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High-throughput method for in process monitoring of 3-*O*-sulfotransferase catalyzed sulfonation in bioengineered heparin synthesis

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ABSTRACT

Bioengineered heparin (BEH) offers a potential alternative for the preparation of a safer pharmacological heparin. Construction of in-process control assays for tracking each enzymatic step during bioengineered heparin synthesis remains a challenge. Here, we report a high-throughput sensing platform based on enzyme-linked immunosorbent assay (ELISA) and enzymatic signal amplification that allows the rapid and accurate monitoring of the 3-OST sulfonation in BEH synthesis process. The anticoagulant activity of target BEH was measured to reflect the degree of sulfonation by testing its competitive antithrombin (AT) binding ability. BEH samples with different sulfonation degrees show different AT protein binding capacity and thus changes the UV response to a different extent. This BEH-induced signal can be conveniently and sensitively monitored by the plate sensing system, which benefits from its high sensitivity brought in by the enzymatic signal amplification. Furthermore, modification convenience and mechanical robustness also ensure the stability of the test platform. This proposed strategy exhibits excellent analytical performance in both BEH activity analysis and 3-OST sulfonation evaluation. The simple and sensitive plate system shows great potential in developing on-chip, high-throughput methods for fundamental biochemical process research, drug discovery, and clinic diagnostics.

1. Introduction

Heparin is a linear anionic polysaccharide, consisting of highly sulfated repeating 1→4 linked disaccharide units of *α*-D-glucosamine (GlcN) and alternating uronic acid residues, either *β*-D-glucuronic acid (GlcA) or *α*-L-iduronic acid (IdoA), →GlcN→GlcA/IdoA→ [1]. Heparin is widely used as an anticoagulant drug and anticoagulant activity depends on its interaction with the serine protease inhibitor, antithrombin III (AT), which binds to heparin and undergoes a conformational change that enhances its ability to irreversibly inhibit coagulation serine proteases, including thrombin and factor Xa [2]. This carbohydrate-protein interaction of heparin-AT binding depends on a specific type of pentasaccharide sequence, →GlcNS6S→GlcA→GlcNS3S6S→IdoA2S→GlcNS6S→ (Fig. 1) within heparin that contains a 3-*O*-sulfo group in its central GlcN residue [1,2].

Pharmacological heparins are comprised of complex mixtures of oligosaccharide and polysaccharide chains that are generally extracted from animal tissues. Due to the poorly regulated supply chain as well as the presence of other associated bioactive entities from animal extracts, such as viruses or prions, pharmacological heparins present unique safety concerns, such as the heparin contamination crisis which occurred in 2007 and was associated with as many as 200 deaths in the U.S [3]. Bioengineered heparin (BEH) [4,5], utilizing a chemoenzymatic approach to mimic the biosynthetic pathway of heparin, offers a potential alternative for the preparation of a safer heparin. This approach relies on heparosan, the *E. coli* K5 capsular polysaccharide (CPS), as the starting material [4]. Chemical or enzymatic de-*N*-acetylation and *N*-sulfonation of heparosan, followed by modification using

Abbreviations: (3-OST), 3-*O*-sulfotransferase; (OST), *O*-sulfotransferases; (BEH), bioengineered heparin; (ELISA), enzyme-linked immunosorbent assay; (AT), antithrombin; (GlcN), glucosamine; (GlcA), glucuronic acid; (IdoA), iduronic acid; (CPS), capsular polysaccharide; (OSCS), oversulfated chondroitin sulfate; (HPLC), high performance liquid chromatography; (MS), mass spectrometry; (SPR), surface plasmon resonance; (NMR), nuclear magnetic resonance; (HRP), horseradish peroxidase; (TMB), 3,3′,5,5′-tetramethylbenzidine

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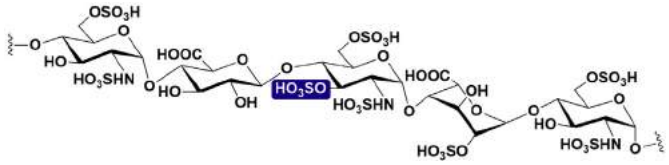


