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Pattern-based sensing of sulfated glycosaminoglycans with a dynamic mixture of iron complexes†

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A dynamic mixture of iron complexes was used as a colorimetric sensor for sulfated glycosaminoglycans. The sensing ensemble was prepared by mixing $[FeCl_2(H_2O)_4]$ with dipicolylamine, the functionalized bipyridyl ligand *N*-(6-aminohexyl)-4′-methyl-2,2′-bipyridine-4-carboxamide, and the dye Evans Blue. Upon addition of the analytes, characteristic changes in the UV-Vis spectrum of the solutions were observed. The spectral changes allowed identifying different sulfated glucosaminoglycans (unfractionated heparin, low molecular weight heparin, dextran sulfate, chondroitin sulfate A, dermatan sulfate, heparan sulfate) with the help of a linear discriminant analysis. Furthermore, it was possible to distinguish mixtures of unfractionated heparin and heparan sulfate with good resolution.

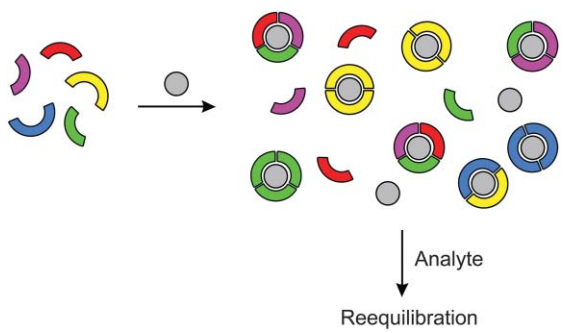
Introduction

Heparin is a sulfated glycosaminoglycan, which is widely used in the clinic as an anticoagulant and antithrombotic agent.[1] In view of its pharmacological importance, it is not surprising that considerable efforts have been made to develop methods for the analysis of heparin.[2] These methods are of interest for the quantification of heparin in biological samples (*e.g.* blood serum) or for the quality control of pharmaceutical preparations. The latter issue became particularly relevant after the discovery that contaminated unfractionated heparin (UFH) had entered the market, which resulted in the death of about 200 patients worldwide.[3]

Colorimetric or fluorescent chemosensors for heparin are appealing because the required instruments are relatively cheap and easy to access. In recent years, a number of such sensors have been reported.[4] In most cases, the goal was to rapidly quantify heparin in blood. Since blood is a complex biological matrix, it was desirable to develop a sensor with a very high selectivity for the target heparin. For a chemosensor that is used in quality control, however, a perfectly selective sensor is not necessarily the best choice. On the contrary, it would be interesting to have a sensor that is able to simultaneously detect heparin and potential contaminants. The group of Nitz has recently reported an interesting approach in this direction.[5] They have demonstrated that an array of indicator displacement assays (IDAs)[6] was able to provide diagnostic patterns for several sulfated polysaccharides. The individual IDAs of the sensor array were obtained by mixing amine-functionalized cyclodextrins with lithocholic acid derivatives containing a quinolinium fluorophore. In the following we describe an alternative approach to create a cross-reactive chemosensor for sulfated glycosaminoglycans. It is shown that a dynamic mixture of Fe(II) complexes can be used to differentiate sulfated polysaccharides by UV-Vis spectroscopy. The assay allows the identification of common glycosaminoglycans with high fidelity. Furthermore, it is possible to distinguish mixtures of UFH and heparan sulfate with good resolution.

Results and discussion

We have recently demonstrated that dynamic mixtures of metal complexes can be used as powerful colorimetric sensors.[7] The basic principle is shown in Scheme 1. A mixture of homo- and heteroleptic metal complexes is formed upon addition of one or more exchange-labile metal ions to a set of ligands. In order to utilize such a mixture as a colorimetric sensor, several species need to be colored. This can be achieved by using colored ligands (*e.g.* metal-binding dyes) or ligands, which give colored metal-complexes. If the addition of an analyte leads to a re-equilibration of the system, a color change will be observed. A pattern-based analysis of the spectral changes can then be used to obtain information about the identity, the quantity, or the purity of the analytes.



Scheme 1 A dynamic mixture of homo- and heteroleptic complexes is formed by reaction of an exchange-labile metal ion (grey circle) with a set of colored ligands. The addition of an analyte, which binds to one or several species of the mixture, will result in a re-equilibration and a characteristic change in color.

