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Surface plasmon resonance analysis to evaluate the importance of heparin sulfate groups' binding with human aFGF and bFGF*

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Abstract: Human acidic and basic fibroblast growth factors (aFGF and bFGF) are classic and well characterized members of the heparin-binding growth factor family. Heparin is generally thought to play an extremely important role in regulating aFGF and bFGF bioactivities through its strong binding with them. In order to unravel the mechanism of the interactions between heparin and FGFs, and evaluate the importance of heparin sulfate groups' binding with FGFs, surface plasmon resonance analyses were performed using IAsys Cuvettes System. Heparin and its regioselectively desulfated derivatives were immobilized on the cuvettes. aFGF and bFGF solutions with different concentrations were pipetted into the cuvettes and the progress of the interaction was monitored in real-time by Windows-based software, yielding kinetic and equilibrium constants for these interactions. In addition, in order to reduce the delicate difference among the cuvettes, inhibition analyses of mixture of FGFs and immobilized native heparin by modified heparins were also done. The data from these two methods were similar, indicating that all sulfate groups at 2-O, 6-O and N- in heparin were required for the binding to aFGF; and that their contribution to the binding was in the order 2-O, N- and 6-O-sulfate group. In contrast, definite contribution of the 6-O-sulfate group to the binding with bFGF was most apparent, while the other two sulfate groups appeared to be necessary in the order 2-O and N-sulfate group. These methods established here can be used for analysing the effect of sulfate groups in heparin on the binding with other human FGF members or other heparin-binding proteins.

Key words: Surface plasmon resonance analyses, Heparin, Sulfate groups, Human aFGF and bFGF
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INTRODUCTION

Fibroblast growth factor (FGF) is an angiogenic and mitogenic polypeptide that can promote cell proliferation and differentiation in a wide variety of cell types (Goldfarb, 1990; Basilico et al., 1992). The FGF family has many members with similar amino acid sequence and biological activities (Fernig et al., 1994). Acidic and basic fibroblast growth factors (aFGF, bFGF) are two typical members well characterized and widely used as models for research on growth factors. As aFGF and bFGF have potentially useful mitogenic properties, naturally, great attention was paid to their functions and applications in the therapeutic field; so extensive studies were carried out recently to determine the mechanism of, and related factors which promote or inhibit, their activities. It was already clear that heparin can, at least to some extent, promote their activity in vitro in cells. Thus, in combination with heparin, application of human aFGF and bFGF in healing wounds, regulating angiogenesis and neovascularization, etc., is considered to be very promising (Sun, 1997; Hakan, 1999; Shane, 1999). However, the addition of exogenous heparin has potentially dangerous side-effect because it can also bind to many bioactive proteins in vivo and subsequently cause abnormal physiological activities. Therefore, clearer understanding of the interactions between heparin and heparin-binding proteins, such as FGFs, is very important.

Human aFGF and bFGF show strong affinity

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to heparin. This binding is believed to favour the stabilization of FGFs through the formation of complexes resistant to inactivation by adverse conditions, such as high temperature, acidic conditions, and proteolysis. Moreover, this binding is also considered to associated with the ability of FGFs to bind to heparan sulfate (HS) present in proteoglycans on the cell surface and in the extracellular matrix. The binding of FGFs to heparin/HS facilitates approach to their receptors on the cell surface. These results aroused increasing interest in the binding of FGFs to heparin/HS. As the sulfate groups in heparin play the central role in these interactions with FGFs, clarification of the differences in the effects of the three main sulfate groups in heparin has theoretical and practical significance. This basic study will be helpful for designing saccharide analogues that activate or suppress individual growth factor action.

Surface plasmon resonance (SPR) is usually used to analyze biomolecular interaction (Liedberg, et al., 1983; Mayo, et al., 1989; Fagerstam, 1990; Myszka, 1997; Ueda et al., 1998), and has advantages over commonly used analysis methods such as fluorescence method and absorption method, as it indicates directly changes in number of molecules that bind to the ligand at the sensor surface as shown in Fig. 1. Samples can be analyzed without labeling in this system, which is a rapid and powerful tool for analysis of the molecular interaction, allowing molecular interactions to be monitored continuously and quantitatively in real-time. Based on the collected data, kinetics and equilibrium constants were estimated by Window-based software. This paper reports the results of analyses of the interactions of aFGF, bFGF with desulfated heparins through this system.