Fig. 1. The antithrombin III-binding pentasaccharide found in heparin.

recombinantly expressed C5-epimerase and *O*-sulfotransferases (OSTs), afford a product that closely resembles the natural heparin and eliminate many undesirable structural artifacts present in the animal-derived heparins, such as oversulfated chondroitin sulfate (OSCS) which was deemed an adulterant and could cause hypotension and cross-talk between the coagulation, kinin–kallikrein, and complement cascades [6].

During bioengineered heparin preparation, the action of 3-*O*-sulfotransferase isoform-1 (3-OST-1) is believed to be the last step on the precursor to form the AT-binding site [7]. Though 3-*O*-sulfo group is present at very low levels in heparin, the content is critical and correlating positively to heparin's anticoagulant activity [7]. Previous research also showed that longer 3-OST treatment would cause over sulfonation on BEH, and finally result in undesired increase of the potency of BEH. Thus, controlling the 3-*O*-sulfo group levels of bioengineered heparin to be consistent with USP heparin is very important. Furthermore, in light of the heparin contamination crisis involving oversulfated chondroitin sulfate (OSCS) [8], it is highly desirable to develop an in-process assay to monitor the 3-OST sulfonation in the last stage of bioengineering heparin synthesis. Tetrasaccharide analysis using high performance liquid chromatography (HPLC) and HPLC/mass spectrometry (MS) can be achievable, but these methods require a time-consuming enzymatic digestion of heparin and the removal of enzymes and buffer salts prior to sample analysis [9,10]. Surface plasmon resonance (SPR) assay was recently developed to evaluate heparin anticoagulant activity in our lab. However, this method could not be applied into high-throughput analysis and highly relying on the SPR instrument. Nuclear magnetic resonance (NMR) was utilized in the past to quantify heparosan production [11], however, it is far away to achieve the in-process monitor for 3-OST sulfonation as media components often interfere with this assay.

In this work, we designed a high-throughput 96-well plate method for monitoring 3-OST sulfonation process based on enzyme-linked immunosorbent assay (ELISA) and enzymatic signal amplification, which was achieved by measuring the activity of BEH product. Scheme 1 illustrates the developed strategy for 3-OST sulfonation evaluation. Streptavidin-coated 96-well plate is well known for constructing fast plate assay with high loading capacity. Taking advantage of the efficient streptavidin − biotin affinity interaction, biotin-labeled USP heparin was successfully immobilized on the inner surface of the plate. After the USP heparin-coated plate was incubated with AT protein or AT-heparin complex, excess AT protein was further bound on the plate through competitive affinity reaction. The resulting plate was then incubated with AT antibody and horseradish peroxidase (HRP)-conjugated IgG secondary antibody step by step. Owing to the enzymatic signal amplification induced by HRP-catalyzed TMB (3,3′,5,5′-tetramethylbenzidine) reaction [12], the activity of bioengineered heparin under 3-OST sulfonation can be easily and sensitively reflected by reading the colorimetric signal, which is inversely proportional to the anticoagulant activity of heparin present in the sample.

2. Methods

2.1. Chemicals and materials

Human antithrombin and antithrombin III monoclonal antibody were bought from Fisher Scientific, US. Horseradish peroxidase (HRP) labeled-Goat anti-mouse IgG secondary antibody (1 mg/mL), EZ-Link™ Amine-PEG3-Biotin and Pierce™ streptavidin coated high capacity plates (black, 96-Well) were purchased from Thermo Scientific, US. 3,3′,5,5′-Tetramethylbenzidine (TMB), sulfuric acid (99.999%), and sodium cyanoborohydride ($NaCNBH_3$) were obtained from Sigma-Aldrich, US. Amicon ultra-0.5 mL centrifugal Filters with 3 KDa Molecular weight cut-off were purchased from Millipore, US. Clear 96-well microplate with flat bottom was obtained from Greiner Bio-One, US. Heparin sodium USP lyophilized powder (from porcine intestinal mucosa, Lot No.: PH-85314) was bought from Celsus Laboratories, Inc. Other regents of analytical grade were obtained from Fisher Scientific, US.