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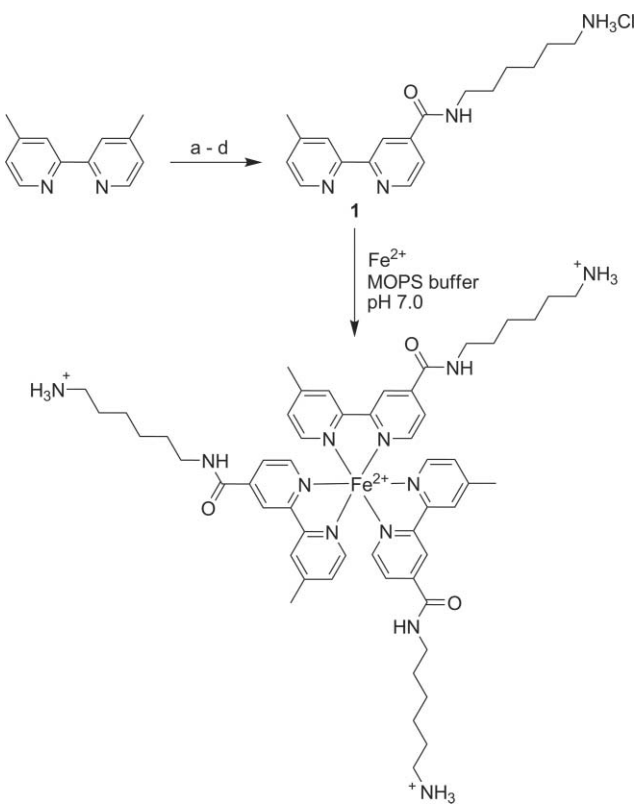
Sensors based on dynamic combinatorial libraries[8,9] of metal complexes have some interesting characteristics. First of all, it is very easy to make the sensors because they are obtained by mixing commercially available or easily accessible building blocks. Secondly, the sensors can rapidly be optimized for a particular sensing problem by variation of the nature and/or the quantity of the metal ions and the ligands. A potential drawback, however, is that these sensors are not very selective. The sensing of analytes in a complex matrix containing interfering substances can thus be problematic, unless variations of the matrix are small (constant background). For the analysis of mixtures of analytes under standardized conditions, however, these sensors are potentially very well suited.

Our starting point for the development of a colorimetric chemosensor for sulfated glycosaminoglycans was the recent discovery that Ru(II) complexes with bipyridyl ligands containing protonated amine side chains are able to bind to heparin in buffered aqueous solution.[4b] We reasoned that by combining a similar ligand with the exchange-labile Fe(II) instead of Ru(II) we would be able to make a dynamic complex with an affinity for negatively charged, polysulfated sugars. Therefore, we synthesized the amine-substituted bipy ligand **1** following a recently reported procedure (Scheme 2).[10] Subsequent reaction with $[FeCl_2(H_2O)_4]$ in buffered aqueous solution gave the homoleptic complex $[Fe(\mathbf{1})_3]^{n+}$ as evidenced by spectrophotometric titrations (see ESI†). It is expected that the primary amine groups are protonated at neutral pH. Therefore, the complex $[Fe(\mathbf{1})_3]^{n+}$ exists predominantly as a pentacation under these conditions ($n = 5$).

For the creation of a dynamic mixture of Fe(II) complexes, we decided to use two additional ligands: dipicolylamine (dpa) and the dye Evans Blue. The tridentate ligand dpa is known to form stable ML_2 complexes with Fe(II).[11] In aqueous solution, $[Fe(dpa)_2]^{2+}$ shows a broad absorption band in the UV/Vis-spectra with a maximum at 431 nm. The complex can thus easily be distinguished from $[Fe(\mathbf{1})_3]^{n+}$, which shows a broad band with a maximum at 540 nm.

