

Kinetic and Structural Studies of Interactions between Glycosaminoglycans and Langerin

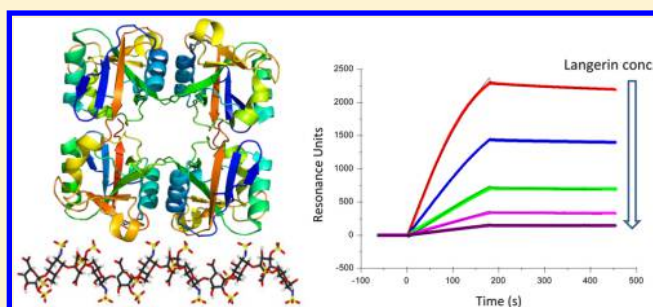
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ABSTRACT: Langerin, a C-type lectin, is expressed in Langerhans cells. It was reported that langerin binds sulfated glycans, which is an important initial step for its role in blocking human immunodeficiency virus (HIV) transmission by capturing HIV pathogens and mediating their internalization into Birbeck granules for their elimination. It is fundamentally important to understand these interactions at the molecular level for the design of new highly specific therapeutic agents for HIV. Surface plasmon resonance (SPR), which allows for the real-time, direct, quantitative analysis of the label-free molecular interactions, has been used successfully for biophysical characterization of glycosaminoglycan (GAG)–protein interactions. In this study, we report kinetics, structural analysis, and the effects of physiological conditions (e.g., pH, salt concentration, and Ca²⁺ and Zn²⁺ concentrations) on the interactions between GAGs and langerin using SPR. SPR results revealed that langerin binds to heparin with high affinity ($K_D \sim 2.4$ nM) and the oligosaccharide length required for the interactions is larger than a tetrasaccharide. This heparin/heparan sulfate-binding protein also interacts with other GAGs, including dermatan sulfate, chondroitin sulfates C–E and KS. In addition, liquid chromatography–mass spectrometry analysis was used to characterize the structure of sulfated glycans that bound to langerin.



Langerin is a membrane-associated C-type lectin exclusively expressed in Langerhans cells (LCs), which are found in the epidermis of skin and mucosal tissue, belonging to the immune system. LCs function as the first barrier against invading pathogens by capturing and processing the antigens for presentation to T cells and have an important role in the adaptive immune response.¹ Initially, langerin was identified as a specific cell marker to distinguish LCs from other human dendritic cells.² Recognized by a monoclonal antibody DCGM4, langerin was found to be involved in a nonconventional pathway for antigen capture in LCs. It can cause the formation of Birbeck granules (BGs, a specific organelle present in only LCs) by superimposition and zippering of cell membranes as an endocytic receptor.³ Langerin recognizes pathogens by binding to glycoconjugates on the surface of microorganisms. It was found to bind to the mannose and β -glucans present on fungal cell walls (*Malassezia furfur*, *Candida*, and *Saccharomyces*) and the mannose-containing O-specific polysaccharides derived from bacterial lipopolysaccharides (*Mycobacterium leprae*).^{4,5} Recently, langerin has attracted more attention because of its importance as a key player in human immunodeficiency virus-1 (HIV-1) transmission by LCs. HIV-1 captured by langerin is internalized into BGs and

degraded, proving that langerin is a natural barrier to HIV-1 infection.⁶ The classical functional region of langerin is an extracellular domain (ECD), which is composed of a neck region and a C-terminal carbohydrate recognition domain (CRD), determining that langerin mediates pathogen capture in a carbohydrate-dependent pathway.⁷ It reportedly binds to many different carbohydrate ligands in both Ca²⁺-dependent and Ca²⁺-independent manners. Within its typical Ca²⁺-binding site in the CRD, langerin is able to recognize oligosaccharides containing terminal mannose, terminal N-acetylglucosamine, and 6-sulfogalactose residues.^{8,9} Langerin shows specificity for monosaccharides very similar to that of another mannose-binding C-type lectin, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN).¹⁰ Recently, langerin has been reported to bind sulfated glycosaminoglycans (GAGs) in a Ca²⁺-independent manner, especially for heparin and heparan sulfate (HS).¹¹

Heparin and HS are linear, polydisperse, anionic, and often highly sulfated GAGs responsible for a variety of critical

