Glycoconj J (2009) 26:577–587
DOI 10.1007/s10719-008-9210-0

A surface plasmon resonance-based solution affinity assay for heparan sulfate-binding proteins

Siska Cochran · Cai Ping Li · Vito Ferro

Received: 16 July 2008 / Revised: 5 October 2008 / Accepted: 27 October 2008 / Published online: 26 November 2008

Abstract A surface plasmon resonance-based solution affinity assay is described for measuring the K_d of binding of heparin/heparan sulfate-binding proteins with a variety of ligands. The assay involves the passage of a pre-equilibrated solution of protein and ligand over a sensor chip onto which heparin has been immobilised. Heparin sensor chips prepared by four different methods, including biotin–streptavidin affinity capture and direct covalent attachment to the chip surface, were successfully used in the assay and gave similar K_d values. The assay is applicable to a wide variety of heparin/HS-binding proteins of diverse structure and function (e.g., FGF-1, FGF-2, VEGF, IL-8, MCP-2, ATIII, PF4) and to ligands of varying molecular weight and degree of sulfation (e.g., heparin, PI-88, sucrose octasulfate, naphthalene trisulfonate) and is thus well suited for the rapid screening of ligands in drug discovery applications.

Keywords Heparan sulfate-binding proteins · heparin · solution affinity assay · surface plasmon resonance

Abbreviations

HS	heparan sulfate
GAG	glycosaminoglycan
SPR	surface plasmon resonance
FGF-1	fibroblast growth factor 1
FGF-2	fibroblast growth factor 2
VEGF	vascular endothelial growth factor
IL-8	interleukin 8
MCP-2	monocyte chemotactic protein 2
PF4	platelet factor 4
ATIII	antithrombin III
ADHZ	adipic acid dihydrazide
NHS	*N*-hydroxysuccinimide
EDC	*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide
LMWH	low molecular weight heparin
NTS	1,3,6-naphthalenetrisulfonate
SOS	sucrose octasulfate

Electronic supplementary material The online version of this article (doi:10.1007/s10719-008-9210-0) contains supplementary material, which is available to authorized users.

S. Cochran · C. P. Li · V. Ferro
Drug Design Group, Progen Pharmaceuticals Ltd,
Darra, QLD, Australia

Present address:
V. Ferro (✉)
School of Physical and Chemical Sciences,
Queensland University of Technology,
GPO Box 2434, Brisbane, QLD 4001, Australia
e-mail: vito.ferro@qut.edu.au

Introduction

Heparin and heparan sulfate (HS) are members of the glycosaminoglycan (GAG) family of linear, polyanionic polysaccharides composed of repeating disaccharide subunits of uronic acid-(1→4)-D-glucosamine [1–3]. They share a common biosynthetic pathway in which numerous modifications are made to these subunits resulting in a large number of complex sequences [4]. The uronic acid component can be either β-D-glucuronic acid (GlcA) or its C-5 epimer, α-L-iduronic acid (IdoA), which can also be sulfated at the 2-*O* position. The glucosamine may be either *N*-acetylated or *N*-sulfated (or in rare cases, unsubstituted) and may contain further sulfation at the 6-*O* and 3-*O* positions.

HS is ubiquitously expressed as a proteoglycan on the surface of most animal cells and as a component of extracellular matrices and basement membranes. HS interacts

with a large range of proteins involved in many biological processes, for example, cell growth and development [5], tumour metastasis and angiogenesis [6], inflammation [7] and viral infection [8]. The more highly sulfated heparin, which has been used clinically as an anticoagulant for decades and is thus widely available, is often used as a model compound for HS. The important role of HS/heparin in mediating these diverse biological functions has made these molecules the focus of much research [9–11]. The potential for mimetics of heparin/HS in the treatment of diseases such as cancer and cardiovascular disease, has been recognized and is an area of much recent interest [12–14]. The evaluation of binding specificities and affinities of potential ligands forms a major component of such drug discovery research.

Surface plasmon resonance (SPR) spectroscopy is an established method for measuring biomolecular interactions and has been successfully used to study the binding affinities and kinetics of heparin–protein interactions [15–20]. SPR-based binding experiments typically involve the immobilisation of one of the binding partners onto a sensor chip surface, followed by injection of the second molecule over the surface. The binding interaction between the two molecules results in a change in the intensity and angle of light reflected from the sensor chip surface, reported as a response increase, from which kinetic parameters can be derived. When studying heparin–protein interactions by SPR, heparin is preferentially immobilised onto the sensor chip rather than the protein because this more closely mimics natural biological systems where HS is found at the cell surface as a proteoglycan and binds to target proteins as they flow past [21, 22]. In drug discovery applications where libraries of compounds are screened against a target protein, however, the immobilisation of the protein is usually required because it is impractical to immobilise each ligand separately, especially if the library is structurally diverse and requires multiple immobilisation chemistries. A drawback of this approach is the requirement of large amounts of available protein for immobilisation onto the sensor chip surface and is limited by the stability of the protein, particularly if harsh conditions are required to regenerate the sensor chip surface.

To overcome some of these limitations, an SPR-based solution affinity assay was developed in which neither the protein nor the ligand of interest are immobilised. Instead, immobilised heparin is used to measure binding kinetics in solution. The principle of this assay is that in a mixture of protein and ligand at equilibrium, immobilised heparin can distinguish between free protein and protein complexed with ligand when the ligand has bound in the heparin binding site. Thus, when a mixture of protein and ligand at equilibrium is injected across a heparin surface, free protein in the mixture binds to immobilised heparin resulting in a binding response. Quantitation of free protein in a series of mixtures containing varying concentrations of ligand enables calculation of the ligand binding affinity. The assay was used to measure the binding of various ligands, including the antiangiogenic drug candidate PI-88 as well as heparin and HS, to the heparin-binding, angiogenic growth factors FGF-1, FGF-2 and VEGF [23]. The assay was subsequently applied to the screening of various heparin-mimetic compounds as potential antiangiogenic, anti-cancer agents [12, 24–28].

In this study, four different methods for the immobilisation of heparin onto sensor chips were investigated and the effects of the different sensor chips on the solution affinity assay were examined. In addition, the generality of this assay and its suitability for drug discovery screening was explored by analysing the binding of several heparin/HS-binding proteins of diverse structure and function with a number of known ligands of varying molecular weight and degree of sulfation.

Material and methods

Materials

Recombinant human FGF-1 (140 amino acid residue, *N*-terminally truncated form), recombinant human FGF-2 (146 amino acid residue, *N*-terminally truncated form), recombinant human VEGF (165 amino acid form), recombinant human IL-8 (77 amino acid form), recombinant human MCP-2 and recombinant human PF4 were purchased from R&D Systems, Inc (Minneapolis, MN). Each of these protein preparations contained 50μg of BSA per μg of growth factor. Human ATIII, heparin (from bovine lung or bovine intestinal mucosa, average mol. wt. ~12.5kDa), adipic acid dihydrazide (ADHZ), 1,4-diaminobutane, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), $NaCNBH_3$, ethanolamine, low molecular weight heparin (LMWH, from porcine intestinal mucosa, average mol. wt. ~3kDa), heparin–albumin–biotin, albumin–biotin, heparin–biotin and 1,3,6-naphthalenetrisulfonate (NTS) were purchased from Sigma. Sucrose octasulfate, potassium salt (SOS) was purchased from Toronto Research Chemicals (Toronto, Canada). PI-88 was supplied by Progen Pharmaceuticals (Brisbane, Australia). Streptavidin (SA), CM5, C1 and CM4 (B1) sensor chips and HBS-EP buffer (10mM HEPES, pH7.4, 150mM NaCl, 3.0mM EDTA and 0.005% (*v*/*v*) surfactant P20) were purchased from BIAcore AB (Uppsala, Sweden). Surface plasmon resonance (SPR) measurements were performed on a BIAcore 3000 (BIAcore) operated using the BIAcore Control Software.

