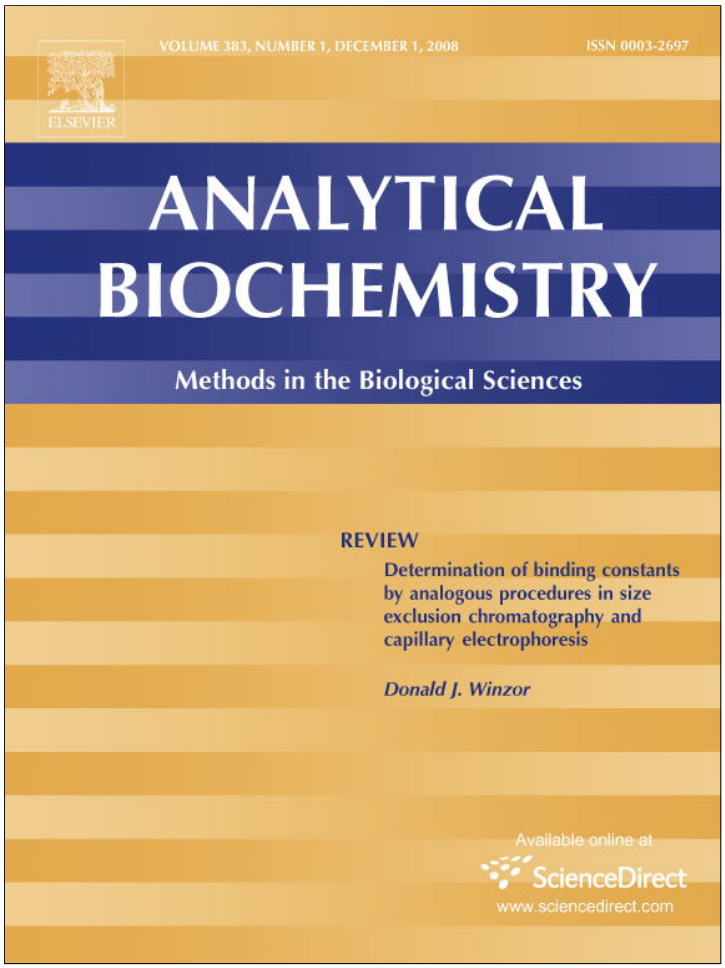

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Signal amplification of target protein on heparin glycan microarray

Tae-Joon Park [a,1], Moo-Yeal Lee [b,1], Jonathan S. Dordick [a,d,*], Robert J. Linhardt [a,c,d,*]

[a] Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
[b] Solidus Biosciences, Troy, NY 12180, USA
[c] Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
[d] Department of Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

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ABSTRACT

A heparin glycan chip (HepGlyChip) with a 4800-fold enhanced signal-to-noise ratio as compared with the control without heparin was developed for high-throughput analysis of heparin–protein interactions for new drug development and for screening biological samples in diagnostic applications. As a proof of concept, a heparin glycan microarray was prepared on a poly(styrene-*co*-maleic anhydride) (PS–MA)-coated glass slide. Heparin was covalently immobilized on poly-l-lysine (PLL) layer with multiple binding sites by sulfo-ethylene glycol bis(succinimidylsuccinate) (sulfo-EGS), increasing the signal-to-noise ratio, minimizing nonspecific binding of target proteins, and resulting in a three-dimensional (3D) structure on the HepGlyChip. This on-chip signal amplification platform was successfully demonstrated by probing the heparin microarray with the highly specific heparin-binding protein antithrombin III (AT III).

The application of combinatorial methods for synthesis in drug discovery enables efficient in vitro and in vivo testing of potential drugs. Advances in genomics, proteomics, and glycomics have dramatically increased developments in high-throughput screening methods, resulting in a direct impact on the evaluation of toxicity tests and metabolic pathways of drug targets [1]. Despite these advances, carbohydrate microarrays still pose considerable challenges because carbohydrate–protein interactions are often relatively weak compared with DNA–DNA, DNA–RNA, and protein–protein interactions [2]. Hence, a new surface chemistry needs to be developed for carbohydrate microarrays that have a high signal-to-noise ratio and a high localized concentration of carbohydrate to which proteins can bind [3].