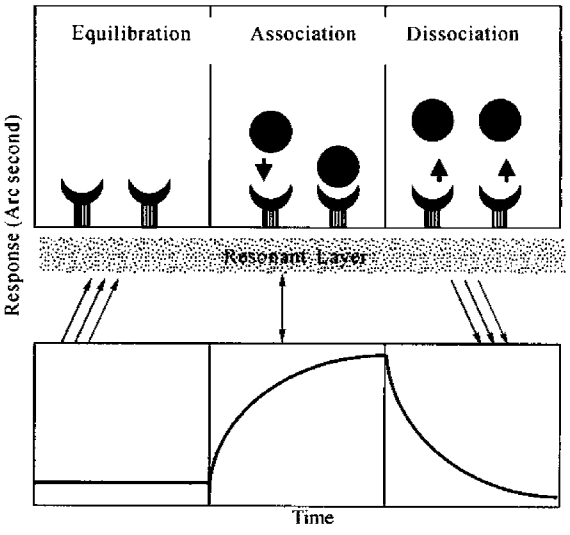

Fig. 1 Principle of FGF-heparin interaction analysis using IAsys. The FGF sample was allowed to bind to heparin immobilized on CM-Dextran surface of IAsys cuvette. The progress of the interaction was monitored by Window-based software.

MATERIALS AND METHODS

Materials

Human recombinant aFGF and bFGF were obtained by expression in the silkworm with recombinant baculovirus harbouring aFGF and bFGF genes (Wu et al., 2001). The purified aFGF and bFGF were subsequently identified by SDS-PAGE and amino acid sequence analyses. Their mitogenic activities were also proved through the in vitro cell culture of human umbilical vein endothelial cells. A series of FGFs solution of various concentrations were prepared with the PBS (10 mmol/L sodium phosphate buffered saline pH 7.4, 138 mmol/L NaCl, 2.7 mmol/L KCl). Measurement of SPR was carried out using an IAsys system (Fisons, England).

Carboxymethylated dextran (CM-Dextran) cuvettes were obtained from Affinity Sensors (Cambridge, UK). Sodium cyanoborohydride was purchased from Aldrich Chemical Company, Inc. Heparin from bovine intestinal mucosa was a product of Viobin (Waunakee, WI, USA). Regioselectively desulfated heparins were prepared according to the method of Minami (2001). 2-O-desulfated (2DS), 6-O-desulfated (6DS), N-desulfated and N-acetylated (NDSNAc) heparin were used as ligand in this experiment.

Glycaminated heparin and desulfated heparins

In order to improve the efficiency of immobilization of heparin and desulfated heparins as described in the following section, the amino group was introduced to their reducing end by the glycamination reaction method as follows. Heparin

or desulfated heparins (20 mg) was dissolved in 1.4 ml of water. Then, ammonium acetate (80 mg) and sodium cyanobrohydride (30 mg) was successively added. After being allowed to incubate at 100 ℃ for 4 hours, the solution was dialyzed against distilled water for 48 hours to remove the remaining chemicals, and lyophilized. The heparins aminated at reducing end were kept at 4 ℃ before the immobilization reaction.

Immobilization of heparins on carboxymethylated dextran (CMD) matrix

Since the standard method for heparin immobilization supplied by the manufacturer appeared to yield only small SPR response possibly due to the small amount of the immobilized ligand, the immobilization of the ligand to CMD on the cuvettes was completed by a modified method in order to increase the amount of immobilized ligand using glycol chitosan as a multi-valent linker. First, the CMD cuvette was equilibrated with PBS and activated for 10 minutes with a mixture of 50 ml of 2.5 mol/L EDC (1-ethyl-3-carbodiimide) and 50 ml of 10 mol/L NHS (N-hydroxysuccinimide), followed by PBS wash for 2-3 times. Then, 100 ml of 0.4 mg/ml glycol chitosan was added and kept for incubation until a response plateau was reached. This process usually takes 3-4 hours; after washing with PBS, 200 ml of 1 mol/L ethanol amine was added to block the activated COOH groups for 10 minutes; then the cuvette was washed with 200 ml of 1% glutaraldehyde solution and a mixture solution (100 ml 0.2 mol/L $NaCNBH_3$ solution + 100 ml 1% glutaraldehyde); followed with PBS wash; finally, 200 ml (10 mg/200 ml) of glycaminated native heparin or desulfated heparin was pipetted into the cuvette for immobilization for 3-6 hours or overnight, followed by washing twice with 200 ml 0.1mol/L $NaBH_4$ solution to reduce the remaining carboxyl groups and equilibrated with PBS before the kinetic experiment.