Immobilisation of heparin–albumin–biotin onto streptavidin sensor chips

The immobilisation of heparin–albumin–biotin onto streptavidin sensor chips has been described previously [23]. A

single injection of a 1μg/mL aqueous solution of heparin–albumin–biotin resulted in an increase in response of 60–200 response units (RU) in flow cells 2 and 4. Subsequent injections of heparin–albumin–biotin at 1–50μg/mL did not result in further immobilisation. The remaining two flowcells were used as negative controls, with albumin–biotin immobilised in these using the above method. This resulted in a response increase of 360–730 RU. As the albumin–biotin does not bind to the proteins of interest here, the higher levels of immobilised albumin–biotin have no effect on the assay.

Immobilisation of heparin–biotin onto streptavidin sensor chips

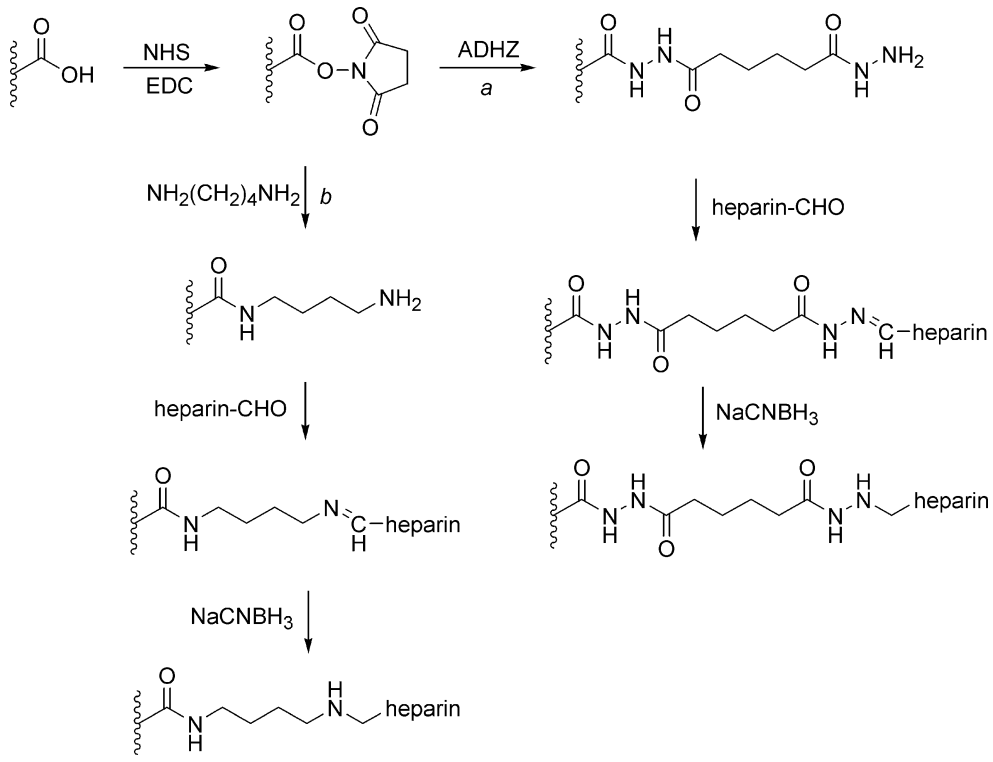
Biotinylated heparin was immobilised using the procedure described above. A single injection of 50μL of 1μg/mL biotinylated heparin at a flow rate of 1μL/min resulted in a response increase of 152 RU. The negative control flowcell remained unmodified.

Immobilisation of heparin onto C1 and CM5 sensor chips *via* reductive amination with adipic acid dihydrazide

Heparin was immobilised onto C1 and CM5 sensor chips using the method described by Satoh and Matsumoto [29] (see Scheme 1). Prior to immobilisation, the C1 sensor chips were cleaned by consecutive injections of 10 and 5μL of 0.1M glycine–NaOH, 0.3% Triton-X100, followed by 5μL of HBS-EP buffer at 5μL/min. CM5 sensor chips were not treated. Flowcells were activated with a mixture of 200μL of 0.2M EDC/0.05M NHS at a flow rate of 5μL/min, and 200μL of a near-saturated solution (approximately 100mg/mL) of ADHZ in H_2O was subsequently injected at the same flow rate. Heparin was immobilised onto the hydrazide group by injecting 150μL of 100mg/mL heparin in 2M guanidine HCl, 7.5mM sodium acetate buffer, pH4, at 1μL/min, followed by injecting 80μL of 1mg/mL $NaCNBH_3$ at 2μL/min. For the C1 sensor chip, the above procedure was repeated to increase the heparin immobilisation level before injection of 200μL of 1M ethanolamine at 5μL/min to block any remaining activated sites. The flowcells were then washed with 40μL of 4M NaCl followed by an HBS-EP buffer injection at 40μL/min. On the C1 and CM5 sensor chips, the negative control flowcells were left unmodified, since the level of non-specific binding of proteins to these flowcells was negligible and did not change following treatment of the flowcells with NHS/EDC and ethanolamine using the method described above. Following the heparin immobilisation procedure, the C1 and CM5 sensor chips were undocked and soaked in HBS-EP buffer at 4°C for at least 1week prior to use.

Immobilisation of heparin onto CM4 sensor chips *via* reductive amination with 1,4-diaminobutane

In this immobilisation procedure, heparin was covalently attached by reductive amination to a surface modified by 1,4-diaminobutane [30] (see Scheme 1). The surface in



Scheme 1 Immobilisation of heparin by reductive amination onto derivatised carboxylated sensor chips (CM5, C1 or CM4). Path ***a***: *via* adipic acid dihydrazide (ADHZ), path *b*: *via* 1,4-diaminobutane

flowcell 2 was activated by injection of 200μL of a 0.05M NHS and 0.2M EDC mixture at 5μL/min, resulting in a response increase of 795 RU. Following activation, 200μL of 1M 1,4-diaminobutane was injected at 5μL/min, and a response decrease of 682 RU was observed. A 200μL solution of 1M ethanolamine at 5μL/min was injected, resulting in no further decrease in response. This suggests that this blocking step may not be necessary because 1,4-diaminobutane is able to block all available activated sites.

After undocking the sensor chip from the instrument, ~250μL of 50mg/mL heparin in water was applied to the sensor chip surface and the sensor chip left to stand overnight at room temperature. The solution was then replaced with ~250μL of 50mg/mL heparin containing 2.5mg/mL $NaCNBH_3$ and left at room temperature for a further 20h. The surface was washed twice with ~500μL of water, dried and then stored in HBS-EP buffer at 2–8°C for 1week.

