JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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A Nanosensor for Ultrasensitive Detection of Oversulfated Chondroitin Sulfate Contaminant in Heparin

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Supporting Information

ABSTRACT: Heparin has been extensively used as an anticoagulant for the last eight decades. Recently, the administration of a contaminated batch of heparin caused 149 deaths in several countries including USA, Germany, and Japan. The contaminant responsible for the adverse effects was identified as oversulfated chondroitin sulfate (OSCS). Here, we report a rapid, ultrasensitive method of detecting OSCS in heparin using a nanometal surface energy transfer (NSET) based gold-heparin-dye nanosensor. The sensor is an excellent substrate for heparitinase enzyme, as evidenced by ~70% recovery of fluorescence from the dye upon heparitinase treatment. However, the presence of OSCS results in diminished fluorescence recovery from the nanosensor upon heparitinase treatment, as the enzyme is inhibited by the contaminant. The newly designed nanosensor can detect as low as 1×10^{-9} % (w/w) OSCS making it the most sensitive tool to date for the detection of trace amounts of OSCS in pharmaceutical heparins.

Heparin has been used as a clinical anticoagulant for several decades in nearly every major surgery and in the treatment of several clotting disorders such as deep vein thrombosis and acute coronary syndrome.[1−3] Structurally, heparin is a highly sulfated glycosaminoglycan (GAG) consisting of sulfated disaccharide repeating units of predominantly iduronic acid/minor glucoronic acid and glucosamine. However, due to its animal origin, heparin lots have been found to contain other naturally occurring sulfated GAGs too. Over 600 million pigs are used in producing more than 100 t of heparin every year. In 2007−2008, adulteration of hog mucosal heparin led to the contamination of the global heparin supply chain and resulted in severe adverse reactions such as angiodema, hypotension, swelling of the larynx, and anaphylactic reactions.[4−6] Worldwide there were 574 reports of adverse effects and at least 149 deaths in the U.S. and Europe alone due to oversulfated chondroitin sulfate (OSCS), a non-natural highly sulfated GAG identified as the principal contaminant in heparins.[4,5,7,8]

To detect OSCS in heparin, several analytical methods have been developed. These analytical techniques include ^{1}HNMR,[9] strong anion exchange (SAX)-HPLC,[10] capillary electrophoresis (CE),[11] polyacrylamide gel electrophoresis (PAGE),[12] near-infrared (NIR)[13] and electrochemical method.[14] The limit of detection (LOD) of these techniques varies from 1% (NIR) to 0.03% (SAX-HPLC) (w/w) OSCS in heparin.[9−14] However, these analytical methods have other drawbacks along with moderate detection limits. To overcome these challenges, several research groups have also developed 96-well microplate assays to detect OSCS contamination. Colorimetric method is routinely used to determine the concentration of heparin.[15,16] Likewise, colorimetric methods are also developed to detect OSCS in heparin; the most sensitive assay to date has an LOD of 0.003% (w/w) OSCS.[17] This method employs water-soluble cationic Leclerc Poly Thiophene Polymer (LPTP), a yellow colored probe that interacts with polyanionic heparin and results in a color change from yellow to red.[18] However, this color change is nonlinear with respect to % (w/w) of OSCS in heparin, and thus, this is less reliable.[17] Therefore, there is an urgent need for the development of a novel sensor that is ultrasensitive, linear and highly reliable in detecting OSCS in heparins.

Here, we report the development of the most sensitive nanosensor to rapidly determine the OSCS contaminant in heparin lots. Compared to the present LOD of 0.003% (w/w) OSCS in heparin reported in the literature, our nanosensor, Au-heparin-dye, can detect 10^{-9} % (w/w) OSCS in heparin in 0.5 h. It is noteworthy that various nanoparticle−GAG conjugates have been employed as imaging agents,[19,20] therapeutics,[21−23] and biochemical tools.[24]

Gold nanoparticles (Au NPs) exert both FRET and NSET on fluorophores that are present on their surfaces. FRET dictates the quenching property of Au NPs at a separation distance of 100 Å between the dye and the Au NP. NSET takes over beyond 100 Å and maintains the quenching character of Au NP to a separation distance of 220 Å.[25,26] We hypothesize that an Au-NP-Hep-dye conjugate would demonstrate strong fluorescence quenching when the dye is conjugated. However, upon enzyme treatment of the heparin linker, the fluorescence should be recovered as dye-heparin fragments are released from the nanoparticle. To test this hypothesis, we first synthesized

