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Detection of Possible Economically Motivated Adulterants in Heparin Sodium and Low Molecular Weight Heparins with a Colorimetric Microplate Based Assay

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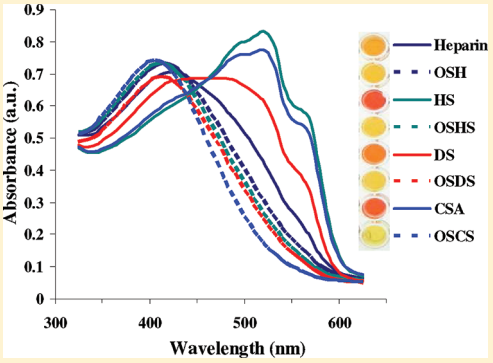
ABSTRACT: Recently, we described a 96-well plate format assay for visual detection of oversulfated chondroitin sulfate A (OSCS) contamination in heparin samples based on a water-soluble cationic polythiophene polymer (3-(2-(*N*-(*N*′-methylimidazole))ethoxy)-4-methylthiophene (LPTP)) and heparinase digestion of heparin. Here, we establish the specificity of the LPTP/heparinase test with a unique set of reagents that define the structural requirements for significant LPTP chemosensor color change. For example, we observed a biphasic behavior of larger shifts to the red in the UV absorbance spectra with decreasing average molecular weight of heparin chains with a break below 12-mer chain lengths. In addition, the oversulfation of chondroitin sulfate A (CSA) to a partially (PSCS) or fully (OSCS) sulfated form caused progressively less red shift of LPTP solutions. Furthermore, glycosaminoglycans (GAGs) containing glucuronic acid caused distinct spectral patterns compared to iduronic acid containing GAGs. We applied the LPTP/heparinase test to detection of OSCS (≥0.03% (w/w) visually or 0.01% using a plate reader) in 10 μg amounts of low molecular weight heparins (LMWHs; i.e. dalteparin, tinzaparin, or enoxaparin). Furthermore, because other oversulfated GAGs are possible economically motivated adulterants (EMAs) in heparin sodium, we tested the capacity of the LPTP/heparinase assay to detect oversulfated dermatan sulfate (OSDS), heparin (OSH), and heparan sulfate (OSHS). These potential EMAs were visually detectable at a level of ∼0.1% when spiked into heparin sodium. We conclude that the LPTP/heparinase test visually detects oversulfated GAGs in heparin sodium and LMWHs in a format potentially amenable to high-throughput screening.



In 2010, a naked-eye detection assay for heparin levels in serum using a water-soluble cationic polythiophene polymer developed by Leclerc's group (Leclerc polythiophene polymer (LPTP)) was described.[1] The change in the optical properties of the LPTP chemosensor was attributed to conformational modification of the polymer backbone with anion association.[2,3] Thus, LPTP has a random coil solution structure with a yellow color, and upon addition of an anion with certain characteristics, a shift to the red occurs caused by the polymer backbone adopting a highly conjugated planar conformation. Any alteration in the backbone conformation because of less than ideal anion characteristics results in less conjugation and a less planar structure with a concomitant decrease in the observed color shift.

For example, a similar version of the LPTP chemosensor allowed selective detection of iodide over a wide range of anions (e.g., F^-, Cl^-, Br^-, HPO_4^{2-}, or SO_4^{2-}[3]). In addition, chondroitin sulfate A (CSA) and heparin association caused a LPTP color change, while little change was observed in the presence of the nonsulfated polysaccharide hyaluronic acid (HA).[1] Notably, the anion characteristics necessary for significant LPTP chemosensor color changes remain to be fully defined.

Subsequently, we developed a 96-well plate format assay for sensitive detection of oversulfated chondroitin sulfate A (OSCS) in heparin active pharmaceutical ingredient (API) and crude heparin based on LPTP and heparinase digestion of heparin.[4] The assay utilized several distinct characteristics of heparin, OSCS, and LPTP, including the inhibition of heparinase I and II activity by OSCS, the dependence of heparin−LPTP spectral shifts on molecular size, and the different associations of heparin fragments and OSCS to LPTP. These factors combine to enable naked-eye detection of the presence of 0.1% spiked OSCS in 10 μg of heparin sodium API. Importantly, the assay was insensitive to the presence of common heparin impurities (e.g., 10% (w/w) dermatan sulfate (DS) or CSA).

In the development of the LPTP assay we observed that, similar to the nonsulfated polysaccharide HA, OSCS does not cause a shift to the red in the absorbance spectrum with LPTP association. In addition, the difference in conformation of the uronic acid residues in DS or CSA (i.e., DS contains iduronic acid, while CSA contains glucuronic acid) causes significantly different shifts in the LPTP−glycosaminoglycan (GAG) absorbance spectra. Thus, to further assess the ability of the

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LPTP/heparinase test to flag contaminated heparin drugs, the absorbance characteristics of LTPT solutions with compounds that might be added for economic reasons (i.e., economically motivated adulterants (EMAs)) needed to be determined.

