a gold-heparin-dye based fluorescent nanosensor for OSCS detection was developed, which was able to detect as low as $1 \times 10^{-9}\%$ (w/w) OSCS in Hep.¹⁹ An adenosine-repeated molecular beacon based system was reported for the detection of 0.01% (w/w) OSCS in Hep.²⁰ By using a Hep specific binding peptide AG73 as the recognition unit, our group has reported two enzyme assisted strategies for OSCS detection.^{21,22}

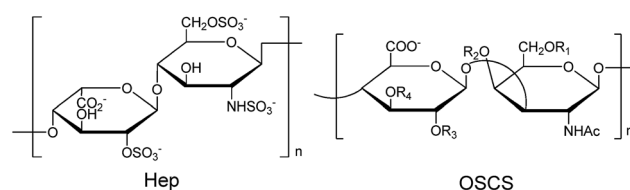
Despite the substantial progress, most of the reported optical probes for OSCS were based on fluorescence “turn on” or “turn off” at a specific wavelength as the reporting signal.²⁴ The ratiometric fluorescent probes for OSCS are still rare.²³ Compared with fluorescence “turn on” or “turn off” probes, ratiometric fluorescent probes can avoid inevitable interferences from fluctuations of probe concentration and detection ambience because they detect analytes by monitoring the changes of fluorescent intensity ratio at two different emission

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Scheme 1 Major disaccharide repeating units of heparin and OSCS.

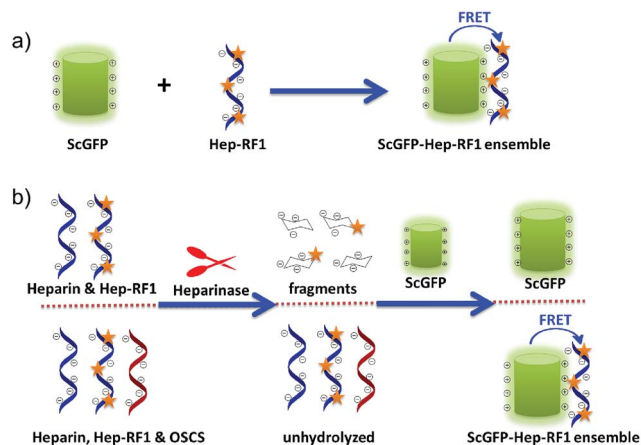


Fig. 1 (a) Construction of ScGFP and Hep-RF1 based FRET system; (b) ScGFP and Hep-RF1 based FRET system for OSCS detection.

wavelengths.²⁵ For the design of ratiometric fluorescent probes, fluorescence resonance energy transfer (FRET) is one of the promising sensing strategies.^{26,27} An effective FRET system requires a reasonable energy donor-acceptor distance and spectral overlap between the emission spectrum of a donor and the absorption spectrum of an acceptor.²⁸ To construct a FRET sensing system, a donor and an acceptor can be linked by either covalent bond or supramolecular interactions, or even both.

In consideration of GAGs' abundant negative charges, positively charged fluorophores are ideal hosts for developing FRET sensors for GAGs.²⁹ In this regard, superpositively charged green fluorescent protein (ScGFP) is of particular interest because of its excellent biocompatibility and high water solubility.²⁹ Recently, ScGFP was used as a powerful and versatile probe for GAGs detection. To the best of our knowledge, no ScGFP based FRET sensing system has been developed for GAGs.³⁰ Here, we developed a FRET system for effective detection of OSCS by using ScGFP as the energy donor and rhodafuor dye labelled heparin (Hep-RF1) as the energy acceptor (Fig. 1a). Upon the electrostatic and hydrogen bonding interactions between positively charged ScGFP and negatively charged Hep-RF1, the process of FRET from ScGFP to Hep-RF1 was observed. The FRET could be diminished by treating the Hep-RF1 with heparinase, which hydrolysed Hep-RF1 into small fragments that can no longer bind effectively with ScGFP. On the other hand, in the presence of OSCS, which is an inhibitor of heparinase enzyme, the FRET from ScGFP to Hep-RF1 could be recovered after heparinase treatment (Fig. 1b). With this ScGFP based FRET sensing platform, as low as 0.001% (w/w) of OSCS in Hep was successfully detected.

