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Sensitive Detection of Oversulfated Chondroitin Sulfate in Heparin Sodium or Crude Heparin with a Colorimetric Microplate Based Assay

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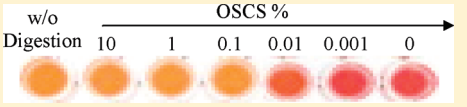
Supporting Information

ABSTRACT: In this work we describe a 96-well microplate assay for oversulfated chondroitin sulfate A (OSCS) in heparin, based on a water-soluble cationic polythiophene polymer (3-(2-(*N*-(*N*′-methylimidazole))ethoxy)-4-methylthiophene (LPTP)) and heparinase digestion of heparin. The assay takes advantage of several unique properties of heparin, OSCS, and LPTP, including OSCS inhibition of heparinase I and II activity, the molecular weight dependence of heparin-LPTP spectral shifts, and the distinct association of heparin fragments and OSCS to LPTP. These factors combine to enable detection of the presence of 0.003% w/w spiked OSCS in 10 μg of heparin sodium active pharmaceutical ingredient (API) using a plate reader and with visual detection to 0.1% levels. The same detection limit for OSCS was observed in the presence of 10% levels of dermatan sulfate (DS) or chondroitin sulfate A (CSA) impurities. In addition, we surveyed a selection of crude heparin samples received by the agency in 2008 and 2009 to determine average and extreme DS, CSA, and galactosamine weight percent levels. In the presence of these impurities and the variable heparin content in the crude heparin samples, spiked OSCS was reliably detected to the 0.1% w/w level using a plate reader. Finally, authentically OSCS contaminated heparin sodium API and crude samples were distinguished visually by color from control samples using the LPTP/heparinase test.



Heparin belongs to a family of naturally occurring negatively charged glycosaminoglycans (GAGs) which are complex linear polysaccharides comprised of repeating disaccharide units.[1,2] GAGs including heparin, heparan, and chondroitin(s) are distinct analytically by degree of sulfation, chain length, and molecular structure and distinct biologically through selective interactions with bioactive mediators.[2,3] Heparin has been used since the 1940s as an anticoagulant due to its broad efficacy enabled by specific interactions with antithrombin and factors in the coagulation, kallikrein, and complement pathways.[4,5]

Heparin for therapeutic use is isolated from mast cells in porcine intestinal mucosa. Generally the crude raw material undergoes extensive physical and chemical clean up under cGMP conditions to generate heparin sodium active pharmaceutical ingredient (API).[6] Maintaining a high quality heparin supply chain is an important health issue because tons of heparin is used every year, few alternative anticoagulants are available as substitutes for some indications and certain patients receive the drug in a chronic manner.[6]

In 2008 the vulnerability of the global heparin supply chain to economically motivated additives (EMAs) was evidenced with tragic effects.[7,8] Ultimately, administration of oversulfated chondroitin sulfate A (OSCS) contaminated heparin to patients led to 574 reports of adverse reactions and 94 deaths[9] in the U.S. and abroad. OSCS is a synthetic material made by oversulfation of chondroitin sulfate A (CSA) and has approximately 20 to 25% of the anticoagulant activity of heparin.[10] The current hypothesis as to the mechanism of the adverse events is that in susceptible individuals, OSCS in the presence of heparin activates the contact system leading to bradykinin release and a concomitant anaphylactic reaction.[11]

In response to this contamination, the U.S. and European Pharmacopeias made revisions to the heparin monographs to include strong-anion-exchange (SAX)-HPLC and 1D-^{1}H NMR tests which were sensitive to the presence of OSCS in the drug.[12] In addition, capillary electrophoresis (CE), polyacrylamide gel electrophoresis (PAGE), near-infrared (NIR) and selective electrodes have been used to detect OSCS in heparin.[13−16] These tests have limits of detection for OSCS in heparin of 0.03% w/w (SAX-HPLC[17]), 0.1% (NMR[18]), 0.1% (CE[13]), 0.2% (PAGE[14]), ~1% (NIR[15]), and 0.5% electrochemically[16] with microgram to milligram sample amounts assayed (Table 1). Importantly, some of these tests are sensitive to the presence of other possible oversulfated EMAs in heparin (e.g., refs 14 and 19) and work for crude heparin analysis.[20] However, a limitation of these tests is that they require specialized equipment and expert operators and are not amenable to automated rapid high-throughput screening.

To address this shortcoming several alternative fluorescent, enzymatic or colorimetric based analytical methods have been developed for the detection of heparin or OSCS in heparin.[21−28] Three of these approaches take advantage of enzyme inhibition by OSCS and have detection limits from 0.1% to 0.5% w/w for oversulfated compounds in heparin sodium API[25,27,29] in a 96-well microplate format. Alternately, Bairstow et al. used a commercially available enzyme immunoassay in a 96-well microplate format and found a limit of OSCS detection of 0.1% w/w.[21] The microplate assays have the advantage over the chromatography or PAGE based tests in that they can be suitable for

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Table 1. Comparison of Analytical Assays (Top) and Microplate Tests to Detect OSCS

analytical assay	[heparin]	LOD w/w %
SAX-HPLC	20 mg/mL	0.03%[17]
	100 mg/mL	0.02%[20]
CE	50 mg/mL	0.05%[40]
	30 mg/mL	0.1%[41]
	1 mg/mL	1%[13]
NMR	25 mg	0.1%[39]
	10 mg	0.5%[14]
IR-Raman	10 mg/mL	~1%[15]
electrochemical	1 mg/mL	0.5%[16]
PAGE	100 μg	0.5%[14]
microplate assays	**[heparin]**	**sensitivity % w/w**
LPTP (this work)	10 μg	0.003%
Heparin EIA	20 μg	0.1%[21]
Polymer-H	1.3 μg	0.5%[29]
OD 232 nm	100 μg	≤0.1%[27]
DNA polymerase	0.6 μg	0.16%[25]

high-throughput screening using commonly available laboratory instrumentation while only requiring microgram quantities of sample for assay.