In the United States, heparin APIs are required to be obtained from porcine intestinal mucosa.[5] Crude heparin is fractionated from intestinal tissue and consists of a mixture of heparin and other related GAGs, including heparan sulfate (HS), DS, HA, and CSA.[6] The less sulfated GAGs are removed from crude heparin during a series of isolation steps in the manufacture of the API form.[5] Heparin sodium can be processed further by chemical or enzymatic means to produce LMWHs.[7]

In 2007−2008, heparin sodium API drug products were found to be contaminated with OSCS, and these drugs were associated with deaths and adverse events.[8] OSCS is a synthetic material made by oversulfation of CSA and has approximately 20−25% of the anticoagulant activity of heparin.[9,10] At the time of the heparin crisis, OSCS synthesized from animal cartilage was inexpensive and readily obtainable in the marketplace as a nutraceutical used to self-treat osteoarthritis.[11] Thus, heparin could have been cut with the less expensive anticoagulant (i.e., OSCS or other EMAs) to increase profit while only marginally decreasing the anticoagulation time observed in the heparin sodium USP monograph sheep plasma test being used at that time.

In 2008 and 2009 the USP monograph tests were revised to be sensitive to the presence of OSCS and other possible GAG contaminants in heparin sodium. However, these tests require expensive equipment (e.g., high-field NMR) with expert operators to identify the wide range of possible GAG-like contaminants in heparin sodium. In addition, these tests are not amenable to high-throughput screening or field testing.

Because of the need to ensure the safety of the heparin supply chain, a number of assays have been applied to detect OSCS and other oversulfated GAGs in heparin sodium.[12] The most sensitive methods for detection of oversulfated forms of CSA, DS, HS, HA, and/or heparin include SAX-HPLC (~0.1% (w/w) OSCS, OSDS, OSHS, or OSH),[13] a heparin immunoassay (0.1% OSCS and OSDS),[14] and a real time PCR assay for Taq polymerase (0.16% OSCS, OSDS, and OSHS).[15] In addition, Zhang et al. evaluated PAGE and NMR for analysis of heparin byproduct as well as many other potential contaminants spiked in heparin sodium and observed limits of detection from 0.5% to 10% for NMR and from 0.1% to 5% for PAGE.[16] None of these assays are easily adaptable to field testing.

We recently showed the capacity of a colorimetric microplate LPTP/heparinase test to detect OSCS in heparin sodium API by the naked eye down to the 0.1% level and in crude heparin down to the 0.1% level with a plate reader.[4] However, the sensitivity of the test for other possible EMAs in heparin or for detection of contaminants in LMWHs has not been evaluated. In this work, with selected reagents, we define the critical structural characteristics necessary for significant LPTP color shifts. In addition, we determine the capacity of the LPTP/heparinase test to detect OSCS spiked into smaller molecular weight heparin drugs. Finally, we show that the LPTP/heparinase assay robustly detects a selection of possible economically motivated adulterants in heparin sodium.

EXPERIMENTAL SECTION

Materials. Chondroitin sulfate A sodium salt from bovine trachea, bovine serum albumin (BSA), and heparinase II (E.C. 4.2.2.8) from *Flavobacterium heparinum* were purchased from Sigma-Aldrich (St. Louis, MO). Heparinase I (E.C. 4.2.2.7) from *Flavobacterium heparinum* was purchased from the Associates of Cape Cod (Falmouth, MA). The heparin sodium identification standard was purchased from USP (Rockville, MD). A nonsulfated polysaccharide standard of MW = 11800 derived from *Aureobasidium pullulans* was purchased from Polymer Laboratories (United Kingdom). The nonsulfated linear polysaccharide consists of repeating maltotriose units with monosaccharides linked 1 → 4 within the triose units and 1−6 between units. Dalteparin, tinzaparin, and enoxaparin were obtained from sources approved to supply the U.S. marketplace. Chondroitin sulfate B (also known as dermatan sulfate) from porcine intestinal mucosa was purchased from Calbiochem (La Jolla, CA). Heparan sulfate fraction I from porcine intestinal mucosa was purchased from Celsus (Cincinnati, OH). Arixtra (fondaparinux sodium, manufactured by GSK) was purchased as an injectable solution at 12.5 mg/mL from Bradley Care Drugs (Bethesda, MD). Porcine heparin molecular weight standards denoted as 15000, 9000, 5000, and 3500 molecular weights were obtained from an industry source. The 20000 molecular weight heparin standard was purchased from Neoparin (Richmond, CA). Nunc Maxisorb microplates were purchased from Fisher (Pittsburgh, PA).

