The effects of metal ions on heparin/heparan sulfate-protein interactions

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Abstract
Heparin/heparan sulfate (HS) interacts with a number of proteins thereby playing an essential role in the regulation of many physiological processes. The understanding of heparin/HS-protein interactions at the molecular level is of fundamental importance to biology and will aid in the development of highly specific glycan-based therapeutic agents. The heparin-binding proteins (HBPs) interact with sulfated domains of heparin/HS chains primarily through ionic attraction between negatively charged groups in HS/heparin chains and basic amino acid residues within the protein. Reports in literature have been shown that heparin molecules have a high affinity for a wide range of metal ions. In the present study, we used surface plasmon resonance (SPR) to study the effects of metal ions (underphysiological and non-physiological concentrations) on heparin/HS-protein interactions. The results showed that under non-physiological concentration, different metal ions showed different effects on heparin binding to fibroblast growth factor-1 (FGF1) and interleukin-7 (IL7). While the effects of individual metal ions at physiological concentrations had little impact on protein binding, the mixed metal ions reduced the FGF1/heparin or IL7/heparin binding affinity, changing its binding profile.

Keywords: Heparin, Protein, Metallon, Interaction, Surface Plasmon Resonance.

Abbreviations
SPR, surface plasmon resonance; GAG, glycosaminoglycan; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; HS, heparan sulfate; RU, resonance unit, FGF1, Fibroblast growth factor 1; IL7, Human interleukin 7.

Introduction
Heparin/heparan sulfate (HS) glycosaminoglycans (GAGs) are anionic and often highly sulfated, polydisperse linear polysaccharides. GAGs are ubiquitous molecules exhibiting a wide range of biological functions by interaction with various growth and differentiation factors and morphogens, extracellular matrix components, protease inhibitors, protease, lipoprotein lipase, and various pathogens [1-4]. Interactions between heparin/HS and proteins mediate diverse pathophysiological processes including: blood coagulation, cell growth and differentiation, host defense and viral infection, lipid transport and metabolism, cell-to-cell and cell-to-matrix signaling, inflammation, angiogenesis and cancer [4-6]. Thus, an understanding of heparin/HS-protein interactions at the molecular level is of fundamental importance to biology and should aid in the development of highly specific glycan-based therapeutic agents [3,5].

Metals play crucial roles in biological processes which are involved in cellular and subcellular functions [7]. For instance, the divalent magnesium and calcium ions play important regulatory roles in cells. Lack of body iron is common in cancer patients and it is associated with complications in surgery and in animal experiments. Metal ions play essential roles in about one third of enzyme interactions [8]. These ions can modify electron flow in a substrate or enzyme, thus effectively controlling an enzyme-catalyzed reaction. They can serve to bind and orient substrate with respect to functional groups in the active site of the enzyme [9]. Copper is recognized as an essential metalloelement and is primarily associated with copper-dependent cellular enzymes. Metal ions function in numerous metalloenzymes, are incorporated into pharmaceuticals and used as inorganic drugs for many diseases [7,10].

The heparin-binding proteins (HBPs) interact with sulfated domains of HS/heparin chains by ionic attraction between negatively charged groups in HS/heparin chains and basic amino acid residues within the protein. Previous study has shown that heparin molecules have a high affinity for a wide range of metal ions [11-19], which suggests the presence of metals may play a significant role in heparin/HS-protein interactions. For example, divalent cations play an important role in regulating the anti-Factor Xa activity of heparin [20]. It has also reported that divalent cations and heparin/heparan sulfate cooperate to control assembly and activity of the fibroblast growth factor [21]. Unfortunately, the effects of metal ions on protein-heparin/HS complexes and their biological activities are largely unknown. Thus, we undertook this study to evaluate the impact of metal ions protein-heparin/HS interaction. The present study uses surface plasmon resonance (SPR) spectroscopy to evaluate the effect of common metal ions on heparin/HS interactions with fibroblast growth factor-1 (FGF1) and interleukin-7 (IL7).

