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ORIGINAL ARTICLE

Interactions between nattokinase and heparin/GAGs

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Abstract Nattokinase (NK) is a serine protease extracted from a traditional Japanese food called natto. Due to its strong fibrinolytic and thrombolytic activity, NK is regarded as a valuable dietary supplement or nutraceutical for the oral thrombolytic therapy. In addition, NK has been investigated for some other medical applications including treatment of hypertension, Alzheimer's disease, and vitreoretinal disorders. The most widely used clinical anticoagulants are heparin and low molecular weight heparins. The interactions between heparin and proteins modulate diverse patho-physiological processes and heparin modifies the activity of serine proteases. Indeed, heparin plays important roles in almost all of NK's potential therapeutically applications. The current report relies on surface plasmon resonance spectroscopy to examine NK interacting with heparin as well as other glycosaminoglycans (GAGs). These studies showed that NK is a heparin binding protein with an affinity of ~250 nM. Examination with differently sized heparin oligosaccharides indicated that the interaction between NK and heparin is chain-length dependent and the minimum size for heparin binding is a hexasaccharide. Studies using chemically modified heparin showed the 6-*O*-sulfo as well as the *N*-sulfo groups but not the 2-*O*-sulfo groups within heparin, are essential for heparin's interaction with NK. Other GAGs (including HS, DS, and CSE) displayed modest binding affinity to NK. NK also interfered with other heparin-protein interactions, including heparin's interaction with antithrombin and fibroblast growth factors.

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Keywords Heparin · Nattokinase · Binding · Surface plasmon resonance

Abbreviations

GAG	Glycosaminoglycan
NK	Nattokinase SPR, surface plasmon resonance
HS	Heparan sulfate
CSA	Chondroitin sulfate A
DS	Dermatan sulfate
CSC	Chondroitin sulfate C
CSD	Chondroitin sulfate D
CSE	Chondroitin sulfate E
SA	Streptavidin
FC	Flow-cell
RU	Resonance unit
dp	Degree of polymerization.

Introduction

Nattokinase (NK) is a protein that is extracted from a popular Japanese food called natto, a vegetable cheese-like food, which is boiled soybeans fermented with *Bacillus subtilis natto*. Natto has been used as a folk remedy for diseases of the heart and cardiovascular diseases for hundreds of years [1]. Modern studies on NK demonstrated that it was not a kinase, but rather a subtilisin-like serine protease, and that this activity was responsible for NK's fibrinolytic effects [2]. NK is regarded as a valuable dietary supplement and/or a nutraceutical since it is sufficiently stable in the gastrointestinal tract to make this enzyme a potentially useful agent for the oral thrombolytic therapy [3]. *In vitro* and *in vivo* studies have demonstrated the potent pro-fibrinolytic effect of the enzyme [4–7]. An open-label, self-controlled clinical trial showed that on oral administration NK could be considered a nutraceutical for the prevention of cardiovascular disease, decreasing plasma levels of fibrinogen, factor VII, and factor VIII [7]. There are other potential applications for NK including treatment of hypertension, Alzheimer's disease, and vitreoretinal disorders [3, 8–11]. A randomized, double-blind, placebo-controlled study, to examine how nattokinase supplementation impacted systolic and diastolic blood pressure in pre-hypertension subjects, showed NK supplementation reduced both. After absorption through the intestine NK retained its protease activity suggesting it could decrease blood pressure through cleavage of plasma fibrinogen. These NK-degradation products, prevent the elevation of plasma angiotensin II level to suppress hypertension [8]. It was reported that NK can assist toxic amyloid fibril catabolism, and that such fibrils can include insulin fibrils in diabetes, fibrils in Alzheimer's Disease, and fibrils prion disease [10]. Based on the preliminary results of amyloid-degrading ability of NK, they suggested it might be useful in the treatment of amyloid-related diseases. However, it is not clear how NK or NK fragments can enter the circulation when taken orally. Many of the basic biochemistry mechanisms behind all the promising medical applications of NK are still reminded to be elucidated.

