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Modulating luminescence of Tb^{3+} with biomolecules for sensing heparin and its contaminant OSCS

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ABSTRACT

The detection of heparin (Hep) and its contaminant oversulfated chondroitin sulfate (OSCS) is of great importance in clinics but remains challenging. Here, we report a sensitive and selective time-resolved luminescence (TRL) biosensing system for Hep by modulating the photoluminescence of Tb^{3+} with guanine-rich ssDNA and Hep-specific AG73 peptide (RKRLQVQLSIRT). With the developed system, Hep including both unfractionated Hep (UFH) and the low molecular weight Hep (LMWH) has been successfully detected with a satisfactory detection limit. Owing to the highly specific interaction between Hep and AG73 peptide, major interfering substances in Hep detection, such as Hep analogs of chondrotin sulfate (Chs) and hyaluronic acid (HA), did not interfere with Hep detection. The established TRL sensing system was then successfully used for monitoring Hep metabolism in living rats by microdialysis. Moreover, the proposed TRL sensing system was further applied to analyze OSCS contaminant in Hep with heparinases treatment by exploring the inhibition effects of OSCS on the activity of heparinases. As low as 0.002% of OSCS in Hep was identified.

1. Introduction

Luminescent materials have attracted enormous attention due to their wide applications in various fields (Guo and Irudayaraj, 2011; Hu et al., 2015; Lei et al., 2014; Wei et al., 2010). Among them, rare earth element-based luminescent materials, especially trivalent lanthanide ions (Ln^{3+})-based ones, have been increasingly explored for diverse bioanalytical and biomedical applications due to their remarkable optical characteristics, such as long photoluminescent lifetime and tunable emission (Bünzli and Piguet, 2005; Bünzli, 2010; Chen et al., 2008; Huang et al., 2014a, 2014b; Li and Lu, 2015; Lin et al., 2014; Zhang et al., 2015a). The long lifetime endows the Ln^{3+}-based probes with time-resolved luminescent (TRL) property, which can effectively eliminate the background luminescence of biological samples (Bünzli, 2010). On the other hand, the intrinsically low photoluminescence of Ln^{3+} can be significantly enhanced by modulating the interaction between the f orbitals of Ln^{3+} and the surrounding antenna ligands (Bünzli and Piguet, 2005).

Interestingly, it has been demonstrated that certain biomolecules (e.g., peptides with Tb^{3+}-chelators and single-stranded DNA (ssDNA) with guanine-rich sequence) are effective antenna ligands to sensitize the luminescence of Tb^{3+} (Clark et al., 1993; Ringer et al., 1978). The enhanced photoluminescence of Tb^{3+} by guanine-rich ssDNA is attributed to the energy transfer from the ssDNA to Tb^{3+}, which is promoted by the coordination interaction between ssDNA's guanine bases and Tb^{3+} as well as the electrostatic binding of phosphate backbone onto Tb^{3+} (Fu and Turro, 1999). Based on this phenomenon, numerous TRL biosensing systems have been developed for metal ions, small molecules and enzymes activity (Han et al., 2015; Zhang et al., 2013, 2014). Despite of the substantial progress, to the best of our knowledge, no studies have been devoted to saccharides detection with ssDNA sensitized Tb^{3+} TRL probes (Koshi et al., 2005; Ouchi et al., 2013; Regueiro-Figueroa et al., 2010). Here, we report a sensitive and selective TRL sensing system for heparin (Hep) and its main contaminant oversulfated chondroitin sulfate (OSCS) by modulating the photoluminescence of Tb^{3+} with guanine-rich ssDNA and Hep-specific peptide (Scheme 1), demonstrating the promise of Tb^{3+}-based TRL sensing system for saccharides.

Hep, a linear glycosaminoglycan (GAG) with the highest negative charge density among biomacromolecules, plays an anticoagulant role by enhancing the inhibitory activity of antithrombin against thrombin and other coagulation factors (Fig. S1) (Esko and Selleck, 2002; Guo et al., 2015, 2012). In clinics, Hep has been extensively used as major injectable anticoagulants during surgeries to effectively prevent thrombosis (Warkentin et al., 1995).