Interaction analysis and determination of kinetic parameters

In this study, IAsys Cuvette System was applied to analyze SPR-based interaction analysis between FGFs and heparins. Running buffer was PBS (phosphate buffered saline)/T (PBS + 0.05% v/v Tween 20). Regeneration buffer used between analyses of different samples of the same ligate was 2 mol/L NaCl, 10 mmol/L Na_2HPO_4, pH 7.2. All experiments were carried out at 24 ℃. For association, a final volume of 200 ml running buffer in the cuvette was used. A range of final concentrations of aFGF (20 – 500 nmol/L) and bFGF (20 – 350 nmol/L) were used. At minimum, five different concentrations per ligate were analyzed. Association was measured for 5 – 10 minutes. Dissociation was carried out in 200 ml running buffer for at least 5 minutes. Regeneration buffer was added for 2 minutes, followed by re-equilibration in running buffer.

Kinetic association (K_{ass}, and dissociation constants K_{diss}) and dissociation equilibrium constant (K_D) were calculated using Window-based software FASTfit program (Fisons).

Competitive binding analysis with a native heparin-immobilized cuvette

In order to improve the experimental precision by neutralising the slight differences in the ligand amount among cuvettes, competitive binding analysis using desulfated heparins was also done. As shown by the working principle in Fig.5a, binding of FGFs to heparin immobilized on the SPR cuvette was competitively inhibited when heparin or desulfated heparin was added to the system. The concentration-dependency of the inhibition was used as an index of the FGF-binding ability of the external heparin or desulfated heparin. The concentration of aFGF and bFGF was constant for all analyses, and was 125 nmol/L, and native and desulfated heparins in a series of concentrations were prepared. First, only FGF was pipetted into the cuvette for binding and the maximum response (R_{max} in arcsecs) was measured. Then different samples of FGF and heparins mixture was analyzed and response (R in arc secs) were obtained. The concentration of desulfated heparins which give R/R_{max} value of 0.5 was measured.

NaCl concentrations required for releasing FGF from immobilized heparin column

To further confirm the above results, the effect of each sulfate group on heparin's for binding to FGFs was also evaluated by using a traditional method (determining NaCl concentrations required for releasing FGF from heparin-immobilized column). The immobilization of native and

chemically modified heparins on Amino-Cellulofine was performed by the method of Sasaki et al. (1987). Native and chemically modified heparins were immobilized on Amino-Cellulofine by reductive amination reaction between the amino group in the matrix and the reducing end of the polysaccharide. The column size used for this experiment was 1.0 ml. Affinity chromatography was carried out using an FPLC system (Pharmacia Biotech, Upsala, Sweden). Recombinant human aFGF or bFGF was loaded onto a heparin- or modified heparin-immobilized column equilibrated with 10 mmol/L Tris-HCl buffer, pH 7.4, and eluted with a 120-min linear gradient of NaCl (0 to 2.0 mol/L) in 10 mmol/L Tris-HCl buffer, pH 7.4 at flow rate of 1.0 ml/min. For detection, the absorbance at 280 nm was monitored. The strength of the affinity to heparin was estimated from the peak top where aFGF or bFGF was eluted. The NaCl concentration was determined in duplicate experiments by measuring conductivity of the eluate.

RESULTS

Immobilization of native and regioseletively desulfated heparins on Iasys Cuvette

Heparin and its chemically modified derivatives were effectiwely immobilized on the IAsys cuvettes using the (COOH-glycol chitosan-glutaraldehyde-heparinNH_2) method. The immobilization was tested by observing the maximum response of binding interaction using the same concentration of ligand. Compared with other methods of immobilization (Minami, 1999), the method established in this study was proved to be quite successful.

Binding profile of aFGF and bFGF to desulfated heparins immobilized on CM-Dextran surface

The interaction profile of FGF with heparins immobilized on a CM-Dextran surface was obtained (Fig.2). For comparison, the aFGF concentration was fixed as 150 nmol/L. Both association and dissociation patterns, as well as the maximum response in arcsecs, were different among the native, 2-O, N-and 6-O-desulfated heparins. While 6DS Hp showed SPR response as strong as native heparin (650 arcsec), while 2DS and NDS-NAc Hp showed the weaker

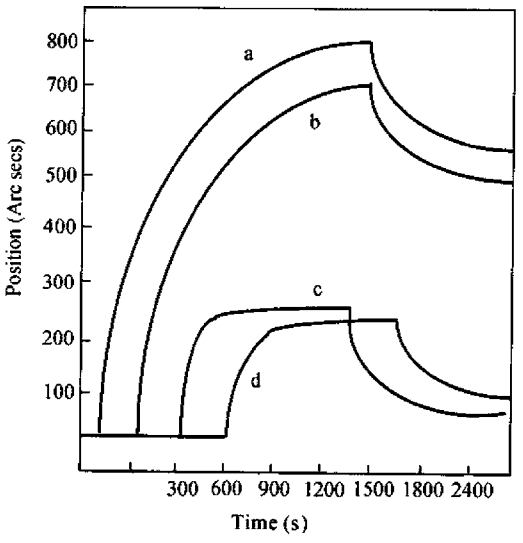

Fig.2 Binding profile of human aFGF to heparin and its desulfated derivatives immobilized on cuvettes. a. native heparin; b. 6DS heparin; c. 2DS heparin; d. NDS heparin. The cuvettes were equilibrated with PBS/T. After aspirating the PBS/T, 50 ml of aFGF solution was pipetted into cuvette for binding. In case of bFGF, it showed a similar pattern.