OSCS was synthesized by sulfation of chondroitin sulfate A following the literature procedure.[10] Preparation of oversulfated forms of DS, heparin, and heparan sulfate and partially sulfated CSA (PSCS) were synthesized as previously described.[13] The percent sulfur content analysis was performed by pyrolysis on samples pressure dialyzed with a 1000 molecular weight cutoff filter versus over 1 L of water as previously described.[13]

Leclerc Polythiophene Polymer. 3-(2-(*N*-(*N*′-Methylimidazole))ethoxy)-4-methylthiophene (LPTP) was synthesized following the literature procedure.[2] Specific details of the synthesis to obtain optimal colorimetric response of the polymer were previously described.[4]

Heparinase Treatment. Heparin sodium/LMWHs 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 stocks. Heparin sodium (10 μg) or LMWHs (10 μg) were digested in microcentrifuge tubes by 0.2 mU of heparinase I plus 0.1 mU of heparinase II or treated with buffer (20 mM ammonium acetate, 2 mM calcium acetate, pH 7.4) for 30 min at 37 °C. For OS-spiking studies, OSCS, OSH, OSDS, or OSHS were serially diluted and added to USP heparin at the indicated percentage (w/w) prior to heparinase treatment. For LMWHs, only OSCS contamination was studied. The samples were boiled for 3 min to terminate the enzyme activity. The optimal incubation time and heparinase concentrations for LMWH digestions were determined in preliminary studies.

Polythiophene Microplate Assay. Polysaccharides were prepared at 0.5−1 mg/mL (10−20 μg/well). Some samples were run neat; others were treated with heparinases in microcentrifuge tubes. A 20 μL volume of each sample was transferred to a Nunc Maxisorb plate, and 180 μL of fresh polymer (0.3 mM, MW 256.75 based on repeat unit) in Milli-Q water (<18 mΩ) was added. The plate was gently tapped to mix, and then the absorbance values from 300 to 625 nm were measured every 5 nm using a BioTek microplate reader (Winoski, VT). The spectral data were exported to Microsoft Excel 2003 for analysis.

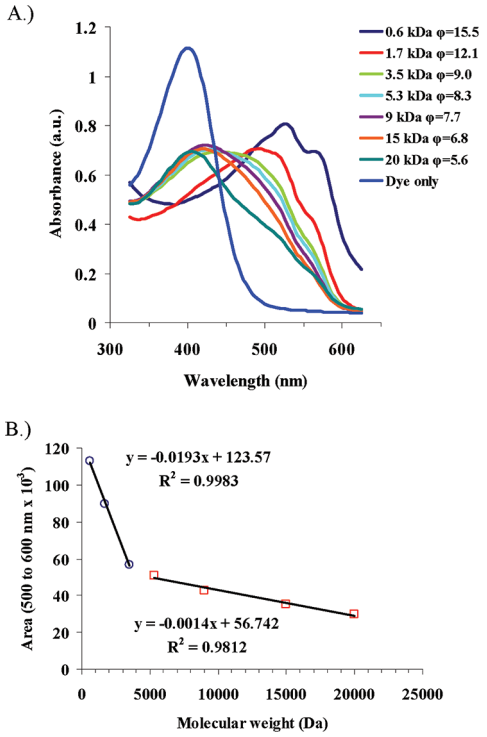
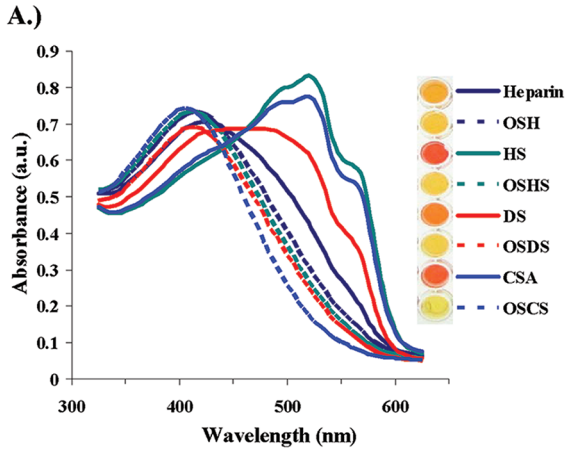


Figure 1. Panel A shows overlaid plots of the absorbance spectra of LPTP solutions in the presence of 10 μg of heparin discaccharide (MW = 600), fondaparinux (MW = 1700), and isolated heparin digestion fractions (MW = 3500−15000). The figure legend shows the φ value for each sample at 525 nm ($\varphi = (I - I_o)/I_o$, where I and I_o are the absorbances at 525 nm in the presence and absence of analyte). Panel B shows the plot of the area under the curve from 500 to 600 nm from the spectra in panel A versus molecular weight. The trendlines, linear equations, and R^2 values from the fits derived from linear regression analysis of the low molecular weight range (600−3500) or the high molecular weight standards (5300−20000) are shown.



B.)