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biological functions.^{12–14} Heparin has a high negative charge density as a result of its high content of negatively charged carboxyl and sulfo groups. It also shows complex micro-heterogeneity because of the numerous structural variations in heparin's disaccharide units. The 2-amino group of the glucosamine residue, the 3- and 6-hydroxyl groups of the glucosamine residues, and the 2-hydroxyl group of the iduronic acid residues can all be substituted.¹⁵ Furthermore, low-molecular weight heparin and heparin-derived oligosaccharides have been developed as heparin substitutes and have demonstrated comparable activities.^{16,17} Heparin, HS, and other membrane-associated sulfated GAGs have been reported to interact with proteins having diverse functions.^{15,18} As a result, many pathophysiological processes can be mediated through protein–heparin/GAG interactions. Such interactions include blood coagulation, cell growth and differentiation, host defense, cell-to-cell and cell-to-matrix signaling, lipid transport and metabolism, inflammation, cancer, and Alzheimer's disease.^{15,18–20} In LCs, sulfated GAGs may play a crucial role in the formation of BGs through their binding to langerin because they have been discovered to act as bridges in the organization of BG membranes.²¹ The interaction of protein and heparin/GAG is complex and structure-dependent. As for langerin, a previous study showed that the binding between langerin and heparin was not simply due to a net charge effect but that the sulfation pattern influences this interaction, such as the content of 6-*O*-sulfo groups or iduronic acid.¹¹ Muñoz-García et al.²² reported two different langerin-binding sites, one for a heparin-like trisaccharide and one for a heparin-like hexasaccharide that were Ca²⁺-dependent and Ca²⁺-independent,¹⁰ respectively. On the basis of these results, it is easy to speculate that the interaction between langerin and heparin is impacted by both the solution conditions and the heparin structure [chain size as the degree of polymerization (dp) and sulfation pattern]. Thus, a detailed understanding of langerin and heparin/GAG binding properties at the molecular level is of fundamental importance to understanding the biological role of the langerin–heparin interaction and also to the design of new highly specific therapeutic agents.

In this study, we utilize a preimmobilized streptavidin (SA) chip to which biotinylated heparin was immobilized onto the surface. Using a Biacore SPR instrument, the kinetics of the interaction between langerin and heparin was studied under different physiologically relevant conditions with various salt species, salt concentrations, and pH conditions. Studies of the competition between the heparin on the chip surface and GAGs in the solution phase were conducted to determine the preferences of binding of langerin to GAGs. Competition studies with heparin-derived oligosaccharides having different chain lengths and with different chemically modified heparins were conducted to determine the chain size dependence and the effect of different sulfo groups on langerin–heparin interactions. In addition, liquid chromatography–mass spectrometry (LC–MS) analysis was used to characterize the structure of sulfated glycans that bound to langerin.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human langerin was from R&D Systems (Minneapolis, MN).

GAGs used were porcine intestinal heparan sulfate (12 kDa) and porcine intestinal heparin (16 kDa) from Celsus Laboratories (Cincinnati, OH), porcine rib cartilage chon-

droitin sulfate A (20 kDa) (Sigma, St. Louis, MO), porcine intestine chondroitin sulfate B (30 kDa) (Sigma), shark cartilage chondroitin sulfate C (20 kDa) (Sigma), whale cartilage chondroitin sulfate D (20 kDa) (Seikagaku, Tokyo, Japan), squid cartilage chondroitin sulfate E (20 kDa) (Seikagaku), and keratan sulfate (14.3 kDa) that was isolated from bovine cornea in the Linhardt laboratory.²³ *N*-Desulfated heparin (*N*-DeS HP, 14 kDa) and 2-*O*-desulfated heparin (2-DeS HP, 13 kDa) were prepared using the methods of Yates et al.²⁴ The 6-*O*-desulfated heparin (6-DeS HP, 13 kDa) was provided by Dr. Wang (University of Georgia, Athens, UGA). Oligosaccharides from heparin include tetrasaccharide (dp4), hexasaccharide (dp6), octasaccharide (dp8), decasaccharide (dp10), dodecasaccharide (dp12), tetradecasaccharide (dp14), hexadecasaccharide (dp16), and octadecasaccharide (dp18). These were prepared from porcine intestinal through heparin controlled partial heparin lyase I treatment and size fractionation. The GAG chemical structures are presented in Figure 1. Biacore (Biacore AB, Uppsala, Sweden) Sensor SA

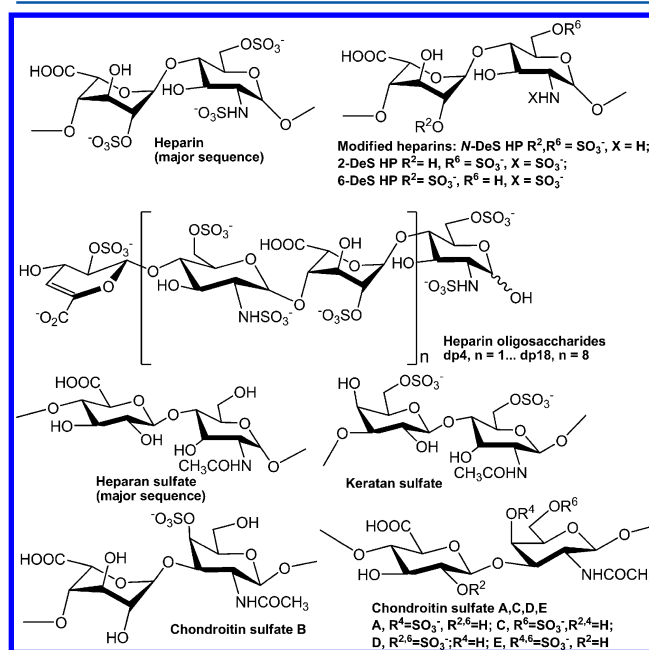


Figure 1. Structures of heparin/oligosaccharides and other GAGs.