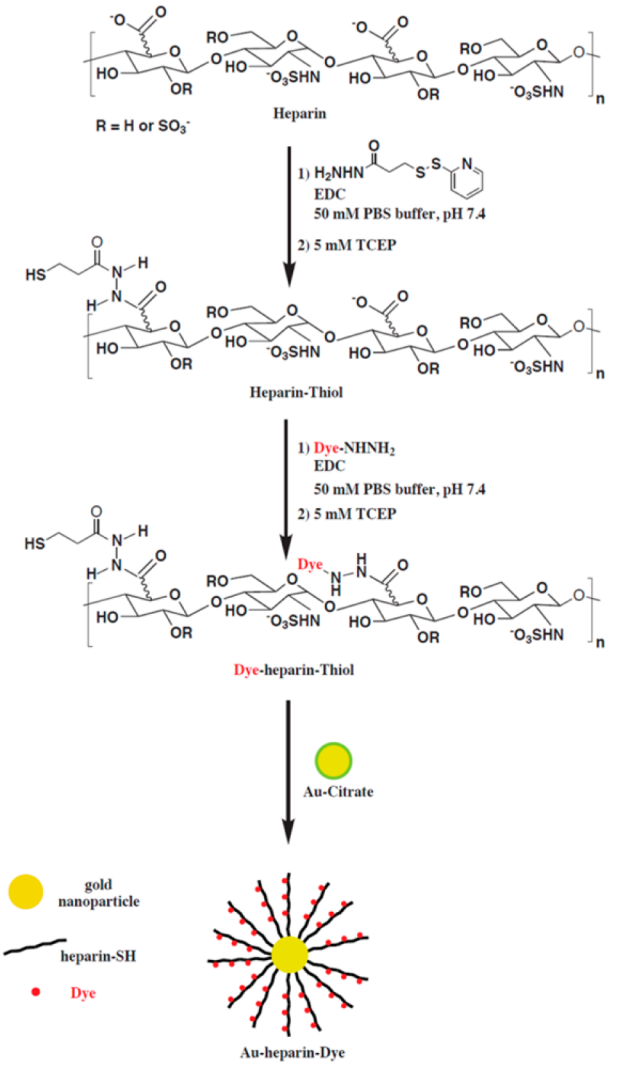
Received: September 4, 2013
Published: October 15, 2013

   dx.doi.org/10.1021/ja409170z | J. Am. Chem. Soc. 2014, 136, 554−557

thiolated heparin-dye stabilized Au NP. The fluorescence quenching property of this conjugate was then analyzed and the fluorescence recovery upon enzyme treatment was observed.

To synthesize Au-Hep-dye nanosensors, a ligand exchange protocol was followed (Scheme 1). The structural morphology

Scheme 1. Construction of the Au-Heparin-Dye Nanosensor



of the nanosensor before and after heparitinase treatment was observed through transmission electron microscopy (Figure 1A,B). The average particle sizes were 18 nm in both cases. Next, the quenching capability of the nanoparticle conjugates was determined using a fluorescence spectrophotometer. We observed an 88% reduction in fluorescence intensity of the dye after conjugation with the Au NPs suggesting an efficient energy transfer from the dye to Au NP (Figure 2A). The Au-heparin-dye nanosensor was then incubated with heparitinase I, II, and III at 37 °C in order to study the efficacy of the enzyme action on the probe. Indeed, a fluorescence recovery of 70% was observed over a period of 4 h (Figure 2B). A heat map of the 96-well microplate imaged using an IVIS imager complemented this observation (Figure 2C).

Once we established that enzyme action causes the fluorescence probe to recover from being quenched, a heparitinase enzyme inhibition study was carried out in the presence of OSCS-contaminated heparin to determine whether

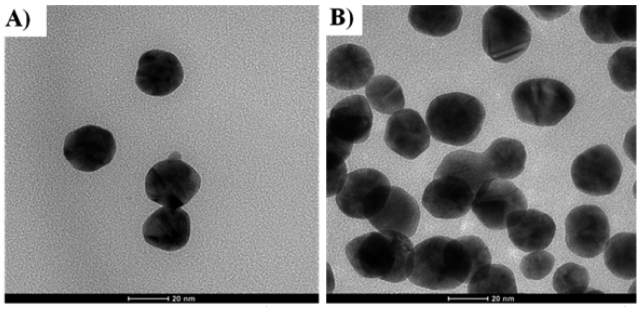


Figure 1. TEM images of (A) Au-Heparin-dye nanosensor and (B) heparitinase- treated Au-heparin-dye nanoparticle after 4 h of incubation. The average size of the particles is ~18 nm in both cases. The scale bar is 20 nm.