response (about 300 arcsec). In addition, the dissociation curves of native and 6DS Hp demonstrated slower while those of 2DS and NDS-NAc Hp showed very rapid decline. The interaction profiles showed that the binding between native, 6DS heparin and aFGF was stronger than that between 2DS, NDS heparin and aFGF, i.e, the loss of 6-sulfate group from heparin did not cause remarkable decrease in the binding ability as 2- or N-sulfate group did. Thus it could be judged primarily that 2-O-sulfate and N-sulfate group are more important than the 6-O-sulfate group for the binding with aFGF. A similar profiles was also observed in the case of bFGF binding.

Kinetic constants of FGF-heparin interactions

Data available from experiments were analyzed using FASTfit, a rapid data analysis program specially designed for analyzing data from the IAsys cuvette system. Fig.3 and 4 show the plot of accumulated data analysis. In theory, the slope represents the association rate constant and

the intercept value on the y-axis represents the dissociation rate constant. The K_D value reflects the interaction performance of binding. Lower K_D value means stronger binding. Table 1 shows the kinetic equilibrium constants for the interaction of FGFs and desulfated heparins. In the case of aFGF, K_D for native heparin was about 1.81×10^{-7} mol/L, lowest value compared with others, while for 6DS Hp, it was 2.31×10^{-7} mol/L, 2DS Hp it, was 3.53×10^{-7} mol/L and for NDS-NAc Hp, it was 4.47×10^{-7} mol/L. This result indicated that the removal of any one of the sulfate groups in heparin could cause decrease in the capacity to bind to aFGF. Moreover, the three sulfate groups in heparin appeared to have different contributions to binding with aFGF. According to the K_D values, it could be generally concluded that their contribution to binding with aFGF was in order, 2-O-sulfate group > N-sulfate group > 6-O-sulfate group. K_D values for bFGF were obtained by the same analysis, 7.13×10^{-8} mol/L for native Hp, 7.16×10^{-8} mol/L for 6DS Hp, 2.17×10^{-7} mol/L for NDS-Nac Hp and 3.27×10^{-7} mol/L for 2DS-Hp. Compared with aFGF, this K_D value was much lower, suggesting that bFGF-heparin bindings were possibly stronger. Although the K_D value for bFGF was in the same order of magnitude as that of aFGF among the four heparins, the 6-O-sulfate group had almost the same K_D value as that of native heparin, indicating that the 6-O-sulfate group was not essential for heparin's binding to bFGF.