2.2. Instruments

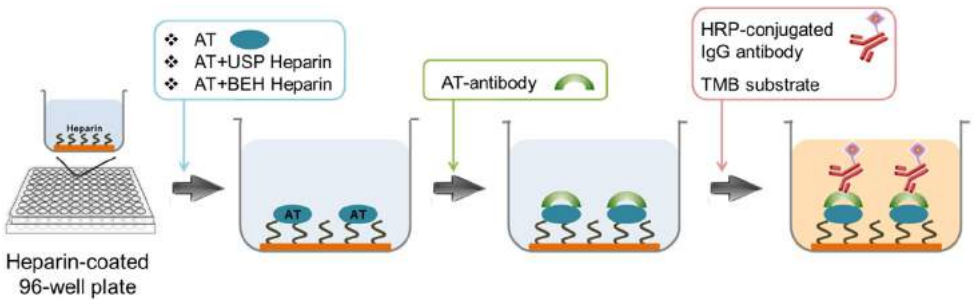
Ultraviolet–visible (UV) spectra were measured on a SpectraMax Microplate Reader (Molecular Devices, LLC) at room temperature.

2.3. Procedure for biotin-labeled heparin synthesis

2 mg Heparin, 2 mg amine-PEG_3-Biotin (Pierce, Rockford, IL) and 10 mg $NaCNBH_3$were dissolved in 200 μL H_2O and reacted at 70 °C for 24 h, following by a further addition of 10 mg $NaCNBH_3$. Then the mixture was reacted at 70 °C for another 24 h. After cooling to room temperature, the mixture was desalted with the 3000 MWCO spin column.

2.4. Procedure for in-process monitoring 3-OST sulfonation

In a typical experiment, heparin-coated plate was prepared by incubating streptavidin-coated plate well with 10 μg/mL biotin-labeled heparin in reaction buffer (20 mM TriS, 200 mM NaCl, 0.2% Tween 20, pH 7.0) for 20 min, following by thoroughly rinsing with reaction buffer. The affinity binding between AT with heparin standard and test sample was carried out in a different clear microplate containing reaction buffer for 4 min with AT concentration of 0.02 μM and heparin standard concentrations varied from 0.5 to 10 U mL^{-1}. The yielded AT-heparin complex was then reacted with heparin-coated plate for 4 min. After rinsing with reaction buffer, the resulted plate was incubated with



Scheme 1. Schematic representation of high-throughput method for in-process monitoring 3-OST sulfonation in bioengineered heparin synthesis.

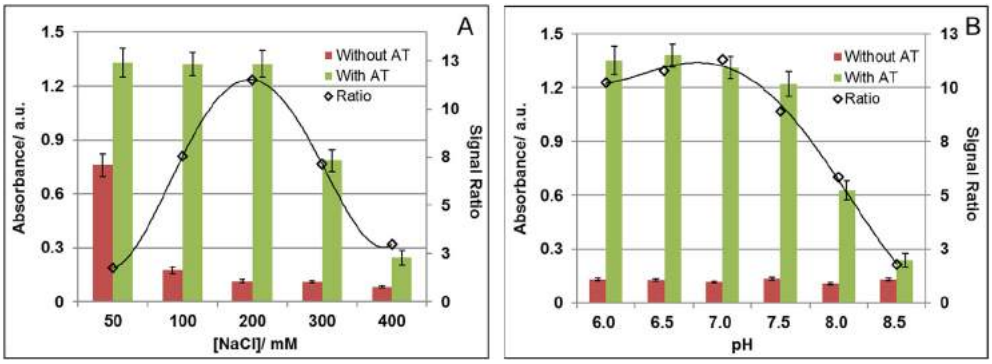


Fig. 2. Assay optimization of (A) NaCl concentration and (B) pH.