Heparin, a linear, polydisperse, negatively charged, and highly sulfated glycosaminolgycan (GAG),[2] and the structurally related low-molecular-weight (LMW) heparins carry out a variety of important biological roles as anticoagulant drugs [4]. Heparan sulfate, a related GAG, also plays a critical role in developmental biology, controlling cell growth, cell differentiation, cellular adhesion, and cell migration [5]. Even for biological activities closely associated with heparin or heparan sulfate, the progression to pharmaceutical and clinical application is slow because of the complex structures of these GAGs. X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, hemagglutination inhibition assay, enzyme-linked lectin assay, surface plasmon resonance (SPR) spectroscopy, and isothermal titration calorimetry (ITC) have been used to monitor carbohydrate–protein interactions [3,5,6]. Since the first carbohydrate microarray appeared in the literature in 2002, such microarrays have been promising tools to probe carbohydrate–protein interactions, particularly in the developing field of glycomics [7–12]. More than 1000 spots on a microarray chip can be conveniently monitored using very small amounts of both ligand and analyte to elucidate the specific antibodies useful in the diagnosis of diseases, to identify inhibitors of carbohydrate–protein interactions, and to study events involving carbohydrate–cellular interactions [7–19]. Despite the importance of measuring binding events on carbohydrate microarrays, this technology has not been applied extensively to probe protein binding to GAGs because GAGs are heterogeneous molecules. GAG biosynthesis is poorly understood and difficult to control, and GAG-based signaling is still in the very early stages of scientific study [8].

Two-dimensional (2D) surface chemistries for site-specific and covalent immobilization generally result in low signal intensity and substantial nonspecific binding of target proteins because of an insufficient number of binding sites and the presence of surface–protein interactions. Chip design using polymers such as hydrogels and dendrimers might provide a three-dimensional (3D) structure with more binding sites required for signal amplification [20]. In this article, we demonstrate for the first time a poly-l-lysine

* Corresponding authors. Fax: +1 518 276 2207 (J.S. Dordick);+1 518 276 3405 (R.J. Linhardt).
E-mail addresses: dordick@rpi.edu (J.S. Dordick), linhar@rpi.edu (R.J. Linhardt).

[1] These authors contributed equally to this work.

[2] *Abbreviations used:* GAG, glycosaminolgycan; LMW, low-molecular-weight; SPR, surface plasmon resonance; ITC, isothermal titration calorimetry; 2D, two-dimensional; 3D, three-dimensional; PLL, poly-l-lysine; HepGlyChip, heparin glycan chip; FITC, fluorescein isothiocyanate; Mes, 2-(4-morpholino)-ethane sulfonic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; NHS, *N*-hydroxylsuccinimide; DMF, dimethyl formamide; AT III, antithrombin III; PBS, phosphate buffered saline; PS–MA, poly(styrene-*co*-maleic anhydride); EGS, ethylene glycol bis(succinimidylsuccinate).

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(PLL, Sigma, St. Louis, MO, USA)-based 3D system to immobilize a sufficient amount of heparins to provide the high signal intensity required for target protein on a heparin glycan chip (HepGlyChip).