Experimental section

Reagents and instruments

Commercially available solvents and reagents were used as received. Heparin sodium salt from hog intestine was purchased from TCI (Shanghai) Development Co., Ltd. Heparinases I, II, and III from *Flavobacterium heparinum* were from Sigma-Aldrich. UV-visible spectra were recorded using a TU-1900 spectrophotometer (Beijing Purkinje General Instrument

Co. Ltd., China). Fluorescence measurements were carried out on a HITACHI F-4600 fluorescence spectrophotometer. The expression and purification of ScGFP were described in the ESI.† The organic dye RF1 for labelling Hep was synthesized according to a reported method.³¹

Synthesis of Hep-RF1

Hep (60 mg) was dissolved in 10 mM pH 7.4 PBS buffer (60 mL), and then treated with 120 mg of EDC and 21 mg of RF1. The reaction mixture was stirred at 25 °C for 48 h. Excess RF1 dye and EDC were removed by dialysis using 3000 MWCO dialysis tubing. As Hep-RF1 was diluted due to dialysis, the final volume of the solution was fixed at 120 mL to obtain $\sim 0.5 \text{ mg mL}^{-1}$ Hep-RF1 stock solution.

Heparinase treatment

Heparinase treatments were performed at 37 °C with an enzyme cocktail solution of heparinase I, II, and III. To obtain the cocktail solution, stock solutions of heparinases I, II and III were first prepared at concentrations of 250 U mL^{-1} , 50 U mL^{-1} and 25 U mL^{-1} , respectively. They were then mixed in a ratio of 1 : 5 : 10 (v : v : v) and diluted for 10 times for use ($\sim 4.7 \text{ U mL}^{-1}$ total heparinase).

Results and discussion

Design and synthesis of Hep-RF1

As discussed above, an effective FRET system requires the spectral overlap between the emission spectrum of an energy donor and the absorption spectrum of an acceptor. Since the maximum fluorescence emission peak of ScGFP was at $\sim 510 \text{ nm}$, we chose a rhodafuor dye (RF1) as the complementary energy acceptor, whose absorption peak was also at $\sim 510 \text{ nm}$ in PBS (Fig. 2). Moreover, the amine of the piperazine moiety in RF1 can form an amide bond with the carboxyl group in Hep, which enabled the labelling of RF1 to Hep.

Hep-RF1 was synthesized by EDC chemistry under mild reaction condition and purified *via* dialysis before use. Upon

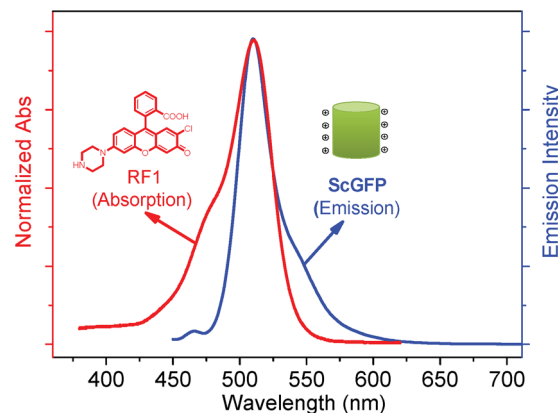


Fig. 2 Spectral overlap between fluorescence emission spectrum of ScGFP (blue curve) and absorption spectrum of RF1 dye (red curve) in PBS (10 mM, pH 7.4). Inset: structure of RF1. $\lambda_{\text{ex}} = 400 \text{ nm}$.

conjugation to Hep, the maximum absorption peak of **RF1** was red shifted from 510 nm to 530 nm, and the maximum emission peak was shifted from 541 nm to 571 nm (Fig. 3). These vivid spectral changes indicated the successful conjugation of **RF1** to the Hep disaccharide chain. Despite of the slight spectral red shift, the absorption spectrum of **Hep-RF1** still overlapped the emission spectrum of **ScGFP** to a large extent. Moreover, red shift of the emission peak of the energy acceptor **Hep-RF1** to 571 nm may provide a FRET signal with a higher resolution. As the emission peak of **ScGFP** is at 510 nm, the gap between the emission peaks of the energy donor and acceptor is 61 nm (as a comparison, the gap between the emission peaks of **ScGFP** and **RF1** is 31 nm).

Construction of the FRET sensing platform

For the construction of **ScGFP** based FRET sensing platform for OSCS, a superpositively charged green fluorescent protein with a net positive charge of 36 was used. We envisioned that the negatively charged **Hep-RF1** chain would bind effectively to positively charged **ScGFP** by both electrostatic attraction interaction and hydrogen bonding. After the binding, the distance between the **ScGFP** and **Hep-RF1** was close enough for FRET. Besides, the efficient spectral overlap between the emission spectrum of **ScGFP** and the absorption spectrum of **RF1** further enabled the effective FRET process.