Heparan sulfate (HS) and heparin are members of the glycosaminoglycan (GAG) family. These are anionic, polydisperse, linear polysaccharides that are often highly sulfated. HS, found on the external cell membrane and also in the extracellular matrix (ECM), can interact with many of protein ligands [12–14]. Many physiological and pathophysiological processes can be modulated through protein-heparin/HS interactions including: blood coagulation, cell growth and differentiation, host defense, viral infection, lipid transport and metabolism, cell-to-cell and cell-to-matrix signaling, inflammation, Alzheimer's disease and cancer [12]. HS-protein interactions have been demonstrated to critically modulate the biological activity of the proteins through various mechanisms, including localization of proteins in the ECM, regulation of enzymatic activities, ligands binding to receptors, and protein protection against proteolysis [13]. Heparin or HS proteoglycans are also essential cofactors for the interaction of fibroblast growth factors (FGFs) with their receptors (FGFRs) [14, 15]. The goal of this study is to analyze molecular interactions of heparin/GAGs with NK. A BIAcore 3000, employed for this study, relies on surface plasmon resonance (SPR) for the direct quantitation, in real-time, of molecular interactions.

Materials and methods

Materials

Nattokinase was purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Porcine mucosal heparin (~16 kDa) as well as porcine mucosal HS were from Celsus Laboratories, (Cincinnati, OH); porcine rib cartilage chondroitin sulfate type A (~20 kDa), porcine mucosal chondroitin sulfate type B (also known as dermatan sulfate, ~30 kDa) and shark cartilage chondroitin sulfate type C (~20 kDa) were from Sigma Chemical (St. Louis, MO); whale cartilage chondroitin sulfate type D (~20 kDa) and squid cartilage chondroitin sulfate type E (20 kDa) were from Seikagaku (Tokyo, Japan). Controlled partial heparin lyase I depolymerization of beef lung heparin (Sigma) with subsequent size-fractionation [17] was used to prepare heparin tetrasaccharide (dp4), heparin hexasaccharide (dp6), heparin octasaccharide (dp8), heparin decasaccharide (dp10), heparin dodecasaccharide (dp12), heparin tetradecasaccharide (dp14), heparin hexadecasaccharide (dp16) and heparin octadecasaccharide (dp18). The 2-*O*-desulfonated IdoA heparin (~13 kDa) and the *N*-desulfonated heparin (~14 kDa) were prepared as previously reported [16]. The 6-*O*-desulfonated heparin (~13 kDa) [17] was a gift from Professor L. Wang of the University of Georgia. Human antithrombin III (AT III) was from Haematologic Technologies (Essex Junction, Vermont). Fibroblast growth factor-1 and -2 (FGF1 and FGF2) were generously provided by Amgen (Thousand Oaks, CA). GE Healthcare Bio-Sciences AB (Uppsala, Sweden) was the source of the SA sensor chips. A BIAcore 3000, running under BIAcore 3000 control and Version 4.0.1 BIAevaluation software, was used for SPR measurements.

Heparin biochip preparation

Biotin-heparin conjugate was synthesized through the reaction of long-chain sulfo-*N*-hydroxysuccinimide biotin