The third ligand, Evans Blue (EB), was chosen because of several favorable characteristics. Similar to dpa, the diazo dye EB is commercially available. In aqueous solution, EB shows a strong absorption at 606 nm, which is well separated from the absorption maxima of the homoleptic complexes with the ligands dpa (λ_{max} = 431 nm) and **1** (λ_{max} = 540 nm). Furthermore, EB was found to form heteroleptic complexes with $[Fe(dpa)_2]^{2+}$ and $[Fe(\mathbf{1})_3]^{5+}$. This was evidenced by spectrophotometric titrations. When aliquots of a stock solution of complex $[Fe(\mathbf{1})_3]^{5+}$ were added to a buffered aqueous solution of EB ([EB] = 7.5 μM, MOPS, pH 7.0), the band at 606 nm decreased with concomitant formation of a new maximum at 556 nm (Fig. 1). The spectral changes level off at a ratio of $[Fe(\mathbf{1})_3]^{5+}$:EB = 1 : 1. Assuming that EB acts as a bidentate ligand and that Fe(II) maintains its coordination number of six, the absorption spectra point to the formation of a complex with the stoichiometry $[Fe(\mathbf{1})_2(EB]$. A similar behavior was observed for experiments with the dpa complex: addition of $[Fe(dpa)_2]^{2+}$ to a solution of EB resulted in a decrease of the absorption band at 606 nm (see ESI†). However, higher concentrations of $[Fe(dpa)_2]^{2+}$ (~ 900 μM, 120 equiv.) were needed to achieve saturation. This data suggest that heteroleptic Fe-dpa-EB complexes are formed as well, but that the mixed complexes are less favored than in the case of ligand **1**.



Scheme 2 Formation of the homoleptic complex $[Fe(\mathbf{1})_3]^{5+}$. The amine–substituted bipy ligand **1** was synthesized from 4,4′-dimethyl-bipyridine as described in the literature:[10] a) SeO_2, dioxane; b) $AgNO_3$/NaOH, water–ethanol; c) CDI, DMF, Boc-NH$(CH_2)_6NH_2$; d) HCl/methanol.

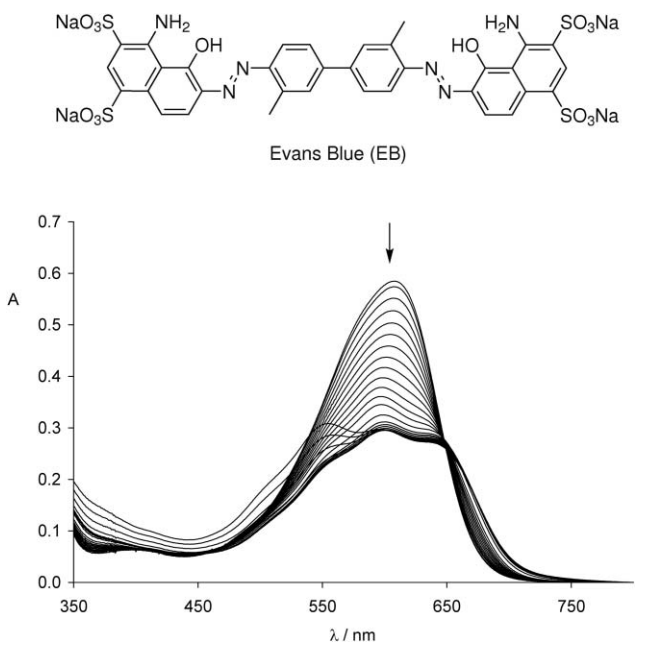
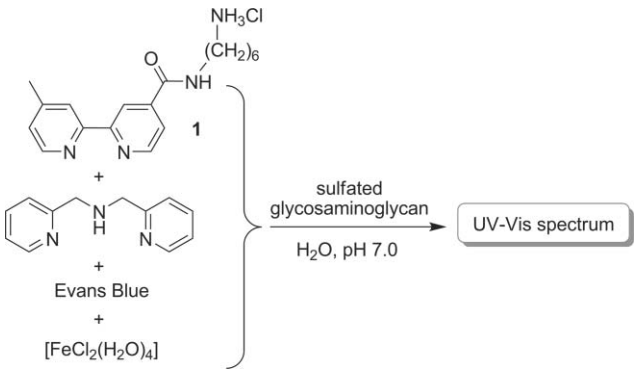


Fig. 1 Absorption spectra of a buffered aqueous solution (MOPS, pH 7.0) containing Evans Blue (7.5 μM) upon addition of increasing amounts of complex $[Fe(\mathbf{1})_3]^{5+}$ (0 – 22.5 μM).

The sensing ensemble was prepared by mixing $[FeCl_2(H_2O)_4]$ with the ligands dpa, **1**, and EB in buffered aqueous solution (MOPS, pH 7.0). After equilibration, aliquots of stock solutions of low molecular weight heparin (LMWH), unfractionated heparin (UFH), dextrane sulfate, chondroitin sulfate A, chondroitin sulfate B/dermatan sulfate, and heparan sulfate were added (Scheme 3). The final concentrations were: [Fe] = 30 μM, [dpa] = 300 μM, [**1**] = 90 μM, [EB] = 3.75 μM, and [analyte] = 25 μg ml^{-1}. The concentrations of the ligands were chosen to take into account differences in stability (dpa[11b] *vs.* the bipyridyl ligand **1**[12]) and the absorption coefficient (high value for EB). However, an extensive optimization of the ligand concentrations was not performed.[13] The samples were analyzed by UV-Vis spectroscopy after an equilibration time of 90 min. For each analyte, six independent analyses were performed.