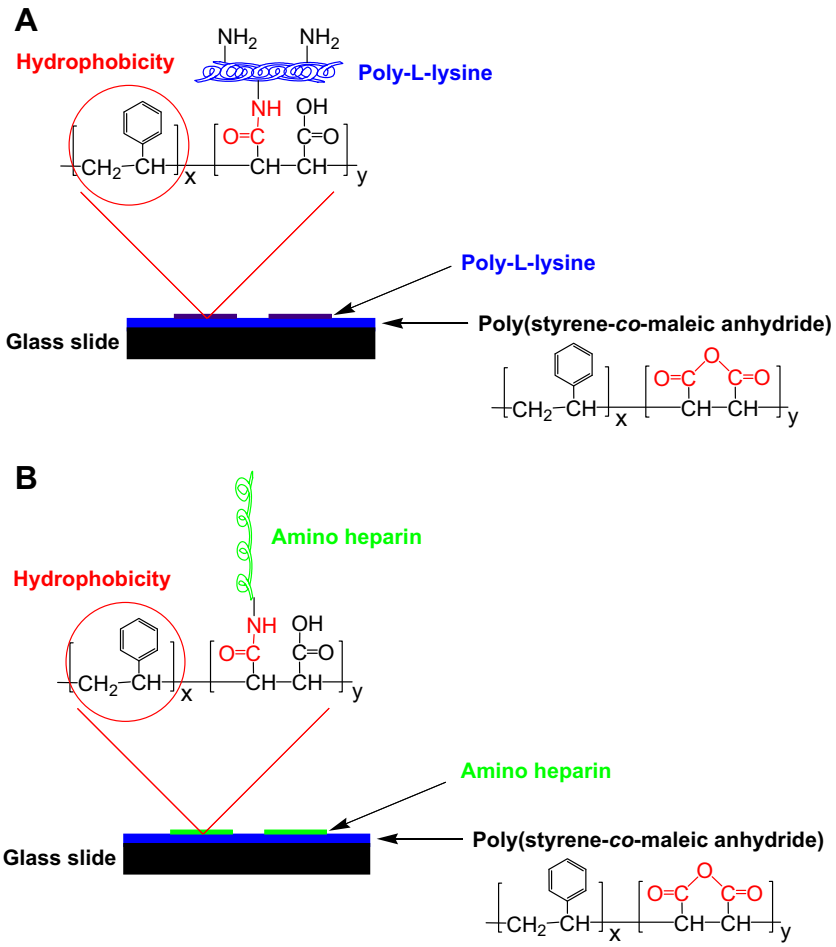
Materials and methods

Preparation of FITC-labeled amino heparin

ω-Hydrazido-adipyl-azo heparin (amino heparin) was prepared from heparin (sodium salt, extracted from porcine intestinal mucosa, USP activity 169 U/mg, Celsus Laboratories, Cincinnati, OH, USA) by reductive amination [21]. Thus, amino heparin contains a single amine group at the reducing end of the heparin chain, allowing the single point-oriented attachment of heparin chains. To prepare fluorescein isothiocyanate (FITC)-labeled amino heparin, 1 ml of 50 mg amino heparin in 0.05 M 2-(4-morpholino)-ethane sulfonic acid (Mes) buffer solution was activated with a mixture of 15 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 9 mg *N*-hydroxylsuccinimide (NHS) for 15 min. Then 0.1 ml of 0.1 M fluorescein amine (Sigma) in dimethyl formamide (DMF) was added to the NHS-activated heparin. This 1.1-ml mixture was incubated overnight in the dark and then dialyzed in the dark for 24 h. A column (2.5 × 80 cm) filled with a cation exchange resin (H^+ form, Sigma) was washed with 1.4 liters (equivalent to 2 packed bed volumes) of 1 M HCl. FITC–amino heparin reaction mixture was loaded in this column, and FITC–amino heparin was eluted by washing with 700 ml of distilled water. The eluted FITC–amino heparin solution was neutralized with NaOH, and then sodium chloride was added to obtain a 16% saline solution. Methanol (80%, v/v) was added to precipitate the FITC–amino heparin, and the recovered precipitate was dissolved in water and dialyzed for 48 h to obtain pure FITC–amino heparin.

Preparation of antithrombin III-immobilized Sepharose beads to separate low- and high-affinity heparins

Antithrombin III (AT III, 2 mg, purified from human plasma, specific activity >6 U/mg, Aniara, Mason, OH, USA) was dialyzed in 200 µl of coupling buffer (0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3) at 4 °C overnight. CNBr-activated Sepharose beads (3 mg, Sigma) were washed and swollen in cold 1 mM HCl for 40 min, washed with distilled water, and then washed with 0.1 M $NaHCO_3$/0.5 M NaCl (pH 8.3) coupling buffer. Then 100 mg of heparin was added to dialyzed AT III solution in the coupling buffer to protect the active site of AT III. The coupling reaction was performed by mixing the AT III solution with CNBr-activated beads for 2 h at room temperature and then for 24 h at 4 °C with gentle shaking. The mixture was filtered using a 0.8-µm polycarbonate filter to recover the beads, and then residual AT III and heparin were immediately removed by washing the beads with the coupling buffer. Unreacted CNBr-activated groups were blocked with 0.2 M glycine (pH 8.0) for 2 h at room temperature. The glycine solution was removed by washing with a basic coupling buffer solution (0.1 M $NaHCO_3$/



Scheme 1. Schematic representation of PS–MA coating. (A) Immobilization of PLL on PS–MA coating for signal amplification system (3D system). (B) Direct immobilization of amino heparin on PS–MA coating (2D system).

0.5 M NaCl) at pH 8.5 and then with an acetate buffer solution (0.1 M, pH 4.0) containing 0.5 M NaCl.

Separation of high- and low-affinity heparin

A column packed with 10 ml of AT III–Sepharose beads was washed with the basic coupling buffer solution and then with the acetate buffer solution. The AT III–Sepharose column was equilibrated in 50 mM Tris equilibrium buffer with HCl (pH 8.5) containing 0.3 M NaCl, and then 10 μl of heparin (10 mg/ml) was loaded. Low- and high-affinity heparins were obtained by eluting with 20 ml of the equilibrium buffer and then 20 ml of 50 mM Tris buffer with HCl (pH 8.5) containing 2.8 M NaCl.