chips were used, and SPR was performed on a Biacore 3000 operated using Biacore 3000 control and BIAevaluation software (version 4.0.1). Unsaturated heparin/HS disaccharide standards were 0S (Δ UA-GlcNAc), NS (Δ UA-GlcNS), 6S (Δ UA-GlcNAc6S), 2S (Δ UA2S-GlcNAc), NS2S (Δ UA2S-GlcNS), NS6S (Δ UA-GlcNS6S), 2S6S (Δ UA2S-GlcNAc6S), and TriS (Δ UA2S-GlcNS6S), purchased from Iduron (Manchester, U.K.). Tributylamine was purchased from Sigma. Ammonium acetate, acetic acid, water, and acetonitrile were of high-performance liquid chromatography grade (Fisher Scientific, Springfield, NJ). Microcon Centrifugal Filter Units (YM-10) were from Millipore (Bedford, MA). *Escherichia coli* expression and purification of recombinant *Flavobacterium heparinum* heparin lyase I, II, and III (EC 4.2.2.7, 4.2.2.X, and 4.2.2.8, respectively) were performed in our laboratory as previously described.²⁵

Preparation of the Heparin Biochip. The biotinylated heparin was synthesized by treating sulfo-*N*-hydroxysuccini-

vide long-chain biotin (Thermo Scientific, Waltham, MA) with free amino groups of unsubstituted glucosamine residues in the polysaccharide chain following a literature method.²⁶ Biotinylated heparin was immobilized to a streptavidin (SA) chip using the manufacturer's protocol. A 20 μL solution of the heparin–biotin conjugate (0.1 mg/mL) in HBS-EP running buffer was injected over the second flow cell of a SA chip at 10 $\mu\text{L}/\text{min}$. Successful immobilization of heparin was confirmed by an ~ 100 resonance unit (RU) increase in the sensor chip. The first flow cell (control) was prepared by a 1 min injection with saturated biotin.

Measurement of Interaction between Langerin and Heparin using BIAcore. The protein samples were diluted in HBS-EP buffer [0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20, (pH 7.4)]. Different dilutions of the protein samples were injected at a flow rate of 30 $\mu\text{L}/\text{min}$. Following sample injection, HBS-EP buffer was passed over the sensor surface for dissociation. The sensor surface was regenerated by injection of 30 μL of 0.25% sodium dodecyl sulfate (in water) after a 3 min dissociation time to fully regenerate the surface. The response was determined as a function of time (sensorgram) at 25 °C.

SPR Solution Competition between Heparin Bound to the Chip Surface and Heparin-Derived Oligosaccharides in Solution. Langerin (20 nM) was mixed with 1000 nM heparin oligosaccharides, including tetrasaccharide (dp4), hexasaccharide (dp6), octasaccharide (dp8), decasaccharide (dp10), dodecasaccharide (dp12), tetradecasaccharide (dp14), hexadecasaccharide (dp16), and octadecasaccharide (dp18), in HBS-EP buffer. These mixtures were injected over the heparin chip each at a flow rate of 30 $\mu\text{L}/\text{min}$. Dissociation and the regeneration steps were performed after each run as described above. For each set of competition experiments via SPR, a control experiment (only protein without heparin or oligosaccharides) was used to ensure the surface had been completely regenerated and that results obtained between runs were comparable.

SPR Solution Competition between Heparin Bound to the Chip Surface and GAGs and Chemically Modified Heparin in Solution. For the testing of inhibition of other GAGs and chemically modified heparins to the langerin–heparin interaction, 20 nM langerin was premixed with 1000 nM GAG or chemically modified heparin and injected over the heparin chip at a flow rate of 30 $\mu\text{L}/\text{min}$. A dissociation period and regeneration protocol was performed as described above after each run.

Measurement of the Effects of Physiological Conditions on the Interaction between Heparin and Langerin Using SPR. Langerin was diluted in HBS-P buffer [0.01 M HEPES, 0.15 M NaCl, and 0.005% surfactant P20 (pH 7.4)]. Langerin samples in buffers with different physiological conditions (pH, salt concentration, and Ca^{2+} and Zn^{2+} concentrations) were injected at a rate of 30 $\mu\text{L}/\text{min}$. The same buffer was passed over the sensor surface to facilitate dissociation at the end of the sample injection. The sensor surface was regenerated by injecting 30 μL of 0.25% SDS to obtain a regenerated surface after a 3 min dissociation time. The response was determined as a function of time (sensorgram) at 25 °C.

Filter Trapping of HS Oligosaccharides Binding to Langerin and Composition Analysis Using LC–MS. HS dp10 was mixed with langerin in buffer with 25 mM Hepes and 150 mM NaCl (pH 7.4) and incubated at room temperature for