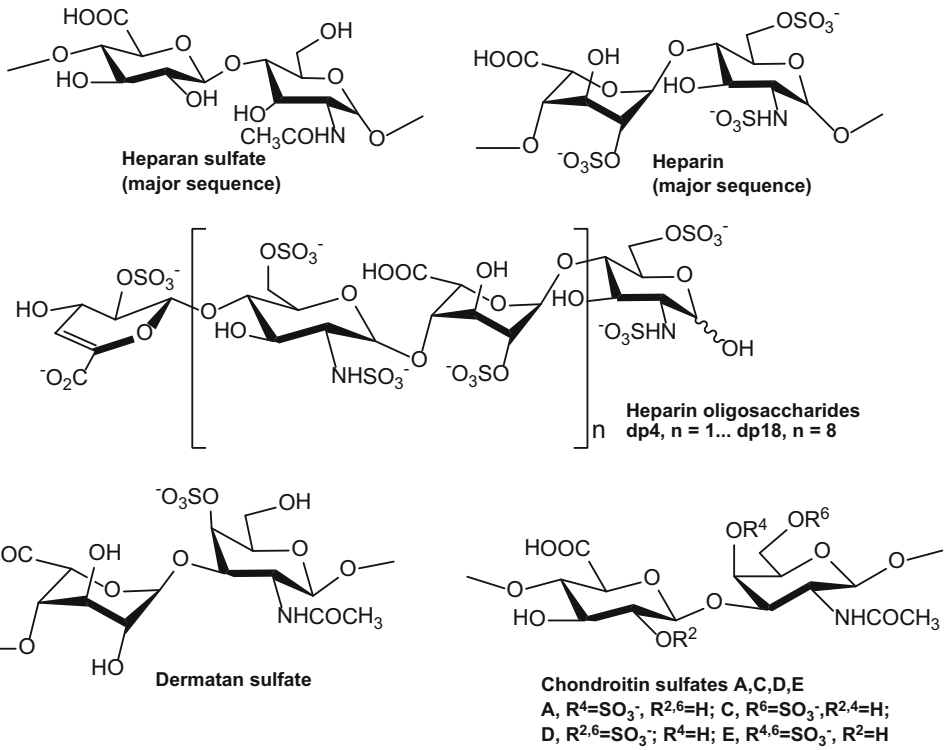

Fig. 1 Chemical structures of heparin, heparin-derived oligosaccharides and other GAGs

(Thermo Fisher, Rockford, IL) with the unsubstituted amino groups of the polysaccharide chain's glucosamine residues based on published methods [18–20]. The biotin-heparin conjugate was then bound to a streptavidin chip. A 20-μl solution of the biotin-heparin conjugate in HBS-EP running buffer (0.1 mg/mL) was injected over SA chip flow cell 2 at 10 μL/min. Immobilization of heparin was indicated through a ~250 resonance unit (RU) increase. Control flow cell (FC1) was prepared through a 1 min injection of saturated biotin.

Measuring heparin- NK interaction using SPR

Protein samples were made up in HBS-EP buffer (0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.15 M sodium chloride, 3 mM ethylenediaminetetraacetic acid, 0.005 % surfactant P20, pH 7.4). Protein samples prepared at different concentrations were injected at 30 μL/min. The HBS-EP buffer was flowed over the sensor surface to facilitate dissociation at the end of the sample injection. After dissociation over a 3 min time period, the surface of the sensor was completely regenerated with 30 μL of 2 M NaCl. Sensorgrams, measuring response as a function of time, were recorded at 25 °C.

Competition SPR NK-binding studies of immobilized heparin and soluble heparin-derived oligosaccharides

A mixture of NK (2.5 μM) and 1 μM of heparin oligosaccharide (dp4 through dp18) in HBS-EP buffer was injected over heparin chip at 30 μL/min. The dissociation and the regeneration, as described above, were performed after each run. A control experiment (protein only with no heparin or heparin oligosaccharides) was performed for each set of competition experiments on SPR in order to make certain that the surface was completely regenerated and that the results between runs were comparable.

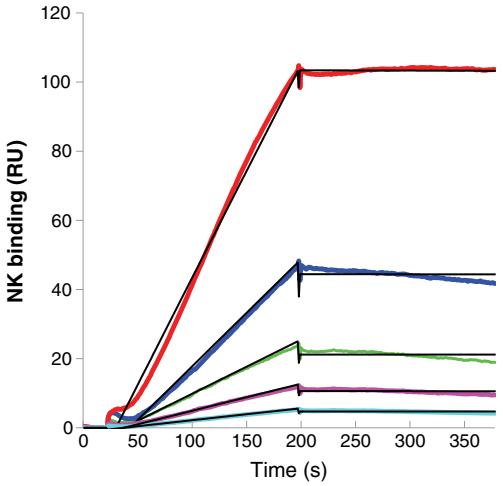

Fig. 2 SPR sensorgrams of NK-heparin interaction. Concentrations of NK (*from top to bottom*): 2500, 1250, 630, 315, and 158 nM, respectively. The black curves are the fitting curves using models from BIAevaluate 4.0.1

Table 1 Summary of kinetic data of NK-heparin interactions

Interaction	k_a *(1/MS)*	k_d *(1/S)*	K_D *(M)*
NK-Heparin	28.5 (±1.3)	7.0×10^{-6} ($\pm6.1\times10^{-7}$)	2.5×10^{-7}

The data with (±) in parentheses are the standard errors (SE) from global fitting

SPR competition study between immobilized heparin on chip surface and solution GAGs

NK (2.5 μM) pre-mixed with 1.0 μM of GAG (or chemical modified heparin) (Fig. 1) was injected over the heparin chip at 30 μL/min to study their inhibition of NK-heparin interaction. After each run, a dissociation period and regeneration protocol was performed.

