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Novel method for measurement of heparin anticoagulant activity using SPR

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ABSTRACT

A novel method has been developed for the easy measurement of heparin's anticoagulant activity using surface plasmon resonance. The anticoagulant activity of target heparin was evaluated by measuring the competitive antithrombin III binding of analyte heparin in the solution phase and USP heparin immobilized on chip surface. Heparins, obtained from different animal sources, and low molecular weight heparins were analyzed. The results were reproducible and correlated well with the results of chromogenic assays (correlation coefficient r = 0.98 for anti-Xa and r = 0.94 for anti-IIa). This protocol provides many advantages, significantly minimizing time, cost and the complications of chromogenic assay methods.

Heparin is a linear, polydisperse, anionic, and highly sulfated polysaccharide derived from animal tissues [1]. Heparin-based anticoagulant drugs have been widely used clinically since 1935. However, an international heparin contamination crisis occurred in 2007–2008 resulting in a rising concern about the quality of heparin. Since then, improved assays for the anticoagulant activities of low molecular weight (LMW) heparins, biosynthetic heparins, bioengineered heparins, and heparins extracted from the organs of different animals have been developed [2–5]. With the development of new heparin-based drugs, novel methods for the quick, easy and accurate measurement of anticoagulant activity are needed to ensure quality control of these heparin products.

The anticoagulant activity of heparin is primarily mediated through its binding and regulation of antithrombin III (AT), which is a serine protease inhibitor that inactivates various activated coagulation serine proteases, including factors IXa, Xa, TF-VIIa complex, and thrombin (factor IIa) [6,7]. The ability of AT to inhibit serine proteases is markedly enhanced in the presence of heparin [8]. Accordingly, the interaction between heparin and AT is a crucial step in the anticoagulation process, which is the key step for the measurement of anticoagulant activity of heparin.

The chromogenic methods for anti-Xa and anti-IIa assay using commercial kits (i.e., HYPHEN BioMed) involve a two-step chromogenic method based on the inhibition of a constant, excess amount of factor Xa/IIa, by the tested heparin in presence of exogenous AT (stage 1), and the hydrolysis of factor Xa/IIa-specific chromogenic substrate, by residual factor Xa/IIa (stage 2). The *p*-nitroaniline (pNA) chromogen is then released from the substrate and the released amount is related to the residual factor Xa/IIa activity. The reactions for the two-step chromogenic method factor Xa activity are:

Stage 1: Hep. + AT → [AT Hep.];
Stage 2: [AT Hep.] + [FXa (excess)] → [FXa-AT-Hep.] + [residual FXa];
[residual FXa] + Substrate → Peptide + pNA (A405 nm)

There is an inverse relationship between the concentration of heparin and color development, measured at 405 nm. These

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chromogenic methods require a high level of skill, are time consuming, and their accuracy can be impacted by the variable quality of available reagents.

Surface plasmon resonance (SPR), a rapidly developing technique for research on molecular interactions, is label-free, real-time, medium-throughput and requires only small quantities of reagents. It uses an optical method to measure the change in refractive index of the medium close to a metal surface to monitor the binding of analyte molecules to receptor molecules, which are immobilized on the metal surface [9]. We found that the binding between heparin and AT is easy to measure using SPR (Biacore 3000, GE healthcare, Uppsala, Sweden) and this can be developed as a new method to quickly evaluate the anticoagulant activity of heparin.