A wide variety of fluorescent chemosensors[26,28,30−38] have been reported for polyanion (e.g., heparin or DNA) sensing. For the most part, the development of these chemosensors has focused on detection of heparin in serum. Of these, Polymer-H, has been evaluated for OSCS detection in a heparin sodium API matrix with common impurities present (e.g., dermatan sulfate (DS) or CSA).[22,29] In this case, Polymer-H[37] was used in a 96-well microplate assay and reported a LOD/LOQ of 0.5%/0.6% w/w for OSCS in heparin. This assay used heparinase I digestion of heparin to detect the presence of polyanions resistant to heparinase (e.g., OSCS). The Polymer H based test was insensitive to lower molecular weight components (M_r < 3000) and those polysaccharides with less than ~1.2 sulfates per disaccharide.[29]

Recently, a naked-eye detection and quantification of heparin in serum with a water-soluble cationic polythiophene polymer developed by Leclerc's group[34,35] (Leclerc PolyThiophene Polymer (LPTP)) was described.[28] Leclerc's group performed studies to understand the basis for the observed color changes in LPTP.[34,35] They attribute the change in the optical properties of the chemosensor to conformational modification of the polymer backbone with anion association. Thus, the uncomplexed LPTP random coil solution structure has a yellow color and, upon optimal anion association, a shift to the red occurs caused by the polymer backbone adopting a highly conjugated planar conformation. Any twist in the backbone conformation because of less than optimal association results in less conjugation and a less planar structure with a concomitant decrease in the observed color shift.

LPTP has been found to be selective for anions with particular characteristics. For example, LPTP solutions change color in the presence of a small counterion[35] or for selected large polymeric glycosaminoglycans.[28] Specifically, a similar version of this chemosensor allowed selective detection of iodide over a wide range of anions (e.g., F^-, Cl^-, Br^-, HPO_4^{2-}, or SO_4^{2-} [35]). In addition, CSA and heparin association caused a LPTP color change while little change was observed in the presence of hyaluronic acid (HA).[28] The basis of LPTP's selectivity remains to be defined. Of note, OSCS or DS were not evaluated in these studies.

As part of a larger agency study to develop facile field deployable tests for assuring the quality of the heparin supply chain, we assessed the LPTP as a chemosensor for OSCS in heparin sodium and crude heparin. Similar to Lühn et al., we utilized heparinase to digest heparin in the presence of OSCS and observed that the test was insensitive to common impurities in heparin. However, while Lühn et al. used only heparinase I, we utilized heparinase I and II to obtain a more complete level of digestion. In addition, in contrast to the chemosensor used by Lühn et al. (Polymer-H[37]), LPTP is sensitive to lower molecular weight components and produces a different spectral shift for OSCS than for heparin. These unique properties of OSCS, heparin, and LPTP resulted in a highly sensitive assay for the presence or absence of OSCS in heparin API or crude heparin.

EXPERIMENTAL SECTION

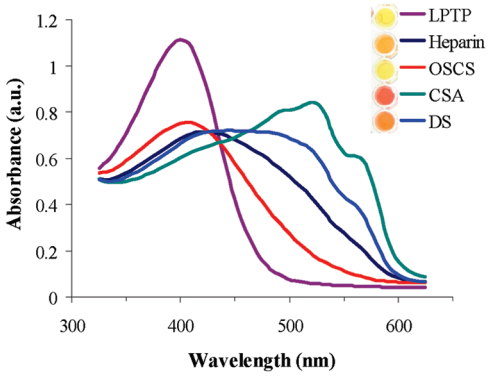
Reagents. *Materials.* Hydrazine (anhydrous, 98%), chloroform (anhydrous, >99%), chondroitin sulfate A sodium salt from bovine trachea, bovine serum albumin (BSA) and heparinase II (E.C. 4.2.2.8) and heparinase III from *Flavobacterium heparinum* were purchased from Sigma Aldrich (St. Louis, MO). Iron(III) chloride (anhydrous, 98%), tetrabutylammonium chloride (95%) and $CDCl_3$ (99.8% atom D) were purchased from Acros Organics (New Jersey). Acetone (HPLC grade), methanol (HPLC grade), extraction thimbles (25 mm × 80 mm, Whatman single thickness cellulose) and Nunc maxisorb microplates were purchased from Fisher (Pittsburgh, PA).

Batches of crude heparin and authentically contaminated heparin API were collected from international markets by the FDA. Alcian blue was purchased from MP Biomedicals. Heparin sodium API samples for the 2009 test set were obtained from sources approved to supply the U.S. marketplace. Standard heparin sodium was purchased from USP (catalog no. 1235853). Chondroitin sulfate B (also known as dermatan sulfate) from porcine intestinal mucosa was purchased from Calbiochem (La Jolla, CA). Arixtra (Fondaparinux sodium, manufactured by GSK) was obtained as an injectable solution at 12.5 mg/mL from McKesson. OSCS was synthesized by sulfation of chondroitin sulfate A following the literature procedure.[10] Heparinase-1 (E.C. 4.2.2.7) from *Flavobacterium heparinum* was purchased from Associates of Cape Cod (Falmouth, MA). Precast 25% Tris-HCL 20 cm × 20 cm gels were obtained from Jule Inc. (Milford, CT).