1 h. The nonbinding oligosaccharides were removed from the mixture using spin columns (MWCO of 10 kDa), which were washed three times with buffer. The high-affinity oligosaccharides were subjected to disaccharide compositional analysis using LC–MS. Samples (100 μg in 5 μL of distilled water) were added to 100 μL of digestion buffer [50 mM NH_4Ac and 2 mM CaCl_2 (pH 7.0)]. Heparin lyase I, II, and III [10 milliunits each in Tris-HCl buffer (pH 7.0)] were added and mixed well. Samples were sufficiently digested in a 37 °C water bath for 12 h. Enzymatic digestion was terminated by removing the enzymes using a spin column (MWCO of 10 kDa). The filtrates were lyophilized and redissolved in distilled water. Reverse phase ion pairing liquid chromatography with online electrospray ion trap mass spectrometry analyses (RPIP–LC–MS) were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Wilmington, DE) equipped with a model 6300 ion trap and a binary pump. A Poroshell 120, EC-C18 column (2.7 μm , 2.1 mm \times 100 mm, Agilent Technologies) was used for separation. Eluent A consisted of water and acetonitrile [85:15 (v/v)], and eluent B consisted of water and acetonitrile [35:65 (v/v)]. Both eluents contained 12 mM tributylamine and 38 mM ammonium acetate with the pH adjusted to 6.5 with acetic acid. A gradient of solution A for 8 min followed by a linear gradient from 8 to 15 min (0 to 80% solution B) was used at a flow rate of 100 $\mu\text{L}/\text{min}$. The column effluent entered the source of the ESI-MS instrument for detection by MS. The electrospray interface was in the negative ionization mode using a skimmer potential of -40.0 V, a capillary exit of -40.0 V, and a source temperature of 350 °C for a maximal abundance of the ions in a full-scan spectrum (200–1500 Da). Nitrogen (8 L/min, 40 psi) was selected as a drying and nebulizing gas. An unsaturated disaccharide standard mixture solution was prepared (each at a concentration of 100 $\mu\text{g}/\mu\text{L}$) for relative quantification.

RESULTS

Kinetic Measurements of Langerin–Heparin Interactions. Several previous reports showed that langerin is a heparin-binding protein.^{11,22} Kinetic measurement of langerin–heparin interaction was performed using a sensor chip with immobilized heparin. Sensorgrams of langerin–heparin interaction are shown in Figure 2. The sensorgrams were globally fit to calculate the apparent on (k_a) and off (k_d) rates for the

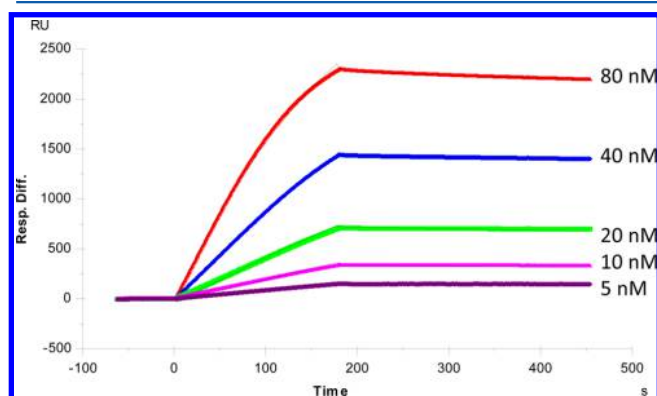


Figure 2. SPR sensorgrams of langerin–heparin interaction. Langerin concentrations (from top to bottom): 80, 40, 20, 10, and 5 nM, respectively. The black fitting curves use models from BIAevaluation version 4.0.1.

binding equilibrium (Table 1), using the BiaEvaluation software based on a 1:1 Langmuir model.

Table 1. Kinetic Data for Langerin–Heparin Interactions

interaction	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_D (M)
langerin–heparin	5.8×10^4 ($\pm 587^a$)	1.4×10^{-4} ($\pm 5.2 \times 10^{-6a}$)	2.4×10^{-9}
langerin–N-DeS HP	NT ^b	NT ^b	1.1×10^{-7c}
langerin–2-DeS HP	NT ^b	NT ^b	6.0×10^{-9c}
langerin–6-DeS HP	NT ^b	NT ^b	8.5×10^{-8c}
langerin–heparin dp16	NT ^b	NT ^b	1.1×10^{-7c}
langerin–heparin dp18	NT ^b	NT ^b	4.3×10^{-9c}

^aStandard deviation obtained from global fitting of five injections.

^bNot tested. ^cSolution-based affinities (K_i) were calculated from IC_{50} values measured from SPR competition experiments using the equation $K_i = IC_{50}/(1 + [C]/K_D)$, where $[C]$ (20 nM) is the concentration of langerin used in the competition SPR, and the langerin–heparin binding affinity (K_D) is 2.4×10^{-9} M.

Solution Competition Study between Heparin (chip surface) and Heparin-Derived Oligosaccharides (in Solution) Using SPR. Solution/surface competition experiments were performed by SPR to examine the impact of the saccharide chain size of heparin on the langerin–heparin interaction. Heparin-derived oligosaccharides of different sizes (from dp4 to dp18) were used in the competition study. Heparin oligosaccharides (1000 nM) were preincubated in langerin solutions that were injected over the heparin chip. Comparable competition effects were observed when 1000 nM oligosaccharide (dp6–dp14) was present in the langerin protein solution. When the size of the oligosaccharide increased to dp16, the level of binding of langerin to the surface heparin significantly decreased (Figure 3). The observed variation of langerin binding suggests that the interaction between langerin and heparin is chain length-dependent and that langerin prefers to bind long-chain heparin.