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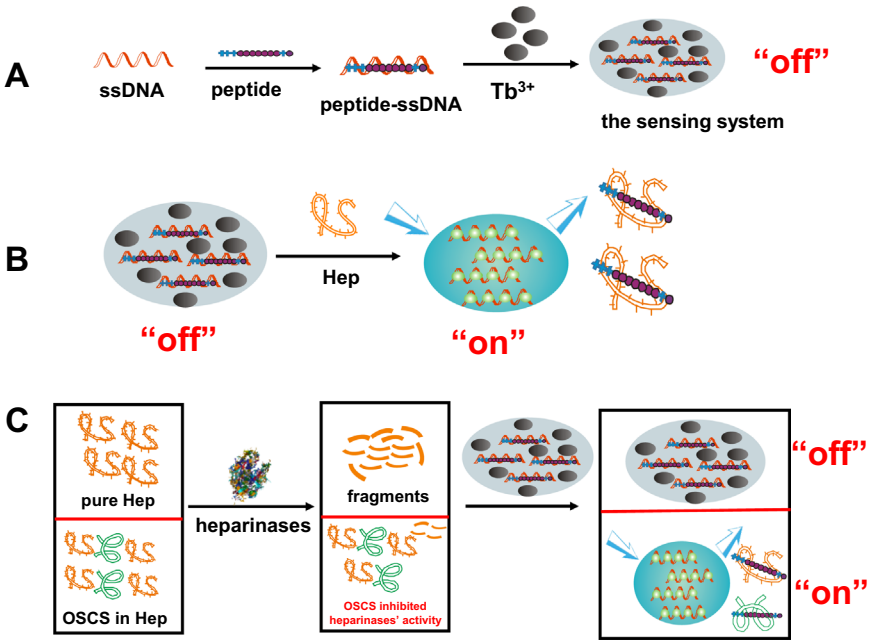

Scheme 1. Modulating the luminescence of Tb^{3+} with biomolecules for sensing Hep and OSCS. (A) Construction of the sensing system by modulating the luminescence of Tb^{3+} with ssDNA and positively charged peptide. (B) "Turn on" TRL sensing system for Hep. (C) "Turn on" TRL sensing system for OSCS contaminant in Hep, assisted with heparinases treatment. (For interpretation of the references to color in this scheme, the reader is referredto the web version of this article.)

However, the overdose of Hep could cause catastrophic complications such as hemorrhage (Warkentin et al., 1995). Therefore, close monitoring the levels of Hep is of great significance. In clinical laboratories, the most common methods for Hep detection include activated partial thromboplastin time assay, activated clotting time assay, and anti-factor Xa activity assay (Bowers and Ferguson, 1994; Cheng et al., 2001). However, these assays are indirect, time-consuming and not sufficiently reliable. Therefore, the development of selective, sensitive and reliable approaches for Hep detection has attracted considerable interest. Recently, a number of methods for Hep assay based on various strategies have been developed (Bromfield et al., 2013; Cai et al., 2011; Chen et al., 2015; Crespo et al., 2012; Ding et al., 2015a; Francoia et al., 2015; Fu et al., 2012; Lühn et al., 2011; Li et al., 2015a, 2015b; Qi et al., 2013; Thirupathi et al., 2015; Wang et al., 2015; Yang et al., 2016; Zhang et al., 2015b; Zhong and Anslyn, 2002). For instance, by labeling a fluorescent dye onto a heparin specific peptide, we recently designed a "turn-on" fluorescent probe for heparin detection (Ding et al., 2015b). Many of the developed fluorescent strategies, however, produced "turn-off" signals (Wright et al., 2005). Thus, the "turn on" luminescent probes for Hep are still highly desired.

On the other hand, OSCS, a non-natural GAG with Hep-like structure, has been identified as the main contaminant in Hep (Fig. S1) (Guerrini et al., 2008; Kishimoto et al., 2008). Due to its severe adverse effects, considerable efforts have been devoted to the identification of OSCS with multiple analytical techniques, such as high performance liquid chromatography, nuclear magnetic resonance spectroscopy, mass spectrometry, colorimetry, electrochemistry, fluorescence, Raman spectroscopy, etc. (Beni et al., 2011; Guerrini et al., 2008; Jagt et al., 2009; Kalita et al., 2014; Kishimoto et al., 2008; Lester et al., 2015; Sommers et al., 2011; Wang et al., 2008). Most of the developed methods are either complicated or high-cost. Hence, it is urgent to develop effective and reliable methods for identifying and quantifying OSCS contaminant in Hep. As shown in Scheme 1, by further combining with heparinases-treatment, our TRL sensing system was also effective for "turn-on" detection of OSCS in Hep, which may provide an alternative approach for Hep quality control.