Scheme 3 Experimental procedure for the sensing of sulfated glycosaminoglycans.

Inspection of the absorption spectra showed that the analytes had induced significant changes in the area between 400 and 700 nm. For subsequent analyses, we focused on six different wavelengths: 431, 545, 585, 612, 632, and 669 nm. The absolute changes at these six wavelengths for the different analytes at a concentration of 25 μg ml^{-1} are shown in Fig. 2.

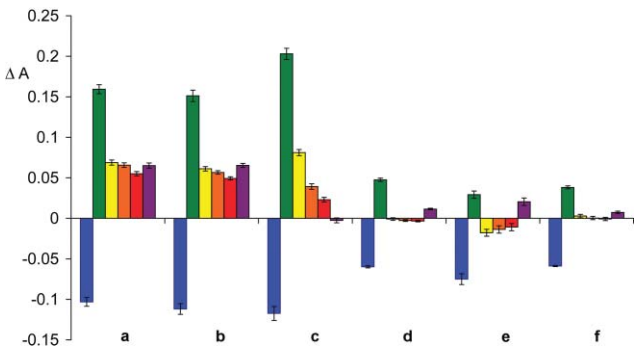


Fig. 2 Response of the sensing ensemble composed of $[FeCl_2(H_2O)_4]$, dpa, **1**, and Evans Blue (for conc. see text) after addition of different glycosaminoglycans (final conc. = 25 μg ml^{-1}). Blue: 431 nm, green: 545 nm, yellow: 585 nm, orange: 612 nm, red: 632 nm, and purple: 669 nm. *a* = LMWH, *b* = UFH, *c* = dextrane sulfate, *d* = chondroitin sulfate A, *e* = dermatan sulfate, *f* = heparan sulfate. The values represent averages of six independent measurements.

The glycosaminoglycans LMWH (Fig. 2a), UFH (Fig. 2b) and dextrane sulfate (Fig. 2c) showed a strong interaction with the assay mixture and therefore a very pronounced response. The absorption at 431 nm decreased, whereas most other bands increased. These changes indicate that a re-equilibration had occurred, which increased the concentration of complexes containing the ligand **1** and Evans Blue on behalf of complex $[Fe(dpa)_2]^{2+}$ (λ_{max} = 431 nm). The thermodynamic basis for this re-equilibration is likely the stabilization of polycationic Fe-ligand **1** complexes by the highly negatively charged analytes. Dextrane sulfate (Fig. 2c) displayed a distinct change at 545 nm but relatively small changes at 612, 632, and 669 nm compared to LMWH (Fig. 2a) and UFH (Fig. 3b). Chondroitin sulfate A (Fig. 2d), dermatan sulfate (Fig. 2e), and heparan sulfate (Fig. 2f) gave a lower overall response due to their reduced charge density. Even though major changes were only observed for 431 and 545 nm, there were also small but significant changes for the values at 669 nm.

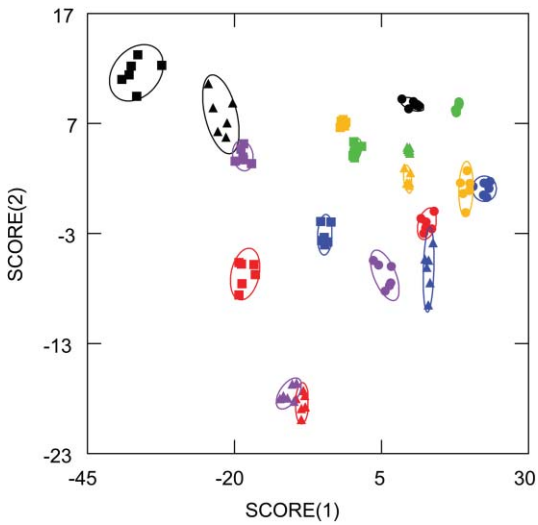


Fig. 3 Score plot of a LDA performed with the sensor data at λ = 431, 545, 585, 612, 632, and 669 nm. The symbols correspond to the following samples: red = UFH, pink = LMWH, yellow = chondroitin sulfate A, blue = dermatan sulfate, green = heparan sulfate, black = dextrane sulfate; circles = 10 μg ml^{-1}, triangles = 25 μg ml^{-1}, squares = 50 μg ml^{-1}.