Solution Competition Study of Various Chemically Modified Heparins. SPR competition sensorgrams and graphs of the competition levels of chemically modified heparin are presented in Figure 4. For langerin, all three chemically modified heparins (*N*-desulfated heparin, 2-*O*-desulfated heparin, and 6-*O*-desulfated heparin) resulted in reduced inhibitory activities. A much greater reduction of inhibitory activities was seen for *N*-desulfated heparin and 6-*O*-desulfated heparin than for 2-*O*-desulfated heparin, suggesting that the 2-*O*-sulfo group of heparin has less impact on the langerin–heparin interaction.

SPR Solution Competition Study of Various GAGs. The SPR competition assay was also applied to determine the binding preference of langerin for different GAGs (Figure 1). SPR competition sensorgrams and graphs of the GAG competition levels are presented in Figure 5. For langerin, heparin produced the greatest inhibition by competing with >95% of the langerin binding to the immobilized heparin signal. Strong inhibitory activities (>80%) were observed for CSB and CSE. Modest inhibitory activities (>50%) were observed for HS, CSC, CSD, and KS. Weak inhibitory activity was observed for CSA.

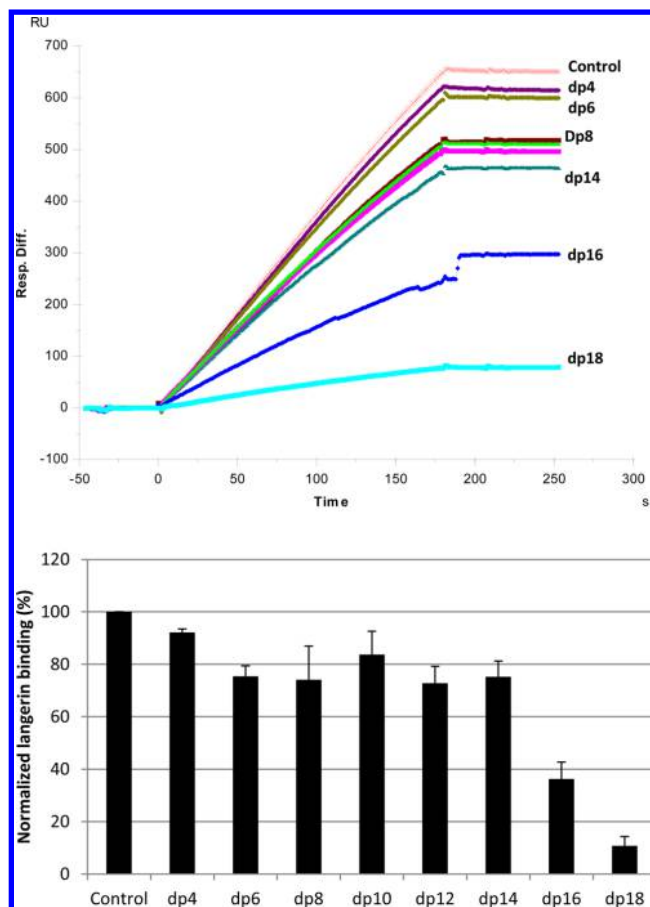


Figure 3. Solution heparin oligosaccharide/surface heparin competition sensorgrams (top). The concentration of langerin was 20 nM, and heparin oligosaccharide concentrations in solution were 1000 nM. Bar graphs (triplicate experiments with SD) of the normalized langerin binding preference for surface-immobilized heparin by competing with heparin oligosaccharides of different sizes in solution (bottom).

Effects of Physiological Conditions on the Interaction between Heparin and Langerin.

Binding buffers at different pH values (pH 7.4, as a control, and pH 6.0, 5.0, and 4.0) and with different salt (NaCl) concentrations (150 mM, as a control, and 300, 500, and 1000 mM) were used for the SPR analysis to examine the effect of buffer physiological conditions on langerin–heparin interaction. The results of langerin–heparin interaction in different buffers are presented in panels A and B of Figure 6. The results showed that acidic pH reduced the level of langerin binding, and no binding was detected at pH 4.0. High salt concentrations (0.5 and 1 M NaCl) inhibited all the binding to heparin, suggesting that this interaction is primarily electrostatically driven.

The impact of Ca^{2+} and Zn^{2+} on langerin–heparin interaction was also assessed. Langerin–heparin interaction was also examined in the presence of $CaCl_2$ or $ZnCl_2$ at concentrations of 0, 10, 100, and 1000 μ M. The results (Figure 6C,D) showed that the level of binding of langerin to heparin increased with addition of 10, 100, and 1000 μ M Ca^{2+} , and 100 μ M Ca^{2+} gave the strongest binding promotion. The impact of Zn^{2+} on langerin–heparin interaction showed a pattern similar to that with Ca^{2+} , except the level of langerin binding was reduced upon addition of 1000 μ M Zn^{2+} .