Testing for heparin immobilisation

To test the integrity of the heparin immobilised on these sensor chips, 25–200μL of 1–3nM FGF-1 in HBS-EP buffer was injected at 5–40μL/min. The sensor chip was deemed suitable for use in experiments if FGF-1 binding resulted in a response increase of >25 RU. Typically, a response increase of 50 RU or more was obtained in C1, CM5 or CM4 sensor chips. The surface was regenerated by injecting 40μL of 4M NaCl at 40μL/min, followed by injection of 40μL of HBS-EP buffer at 40μL/min.

Testing for mass transport

The principle of the solution affinity assay method has been described previously [23]. For successful application, this assay must be performed either under mass transport conditions, or the protein concentration used in the assay must be ten-fold below its K_d with heparin, because only under these conditions are the binding responses linearly proportional to free protein concentration in the equilibrium mixture [31]. Mass transport conditions are preferred because binding responses are linearly proportional over a wider range of protein concentrations. Additionally, if the K_d is very low, use of a ten-fold lower protein concentration would give a very low response.

To test whether or not mass transport conditions were established, standard curves were generated by injecting 25–200μL of standard protein solutions at varying concentrations in buffer (HBS-EP buffer for FGF-1, VEGF, MCP-2, IL-8 and ATIII, and HBS-EP buffer containing 0.3M NaCl for FGF-2) at 5–40μL/min. Prior to injection, standard solutions were maintained at 4°C to maximize protein stability, and the surface binding experiments were performed at 25°C. The surface was regenerated by injection of 40μL of 4M NaCl at 40μL/min, followed by injection of 40μL of buffer at 40μL/min. Carry-over between injections was eliminated by including a DIPNEEDLE command between injections, and an EXTRACLEAN command after each injection. The standard curves obtained were linear and passed through the origin thus confirming that the assays were under mass transport conditions [32] (see Fig. 1).

Derivation of K_d values

K_d values were derived as described previously [23]. Briefly, 100–250μL solutions were prepared containing 1.29–3nM FGF-1, 0.5–3nM FGF-2, 3nM VEGF, 45nM IL-8, 4.4nM MCP-2, 1nM ATIII and 5nM PF4 and varying concentrations of the ligand in buffer on ice. For each assay mix, 25–200μL of solution was injected at 5–40μL/min and the relative response was measured. All surface binding experiments were performed at 25°C. Data analyses were carried out using the BIAevaluation version 3.0 software. The binding rates or responses for FGF-1, FGF-2, VEGF, IL-8, MCP-2, ATIII and PF4 were converted to free protein concentration using the method described by Karlsson [23, 31].

A stoichiometry of 1:1 was assumed for the protein/ligand complex formed in solution prior to injection:

$$P + L \rightleftharpoons PL \tag{1}$$

where P corresponds to the protein, L is the ligand and PL is the protein/ligand complex. The equation for the equilibrium constant is:

$$K_d = \frac{[P][L]}{[PL]} \tag{2}$$

and the equation relating K_d to free protein concentration can be expressed as:

$$[P] = [P]_{total} - \frac{\left(K_d + [L]_{total} + [P]_{total}\right)}{2} + \sqrt{\frac{\left(K_d + [L]_{total} + [P]_{total}\right)^2}{4} - [L]_{total}[P]_{total}} \tag{3}$$

where $[P]_{total}$ and $[L]_{total}$ represent the total concentrations of protein and ligand, respectively, in the injected solution [23].

Under conditions of mass transport, standard curves relating the relative binding response to the injected protein concentration are linear [32]. The relative binding response for each injection can, therefore, be converted to free protein concentration using the equation:

$$[P] = \frac{r}{r_m}[P]_{total} \tag{4}$$

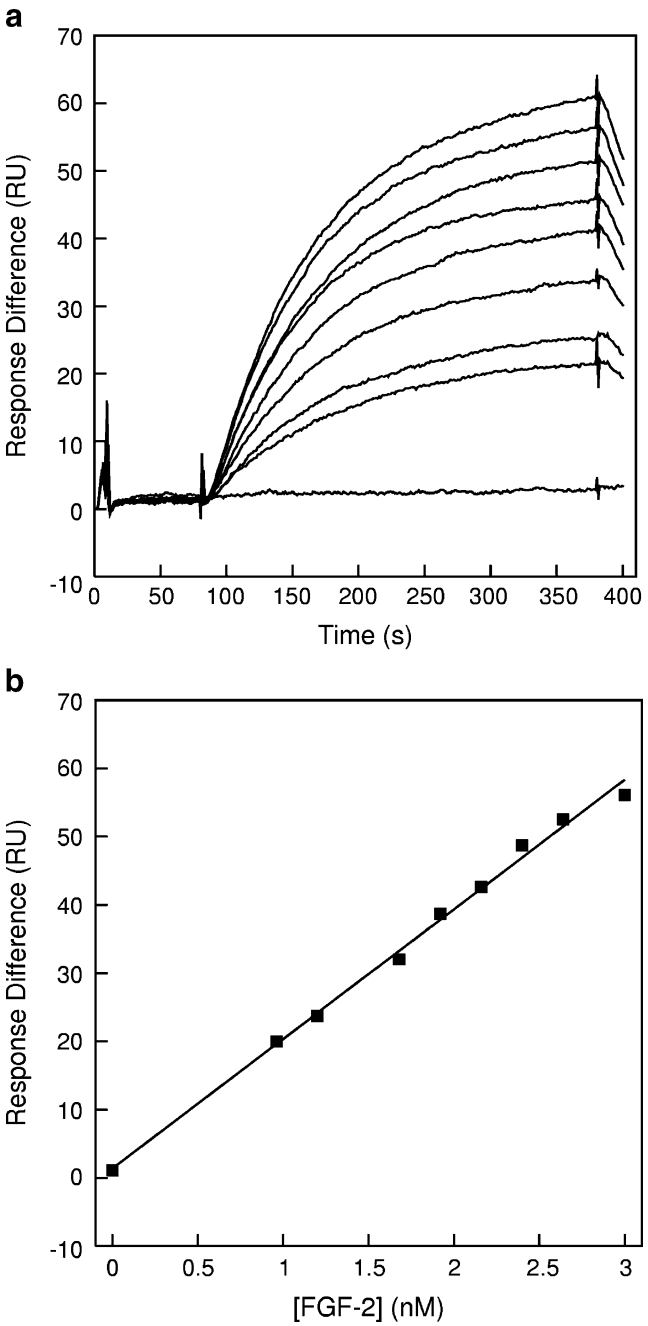


Fig. 1 Representative protein standard curve determined for FGF-2. **a** SPR sensorgrams demonstrating the change in binding response (in RU) upon injection of varying concentrations of FGF-2 from 0 to 3 nM in HBS-EP running buffer containing 0.3 M NaCl over a heparin surface. **b** Protein standard curve constructed by plotting the observed binding response against the protein concentration

where r is the relative binding response and r_m is the maximal binding response (both responses were measured at 10s before to the end of injection). A plot of [P] *versus* $[L]_{total}$ and fitting of Eq. 3 enables the determination of K_d (Fig. 2). Initial binding rates can also be used instead of relative binding response to measure [P]. However, if the initial binding rates are very fast then the traces may contain artifacts from buffer mixing [31].