GAGs	λ Max (nm)	φ (525 nm)	% Sulfur[a]
Heparan Sulfate	520	12.9	5.0
CSA	520	11.9	5.4
DS	440	9.3	6.3
OSHS	410	3.5	10.5
Heparin	420	6.2	10.9
OSDS	415	3.2	12.5
OSCS	405	2.1	14.4
OS Heparin	415	4.1	14.9

[a]By pyrolysis w/w%, see reference[24]

Figure 2. Panel A shows overlaid plots of absorbance spectra of LPTP solutions in the presence of 20 μg of heparin sodium, heparan sulfate, dermatan sulfate, and chondroitin sulfate A and their oversulfated forms OS-heparin, OSHS, OSDS, and OSCS. The inset shows a photograph of the LPTP solutions. Panel B shows the wavelength of peak absorbance (λ Max) and a normalized absorbance (φ) at 525 nm arranged in order of increasing weight percent sulfur content.

Plates were placed in a CAMAG Reprostar 3 light box and photographed using a Canon Powershot SX10 IS.

Statistical Analysis. Results are expressed as the mean plus or minus the standard deviation (SD) from at least three independent experiments each containing triplicate samples per group. Statistical significance was assessed by using a one-tail $P(T \leq t)$ t test ($p < .05$) for comparison with the appropriate control group (e.g., no OSCS, digest).

RESULTS AND DISCUSSION

LMWH−LPTP Characterization. We previously demonstrated that addition of OSCS, heparin, DS, or CSA to LPTP solutions resulted in varying amounts of color change (yellow to red) with concomitant shifts of the peak absorption wavelength to 405, 420, 440, and 520, respectively.[4] Furthermore, lower molecular weight heparins formed upon heparinase digestion and elicited an LPTP spectral shift which was larger than that observed for the corresponding nondigested higher molecular weight species. Given these observations, we developed and validated an assay using LPTP for detection of OSCS in heparin based on the ability of OSCS to inhibit heparinase I and II activity and the molecular weight dependence of heparin fragment−LPTP spectral shifts. Here, to define the requirements for significant LPTP spectral shifts, we examined the effect of the GAG chain length and sulfation state on the LPTP solution absorbance spectra and color (Figures 1 and 2).

Molecular Weight Dependence. For heparin, the most abundant disaccharide is the trisulfonated L-iduronic acid sulfated at carbon 2, linked β-1−4 to D-glucosamine that is *N*-sulfated and sulfated at the 6 carbon (also known as $I_{2S}-A_{NS,6S}$).[17,18] We obtained $I_{2S}-A_{NS,6S}$ disaccharide from a commercial source. In addition, we performed complete heparinase digestion of heparin to obtain a mixture of heparin-derived disaccharides (see ref 17 for the composition of the disaccharide mixture). In addition, we purchased a heparin-like pentamer (fondaparinux) and obtained several fractions purified from heparin sodium ranging in average MW from 3500 to 20000.

As shown in Figure 1A, LPTP solutions of different average molecular weight heparin standards show a progressively larger red absorbance shift with decreasing size. To compare the magnitude of the LPTP shifts between these heparin standards, we used normalized absorbance ($\varphi = (I - I_o)/I_o$, where I and I_o are the absorbances at 525 nm in the presence and absence of analyte). The greatest φ value of the saccharides tested is observed for $I_{2S}-A_{NS,6S}$ disaccharide, consistent with the

similar φ values observed for disaccharide level heparin digests.[4]

Because the LPTP polymer solutions had minimal absorbance in the 500−600 nm region of the spectrum, we assessed the relationship between the molecular weight and the magnitude of the LPTP spectral shifts upon mixing with heparin molecular weight standards using the change in the area under the curve over this 100 nm wavelength range. The plot of the area under the curve versus molecular weight shows a biphasic behavior; a steep linear response is observed between MW = 577 and MW = 3500, while a shallower linear response is noted for the higher molecular weight range (5300−20000) (Figure 1B). The molecular weight of the most abundant heparin disaccharide is 577. Thus, the break point observed between these two ranges is below a 12-mer chain length (i.e., six disaccharide pairs). Presumably, the heparin chains shorter than ∼12-mers associate with LPTP chains to cause greater changes in chromophore molar absorptivity and/or highly planar conjugated backbone conformation than larger heparin chain lengths.

By contrast, other chemosensors applied to heparin assays require molecular weights above a certain size for effective interactions (e.g., see refs 19−21). For example, the observed increased fluorescence of the heparin sensor polymer-H requires a degree of sulfation greater than ∼1.2 and a molecular weight above 3000.[20] In another example, the fluorescence quenching observed with a ruthenium compound based heparin assay was larger for heparin sodium (MW ≈ 17000) than for a low molecular weight heparin (∼4000), and no quenching was observed with fondaparinux (MW = 1700).[21] Furthermore, in the heparin ELISA test,[14] the sensitivity of the assay to low molecular weight heparin (∼6000) is 3−4-fold less than that to heparin sodium, which indicates a higher minimal molecular weight requirement.

