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Naked-Eye Detection and Quantification of Heparin in Serum with a Cationic Polythiophene

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A strategy for naked-eye detection and quantification of heparin in biological media, such as fetal bovine serum (FBS) is demonstrated by monitoring the absorbance change of a water-soluble cationic polythiophene. The negatively charged heparin interacts with positively charged polythiophene through electrostatic interaction, which leads to polymer conformation and color change from yellow to orange in solution. Under optimized conditions, addition of heparin derivatives, such as hyaluronic acid or chondroitin 4-sulfate to the same polymer solution leads to less change in polymer conformation and solution color due to their lower charge density as compared to that of heparin. Increasing the detection temperature or simply adding some organic solvent to the aqueous media reduces the polymer−polymer interchain π stacking, and the polymer color change can be used to clearly differentiate heparin from its analogues in homogeneous solutions. Quantification of heparin is also demonstrated by correlating the changes in polymer absorbance to the heparin concentration. A linear calibration curve is observed in the 0−6.7 U/mL and 0−2.2 U/mL ranges for heparin quantification in pure water and in FBS, respectively.

Heparin is a highly sulfated linear acidic polysaccharide.[1] It plays an important role in physiological processes through electrostatic interaction with diverse proteins such as antithrombin, fibroblast growth factor, extracellular superoxide dismutase, lipoprotein, etc.[2−4] Heparin has long been used as an anticoagulant to convert antithrombin into suicide substrate for procoagulant proteinase.[5] During the anticoagulant therapy and surgery, close monitoring of heparin levels is of vital importance to avoid complications such as hemorrhage or thrombocytopenia induced by heparin overdose.[6−8] The therapeutic dosing level of heparin is 2−8 U/mL (17−67 μM) during cardiovascular surgery and 0.2−1.2 U/mL (1.7−10 μM) in postoperative and long-term care. The development of quick and reliable methods that allow heparin detection and quantification is of practical importance.

Many assays for heparin quantification and/or detection have been established. Traditional laboratory assays for heparin quantification are indirect, which rely on monitoring of the activated coagulation time (ACT) or the activated partial thromboplastin time (aPTT).[9] These assays are not sufficiently accurate and reliable because of their lack of specificity and potential interference from other factors.[10] Recently, a wide variety of fluorescent and colorimetric methods have been reported for heparin sensing.[11−20] Synthetic cationic chromophores, such as tripodal boronic acids,[11]polycationic calyx[8]arenes,[12] polymethinium salts,[13] and a chromophore-tethered flexible copolymer,[14] have been used as heparin indicators. Most of these assays adopt fluorescence quenching as signal output, which is undesirable for visual detection of heparin. Subsequently, a peptide based sensor was reported to show fluorescence increase upon interaction with heparin.[15] However, the detection window of the fluorescence turn-on assay (0−0.4 U/mL) is out of the clinical range. More recently, a cationic silole derivative has been reported to show a wide range response to heparin, although it tends to aggregate in the presence of nonspecific proteins to give strong background signals.[16] To further differentiate heparin from its derivatives, an array of self-assembled supramolecular fluorescent receptors based on a modified cyclodextrin host−guest complex has been developed.[17] However, among these methods reported in the literature, very few can operate in complex biological media, such as plasma or serum to give a wide quantification range as needed for clinical treatment.[18]

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Conjugated polymers have been widely used for chemical and biological sensing.[21] Conjugated polymers are superior to small molecules in responding to minor environmental perturbs, due to their large delocalized π conjugated system and efficient inter/intrachain energy transfer.[21] Recently, we designed two benzothiadiazole (BT) containing conjugated polymers for naked-eye detection and quantification of heparin.[22,23] The first is a cationic poly(fluorene-*co*-phenylene) derivative with a small fraction of BT, which changes its fluorescent color from blue to orange upon interaction with heparin in solution.[22] The second is a cationic poly(fluorene-*co*-benzothiadiazole), which shows fluorescence turn-on response (from dark to orange) to heparin.[23] Both polymers are able to quantify purified heparin in nearly the whole clinical detection range. However, they are not suitable for heparin sensing in plasma or serum as the polymer fluorescence is greatly affected by proteins in these biological media. It is highly desirable to develop new assays for real-time naked-eye detection and quantification of heparin in complex biological media.

Water-soluble polythiophenes have been widely used for the detection of nucleic acids, polysaccharides, and small biomolecules.[23−29] These polymers can undergo unique conformational changes upon interaction with different analytes through electrostatic interaction, resulting in absorbance and solution color changes. In addition, by binding with helical biomolecules or asymmetric small molecules, the induced circular dichroism (ICD) signals of polythiophene can be observed in the $\pi \rightarrow \pi^*$ transition region.[25,26,28−31] In this contribution, we take advantages of the optical changes of cationic poly(1-methyl-3-[2-[(4-methyl-3-thienyl)oxy]ethyl]-1*H*-imidazolium) (P4Me-3TOEIM) to develop a new assay for visual heparin detection and quantification in blood serum. Attempts have also been made to understand the P4Me-3TOEIM/polysaccharides supercomplex formation by the use of different techniques and through fine-tuning experimental conditions.