Effect of NK on other heparin-protein interactions

NK (1000 nM) was added to other heparin binding proteins (AT III, FGF1 and FGF2) to see the effect of NK on other heparin-protein interactions. Different protein samples were injected to a heparin chip at 30 μL/min and a dissociation period and regeneration protocol were performed after each injection.

Statistical analysis

The data obtained was subjected to *T*-test. Statistical differences were calculated using software SPSS version 14.0 (SPSS Inc., Chicago, USA) at the 5 % probability level ($p<0.05$).

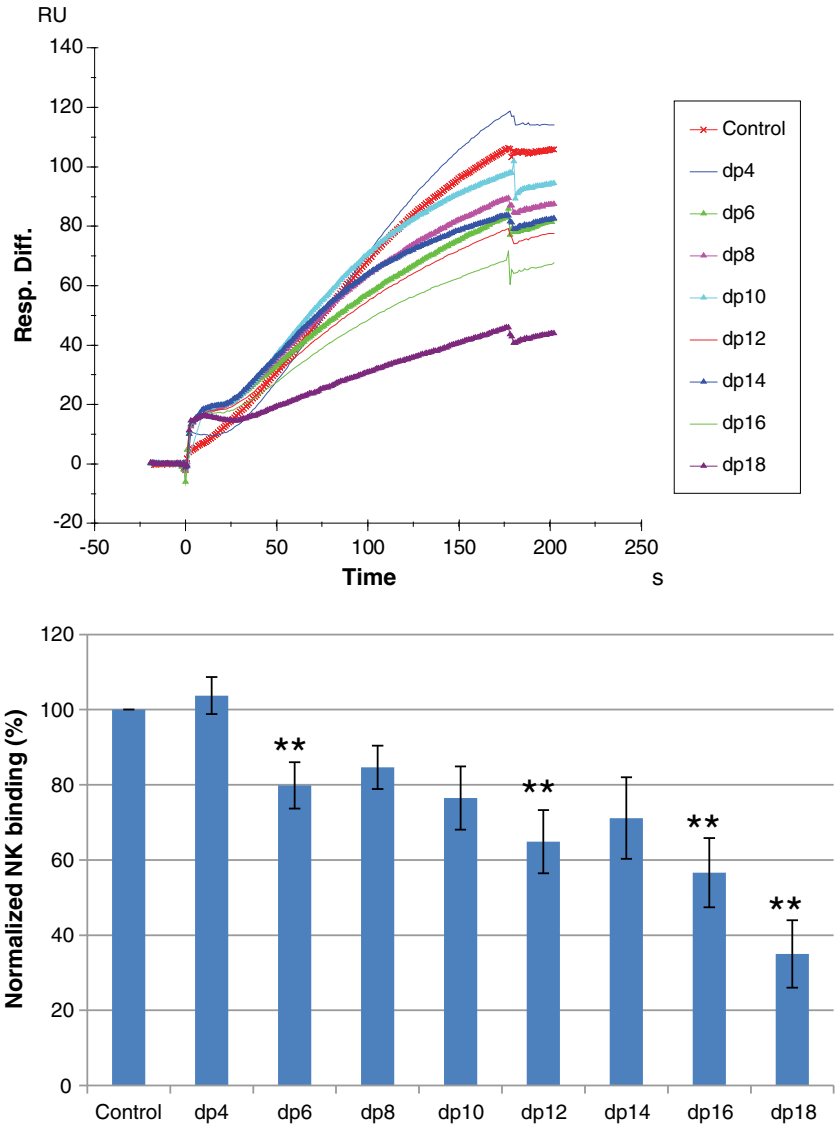

Fig. 3 SPR competition study using heparin oligosaccharides. *Top*: Sensorgrams of solution heparin oligosaccharides/surface heparin competition. NK concentration was 2500 nM, and concentrations of heparin oligosaccharides in solution were 1000 nM). *Bottom*: Bar graphs (based on triplicate experiments with standard deviation) of normalized NK binding to surface heparin by competing with different size of heparin oligosaccharides in solution. Statistical differences were calculated using software SPSS version 14.0 at the 5 % probability level (**, $p<0.05$)

