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# Development and evaluation of a fluorescence microplate assay for quantification of heparins and other sulfated carbohydrates

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ABSTRACT

Due to their complex composition, quantification of heparins is difficult. On the one hand there are many biological tests, which only indirectly detect effects of the antithrombin-binding material. On the other hand direct quantitative methods are available but they are often insensitive, challenging, time-consuming or expensive. The aim of this study was to develop a sensitive, rapid, simple as well as inexpensive direct quantification assay suitable for routine analysis.

Based on Polymer-H, a novel heparin complexing, fluorescent labeled synthetic polymer ($\lambda_{(ex)}$ 320 nm, $\lambda_{(em)}$ 510 nm), a microplate assay was developed and optimized. The specificity of the assay was evaluated by structure–assay response relationships studies using structurally defined glucan sulfates, heparins, and other natural and synthetic sulfated carbohydrates.

The fluorescence intensity of Polymer-H (7.5 μg/ml) showed to be concentration-dependently amplified by heparins as well as by other sulfated carbohydrates. The best sensitivity, accuracy and linearity were observed in a range from 0.63 to 5.0 μg/ml heparins. No differences in the fluorescence between various heparins were observed, so that only one calibration curve is needed. In addition, all types of carbohydrates with a degree of sulfation (DS) > ~1.2 and a $M_r$ > 3000 can be quantified as well. By own calibration curves also other sulfated carbohydrates like fondaparinux or other glycosaminoglycans (DS > 0.4) can be determined.



## 1. Introduction

Heparins still represent the antithrombotic drugs of choice in short- and medium-term prophylaxis and therapy of thromboembolic diseases [1]. Both unfractionated heparin (UFH) and low molecular weight heparins (LMWHs) are porcine extract preparations and consist of heterogeneous mixtures of highly sulfated glycosaminoglycans, which considerably differ in their individual structure [2]. The complex composition and pronounced batch-to-batch variability rule out heparin dosing in milligram [3]. Instead, heparins are indirectly quantified, namely according to their in vitro anticoagulant effect indicated in international units (IU) [4].

The latter is mainly based on their antithrombin-mediated inhibition of factor Xa and thrombin [5]. However, only about 30–50% of the UFH molecules and even less than 20% of the LMWH molecules contain the essential antithrombin-binding pentasaccharide [6]. Accordingly, quantification by anti-factor Xa (aXa-) activity records only a small part of a heparin (the high-affinity material), whereas more than 50% or 80% (the low-affinity material), respectively, are neglected. The same is true for all the other functional methods used in clinical practice and research (e.g. APTT, PiCT, thromboelastography). The detection of only a small portion of the applied heparin may be one reason for the poor correlation between the ex vivo anticoagulant activity and the in vivo efficacy and safety of heparins [4].

Another disadvantage of the quantification in IU is that any measurement needs a calibration curve established with the reference standard for the corresponding heparin.

Moreover, heparins as well as other sulfated polysaccharides exhibit a wide range of additional biological activities like antithrombin-independent antithrombotic actions, antiinflammatory, antimetastatic and/or antiangiogentic effects [7–9]. Such actions are supposed to contribute to the overall therapeutic efficacy of heparins, e.g. causing a prolonged survival of tumor patients [10]. Several studies showed that the antithrombin-independent activities of heparins and other sulfated carbohydrates do not correlate with their respective anticoagulant activity [11–13]. The latter

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is therefore inadequate for the quantification of such compounds in pharmacological studies.

Finally, the recent heparin scandal caused by UFH contaminated with oversulfated chondroitin sulfate (OSCS) demonstrates that current methods for quality control of heparin are insufficient [14]. The contaminant was neither detected by the APTT nor by the aXa-assay, which are the Pharmacopoeia quantification assays for UFH and LMWH, respectively.

Consequently, there is a need for a direct quantitative assay, which detects the chemical substance heparin and not only a pharmacological effect. Corresponding efforts have been made, but the methods available at present are unsuitable for rapid and sensitive routine analysis. Colorimetric determinations of the anionic groups, the uronic acids [15] or the sugar units are insensitive, often unreliable and also destructive. Potentiometric [16], conductometric [17], or electrophoretic methods [18] are more sensitive, but very time-consuming.

In recent years, the development of so-called heparin sensors has attracted attention. These are devices or molecules causing a signal in the presence of heparin, e.g. electrochemical sensors [19] and colorimetric sensors [20,21].

At present, research focuses on the development of fluorescent sensors for heparins. Sauceda et al. [22] synthesized a heparin-specific peptide, which however only quantifies heparin concentrations > 3 IU/ml. From a benzimidazolium dye library, the two heparin sensors "Heparin Orange" and "Heparin Blue" have been discovered [23] and cationic silacyclopentadiene derivatives were described as turn-on sensors, since they become highly fluorescent by aggregation with heparin [24].

Another popular heparin interacting structure is boronic acid. Based on the findings of the Anslyn group [25] and an approach originally devoted to protein surface recognition [26] we synthesized polymeric fluorescent sensors [27]. Polymer-H, the copolymer with the highest affinity to heparin, consists of three types of functional monomers based on a methacrylamide skeleton: (1) dansyl monomers representing the fluorescence labels, (2) ortho-aminomethylphenylboronate forming cyclic esters with glycols, and (3) ethylammonium units exhibiting additional electrostatic attraction (Fig. 1). Binding studies with neutral sugars produced a marked increase in fluorescence intensity (FI) of Polymer-H, whereas heparin led to significant concentration-dependent fluorescence quenching. With a detection limit of ~0.09 μg/ml LMWH (~0.01 aXa-IU/ml), Polymer-H showed a very high sensitivity for heparin.