Degree of Sulfation: LPTP Characterization. Comparisons of the effects of the number of sulfate groups per GAG repeating unit on LPTP color shifts have been complicated by a lack of reagents with differences solely in the number of sulfate groups but not in conformational elements. Here we attempted to assemble a set of reagents to help define the GAG structural elements that lead to robust color change of LPTP. Therefore, we briefly review the structure and composition of the reagents studied in this work.

As stated above, heparin is a linear polysaccharide of primarily trisulfated $I_{2S}-A_{NS,6S}$ (∼60−70%) repeating units where the I-ring is α-L-iduronic acid sulfated at the carbon in position 2 and the A-monosaccharide is an *N*-sulfated and 6-sulfated glucosamine. Heparin also contains populations of A-monosaccharides with *N*-acetyl groups (∼15%) and G-monosaccharides (β-D-glucuronic acid, ∼15%[18]). In contrast with heparin, heparan sulfate is monosulfated on average and contains ∼66% G-monosaccharides (with a lesser amount of I-monosaccharide) linked from position 1 to position 4 to *N*-sulfated or *N*-acetylated glucosamine A-rings.[18]

For the chondroitins, CSA is ∼100% monosulfated [$-G_{2OH}$-(1,3)-$A_{NAc,4S}-$] or [$-G_{2OH}$-(1,3)-$A_{NAc,6S}-$] (i.e., a G-ring with a hydroxyl group at carbon 2, linked 1 → 3 to an *N*-acetylated and 4-sulfated or 6-sulfated substituted galactosamine A-ring). The G 1 → 3 A-linked monosaccharides of the chondroitins are linked 1 → 4 between disaccharide units. In contrast with CSA, DS is primarily composed of monosulfated [$-I_{2OH}$-(1,3)-$A_{NAc,4S}-$] or [$-I_{2OH}$-(1,3)-$A_{NAc,6S}-$]. In addition, HA is primarily nonsulfated [$-G_{2OH}$-(1,3)-$A_{NAc}-$] with a 1 → 4 linkage between disaccharides.

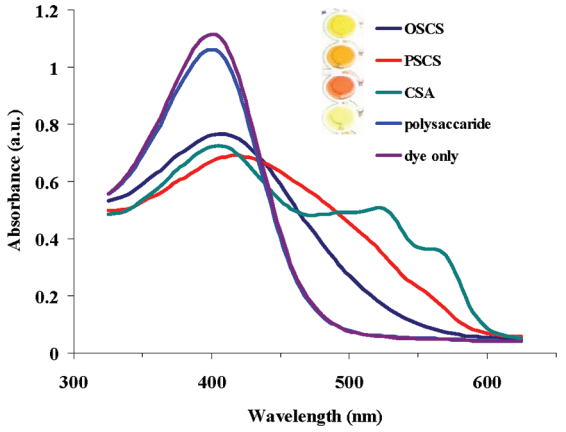


Figure 3. Overlaid plots of absorbance spectra of LPTP solutions in the presence of CSA, OSCS, PSCS, and an unsulfated polysaccharide (MW = 11800). The inset shows a photograph of the LPTP solutions.

The synthesis of fully oversulfated versions of these GAGs has been shown to cause conformational changes in the uronic acid moieties.[10,22] For example, the oversulfation of CSA ([$-G_{2OH}$-(1,3)-$A_{NAc,4S}-$]) to fully sulfated CSA ([$-G_{2S,3S}$-(1,3)-$A_{NAc,4S,6S}-$]) has been reported to alter the G-ring conformational preference from $^4C_1$ to $^1C_4$ because of repulsion of proximal 2-*O*- and 3-*O*-sulfate groups. The $^1C_4$ glucuronic acid was proposed to closely resemble the 2-*O*-sulfonated iduronate residue found in heparin. Similarly, Toida et al. present data that indicate that the full oversulfation of DS, HA, or HS leads to distortion of the G- or I-monosaccharide conformations in these GAGs.[22] Notably, these conformational changes are associated with significant increases (40−60%) in the anti-factor IIa activity of these molecules. By contrast, oversulfation of heparin (to 4.7 sulfates/disaccharide) decreases its anti-factor IIa activity by ∼80%.[22]

For the set of reagents examined here, the ascending order of normalized absorbance values was OSCS (φ = 2.1), OSDS (φ = 3.2), OSHS (φ = 3.5), OSH (φ = 4.1), heparin (φ = 6.2), dermatan sulfate (φ = 9.3), CSA (φ = 11.9), and heparan sulfate (φ = 12.9). In addition, all the solutions of LPTP and the oversulfated compounds tested remained yellow, while LPTP solutions of their progenitor molecules resulted in colors ranging from orange to red (Figure 2A, inset).