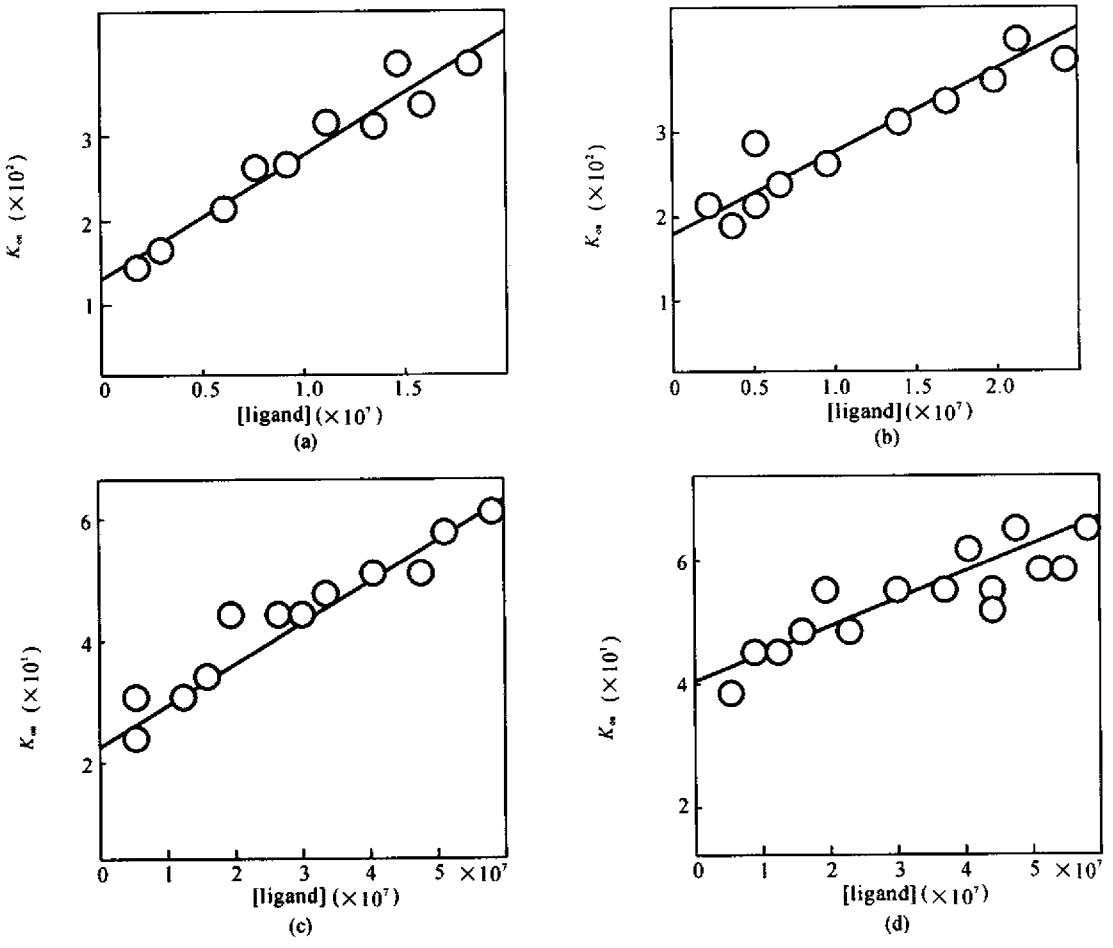

Fig.3 Plot of Fit accumulated data analysis of aFGF-heparin interactions. The slope of the line in figure is the association rate constant and in theory the intercept value on the y-axis is the dissociation rate constant.

(a) native heparin; (b) 6DS heparin; (c) NDS, NAc-heparin; (d) 2DS heparin.