EXPERIMENTAL SECTION

Materials. All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Heparin sodium salt from bovine intestinal mucosa (Fluka) has 170 U/mg. The molecular weight of heparin was determined by disaccharide (644.2 g/mol), and 1 μM heparin corresponded to 0.11 U/mL. Chondroitin 4-sulfate sodium salt from bovine trachea (BioChemika) and hyaluronic acid salt from *Streptococcus equi* (BioChemika) were used as received. P4Me-3TOEIM was synthesized according to the literature.[25] The fresh stock solution (2 mM) of P4Me-3TOEIM in water was prepared based on repeat unit. Stock solutions of heparin, chondroitin 4-sulfate, and hyaluronic acid in water were prepared based on disaccharide units. Fetal bovine serum (FBS, HyClone) was stored at −20 °C and was defreezed at room temperature before use. Milli-Q water (18.2 MΩ) was used for experiments.

Instruments. Absorption spectra were recorded on a Shimadzu UV-1700 spectrometer. The software was UV Probe Version 2.21 and scan speed was "fast". The temperature was controlled by Shimadzu temperature controller, using "normal" mode. Either a 3 mL quartz cuvette or a 200 μL 8 microcell cuvette was used for experiments. Laser light scattering measurements were performed using a Brookhaven Instruments Corporation (BIC) 90 plus instrument with λ = 659 nm at a scattering angle of 90 °C. The particle diameters were calculated using Zeta Plus Particle Sizing software. Circular dichroism spectra were recorded on Jasco J-810 Circular Dichroism Spectropolarimeter equipped with a Peltier thermoelectric type temperature control system. The instrument was controlled by Jasco's Spectra Manager software, version 1.53.01. Two channels were used to detect circular dichroism and absorption spectra simultaneously. Photographs of the polymer solutions were taken using a Canon EOS 400D digital camera.

Polysaccharide Detection at Room Temperature. P4Me-3TOEIM stock solution (480 μL, 2 mM) and 2520 μL of water were mixed and transferred to a 3 mL quartz cuvette to yield final [P4Me-3TOEIM] = 0.32 mM. Polysaccharide solution (90 μL, 2 mM) was then added dropwise to each cuvette at an interval of 3 μL. Upon each addition, the mixture was gently shaken at room temperature before UV−vis measurement. The absorption spectra were collected in the range of 300−700 nm at room temperature.

Polysaccharide Detection at High Temperature. P4Me-3TOEIM stock solution (48 μL, 2 mM) and 252 μL of water were mixed to yield final [P4Me-3TOEIM] = 0.32 mM. Polymer solution (100 μL) was added to each microcell and the 8-microcell cuvette was warmed up to 70 °C in 5 min. Each polysaccharide (7.5 μL) solution (0.8 mM) was then added dropwise to the corresponding microcell at an interval of 0.5 μL. Upon each addition, the mixture was gently mixed using pipet before UV−vis analysis. The absorption spectra were collected in the range of 300−700 nm at 70 °C.

Laser Light Scattering Measurement (LLS). P4Me-3TOEIM stock solution (160 μL, 2 mM), 840 μL of water, and 22.5 μL of heparin or chondroitin 4-sulfate stock solution (2 mM) were mixed and transferred to a 3 mL plastic cuvette and further diluted with 2000 μL of water to yield a final [P4Me-3TOEIM] = 0.11 mM and [polysaccharide] = 15 μM. The temperature was set manually from 30 to 70 °C, and the data were the average of 8 scans. Control experiments were conducted for each of the 0.11 mM polythiophene or 15 μM heparin or chondroition sulfate solution at 30 °C. In addition, LLS for the mixture of 0.11 mM P4Me-3TOEIM and 15 μM heparin or chondroitin sulfate in water/methanol (v/v = 2:3) was also studied at 30 °C under the same experimental conditions.

Circular Dichroism Measurement (CD). P4Me-3TOEIM stock solution (480 μL, 2 mM), 2520 μL of water, and 90 μL of heparin or chondroitin 4-sulfate stock solution (2 mM) were mixed and transferred to a 3 mL quartz cuvette to yield a final [P4Me-

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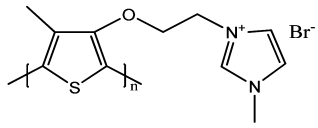
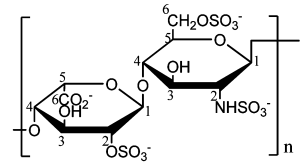
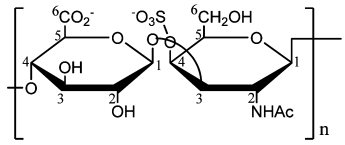


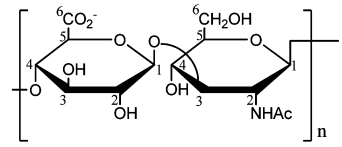
P4Me-3TOEIM



Heparin (Major unit)



Chondroitin 4-sulphate



Hyaluronic acid

Figure 1. Chemical structures of P4Me-3TOEIM, Hep, ChS, and HA.