Leclerc Polythiophene Polymer (LPTP). 3-(2-(*N*-(*N*′-methylimidazole))ethoxy)-4-methylthiophene (LPTP) was synthesized following the literature procedure.[34] Details in the synthesis procedure that were found to be important to obtain the best colorimetric response of the polymer are described in the Supporting Information. The dry polymer is stable when stored in the dark at room temperature. The solution remains stable for weeks as observed by Zhan et al.[28]

Heparinase Treatment. Heparin sodium, crude heparin, and all other polysaccharides were prepared as 10 mg/mL stocks in milli-Q water. Heparinase-I and heparinase-II were reconstituted in 0.1% BSA as 1 U/mL stock. Heparin sodium (10 μg) or crude heparin (20 μg) were digested in microcentrifuge tubes by 0.2 milli-Units (mU) of heparinase-I plus 0.1 mU of

A.)



B.)

GAGs	Max nm	φ
CSA	520	13
DS	440	9.3
Heparin	420	6.2
OSCS	405	2.1
Polymer Alone	400	0

C.)

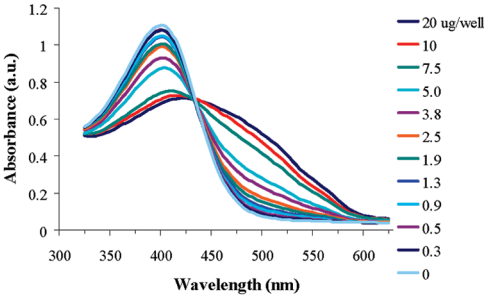


Figure 1. Panel A shows the overlaid plots of absorbance spectra for LPTP in the presence of buffer alone, heparin sodium, OSCS, CSA, or DS (20 μg/well). The colors of the solutions in the plate wells are shown in the inset for LPTP, heparin, OSCS, CSA, or DS. Panel B shows the wavelength of peak absorbance (maximum nanometer) and a normalized absorbance (φ), where φ = absorbance with GAG at 525 minus the absorbance without GAG present divided by the absorbance without GAG. Panel C shows the overlaid plots of absorbance spectra of LPTP solutions with a range of heparin sodium levels (0−20 μg/well).

heparinase-II or treated with buffer (20 mM ammonium acetate, 2 mM calcium acetate, pH 7.4) for 30 min or 2 h (crude samples) at 37 °C. For OSCS-spiking studies, OSCS was serially diluted and added to USP heparin at the indicated percentage (w/w) prior to heparinase treatment. The reaction was quenched by heating on a 97 °C hot plate for 3−5 min. The concentration and reaction time of heparinase-I and heparinase-II was optimized to yield a maximal spectral shift with polythiophene dye in preliminary studies (Supplemental Figure 1A in the Supporting Information). For PAGE analysis, heparinase-treated samples were combined with an equal volume of 100% (w/v) sucrose and a trace of bromophenol blue dye for visualization of the ion front during electrophoresis. PAGE analysis demonstrated that the sample was significantly depolymerized (Supplemental Figure 1B in the Supporting Information).

Polythiophene Microplate Assay. Heparin was serially diluted in a 96-well plate for concentration studies (0−1 mg/mL, 0−20 μg/well) or treated with heparinases in microcentrifuge tubes. A volume of 20 μL of each sample was transferred to a Nunc maxisorb plate, and 180 μL of fresh LPTP (0.3 mM; MW = 256.75, based on repeat unit) in milli-Q water was added. The plate was gently tapped to mix, and then absorbance spectra from 300 to 625 nm were captured every 5 nm using a BioTek Microplate reader (Winoski, VT). The spectral data were exported to Microsoft Excel 2003 for analysis. Plates were placed in a CAMAG Reprostar 3 light box and photographed using a Canon Powershot SX10 IS.

Analytical Methods for Crude Survey. A set of approximately 50 crude heparin samples were selected from over 100 samples received at the agency for analysis based on the quantity of sample obtained and a diversity of sources. SAX-HPLC and ^{1}H NMR analyses of the crude heparin samples were performed as previously described to obtain a measure of the average and extreme properties of crude heparin.[20] Specifically we estimated heparin content and quantified OSCS content by SAX-HPLC. Separately, 1D-^{1}H NMR was used to estimate the DS and CSA content of crude heparin.

From the NMR data, the peak heights *N*-acetyl methyl proton signals of OSCS, DS, heparin, and CSA were used to calculate an estimate of the weight percent levels of these compounds in heparin using the method of McEwen et al.[18] (eqs 1 and 2) to quantify the level of OSCS and DS as follows:

$$\text{OSCS w/w\%} = (\text{OSCS peak height (at 2.15 ppm)}/\text{heparin (at 2.05 ppm)}) \times 0.047 \quad (1)$$

or

$$\text{DS w/w\%} = (\text{DS peak height (at 2.08 ppm)}) - y\{(10\% \text{ of heparin peak height(at 2.05 ppm)})/(0.07 \times \text{heparin peak height (at 2.05 ppm)})\} \quad (2)$$

Several factors reduce the accuracy of the values measured by this approach, including variation in the degree of heparin acylation,