Preparation of FITC-labeled AT III

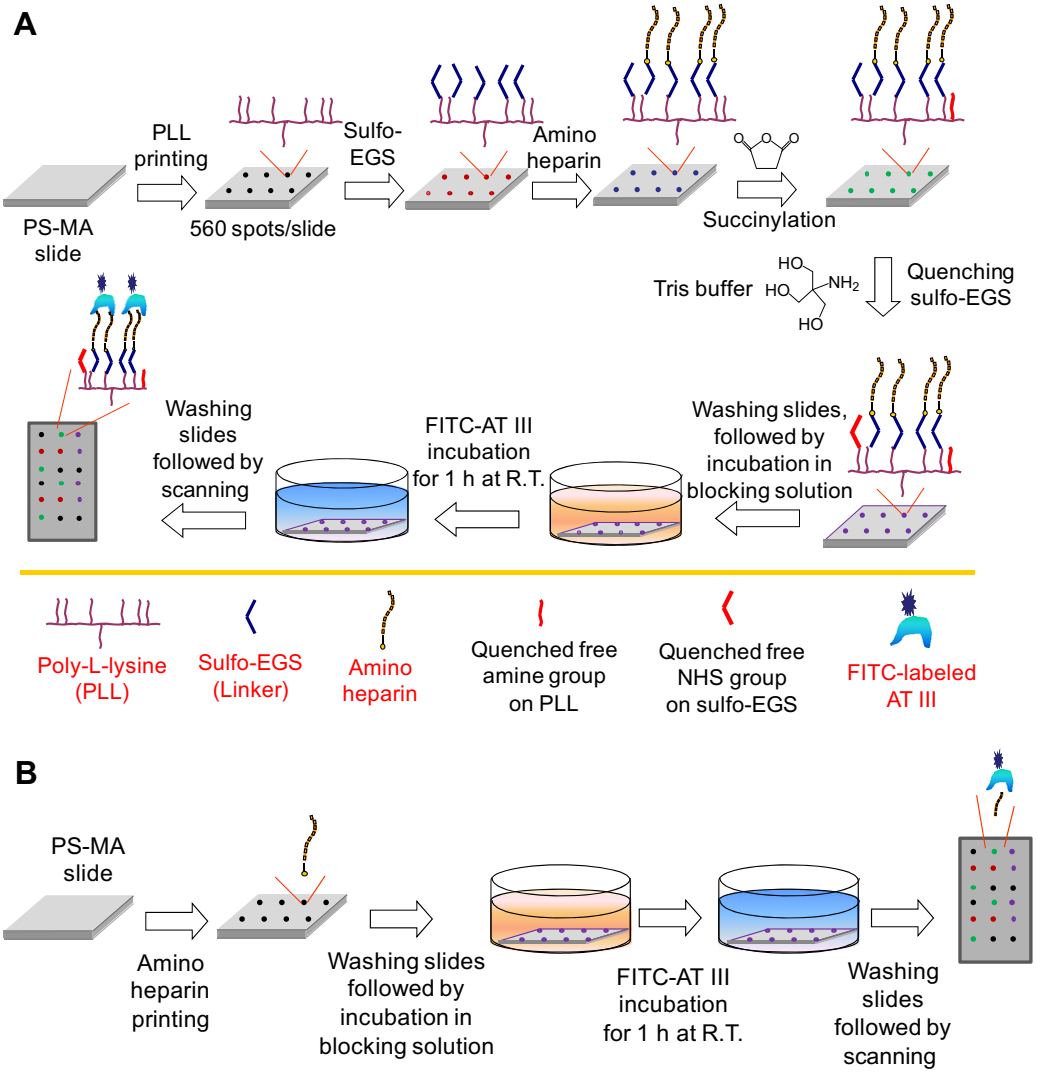
AT III (1 ml, 2 mg/ml) was exchanged into 50 mM sodium borate buffer (pH 8.5) by dialysis. A fluorescein labeling reagent, NHS–fluorescein (830 nmol, Pierce, Rockford, IL, USA) in 100 μl of DMF, was added to the AT III solution (24-fold molar excess). The reaction mixture was mixed well and incubated for 1 h at room temperature and then dialyzed against distilled water.

Purification of FITC-labeled AT III by heparin–agarose beads

A column packed with 2 ml of heparin–agarose (Sigma) was equilibrated with 6 ml of 20 mM Tris buffer with HCl (pH 7.3) containing 0.15 M NaCl. FITC-labeled AT III in the equilibration buffer solution was applied to the column and then washed with 4 ml of the equilibration buffer to remove inactivated AT III. The active FITC-labeled AT III bound to the column was eluted with 6 ml of 20 mM Tris buffer with HCl (pH 7.3) containing 2 M NaCl and then dialyzed against 10 mM phosphate-buffered saline (PBS) containing 15 mM NaCl (pH 7.1).