2. Experimental

2.1. Materials and apparatus

Commercially available buffers and reagents were used as received. The purified guanine-rich oligonucleotides 5′-$(GGGGA)_4$-3′ (**G16**), 5′-$(GGGGA)_7$-3′ (**G28**), 5′-$(GGGGA)_{10}$-3′ (**G40**) and 5′-$(GGGGA)_{15}$-3′ (**G60**) were obtained from Sangon Biotech. (Shanghai, China) (Han et al., 2015). The purified peptide N′-Arg-Lys-Arg-Leu-Gln-Val-Gln-Leu-Ser-Ile-Arg-Thr-C′ (AG73 peptide) was obtained from GenScript Biotechnology Co. Ltd. (Nanjing, China) (Ding et al., 2015b; Hoffman et al., 2001). Heparin (Hep) sodium salt from hog intestine was from TCI (Shanghai) Development Co., Ltd. Chondroitin sulfate sodium salt from bovine trachea, lysozyme from chicken egg, hyaluronic acid sodium salt from *Streptococcus equi*, lipopolysaccharides from *Escherichia coli* 0111:B4, and heparinases I and II from *Flavobacterium heparinum* were purchased from Sigma-Aldrich. Oversulfated chondroitin sulfate (OSCS) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). 5′-ATP-Na_2, 5′-ADP-Na_2, 5′-AMP-Na_2, Tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) and Terbium (III) chloride hexahydrate ($TbCl_3 \cdot 6H_2O$) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Glucose anhydrous, magnesium sulfate ($MgSO_4$), calcium chloride ($CaCl_2$), sodium chloride (NaCl) and sodium sulfate anhydrous (Na_2SO_4) were purchased from Nanjing Chemical Reagent Co., Ltd. Bovine serum albumin (BSA) was from Biosharp and potassium nitrate (KNO_3) was from Sinopharm Chemical Reagent Co. Ltd. All aqueous were prepared with deionized water (18.2 $M\Omega \cdot cm$, Millipore).

The luminescence emission spectra were obtained on a Hitachi F-4600 fluorescent spectrometer (Japan) with phosphorescent mode. The delay time is 1 ms. All of samples were measured in 20 mM Tris-HCl buffer (pH=7.4), with excitation wavelength at 290 nm. The slit width was 5 nm, the PMT voltage was 950 V, and

the scanning range from 450 nm to 650 nm.

2.2. Detection of Hep with the Tb^{3+}-based TRL sensing system

AG73 peptide (6 μM) and G40 (1 μM) were pre-incubated for 10 min, then 6 μM of Tb^{3+} was added and incubated for another 40 min, which resulted in the Tb^{3+}-based TRL sensing solution. Hep (including UFH from hog intestine and LMWH) was then introduced to the as-prepared Tb^{3+}-based TRL sensing solution with 30 min reaction for luminescence measurements.

2.3. Detection of OSCS contaminants in Hep assisted with heparinases treatment

For the heparinases treatments, cocktail solution of heparinases I and II was prepared before use. Stock solutions of heparinases I and II were prepared at concentrations of 250 U/mL and 50 U/mL. Enzyme cocktail solution was obtained by mixing the stock solutions of heparinases I and II at a ratio of 1:10 (v:v), and then diluted for 10 times (~6.8 U/mL total heparinase). Then, different percentages of spiked OSCS contaminant in Hep (2 μg/mL OSCS and Hep in total) were treated with heparinases for 45 min.

The enzyme-treated samples were added into the above-mentioned TRL sensing system and further incubated for 30 min at room temperature. Then the mixture solution was used for luminescence measurement.