The aim of the present study was to develop a direct, rapid, accurate and sensitive assay based on Polymer-H, which is suitable for routine analysis. Further, structure–assay response relationships should be established using structurally defined glucan sulfates as well as various heparins, other natural glycosaminoglycans, and the heparin contaminant OSCS.

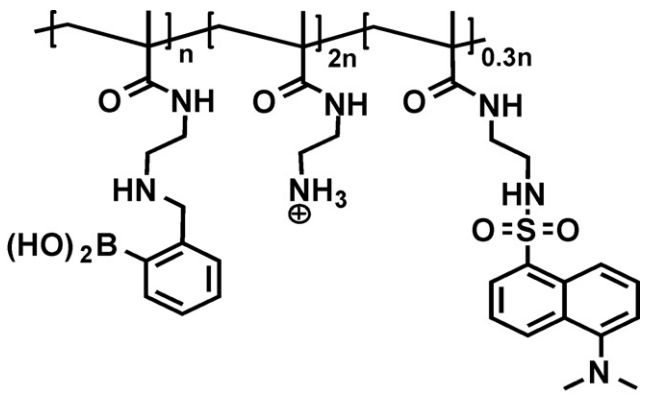


**Fig. 1.** Schematic structure of polymer fluorescent (dansyl), tailored for heparin complexation by electrostatic ($NH_3^+$) and covalent interactions (boronic acid).

## 2. Experimental

### 2.1. Fluorescent sensor and test compounds

#### 2.1.1. Polymer-H

Polymer-H has been synthesized as previously described [27]. In short, first the three comonomer units, i.e. methacrylamide derivatives of (1) the dansyl, (2) the ortho-aminomethylphenylboronic acid and (3) the ethylammonium moiety were prepared. These monomers were subjected to conventional radical copolymerization with AIBN (2,2′-azobisisobutyronitrile), which resulted in Polymer-H containing the three functional monomers in the ratio (1) 0.3: (2) 1.0: (3) 2.0 (Fig. 1). Its hydrodynamic volume $MW_{HD}$ amounts to 116,000 as determined by aqueous gel filtration chromatography using polyethylene oxide and polyethylenglycol as standards [27]. Binding studies performed in HEPES-buffered (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.1; 25 mM) solutions revealed an association constant $K_a$ with a LMWH (relative molecular mass $M_r$ of 3000) of $\sim 3 \times 10^7\ M^{-1}$ [27].

#### 2.1.2. Heparins and fondaparinux

UFH (from porcine intestinal mucosa, characteristic $M_r$ 15,000), medium molecular weight heparin with a narrow molecular weight (MMWH, mass-average $M_r$ 9500–11,500, characteristic $M_r$ 10,500) [28], three low molecular weight heparin fractions LMWH-I, -II, -III (characteristic $M_r$ 6600, 8000 and 9500, respectively), the heparin fragments A (decasaccharide mixture) and B (pentasaccharide mixture), and certoparin sodium (Mono-Embolex®, mass-average $M_r$ 4200–6200, characteristic $M_r$ 5000) were kind gifts from Novartis (Nürnberg, Germany). Further, UFH with a degree of sulfation (i.e. number of sulfate groups per monosaccharide unit, DS) of 1.20 was used to produce both 6-O-desulfated heparin (DS 0.49, conditions: solvent N-methylpyrrolidinone:water 9/1, reaction time 24 h, reaction temperature 90 °C) and – starting from the latter – resulfated heparin (DS 0.99) according to Baumann et al. [29]. Nadroparin calcium (Fraxiparin®, mass-average $M_r$ 3600–5000, characteristic $M_r$ 4420) and fondaparinux sodium (Arixtra®, DS 1.60, $M_r$ 1728) were kind donations from Glaxo Smith Kline (Notre Dame de Bondville, France). Enoxaparin sodium (mass-average $M_r$ 3800–5000, characteristic $M_r$ 4550) was the chemical reference substance (CRS, Ref. Code E0180000) ordered from the European Directorate for the Quality of Medicines & HealthCare (EDQM, Strasbourg, France).

#### 2.1.3. Other glycosaminoglycans, OSCS

Hyaluronate sodium (Hya-ject®, produced by fermentation) was obtained from Ormed GmbH & Co. KG (Freiburg, Germany). Chondroitin sulfate B (dermatan sulfate, sodium salt from porcine intestinal mucosa, C3788), chondroitin sulfate C (sodium salt from shark cartilage, C4384), chondroitin sulfate A (sodium salt from bovine trachea, 27042) and heparan sulfate (sodium salt from bovine kidney, H7640) were purchased from Sigma (St. Louis, Missouri, USA).

According to Maruyama et al. [30], we synthesized OSCS by sulfating chondroitin-4-sulfate (i.e. chondroitin sulfate A).

#### 2.1.4. Semisynthetic glucan sulfates

Three series of structurally defined linear glucan sulfates, i.e. phycarin sulfates, curdlan sulfates, and pullulan sulfates, were produced by semisynthetic modifications of neutral, linear glucans under specified conditions as previously described [31,32] to obtain glucan sulfates with distinct DS and molecular weights. The β-1,3-glucans phycarin (degree of polymerization 23–25, determined by electrospray ionization mass spectrometry, Goemar

Laboratories, St. Malo, France), curdlan (high $MW_{HD}$, Wako Pure Chemical Industries, Osaka, Japan) and the α-1,4/1,6-glucan pullulan (thermally degraded and fractionated [31], Wacker Chemie, München, Germany) were used as starting carbohydrates. Sulfation was performed with sulfur trioxide-pyridine complex dissolved in dimethylformamide.