Modification of a microscopic glass slide

Borosilicate microscope slides (25 × 75 mm^2, Fisher, Pittsburgh, PA, USA) were pretreated with ethanol, followed by concentrated H_2SO_4, overnight to remove dust and oil from the glass surface. The slides were then sonicated for 30 min, rinsed with distilled water five times, and immersed twice in acetone. The acid-cleaned slides were dried for 30 min in an oven at 120 °C. Finally, the acid-cleaned slides were spin-coated (WS-400B-6NPP/Lite, Laurell, North Wales, PA, USA) with 1.5 ml of 0.1% (w/v) poly(styrene-*co*-maleic anhydride) (PS–MA, Sigma) in toluene for 30 s at



Scheme 2. Preparation of 3D and 2D HepGlyChips for heparin–AT III interaction. (A) 3D HepGlyChip constructed on PS–MA coating–PLL–EGS–heparin. (B) 2D HepGlyChip prepared on PS–MA coating–heparin. R.T., room temperature.

3000 rpm. The PS–MA coating provides a reactive functionality to covalently attach PLL while enhancing hydrophobicity of the slide surface [22].

Microarraying of amino heparin to prepare the HepGlyChip

To immobilize amino heparin on PLL (3D system), 30 nl of 0.01% PLL solution (mol wt 70,000–150,000, Sigma) was spotted onto the PS–MA-coated slides (40 × 14 spot array, 560 spots/slide) using a MicroSys 5100-4SQ microarrayer (DIGILAB Genomic Solutions, Ann Arbor, MI, USA) (Schemes 1A and 2A). Following spotting and drying of PLL spots, 30 nl of up to 10 mg/ml sulfo-ethylene glycol bis(succinimidylsuccinate) (EGS, Pierce) was spotted atop each PLL spot. Amino heparin was covalently attached by spotting 30 nl of up to 10 mg/ml amino heparin on top of PLL–EGS spots (Scheme 2A). To quench unreacted amine groups on PLL and remaining reactive groups on sulfo-EGS, 30 nl of succinic anhydride (0.2 mg/ml) and 30 nl of Tris buffer (2 mg/ml) were spotted sequentially. The 3D HepGlyChip was rinsed three times in a washing buffer (10 mM PBS, pH 7.1) for 30 min, followed by incubation for 24 h at 4 °C in a blocking solution (SuperBlock, Pierce). Amino heparin was covalently attached by spotting 30 nl of up to 10 mg/ml amino heparin on PS–MA slides to directly immobilize it in the 2D system (Scheme 1B). Spots containing no amino heparin were used as control. The 2D HepGlyChip was rinsed in the washing buffer, followed by incubation in the blocking solution (Scheme 2B).

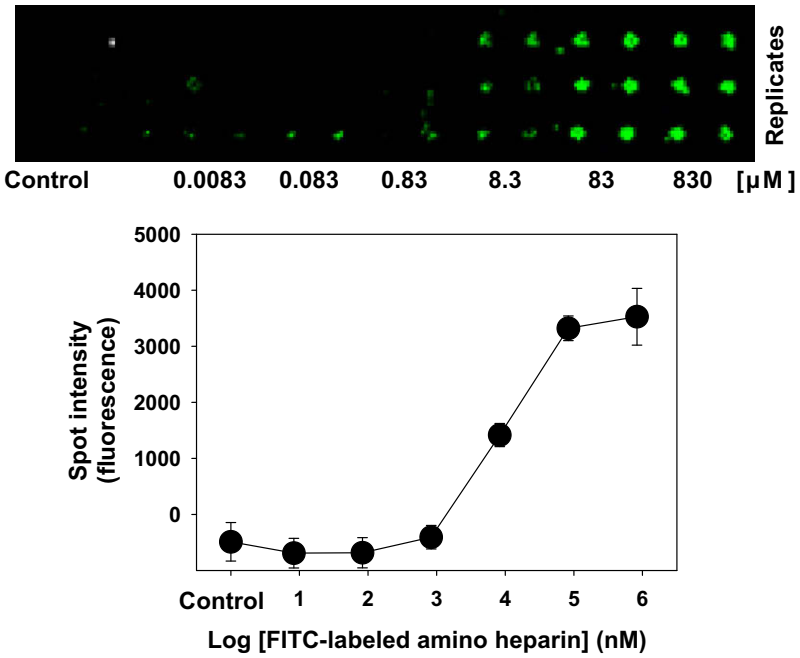


Fig. 1. Fluorescent intensity of immobilized FITC-labeled amino heparin as a function of spotted heparin concentration. The concentrations of printed FITC-labeled amino heparin in the spots ranged from 8.3 nM (100 ng/ml) to 830 μM (10 mg/ml). Spots containing no FITC-labeled amino heparin were used as control.