Next, we have made a second sensing ensemble, in which ligand **1** was replaced by the standard 2,2-bipyridine (concentration as described above). No significant change in the absorption spectrum was observed when UFH was added to this mixture at a concentration of 25 μg ml^{-1}. This result confirmed that the amino-substituted ligand **1** containing a positively charged side chain was a crucial component of the sensor.

From the data shown in Fig. 3 it is apparent that a characteristic response was obtained for each glycosaminoglycan. Analyte-specific patterns were also found for samples containing the glycosaminoglycans at a lower concentration of 10 μg ml^{-1} or a higher concentration of 50 μg ml^{-1} (see ESI†). To determine whether the variance of the data was sufficient to distinguish all samples (6 analytes × 3 concentrations), we have performed a linear discriminant analysis (LDA).[14] A graphic representation of this analysis in form of a score plot is shown in Fig. 3. Most of the data of the different glycosaminoglycans appear in well separated clusters. The good discrimination was confirmed by "jack-knifed" matrix validation procedure, in which one measurement at a time was omitted. The remaining data were used as a training set and

the omitted observation was evaluated. This procedure gave a correct classification in all cases. The colorimetric response of the dynamic mixture of Fe complexes can thus be used to identify the respective glycosaminoglycans *and* to obtain information about the quantity of the analyte. It should be pointed out, however, that we have used only three different analyte concentrations. A more precise quantitative analysis might lead to data overlap.

Subsequently, we have explored the possibility to use the sensor for the discrimination of mixtures of glycosaminoglycans. For that purpose, we have examined the sensor response for samples containing different ratios of UFH and heparan sulfate ([UFH] = $(25-x)$ μM, [heparan sulfate] = x μM, with $x = 0, 5, 10, 15, 20, 25$). The sensing experiments were performed under similar conditions as described above and the absorption data were evaluated by a LDA. From the resulting score plot (Fig. 4) it is clear that all six mixtures are clearly separated. A single UV-measurement is thus sufficient to differentiate mixtures of the glycosaminoglycans UFH and heparan sulfate with high precision.

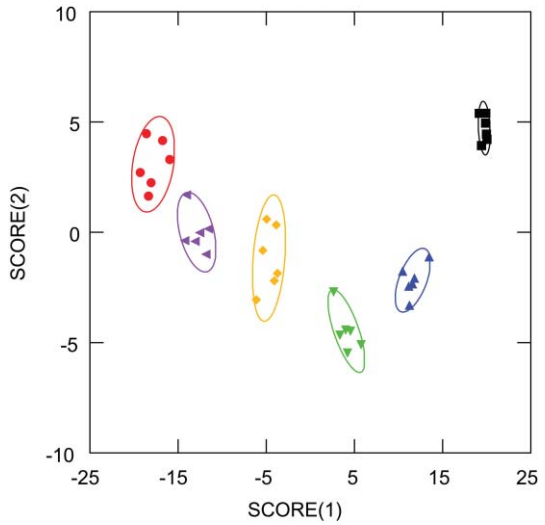


Fig. 4 Score plot of a LDA performed with the sensor data at $\lambda = 431$, 545, 585, 612, 632, and 669 nm. The symbols correspond to the following samples: red = pure UFH, pink = UFH/heparan sulfate 4 : 1, yellow = UFH/heparan sulfate 3 : 2, green = UFH/heparan sulfate 2 : 3, blue = UFH/heparan sulfate 1 : 4, black = pure heparan sulfate; $[\text{analyte}]_{tot}$ = 25 μg ml^{-1}.

Conclusions

A dynamic mixture of homo- and heteroleptic iron complexes was obtained by mixing $[FeCl_2(H_2O)_4]$ with the ligands dpa, **1**, and EB. When sulfated glycosaminoglycans were added to this mixture, a re-equilibration occurred, which favored the formation of complexes containing the ligand **1**.[15] The re-equilibration was associated with a change of color. The mixture could thus be used as a colorimetric sensor for sulfated glycosaminoglycans. The sensor response was most pronounced for the highly charged LMWH, UFH, and dextrane sulfate, but other glycosaminoglycans could be detected as well. Each analyte gave rise to a characteristic colorimetric response. It was therefore possible to use the UV-Vis spectrum as a 'fingerprint' for the respective glycosaminoglycans. Analysis of these color patterns by a LDA allowed to identify glycosaminoglycans and to obtain information about the quantity and the purity of the analytes.