By protecting position 6 of the glucose units of the β-1,3-glucan laminarin with an adamantoyl group, a laminarin sulfate selectively sulfated in position 2 and 4 (average $MW_{HD}$ 11,500, DS 1.15) was produced [32]. In contrast to MMWH, it does not show any affinity to antithrombin.

#### 2.1.5. Dextran sulfates, curdlan phosphate

The low molecular weight dextran sulfate sodium (dextran sulfate-2, DS 1.70, $MW_{HD}$ 9000) was purchased from Sigma (St. Louis, Missouri, USA) and the high molecular weight dextran sulfate sodium (dextran sulfate-1, DS 1.50, $MW_{HD}$ 500,000) from Serva (Heidelberg, Germany).

Curdlan phosphate (5.02291) was obtained from CarboMer, Inc., San Diego, California, USA.

### 2.2. Microplates

Seven types of microplates were evaluated. Three of them were from Greiner Bio-One GmbH (Frickenhausen, Germany): the top reading plates Fluotrac® 200 (655076) and Fluotrac® 600 (655077) and the bottom reading plate μClear® (655090). The other four were from Nunc GmbH & Co. KG (Langenselbold, Germany): the top reading standard plate (237108), MaxiSorp® (437111) and a cell-culture plate (137101) as well as the bottom reading cell-culture plate (165305).

### 2.3. Analytical methods

The molecular weights of the glucan sulfates were determined as $MW_{HD}$ by gel filtration chromatography on a Sephadex S75 column (Amersham Biosciences Europe, Freiburg, Germany) using a fast protein liquid chromatography system (Amersham Biosciences Europe) and a refractive index detector (Waters, Eschborn, Germany). The system was calibrated with neutral pullulans with defined molecular mass, stachyose and glucose as reference standards.

The DS of the glucan sulfates were determined by ion chromatography on a HPLC system with conductivity detection, whereby the sulfate ions, which were released by hydrolysis of the glucan sulfates with trifluoroacetic acid, were quantified [32].

The sulfation pattern of the glucan sulfates was examined by a modified methylation procedure [33]. The resulting methylated, ethylated alditol acetates were analyzed by combined gas–liquid chromatography mass spectrometry, providing qualitative and quantitative information about the carbohydrate structure and the distribution of the sulfate groups.

Excitation and emission spectra of Polymer-H were recorded with a Luminescence spectrometer (LS50B, PerkinElmer, Wiesbaden, Germany).

### 2.4. Polymer-H fluorescence assay procedure

All test compounds and Polymer-H were dissolved in 0.9% aqueous sodium chloride (NaCl). Sample aliquots of 180 μl were given into wells of a microplate (MaxiSorp® 437111, Nunc) and 20 μl Polymer-H (75 μg/ml) were added. After incubation (10 min, room temperature) in the microplate reader Optima (BMG LABTECH GmbH, Offenburg, Germany), the fluorescence signals were measured by top reading ($\lambda_{(ex)}$ 320 nm (±10 nm), $\lambda_{(em)}$ 510 nm (±10 nm)). Certoparin (0.16–10.0 μg/ml) was used as standard for the assay development. Aqueous NaCl (0.9%) served as control.

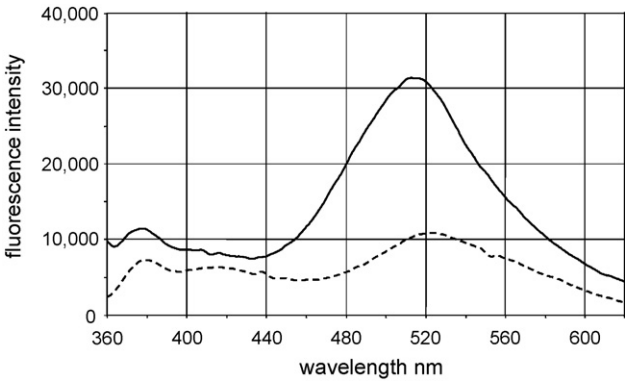


**Fig. 2.** Emission spectra of a Polymer-H solution. The fluorescence intensity of a Polymer-H solution (3 ml, 0.07 mg/ml in 0.9% NaCl) was measured before (- - Polymer alone) and after the addition of certoparin (final-concentration: 2.2 μg/ml, – Polymer and certoparin) after excitation at 334 nm (excitation-/emission-slit: 12.5 nm, scan speed 500 nm/min). Chinin sulfate (0.1 μM in 0.5 M sulfuric acid, 1:1000 dilution with 0.9% NaCl) served as control.

Each sample was measured twice. The presented FI values were obtained by subtracting the control FI (Polymer-H) from the measured values.

## 3. Results and discussion

### 3.1. Assay development

To develop a quantification assay for heparins based on Polymer-H, we evaluated the optimal Polymer-H concentration and buffer conditions. Further parameters, which might influence the results, were examined to define the final assay procedure.

#### 3.1.1. Increase and quenching of the FI of Polymer-H

Excitation and emission spectra of Polymer-H dissolved in 0.9% NaCl were recorded. In the presence of the LMWH certoparin, the FI of Polymer-H was increased by approximately three times and its emission maximum was slightly shifted from 522 to 513 nm (Fig. 2). The FI increase by heparin is in contrast to the previously described quenching of Polymer-H in HEPES buffer [27]. The observation that certoparin increased the FI, when the measurements were performed in 0.9% aqueous NaCl suggests that HEPES itself is responsible for the FI decrease of Polymer-H by heparins.

This seems plausible, since the small HEPES molecule ($M_r$ 238) contains a sulfonate anion as well as a hydroxyl group like heparins and may interact with Polymer-H and increase its FI. As obvious from the previously found quenching effect of a LMWH (30–150 nM) [27], heparins successfully compete with HEPES (25 mM) for binding to Polymer-H, but enhance its FI less than the small HEPES molecules.