3. Results and discussion

3.1. Principle of Tb^{3+}-based TRL sensing system for Hep

The TRL sensing system for Hep was constructed by modulating the luminescence of Tb^{3+} with guanine-rich ssDNA and a positively charged peptide AG73 (Scheme 1A). Tb^{3+} alone in aqueous solution exhibited extremely low phosphorescence due to the non-radiative deactivation by O-H from metal-bound water molecules (Figs. 1 and S2) (Armelao et al., 2010). The interaction with a guanine-rich ssDNA (i.e., **G40**), however, enhanced the emission of Tb^{3+} by more than 300 folds (Fig. 1). The enhancement was due to the strong binding of Tb^{3+} onto guanine-rich ssDNA and the resulted efficient energy transfer from the triplet energy state of guanine bases to $^5D_4$ emissive state of Tb^{3+} (Armelao et al., 2010). The presence of a positively charged peptide would compete for binding onto ssDNA against Tb^{3+}, which in turn would liberate Tb^{3+} and quench its luminescence. Here, the AG73 peptide (sequence: RKRLQVQLSIRT), a Hep-specific binding peptide with a pI of 12.4 and a net charge of +4 at pH 7.4, was employed to modulate the ssDNA-sensitized Tb^{3+} luminescence (Hoffman et al., 2001). Because of the electrostatic interaction, the AG73 peptide formed a stable complex with the ssDNA, resulting in a remarkable luminescence quenching (more than 90%) (green curve in Fig. 1). As shown in Scheme 1B, the presence of Hep in detection samples would specifically recognize AG73 peptide and trigger the ssDNA release from the ssDNA/peptide complex. The released guanine-rich ssDNA would rebind to Tb^{3+} and restore its phosphorescence. Based on this mechanism, a "turn on" TRL sensing system for Hep was proposed. As shown in Fig. 1, the addition of 5 μg/mL Hep into the detection solution containing guanine-rich ssDNA/AG73 peptide/Tb^{3+} resulted in more than 10-fold emission enhancement (green and blue curves), demonstrating the feasibility of the proposed sensing strategy for Hep detection.

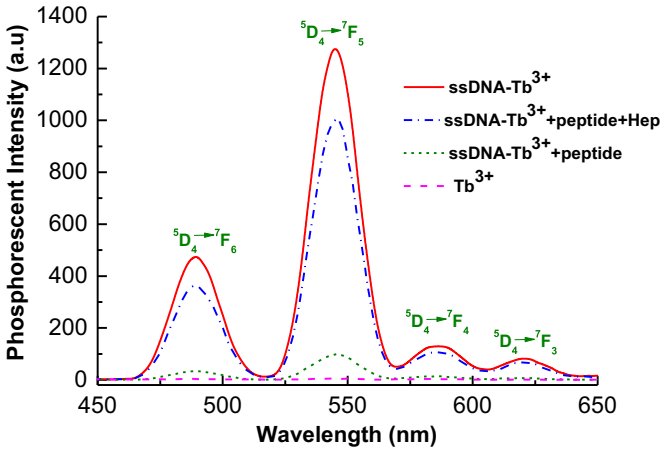

Fig. 1. Luminescence spectra of Tb^{3+}, guanine-rich ssDNA/Tb^{3+} complex, guanine-rich ssDNA/Tb^{3+} complex with AG73 peptide, and guanine-rich ssDNA/AG73 peptide/Tb^{3+} with Hep in 20 mM, pH=7.4 Tris-HCl buffer. Guanine-rich ssDNA used was **G40**. λ_{ex}=290 nm. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

3.2. Sensitive and selective detection of Hep with the TRL sensing system

After establishing the feasibility of the Tb^{3+}-based TRL sensing system for Hep detection, several parameters (such as the ratio of guanine-rich ssDNA to Tb^{3+}, the ratio of AG73 peptide to ssDNA/Tb^{3+} complex, the length of guanine-rich ssDNA, the reaction time, etc.) were then optimized to obtain the best sensing performance. As shown in Fig. S3, the strongest emission was observed when the ratio of ssDNA to Tb^{3+} was 1:6. To minimize the background signal of the sensing system, the concentration of AG73 peptide was also optimized. As shown in Fig. S4, 6 μM AG73 peptide could efficiently quench the luminescence emission of 1 μM ssDNA and 6 μM Tb^{3+}. Therefore, the sensing system consisting of 1 μM ssDNA, 6 μM Tb^{3+} and 6 μM AG73 peptide with weak background luminescence was chosen for the subsequent measurements. The effects of the length of ssDNA, the solution pH, the solution ionic strength and the reaction time were also studied and discussed in Figs. S5–S7.

Under the optimized conditions, the titration of Hep into the proposed TRL sensing solution gradually turned on the phosphorescence of Tb^{3+} (Fig. 2A). For the four dominant emission peaks at around 490 nm, 546 nm, 586 nm and 620 nm, the peak of 546 nm exhibited the strongest emission and was therefore employed for further quantitation analysis. A wide dynamic range for Hep from 0 to 10 μg/mL was shown in Fig. 2B. More, a linear response range from 0.01 μg/mL to 1 μg/mL was obtained (Fig. 2B, Inset). The calculated limit of detection (LOD) based on 3σ/s for Hep was 4.6 ng/mL, which was much lower than the clinically demanded concentration of Hep. A slightly higher LOD for Hep was obtained (i.e., 14.8 ng/mL) in the presence of 10 μg/mL chondrotin sulfate (Chs), showing the robustness of the current method (Fig. S8).