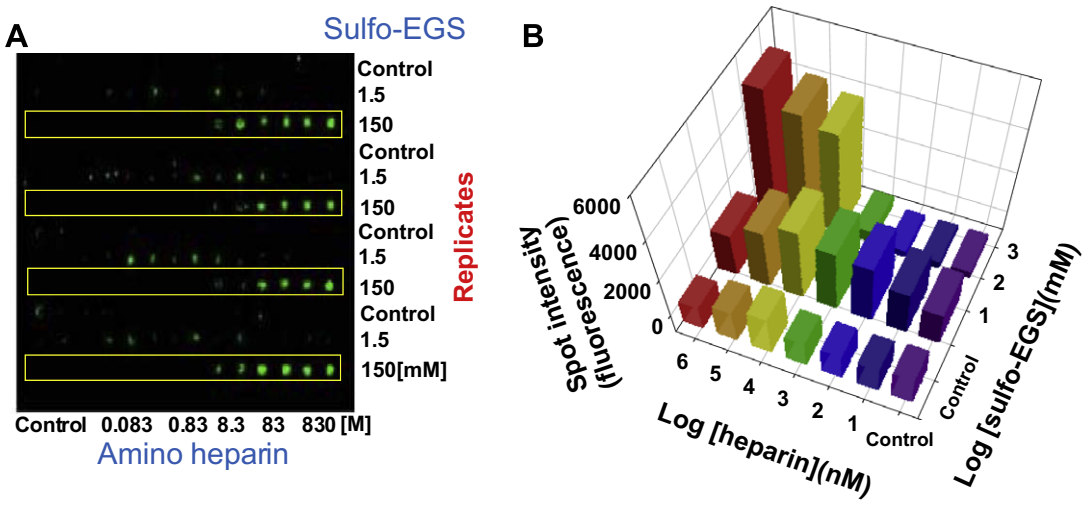


Fig. 2. Optimization of fluorescence intensity of bound FITC-labeled AT III as a function of sulfo-EGS and amino heparin concentrations. (A) Scanning image of the slide (3D system) where the printed concentrations of sulfo-EGS and amino heparin ranged from 1.5 mM (0.1 mg/ml) to 150 mM (10 mg/ml) (including no sulfo-EGS used as control) and from 8.3 nM (100 ng/ml) to 830 μM (10 mg/ml) (including no amino heparin used as control), respectively. (B) Average of fluorescent intensity of bound FITC-labeled AT III in the spots at different concentrations of sulfo-EGS and amino heparin printed (3D system).