3TOEIM] = 0.32 mM and [polysaccharide] = 60 μM. The temperature was manually set from 30 to 70 °C, and the scanning speed was 200 nm/minute. The CD and the absorption spectra were collected in the range of 300−700 nm.

Polysaccharide Detection in Solvent Mixture. P4Me-3TOEIM stock solution (480 μL, 2 mM), 1800 μL of methanol, and 720 μL of water were mixed and transferred to a 3 mL quartz cuvette to yield a final [P4Me-3TOEIM] = 0.32 mM. The polysaccharide stock solution (90 μL, 2 mM) was added dropwise at an interval of 6 μL. Upon each addition, the mixture was gently shaken at room temperature before UV−vis analysis. The absorption spectra were collected in the range of 300−700 nm at room temperature. The reference sample was a mixture of methanol and water (v/v = 3:2).

Polysaccharide Detection in Fetal Bovine Serum (FBS). The P4Me-3TOEIM stock solution (48 μL, 2 mM), 30 μL of FBS, and 252 μL of water were mixed to a final [P4Me-3TOEIM] = 0.32 mM in 10% FBS. The mixture was divided into three equal portions and was added to three microcells of an 8-microcell cuvette incubated at 23 °C for 5 min. Each polysaccharide (0−7.5 μL, 0.8 mM) was added separately to each microwell at a 0.5 μL interval. Upon each addition, the mixture was gently mixed using a pipet before UV−vis measurement. The absorption spectra were collected in the range of 300−700 nm at 23 °C. For high temperature measurement, the same experiments were conducted at 70 °C.

RESULTS AND DISCUSSION

Structures of P4Me-3TOEIM and the Polysaccharide Targets. The chemical structures of P4Me-3TOEIM and the polysaccharides are shown in Figure 1. Cationic P4Me-3TOEIM was selected as the polymer probe for this study. The conformational change of the polymer refers to the transition between a planar form and a nonplanar structure of its backbone. The presence of the 4-methyl group on each thiophene ring is essential for the polymer to form a planar structure upon complexation with negatively charged molecules. The polymer will adopt either a planar or nonplanar conformation according to different types of analytes.[32,33] The polysaccharides selected for this study are heparin (Hep), chondroitin 4-sulfate (ChS), and hyaluronic acid (HA), the latter two are often found as contaminates for clinical

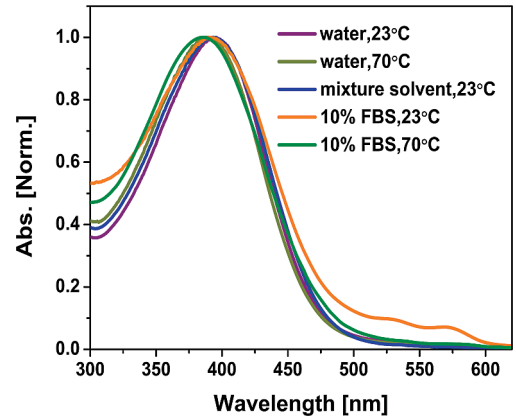


Figure 2. Absorption sepctra of P4Me-3TOEIM under different conditions. Mixture solvent: methanol/water (v/v) = 3:2.

heparin. The main unit of Hep is 1→4 linked uronic acid and glucosamine repeating disaccharide unit (iduronic-acid-2-sulfate→glucosamine-2, 6-disulfate). On average, Hep carries three sulfate groups and one carboxylate group per disaccharide unit. ChS consists of the disaccharide unit formed by 1→3 linked *N*-acetylgalactosamine and glucuronic acid, modified by sulfation in the position 4. ChS possesses one sulfate and one carboxylate moiety per disaccharide unit. HA is formed by 1→3 linked glucuronic acid and *N*-acetylglucosamine, which has only one carboxyl group per disaccharide unit.

Optical Properties of P4Me-3TOEIM. The absorption spectra of P4Me-3TOEIM in different media at room temperature (23 °C) and at 70 °C are shown in Figure 2. At room temperature, the polymer has an absorption maximum at 393 nm, which corresponds to the random coil conformation of the backbone structure. The increase in absorption at 500 nm in 10% FBS is due to polymer conformation changes upon binding to protein mixtures in the medium. When the temperature is increased to 70 °C, the absorption maxima slightly blue-shifted for about 5 nm in both water and 10% FBS, as compared to that at 23 °C. The polymer absorption maximum remains unchanged in a mixture

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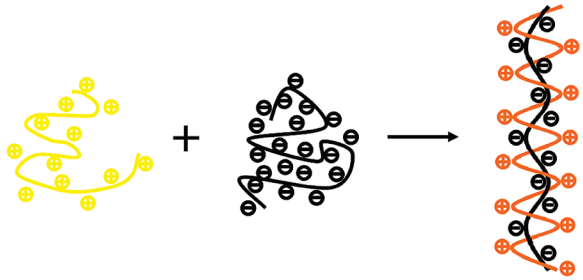
Scheme 1. Schematic Illustration of Heparin Detection



solvent of methanol/water (v/v) = 3:2 at room temperature, Under all conditions, the solution color is light yellow.