Structural Analysis on the Specific Oligosaccharides Binding to Langerin. A “fishing” filter trapping experiment

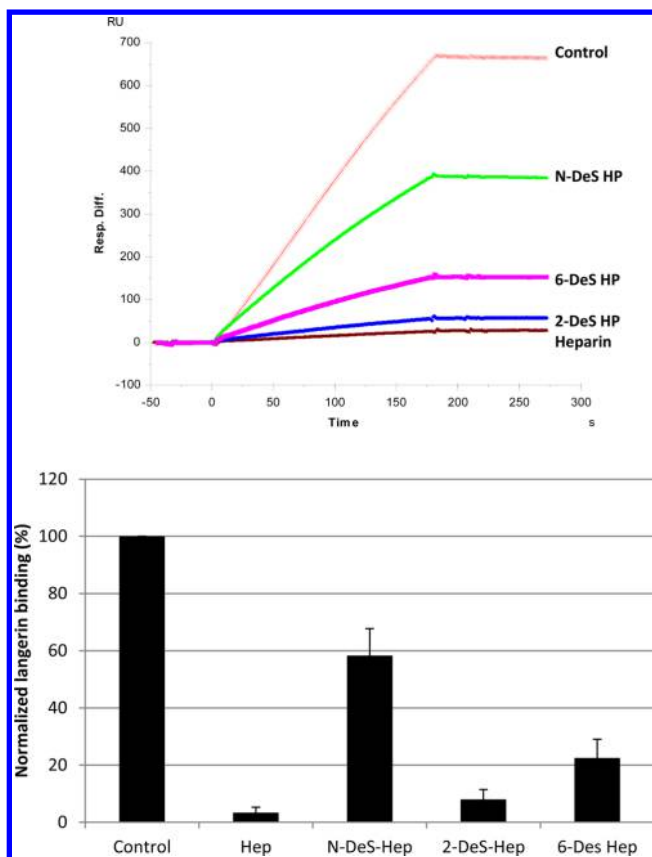


Figure 4. Solution chemically modified heparin/surface heparin competition sensorgrams (top). The langerin concentration was 20 nM, and concentrations of chemically modified heparin in solution were 1000 nM. Bar graphs (triplicate experiments with SD) of normalized langerin binding preference for surface-immobilized heparin by competing with different chemically modified heparins in solution (bottom).

was conducted using langerin as “bait” to catch the preferred heparin/HS from HS-derived oligosaccharide to further investigate the fine structural specificity of heparin/HS for the langerin interaction. Our previous report showed HS dp10 oligosaccharide with diversity of the disaccharide compositional structures is a good candidate for the filter trapping experiment.²⁷ The high-affinity oligosaccharides caught by langerin were subjected to disaccharide compositional analysis using LC–MS (Figure 7). The results showed the HS dp10 that bound to langerin was substantially enriched in TriS, NS2S, and NS6S disaccharide in comparison to the original HS dp10.

DISCUSSION

Glycans can mediate many physiological or pathophysiological functions (e.g., blood coagulation, cell-to-cell and cell-to-matrix signaling, cell growth and differentiation, inflammation, host defense, and viral infection) by interacting with glycan-binding proteins (GBPs). The GBPs can be broadly classified into the major groups of lectins and glycosaminoglycan (GAG)-binding proteins. Most interactions between lectins and glycans feature defined “carbohydrate recognition domains” (CRDs) with specific amino acid sequences or three-dimensional structures on lectins. CRDs bind to carbohydrate ligands mostly through hydrogen bonding interactions.²⁸ For interactions of protein with sulfated GAGs, most of the binding occurs primarily

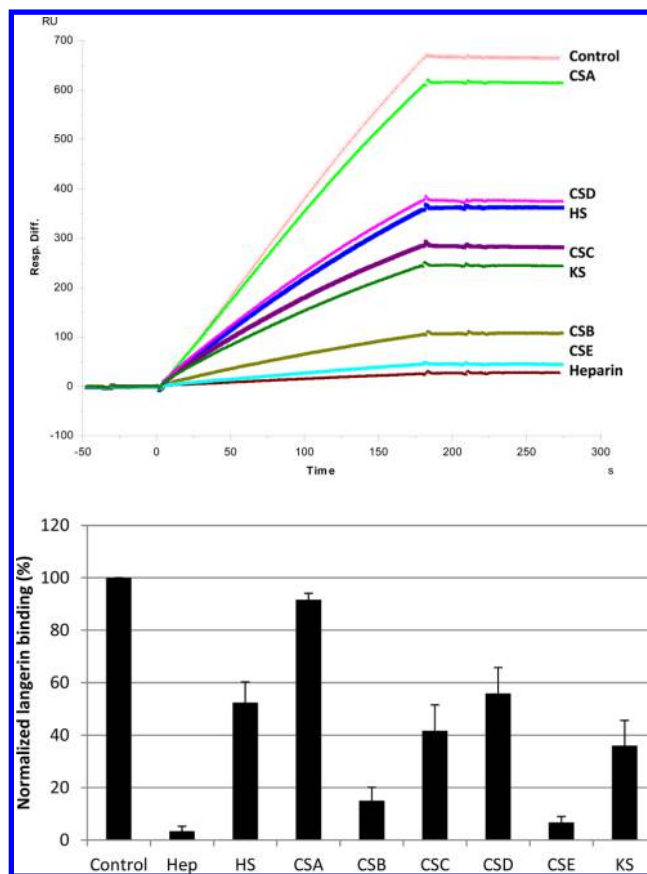


Figure 5. Solution GAG/surface heparin competition sensorgrams (top). The langerin concentration was 20 nM, and concentrations of solution GAGs were 1000 nM. Bar graphs (triplicate experiments with SD) of normalized langerin binding preference for surface-immobilized heparin by competing with different GAGs (bottom).

through ion pairing interactions between positively charged amino acids and anionic GAG chains.¹⁵ Langerin is a C-type lectin with CRD that shows specificity for mannose, GlcNAc, and fucose.^{7–10} It has binding specificity to sulfated glycans such as Lewis x analogues, sulfated dextrans, galactose 6-sulfated oligosaccharides, and keratan sulfate.⁸ Recently, it was reported that langerin is able to bind sulfated GAGs, especially for heparin and HS.¹¹ While these studies provide some level of understanding of langerin-sulfated GAG interaction, there is an absence of detailed structural and biophysical data for these interactions.