Table 1: Physiological metal ions concentration reference ranges for blood tests

<table>
<thead>
<tr>
<th>Metal</th>
<th>Lower limit conc.</th>
<th>Upper limit conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg²⁺</td>
<td>10 μM</td>
<td>100 μM</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>10 μM</td>
<td>20 μM</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>10 μM</td>
<td>35 μM</td>
</tr>
<tr>
<td>K⁺</td>
<td>1000 μM</td>
<td>3500 μM</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1000 μM</td>
<td>1300 μM</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>10 μM</td>
<td>24 μM</td>
</tr>
</tbody>
</table>
**Materials and Methods**

**Materials.** Porcine intestinal heparin (16 kDa) and porcine intestinal heparan sulfate (12 kDa) were obtained from Cerus Laboratories (Cincinnati, OH). Sensor SA chips were from GE Healthcare (Uppsala, Sweden). Fibroblast growth factor 1 (FGF1) was a gift from Amgen (Thousand Oaks, CA). Human interleukin 7 (IL7) was provided by Dr. Walsh (Center for Advanced Research in Biotechnology, University of Maryland Biotechnology Institute). SPR measurements were performed on a BIAcore 3000 (GE Healthcare, Uppsala, Sweden) operated using BLACore software (version 4.0.1).

**Preparation of heparin biochip.** Biotinylated heparin or HS was prepared by reaction of sulfo-N-hydroxysuccinimide long-chain biotin (Piecz, Rockford, IL) with free amino groups of unsubstituted glucosamine residues in the polysaccharide chain following a published procedure [22]. Biotinylated heparin was immobilized to streptavidin chip based on the manufacturer’s protocol. In brief, 20-µl solution of the heparin-biotin conjugate (0.1 mg/ml) in HBS-EP running buffer was injected over flow cell 2 of the streptavidin chip at a flow rate of 10 µl/min. The successful immobilization of heparin was confirmed by the observation of a ~250 resonance unit (RU) increase in the sensor chip. The control flow cell was prepared by 1 min injection with saturated biotin.

Measurement of the effects of metal ions on the interaction between heparin/HS and protein (FGF1 or IL-7) Using SPR. The protein samples were diluted in HBS-P buffer (0.01 M HEPES, 0.15 M NaCl, 0.005% surfactant P20, pH 7.4). Different dilutions of protein samples with or without addition of metal ions were injected at a flow rate of 30 µl/min. At the end of the sample injection, the same buffer was flowed over the sensor surface to facilitate dissociation. After a 3 min dissociation time, the sensor surface was regenerated by injecting 30 µl of 2 M NaCl to obtain a regenerated surface. The response was monitored as a function of time (sensorgram) at 25 °C.

The additions of metal ions to the heparin/HS and protein binding measurement were in three categories: 1) addition of CaCl2, ZnCl2, FeCl3, MgCl2, and KCl in concentration of 0, 10, 100 and 1000 µM, respectively; 2) addition of metal ions in physiological lower/upper limit concentrations (Table 1); 3) the addition of mixed metals ions with Mg2+ (50µM), Zn2+ (15µM), Fe3+ (20µM), K+ (2000µM), Ca2+ (1150µM), and Cu2+ (15µM) in physiological concentrations.

**Results and Discussion**

**The effects of metal ions on heparin/HS-protein interactions in non-physiological concentrations.**

The first set of SPR measurements on heparin/HS-protein interaction were conducted through the addition of CaCl2, ZnCl2, FeCl3, MgCl2, and KCl at concentration of 0, 10, 100 and 1000 µM, respectively. FGF1 which is well known as a heparin-binding protein (HBP), was used in this initial experiment. The results (Figures 1 to 3) showed that at non-physiological concentrations, different metal ions showed different effects on the heparin/HS-protein binding. The metal ions showed a greater effect on the HS-FGF1 interaction than on the heparin-FGF1 interaction and most of the effects of most of the metal ions were concentration dependent. FGF1 binding to heparin/HS was reduced with addition of Ca2+ or Mg2+ at 10 µM and the effects were decreased at 100 and 1000 µM concentrations of Ca2+ or Mg2+. FGF1 binding to heparin/HS was unaffected with addition of 10 µM Zn2+ but binding was dramatically reduced at Zn2+ concentrations of 100 and 1000 µM. FGF1 binding to heparin/HS was reduced at 10 µM Fe3+ and further decreased at 100 µM Fe3+ and no binding was detected at 1000 µM Fe3+. FGF1 to heparin/HS was greatly reduced at all concentrations of K+, ranging from 10 to 1000 µM. Some studies have reported that physiological metal ions such as sodium, calcium, and magnesium bind to heparin based on the polyelectrolyte theory [23-26].