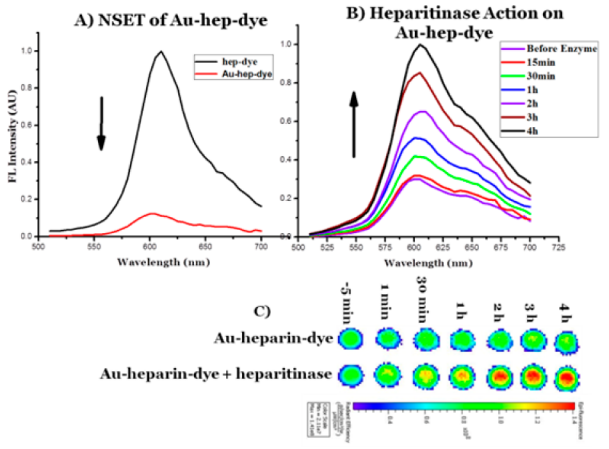


Figure 2. (A) Au-heparin-dye nanosensor shows ~88% reduction in fluorescence due to NSET. (B) Incubation of the nanosensor with heparitinase enzyme at different time points (before addition of enzyme, 30 min, 1 h, 2 h, 3 h, and 4 h) shows a gradual increase in fluorescence intensity. A recovery of ~70% fluorescence intensity is recorded over a period of 4 h. (C) A heat map of fluorescence increase is captured through the IVIS's CCD camera using a Ds red filter (575−656 nm). The epifluorescence scale bar represents the increasing order of radiant efficiency [(photons/s/cm^2/str)/(μW/cm^2)] from blue to red.

OSCS inhibits heparitinase activity. For this study, the following samples were incubated with heparitinases: (a) heparin alone; (b) heparin with 10% (w/w) chondroitin sulfate A and C (CS A and C); and (c) heparin with 10% (w/w) OSCS. The resulting fragments were then analyzed on an analytical HPLC. The results show that 10% (w/w) OSCS is a powerful inhibitor of heparitinase enzyme activity (Figure S1, see Supporting Information). Thus, in the presence of 10% (w/w) OSCS, the heparitinases should not act upon the Au-heparin-dye nanosensor. On the basis of these results, the enzyme activity on the nanosensor should be maximal at low OSCS concentration and minimal at high OSCS concentration. Thus, the fluorescence quenching of the dye should be minimal at low OSCS concentration and maximal at high OSCS concentration. We next tested the quenching of the dye present on the Au-Hep-dye nanosensor in the presence of OSCS upon heparitinase treatment. To develop an analytical method for determining levels of this contaminant in heparin, serial dilutions of OSCS were performed at log increment concentrations from 0.1 μg/μL to 0.1 fg/μL. Standard heparin solutions (10 μg) were spiked serially with OSCS solutions

dx.doi.org/10.1021/ja409170z | J. Am. Chem. Soc. 2014, 136, 554−557

from 1 μg (10% w/w) to 0.1 fg (10^{-9} % w/w) contamination resulting in 11 separate tests. Heparin (10 μg) and OSCS (10 μg) were utilized as positive and negative controls, respectively. When these OSCS-spiked heparin samples were incubated with heparitinases in the presence of nanosensor, the fluorescence intensity of the dye recovered quickly in samples with limited contamination (Figure 3A, see Supporting Information for details). Differences in the enzyme activity could be observed within 30 min of incubation owing to the rapid nature of the assay even at femtomole concentrations of OSCS-contamination (Figure S2, see Supporting Information).