Detection Mechanism. The schematic illustration of heparin detection and quantification is shown in Scheme 1. One starts with P4Me-3TOEIM in solution. The polymer takes a random coil conformation, and the solution color appears light yellow. Addition of negatively charged targets induces complexation between the polymer and the targets, the extent of which is dependent on the charge density of the analytes. The polymer/analyte complex formation leads to polymer conformational changes, which are reflected by spectral shift and solution color variations (yellow to orange). Due to different charges and charge arrangement of polysaccharides, the binding of P4Me-3TOEIM to the polysaccharides are expected to take different conformation and aggregation modes and consequently different colors for heparin and its analogues.

Polysaccharide Detection in Water at Room Temperature. Titration of P4Me-3TOEIM with Hep in water at room temperature was monitored by UV−vis spectroscopy, and the spectra are shown in Figure 3A. In these experiments, [P4Me-3TOEIM] = 0.32 mM, and [Hep] varied from 0 to 60 μM at intervals of 2 μM. Upon addition of increased amount of heparin, the absorption maximum is gradually shifted to 420 nm and the absorbance at the longer wavelength gradually increases. An isosbestic point is observed at 430 nm. This transformation in the absorption spectra of P4Me-3TOEIM reflects the gradually increased conformational change of P4Me-3TOEIM upon P4Me-3TOEIM/Hep complex formation. At [Hep] = 60 μM, the solution remains stable for weeks. At this point, the concentration of negative charges from heparin is 240 μM, while the concentration of positive charges from P4Me-3TOEIM is 320 μM. Further addition of heparin will not cause obvious spectral changes; however, the solution becomes less stable due to more complete charge neutralization of the complexes. The solution color changes from yellow to orange accompanying the transition of the spectra, indicating the potential application of P4Me-3TOEIM as a colorimetric probe for Hep sensing.

To study the selectivity of P4Me-3TOEIM toward other polysaccharides, the changes in the absorption spectra of P4Me-3TOEIM in the presence of HA and ChS are studied. As shown in Figure 3B, at [P4Me-3TOEIM] = 0.32 mM, upon addition of 60 μM HA, a very small absorption tail is observed at 500 nm and there is almost no bathochromic shift observed. The solution remains yellow in the presence of HA. This is simply due to the low charge density per disaccharide unit of HA, and the electrostatic attraction between HA and P4Me-3TOEIM is significantly weaker than that between Hep and P4Me-3TOEIM. However, upon addition of 60 μM ChS to P4Me-3TOEIM, obvious long

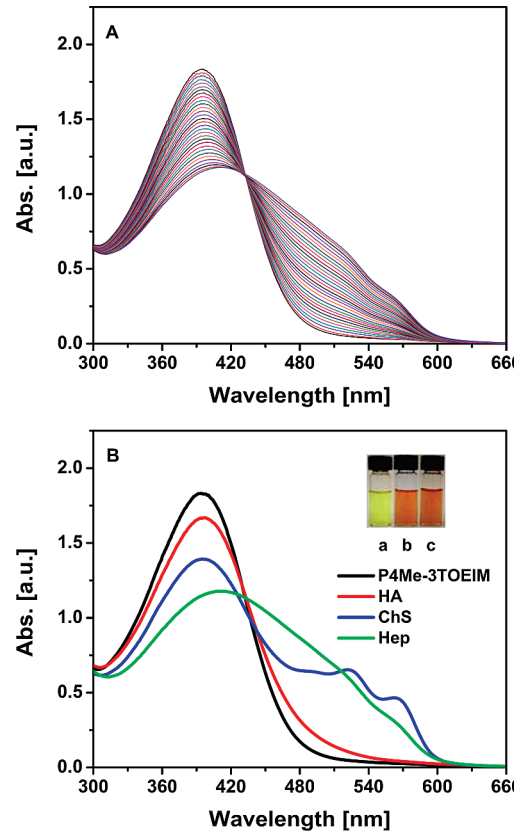


Figure 3. (A) Absorption spectra of P4Me-3TOEIM at [P4Me-3TOEIM] = 0.32 mM in water upon addition of Hep from 0 to 60 μM at intervals of 2 μM. (B) Absorption spectra of P4Me-3TOEIM at [P4Me-3TOEIM] = 0.32 mM in water in the presence of [Hep] = [ChS] = [HA] = 60 μM. Inset shows the photographs of polymer solutions with 60 μM HA (a), ChS (b), and Hep (c).

wavelength absorption peaks at 523 and 576 nm are observed. These vibronic transitions indicate the formation of intermolecular π stacking of the P4-Me-3TOEIM/ChS complex.[28,34,35] Similar to that of Hep, the solution color appears orange. These experiments indicate that at room temperature, the polymer can only differentiate Hep from HA but not Hep from ChS. As a consequence, two strategies have been proposed to solve the problem. One is to add methanol to the detection medium, and the other is to increase the detection temperature.