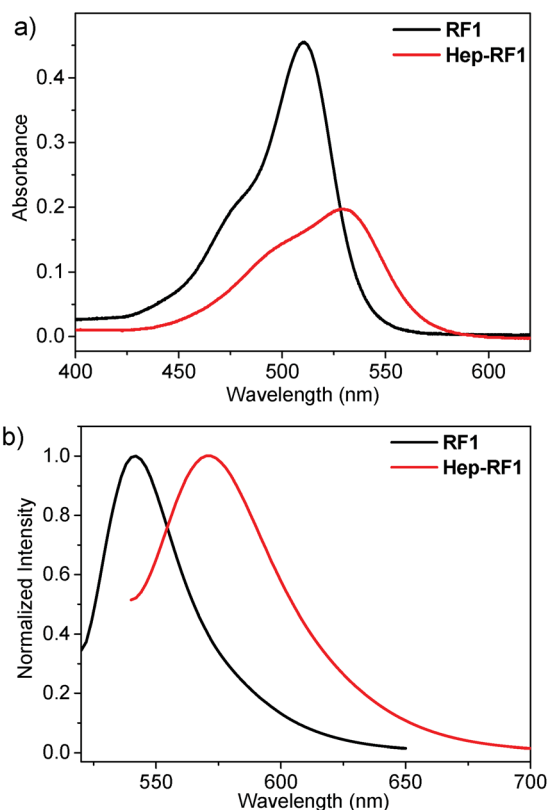


Fig. 3 Characterization of dye labelled Hep. (a) UV-visible absorption spectra of **RF1** ($10 \mu\text{M}$) and **Hep-RF1** ($15 \mu\text{g mL}^{-1}$); (b) normalized emission spectra of **RF1** and **Hep-RF1**, the excitation wavelengths were fixed at corresponding maximum UV-visible absorption peaks.

The interaction between **ScGFP** and **Hep-RF1** was investigated using a fluorescence spectrometer. As shown in Fig. 4, the successive addition of **Hep-RF1** into the PBS solution of **ScGFP** ($10 \mu\text{g mL}^{-1}$) resulted in vivid decrease of the emission peak at ~ 510 nm, accompanied with the development of a new sharp emission peak at ~ 552 nm. We noticed that the original emission peak of **Hep-RF1** at 571 nm was blue shifted to 552 nm in the **ScGFP-Hep-RF1** ensemble. This data indicated that one or more hydrogen bonds may be formed between dye **RF1** in **Hep-RF1** with **ScGFP**, which affected the emission properties of **RF1**. The saturation of the emission spectral changes required $\sim 15 \mu\text{g}$ of **Hep-RF1** (Fig. 4, inset). The reaction speed between **ScGFP** and **Hep-RF1** was monitored at the emission intensity at 552 nm, which indicated that ~ 20 min were required to form the **ScGFP-Hep-RF1** supramolecular ensemble (Fig. S1†). This ratiometric fluorescent spectral change indicated the successful FRET from **ScGFP** to **Hep-RF1**.

Enzyme assisted OSCS detection

Above results indicated the successful construction of a FRET platform using **ScGFP** as the energy donor and **Hep-RF1** as the energy acceptor. Interestingly, if **Hep-RF1** was treated with heparinase before interaction with **ScGFP**, the FRET process would be greatly inhibited. As shown in Fig. 5, the addition of heparinase pre-treated **Hep-RF1** to **ScGFP** resulted in only 17.6% FRET efficiency compared to that of **Hep-RF1** without heparinase treatment. This is probably because **Hep-RF1** was hydrolysed into small fragments under heparinase treatment, and the resulted small fragments of **Hep-RF1** were not able to bind to **ScGFP** effectively. Note, the hydrolysis of **Hep-RF1** into small fragments in the presence of heparinase has been previously confirmed by us using a gel electrophoresis experiment.²²

With heparinase treatment, the **ScGFP-Hep-RF1** ensemble can be used as a sensing platform for OSCS detection in Hep. It has been proved that OSCS is an inhibitor of heparinase activity.^{32,33} Thus the presence of OSCS in Hep sample would prevent hydrolysis of **Hep-RF1** under heparinase treatment, and the FRET from **ScGFP** to **Hep-RF1** should be maintained at