For the equilibrium in which a ligand binds cooperatively to the protein:

$$P + nL \rightleftharpoons PL_n$$

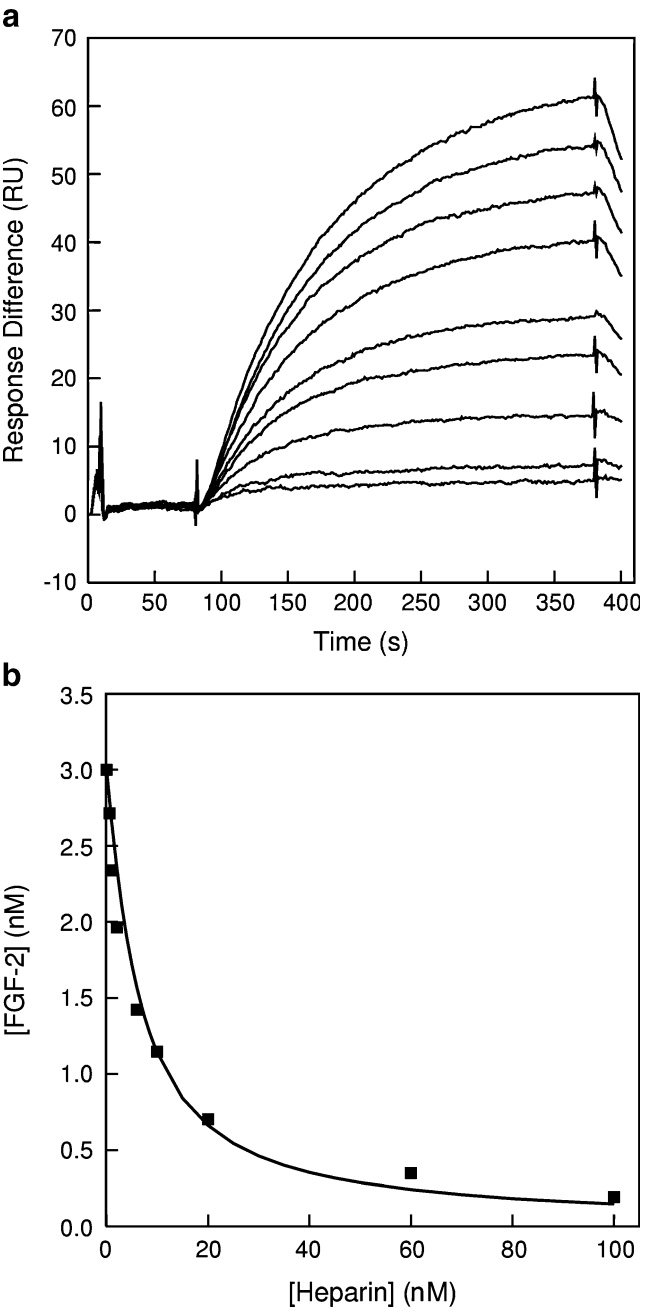


Fig. 2 Representative K_d measurement of heparin binding to FGF-2. **a** SPR sensorgrams showing the change in binding response (in RU) upon injection of 3 nM FGF-2 mixed with varying concentrations of heparin from 0 to 100 nM in HBS-EP running buffer containing 0.3 M NaCl over a heparin surface. **b** Binding curve constructed by plotting free FGF-2 concentration against varying concentrations of heparin. Values for free FGF-2 concentration were calculated as described in "Material and methods"

the equilibrium equation is:

$$K_d = \frac{[P][L]^n}{[PL_n]}. \quad (5)$$

The binding equation can be derived as above to give:

$$[P] = [P]_{total} - \frac{\left(K_d + [L]^n_{total} + [P]_{total}\right)}{2} + \sqrt{\frac{\left(K_d + [L]^n_{total} + [P]_{total}\right)^2}{4} - [L]^n_{total}[P]_{total}}. \quad (6)$$

Results and discussion

Linear standard curves, which passed through the origin, relating relative responses to protein concentration in the absence of ligand, were obtained for each protein tested, indicating that all SPR measurements were performed under mass transport conditions [23, 31].

The use of a streptavidin–biotin–albumin–heparin sensor chip, prepared by passing a solution of commercially available heparin–albumin–biotin over a streptavidin chip, has been detailed previously for the solution affinity assay [23]. In the present study the use of a streptavidin–biotin–heparin sensor chip (which contains no albumin), similarly prepared by passing a solution of commercially available heparin–biotin over a streptavidin chip, is also described. In most previous SPR studies of heparin/HS-binding proteins, biotinylated heparin was similarly bound to streptavidin or avidin immobilised on the chip surface [15, 16, 18, 20]. However, the method of biotinylation of heparin can affect its binding to the protein [21] and many heparin-binding proteins also interact non-specifically with avidin and streptavidin [22]. The use of avidin can also be problematic because it is itself a heparin-binding protein [33]. Furthermore, the streptavidin–biotin–heparin chip is not stable to the harsh regeneration conditions required for the removal of proteins that bind tightly [22]. It was similarly found in this study that the streptavidin–biotin–(albumin)–heparin chips, in which the heparin is not covalently bound to the sensor chip, are not especially stable to harsh regeneration conditions. Additionally, the biotinylated species will dissociate from the streptavidin if the sensor chip is stored in HBS-EP buffer. Therefore, methods were sought to attach heparin covalently to the sensor chip, so that the sensor chip can better withstand harsh regeneration conditions and can be stored in HBS-EP buffer for long periods of time without losing its binding capacity.

Heparin has been covalently bound to a sensor chip *via* a heparin–albumin conjugate with immobilisation through the primary amino groups of the albumin [22]. However, the covalent attachment of heparin directly onto a sensor chip *via* its reducing end should present the heparin in a manner that more closely resembles a proteoglycan [21]. The methodology for this type of attachment has recently been reviewed [34], and the method of Satoh and Matsumoto [29, 35] was successfully applied here (Scheme 1). Briefly, hydrazide groups were firstly introduced onto an (EDC/NHS)-activated carboxylated dextran matrix of a CM5 sensor chip with adipic acid dihydrazide. Heparin was then immobilised onto the hydrazide groups *via* reductive amination of its reducing end aldehyde group. The method was also successfully applied to C1 sensor chips which have a carboxylated surface similar to CM5 chips but lack the dextran matrix. Kamei and co-workers [30] used a strategy similar to that of Satoh and Matsumoto to immobilise heparin onto the carboxymethylated dextran surface of evanescent wave biosensor cuvettes, utilising amino groups (introduced using 1,4-diaminobutane) instead of hydrazide groups. This method was successfully adapted to preparing sensor chips using CM4 chips (previously known as B1 chips), which are similar to CM5 chips but with a lower degree of carboxylation (Scheme 1). The lower density of negative charge associated with the CM4 chip facilitates the approach of heparin to the surface to react with the amino groups by minimising the charge–charge repulsion. In our experience, these two immobilisation methods, particularly the latter, provide reproducible and stable sensor chips that can be used continuously for up to 6 months.