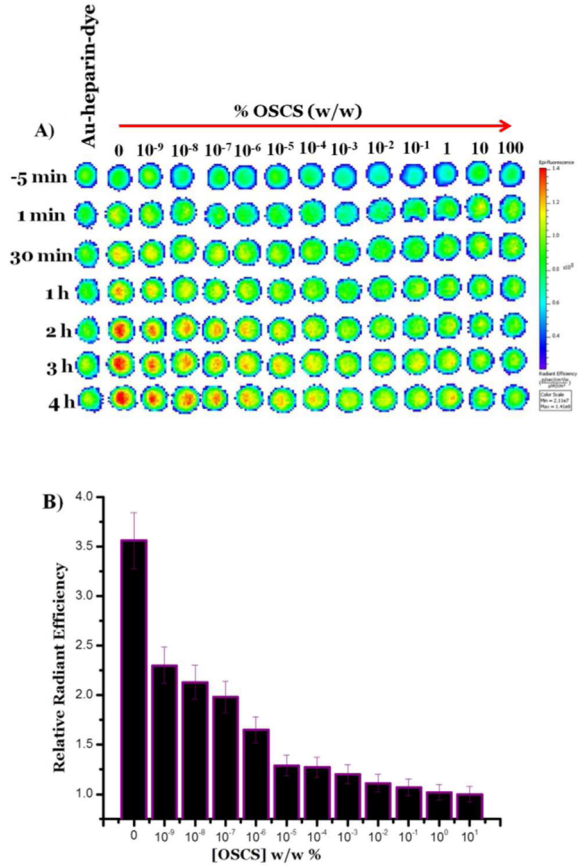


Figure 3. (A) The heat map of the 96-well plate is captured after exciting the wells with 535 nm and recording the emission with a Ds red filter (575–656 nm). The images are recorded before addition of heparitinase, and 1 min, 30 min, 1 h, 2 h, 3 h, and 4 h after incubating the nanoprobe with the heparitinase enzyme treated heparin samples that are spiked with OSCS. (B) A plot of relative average radiant efficiencies of the wells (after incubation with the enzyme cocktail for 30 min) versus [OSCS] (w/w)% in heparin shows that the CCD camera effectively detects 1 fg of OSCS contaminant in 10 μg of heparin. This plot strongly reaffirms the ultrasensitive LOD of the nanosensor.

It is known that other glycosaminoglycans such as chondroitin sulfates (CS-A and CS-C) and dermatan sulfate are commonly found as minor impurities in pharmaceutical heparin. To determine whether the newly developed nanosensor system is susceptible to interference from these other glycosaminoglycans, we treated heparin samples containing 10% (w/w) CS-A, CS-C, or dermatan sulfate with heparitinases in the presence of nanosensor. The fluorescence intensity of the dye was recovered quickly in heparin samples containing natural glycosaminoglycan impurities but not in the heparin sample containing OSCS contaminant (Figure S3, see Supporting Information). Therefore, the nanosensor described here is robust and reliable for routine screening of commercial heparin lots to detect trace levels of OSCS contaminant.

Next, an imaging experiment with an IVIS Lumina II system was designed to capture the emitting photons after treating the nanoprobe with heparitinase/OSCS spiked heparin. The images were taken by exciting the samples at 535 nm and recording emission using a Ds red filter (575–650 nm). A comparative heat map confirmed the gradient decrease in photons as OSCS contamination increases (Figure 3A). When the relative radiant efficiency (radiant efficiency after enzyme treatment/radiant efficiency before enzyme treatment) of each sample is plotted versus (w/w) % OSCS contaminant in heparin, 10^{-9}% (w/w) contaminant was detectable within 30 min. The plot also demonstrates the superior sensitivity of the nanosensor reflected in the relative radiant efficiencies when there is no OSCS contamination (3.56) and 10^{-9}% (w/w) OSCS contamination (2.3) (Figure 3B). With the use of the IVIS scanner, the LOD of this assay system is improved even further.

In summary, the Au-Hep-dye nanosensor described herein can be used to rapidly screen heparin stocks and detect femtogram levels of OSCS contaminants within half an hour. This probe also has potential application in forensic science.[27] We envision that the use of this nanosensor will greatly enhance our ability to maintain a high quality global heparin supply chain, thereby saving human lives.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures, materials, and method. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge funding support from NIH grants (HL107152 and GM075168). We would like to acknowledge David Belnap of the Electron Microscopy Core Laboratory, University of Utah for helping us with the TEM images. We also thank Marla Pyle of Kansas State University for her helpful suggestions in the preparation of the manuscript.

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