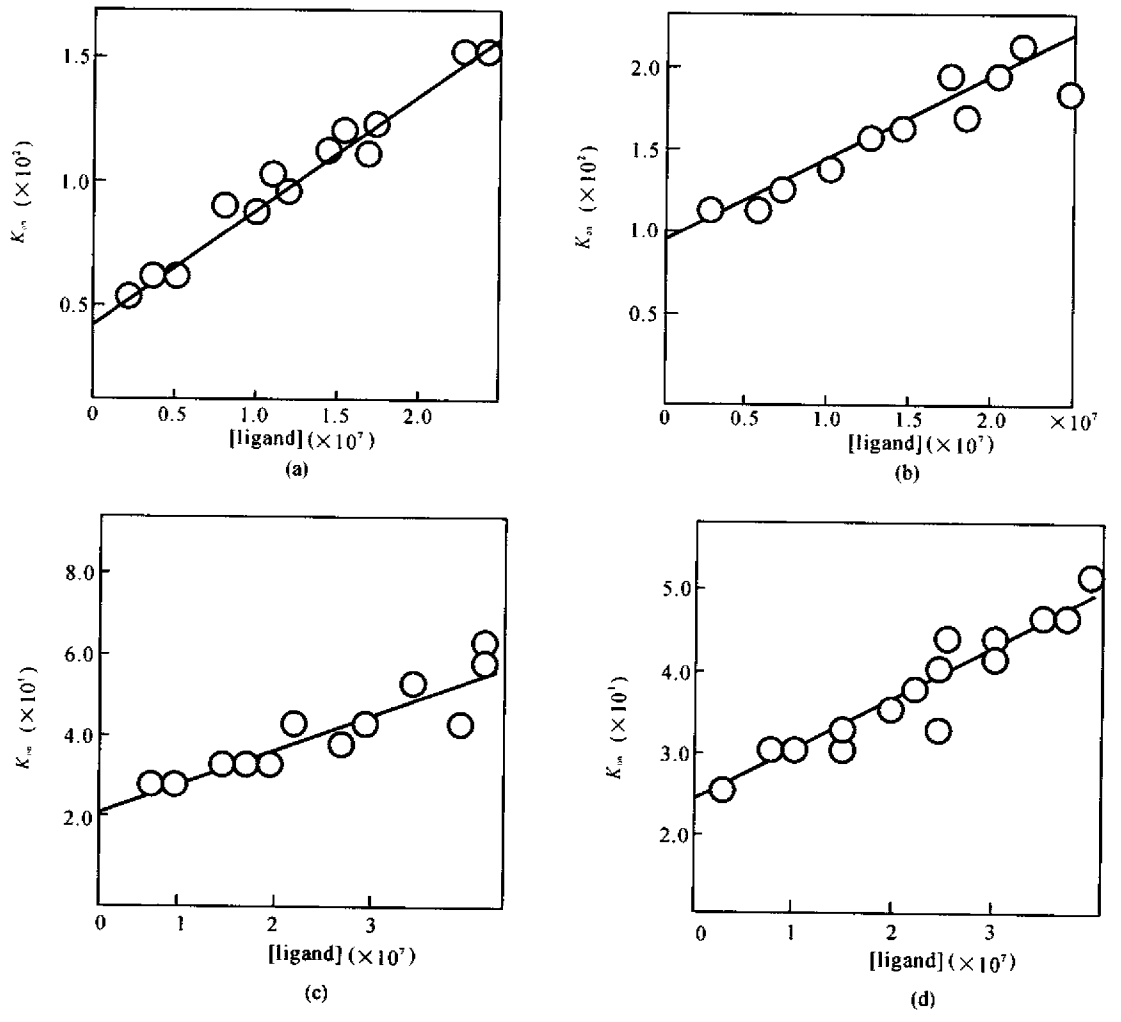

Fig.4 Plot of Fit accumulated data analysis of bFGF-heparin interactions. The slope of the line in the figure is the association rate constant and in theory the intercept value on the *y*-axis is the dissociation rate constant.

(a) native heparin; (b) 6DS heparin; (c) NDS, NAc-heparin; (d) 2DS heparin.

Table 1 Kinetic measurement for interactions of human aFGF and bFGF with heparin and its desulfated derivatives.

	Cuvettes	K_{ass} (mol/L•s)$^{-1}$	K_{diss} (s^{-1})	K_D (mol/L)
aFGF	native heparin	$8.57 \pm 0.7 \times 10^{4}$	$1.55 \pm 0.2 \times 10^{-2}$	$1.81 \pm 0.3 \times 10^{-7}$
	6DS-Hp	$7.07 \pm 0.7 \times 10^{4}$	$1.63 \pm 0.1 \times 10^{-2}$	$2.31 \pm 0.3 \times 10^{-7}$
	NDS, NAc-Hp	$6.35 \pm 0.8 \times 10^{5}$	$2.24 \pm 0.2 \times 10^{-1}$	$3.53 \pm 0.5 \times 10^{-7}$
	2DS-Hp	$6.27 \pm 0.9 \times 10^{5}$	$2.80 \pm 0.3 \times 10^{-1}$	$4.47 \pm 0.7 \times 10^{-7}$
bFGF	native heparin	$5.40 \pm 0.7 \times 10^{4}$	$3.85 \pm 0.9 \times 10^{-3}$	$7.13 \pm 2.0 \times 10^{-8}$
	6DS-Hp	$5.26 \pm 0.5 \times 10^{4}$	$3.77 \pm 0.9 \times 10^{-3}$	$7.16 \pm 1.8 \times 10^{-8}$
	NDS, NAc-Hp	$1.00 \pm 0.2 \times 10^{6}$	$2.17 \pm 0.5 \times 10^{-1}$	$2.17 \pm 0.6 \times 10^{-7}$
	2DS-Hp	$0.62 \pm 0.1 \times 10^{6}$	$2.03 \pm 0.3 \times 10^{-1}$	$3.27 \pm 0.8 \times 10^{-7}$

Note: K_{on} (s^{-1}), Measured on-rate constant, obtained from association analysis;
K_{ass} ((mol/L•s)$^{-1}$), Derived association rate constant. Obtained from the slope of the plot of K_{on} versus concentration of sample;
K_{diss} (s^{-1}), Derived dissociation rate constant. Obtained from the intercept of the plot of K_{on} versus concentration of sample;
K_D (mol/L), Dissociation equilibrium constant or dissociation constant ($K_D = 1/K_A = K_{diss}/K_{ass}$).