The four sensor chips described above were used in K_d determinations for four known ligands (heparin, PI-88, SOS and NTS) [18, 23, 36, 37] binding to the HS-binding growth factors FGF-1, FGF-2 and VEGF (Table 1). In this way the effects of using different heparin sensor chips on the solution affinity assay was examined. The ligands chosen are of diverse structure, molecular weight range [434 (NTS) to ~12,500 (heparin)] and degree of sulfation. The affinity of the compounds for the growth factors ranged from low nanomolar to high micromolar and the K_d values obtained by using the four different sensor chips gave similar results for each protein–ligand pair. It is noteworthy that measuring such a large range of K_d values is normally not possible using direct binding kinetics on the BIAcore.[1] This suggests that the assay is robust and the method of heparin immobilisation has little impact on the measurement of K_d values. This can in part be explained by the fact that the function of the immobilised heparin is to bind to free protein in the equilibrium solution, and does not depend on the activity of the heparin on the sensor chip as some kinetic models require [21].

[1] Typical range of K_d values is 200 nM to 200 pM. BIAapplications Handbook, version AB, 1998.

Table 1 K_d values of selected compounds with FGF-1, FGF-2 and VEGF using different sensor chips

Ligand	Growth factor	SA chip 1[a]	SA chip 2[b]	CM5 or C1 chip[c]	CM4 (B1) chip[d]
Heparin	FGF-1	4.2±2.9 nM	5.8±0.5 nM	5.1±1.4 nM	1.7±0.3 nM
	FGF-2	5.3±0.7 nM	1.6±0.7 nM	7.5±1.6 nM	5±0.9 nM
	VEGF	42.1±4.5 nM	85.7±11.9 nM	40.1±8.4 nM	83±8 nM
PI-88	FGF-1	307±128 pM	244±26 pM	286±108 pM	192±25 pM
	FGF-2	125±12 nM	550±90 nM	318±32 nM	113±3 nM
	VEGF	2.8±1 nM	2.5±0.6 nM	2.1±0.5 nM	845±285 pM
SOS	FGF-1	416±94 nM	248±8 nM	509±124 nM	650±217 nM
	FGF-2	3.4±0.4 μM	11.1±2 μM	6.3±0.9 μM	2.6±0.2 μM
	VEGF	137±17 μM	154±15 μM	n.d.	57±14 μM
NTS	FGF-1	n.d.	770±50 μM	765±334 μM	470±139 μM
	FGF-2	n.d.	230±70 μM	193±16 μM	150±40 μM
	VEGF	n.d.	175±12 μM	196±34 μM	130±13 μM

[a] *Via* heparin–albumin–biotin
[b] *Via* heparin–biotin
[c] *Via* ADHZ
[d] *Via* 1,4-diaminobutane

To further demonstrate the generality of the solution affinity assay to HS-binding proteins, four HS-binding proteins of diverse structure and function were selected for study. The proteins were interleukin 8 (IL-8), a pro-inflammatory CXC chemokine, platelet factor 4 (PF4), a CXC chemokine released by activated platelets, antithrombin III (ATIII), a coagulation cascade serpin, and monocyte chemotactic protein 2 (MCP-2), a CC chemokine which plays a role in the inflammatory response of blood monocytes. The K_d values of binding to the ligands heparin, LMWH and PI-88 were determined and the results are presented in Table 2. The K_d values presented in Tables 1 and 2 range from micromolar to picomolar and compare reasonably well with previously published data determined by various methods (Table 3). The range of K_d values reported in the literature vary considerably, particularly for heparin and LMWH as ligands. The reported values depend on the method used to determine them, the ionic strength of the buffer, the source of the heparin or LMWH and the resultant variability in molecular weight and charge distribution. The results obtained *via* the solution affinity assay are generally within or close to the reported ranges, indicating the applicability of the assay for studying these types of interactions.

Most of the commercial protein preparations used in this study contained a large excess of BSA (typically 50μg of BSA per μg of protein). To ensure that the BSA had no effect on the assay, solutions of BSA in buffer were passed over the sensor chip surface. The responses observed were typically <5 RU, indicating that BSA did not bind significantly to the sensor chip surface and thus its presence does not adversely affect the assay. As a further check, the assay was used to measure the affinity of heparin to recombinant FGF-1 prepared in house containing no BSA. The K_d value obtained (1.3 ± 0.6nM) is consistent with that obtained in the presence of BSA (2.4 ± 0.1nM), confirming the benign nature of BSA in this assay.

The solution affinity assay involves the mixing of protein, held at a constant concentration, with ligand at varying concentrations. After incubation, the mixture is injected over a surface onto which heparin has been immobilised. The binding of free protein to heparin is detected as an SPR response which decreases with increasing ligand concentration. It is important to note that this can only be observed when the interaction involves the heparin binding site, thus eliminating the possibility that non-specific binding is being evaluated. Thus, the K_d values measured in this study apply only to the interaction of ligand with the protein in its heparin-binding site. This is an important feature of this assay and makes it well suited to drug discovery where the main objective is the identification of ligands that can compete with heparin for binding to target proteins.

During the assay it is assumed that exposure of the protein/ligand mixture to the heparin surface does not

Table 2 K_d values of heparin, LMWH and PI-88 with IL-8, MCP-2, PF4 and ATIII

Ligand	MCP-2	IL-8	PF4	ATIII
Heparin	80±4 nM	350±40 nM	160±30 pM	21.5±1.0 nM
LMWH	6.5±1.8 μM	8.5±0.6 μM	7.4±0.8 nM	163±10 nM
PI-88	460±40 nM	30±4 μM	16.0±1.9 nM	35.9±2.0 μM

Table 3 Literature K_d values determined by various techniques for ligand and protein combinations used in this study

Ligand	FGF-1	FGF-2	VEGF	IL-8	PF4	ATIII
Heparin	505 nM [41]	7.9 nM [44]	157 nM [47]	37 μM [48]	4.4 nM [49]	48.8 nM [52]
	~1 nM [42]	2.2 nM [45]	165 nM [46]		20 nM [50]	11 nM [45]
	5.0 nM [43]	~1 nM [42]			30 nM [51]	57.4 nM [53]
	180 nM [30]	71 nM [30]				
		23 nM [46]				
LMWH	461 nM [41]	470 nM [54]		597 nM [55]	27 nM [56]	16 nM [45]
	91 nM [45]	2.0 nM [45]		5.5 μM [56]		100 nM [53]
PI-88		10.3 nM [44]				
SOS	3.4 μM [41]	280 nM [57]				
		170 μM [58]				
NTS	50 μM [36]					

significantly affect the pre-established equilibrium between the protein and ligand. That is, only insignificant dissociation of the protein/ligand complex occurs during the time of contact of the mixture with the heparin surface [38]. The flowcell in which heparin is immobilised has a volume of 20nL. At the flowrate of 5μL/min (the worst case scenario)[2] used during injection of the protein/ligand mixture, the contact time is calculated to be 0.24s. If 1% dissociation of the complex is allowed in this time, the maximum permissible rate for dissociation can be calculated using the equation:

$$[PL]_t = [PL]_0 \times e^{-k_{diss} \times t}$$

where $[PL]_t$ is the complex present at time t, and $[PL]_0$ at time $t = 0$. Allowing 1% dissociation in 0.24s gives:

$$0.99 = e^{-k_{diss} \times 0.24}.$$

Thus, the maximum permissible dissociation rate, k_{diss}, is $0.042s^{-1}$. The solution affinity assay does not allow measurement of the dissociation rate for a given protein/ligand complex. However, the k_{diss} reported for the interaction of FGF-1 with heparin ($0.016s^{-1}$ [30]; $0.015s^{-1}$ [22]), as well as a preliminary kinetic experiment on FGF-1 binding to heparin in the current study ($4.41 \pm 0.02 \times 10^{-3}s^{-1}$, data not shown) indicated that the rate constant k_{diss} was well below the maximum permissible rate. On this evidence, the assumption that the pre-established equilibrium is not altered is valid.