Our SPR method is mainly based on a competitive effect between standard USP heparin that is biotinylated and immobilized on the SA chip (GE healthcare, Uppsala, Sweden) and a heparin sample that is pre-mixed with AT in the solution (Fig. 1 A). The biotinylated heparin was synthesized by reacting sulfo-*N*-hydroxysuccinimide long-chain biotin (Thermo Scientific, Waltham, MA) with the free amino groups of unsubstituted glucosamine residues in the heparin chain following a published procedure [10]. Once the heparin-binding sites of AT are occupied by heparin in the solution phase, AT binding to the surface-immobilized heparin should decrease, resulting in a reduction in the RU of the SPR signal. This well-established competition SPR method has been previously used in many studies for characterizing heparin/protein interaction [11–13]. The SPR method first requires the immobilization of standard USP heparin on a streptavidin (SA) chip following the manufacturer's protocol. In brief, a 20 μL solution of the heparin-biotin conjugate in HBS-EP running buffer [0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20, (pH 7.4)] is injected over the flow cell of a SA chip at a flow rate 10 μL/min. Successful immobilization of heparin can be confirmed by a ~200 resonance unit (RU) increase in the sensor chip. USP heparin was immobilized to the three flow cells of an SA chip and one flow cell, serving an as a control, was prepared by a 20 μL injection with a saturated solution of biotin. Different dilutions of heparin analyte, having different total activities, were pre-mixed with AT solution just prior to injection. At the end of the each sample injection, the sensor surface was regenerated by injecting 30 μL of 2 M NaCl. The response was monitored as a function of time (sensorgram) at 25 °C. AT solution without heparin was flowed across the chip's surface as a control experiment. The competitive effect of different heparin samples was measured though the decrease of the RU resulting from the addition of heparin into AT solution (Fig. 1B , C and D). This protocol requires only standard USP heparin as reference and AT as reactant and does not require the various chemical and protein reagents used in chromogenic methods. In addition, it is much easier to perform SPR than a chromogenic assay, which depends on sequential addition of reagents and the strict control of reaction times and environmental conditions.

In this study, heparin samples from several animal sources: porcine intestinal heparins from Scientific Protein Laboratories (SPL, Madison, WI), bovine lung heparin (BL1, provided by Dr. J. Fareed in Loyola University) and LMW heparins (LMW1, from Sandoz) were determined based on the SPR method. The results were then compared with activities measured by chromogenic methods, using anti-Xa and anti-IIa kit of HYPHEN BioMed (West Chester, OH). SPR sensorgrams from different dilutions of the