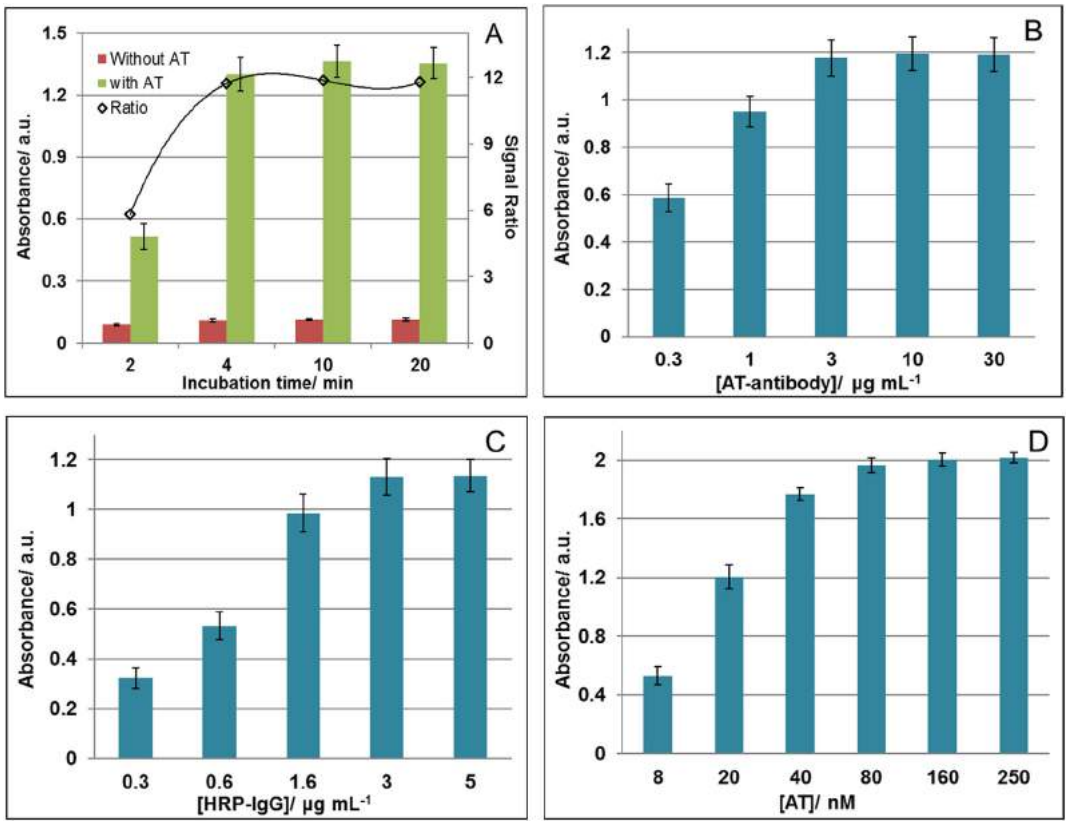


Fig. 3. Assay optimization of (A) incubation time of each step, concentrations of (B) AT-antibody, (C) HRP-conjugated IgG and (D) AT protein.