In this study, we utilized a SPR system to measure the binding kinetics and affinity of langerin–heparin interaction using a heparin chip. SPR analysis demonstrated that langerin bound to heparin with nanomolar ($K_D \sim 2.4$ nM) affinity (Figure 2). Our affinity data agree with the previous report,¹¹ suggesting heparin is the strongest binding glycan ligand ever described for langerin. Most langerin–glycan (e.g., mannose, GlcNAc, and fucose) binding affinities were in the millimolar range.⁷ It is noteworthy that SPR has some limitations when determining the stoichiometry of molecular interactions. We used a simple 1:1 Langmuir bimolecular reaction model, which is most commonly selected to process the binding data, and the sensorgrams fit well with this model. More experiments, including isothermal titration calorimetry (ITC), might be useful for determining the stoichiometry of heparin–langerin interaction.

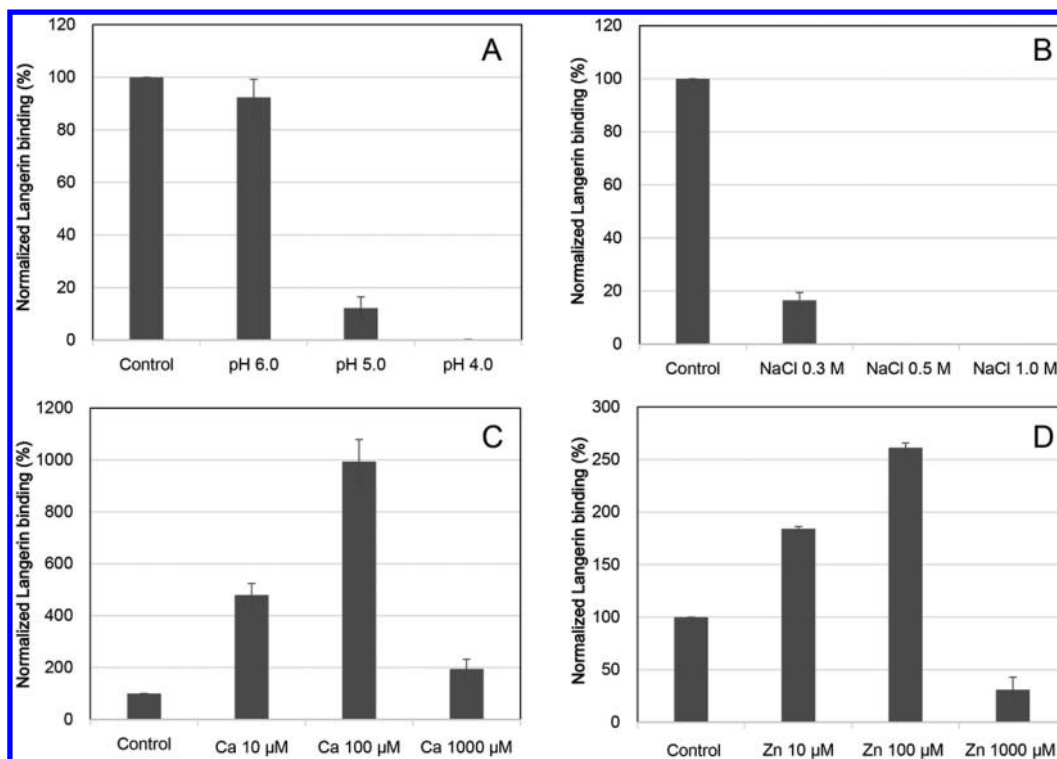


Figure 6. SPR analysis of the effects of physiological conditions on the interactions between heparin and langerin. (A) Effect of buffer pH on the heparin–langerin interaction. (B) Effect of salt (NaCl) concentration on the heparin–langerin interaction. (C) Heparin–langerin interaction with the addition of CaCl₂ (0, 10, 100, and 1000 μM). (D) Heparin–langerin interaction with addition of ZnCl₂ (0, 10, 100, and 1000 μM).

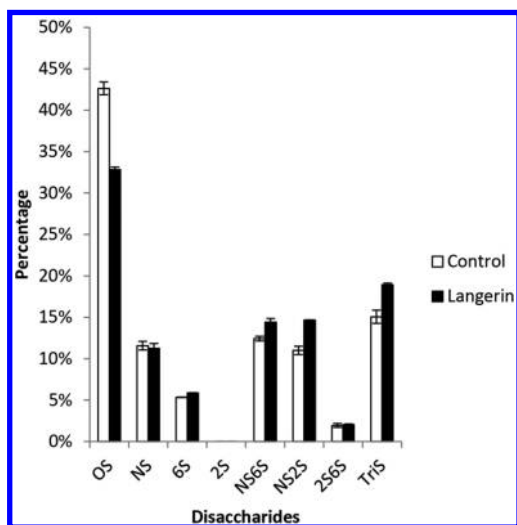


Figure 7. Disaccharides compositional analysis of HS dp10 binding to langerin using LC–MS.