Polysaccharide Detection in Methanol/Water at Room Temperature. Although both methanol and water are good solvents for cationic polythiophenes, they show preferred solvation for different parts of polymers. Methanol is a better solvent for the hydrophobic backbone with respect to water, while water shows better affinity to the terminal charged side chains.[28] Previous study of folic acid induced polymer aggregation of a cationic polythiophene reveals that addition of methanol to the polymer aqueous solution can weaken $\pi-\pi$ stacking of the polymer backbone due to solubility improvement of the hydrophobic moieties.[28] In this regard, it is reasonable to expect that when the $\pi-\pi$ stacking of P4Me-3TOEIM within the complexes

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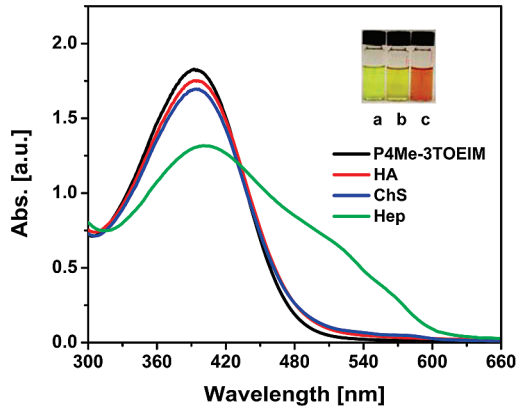

Figure 4. Absorption spectra of P4Me-3TOEIM in the absence and presence of 60 μM HA, ChS, and Hep. [P4Me-3TOEIM] = 0.32 mM, methanol/water (v/v) = 3:2 at room temperature. Inset shows the corresponding photographs of polymer solutions with HA (a), ChS (b), and Hep (c).

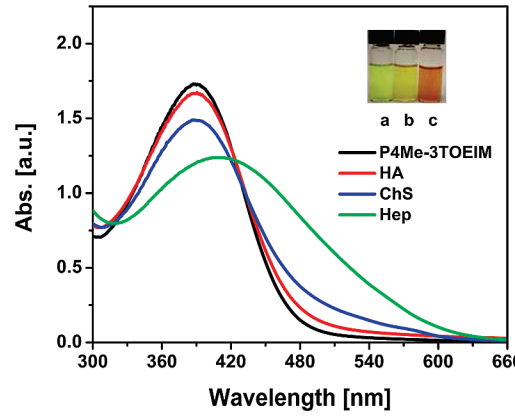

Figure 5. Absorption spectra of P4Me-3TOEIM in the absence and presence of 60 μM HA, ChS, and Hep at 70 °C in water. [P4Me-3TOEIM] = 0.32 mM. The inset shows the corresponding photographs of polymer solutions with HA (a), ChS (b), and Hep (c).

is reduced, the absorption spectrum of P4Me-3TOEIM will change subsequently. Discrimination between Hep and ChS is, thus, studied at room temperature in a mixture of methanol and water (v/v = 3:2). As shown in Figure 4, when 60 μM HA, ChS, or Hep is added to 320 μM P4Me-3TOEIM, only Hep shows the long wavelength absorption band. There is almost no difference in spectral shape and the maximum absorption wavelength for solutions containing HA or ChS, as compared to that for pure P4Me-3TOEIM. As a consequence, only the solution with Hep shows an orange color and others remain light yellow. When one compares Figure 4 to Figure 3B, it is significant that there is almost no obvious change in the absorption spectrum of P4Me-3TOEIM/Hep in different media, while the vibronic spectrum for P4Me-3TOEIM/ChS disappears in the presence of 60% methanol.

Thermochromic Property of P4Me-3TOEIM. *Temperature Dependent UV−vis.* The temperature dependent absorption spectra were also studied to understand the thermal stability of the complexes formed between P4Me-3TOEIM and different polysaccharides. In these studies, the final solutions contain 0.32 mM P4Me-3TOEIM and 60 μM each of HA, ChS, or Hep. The absorption spectrum for each solution at 70 °C is shown in Figure 5. As compared to Figure 3B, there is no obvious change observed for P4Me-3TOEIM/Hep solution at different temperatures and the solution color remains orange. However, for the P4Me-3TOEIM/ChS solution, increasing the solution temperature from 23 to 70 °C significantly affects the vibronic peaks at 523 and 576 nm, and these long wavelength absorption peaks are almost disappeared at 70 °C. The final solution gives a yellow color, which is significantly different from that of P4Me-3TOEIM/Hep. To understand the origin of solution spectral change with temperature and solvent, laser light scattering and CD spectra were collected for each solution.

Temperature Dependent LLS. Due to the detector response limits, the sample concentration used for LLS study is one-third of the absorption study. The LLS for 0.11 mM P4Me-3TOEIM and each of 20 μM Hep and ChS was studied first. No signal was found for any solution, which indicated that all starting materials were well dissolved in water, with almost no aggregation in solution. The dynamic radius for the P4Me-3TOEIM/Hep complex was measured to be 125 nm at 30 °C, which did not change