Due to the pronounced enlargement of the linear measurement range and higher flexibility concerning useable buffers, we preferred to develop an assay based on the FI increase instead of the quenching by heparins.

#### 3.1.2. Polymer-H concentration

To identify the optimal Polymer-H concentration in 0.9% NaCl, the FI was measured at concentrations ranging from 0.025 to 25 mg/ml. The FI concentration-dependently increased, and linearity was observed up to 1.56 mg/ml ($R^2$ = 0.998) (data not shown). Higher Polymer-H concentrations were associated with self-absorption and self-quenching demonstrating the so-called

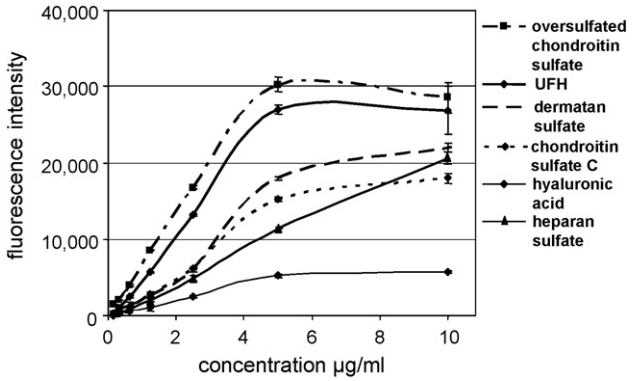


**Fig. 3.** Influence of glycosaminoglycans on the Polymer-H fluorescence intensity.

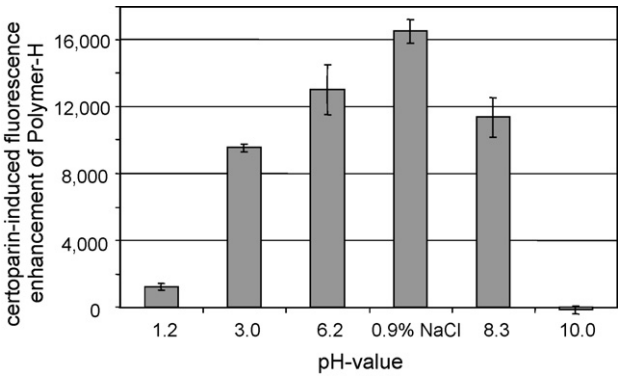


**Fig. 4.** Certoparin-induced fluorescence intensity enhancement of Polymer-H in dependence on the pH-value. Certoparin was used in a concentration of 2 µg/ml. The pH-values were adjusted using adequate buffers: citrate buffers (pH 3.0 and 6.2; 0.1 M), TRIS buffer (pH 8.3; 0.05 M), sodium hydrogen carbonate/carbonate buffer (pH 10.0; 0.2 M), hydrochloric acid/potassium chloride (pH 1.2; 0.1 M) and 0.9% sodium chloride (pH 4.5–7.0).

inner filter effect. Subsequently, standard curves of certoparin (0.016–20.0 µg/ml) were established with different Polymer-H concentrations (1.2, 1.0, 0.75, 0.5, 0.25, 0.1, 0.075, and 0.05 mg/ml). All of them revealed a concentration-dependent increase of FI up to a certain certoparin concentration (example UFH shown in Fig. 3). With decreasing Polymer-H concentrations, the linear range of the curves was shifted to lower certoparin concentrations, which implies increased sensitivity. For example, at Polymer-H concentrations of 250 and 75 µg/ml, linear FI increases were observed in the range from 1.25 to 20 µg/ml certoparin ($R^2$ = 0.998), and 0.63 to 5.0 µg/ml certoparin ($R^2$ = 0.999), respectively.

A Polymer-H concentration of 75 µg/ml (i.e. final assay concentration: 7.5 µg/ml) revealed optimal sensitivity, accuracy as well as linearity of certoparin quantification.

### 3.1.3. Influence of pH-value and salt concentration

There are two conceivable molecular mechanisms for the FI enhancement of Polymer-H by heparins and other sulfated carbohydrates observed in other buffers than HEPES:

a) On the one hand, more rigid structures in fluorophors are known to be associated with higher FI, as conformational restriction reduces internal conversion, intersystem crossing or radiative decay [34]. Due to electrostatic interactions and presumed covalent boronic ester formation, sulfated carbohydrates may cause conformational restriction of Polymer-H and thus increase its FI.
b) On the other hand, Polymer-H contains ortho-aminomethylphenylboronic acid, which increases the FI of the fluorophor by boronic ester formation with carbohydrates. Critical to the FI modulation is the amino group next to the boron atom (Fig. 1). At neutral pH and in the absence of carbohydrates, its nitrogen ion pair electrons quench the fluorescence through photoelectron transfer (PET). In the presence of carbohydrates, formation of complexes between diols and boronic acid suppresses fluorescence quenching through PET and so increases the FI, when the pH of the solution is between the two p$K_a$-values of the boronic acid unit. Originally, it was proposed that the FI increase by carbohydrates is based on the formation of a B–N bond, which makes the nitrogen ion pair electrons less available for fluorescence quenching through PET. A newer explanation is the hydrolysis mechanism [35]: If the reactions are carried out in a protic solvent such as water, complex formation induces solvolysis, which results in the protonation of the amine nitrogen. Also this protonation prevents PET resulting in reduced quenching.