Although a stoichiometry of 1:1 was assumed for all protein/ligand interactions, it is well known that heparin/HS molecules contain many binding sites for a given protein that range from low to high affinity. Direct binding experiments of both FGF-1 and FGF-2 to immobilised heparin using SPR have previously been described, and relatively poor fits of the data to a simple 1:1 interaction model is evident [30]. It has been suggested that the heterogeneity in FGF-1 binding to heparin may reflect the structural heterogeneity of the immobilised heparin. Alternatively, a single heparin molecule could contain multiple binding sites with varying degrees of affinity for FGF-1 and FGF-2 [30]. For FGF-1 and FGF-2 binding to heparin, both earlier [18] and more recent data [39, 40] indicate that the stoichiometries may be far higher, up to 16:1 for FGF-1. While this complex binding behaviour is apparent in direct binding assays, it cannot be observed in solution affinity assays. The K_d observed in the solution affinity assay is, therefore, a product of the individual binding equilibria between protein and heparin and in this case may be best described as an apparent K_d. Alternatively, the data can be expressed as an IC_{50} value. Like heparin/HS, the binding of protein to PI-88 (a complex mixture of sulfated oligosaccharides) is also a mixture of binding equilibria, and here also the observed K_d is a product of the binding of protein molecules to the individual components of the mixture.

IL-8 was highly unstable upon dilution into HBS-EP buffer. Injection of freshly prepared 4.5nM IL-8 resulted in a response increase until the injection time reached ~1.5min, after which the binding response decreased rapidly. Incubation of the diluted protein for up to 5min on ice prior to injection resulted in complete loss of binding signal. Addition of BSA or DTT or combinations of these to the diluted protein did not increase its stability. Moreover, the concentrated protein sample was found to

Table 4 Effects of NaCl in the running buffer on K_d values for FGF-1 and FGF-2 with selected compounds

Ligand	K_d FGF-1 (150 mM NaCl)	K_d FGF-1 (300 mM NaCl)	K_d FGF-2 (180 mM NaCl)	K_d FGF-2 (300 mM NaCl)
Heparin	2.4±0.1 nM	20.4±1.8 nM	160±60 pM	5.0±0.2 nM
LMWH	17.3±1.6 nM	260±15 nM	n.d.	84.1±2.2 nM
PI-88	237±11 pM	8.4±0.8 nM	4.5±0.7 nM	130±3 nM

[2] Most assays are performed at flow rates of 20–40 μL/min which will further reduce the contact time.

be stable. K_d values for the ligands binding to IL-8 were, therefore, measured by preparing fresh dilutions of protein for each assay mixture. The final concentration of IL-8 used in the assay mixes is relatively high at 45nM compared with those used for the other proteins, however, at this concentration IL-8 is most likely still in its monomeric state as confirmed by the magnitude of the K_d values for heparin and LMWH [48, 55].

MCP-2 is dimeric [59], but stable upon dilution. When analysing the binding results for MCP-2, however, it was noticed that the fitted Eq. 3 deviated significantly from the data points. Deviations from best fit were observed for all ligands except heparin. Inclusion of a Hill coefficient in the binding equation (*i.e.* Eq. 6) enabled a much better fit to the data. The Hill coefficient refined to a value of ~1.5 for all the ligands except heparin (for which it was set to 1.0 by using Eq. 3), suggesting that there is more than one binding site for the ligand on the protein. The results suggest, therefore, that there are two binding sites for the ligand on the protein dimer that bind the ligand cooperatively.

The effect of buffer components, in particular NaCl, on the binding equilibrium was also considered. For all proteins except FGF-2 the binding affinities were measured in HBS-EP buffer at pH7.4. This buffer is frequently used for binding assays and is designed to mimic physiological conditions. For FGF-2 the NaCl concentration in the buffer was increased from 150 to 300mM to reduce non-specific binding to the sensor chip surface. The equilibrium considered here is only that of the interaction between protein and ligand. Interaction of components of the buffer with either the protein or ligand almost certainly exist as well, and these will have some effect on the protein/ligand equilibrium. In the case of FGF-2 where non-specific binding of the protein is reduced by the addition of NaCl, the effect of Na^+ and Cl^- ions on the ligand binding equilibrium is likely to be greater.

To measure the magnitude of this effect, several binding experiments with FGF-1 were conducted in HBS-EP buffer with NaCl concentration increased to 300mM (see Table 4). Of the three ligands measured (heparin, PI-88 and LMWH), the effect was greatest on PI-88. The K_d for this ligand binding to FGF-1 increased 35-fold, while those for heparin and LMWH binding to FGF-1 increased by 8.5 and 15-fold, respectively. A large effect on the K_d values due to NaCl is expected since both the ligand and protein are charged and their interaction has a significant ionic component. Since PI-88 has a higher number of sulfate groups per monosaccharide compared to heparin and LMWH, the effect of NaCl on PI-88 binding is expected to be higher. Despite the increase in the K_d value for PI-88 binding to FGF-1, it is 15-fold below that measured for FGF-2, indicating that PI-88 interacts preferentially with the former. Subsequently it was found that if the CM4 sensor chip is used for assaying FGF-2, the amount of salt in the running buffer can be significantly reduced to 180mM (see Table 4). This is possibly because the lower density of carboxyl groups on the CM4 chip causes less non-specific binding.

Conclusions

In summary, we have reported a robust biochemical assay to determine the affinity of various ligands for heparin-binding proteins. Four different methods of heparin immobilisation onto the sensor chips were utilised and the dissociation constants for some known ligands were obtained. It was found that the K_d values obtained with different sensor chips were similar, however, sensor chips prepared by covalent attachment of heparin to the chip surface are preferred because they are more stable. Furthermore, the applicability of this assay method to a variety of other heparin-binding proteins was demonstrated. The fact that a single sensor chip can be used for measuring the affinity of ligands of various molecular weight and degree of sulfation for a variety of heparin-binding proteins, significantly reducing the sensor chip preparation time and reducing the protein consumption, makes this assay well suited to heparin/HS mimetic drug discovery applications.

Acknowledgements We wish to thank Mr. Rick Filonzi (Biacore AB, Australia) for his technical support, Ms. Anna Bezos and Prof. Chris Parish (John Curtin School of Medical Resarch, Australian National University) for useful discussions and Dr. A. Satoh (Ochanomizu University, Japan) for his technical advice on the immobilisation of heparin onto the CM5 sensor chip. We also thank Dr. Ian Bytheway (Progen) for useful discussions, critical reading of this manuscript, and the preparation of the figures.