For LPTP solutions of these GAGs, the largest absorbance wavelength maximum shifts (from 400 nm for dye alone to bands at 520 and 570 nm) were observed for polysaccharides that contain high percentages of β-D-glucuronic acid as opposed to α-L-iduronic acid (i.e., CSA and HS, parts A and B, respectivley, of Figure 2). A direct comparison of I- versus G-monosaccharide containing GAGs is the relative LPTP shifts observed with solutions of CSA versus DS. In addition, the presence of a G-monosaccharide and a sulfate group was necessary for larger LPTP shifts because the nonsulfated G-containing GAG HA ([$-G_{2OH}$-(1,4)-$A_{NAc}-$]) has been shown not to cause a significant red shift with LPTP.[1]

The monosulfated GAGs (CSA, DS, and HS) were the most shifted, while the oversulfated forms of these compounds (∼4−5 sulfates/disaccharide) resulted in significantly decreased LPTP spectral shifts. Heparin averages ∼2.5 sulfates/disaccharide and

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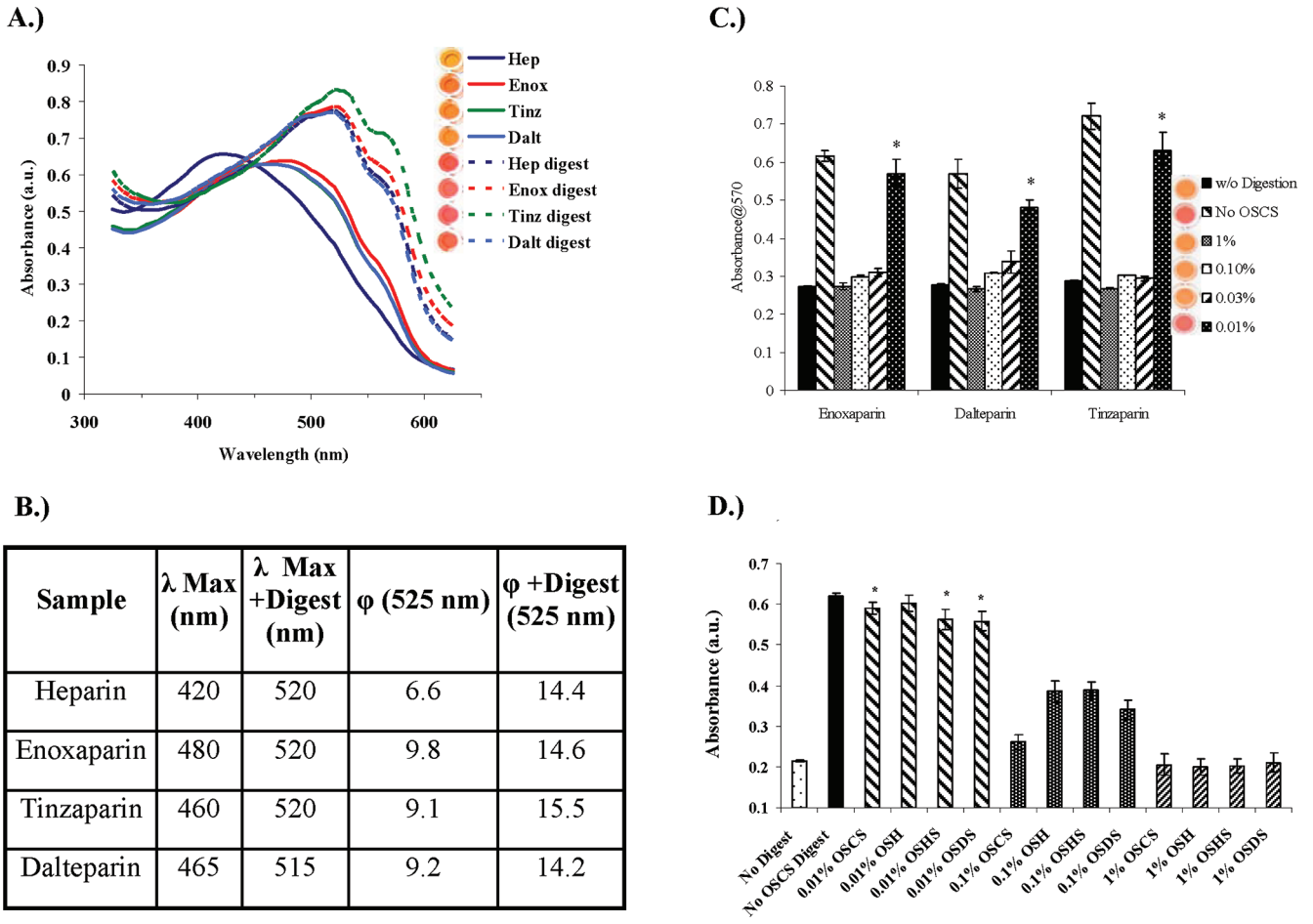