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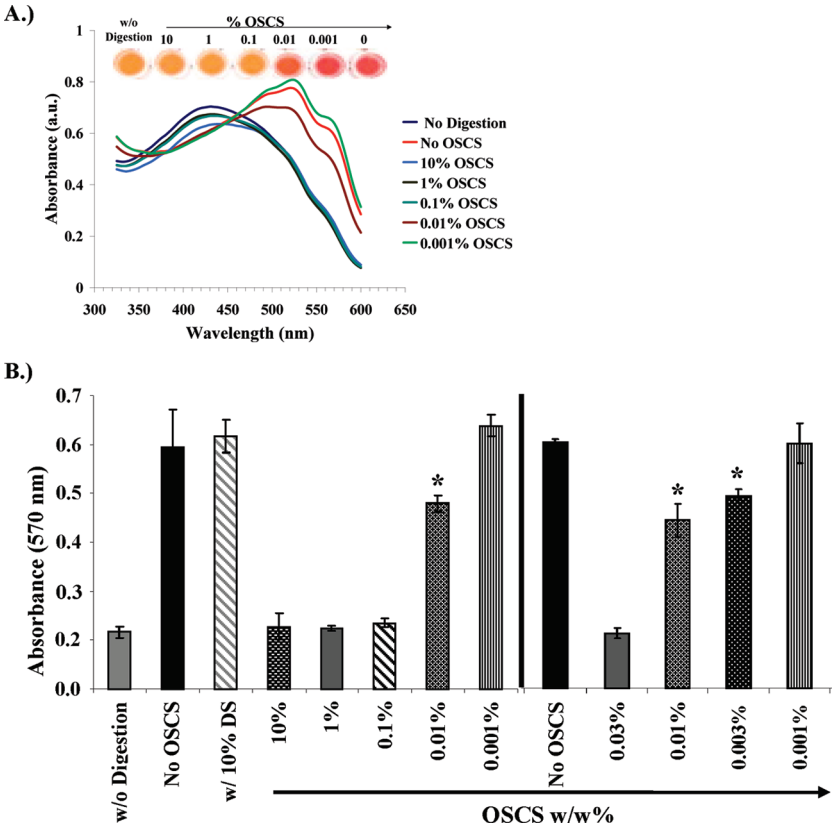

Figure 2. Panel A shows overlaid plots of absorbance spectra of LPTP solutions in the presence of heparinase I and II treated USP heparin (10 μg) spiked with OSCS (0−10% w/w). The w/o digestion values are from heparinase untreated USP heparin. The inset shows a photograph of LPTP solutions in plate wells with or without heparinase-treated heparin containing varying levels of OSCS. Panel B shows the mean absorbance values (570 nm) ± standard deviation (SD) of triplicate OSCS spiked at log increment concentrations and unspiked samples digested and mixed with LPTP. The left panel represents the mean absorbance levels derived in part from the spectra shown in panel A. The right panel is from a separate set of experiments using half-log increment OSCS levels. Statistical analysis was assessed with use of a one-tail P(T ≤ t) t-Test (∗, $p < 0.01$) for comparison with the no OSCS groups. No statistically significant differences are observed between "No OSCS", 0.01%, and 0.001% OSCS values in the right and left panels (see text for details). In addition, no statistically significant differences are observed between the "No OSCS" in the right and left panels and the no OSCS "w/10% DS" value obtained in the left panel.

broad signals which overlap and the error in weighing heparin caused by differences in water, residual processing solvents, or salt content in heparin sodium APIs. With these caveats in mind, the approach is a simple way to get a rough estimate (∼30% RSD) for OSCS or DS in crude heparin. The LODs for OSCS and DS were reported as 0.1% and ∼0.3% w/w, respectively, for this method.[18]

We developed a method similar to that of McEwen et al. to obtain an estimate of the amount of CSA present in the crude samples by NMR. We used NMR peak height of the CSA *N*-acetyl methyl signal at (2.02 ppm) data obtained on two "clean" API heparins spiked with bovine trachea CSA (Sigma) at 1%, 5%, or 10% w/w% levels ($n = 6$). Because the CSA peak at 2.02 ppm is a shoulder on the heparin peak at 2.05 ppm, we subtracted 10% of the heparin peak height from the CSA peak height as was done for the DS measurement (eq 3).

$$\text{CSA w/w\%} = (\text{CSA peak height (at 2.02 ppm)}) - y\{(10\%\text{of heparin peak height (at 2.05 ppm)})/5.8\} \quad (3)$$

% Galactosamine in Total Hexosamine. Weight percent galactosamine (% Gal) in total hexosamine content was determined as described in USP PF Volume 35(2) [March−April 2009] Heparin Sodium. In total, 12 mg aliquots of various crude heparin sodium samples were used. We found that the acid hydrolysis used for this procedure eliminated the large amount of insoluble material normally found in aqueous solutions of crude heparin. This test gives a measure of the total chondroitin content of the samples and does not discriminate between the different forms or their sulfation states.

Statistical Analysis. Results are expressed as means plus or minus the standard deviation (S.D.) of triplicate samples of a representative experiment ($n = 3$) or a combination of data ($n = 6$) from multiple experiments. Statistical significance was assessed by using a one-tail P(T ≤ t) t-Test (∗, $p < 0.01$; ∗∗, $p < 0.001$) for comparison with the appropriate control group (no OSCS, mean API, average crude).