There are mainly two forms of Hep available for clinical use (i.e., UFH and LMWH), and the one tested above was UFH from hog intestine. To demonstrate the versatility of the proposed sensing strategy, it was also used for detecting the other form of Hep (i.e., LMWH). As shown in Figs. S9 and S10, the Tb^{3+}-based TRL sensing system exhibited excellent responses to other two LMWH (Enoxaparin and Dalteparin) with wide dynamic range and high sensitivity, indicating that the proposed TRL sensing system has the general capability for Hep sensing.

The selectivity of the Tb^{3+}-based TRL sensing system was then evaluated. It is well-known that GAG analogs are the major interfering substances in Hep detection, especially chondrotin sulfate (Chs) and hyaluronic acid (HA). Fortunately, due to the highly specific interaction between Hep and the AG73 peptide, even as

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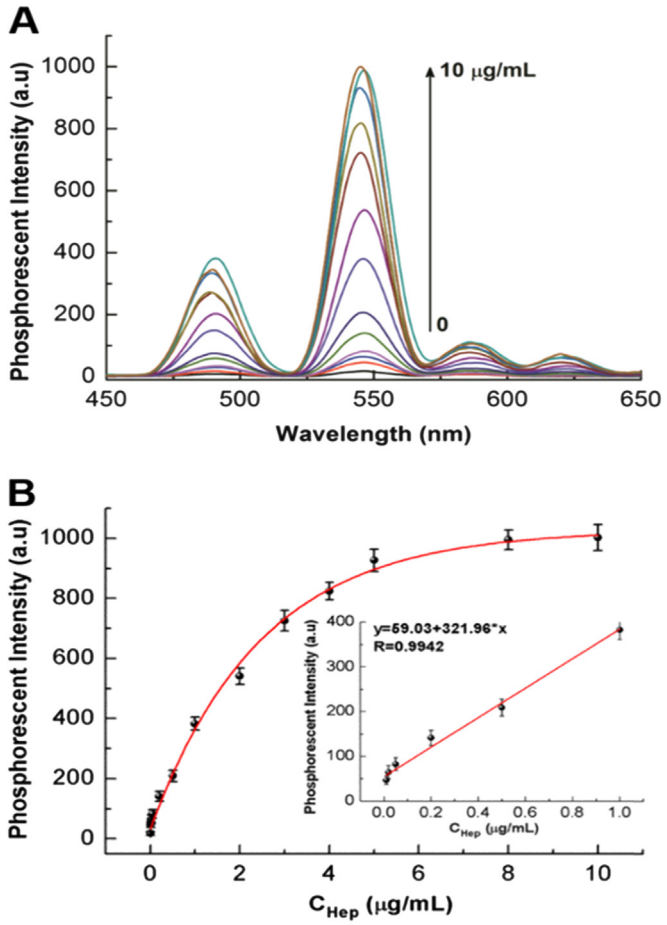

Fig. 2. Hep detection with the Tb^{3+}-based TRL sensing system. (A) Luminescence titration curves with increasing amount of Hep in 20 mM, pH=7.4 Tris-HCl; (B) Luminescence intensity at 546 nm versus Hep concentration. Inset: linear response curve of Hep. λ_{ex}=290 nm. (For interpretation of the references to color in this figure, the reader is referredto the web version of this article.)