Sample	λ Max (nm)	λ Max +Digest (nm)	φ (525 nm)	φ +Digest (525 nm)
Heparin	420	520	6.6	14.4
Enoxaparin	480	520	9.8	14.6
Tinzaparin	460	520	9.1	15.5
Dalteparin	465	515	9.2	14.2

Figure 4. Overlaid plots of absorbance spectra of LPTP in the presence of (A) untreated heparin sodium (UFH), enoxaparin, tinzaparin, or dalteparin (solid lines) or heparinase I and II treated samples (dashed lines). The colors of polymer solutions for a representative of each sample are shown in the inset. Panel B shows the wavelength of peak absorbance (Max, nm) and a normalized absorbance (φ). Panel C shows the mean absorbance values (570 nm) ± SD of six OSCS spiked and unspiked samples digested and mixed with LPTP. The inset photo shows an example of the plate well colors of enoxaparin spiked with different levels of OSCS. Panel D shows the mean absorbance values (570 nm) ± SD of OSCS, heparin (H), HS, and DS spiked and unspiked samples digested and mixed with LPTP. The digest group refers to heparin without the addition of OS forms. Results are from three independent experiments of triplicate samples. Statistical analysis was assessed with use of a one-tail $P(T \leq t)$ t test (*, $p < .05$) for comparison with the no OS group.

has a spectral shift intermediate between those of the monosulfated and fully oversulfated GAGs tested here. For a more direct comparison, we evaluated the LPTP interactions with OSCS, a partially sulfated CSA, CSA, and a nonsulfated polysaccharide (Figure 3). The weight percent sulfur determined by pyrolysis of CSA was 5.4%, the partially sulfated CSA was 11%, and OSCS was 14.4%.[13] As shown in Figure 3, CSA has the greatest spectral shift, followed by the partially sulfated form and then the oversulfated form.

The combination of LPTP and a nonsulfated noncarboxylated linear polysaccharide (MW = 11800) showed almost no change from solutions of LPTP alone, confirming the requirement for some sulfated or anionic groups to bind to the cationic polymer (Figure 3A). Furthermore, HA, which consists of primarily nonsulfated carboxylated [$-G_{2OH}$-(1,4)-$A_{NAc}-$] disaccharides was shown not to shift the LPTP absorbance spectra significantly, indicating the preference for sulfated G-monosaccharide groups for LPTP association.[1] A combination of equal amounts of CSA and OSCS with LPTP resulted in a spectral profile similar to that of the partially sulfated form (data not shown). On the basis of the properties of the LPTP polymer established with this unique set of reagents, we evaluated the capacity of the LPTP/heparinase test to detect OSCS in LMWHs.

Detection of OSCS in Low Molecular Weight Heparins by the LPTP/Heparinase Test. Here, to evaluate whether the assay could also be used to detect OSCS in LMWH, we evaluated the effect of heparinase I and II treatment of three different LMWHs (enoxaparin, dalteparin, and tinzaparin). These LMWHs have weight average molecular weights ranging from ca. 4000 to 6000. Significant spectral shifts and concomitant color changes were observed when USP heparin or the LMWHs were treated with heparinase I and II at 37 °C for 30 min as shown in Figure 4A. The inset shows the color of the wells with (dark orange-red) and without (pale orange) heparinase treatment.

Consistent with their smaller size, each of the LMWH−LPTP complexes, without heparinase treatment, had higher φ values (φ = 9.1−9.8) than the heparin sodium−LPTP complex (φ = 6.6) (Figure 4B). Following heparinase treatment, the LMWH

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and heparin−LPTP complexes had much higher φ values ranging from 14.2 to 15.5. Interestingly, for reasons which are not clear, tinzaparin, which is manufactured from heparin sodium by a heparinase-mediated cleavage reaction,[7] had the highest normalized absorbance value upon additional heparinase treatment in our assay (φ = 15.5) compared to digests of dalteparin, enoxaparin, or heparin sodium (φ = 14.2−14.6).

In this study, we assessed the effect of the presence of OSCS (1.0−0.01% OSCS in 10 μg of LMWHs) on enoxaparin, dalteparin, and tinzaparin digestions and LPTP spectral properties. OSCS inhibited LPTP color changes and spectral shifts with each of the LMWHs, and the inhibition of the color change could be observed visually at levels of 0.03% OSCS (Figure 4C). Significant differences from non-OSCS-containing enoxaparin, dalteparin, and tinzaparin control digestions (hatched bars) were observed using a plate reader for analysis at the 0.01% level (Figure 4C, $p < 0.05$). For comparison, Luhn et al. reported an OSCS detection limit of 0.5% (w/w) in a polymer-H/enoxaparin digest.[19]

Possible EMA Detection in Heparin. The oversulfation of CSA, DS, HA, or HS has been shown to *increase* the anticoagulation activity of these compounds.[22] By contrast, oversulfation of heparin has been shown to *decrease* the anticoagulation activity of heparin.[22] Thus, in addition to OSCS, if OSDS, OSHA, or OSHS were inexpensively available, they would be candidates for possible economically motivated adulterants to the heparin supply chain. Furthermore, GAG waste and tank bottom fractions from the crude heparin purification process have been proposed as possible sources of inexpensive starting materials for oversulfation.[6] The tank bottom consists primarily of DS, while GAG waste consists of primarily DS, heparin, and HS. Therefore, oversulfation of these side stream products would produce OSDS, OS-heparin, and OSHS.