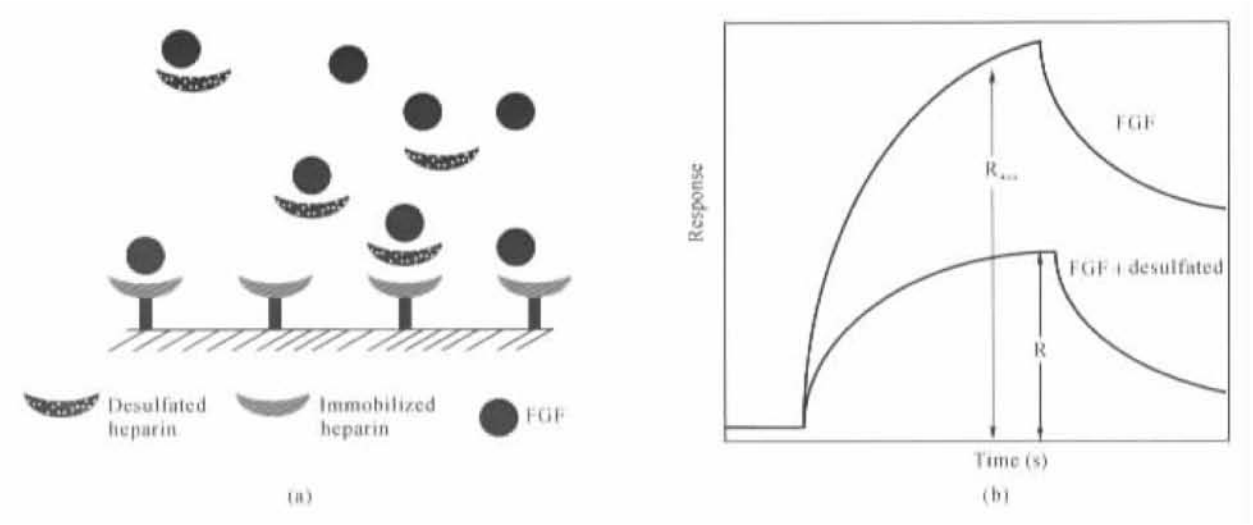

Fig.5 (a) Glimpse of the competitive binding analysis model; (b) the concentrations of desulfated heparins (μg/ml) required for $R/R_{max} = 0.5$

Insights from competitive binding analysis using a native heparin-immobilized cuvette

As pointed out above, in order to neutralize the slight differences among various cuvettes, competitive binding analysis using native heparin-immobilized cuvette was performed. From the measurement of desulfated heparin's inhibition of competitive binding between FGF and immobilized native Hp, it was possible to evaluate the importance of sulfate groups in binding to FGFs. The analysis data are showed in Fig.6. The concentrations of various heparins required for inhibiting 50 % of the aFGF-native Hp binding were about 0.83 μg/ml for native heparin, 3.90 μg/ml for 6DS Hp, 44.44 μg/ml for NDS-NAc Hp and 58.92 μg/ml for 2DS Hp. In the case of bFGF, the concentrations were 0.195 μg/ml for native heparin, 0.47 μg/ml for 6DS Hp, 38.70 μg/ml for NDS-NAc Hp and 56.25 μg/ml for 2DS Hp. The importance of heparin sulfate groups can be clearly deduced based on the above

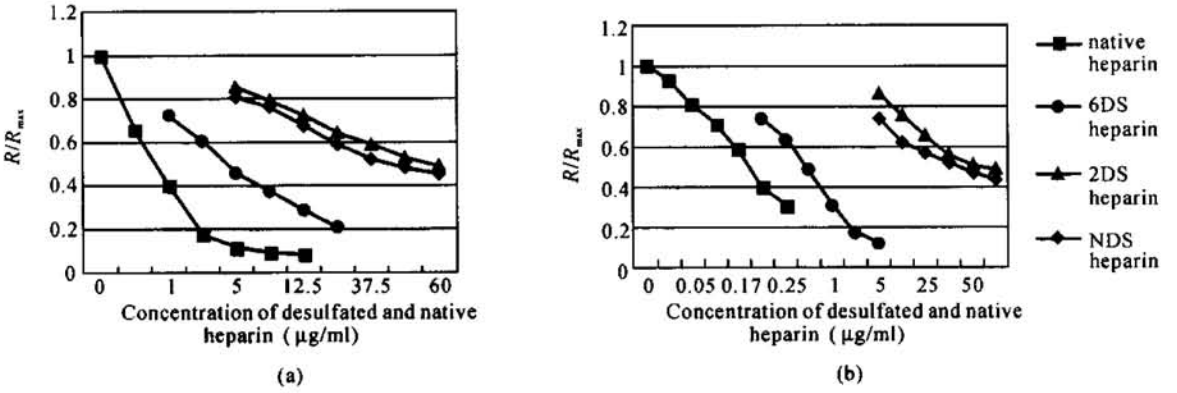

Fig.6 Binding of aFGF(a), bFGF(b) to immobilized native heparin in the presence or absense of native or regioselectively desulfated heparin. The changes in resonant angle on Iasys were measured when fixed concentrations of bFGF and aFGF were added to the native heparin-immobilized cuvette in the presense of various concentrations of native or desulfated heparin. The plot of $R_{max}/R_{max}0$ versus the concentration of native or regioselectively desulfated heparin added together with protein were plotted. $R_{max}0$ and R_{max} are the responses at the plateau in the absense or presense of native or regioselectively desulfated heparin, respectively

data indicating that 2-O-sulfate group played the most important role in binding to both aFGF and bFGF; and that the N-sulfate group was of second importance; while the 6-O-sulfate group gave weaker contribution to the binding. In particular, for bFGF, the 6-O-sulfate group gave negligible contribution judging from the fact that the concentration of 6DS Hp for inhibiting the binding to half was very close to that of the control group native heparin. These results accorded well with the results from IAsys kinetic analyses.