Using atomic absorption and spectrophotometry, it was reported the overall trend for heparin–metal affinity to be Mn2+ > Cu2+ > Ca2+ > Zn2+ > Co2+ > Na+ > Mg2+ > Fe3+ > Ni2+ > Al3+ > Sr2+. There is evidence that divalent metal ions (Ca2+, Cu2+ and Zn2+) are necessary in many protein-heparin interactions thus influencing the affinity, specificity and stability of these complexes [27-28]. A previous study by our group showed the conformational changes induced by calcium ions are necessary for the interaction between heparin and annexin V [25].

**The effects of metal ions on heparin/HS-protein interactions in physiological concentrations.**

Next, heparin/HS FGF1 and heparin/HS-IL7 interactions were studied with the addition of metal ions at the physiological lower/upper limit concentrations (Table 1) [29]. The results (Figures 4 and 5) showed effects of most individual metal ions at physiological lower/upper limit concentrations, on these interactions were minimal. One exception was the effect of Cu2+ on the interaction of heparin/HS with FGF1 (Figure 4). A second exception was the effect of Fe3+, at its upper limit concentration, on the interaction of heparin/HS with IL7 (Figure 5) obviously reduced with the addition of Fe3+ (Figure 5). We previously reported the formation of a Cu2+-heparin complex gave extremely sensitive detection of heparin, permitting the analysis of as low as 10 ng with capillary electrophoresis [30]. Copper, along with FGF, [31] plays an important role in promoting physiological and malignant angiogenesis, the formation of new blood vessels by a tumor, enabling tumor growth, invasion, and metastasis [22]. It also has been reported that the heparin-copper complex is angiogenic in vivo and stimulates migration of capillary endothelium in vitro [32].
The effects of mixed metal ions on heparin-protein interactions. Finally, we measured the effect of physical concentrations of mixed metals ions in physiological concentrations, i.e. Mg2+ (50µM), Zn2+ (15µM), Fe3+ (20µM), K+ (2000µM), Ca2+ (1150µM), and Cu2+ (15µM), on the heparin-FGF1 interaction. Sensorgrams of heparin and FGF1 interactions are shown in Figure 6. The kinetic parameters (Table 2) of FGF1/heparin interactions were obtained by fitting the sensorgrams with a Langmuir 1:1 binding model. The SPR data showed different FGF1/heparin binding profiles (Figure 6 A, and B) with and without the added mixed metals ions. Without the addition of mixed metals ions (control), the KD for FGF1/heparin interaction was 22 nM, with the addition of mixed metals ions, the KD for FGF1/heparin interaction was 350 nM. SPR analysis also showed different binding kinetics for FGF1/heparin interaction in the absence and presence of mixed metals ions. Without the added mixed metals ions, FGF1/heparin interaction exhibited $k_a = 4.5 \times 10^5$ (1/Ms), and $k_d = 0.01$ (1/s), while FGF1/heparin interaction exhibited $k_a = 1.6 \times 10^4$ (1/Ms) and $k_d = 5.7 \times 10^{-3}$ (1/s) the presence of mixed metals ions.