The FI of boronic acid-based sensors is dependent on the pH, so we examine the FI of Polymer-H in the presence and absence of certoparin (2 µg/ml) at different pH-values adjusted with appropriate buffers (Fig. 4). At pH 1.0 (below the first p$K_a$-value of the boronic acid unit) Polymer-H showed no difference in its FI in the presence or absence of certoparin. In both cases, the nitrogen of the boronic acid unit is protonated, which suppresses fluorescence quenching through PET. In addition, heparins are uncharged at pH 1 and thus not capable of ionic interactions with Polymer-H. At pH-values between the first and second p$K_a$-value of the boronic acid unit, certoparin increased the FI of Polymer-H, whereby the strongest effect was found at neutral pH (0.9% NaCl). According to Ni et al. [35], the deprotonated nitrogen becomes protonated by carbohydrates resulting in suppression of PET and so in increased FI. At pH 10.0 (i.e. above the second p$K_a$-value), Polymer-H showed again no difference in the FI in the presence or absence of certoparin, since in both cases, nitrogen is deprotonated and boron is hydroxylated thus enabling PET.

Heparin quantification of Polymer-H is possible over a wide pH-range. Measurements at neutral pH (0.9% NaCl) resulted in both the highest FI increase and the largest linear concentration range and thus turned out optimal.

Beside the pH-value, the salt concentration may influence the formation of resonance structures and ions and so affect the FI of Polymer-H. To exclude potential quenching by chloride ions, we investigated the influence of 0.23, 0.45, 0.90, 1.80 and 3.60% NaCl on the FI increase by certoparin (4 µg/ml) (data not shown). Also concerning this parameter, the assay proved good robustness. Only at the highest NaCl concentration, the FI was significantly reduced by ~16%.

### 3.1.4. Influence of temperature and measurement time

Since fluorescence properties are known to be temperature-sensitive, we compared the FI increase by certoparin measured at room temperature with that at 37 °C. Only at higher concentrations the 37 °C-curve flattened resulting in a 50% lower FI at 5.0 µg/ml compared with that achieved at room temperature (data not shown). Due to increased flexibility of the molecules at higher temperature, external conversion increases and rigidness decreases resulting in lower FI. Thus, the performance of the assay is better at room temperature than at 37 °C.

To evaluate the optimal time of measurement, kinetics of the FI change of Polymer-H by certoparin (0.08–5.0 µg/ml) were recorded. Up to 2.5 µg/ml certoparin, FI remained constant over the measurement time of 28 min. Only at 5.0 µg/ml certoparin, the FI slowly increased as a function of time (Fig. 5). Measuring the microplate again after 6 h revealed a FI decrease of 16% at 1.25 and 2.5 µg/ml certoparin, so that linearity was abrogated (data not

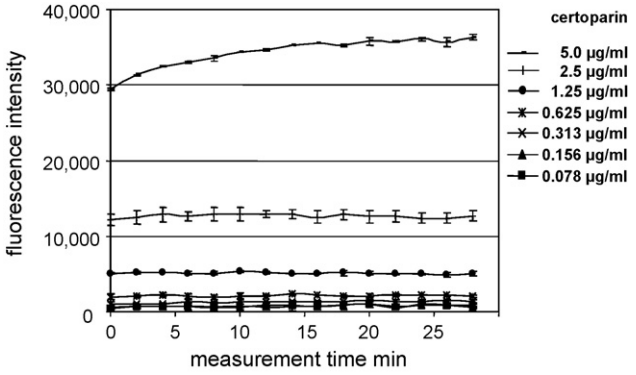


**Fig. 5.** Kinetics of Polymer-H fluorescence induced by various certoparin concentrations. Up to 2.5 µg/ml certoparin, fluorescence intensities are constant over a measurement time of 28 min.

shown). Based on these results, an incubation time of 10 min was chosen, which ensures equilibration and optimal orientation of the molecules as well as enables rapid testing and low coefficients of variation (CVs < 4%).

### 3.1.5. Evaluation of optimal type of microplate

It is often neglected that accuracy and sensitivity of an assay may considerably depend on the type of the used microplate. To identify the most suitable one for this assay, we tested two optical bottom plates and five black bottom plates differing in their surface characteristics (Table 1). Further, with the optical bottom plate (OBP) from Nunc, both top and bottom readings were conducted. Compared with the Nunc plates, the three Greiner plates performed poorly. Linearity of the FI increase by certoparin was only partly given and CVs were considerably higher. For example, the high-binding Fluotrac 600® resulted in a smaller FI amplitude and higher CVs than the high-binding MaxiSorp® from Nunc. With the cell-culture plate µClear® from Greiner, no linear FI increase was measured. Similarly, the black bottom cell-culture plate from Nunc revealed low accuracy and sensitivity. Among the other three Nunc plates, the cell-culture OBP showed the best sensitivity (at bottom reading, whereas top reading was associated with ~50% reduction of FI and a lower slope, data not shown), followed by the MaxiSorp® plate. However, due to pronounced autofluorescence of the cell-culture OBP, its background emission was much higher and also the CVs (2–19%) were higher than those obtained with the MaxiSorp® plate (0–8%). Therefore, the MaxiSorp® plate was considered most qualified for the assay.

### 3.1.6. Assay performance

The established assay procedure enables sensitive determination of heparin concentrations. For all heparins (see Section 3.3.1), the limit of detection amounts to 0.1 µg/ml and the limit of quantification to 0.31 µg/ml, both calculated by means of the standard deviations of the controls according to the ICH guideline [36]. Linearity of the method is given up to 5 µg/ml heparin ($R^2$ = 0.998). Certoparin standard curves revealed low intra- and inter-assay variability with CVs < 4% ($n$ = 8) and 8% ($n$ = 8, measured on different days), respectively.

A concentration of 5 µg/ml corresponds to ~1.0 IU/ml UFH and ~0.5 aXa-IU/ml LMWHs. Prophylactic and therapeutic concentrations of UFH and prophylactic ones of LMWHs are thus within the linear measurement range of the assay. If required, the linear range can be easily shifted to higher concentrations by using higher Polymer-H concentrations or higher concentrations can be quantified by diluting the samples.