DISCUSSION

In order to further confirm the results from IAsys analysis, an experiment applying the traditional method was also done by using affinity chromatography. Table 2 giving the NaCl concentrations for releasing aFGF and bFGF from heparin or its derivatives-immobilized columns shows that, taking native Hp as control, the removal of 6-O-sulfate group, N-sulfate group and 2-O-sulfate group decreased the capacity to bind to aFGF by 24.11%, 50.59% and 51.42%, and to bFGF by 0.38%, 50.25% and 52.84% respectively. This result clearly showed the importance of the three heparin sulfate groups for the binding to aFGF and bFGF, indicating that N-sulfate group and 2-O-sulfate group were important for both aFGF and bFGF, while the 6-O-sulfate group played different roles in binding to aFGF and bFGF. For binding to aFGF, the 6-O-sulfate group was essential; while for binding to bFGF it was virtually nonessential. These results agreed with those from IAsys analyses, especially with the result from competitive binding analysis. As the analysis using affinity chromatography required a large amount of sample, which is usually expensive or in some cases not easily available, and takes much time, IAsys analysis has many advantages, such as saving in time and not requiring of a large amount of samples. Therefore, for experiments evaluating the importance of heparin sulfate groups in binding to other members of FGFs, IAsys cuvette system is very practicable. It should be pointed out particular that the competitive binding method designed and established in this study is quite simple, rapid and precise.

Table 2 NaCl concentrations for releasing human aFGF and bFGF from immobilized heparin derivatives resins. The measurement method is described in the text.

Heparin derivatives	NaCl concentrations (mol/L)	
	aFGF	bFGF
Native heparin	0.85	1.62
6DS-Hp	0.65	1.61
NDS, NAc-Hp	0.42	0.80
2DS-Hp	0.41	0.76

Heparan sulfate(HS) is sulfated polysaccharide which occurs in most animal cells; is present mainly presents in proteoglycans at the cell surface and in the extracellular matrix; is thought to play a prominent role in regulating cell growth by modulating the activity of various growth factors including FGFs(Conrad, 1997); and is structurally similar to heparin; but less highly sulfated than heparin. However, the N-sulfate group highly exists abundantly in HS. The results from this experiment clarify why the N-sulfate group was important for binding to both aFGF and bFGF. It could also be deduced that the N-sulfate group may be essential for binding to other members of the FGF family.

It seemed that the capacity of heparin to bind to FGFs was closely related to the activation of FGFs by heparin; as our results regarding the importance of heparin sulfate groups for binding to aFGF and bFGF accorded well with findings reported by Ishihara (1995, 1997) that a high content of 6-O-sulfate group in N-sulfated glucosamine (GlcNS) residues was required for activation of aFGF (FGF-1), but not bFGF (FGF-2); and that a high content of 2-O-sulfate group in uronate residues of heparin was required to promote the mitogenic activities of both FGF-1 and FGF-2. From a pharmaceutical perspective, studies to gain understanding of the molecular basis of the specific interaction between each growth factor and heparin/HS might faciliate designing of saccharide analogues that activate or suppress individual growth factor action.

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The characteristics and requirements of world-class journal were considered in establishing a rigid peer review system for scientific papers submitted for publication in the English-language *Journal of Zhejiang University SCIENCE* from 2002 onward. We sent the over 408 contributions received from January to December in 2002 to the U.S.A., the U.K., Ireland, France, Canada, Australia, Austria, Germany, New Zealand, the Netherlands, Finland, Poland, Portugal, Italy, Israel, Spain, Belgium, Sweden, Switzerland, Denmark, Japan, Singapore, Slovak, India, Greece, Czech, Mexico, Hong Kong, Macao, Taiwan, etc., for pre-publication review by topnotch international scientists there in their respective specialties. Experience in scientific papers publication has shown that an international peer review system plays an important part in ensuring the high quality of a journal's contents and helping it to be known by worldwide.

Every little improvement of this journal depends on the strong support from the reviewers. We take this opportunity to give our heartfelt thanks to reviewers in China and abroad for their help since the establishment of the journal.