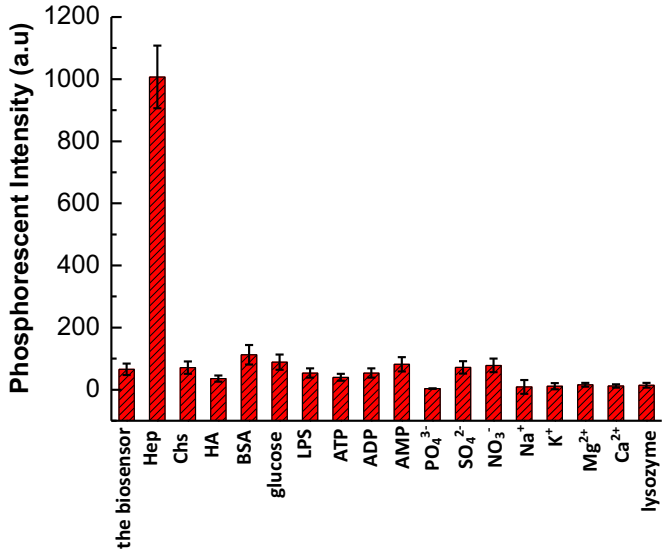

Fig. 3. Selective detection of Hep with the Tb^{3+}-based TRL sensing system. The concentrations of Hep, Chs, HA, LPS and lysozyme were 10 μg/mL; the concentrations of ATP, ADP and AMP were 10 μM; the concentration of BSA was 500 μg/mL; the concentration of PO_4^{3-} was 1 mM; and the concentrations of all other ions were 100 μM.

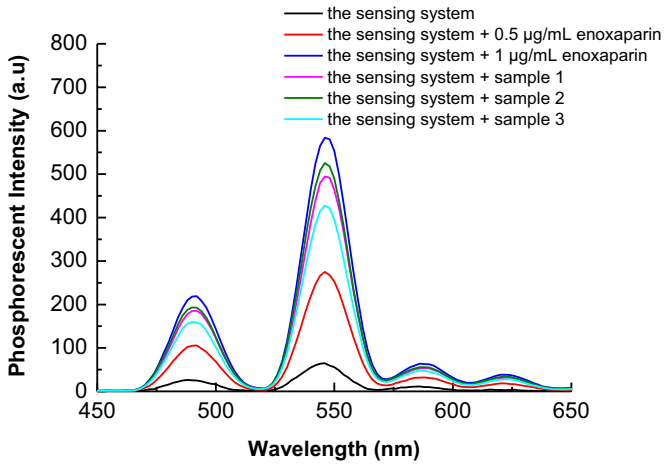

Fig. 4. Luminescence spectra of the Tb^{3+}-based TRL sensing system for detection of enoxaparin in living rats. Black curve, the sensing system alone; red curve, the sensing system with 0.5μg/mL enoxaparin standard sample; blue curve, the sensing system with 1μg/mL enoxaparin standard sample; magenta, olive and cyan curve, the sensing system with the samples from different rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

high as 10 μg/mL Chs and HA did not produce any significant interference towards Hep detection (Fig. 3) (Hoffman et al., 2001). As illustrated in Fig. 3, other interferents (such as LPS (lipopolysaccharide), BSA (bovine serum albumin), glucose, ATP, ADP, AMP, PO_4^{3-}, SO_4^{2-}, NO_3^-, Na^+, K^+, Mg^{2+}, Ca^{2+} and lysozyme) did not affect Hep detection either, indicating the high selectivity of the proposed TRL sensing strategy for Hep detection (Fig. S11).

3.3. Monitoring the metabolism of enoxaparin in living rats with the TRL sensing system

To show the promising application of the TRL sensing system, the heparin metabolism in living rats was monitored. After intraperitoneal administration of enoxaparin (2mL 100μg/mL) for 20min, the serum samples were collected and further dialyzed. Then the 10-fold diluted samples were used for measurements. As shown in Fig. 4 and Table S1, 8.27 ± 0.95μg/mL of enoxaparin was detected from the dialyzed serum samples, demonstrating the feasibility of the current sensing system for living organisms.

3.4. OSCS detection assisted with heparinases treatment

We further proposed a strategy for identifying and quantifying OSCS contaminant in Hep using the above demonstrated TRL sensing system, which was assisted with heparinases treatment (Scheme 1C). In the absence of OSCS, a highly potent inhibitor of heparinases, the heparinases treatment would digest Hep into small fragments and thus liberate the AG73 peptide (Fig. S12). The liberated peptide would in turn rebind onto the ssDNA and thus lead to the luminescence quenching (see black and green curves in Fig. 5A). The presence of OSCS in Hep, however, would effectively inhibit heparinases' activity. The undigested Hep would capture AG73 peptide and thus regenerate the luminescent ssDNA-Tb^{3+} complex (see green and blue curves in Fig. 5A). (Note, the effect of OSCS itself on the TRL sensing system is discussed in SI, see Fig. S13 and associated discussion.) Therefore, with the help of heparinases treatment, OSCS in Hep could be identified using the TRL biosensing system (Scheme 1C).