## 3.2. Structure-dependent FI increase of Polymer-H

To approve the assay, the effects of structurally defined semisynthetic glucan sulfates, β-1,3-glucan phosphate as an example for an organic polyphosphate and sodium sulfate on the FI of Polymer-H were investigated. Neither unbound sulfate ions nor β-1,3-glucan phosphate (data not shown) increased the FI. This suggests that the assay specifically detects sulfated carbohydrates. The used glucan sulfates, i.e. phycarin sulfates, curdlan sulfates and pullulan sulfates, differ in their DS, $MW_{HD}$ and/or their type of glycosidic linkage (Table 2). Like certoparin, the glucan sulfates generally caused a concentration-dependent increase of the FI of Polymer-H.

### 3.2.1. Influence of the DS

Since the FI increase of Polymer-H by glucan sulfates is assumed to be partly based on ionic interactions, a higher DS may imply better binding and thus higher FI. Comparing the concentration-dependent FI curves of the various series of glucan sulfates, those with DS > 1 exhibited an almost linear FI increase up to 5.0 µg/ml, whereas those with DS < 1 showed an approximately linear increase up to 10.0 µg/ml (data not shown). The FI values observed at 5 µg/ml (Fig. 6) demonstrate that glucan sulfates with a DS < 0.4 did not markedly enhance the FI, whereas glucan sulfates with a DS between 0.4 and 1.26 (curdlan sulfate) or 1.48 (phycarin sulfate), respectively, showed a DS-dependent FI increase. Above a DS of 1.4 no further FI increase was observed.

**Table 1**
Influence of the type of microplate on the increase of the Polymer-H fluorescence intensity by certoparin.

| Microplate characteristics[a] | | | Measurement results with certoparin 0.63–5.0 µg/ml | | |
|---|---|---|---|---|---|
| Company | Branded name | Surface/hydrophilic character | Slope[b] | $R^2$ | CV range % |
| Greiner top[c] | Fluotrac® 200 | Med-bind/medium | 4070 | 0.979 | 9–55 |
| | Fluotrac® 600 | High-bind/high | 3170 | 0.998 | 9–29 |
| Greiner bottom[c] | µClear®, OBP[d] | Cell-culture/very high | No linearity[e] | 7–116 | |
| Nunc top[c] | Standard | Untreated/low | 3582 | 0.999 | 6–23 |
| | **MaxiSorp®** | **High-bind**/high | **3875** | **0.997** | **0–8** |
| | Cell-culture | Cell-culture/very high | 2557 | 0.989 | 5–10 |
| Nunc bottom[c] | Cell-culture, OBP[d] | Cell-culture/very high | 6145 | 0.999 | 2–19 |

[a] All tested plates were black, had 96 wells and a flat bottom.
[b] Fluorescence intensity increase/(µg/ml certoparin).
[c] Top = plates measured by top reading; bottom = plates measured by bottom reading.
[d] OBP = optical bottom plate has a transparent bottom in contrast to the others having a black bottom.
[e] Fluorescence intensity increase of Polymer-H by 0.63 µg/ml certoparin was higher than that by 2.5 µg/ml certoparin.
To identify the most suitable microplate, standard curves of certoparin were compared by means of the slopes of the curves, their coefficients of determination ($R^2$), and the coefficients of variations (CVs), whereby the latter are given as range of the minimum and maximum CV value for each concentration of the curve.

**Table 2**
Structural parameters of the tested glucan sulfates.

| Glucan sulfate | Types of glycosidic bonds | Average $MW_{HD}$ | DS |
|---|---|---|---|
| Phycarin sulfate-1 | β-1,3-glucan | 5600 | 0.00 |
| Phycarin sulfate-2 | | 16,000 | 0.64 |
| Phycarin sulfate-3 | | 15,400 | 0.75 |
| Phycarin sulfate-4 | | 19,000 | 1.48 |
| Phycarin sulfate-5 | | 19,000 | 1.80 |
| Phycarin sulfate-6 | | 19,000 | 1.84 |
| Phycarin sulfate-7 | | 19,000 | 2.21 |
| phycarin sulfate-8 | | 19,000 | 3.09 |
| Curdlan sulfate-1 | β-1,3-glucan | >1,000,000 | 0.16 |
| Curdlan sulfate-2 | | 160,000 | 0.64 |
| Curdlan sulfate-3 | | 90,000 | 1.26 |
| Curdlan sulfate-4 | | 90,000 | 1.57 |
| Curdlan sulfate-5 | | 160,000 | 1.74 |
| Curdlan sulfate-6 | | 90,000 | 1.78 |
| Pullulan sulfate-1 | α-1,4/1,6-glucan | 5800 | 0.25 |
| Pullulan sulfate-2 | | 7000 | 0.60 |
| Pullulan sulfate-3 | | 12,000 | 1.50 |
| Pullulan sulfate-4 | | 12,000 | 2.00 |
| Dextran sulfate-1 | Branched α-1,6-glucan | 500,000 | 1.50 |
| Dextran sulfate-2 | | 9000 | 1.70 |

The β-1,3-glucans phycarin (degree of polymerization 23–25) and curdlan (higher molecular mass) and the α-1,4/1,6-glucan pullulan were sulfated and specified as described in Section 2.3.