RESULTS

LPTP−Polyanion Complex Characterization. Addition of certain negatively charged species to LPTP results in spectral shifts and solution color changes (yellow to red) with a corresponding shift of the absorption maximum to longer wavelengths.[35] For example, Zhan et al. showed different colors for heparin, CSA, and HA solutions in the presence of LPTP.[28]

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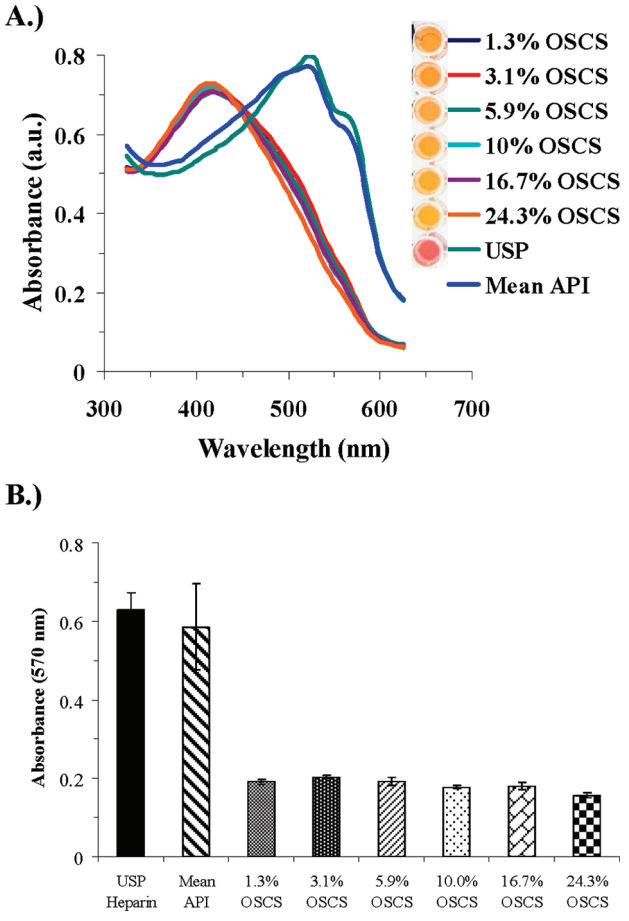

Figure 3. Panel A shows the overlaid plots of absorbance spectra of LTPT solutions in the presence of 10 μg each of six heparinase I + II treated authentically contaminated heparin sodium active pharmaceutical ingredient (API) samples. The contaminated APIs were selected with OSCS (w/w%) levels ranging from 1.3 to 24.3% as determined by SAX-HPLC. The spectra labeled "Mean API" is the average of absorbance spectra (n = 6) from heparinase treated heparin sodium APIs obtained from the six suppliers of heparin sodium to the U.S. market. The inset shows a photograph of LPTP-heparin solutions in the microplate wells. Panel B shows the mean ± SD of triplicate samples of LPTP-heparin solution absorbance at 570 nm. The results are representative of experiments performed on two separate days. Statistical analysis was assessed with use of a one-tail P(T ≤ t) t-Test for comparison with the mean API group, and all APIs had p values of less than 0.0001.

Zhan et al. reported a LPTP alone and LPTP−heparin complex maxima of 393 and 420 nm, respectively.

Here, we synthesized LPTP to evaluate the spectral properties of heparin−, OSCS−, DS−, and CSA−LPTP complexes in a 96-well plate format. Initial experiments assessed the spectra of these complexes with 0.3 mM solution of LPTP and 20 μg of heparin, OSCS, CSA, or DS per well (Figure 1A). The peak absorbance wavelength for LPTP alone was 400 nm, which is similar to the value of 393 nm observed by Zhan et al.[28] where the data were obtained with a higher resolution instrument than our microplate reader.

Polymer complexes with 20 μg/well OSCS, heparin, DS, or CSA shifted the peak absorbance wavelength to 405, 420, 440, and 520 nm, respectively, and increased the absorbance intensity at longer wavelengths. The spectral shifts were accompanied by color changes from yellow (LPTP alone) to red (CSA) (Figure 1A, insert). To compare the polysaccharide−polymer complexes at a fixed wavelength, absorbance was normalized (φ) using $(I - I_o)/I_o$ where I and I_o are the absorbance at 525 nm in the presence and absence of analyte, respectively. CSA complexes had the highest φ value of 13 followed by DS (9.3), heparin (6.2), and OSCS (2.1) (Figure 1B). In subsequent experiments, a 0.3 mM solution of LPTP was added to a plate containing 0−20 μg/well heparin as shown in Figure 1C. The linear detection range for heparin was 0.32−20 μg/well; above 20 μg/well the spectra no longer change significantly.

The tertiary structure of the polysaccharide appears to be more important than the degree of sulfation for enabling LPTP color change. OSCS is synthesized from CSA and has ~four sulfates per disaccharide whereas CSA has one. In addition, the primary difference between CSA and DS is the orientation of the 5′ carboxylic acid group (i.e., DS contains iduronic acid while CSA contains glucuronic acid). However, CSA or DS cause distinct color changes upon LPTP binding while OSCS causes little color change. Importantly, OSCS in heparin API solutions inhibited the heparin-LPTP color change. These data suggest that OSCS does associate with LPTP but does not contain the appropriate tertiary structure elements to result in the LPTP conformational change needed to cause a color change.