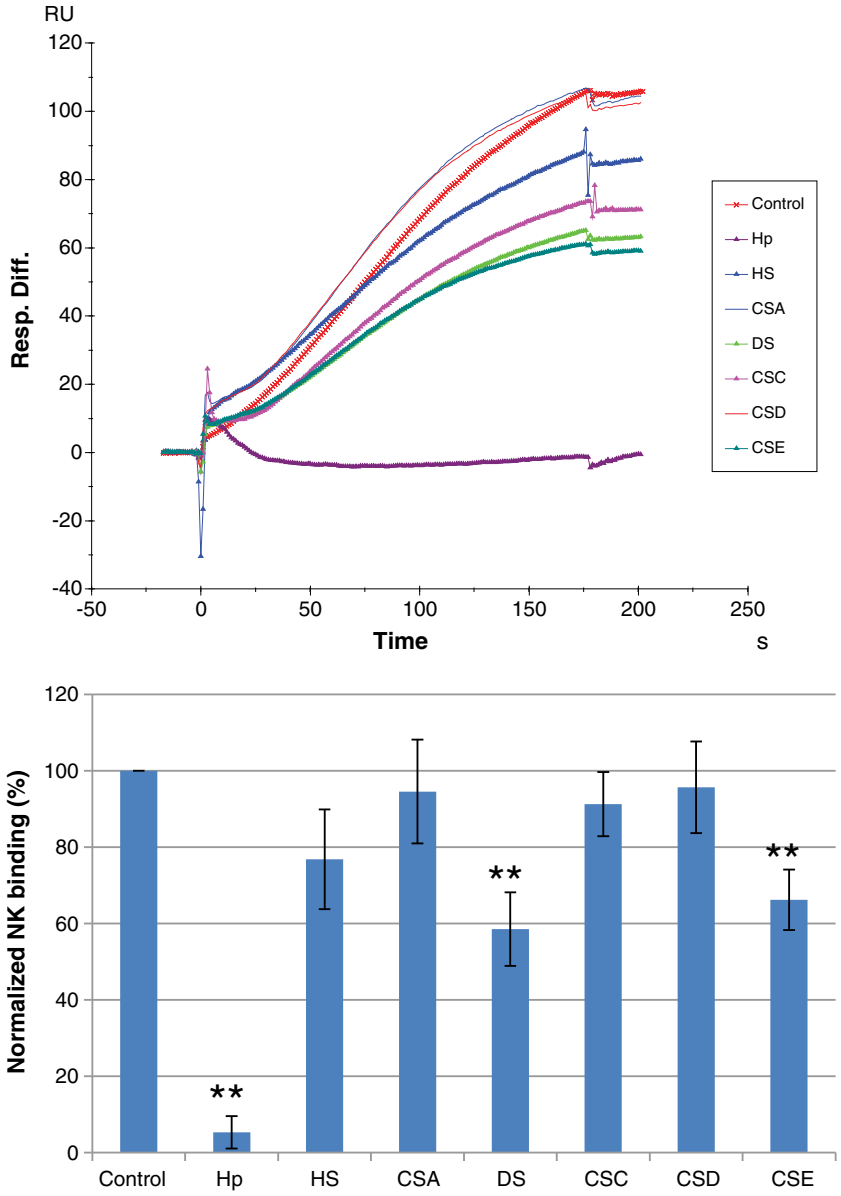

Fig. 4 SPR competition study using different GAGs. *Top*: Sensorgrams of solution GAGs/surface heparin competition. NK concentration was 2500 nM, and concentrations of GAGs in solution were 1000 nM). *Bottom*: Bar graphs (based on triplicate experiments with standard deviation) of normalized NK binding to surface heparin by competing with different GAGs in solution. Statistical differences were calculated using software SPSS version 14.0 at the 5 % probability level (**, $p<0.05$)

Results and discussion

Kinetics measurement of NK-heparin interactions

Sensorgrams of NK-heparin interaction were obtained by injecting different concentrations of NK on a heparin sensor chip to afford binding kinetics (Fig. 2). The sensorgrams were globally fit by a 1:1 Langmuir model at various NK concentrations. The binding kinetics are presented in Table 1. The interaction of NK to heparin displays a slow on-rate (ka) of 28.5 M^{-1} s^{-1} and slow off-rate (kd) of 7.0×10^{-6} s^{-1}. The binding equilibrium dissociation constant (K_D=kd/ka) for the NK/heparin interaction is ~250 nM demonstrating that NK was a heparin-binding protein. This is the first report of the heparin binding properties of NK and may be useful in explaining the different bioactivities (such as fibrinolytic and thrombolytic activity) of NK.