The current USP monograph assays for heparin sodium contain tests for the structure and composition of the drug that are sensitive to charge (i.e., strong anion exchange HPLC) and structure (i.e., 1D ^{1}H NMR) and thus robustly detected EMAs.[13] However, a simple inexpensive high-throughput assay for possible EMAs is desirable to screen more samples in a shorter time frame. Therefore, in this study, we assessed the effect of the presence of OSCS, OSDS, OSH, and OSHS (1.0−0.01% OS (w/w) in 10 μg of USP heparin) in the LPTP/heparinase assay. All the OS forms spiked in heparin at 1% (w/w) completely inhibited LPTP color changes and spectral shifts as shown in Figure 4D. OSCS produced significantly greater inhibition of mean absorbance than the other OS forms at 0.1%. Differences from the non-OS control could be detected in the 0.01% spike with OSCS, OSHS, and OSDS ($p < 0.05$) but not with OS-heparin.

For comparison to other assays, Bairstow et al. showed the detection of OSCS or OSDS (0.1%) in a heparin ELISA test was about a log more sensitive than the detection of OS-heparin.[14] In a Taq-polymerase-based assay, OSCS was detected about 3-fold more sensitively (0.16%, at the limit of detection of 500 pg of OSCS) than OSDS or OSHS.[15] Here we demonstrate that the LPTP/heparinase assay has 0.1% (w/w) naked-eye detection sensitivities for oversulfated GAGs which are similar to the LODs observed using the USP heparin sodium monograph SAX-HPLC approach.

CONCLUSIONS

We utilized a chemosensor that forms distinct colored complexes with heparin/heparin fragments and the known property of OSCS and other oversulfated GAGs to inhibit heparinases to develop a microplate assay with good sensitivity for the detection of OSCS in heparin. Here we present data to establish structural features (e.g., size, sulfation state, and conformation) necessary for significant color change of LPTP solutions. For example, we quantify the molecular weight dependence of the LPTP spectral shifts which are largest for disaccharide level digests of heparin.

Because of the increasing use of LMWHs and the OSCS contamination of enoxaparin in 2008, we evaluated whether the LPTP/heparinase test could be used for detection of oversulfated agents in LMWHs. OSCS was detected in three different LMWHs to a level of ≥0.03% visually and to a level of 0.01% with a plate reader. We note that the sensitivity of the assay is dependent upon the heparinase I and II concentrations and incubation time with a steep response. Thus, to obtain optimal response at low levels of contamination (<0.01%), assay protocols need to be strictly followed. However, the sensitivity of visual detection of OSCS in LMWHs was robust with the 0.1% level 10-fold above the detection threshold of the assay using the plate reader.

We evaluated the role of sulfation levels on the spectral profiles of chemosensor complexes. The presence of at least one sulfate group was necessary to obtain LPTP spectral shifts. All of the synthetic oversulfated reagents used in this study (OSCS, OSH, OSHS, OSDS) combined individually with LPTP remained yellow in color as did the nonsulfated polysaccharide tested. These data suggest that one sulfate per disaccharide is optimal and the addition of further sulfates decreases LPTP spectral shifts.

In addition, we observe that G-monosaccharide containing GAGs resulted in greater LPTP spectral shifts than those containing I-monosaccharides. Thus, the orientation of the carboxylate moiety affects the magnitude of the LPTP spectral shift, with the G-monosaccharide association resulting in distinct peaks in the absorbance spectra (i.e., maxima at 520 and 570 nm).

We found the LPTP/heparinase assay amenable to detection of several oversulfated GAGs which are possible economically motivated adulterants in heparin sodium. On the basis of the results presented here, the heparin digest−LPTP test is a robust and sensitive test for oversulfated contaminates in heparin API and LMWH and is potentially amenable to field testing. In future work we will assess how robust stabilized lyophilized enzymes are under field conditions.

The enzymes are a critical practical issue for the cost and routine use of the LPTP−heparinase assay for monitoring the heparin supply chain. In this work and in our previous work, we show that the LPTP/heparinase test can visually detect OSCS and other oversulfated GAGs in the presence of crude heparin, heparin sodium, and low molecular weight heparin types. We have optimized the heparinase concentrations, reaction time, and storage conditions to minimize the variability with good results. We estimate the cost of running a 96-well plate with the current commercial sources is approximately $300 per plate. This compares favorably to the cost of performing ELISA with a much shorter assay time for the LPTP/heparinase test.

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