Heparinase Treatment. We evaluated the effect of shorter chain length heparins on LPTP binding with a pentasaccharide (fondaparinux) and heparinase digestion of heparin sodium. We observed that fondaparinux or heparin digests containing disaccharides bound LPTP with a concomitant spectral shift which was larger than that observed for undigested heparin (Supplemental Figure 2 in the Supporting Information). Therefore, USP heparin (10 μg) was treated with various combinations of heparinase I, II, or III for 30 min versus 18 h at 37 °C, and the absorbance profiles, color changes, and PAGE profiles were evaluated.

Digestion of heparin by heparinase I alone prior to the addition of LPTP solutions produced an absorbance shift (490 nm) while heparinase II or heparinase III alone resulted in little observed shift. The combination of heparinase I and II produced maximal absorbance shift of LPTP to longer wavelengths following 30 min or 18 h treatment (540 nm, Supplemental Figure 1A in the Supporting Information). The combination of heparinase I and heparinase III showed a slightly smaller effect on the absorbance shift than the combination of heparinases I and II (data not shown). The augmented color and absorbance shifts of polymer in the presence of heparinase I or I and II treated heparin were associated with high degrees of depolymerization as confirmed by PAGE analysis using alcian blue to track intact heparin and depolymerized bands (Supplemental Figure 1 in the Supporting Information). Thus, on the basis of these data, to maximize the heparin color shift with the minimum amount of lyase enzyme in a 30 min digestion, we selected a combination of 0.2 mU of heparinase I and 0.1 mU of heparinase II for 30 min at 37 °C as the digest conditions for the assay.

OSCS Detection in USP Heparin. Because OSCS has been shown to be insensitive to digestion by heparinase and also acts as a heparinase inhibitor,[29] we assessed the effect of the presence of OSCS on USP heparin digestions and LPTP spectral properties. In initial studies, OSCS at 0.001% to 10% w/w in log increments was spiked into USP heparin, and then this solution was treated with heparinase at 37 °C for 30 min before quenching the enzyme activity by heat inactivation. These solutions were mixed with 0.3 mM solutions of LPTP in a 96-well microplate and the

Table 2. Average Crude Heparin Properties Determined from Analysis of 39−48 Crude Samples and the Characteristics of the Crude Heparin Samples Selected to Test the Heparin-Digestion-LPTP Assay

crude	% Gal	% OSCS SAX-HPLC[b]	% Hep SAX-HPLC[c]	% DS NMR[d]	% CSA NMR[e]
average crude	18.7 ± 6.7 (n = 47)	<0.02 (n = 48)	27 ± 11 (n = 48)	5.5 ± 1.8 (n = 39)	5.3 ± 4.0 (n = 39)
A1[a]	17.3	<0.02	39	4.5	3.8
A2[a]	20.1	<0.02	28	4.2	9.9
A3[a]	16.0	<0.02	23	3.9	6.3
high DS (hDS) crude	35.2	<0.02	24	14.2	6.4
high CSA (hCSA) crude	28.5	<0.02	31	5.0	15.5
C1	19.9	1.6	29	5.0	<1
C2	23.2	48	22	8.1	<1
C3	18.0	0.9	14	5.8	1.5
C4	22.6	13.4	23	5.1	<1

[a] These individual "average crudes" crudes were selected based on comparison of % Gal values determined by the USP method. [b] Determined as described in ref 20 (LOD = 0.02%). [c] An estimate of the heparin crude content based on the percentage of the total SAX-HPLC chromatogram area eluting at ∼20.4 min. This area is not corrected for the relative response factors of heparin or the possible impurities or contaminants at 215 nm. [d] Measured by the method of McEwen et al.[18] (LOD ∼ 0.1%). [e] Measured as described in the methods (LOD ∼ 1%).

UV−vis spectral properties measured. Significant visual color differences from non-OSCS containing USP heparin control digestions were observed by eye at the 0.1% level and by absorbance measurement at 570 nm to the 0.01% level in these experiments (Figure 2B, left panel).

To further investigate the sensitivity of the assay, a second set of experiments was performed with OSCS spiked USP heparin solutions at a lower range of concentrations with half log concentration increments (i.e., 0.03−0.001% OSCS in 10 μg of heparin). Significant differences from non-OSCS containing USP heparin control digestions were observed at the 0.003% OSCS level (p < 0.005, Figure 2B, right panel). Comparison of the "No OSCS" control, 0.01% and 0.001% OSCS sample LPTP/heparinase test mean absorbance values from the right and left panel of Figure 2B shows these separate experiments yield the same values within experimental error (i.e., No OSCS, 0.59 (±0.08) left, 0.58 (±0.01) right; at 0.01% OSCS 0.48 (±0.02) left, 0.43 (±0.03) right; and at 0.001% OSCS 0.64 (±0.02) left, 0.58 (±0.04) right). Thus, experiments run from three separate digest on two different days yield similar values.

Importantly, the test is insensitive to the presence of impurities (i.e., CSA or DS) which are known to be copurified with heparin. The current USP specification allows for NMT 1% levels (percent galactosamine in total hexosamine test) of the total of these impurities in heparin sodium. Here, the inhibition of the color change was observed down to the 0.1% OSCS level visually and at the 0.003% OSCS level with plate reader measurements in the presence of 10% levels of DS or CSA. To our knowledge, these data establish that the LPTP/heparinase digest assay is the most sensitive test for the presence of OSCS contamination in the presence of high levels of impurities of any currently published method (Table 1).