SPR competition study of the interaction between NK in solution and immobilized heparin in competition with heparin oligosaccharides in solution

SPR competition experiments were performed to study the impact of heparin oligosaccharide chain size on the heparin-NK interaction. Heparin oligosaccharides of sizes from dp4 to dp18 were used in the competition study. The same concentration (1 μM) of heparin oligosaccharide was present in the NK/heparin interaction solution. No competition effect was observed using 1 μM of tetrasaccharide. The remaining oligosaccharides

tested (dp 6 through dp18) showed decreased NK binding to surface immobilized heparin with increasing oligosaccharide size (Fig. 3). This result suggests that the NK-heparin interaction is chain-length dependent and the minimum size of heparin required for such an interaction is larger than tetrasaccharide.

SPR solution competition study of different GAGs

SPR competition studies were also used to determine binding preference of NK to a variety of GAGs. The sensorgrams and bar graphs showing GAG competition are presented in Fig. 4. The results show that heparin produced the strongest inhibition by competing >90 % of the NK binding to immobilized heparin signal. Modest inhibitory activities were observed for HS, DS and CSE. CSA, CSD and CSC were unable to compete with surface heparin/NK interaction at the concentrations examined.

Study of chemical modified heparins by SPR competition experiments

Chemically modified heparins (1 μM), studied by SPR competition experiments, are presented in Fig. 5 (top). All the three chemically modified heparins (6-*O*-desulfonated heparin, *N*-desulfonated heparin and 2-*O*-desulfonated heparin) show reduced competition when compared with heparin (Fig. 5). The greatly reduced inhibitory effect of 6-*O*-desulfonated and *N*-desulfonated heparin suggests the importance of heparin fine structure in NK interaction. In contrast to the 2-*O*- sulfo groups, 6-*O*-sulfo and *N*-sulfo groups are apparently important in NK-heparin binding.

The effect of NK on other heparin-protein interactions

NK (1000 nM) was added to other heparin binding proteins (AT III, FGF1 and FGF2) to see the effect of NK on other heparin-protein interactions. The results (Fig. 6) showed that AT III