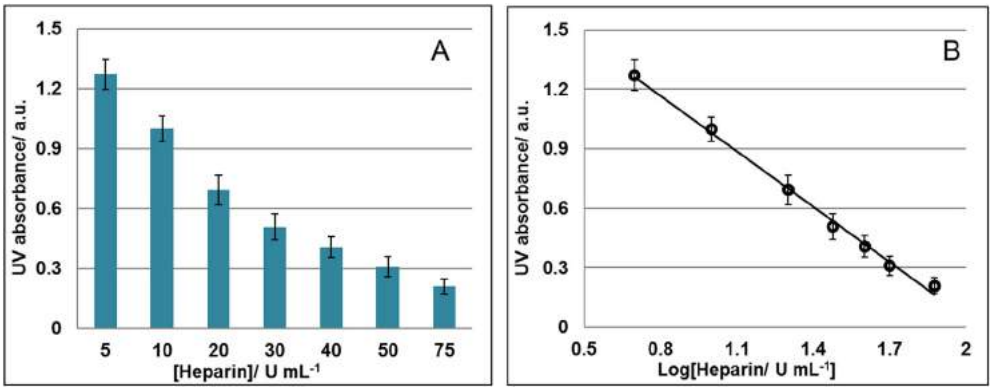


Fig. 4. (A) Dose response of the as-proposed assay. (B) Dependence of UV absorbance on the logarithm of heparin concentration.

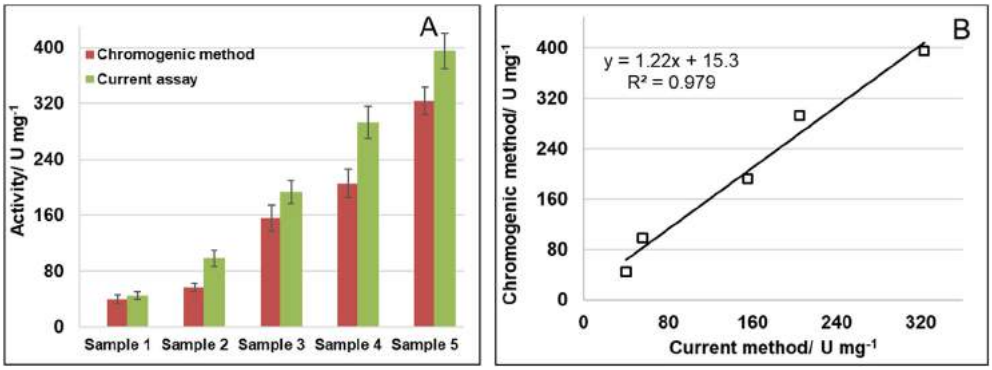


Fig. 5. (A) Anticoagulant activity (anti-Xa) of different heparin samples determined by chromogenic method and as-proposed assay. (B) Correlation between chromogenic method and the as-proposed assay.

3 μg/mL ATIII-antibody and then with 3 μg/mL HRP-conjugated IgG step by step. After the last wash, 30 μL TMB solution was added into each well and reacted for 4 min. Finally, each well was mixed with 20 μL stop solution (0.05 M H_2SO_4) and then placed on a microplate reader for UV measurement at 450 nm immediately. For sample to be tested, it typically contained 0.2 mg/mL heparin. All the affinity-binding steps were conducted on a plate shaker with a speed of 500 rpm under room temperature.

3. Results

3.1. Assay optimization of NaCl concentration and buffer pH

The affinity binding between AT protein and the heparin-coated plate is the first key step for this assay. Two important parameters, salt concentration and buffer pH were optimized (Fig. 2) since they have been reported to be of significant impact on the affinity interaction between AT protein and heparin [13,14]. Here, we used signal ratio ($Abs_{with\ AT}/Abs_{without\ AT}$) to evaluate the affinity binding efficiency, where $Abs_{with\ AT}$ and $Abs_{without\ AT}$ are the UV absorbance at 450 nm of heparin-modified plate with or without the treatment of AT protein, respectively. Higher signal ratio suggests better analytical interface. It is observed that the maximum signal-to-background ratio appears in 200 mM NaCl solution buffered to pH 7, which indicates the best environmental condition for the multi-step affinity interactions within the whole plate system (Fig. 2).