OSCS Detection in Authentically Contaminated Heparin API. During 2008, the agency tested a number of authentically contaminated heparin sodium APIs (for a review, see ref 12). Six samples selected with OSCS contamination levels from 1.3 to 24.3% (*vida* SAX-HPLC) were tested in triplicate and all were found to be visually distinct from the USP heparin control digestion color (Figure 3A) and by relative absorbance at 570 nm (Figure 3b).

All of these assays were performed with a USP heparin identification reference standard as the control. In addition, because heparin APIs are purified from crude heparin by a number of different proprietary processes, we established the range of variation in the heparin digestion-LPTP assay across manufacturers which supply heparin sodium API to the U.S. market. Thus, as an additional control, six OSCS-free heparinase-treated heparin sodium APIs from six different sources were run in triplicate and used to measure the "mean API" digest 570 nm absorbance values. The mean API curve was very similar to that of USP heparin but had a higher standard deviation (Figure 3A,B). The heparinase-induced LPTP spectral shifts in the presence of authentically OSCS contaminated APIs were all significantly inhibited compared with those from control mean API or USP heparin digests (Figure 3A, p < 0.0001).

Crude Sample Matrix. To help ensure a quality heparin drug, tests at multiple steps in the supply chain are desirable. For example, OSCS is carried over into the heparin sodium and low-molecular weight heparin APIs manufactured from contaminated crudes. Therefore, we also investigated the heparin-digest-LPTP assay as a tool to test crude heparins that were found to contain the OSCS contaminant.

Initial studies focused on assessing the variability of the composition of the U.S. crude heparin supply. For the U.S. market, crude heparin is obtained from pig intestinal mucosa by multiple different processes and suppliers. Therefore the crude material contains varying levels of impurities and insoluble material. The agency collected over 100 lots of crude heparin from international markets in 2008 and 2009 for detailed analysis. Here the average levels of CSA, DS (NMR) and percent galactosamine in total hexosamine (% Gal) in a set of crude samples are shown in Table 2. In addition, we selected four crude samples that were found to be OSCS contaminated by SAX-HPLC and NMR analysis for testing by the colorimetric assay.

Because of the high background levels of impurities in the crude heparins, initial studies assessed the spectral properties of crude heparin−LPTP mixtures (75 μg) without heparinase treatment. The wavelength of peak polymer absorbance in the presence of crude samples was much broader (420−450 nm) compared to the peak in the presence of USP heparin (420 nm), reflecting the higher levels of CSA and DS in crudes (Figure 4A). In general, the absorbance profiles of all the crudes were similar and overlapping.

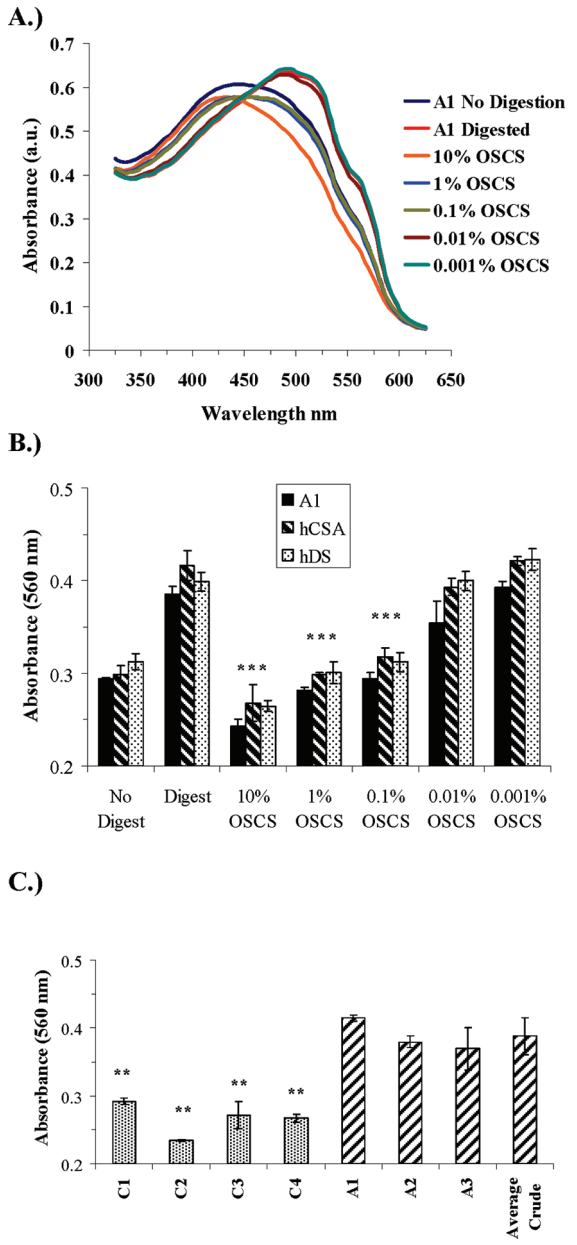

Figure 4. Panel A shows overlaid plots of absorbance spectra of LPTP in the presence of 20 μg of an average crude sample digested with heparinase I and II (Table 2, A1 designates an average crude) spiked with a range of OSCS levels (0−10% w/w). The "No Digestion" spectrum was obtained from A1 not treated with heparinase. Panel B shows the mean LPTP−anion complex absorbance at 560 nm in the presence of heparinase-treated crude samples spiked with OSCS. High CSA (hCSA) contains 15.5% CSA, and high DS (hDS) contains 14.2% DS compared to average values (Table 2). Results are expressed as a mean ± SD of six samples. Statistical analysis was assessed with use of a one-tail P(T ≤ t) t-Test (***, $p < 0.001$) for comparison with their respective uncontaminated digest groups. Panel C shows authentic OSCS contaminated crude heparins assayed by the heparin-digest-LPTP test. A plot of the mean LPTP−polyanion complex absorbance values at 560 nm of the contaminated crudes (C1, C2, C3, and C4) against several individual "average crude heparin" controls (A1, A2, and A3) after digestion. Results are expressed as a mean ± SD of triplicate samples from a representative experiment of two independent studies. Statistical analysis was assessed with use of a one-tail P(T ≤ t) t-Test (**, $p < 0.001$) versus the composite average crude mean absorbance value.