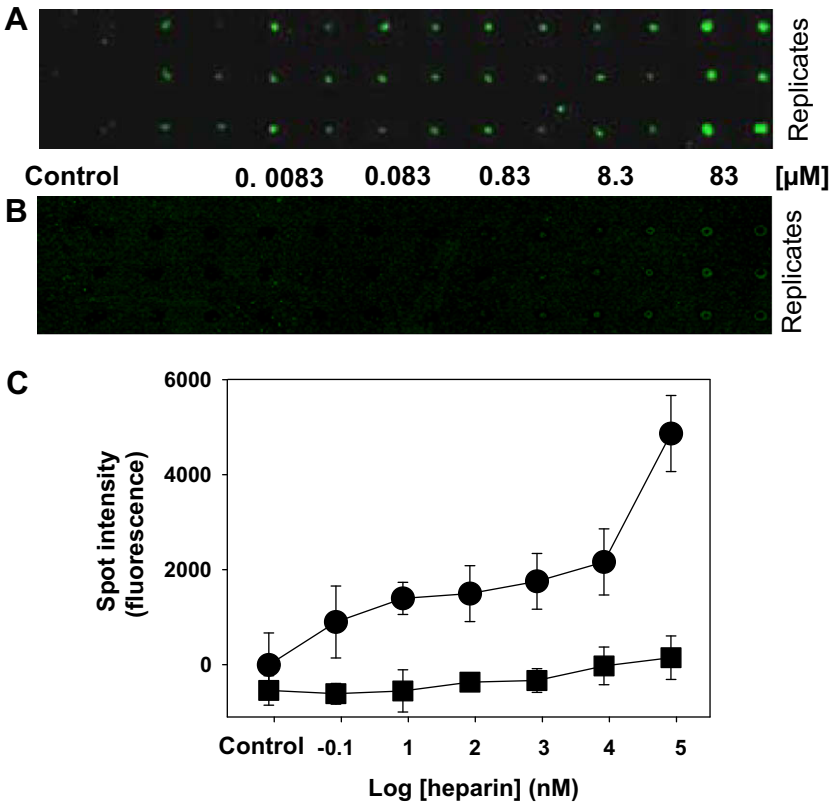


Fig. 3. Comparison of fluorescent intensity of bound FITC-labeled AT III on the 3D and 2D system. (A) Scanning image of the spots containing FITC-labeled AT III bound on PLL–heparin structure (3D system). (B) Scanning image of the spots containing FITC-labeled AT III bound on heparin directly attached on the glass surface (2D system). (C) Comparison of fluorescent intensity on 3D amplification system (●) and 2D system (■). The spots containing 0.78 mM PLL were activated with 150 mM sulfo-EGS, followed by printing amino heparin at a range from 0.83 nM (10 ng/ml) to 83 μM (1 mg/ml). Spots containing no FITC-labeled amino heparin were used as control. The slide was incubated in an aqueous solution containing 8 μM AT III for 1 h to probe heparin-specific protein interaction.

Incubation of the HepGlyChip in FITC-labeled AT III solution to study heparin–protein interaction

The HepGlyChip washed with the blocking solution was rinsed three times in the washing buffer solution for 30 min before protein binding. The HepGlyChip was incubated in 1 ml of 10 μg/ml FITC-labeled AT III solution for 1 h at room temperature, followed by rinsing three times in the washing buffer for 30 min and then washing three times with distilled water for 5 min. To detect the location of each heparin spot where heparin–AT III interactions occurred, the entire HepGlyChip was scanned using a blue laser (excitation = 488 nm) and a standard blue filter (emission = 508–560 nm) for green FITC dye in a GenePix Professional 4200A scanner (Molecular Devices, Sunnyvale, CA, USA). The green fluorescence intensity of the spots was quantified from the scanning image using GenePix Pro 6.0 (Molecular Devices). The data points were plotted from an average of six spots (Figs. 1, 3 and 4) or eight spots (Fig. 2), and the standard errors are presented.

Results and discussion

The most critical issue in chip technologies designed to study heparin–protein interactions is a low signal-to-noise ratio resulting from the nonspecific binding of target proteins to the surface [2,3,5,6,20]. A 3D microarray system, prepared using PLL with multiple amino groups, was used to address this problem. This 3D system provides higher heparin-binding capacity for probing heparin-specific proteins, thereby reducing nonspecific interactions between the slide surface and the proteins. PLL microarrays on the PS–MA coating were prepared by a simple spotting and drying technique. A highly hydrophobic amino-reactive PS–MA coating was used to covalently immobilize PLL while simultaneously preventing the spreading of aqueous spots on the surface of the glass slide (Scheme 1A). PLL increases the number of binding sites for heparin, amplifying signal intensity. Immobilized PLL was next

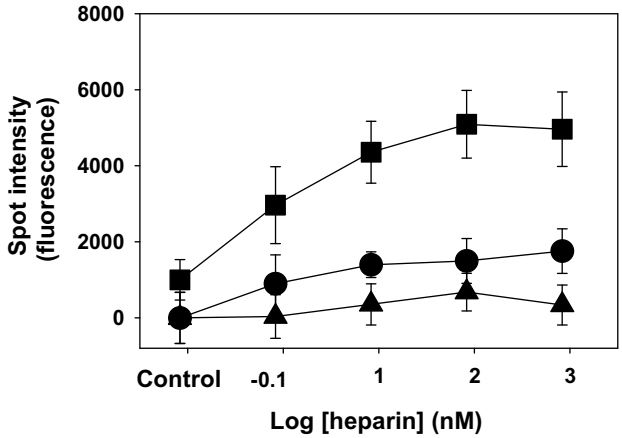


Fig. 4. Fluorescence intensity of bound FITC-labeled AT III in the spots containing immobilized heparin with different percentages of high-affinity chains: high-affinity heparin (■), unfractionated heparin (●), and low-affinity heparin (▲). The spots containing 0.78 mM amine groups on PLL were activated with 150 mM sulfo-EGS, followed by covalent immobilization of heparin with different affinity at a range from 0.83 nM (10 ng/ml) to 0.83 μM (10 μg/ml). Spots containing no FITC-labeled amino heparin were used as control. The slide was incubated in an aqueous solution containing 8 μM AT III for 1 h to probe heparin-specific protein interaction.

modified with a homo-bifunctional cross-linker, sulfo-EGS, and then an amino heparin derivative, ω-hydrazido-adipyl-azo heparin, was covalently attached (Scheme 2).