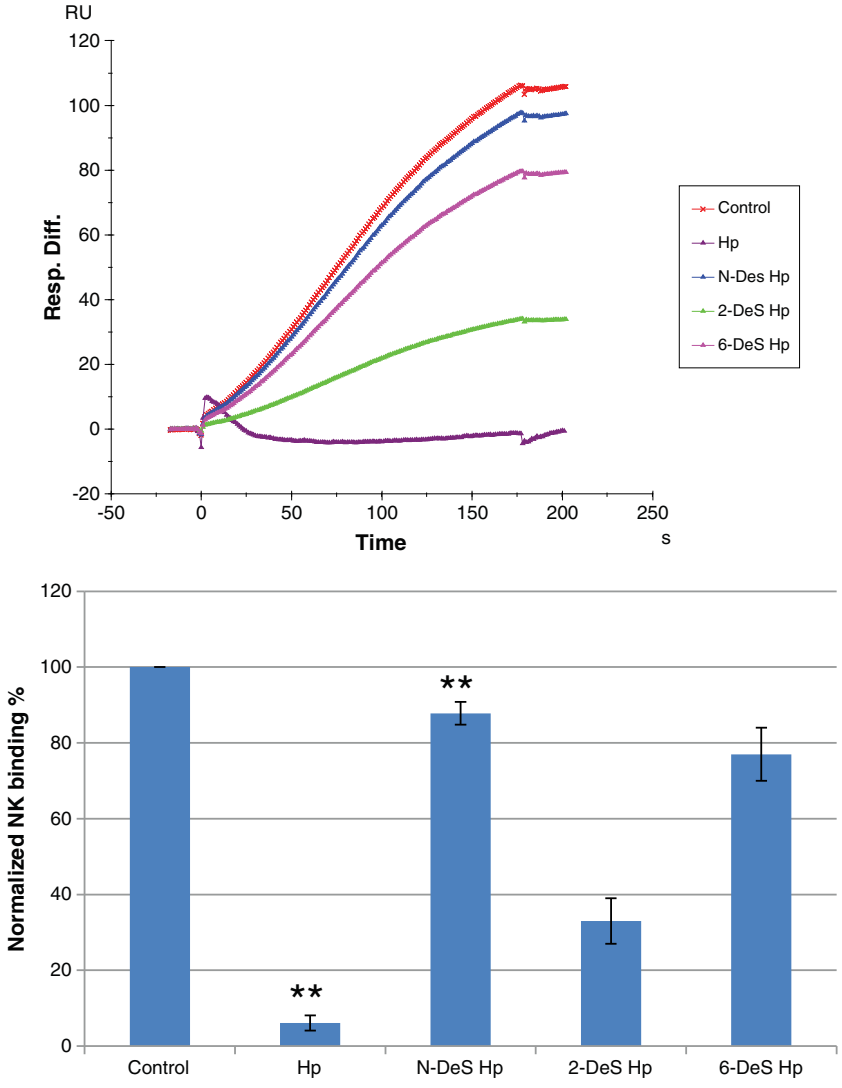

Fig. 5 SPR competition study using chemical modified heparins. *Top*: Sensorgrams of solution chemical modified heparin/surface heparin competition. NK concentration was 2500 nM, and concentrations of chemically modified heparin in solution were 1000 nM). *Bottom*: Bar graphs (based on triplicate experiments with standard deviation) of normalized NK binding to surface heparin by competing with different chemically modified heparins in solution. Statistical differences were calculated using software SPSS version 14.0 at the 5 % probability level (**, $p<0.05$)