The ratio of $(P-P_0)/P$ was employed as the output signal for OSCS contaminant detection, where P is the luminescence intensity of different amounts of OSCS in Hep after heparinases cocktail treatment while P_0 is the luminescence intensity of pure

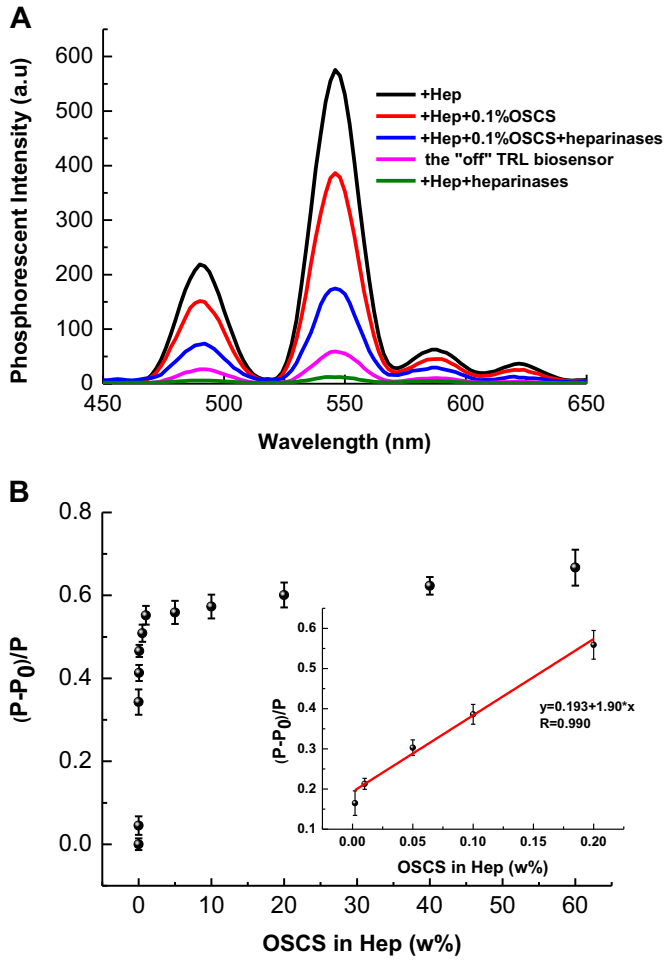

Fig. 5. Detection of OSCS contaminant in Hep using the TRL sensing system assisted with heparinases treatment. (A) Luminescence spectra of the Tb^{3+}-based TRL sensing system in the absence and presence of different analytes. λex =290 nm. (B) The output signal $(P-P_0)/P$ versus the amount (w%) of OSCS in Hep. Inset: linear response curve of the amount (w%) of OSCS in Hep. P, the phosphorescent intensity of the sample treated with heparinases; P_0, the phosphorescent intensity of pure Hep after heparinases treatment. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

Hep after enzyme treatment. It showed that the output signal $(P-P_0)/P$ increased with the increasing amount of OSCS in Hep after heparinases treatment for 45 min (Fig. 5B). More, the amount (w%) of OSCS in Hep showed a good linear relationship with luminescence intensity from 0.002% to 0.2% (Fig. 5B, Inset). As low as 0.002% of OSCS in Hep has been successfully detected.

4. Conclusions

In summary, by modulating the luminescence of Tb^{3+} with guanine-rich ssDNA and Hep-specific peptide, we developed a facile "turn on" TRL sensing strategy for detection of Hep and its contaminant OSCS. The proposed TRL sensing system was successfully used for Hep detection with excellent sensitivity and high selectivity. Both of the two clinically used Hep (i.e., UFH and LMWH) were successfully detected. More, by combining with heparinases treatment, OSCS contaminant in Hep was monitored with the TRL sensing system, which was based on the inhibitory effects of OSCS on heparinases' activity. With the proposed sensing strategy, as low as 0.002% of OSCS in Hep has been identified. The current strategy is advantageous in several aspects: (1) it is a simple and direct assay for Hep detection; (2) the long luminescence lifetime could minimize the background interference; and (3) the "turn on" signaling could avoid false responses. Overall, this work demonstrated the promising use of lanthanide-based TRL sensing system for saccharides. The designed strategy could be applicable to other biologically important targets such as glycans on tumor cells.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.07.085.

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