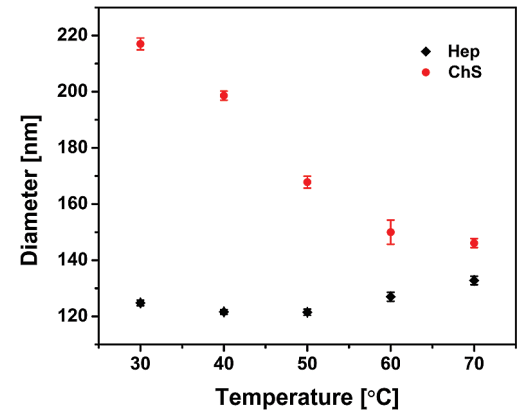

Figure 6. Effective diameters of Hep/P4Me-3TOEIM and ChS/P4Me-3TOEIM aggregates at different temperatures. [P4Me-3TOEIM] = 0.11 mM, [Hep] = [ChS] = 20 μM in pure water.

obviously with the increased temperature (Figure 6). On the other hand, there was a significant decrease in the size of P4Me-3TOEIM/ChS complex from 225 to 150 nm when the temperature was increased from 30 to 70 °C. Previous studies on polycations and DNA interactions have shown that a reduction in polycation charge density weakens the electrostatic attraction between the polymers and DNA, which simultaneously attenuates DNA compaction within the complexes.[25] This observation agrees with our results where smaller aggregation is formed for polysaccharides with a higher charge density, which explains that the complexes formed between Hep and P4Me-3TOEIM are smaller and more stable against temperature variation between 30 to 70 °C. On the other hand, due to the lower charge density of ChS, the electrostatic interaction between ChS and P4Me-3TOEIM is relatively weaker as compared to that for P4Me-3TOEIM/Hep. The loosely packed P4Me-3TOEIM/ChS has a larger aggregation size, and the effective diameter decreases with increased temperature. Even at 70 °C, the measurable effective diameter indicates that P4Me-3TOEIM/ChS or P4Me-3TOEIM/Hep remain associated with each other through electrostatic interaction.

Temperature Dependent CD. To gain more insight into the conformational change of the polymer in P4Me-3TOEIM/polysaccharide complexes during heating, temperature dependent CD spectra were studied. P4Me-3TOEIM has neither a chiral center

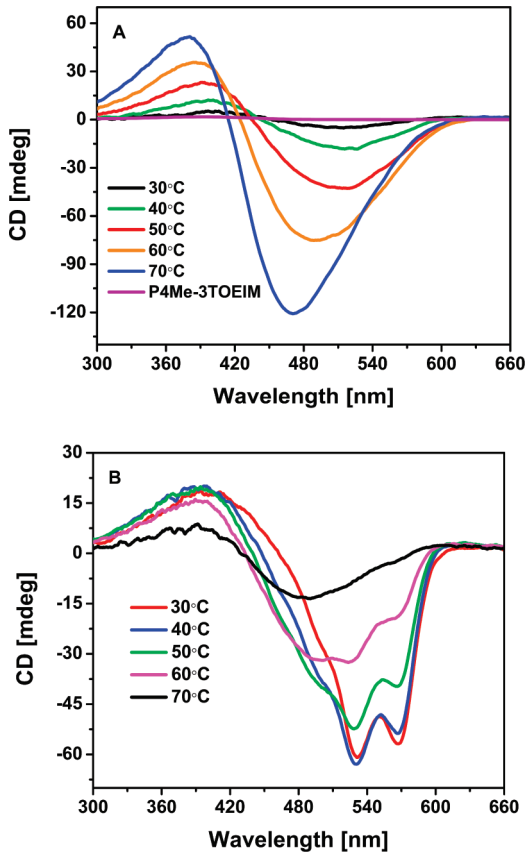

Figure 7. (a) CD spectra of 0.32 mM P4Me-3TOEIM in water and 0.32 mM P4Me-3TOEIM in the presence of 60 μM Hep in water with temperature increased from 30 to 70 °C. (b) CD spectra of 0.32 mM P4ME-3TOEIM at in the presence of 60 μM ChS in water with temperature increased from 30 to 70 °C.

nor ordered chain conformation in solution. This intrinsic optical-inactive property leads to silent CD pattern in the $\pi \rightarrow \pi^*$ transition region for P4Me-3TOEIM (Figure 7A). Due to the asymmetric center of sugar residues, Hep functions as a chiral template for P4Me-3TOEIM to induce CD signals in the visible region. As the CD signal for heparin is below 230 nm,[36] the results shown in Figure 7 indicate that the introduction of Hep into P4Me-3TOEIM solution results in a split type induced circular dichroism (ICD) signal. The sign order of the exciton Cotton effects with a long wavelength negative and a short wavelength positive, corresponding to preferentially left-handed helical arrangement of P4Me-3TOEIM in the P4Me-3TOEIM/Hep complex with a low intensity at 30 °C. The ICD intensity increases with increased temperature from 30 to 70 °C, which indicates that the annealing process greatly facilitates the rearrangement of P4Me-3TOEIM backbone conformation to adopt a more predominant left-handed helical conformation.[26] On the other hand, as shown in Figure 7B, upon addition of ChS to P4Me-3TOEIM at 30 °C, the ICD spectrum has shown strong CD bands with vibronic structures at the long wavelength. Such a spectroscopy characteristic is generally believed to be due to the formation of helical backbone and/or the helical packing of predominantly planar chains.[34,37] With increased temperature, the ICD band intensity for P4Me-3TOEIM/