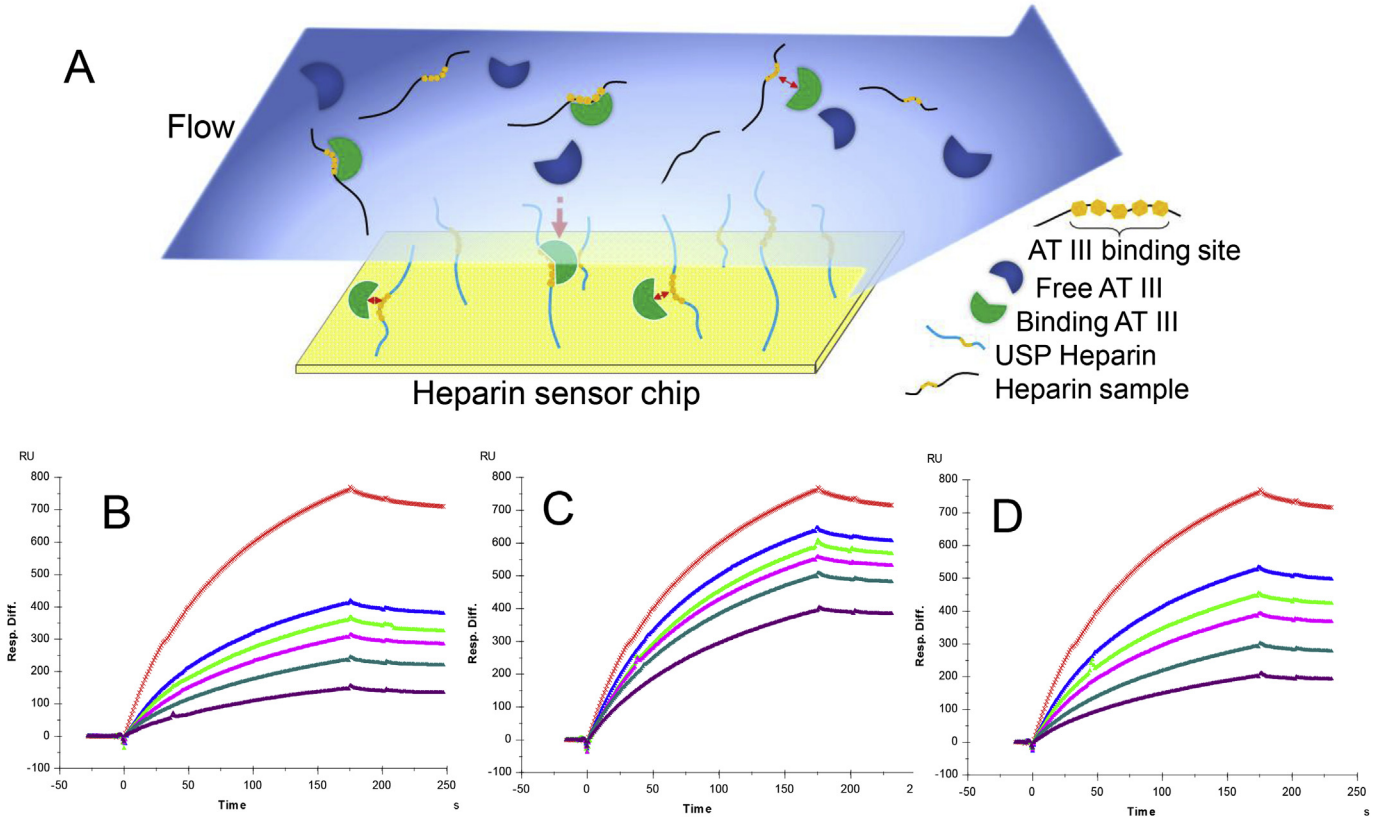

Fig. 1. A. Diagram of SPR solution competition experiment for anticoagulant activity measurement of heparin samples. **B to D:** SPR sensorgrams of AT binding to heparin surface competing with different heparin samples. **B:** USP heparin; **C:** LMW heparin, **D:** bovine lung heparin. The concentration of AT was 62.5 nM. Heparin concentrations in solution (from top to bottom) were 0, 3.13, 6.25, 12.5, 25, and 50 μg/mL, respectively.

heparin samples are shown in Fig. 1. The highest RU was obtained in control experiment (red curve) when there was no heparin sample in the solution. The signal from AT solution premixed with heparin was significantly lower than control, even at the lowest heparin concentration tested. As the concentration of heparin in the solution increased, the sensor surface RU decreased. The set of curves illustrates the competitive effect, between heparin in the solution and on the chip, is concentration dependent. Thus, we speculated that it would possible to observe a proportional relationship between SPR binding signal and heparin concentration.

The RU values at the highest points of each curve, representing the maximum binding, were determined and are referred to as RU_{max}. ΔRU was calculated by the subtraction of RU_{max} between control and analyte samples. As shown in Fig. 2, the logarithm of ΔRU and the related logarithm of heparin concentration fit well by linear regression (all $r > 0.97$). Different types of heparin afforded different slopes in their linear equation, which can be used to evaluate the activity by the equation:

$$Activity_x[Uint/mg] = \frac{Slope_s}{Slope_x} \times Activity_s[Uint/mg] \quad (1)$$

in which $Slope_x$ represents the slope of an analyzed heparin sample, $Slope_s$ represents the slope of standard USP heparin, $Activity_x$ represents the activity of an analyzed heparin sample, and $Activity_s$ represents the activity of standard USP heparin, which was 200 Unit/mg.

The anticoagulant activity calculated for each sample is summarized in Table 1, and compared to the results of anti-Xa and anti-IIa chromogenic assays. The correlation analysis between the SPR assay and the chromogenic assays are shown in Fig. 3. Correlation coefficients of $r = 0.98$ and $r = 0.94$ were obtained for the plots of SPR assay as a function of anti-Xa and anti-IIa assays, respectively.