### 3.2.2. Influence of the molecular mass

To avoid any disturbing effect by the DS, only glucan sulfates with DS ≥ 1.0 were compared to evaluate the influence of the molecular mass on the FI increasing potency. In addition to linear glucan sulfates, two dextran sulfates, i.e. highly branched α-1,6-glucan sulfates with $MW_{HD}$ of 9000 and 500,000, were included. The findings with both the linear and branched glucan sulfates (Fig. 7) allow the conclusion that the FI increasing potency of glucan sulfates with DS ≥ 1.0 and $MW_{HD}$ ≥ 9000 is independent of the molecular mass.

### 3.2.3. Influence of the glucan structure

Since the two dextran sulfates exhibited similar effects as the semisynthetic linear glucan sulfates, glycosidic branching of the glucan backbone does not modify the FI increasing potency of glucan sulfates on Polymer-H (Fig. 7). Notably, the heparin MMWH and the laminarin sulfate provided nearly identical concentration-dependent fluorescence curves (Fig. 8). These two compounds have comparable molecular masses and DS, but considerably differ in their glycosidic structure. Therefore, the results suggest that the glycosidic structure of sulfated carbohydrates has no significant influence on their potency to enhance the FI of Polymer-H. Finally,

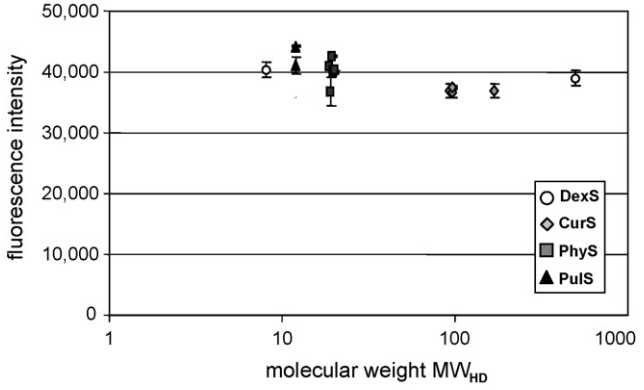


**Fig. 7.** Dependence of the Polymer-H fluorescence intensity on the molecular weight of various sulfated sugars. Semisynthetic glucan sulfates with defined $MW_{HD}$ and a DS > 1.0 were used in concentrations of 5.0 μg/ml. The differences between the $MW_{HD}$ of phycarin sulfates and pullulan sulfates result from the different DS, but the degree of polymerization is the same. No marked differences were observed.

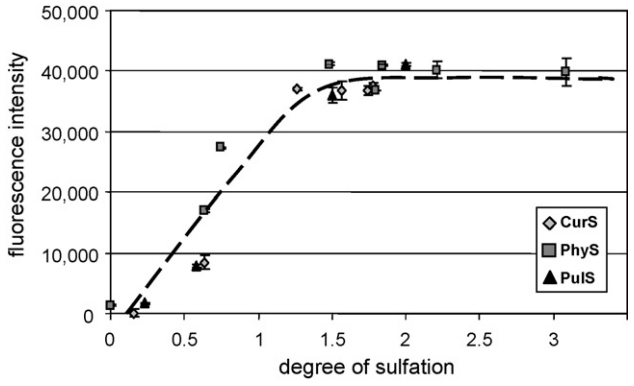


**Fig. 6.** Dependence of the Polymer-H fluorescence intensity on the DS. The line presents the best-fitted curve. Semisynthetic glucan sulfates with defined DS were used in a concentration of 5.0 μg/ml. A saturable increase of the fluorescence signal as a function of the DS was observed for all three types of glucan sulfates.

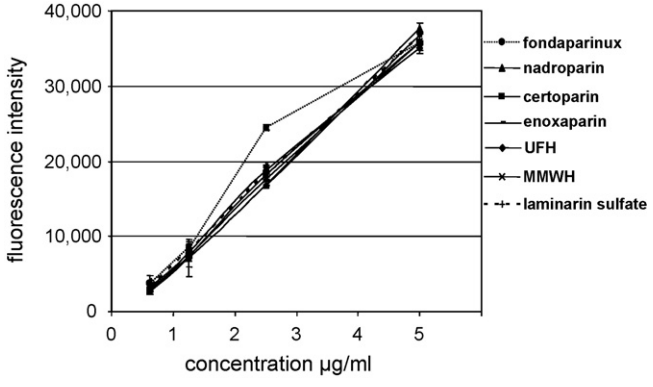


**Fig. 8.** Influence of various heparins, fondaparinux and laminarin sulfate on the Polymer-H fluorescence intensity. Laminarin sulfate is a specifically 2,4-sulfated β-1,3-glucan sulfate with a similar molecular mass and DS as MMWH, but without any affinity to antithrombin.

this example proves that the interaction with Polymer-H is independent of the antithrombin-binding site.

### 3.3. Quantification of heparins

#### 3.3.1. Comparison of different heparins

Heparins may more or less differ in their structural characteristics [2,4,10]. The question was whether any structural differences of heparins influence the FI increase of Polymer-H. To clarify this, various heparins were examined. As shown in Fig. 8, UFH, MMWH and the three LMWHs certoparin, enoxaparin and nadroparin did not differ in their FI increasing effect. The different depolymerization procedures applied to produce LMWHs are associated with distinct structural changes at the ends of the heparin chains. Typical for nadroparin and certoparin, which are obtained by nitrous acid depolymerization, is a 2,5-anhydro-mannitol moiety at the reducing end. In contrast, enoxaparin, which is produced by alkaline depolymerization of benzylated heparin, is characterized by a 4-enopyranose uronate structure at the reducing end. Notably, this unsaturated structure element showed to have no impact on the FI increase of Polymer-H.

All the clinically applied heparins have a DS of 1.0–1.25 [4] implying that no differences had to be expected. But due to the DS dependence (Section 3.2.1), we additionally investigated 6-O-desulfated and afterwards resulfated heparin. The FI values of the resulfated UFH were only slightly lower than those of the original UFH (∼1.3%), whereas the desulfated heparin induced only a FI increase of ∼20% compared with the resulfated heparin (data not shown).