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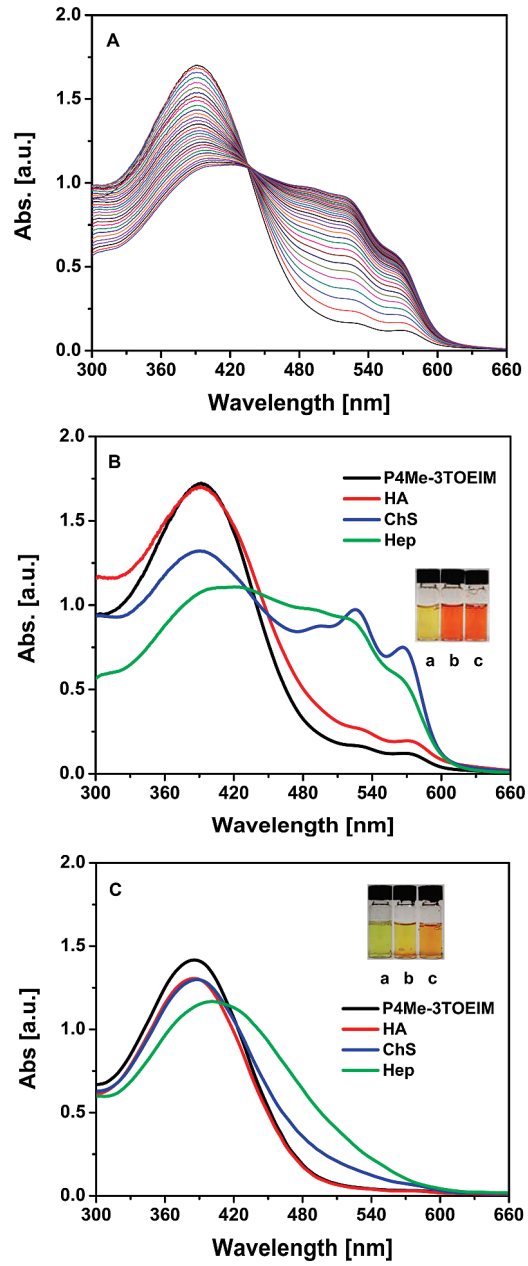

Figure 8. (A) Absorption spectra of 0.32 mM P4Me-3TOEIM in 10% FBS upon addition of 0−60 μM Hep at intervals of 2 μM at 23 °C. The absorption spectra of 0.32 mM P4Me-3TOEIM in 10% FBS in the presence of [Hep] = [ChS] = [HA] = 60 μM at (B) 23 °C and (C) 70 °C, respectively. The insets show the photographs of P4ME-3TOEIM solution with 60 μM HA (a), ChS (b), and Hep (c) at (B) 23 °C and (C) 70 °C, respectively.

ChS decreases and the vibronic bands gradually disappear. It is important to note that even at 70 °C, the optically active P4Me-3TOEIM/ChS complex still exhibits 25% ICD intensity as compared to that at 30 °C, indicating that the formed complexes remain associated, which agrees with the light scattering studies. These results confirm that the P4Me-3TOEIM/ChS complexes are destabilized with increased temperature.

Heparin Detection in Fetal Bovine Serum Medium. To demonstrate the potential application of the P4Me-3TOEIM probe for heparin detection in biological media, 10% FBS in PBS buffer was used as the medium for further studies. As shown in Figure 8A, addition of Hep to a 0.32 mM P4ME-3TOEIM solution results

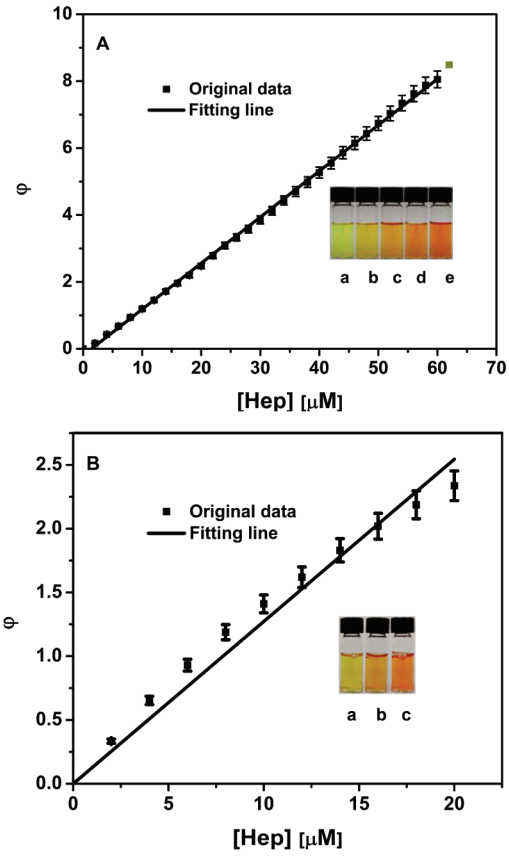

Figure 9. (A) φ as a function of Hep concentration for 0.32 mM P4ME-3TOEIM solution in pure water at room temperature. Inset shows the photographs of P4ME-3TOEIM solutions by adding 0 (a), 15 (b), 30 (c), 45 (d), and 60 μM (e) heparin in pure water. (B) φ as a function of Hep concentration for 0.32 mM P4ME-3TOEIM solution in 10% FBS at room temperature. Inset shows the photographs of P4ME-3TOEIM solutions by adding 0 (a), 10 (b), and 20 μM (c) Hep in 10% FBS at 23 °C, respectively.