SPR competition experiments were undertaken (1) to determine the heparin chain length preference of binding of langerin to heparin, (2) to understand the impact of heparin structure (e.g., sulfation level and position) on the interactions, and (3) to test the specificities of the heparin/HS and other GAGs. The competition SPR studies with different sized heparin oligosaccharides revealed the langerin–heparin interaction is chain length-dependent and demonstrate the minimal length heparin oligosaccharide required for binding as greater than a tetrasaccharide (dp4). The data also showed that langerin prefers to bind longer-chain heparin oligosaccharide (such as dp16 and longer). The result agrees with the reported

in situ model,¹¹ suggesting a longer heparin chain is favorable for the binding and can bridge multiple binding regions on the protein. The SPR competition experiments with chemically modified heparin clearly showed langerin–heparin interaction depends on sulfation level and sulfo group position: 2-*O*-sulfo groups on heparin have an effect on the interaction weaker than that of 6-*O*-sulfo or *N*-sulfo groups. The data are clearly in line with the *in situ* model¹¹ in which electropositive amino acids (Lys and Arg) of the protein accommodate well with negatively charged groups (from 2-*O*-sulfo, 6-*O*-sulfo, or *N*-sulfo) on heparin (Figure 1) well. These data are in agreement with the results from the filter trapping experiment indicating the HS fragments with TriS, NS2S, and NS6S disaccharide are tighter-binding ligands for langerin. Similar effects of sulfation pattern on heparin/HS protein interactions were observed in our previous studies, including effects on Robo1–heparin and TIMP-3–heparin interactions.^{29,30}

The SPR competition assay using various other GAGs revealed the binding preference of langerin: heparin > chondroitin sulfate E > chondroitin sulfate B (DS) > keratan sulfate and chondroitin sulfate C > heparan sulfate > chondroitin sulfate D and A. In a similar SPR competition study reported by Chabrol et al.¹¹ showing the langerin preferentially binds to HS GAGs, with free heparin and HS being the most potent inhibitors, CS/DS-type GAGs showed a much lower level of inhibition with CS-C as the best inhibitor, followed by DS and CS-A; they could not measure the KS to inhibit langerin–heparin interaction. Our results partially agree with the reported langerin binding preference for the GAGs.¹¹ The difference might be due to the different source of GAGs we used. It should be noted that KS has been reported as a langerin-binding ligand in a previous study⁸ and that this is the first time CSE is reported showing strong binding to langerin.

Langerin–heparin interactions are greatly affected by the binding buffer conditions. The interactions are not favored in acidic pH buffer or high-salt (NaCl) conditions, which suggests that this interaction is primarily electrostatically driven. Lower pH values reduce the negative charges on heparin chains and might weaken the molecular electrostatic attraction. It was reported that the interactions between langerin and glycans were pH-dependent.⁷ There are several heparin/HS–protein interactions that are regulated by pH, including β -amyloid peptide (A β)³¹ and stromal cell-derived factor-1 (SDF-1).³² There is an obvious impact of Ca²⁺ and Zn²⁺ on langerin–heparin interaction: langerin binding to heparin was enhanced upon addition of Ca²⁺, although it showed strong binding to heparin ($K_D \sim 2.4$ nM) in Ca²⁺-free buffer. This is in agreement with a previous study²² that reported that there are Ca²⁺-dependent and Ca²⁺-independent heparin-binding sites on langerin.

In conclusion, our analysis shows that langerin is a heparin-binding lectin with a high affinity ($K_D \sim 2.4$ nM). Solution competition studies show that binding of langerin to heparin is dependent on chain length and langerin prefers to bind heparin polysaccharide or large oligosaccharides, with 16–18 sugars. High sulfation levels of GAGs enhance binding affinities; i.e., CSE showed high binding affinity for langerin as heparin, and the low-sulfation level GAGs (HS, CSA, CSB, CSC, CSD, and keratan sulfate) showed modest binding affinity. *N*-Sulfo and 6-*O*-sulfo groups on heparin are needed for the langerin–heparin interaction. The interactions are greatly affected by the buffer conditions (pH, salt concentration, and Ca²⁺ and Zn²⁺ concentrations). The interaction studies and structural characterization conducted here should be useful for understanding the mechanisms and roles of langerin and langerin–heparin complexes in mediating LC processes.

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ABBREVIATIONS

GAG, glycosaminoglycan; SPR, surface plasmon resonance; HS, heparan sulfate; CSA, chondroitin sulfate A; CSB, chondroitin sulfate B; CSC, chondroitin sulfate C; CSD, chondroitin sulfate D; CSE, chondroitin sulfate E; KS, keratan sulfate; SA, streptavidin; dp, degree of polymerization; MWCO, molecular weight cutoff; SD, standard deviation.

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