Three heparin samples with different anticoagulant activities (SPL, BL1, and LMW1 heparin) were selected for triplicate analysis to test the reproducibility of this method. The standard deviation (STDEV) and relative standard deviation (RSD) are shown in Table 1. The RSD for SPL, BL1, and LMW1 heparin were 1.1%, 6.6%, and 3.4%, respectively. The reproducibility is comparable to a chromogenic assay of heparin activity using chromogenic substrate developed by Cate et al., which afforded a RSD of 3.3% for these assays [14]. The chromogenic method by Naga Reveendra et al., for determination of anti factor IIa potency of heparin sodium afforded an RSD of 0.643% with an average activity of 215 USP heparin Units/mg [15], similar to that obtained for SPL heparin determined by SPR method, which afforded an RSD of 1.1% and an average activity of 226 Units/mg.

Determination of heparin anticoagulant activity using SPR can provide investigators with an additional option for testing the anticoagulant activity of both unfractionated heparin and LMW heparin. This protocol provides for accurate, reproducible and easy operation. This protocol requires only USP heparin reference standard and AT protein as reagents and avoids a series of complicated chemical reagents used in other methods. The samples are easy to prepare without any pretreatment and only require commercially available SPR running buffer. In contrast with the strict control of reaction times and environmental conditions, the SPR process is more stable than other bioassays.