References

1. Casu, B., Lindahl, U.: Structure and biological interactions of heparin and heparan sulfate. Adv. Carbohydr. Chem. Biochem. **57**, 159–206 (2001)
2. Whitelock, J.M., Iozzo, R.V.: Heparan sulfate: a complex polymer charged with biological activity. Chem. Rev. **105**, 2745–2764 (2005)
3. Ori, A., Wilkinson, M.C., Fernig, D.G.: The heparanome and regulation of cell function: structures, functions and challenges. Front. Biosci. **13**, 4309–4338 (2008)
4. Esko, J.D., Lindahl, U.: Molecular diversity of heparan sulfate. J. Clin. Invest. **108**, 169–173 (2001)
5. Bishop, J.R., Schuksz, M., Esko, J.D.: Heparan sulphate proteoglycans fine-tune mammalian physiology. Nature **446**, 1030–1037 (2007)
6. Sasisekharan, R., Shriver, Z., Venkataraman, G., Narayanasami, U.: Roles of heparan-sulphate glycosaminoglycans in cancer. Nat. Rev. Cancer **2**, 521–528 (2002)
7. Parish, C.R.: The role of heparan sulphate in inflammation. Nat. Rev. Immunol. **6**, 633–643 (2006)
8. Liu, J., Thorp, S.C.: Cell surface heparan sulfate and its roles in assisting viral infections. Med. Res. Rev. **22**, 1–25 (2002)

9. Capila, I., Linhardt, R.J.: Heparin-protein interactions. Angew. Chem. Int. Ed. **41**, 391–412 (2002)
10. Coombe, D.R., Kett, W.C.: Heparan sulfate-protein interactions: therapeutic potential through structure-function insights. Cell. Mol. Life Sci. **62**, 410–424 (2005)
11. Raman, R., Sasisekharan, V., Sasisekharan, R.: Structural insights into biological roles of protein-glycosaminoglycan interactions. Chem. Biol. **12**, 267–277 (2005)
12. Ferro, V., Dredge, K., Liu, L., Hammond, E., Bytheway, I., Li, C., Johnstone, K., Karoli, T., Davis, K., Copeman, E., Gautam, A.: PI-88 and novel heparan sulfate mimetics inhibit angiogenesis. Semin. Thromb. Hemost. **33**, 557–568 (2007)
13. Presta, M., Leali, D., Stabile, H., Ronca, R., Camozzi, M., Coco, L., Moroni, E., Liekens, S., Rusnati, M.: Heparin derivatives as angiogenesis inhibitors. Curr. Pharm. Des. **9**, 553–566 (2003)
14. Avci, F.Y., Karst, N.A., Linhardt, R.J.: Synthetic oligosaccharides as heparin-mimetics displaying anticoagulant properties. Curr. Pharm. Des. **9**, 2323–2335 (2003)
15. Capila, I., VanderNoot, V.A., Mealy, T.R., Seaton, B.A., Linhardt, R.J.: Interaction of heparin with annexin V. FEBS Lett **446**, 327–330 (1999)
16. Dong, J., Peters-Libeu, C.A., Weisgraber, K.H., Segelke, B.W., Rupp, B., Capila, I., Hernaiz, M.J., LeBrun, L.A., Linhardt, R.J.: Interaction of the N-terminal domain of apolipoprotein E4 with heparin. Biochemistry **40**, 2826–2834 (2001)
17. Ibrahimi, O.A., Zhang, F., Hrstka, S.C., Mohammadi, M., Linhardt, R.J.: Kinetic model for FGF, FGFR, and proteoglycan signal transduction complex assembly. Biochemistry **43**, 4724–4730 (2004)
18. Mach, H., Volkin, D.B., Burke, C.J., Middaugh, C.R., Linhardt, R.J., Fromm, J.R., Loganathan, D., Mattsson, L.: Nature of the interaction of heparin with acidic fibroblast growth factor. Biochemistry **32**, 5480–5489 (1993)
19. Marks, R.M., Lu, H., Sundaresan, R., Toida, T., Suzuki, A., Imanari, T., Hernaiz, M.J., Linhardt, R.J.: Probing the interaction of dengue virus envelope protein with heparin: assessment of glycosaminoglycan-derived inhibitors. J. Med. Chem. **44**, 2178–2187 (2001)
20. Ricard-Blum, S., Feraud, O., Lortat-Jacob, H., Rencurosi, A., Fukai, N., Dkhissi, F., Vittet, D., Imberty, A., Olsen, B.R., van der Rest, M.: Characterization of endostatin binding to heparin and heparan sulfate by surface plasmon resonance and molecular modeling: role of divalent cations. J. Biol. Chem. **279**, 2927–2936 (2004)
21. Osmond, R.I., Kett, W.C., Skett, S.E., Coombe, D.R.: Protein-heparin interactions measured by BIAcore 2000 are affected by the method of heparin immobilization. Anal. Biochem. **310**, 199–207 (2002)
22. Zhang, F., Fath, M., Marks, R., Linhardt, R.J.: A highly stable covalent conjugated heparin biochip for heparin-protein interaction studies. Anal. Biochem. **304**, 271–273 (2002)
23. Cochran, S., Li, C., Fairweather, J.K., Kett, W.C., Coombe, D.R., Ferro, V.: Probing the interactions of phosphosulfomannans with angiogenic growth factors by surface plasmon resonance. J. Med. Chem. **46**, 4601–4608 (2003)
24. Bytheway, I., Cochran, S.: Validation of molecular docking calculations involving FGF-1 and FGF-2. J. Med. Chem. **47**, 1683–1693 (2004)
25. Cochran, S., Li, C.P., Bytheway, I.: An experimental and molecular-modeling study of the binding of linked sulfated tetracyclitols to FGF-1 and FGF-2. ChemBioChem **6**, 1882–1890 (2005)
26. Karoli, T., Liu, L., Fairweather, J.K., Hammond, E., Li, C.P., Cochran, S., Bergefall, K., Trybala, E., Addison, R.S., Ferro, V.: Synthesis, biological activity, and preliminary pharmacokinetic evaluation of analogues of a phosphosulfomannan angiogenesis inhibitor (PI-88). J. Med. Chem. **48**, 8229–8236 (2005)
27. Liu, L., Bytheway, I., Karoli, T., Fairweather, J.K., Cochran, S., Li, C., Ferro, V.: Design, synthesis, FGF-1 binding, and molecular modeling studies of conformationally flexible heparin mimetic disaccharides. Bioorg. Med. Chem. Lett. **18**, 344–349 (2008)
28. Liu, L., Li, C.P., Cochran, S., Ferro, V.: Application of the four-component Ugi condensation for the preparation of sulfated glycoconjugate libraries. Bioorg. Med. Chem. Lett. **14**, 2221–2226 (2004)
29. Satoh, A., Miwa, H.E., Kojima, K., Hirabayashi, J., Matsumoto, I.: Ligand-binding properties of annexin from Caenorhabditis elegans (annexin XVI, Nex-1). J. Biochem. **128**, 377–381 (2000)
30. Kamei, K., Wu, X., Xu, X., Minami, K., Huy, N.T., Takano, R., Kato, H., Hara, S.: The analysis of heparin-protein interactions using evanescent wave biosensor with regioselectively desulfated heparins as the ligands. Anal. Biochem. **295**, 203–213 (2001)
31. Karlsson, R.: Real-time competitive kinetic analysis of interactions between low-molecular-weight ligands in solution and surface-immobilized receptors. Anal. Biochem. **221**, 142–151 (1994)
32. Karlsson, R., Roos, H., Fägerstam, L., Persson, B.: Kinetic and concentration analysis using BIA technology. Methods: A Companion to Methods Enzymol. **6**, 99–110 (1994)
33. Kett, W.C., Osmond, R.I., Moe, L., Skett, S.E., Kinnear, B.F., Coombe, D.R.: Avidin is a heparin-binding protein. Affinity, specificity and structural analysis. Biochim. Biophys. Acta **1620**, 225–234 (2003)
34. Gemma, E., Meyer, O., Uhrín, D., Hulme, A.N.: Enabling methodology for the end functionalisation of glycosaminoglycan oligosaccharides. Mol. BioSystems **4**, 481–495 (2008)
35. Satoh, A., Matsumoto, I.: Analysis of interaction between lectin and carbohydrate by surface plasmon resonance. Anal. Biochem. **275**, 268–270 (1999)
36. Lozano, R.M., Jimenez, M., Santoro, J., Rico, M., Gimenez-Gallego, G.: Solution structure of acidic fibroblast growth factor bound to 1,3, 6- naphthalenetrisulfonate: a minimal model for the anti-tumoral action of suramins and suradistas. J. Mol. Biol. **281**, 899–915 (1998)
37. Volkin, D.B., Verticelli, A.M., Marfia, K.E., Burke, C.J., Mach, H., Middaugh, C.R.: Sucralfate and soluble sucrose octasulfate bind and stabilize acidic fibroblast growth factor. Biochim. Biophys. Acta **1203**, 18–26 (1993)
38. Nieba, L., Krebber, A., Pluckthun, A.: Competition BIAcore for measuring true affinities: large differences from values determined from binding kinetics. Anal. Biochem. **234**, 155–165 (1996)
39. Goodger, S.J., Robinson, C.J., Murphy, K.J., Gasiunas, N., Harmer, N.J., Blundell, T.L., Pye, D.A., Gallagher, J.T.: Evidence that heparin saccharides promote FGF2 mitogenesis through two distinct mechanisms. J. Biol. Chem. **283**, 13001–13008 (2008)
40. Robinson, C.J., Harmer, N.J., Goodger, S.J., Blundell, T.L., Gallagher, J.T.: Cooperative dimerization of fibroblast growth factor 1 (FGF1) upon a single heparin saccharide may drive the formation of 2:2:1 FGF1.FGFR2c.heparin ternary complexes. J. Biol. Chem. **280**, 42274–42282 (2005)
41. Spivak-Kroizman, T., Lemmon, M.A., Dikic, I., Ladbury, J.E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J., Lax, I.: Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. Cell **79**, 1015–1024 (1994)
42. Li, L.Y., Seddon, A.P.: Fluorospectrometric analysis of heparin interaction with fibroblast growth factors. Growth Factors **11**, 1–7 (1994)
43. Mach, H., Middaugh, C.R.: Probing the affinity of polyanions for acidic fibroblast growth factor by unfolding kinetics. Arch. Biochem. Biophys. **309**, 36–42 (1994)
44. Francis, D.J., Parish, C.R., McGarry, M., Santiago, F.S., Lowe, H. C., Brown, K.J., Bingley, J.A., Hayward, I.P., Cowden, W.B.,