The cross-reactive sensor that we have developed complements more traditional chemosensors for glycosaminoglycans, which are optimized for signaling the quantity of a particular analyte (mostly heparin). The possibility to distinguish mixtures from pure samples could be of interest for the quality control of semi-synthetic glycosaminoglycans. With respect to potential applications it should also be noted that minor efforts are required for the preparation of our sensor: the synthesis of ligand **1** is straightforward and all other components are commercially available.

Experimental

General

Dipicolylamine (Sigma), Evans Blue (Fluka), $[FeCl_2(H_2O)_4]$ (Sigma), MOPS buffer (Fluka), low molecular weight heparin (Fluka), unfractionated heparin (Applichem), dextran sulfate (Sigma), heparan sulfate (Sigma), chondroitin sulfate A (Sigma) and dermatan sulfate (Sigma) were used as received. The ligand *N*-(6-aminohexyl)-4′-methyl-2,2′-bipyridine-4-carboxamide hydrochlorid (**1**) was synthesized starting from 4,4′-dimethyl-bipyridin (Fluka) as described in the literature.[10] Stock solutions of the ligands and the metal salt were prepared with a concentration of 10 mM in bidistilled water. Stock solutions of the sulfated glycosaminoglycans were prepared with a concentration of 1 mg ml^{-1}. MOPS buffer (100 mM, pH 7.0) was prepared with bidistilled water and used for all experiments. All UV/Vis spectra were recorded on a Lambda 35 spectrometer (Perkin Elmer) using disposable cuvettes (Brand). Quartz cuvettes (Hellma) were used for spectrophotometric titrations.

Spectrophotometric titration

A stock solution (1.0 mM) of $[Fe(\mathbf{1})_3]^{n+}$ was prepared by dissolving the appropriate amounts of $[FeCl_2(H_2O)_4]$ and ligand **1** in water. The solution was equilibrated for 24 h at room temperature before utilization. The titration was performed by adding aliquots of this stock solution to a buffered aqueous solution of Evans Blue ([EB] = 7.5 μM, [MOPS] = 10 mM, pH 7.0). Each aliquot contained 0.067 equivalents of $[Fe(\mathbf{1})_3]^{n+}$ with respect to EB. After each addition, the solution was equilibrated for 30 min at room temperature before measuring the absorption spectra in the range of 200 to 800 nm. Information about a similar titration with $[Fe(dpa)_2]^{2+}$ can be found in the ESI.†

Sensing experiments

The sensor was prepared by mixing appropriate amounts of stock solutions containing the ligands, the metal salt and the buffer. After equilibration for 15 h at room temperature, a UV/Vis spectrum was recorded. Subsequently, the analytes were added and a second UV/Vis spectrum was recorded after equilibration for 90 min. Each measurement was repeated six times. The final concentrations were: [**1**] = 90 μM, [dpa] = 300 μM, [Evans Blue] = 3.75 μM, $[Fe^{2+}]$ = 30 μM, and [MOPS] = 10 mM. The concentrations of the polysulfated sugars were either 10, 25, or 50 μg ml^{-1}. For the analysis of mixtures UFH and heparan sulfate,

the samples contained a total concentration of 25 μg ml^{-1}. The ratios between UHS and heparan sulfate were 4 : 1, 3 : 2, 2 : 3, and 1 : 4. For all analyses, the difference in absorption before and after addition of the analyte at the following six wavelengths was used: 431, 545, 585, 612, 632 and 669 nm. The data were analyzed with the commercially available statistics program SYSTAT (version 11.0) using a linear discriminant algorithm.

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13 The modular nature of sensors based on dynamic mixtures of metal complexes allows optimizing the sensor response by variation of the relative amounts of the sensor components (metal, ligands). For a more detailed discussion see ref. 7*c*.

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15 The glycosaminoglycans were not expected to act as competitive ligands for the binding to Fe(II). This was substantiated by the following experiment: the addition of UFH (final conc.: 15 μM) to a buffered aqueous solution of $[Fe(\mathbf{1})_3]^{n+}$ (final conc.: 15 μM) did not result in a change of color.