Table 2: Summary of kinetic data of protein-heparin interactions in present of mixed metal ions

<table>
<thead>
<tr>
<th>Interaction</th>
<th>$k_a (1/\text{Ms})$</th>
<th>$k_d (1/s)$</th>
<th>$K_D (\text{M})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF1/Heprin (control)</td>
<td>$4.5 \times 10^5$</td>
<td>0.01</td>
<td>$2.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>FGF1/Heprin present of mixed metal ions</td>
<td>$1.6 \times 10^4$</td>
<td>$5.7 \times 10^{-3}$</td>
<td>$3.5 \times 10^{-7}$</td>
</tr>
<tr>
<td>IL7/Heprin (control)</td>
<td>$2.7 \times 10^4$</td>
<td>$6.6 \times 10^{-3}$</td>
<td>$2.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>IL7/Heprin present of mixed metal ions</td>
<td>$1.0 \times 10^3$</td>
<td>0.025</td>
<td>$2.5 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

Figure 1

![Image A](image_a.png)

![Image B](image_b.png)

![Image C](image_c.png)

![Image D](image_d.png)
In conclusion, the results of this study clearly show different metal ions can have different effects on the heparin/HS-protein binding at non-physiological concentrations. Metal ions in the range of physiological concentrations with few exceptions generally show little impact on heparin/HS-protein interactions. However, mixed metal ions can alter binding affinity in the case of FGF1/heparin or IL-7/heparin binding. This study provides useful information for the formulation of heparin/HS-based agents to promote or block biological processes with heparin-protein interactions.

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**Figure 1.** SPR sensograms of heparin-FGF1 interaction with the addition of metal ions. FGF1 concentration was 500 nM. A: heparin-FGF1 interaction with the addition of CaCl2 (0, 10, 100 and 1000 µM); B: heparin-FGF1 interaction with the addition of ZnCl2 (0, 10, 100 and 1000 µM); C: heparin-FGF1 interaction with the addition of FeCl3 (0, 10, 100 and 1000 µM); D: heparin-FGF1 interaction with the addition of MgCl2 (0, 10, 100 and 1000 µM); E: heparin-FGF1 interaction with the addition of KCl (0, 10, 100 and 1000 µM).
Figure 2. SPR sensograms of HS-FGF1 interaction with the addition of metals ions. FGF1 concentration was 500 nM. A: HS-FGF1 interaction with the addition of CaCl2 (0, 10, 100 and 1000 µM); B: HS-FGF1 interaction with the addition of ZnCl2 (0, 10, 100 and 1000 µM); C: HS-FGF1 interaction with the addition of FeCl3 (0, 10, 100 and 1000 µM); D: HS-FGF1 interaction with the addition of MgCl2 (0, 10, 100 and 1000 µM); E: HS-FGF1 interaction with the addition of KCl (0, 10, 100 and 1000 µM).

Figure 3. A: Normalized FGF1 binding to heparin in the presence of different concentration of metal ions. B: Normalized FGF1 binding to HS in the presence of different concentration of metal ions.

Figure 4. A: FGF1 binding (RU) to heparin in the presence of physiological lower/upper limit concentrations of metal ions. FGF1 concentration was 500 nM; B: FGF1 binding (RU) to HS in the presence of physiological lower limit/upper limit concentrations of metal ions. FGF1 concentration was 500 nM.
Figure 5. A: IL-7 binding (RU) to heparin in the presence of physiological lower/upper limit concentrations of metal ions. IL-7 concentration was 500 nM; B: IL-7 binding (RU) to HS1 in the presence of physiological lower/upper limit concentrations of metal ions. IL-7 concentration was 500 nM.

Figure 6. SPR sensorgrams of heparin-FGF1 or IL-7 interaction with the addition of mixed metals ions in physiological concentrations. A: SPR sensorgrams of heparin-FGF1 interaction without addition of mixed metals ions; B: SPR sensorgrams of heparin-FGF1 interaction with addition of mixed metals ions; Concentrations of FGF1 (from top to bottom): 500, 250, 125 and 63 nM, respectively. C: SPR sensorgrams of heparin-IL7 interaction without addition of mixed metals ions; D: SPR sensorgrams of heparin-IL7 interaction with addition of mixed metals ions; Concentrations of IL7 (from top to bottom): 500, 250, 125 and 63 nM, respectively. The black curves are the fitting curves using models from BIAevaluate 4.0.1.
References

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