Three samples (A1−A3) were selected to represent an average matrix based on comparison of the results from ∼50 crude samples. Similarly, two samples were selected, one with high DS (14.2%; hDS) and one with high CSA (15.5%; hCSA) as extreme examples of impurity levels. As shown in Figure 4A, the polymer absorbance shift from a heparinase-treated average crude sample is much less robust than API heparin sodium (Figure 2). Nevertheless, OSCS inhibited the heparinase induced spectral change with a sensitivity of at least 0.1% ($p < 0.0001$) using the plate reader (Figure 4B). Samples with high levels of CSA or DS did not diminish the sensitivity of OSCS detection (Figure 4B).

We note that a wavelength of 560 nm was selected to assess differences in crude heparins while a wavelength of 570 was used for heparin sodium API studies. The largest difference between control and heparinase treated samples at longer wavelengths was observed at 560 nm for crudes and at 570 nm for heparin sodium spectra. Although we choose to use a single point for our intensity comparisons, other wavelengths or multiple wavelengths will also give differences that can be used to detect contamination.

OSCS Detection in Authentically Contaminated Crudes. Four samples (C1−C4) were identified having OSCS levels ranging from 0.9 to 48% (Table 2). The heparinase-induced spectral profile shifts and polymer absorbance at 560 nm of OSCS contaminated crudes were significantly inhibited (Figure 4C, $p < 0.0001$) compared with the individual or pooled average crude samples.

CONCLUSIONS

The level of OSCS which causes adverse effects in humans is unknown. The USP specification tests for heparin identity allow no detectable amount of this contaminating material in the drug. The most sensitive USP monograph test specific for OSCS contamination is the SAX-HPLC method (LOD of 0.03%). With the use of the SAX-HPLC test, the agency has found authentically contaminated samples at a wide range of levels in samples collected in 2007−2008.[12] For example, in 90 heparin sodium samples, OSCS levels were observed from 0.3% to 26% with a mean of 6.6% OSCS present in the contaminated samples.[12] In a similar study from Europe, the range was 0.1% to 81% OSCS contamination with a mean of 11%.[39] The presence of contaminants like OSCS even at trace amounts is an indication of contamination of the drug supply chain. Thus, the most sensitive assay possible should be used for surveillance purposes to ensure drug quality.

In the aftermath of the recent heparin contamination crisis, many research groups have worked to develop better methods to rapidly and thoroughly screen API heparins in order to prevent a reoccurrence of such an event. One venue of exploration has been the development of assays in a 96-well plate format that can be read with relatively inexpensive and commonly available laboratory equipment. Here we present data to show, to our knowledge, the most sensitive assay for OSCS in heparin reported for any published test using a 96-well plate format.

We utilize the unique properties of a cationic polythiophene polymer developed by Leclerc's laboratory that had been shown to change color in the presence of heparin. We found that LPTP associates with OSCS, intact heparin and small heparin fragments to form solutions with distinct colors; yellow, orange, and red, respectively. In addition, OSCS and other oversulfated GAGs are known to inhibit heparinases. Thus, in the absence of OSCS, heparin is digested and a distinct color change from intact

heparin is observed (i.e., orange to red). If OSCS is present, the enzymes are inhibited and heparin, heparin digest fragments and OSCS compete for association with LPTP, blocking the color change (i.e., the solution remains orange). The combination of these properties forms the basis for a very sensitive pass/fail colorimetric test for OSCS contamination in heparin.

The best detection limits for the LPTP-heparinase assay (~0.003% w/w) require the use of a plate reader. However, the color change inhibition compared to the control can be observed visually in heparin sodium APIs down to at least the 0.1% OSCS levels. In addition, we established a normal range of variability for the API heparin sodium digest-LPTP control across all the manufacturers supplying the U.S. market. Importantly, the assay detection limits were unaffected by the presence of common impurities (i.e., DS or CSA) in heparin sodium up to the 10% w/w level. On the basis of these results, we examined the sensitivity of this approach to test crude heparin samples.

Because the range of impurities in crude heparin has not been reported, we analyzed a set of approximately 50 crude samples to establish average and extreme levels of common impurities. These samples were selected from over 100 samples obtained in 2008 and 2009 by the agency in the regular monitoring of the heparin supply entering the U.S. market. On the basis of the survey data, we selected average and extreme crudes to spike with OSCS to see how the heparin digest-LPTP assay performed with high background interference. In this case, with plate reader measurement, OSCS levels down to 0.1% could be reliably detected.

Finally we tested authentically contaminated heparin sodium API and crude heparin samples analyzed by the agency in 2008. All of these samples showed visually distinct color from control digests. On the basis of the results presented here, the heparin digest-LPTP pass/fail test is more sensitive than the USP SAX-HPLC or other published tests for OSCS contamination, robust to common heparin impurities, and amenable to high-throughput screening with widely available laboratory equipment.

■ ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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