Campbell, J.H., Campbell, G.R., Chesterman, C.N., Khachigian, L.M.: Blockade of vascular smooth muscle cell proliferation and intimal thickening after balloon injury by the sulfated oligosaccharide PI-88: phosphomannopentaose sulfate directly binds FGF-2, blocks cellular signaling, and inhibits proliferation. Circ. Res. **92**, e70–77 (2003)
45. Lee, M.K., Lander, A.D.: Analysis of affinity and structural selectivity in the binding of proteins to glycosaminoglycans: development of a sensitive electrophoretic approach. Proc. Natl. Acad. Sci. U. S. A. **88**, 2768–2772 (1991)
46. Ashikari-Hada, S., Habuchi, H., Kariya, Y., Itoh, N., Reddi, A.H., Kimata, K.: Characterization of growth factor-binding structures in heparin/heparan sulfate using an octasaccharide library. J. Biol. Chem. **279**, 12346–12354 (2004)
47. Krilleke, D., DeErkenez, A., Schubert, W., Giri, I., Robinson, G. S., Ng, Y.S., Shima, D.T.: Molecular mapping and functional characterization of the VEGF164 heparin-binding domain. J. Biol. Chem. **282**, 28045–28056 (2007)
48. Krieger, E., Geretti, E., Brandner, B., Goger, B., Wells, T.N., Kungl, A.J.: A structural and dynamic model for the interaction of interleukin-8 and glycosaminoglycans: support from isothermal fluorescence titrations. Proteins **54**, 768–775 (2004)
49. Dudek, A.Z., Pennell, C.A., Decker, T.D., Young, T.A., Key, N.S., Slungaard, A.: Platelet factor 4 binds to glycanated forms of thrombomodulin and to protein C. A potential mechanism for enhancing generation of activated protein C. J. Biol. Chem. **272**, 31785–31792 (1997)
50. Mayo, K.H., Roongta, V., Ilyina, E., Milius, R., Barker, S., Quinlan, C., La Rosa, G., Daly, T.J.: NMR solution structure of the 32-kDa platelet factor 4 ELR-motif N-terminal chimera: a symmetric tetramer. Biochemistry **34**, 11399–11409 (1995)
51. Kolset, S.O., Mann, D.M., Uhlin-Hansen, L., Winberg, J.O., Ruoslahti, E.: Serglycin-binding proteins in activated macrophages and platelets. J. Leukoc. Biol. **59**, 545–554 (1996)
52. Watton, J., Longstaff, C., Lane, D.A., Barrowcliffe, T.W.: Heparin binding affinity of normal and genetically modified antithrombin III measured using a monoclonal antibody to the heparin binding site of antithrombin III. Biochemistry **32**, 7286–7293 (1993)
53. Conrad, H.E.: Antithrombin, the prototypic heparin-binding protein. In Heparin-Binding Proteins, pp. 203–238. Academic, San Diego (1998)
54. Thompson, L.D., Pantoliano, M.W., Springer, B.A.: Energetic characterization of the basic fibroblast growth factor-heparin interaction: identification of the heparin binding domain. Biochemistry **33**, 3831–3840 (1994)
55. Goger, B., Halden, Y., Rek, A., Mosl, R., Pye, D., Gallagher, J., Kungl, A.J.: Different affinities of glycosaminoglycan oligosaccharides for monomeric and dimeric interleukin-8: a model for chemokine regulation at inflammatory sites. Biochemistry **41**, 1640–1646 (2002)
56. Witt, D.P., Lander, A.D.: Differential binding of chemokines to glycosaminoglycan subpopulations. Curr. Biol. **4**, 394–400 (1994)
57. Forsten, K.E., Wang, N., Robinson, R.M., Nugent, M.A.: A simple assay for evaluating inhibitors of proteoglycan-ligand binding. Ann. Biomed. Eng. **28**, 119–127 (2000)
58. Arakawa, T., Wen, J., Philo, J.S.: Densimetric determination of equilibrium binding of sucrose octasulfate with basic fibroblast growth factor. J. Protein Chem. **12**, 689–693 (1993)
59. Blaszczyk, J., Coillie, E.V., Proost, P., Damme, J.V., Opdenakker, G., Bujacz, G.D., Wang, J.M., Ji, X.: Complete crystal structure of monocyte chemotactic protein-2, a CC chemokine that interacts with multiple receptors. Biochemistry **39**, 14075–14081 (2000)