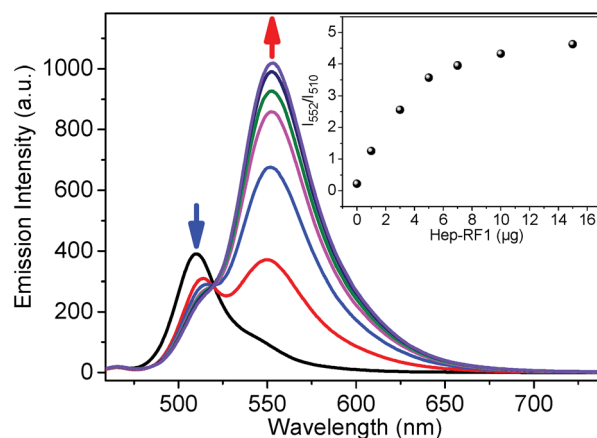


Fig. 4 Fluorescence titration profile of **ScGFP** ($10 \mu\text{g mL}^{-1}$) upon successive addition of **Hep-RF1** (0 – $15 \mu\text{g}$) in 10 mM, $\text{pH } 7.4$ PBS buffer. $\lambda_{\text{ex}} = 400$ nm.

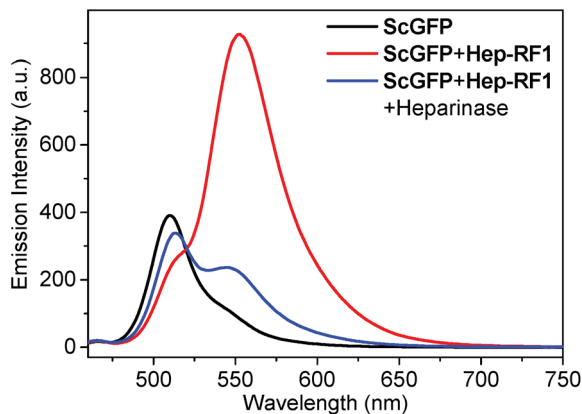


Fig. 5 Heparinase induced FRET quenching. Hep-RF1 (7 μg) was treated with (blue line) or without (red line) heparinase before addition to ScGFP ($10 \mu\text{g mL}^{-1}$) solution in PBS. $\lambda_{\text{ex}} = 400 \text{ nm}$.

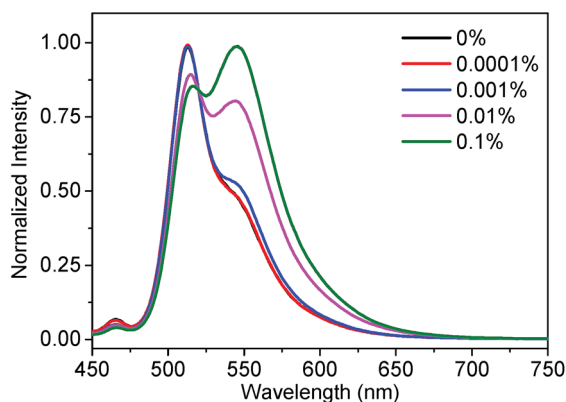


Fig. 6 Heparinase assisted sensing of OSCS with ScGFP based FRET sensing platform. Note, the 0% line overlaps with the 0.0001% line.

a relative high level. To verify this idea, different percentages (wt%: 0.0001, 0.001, 0.01, 0.1, and 0) of OSCS were spiked into pure Hep to obtain the contaminated Hep samples. Then, 7 μg of each contaminated samples was added into the solutions of Hep-RF1 (7 μg), respectively. The mixed solutions were then treated with the heparinase cocktail for 4 h and dialyzed to remove hydrolysed small fragments. The resulting solutions were added into ScGFP ($10 \mu\text{g mL}^{-1}$) for fluorescence measurements, respectively. We discovered that the presence OSCS in Hep indeed inhibited the enzyme activity, as the FRET ratiometric signal became stronger with the increase of OSCS in Hep (Fig. 6). Even the presence of 0.001% of OSCS in Hep induced vivid FRET signal, suggesting that the ScGFP-Hep-RF1 based sensing system was able to detect as low as 0.001% of OSCS in Hep.³⁴

Conclusions

In summary, we designed an ensemble of ScGFP and Hep-RF1 as a FRET sensing platform, in which ScGFP was used as the energy donor and Hep-RF1 as the energy acceptor. The

formation of the ScGFP-Hep-RF1 ensemble was resulted from both hydrogen bonding and electrostatic interactions between positively charged ScGFP and negatively charged Hep-RF1. The FRET from ScGFP to Hep-RF1 could be inhibited *via* treatment of Hep-RF1 with heparinase, with which Hep-RF1 was hydrolyzed into small fragments. Taking the advantage that OSCS is an inhibitor of heparinase, the presence of OSCS in Hep was successfully detected by the ScGFP based FRET sensing platform. Considering these, we believe that this work provided a supramolecular approach to utilize biomacromolecules such as fluorescent proteins to construct an efficient FRET system for analytical application.

Conflicts of interest

There are no conflicts of interest to declare.

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