Similar to the semisynthetic glucan sulfates, the $M_r$ of heparin has no impact in this assay. This was confirmed by the FI curves of UFH and the LMWHs as well as of three heparin fragments LMWH-I, LMWH-II and LMWH-III, which only differed in their $M_r$ (6600, 8000 and 9500, respectively) (data not shown).

#### 3.3.2. Comparison of heparin oligosaccharides and fondaparinux

As demonstrated by heparin oligosaccharides a minimum chain length is necessary for complex formation between heparin and Polymer-H. This was proven by two heparin fragments A (decasaccharide mixture) and B (pentasaccharide mixture). The FI curve of fragment A was similar to that of all the other heparins, whereas that of fragment B was flatter with a ∼30% lower FI (data not shown).

However, the synthetic pentasaccharide fondaparinux (Arixtra®), which contains 8 sulfate groups (DS 1.6), strongly contrasted with the heparin fragments. The slope of its concentration-dependent FI curve was even steeper than that of the heparins resulting in a smaller range of linearity at the used Polymer-H concentration (Fig. 8). This suggests that the DS becomes still more important below a critical chain length ($M_r \sim 3000$).

#### 3.3.3. Direct quantification

Compared with functional heparin assays, the most striking difference of this fluorescence assay is that it measures real concentrations of heparins instead of any pharmacological activity. Pharmacological determinations of heparin have several disadvantages: Firstly, only a small portion of the heparin molecules is detected. Secondly, the results are known to strongly depend on the used assay system. Thirdly, the percentage of the detected high-affinity material considerably varies between different heparins as well as between different batches of one heparin [2,4,10]. In fact, 1 aXa-IU of unspecified LMWH corresponds to an amount ranging from 6 to 14 μg and 1 IU of UFH may be 3.3–6.7 μg heparin substance. Especially in research the latter point may lead to false conclusions, when different heparins are compared on the basis of units. So 1 IU of a LMWH may apparently be more active than 1 IU of UFH, but its superiority is just constituted by the higher gravimetric concentration (and still higher molar concentration) [11]. For that reason, a simple direct quantification assay represents a substantial progress. For example, when the heparin content of an ampoule or syringe is only given in IU, the gravimetric concentration can be easily determined. Moreover, the developed assay could simplify certain steps of the quality control of heparin preparations like the determination of the deviation of the heparin content in syringes or ampoules from the nominal content.

Unfortunately, initial experiments on the quantification in plasma revealed poor signal-to-background ratio, so that further investigations for this application are necessary.

#### 3.3.4. Calibration

The heparin data (Fig. 8) confirm and supplement those attained with the semisynthetic glucan sulfates on the structure-dependent FI increase of Polymer-H. Above a $M_r$ of ∼3000 and a DS of 1.0–1.4, the enhancing effect of a sulfated carbohydrate on the FI of Polymer-H is independent of both its $M_r$ and its DS. Consequently, only one calibration curve is needed to quantify all types of heparins. This is an advantage over the usual heparin assays, because they all require own calibration curves for each heparin, whereby the differences between the calibration curves for the various heparins additionally vary depending on the type of coagulation or chromogenic assay [2,10].

Due to its pronounced FI increasing effect, fondaparinux and other highly sulfated oligosaccharides can be quantified by Polymer-H as well, but require an own calibration curve.

### 3.4. Quantification of other glycosaminoglycans and sulfated carbohydrates

The FI increase of Polymer-H by glucan sulfates demonstrates that the assay does not selectively detect heparins, but can be used to quantify all types of sulfated carbohydrates. In case of sulfated polysaccharides with a DS > ∼1.2 and a $M_r$ > 3000, just a single calibration curve is needed. It was of interest to clarify whether the assay allows differentiating between heparins and other glycosaminoglycans. As presented in Fig. 3 the non-sulfated hyaluronic acid induced only a negligible FI increase. Dermatan sulfate, chondroitin sulfate C and heparan sulfate showed roughly 50% of the response of UFH, whereby their ranking corresponded to their slightly different DS. In contrast, the highly sulfated OSCS (DS 2.0), meanwhile famous as contaminant in counterfeit heparin [14], showed some higher FI values compared with UFH (Fig. 3). Accordingly, discrimination between heparins and other glycosaminoglycans is possible due to their different DS.

However, mixtures of heparin with other glycosaminoglycans or OSCS cannot be recognized. The Polymer-H assay in the presented form is therefore not qualified for the purity control of heparin. Meanwhile we succeeded in removing this limitation by introducing a simple enzymatic pre-treatment of the heparin sample [37]. By this modification, the Polymer-H assay offers a promising alternative to the currently mandatory heparin purity tests by nuclear magnetic resonance spectrometry and capillary electrophoresis or strong anion exchange chromatography method [38,39].

Nevertheless, the Polymer-H assay may be applied for sulfated carbohydrates used as drugs or food supplements. Due to its DS dependence, corresponding compounds like heparin, fondaparinux or chondroitin sulfates can additionally be distinguished from each other by means of their specific FI increase. For example, the FI of 2.5 μg/ml heparin, fondaparinux, chondroitin sulfate C amounted to 18,766 ± 665, 24,506 ± 296, 6184 ± 17, respectively.

## 4. Conclusion

In summary, this microplate assay represents a rapid, sensitive, accurate, simple as well as inexpensive test for the direct quantification of both heparins and other sulfated carbohydrates. It is ready to use for research purposes. In quality control, it may be applied as identification assay for sulfated carbohydrates and the quantitative determination of sulfated carbohydrates in finished products.

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