3.2. Assay optimization of incubation time and concentrations

Four major factors influencing the signal response were further investigated to improve the heparin detection performance (Fig. 3). All assays were performed in triplicate. Incubation time for each step is a crucial parameter for affinity binding process on the plate. Shorter incubation time can greatly improve the assay efficiency. However, insufficient interaction will also lead to poor analytical performance. With the increase of the incubation time, the UV signal gradually increased and finally reached a plateau in 4 min, suggesting a tendency to complete the affinity binding process (Fig. 3A). Thus, an optimal reaction time for each step was chosen as 4 min. The concentrations of AT-antibody and HRP-conjugated IgG play key roles in the signal amplification. The UV absorbance increased with the growing concentrations of both AT-antibody and HRP-conjugated IgG as shown in Fig. 3B and C. The signal value reached its maximum at the concentrations of the AT-antibody and HRP-conjugated IgG for both 3 μg/mL. Additionally, another important parameter, the amount of the AT protein was also optimized in our assay. As can be seen from Fig. 3D, the UV absorbance was close to its maximum at the AT protein concentration of 20 nM, which is strong enough to achieve the heparin activity detection. Although a fully AT-saturated plate enhances the detection sensitivity, excess AT protein requires a greater sample amount for the test, and also leads to UV absorbance saturation, which can adversely impact response linearity. Thus, an optimal concentration of AT-antibody, HRP-conjugated IgG and AT protein was determined as 3 μg/mL, 3 μg/mL and 20 nM, respectively.

3.3. Activity investigation of heparin at different concentrations

On the basis of the optimal experimental conditions, this assay was applied to investigate the activity of heparin at different concentrations. Measurements were conducted three times for each test, and the results are presented in Fig. 4A. As can be seen, the UV absorbance decreased along with the increasing heparin units varied from 5 to 75 U mL^{-1}. The dependence of the UV absorbance on the heparin concentration is shown in Fig. 4B. The UV absorbance exhibits a linear correlation to the logarithm of heparin concentration range from 5 to 75 U mL^{-1}. The linear relationship can be described as A = 1.92–0.935 log c with the correlation coefficient of R^2 = 0.996, where A is the UV absorbance at 450 nm and log c is the logarithm of the heparin concentration. The detection limit of heparin was 5 U mL^{-1} (signal-to-noise ratio of 3), which is comparable to the results obtained from other assays [7,9–11]. These results indicate that sensitive and accurate heparin activity measurement can be achieved through the ELISA and enzymatic signal amplification-based sensing platform.

3.4. Activity investigation of BEH samples

BEH samples 1 to sample 5, treated with 3-OST in an increasing gradient time, were measured by both chromogenic and our assay to investigate the assay feasibility for monitoring the 3-OST sulfonation process. The chromogenic assay, conducted via BIOPHEN HEPARIN Anti-Xa kit, involves two chromogenic steps: (stage 1) inhibition of a constant amount of factor Xa, by the tested heparin in presence of exogenous AT; (stage 2) hydrolysis of factor Xa specific chromogenic substrate, by excess factor Xa [15]. As we expected, the activity of BEH grows rapidly as the enzyme reaction time prolongs (Fig. 5A). We further compared our assay with traditional chromogenic method with the same set of samples. A strong correlation, R^2 = 0.979, was observed between these two methods, which demonstrates the accuracy of our current assay (Fig. 5B). Also this new method for evaluating the anticoagulant activity of heparin exhibits many advantages, significantly minimizing time, cost and complications compared to traditional chromogenic method.

4. Conclusion

In conclusion, a high-throughput sensing platform based on ELISA and enzymatic signal amplification has been developed that allows the in-process monitoring of the 3-OST sulfonation in the last stage of

bioengineering heparin synthesis. The major factors influencing signal response, including the concentrations of salt, AT-antibody, HRP-conjugated IgG and AT protein, as well as buffer pH and incubation time were optimized to improve the detection performance. This method provides an alternative for sensitive and accurate trace of 3-OST sulfonation process with significantly minimized time, cost and complications. As 3-*O*-sulfation level is correlating positively to heparin's anticoagulant activity, monitoring 3-OST sulfonation is crucial in the synthesis of bioengineered heparin to guarantee the anticoagulant activity is consistent with the drug, USP heparin, thus, enable to prepare a safe pharmaceutical heparin. We believe that our assay has great potential for use in biological process research, drug discovery, and diagnostic applications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2019.113419.

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