in a gradual continuous increase in the absorbance at long wavelength. The saturation of the intensity at 500 nm occurs at [Hep] = 30 μM. Further addition of Hep does not lead to obvious changes of the absorbance at long wavelength. The heparin saturation concentration in serum is lower, as compared to that shown in Figure 3A. This is due to charge pairing between the proteins in serum and P4Me-3TOEIM, which partially blocks the interaction between P4Me-3TOEIM and Hep. When the same experiments were conducted for HA and Chs, similar spectral characteristics as those shown in Figure 3B were observed in serum (Figure 8B). Further increasing the detection temperature from 23 to 70 °C significantly reduces the long wavelength absorption for P4Me-3TOEIM/ChS complexes. As shown in the inset of Figure 8C, distinguishable colors are observed for HA, Hep, and ChS, respectively. These results clearly indicate that the cationic P4Me-3TOEIM could be used for naked detection of Hep in serum.

Heparin Quantification. To quantify heparin in solution, the changes in absorption spectra as shown in Figures 3A and 8A are correlated to the analyte concentration using $\varphi = (I - I_0)/I_0$, where I_0 and I are the absorbance at 500 nm in the presence and absence of Hep, respectively. The calibration factor (φ) is defined to minimize the influence of the absorption background in the absence of Hep. Figure 9A,B shows φ as a function of Hep concentration in water and in serum, respectively. The detection range for Hep in water is 0−60 μM, which corresponds to 0−6.7 U/mL and covers the majority of the clinical range; while in the presence of serum, P4Me-3TOEIM gives a linear response for Hep in 0−20 μM (0−2.2 U/mL), which is suitable for heparin monitoring during postoperative and long-term care. In addition, the distinguishable colors for polymer solutions at different heparin concentration (insets of Figure 9A,B) allow naked-eye quantification of heparin in solutions. The limits of detection for Hep in water and serum are 0.01 and 0.15 U/mL, respectively, based on $3 \times S_0/S$, where S_0 is the standard deviation of background and S is the sensitivity. The correlation coefficients are 0.99961 and 0.99288, respectively.

As compared to the recently reported fluorescent/colorimetric Hep assays (Table 1), this method represents the first heparin assay that allows instrument-free heparin detection and quantification with the naked-eye. In addition, this assay also represents one of the few that could be used to clearly differentiate heparin from its derivatives.[17−19] As compared to other conjugated polymer based methods, the assay allows heparin detection and quantification in biological media for the first time. The assay also

Table 1. Comparison of Fluorescent/Colorimetric Assays for Hep Detection/Quantification

receptor	method	work mode	range of quantification (U/mL)	media
polythiophene (this work)	colorimetric (naked-eye)		0−6.7	water
			0−2.2	10% serum
benzimidazolium dyes[18]	fluorescent (naked-eye with UV excitation)	on	0−1.2	buffer
			0−2	20% plasma
silole derivative[16]	fluorescent	on	0.06−32.5	buffer
			0−15	0.4% horse serum
quinolinium labled peptide[15]	fluorescent	on	0−0.4	buffer
			3.3−11.6	10% bovine serum
tripodal boronic acid / pyrocatechol violet[19]	colorimetric			buffer
tripodal boronic acid[11]	fluorescent	off	0.2−9	19% serum
dye labeled protamine[20]	fluorescent	off	0−0.65	buffer
			0−0.26	4.0 mg/mL BSA
			0−0.52	10% bovine plasma
PFBT[22]	fluorescent (naked-eye with UV excitation)	on	0−5	buffer
PFBT[38]	fluorescent (naked-eye with UV excitation)	on	0−8	buffer
Calyx[8]arene/eosin Y[12]	fluorescent	on	0−20	buffer
chromophore-tethered copolymer[14]	fluorescent	off	0.075−0.55	buffer
molecular patterns[17]	fluorescent	on	0−2.0	buffer

shows a reasonably good heparin quantification range with potential applications in postoperative and long-term care after heparin medication.

CONCLUSION

In conclusion, we have successfully developed a simple colorimetric assay for heparin detection and quantification by taking advantage of the conformational change of a cationic polythiophene upon complex formation. Under optimized experimental conditions, the polymer has shown distinguishable color difference between Hep and ChS or HA. The temperature dependent laser light scattering and CD study further reveals the conformational change of the polymer within different complexes with each polysaccharide. The linear response of the polymer absorbance change at 500 nm vs Hep concentration allows efficient and accurate heparin quantification in a range of 0 to 60 μM (~6.7 U/mL) in water and from 0 to 20 μM (~2.2 U/mL) in serum. The most significant advantage of this assay is the feasibility of naked-eye detection and quantification of Hep in real time. The assay reported herein may find applications in research requiring rapid detection and quantification of purified Hep samples or Hep in biological media.

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