FITC-labeled amino heparin, at concentrations ranging from 8.3 nM (100 ng/ml) to 830 μM (10 mg/ml), was printed on top of the spots containing EGS-coupled PLL on the PS–MA slides to demonstrate immobilization of heparin on PLL (Fig. 1). After immobilizing FITC-labeled amino heparin, the residual amino groups on PLL were quenched by succinylation with succinic anhydride to remove potential ionic interactions between PLL and FITC-labeled amino heparin. As shown in Fig. 1, after extensive washing in the PBS solution, the green fluorescence intensity of the spots was found to increase with increasing concentrations of FITC-labeled amino heparin. The fluorescence intensity of FITC-labeled amino heparin was more than three orders of magnitude higher than the control without heparin. Having dramatically increased the signal-to-noise ratio with PLL, the surface chemistry was next optimized by varying the sulfo-EGS and amino heparin concentrations (Fig. 2). In each spot, containing 0.78 mM amine groups on PLL, the concentrations of sulfo-EGS and amino heparin were varied from 1.5 mM (0.1 mg/ml) to 150 mM (10 mg/ml) and from 8.3 nM (100 ng/ml) to 830 μM (10 mg/ml), respectively. The spots without sulfo-EGS and amino heparin were used as controls. After incubation of the slide in FITC-labeled AT III solution for 1 h and extensive rinsing in the wash buffer, the fluorescence intensity of FITC-labeled AT III in the spots where heparin-AT III interactions took place was quantified using a microarray scanner. As a result, fluorescence intensity of FITC-labeled AT III was increased more than 4800-fold as compared with that of the control (i.e., background fluorescent intensity representing nonspecific protein binding).

Fluorescence intensity of AT III in the microarray spots containing heparin covalently attached on PLL (3D system) were compared with those in the spots containing heparin directly immobilized on the PS–MA slide (2D system) to investigate our on-chip amplification system using the 3D PLL–heparin structure. As shown in Fig. 3, the 3D amplification system provided a 100-fold increased signal-to-noise ratio compared with the 2D system.

Heparins having different affinity toward AT III were immobilized to further validate the 3D amplification system. To this end, we prepared microarray spots containing covalently immobilized high affinity heparin (>95% high-affinity chains), unfractionated heparin (containing ~ 30% high-affinity chains and ~ 70% low-affinity chains), and low-affinity heparin (<1% high-affinity chains) on the PLL–sulfo-EGS sites (Fig. 4). Thus, high-affinity heparin should theoretically exhibit a threefold higher fluorescence intensity than that of unfractionated heparin. As a result, fluorescent intensities of AT III in the spots increased as affinities and concentrations of heparin increased. Specifically, the intensity of low-affinity heparin was close to 0, unfractionated heparin was approximately 1500, and high-affinity heparin was approximately 5000 in the spots where 830 nM heparin was spotted. This result indicates that the difference of fluorescence between unfractionated heparin and high-affinity heparin is close to the theoretical ratio. The relative proportion of high-affinity heparin chains in the spots was consistent with fluorescent intensities of FITC-labeled AT III at all concentrations tested. Thus, the surface chemistry we developed could be used to access affinity of heparin to a variety of heparin-binding proteins.

Conclusions

The HepGlyChip has been developed on PS–MA-functionalized slides to detect interactions between heparin and target proteins in a high-throughput manner. As a proof of concept, we successfully demonstrated specific interactions between heparin and AT III on the HepGlyChip, and the PLL-based 3D structure in the microarray spots provided an enhanced signal-to-noise ratio and minimized nonspecific binding of AT III. We are currently performing on-chip chemoenzymatic synthesis of heparin in the spots for high-throughput screening of heparin analogs. This approach may provide valuable information on various structure–function relationships of approximately 1000 different glycans in microarrays on a single microscopic slide. Thus, the HepGlyChip platform might facilitate the development of the next generation of heparin-based drugs for anticoagulant/antithrombotic applications and beyond. We envision that in the near future the HepGlyChip platform could be applied to glycomic studies for high-throughput screening of interactions with heparin-binding proteins. Printing drug candidates and various heparin-binding proteins together on a chip of heparin chains having different sequences might be useful in new drug development. Such microarray technology might have utility in evaluating small-molecule drugs that antagonize with heparin interaction with heparin-binding proteins. Finally, the HepGlyChip may facilitate rapid screening of biological samples for the diagnostic identification of heparin and heparin-binding proteins in biological fluids and tissues.

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