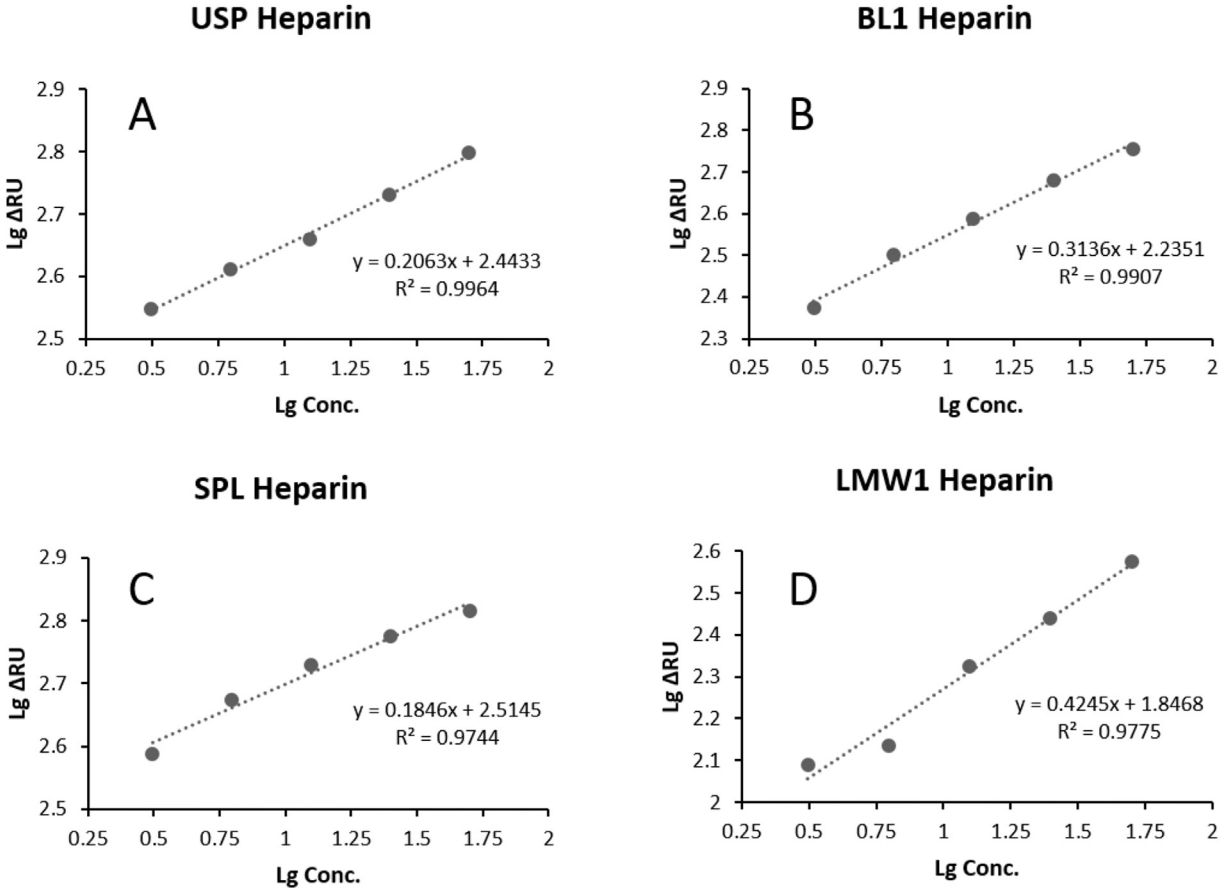

Fig. 2. Linear regression graph of different heparin samples (**A:** USP heparin; **B:** Bovine lung heparin; **C:** SPL heparin; **D:** LMW heparin) for anticoagulant activity measurements: x-axis (Lg Conc): logarithm of heparin concentration: 50, 25, 12.5, 6.3, 3.2 μg/ml; y-axis (Lg ΔRU): logarithm of ΔRU.

Table 1
Anticoagulant activity (anti-Xa) of three heparin samples determined by SPR and Chromogenic method.

Method	Sample	Test 1	Test 2	Test 3	AVG	STDEV	RSD (%)
SPR (Unit/mg)	SPL heparin	228	224	228	226	2.3	1.1
	BL1 heparin	144	132	150	142	9.2	6.6
	LMW1 heparin	92	97	98	96	3.2	3.4
Chromogenic method (Unit/mg)	SPL heparin	208	201	219	209	9.1	4.3
	BL1 heparin	106	107	101	105	3.2	3.1
	LMW1 heparin	85	72	75	77	6.8	8.8

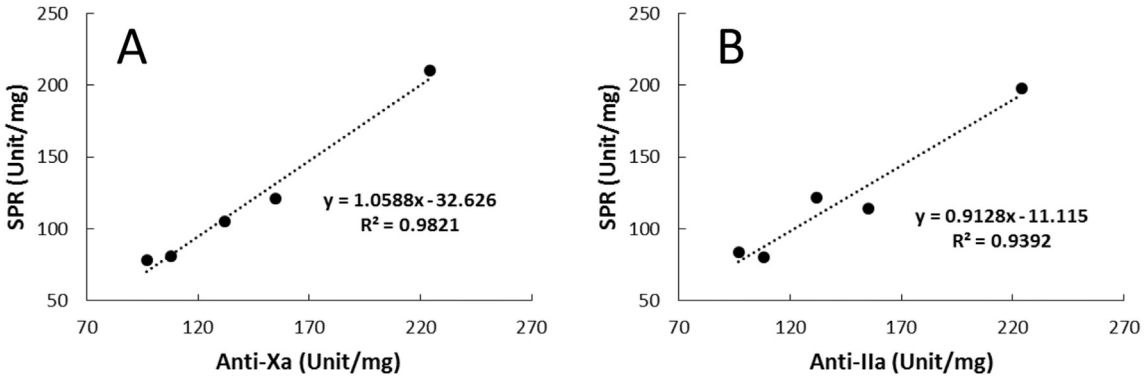

Fig. 3. Correlation analysis of anticoagulant activity determined by SPR and chromogenic methods. Five samples used in the correlation analysis were BL1 heparin, SPL heparin, BM3 heparin, and two low molecular weight heparin (LMW1 and LMW2).

In summary, this new method for evaluating the anticoagulant activity of heparin provides many advantages while significantly minimizing time, cost and complications of traditional chromogenic methods.

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