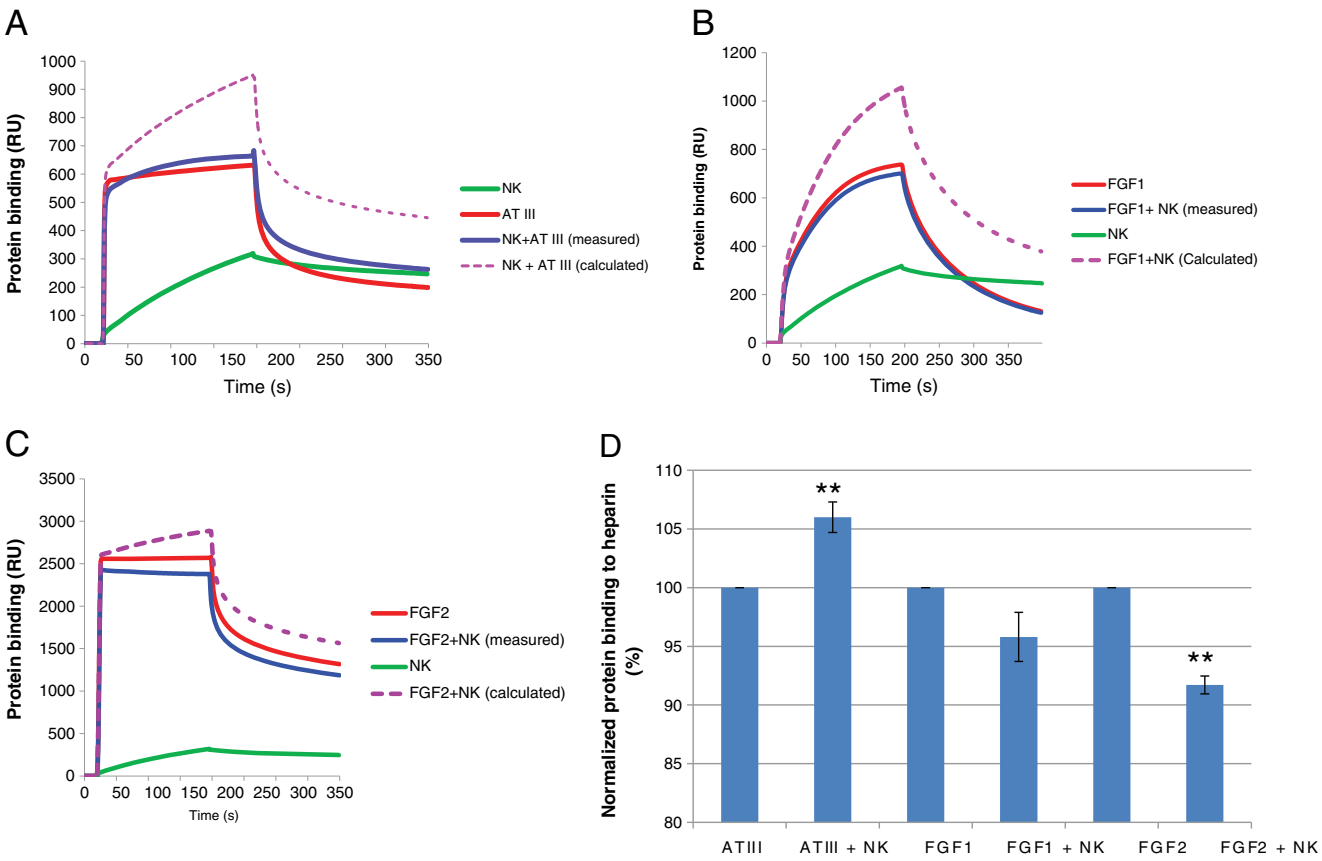

Fig. 6 Interference of NK on other heparin-protein interactions. **a** SPR sensorgrams of NK added in AT III-heparin interaction system; **b** SPR sensorgrams of NK added in FGF1-heparin interaction system; **c** SPR sensorgrams of NK added in FGF2-heparin interaction system. **d** Bar graphs (based on triplicate experiments with standard deviation) of normalized protein binding to surface heparin by adding NK. Statistical differences were calculated using software SPSS version 14.0 at the 5 % probability level (**, $p<0.05$)

binding to heparin was enhanced when NK added in AT III-heparin interaction system, whereas FGF1 and FGF2-binding to heparin was inhibited when NK added in FGF1- heparin or FGF2-heparin interaction system. The calculated sensorgrams (dot curves, based on the signals from the individual protein binding) show that the shape of the sensorgrams measured was similar to the individual ATIII, FGF1 and FGF2 binding suggesting interactions of AT III, FGF1 and FGF2 to heparin were predominant in the two protein binding system (NK+ ATIII, NK+FGF1 and NK+FGF2).

AT III, a member of the Serpin superfamily of serine protease inhibitors, is a key regulatory protein of intrinsic blood coagulation and exerts its primary effect on intrinsic blood coagulation by inhibiting the serine proteinases, thrombin and Factor Xa, of the clotting cascade [21–23]. Heparin functions anticoagulant activity is derived primarily by interacting with AT III, enhancing AT III mediated inhibition of thrombin and factor Xa. The inactivation of these proteases by AT III is greatly accelerated by the binding of heparin. Heparin binds in a ternary complex to thrombin and AT III, increasing the bimolecular rate constant for thrombin inhibition by a factor of 2000 [24]. The thrombolytic activity of NK may attribute to its interaction with heparin-ATIII complex since the SPR data showing that AT III binding to heparin was enhanced with the addition of NK.

FGFs are members of an important family of growth factors involved in developmental and other physiological processes such as cell proliferation, morphogenesis, differentiation, and angiogenesis [25, 26]. The FGF heparin-binding proteins have a high affinity for cell surface HS proteoglycans. FGF1, acidic fibroblast growth factor, and FGF2, basic fibroblast growth factor, are the first two members of this family to be discovered, thus, the kinetics and thermodynamics of their heparin interaction have been studied extensively [27, 28]. The FGFs display their biological activities through binding different, cell surface fibroblast growth factor receptors (FGFRs). FGFRs also bind to heparin, thus, the three components FGF, FGFR, and HS simultaneously interact to stimulate signal transduction [29, 30]. Cell membrane HS binds multiple FGF molecules promoting FGFR dimerization and signal transduction. Our SPR data indicates that the bindings of FGF1 and FGF2 to heparin are inhibited by the addition of NK, suggesting NK may affect the FGF signal signaling pathway.

To conclude, SPR analysis shows that NK is a heparin-binding protein with a binding affinity of approximately 250

nM. The interaction between NK and heparin is chain-length dependent and the minimum size of heparin should be larger than tetrasaccharide. The 6-*O*-sulfo and *N*-sulfo groups of heparin are of greater importance in its interaction with NK than the 2-*O*-sulfo groups. Some other GAGs (HS, DS, and CSC) displayed modest binding affinity to NK. NK also showed different fashions of effects on other heparin-protein (AT III, FGF1 and FGF2) interactions. These data may be useful to explain the different bioactivities of NK.

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