

## Theme Issue Article

# Detection and neutralisation of heparin by a fluorescent ruthenium compound

Helga Szelke<sup>1</sup>; Job Harenberg<sup>2</sup>; Roland Krämer<sup>1</sup>

<sup>1</sup>Anorganisch-Chemisches Institut, Universität Heidelberg, Heidelberg, Germany; <sup>2</sup>Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universität Heidelberg, Medizinische Fakultät Mannheim, Mannheim, Germany

### Summary

Heparin and low-molecular-weight heparin (LMWH) are commonly monitored by determination of activated clotting times or chromogenic assays. Despite their wide use, these assays determine the biological activity and not the concentration of the anticoagulants. They may be inaccurate in some circumstances such as certain disease states. In addition, there is a significant interest in alternative tests for the point-of-care detection of heparin and LMWH. Their binding to small molecules for the detection in biological matrices is poorly explored. We describe

here a new optical molecular probe for the detection of LMWH in serum samples. The polycationic ruthenium compound 1 is applicable to the quantification of heparin by monitoring 630 nm fluorescence. In addition, compound 1 is a rare example of a non-polymeric low molecular weight compound which neutralises the anticoagulant activity of heparin and LMWH in plasma samples. Limitation of the method is its low sensitivity currently being improved by structural modification of compound 1.

### Keywords

Heparin, heparin assay, heparin antagonists, fluorescence, ruthenium

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## Introduction

### Methods for the quantification of heparin

Heparin, a linear polysulphated oligosaccharide, is the most widely used drug to prevent blood clotting. In a clinical setup, it is critical to maintain heparin levels that on the one hand are sufficient to prevent thrombosis but on the other hand avoid risks of bleeding. Considering the fact that more than half a billion heparin doses are used annually, there have been intensive efforts to develop simple and reliable assays and sensor systems that could detect heparin directly in blood or serum samples (1). During surgery or postoperative therapy, heparin is commonly monitored *indirectly* in plasma samples by determination of the activated clotting time or chromogenic factor Xa assay. Despite their wide use, however, these assays are affected by factors other than heparin (2) and are therefore inaccurate in some circumstances such as certain disease states. In addition, there is a significant interest in fast, simple and reliable tests which have a potential for monitoring heparin levels in clinical serum and plasma

samples in point-of-care detection, for example during dialysis. Therefore, more recent research has focused on the direct detection of heparin by its binding to a probe or sensing device (3). Electrochemical methods such as polymer membrane based ion-selective electrodes or ion-selective field effect transistors, avoid the inaccuracy caused by indirect assays, but response is intrinsically dependent on the sample activity of other ions (chloride, bicarbonate), making sensor calibration difficult. Other disadvantages are the irreversibility detection (in case of polymer membrane electrodes) and potential drifts (in case of field effect transistors). Quartz crystal microbalances having protamine adsorbed surfaces have been successfully applied to heparin detection but reach steady state conditions only after relatively long incubation times. Only few optical heparin assays which simply rely on direct interaction of a molecular probe with heparin in a serum or plasma sample have been reported. The fluorescence of a phenylboronic acid molecular receptor (4) decreases on heparin binding. However, fluorescence emission is detected in the UV range (357 nm), less favourable for direct analyses of bio-

### Correspondence to:

Roland Krämer, PhD  
Universität Heidelberg  
Anorganisch-Chemisches Institut  
Im Neuenheimer Feld 270  
69120 Heidelberg, Germany  
Tel: +49 62 21 54 84 38, Fax: +49 62 21 54 85 99  
E-mail: Roland.Kraemer@urz.uni-heidelberg.de

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logical samples due to interference with background fluorescence. The fluorescence signal to background ratio of this probe is relatively poor in serum samples. More recently, benzimidazolium dyes "Heparin Orange" and "Heparin blue" have been described as visible light (emission at 595 and 480 nm) fluorescent probes for heparin detection but in plasma still lack good signal-to-background ratio for the lower range of clinically relevant heparin levels (5). The rationale of the fluorescence based methods is to determine the concentration of heparin and low-molecular-weight heparin (LMWH) in human serum.

### Neutralisation of heparin

Bleeding is the primary complication of anticoagulant therapy. Protamine sulphate, a polycationic polypeptide, reverses the anticoagulant effects of unfractionated heparin (UFH) and (partially) LMWH, and is a widely used specific antidote. However, since protamine may cause severe side effects (6) the finding of save and efficacious heparin antagonists is currently a goal of great clinical importance. With this aim, synthetic medium sized peptides (7), polypeptides (8), as well as low molecular weight protamine (9) and, more recently, foldamers (10) have been reported. Moreover, proteins such as lactoferrin (11), histones (12) and antibodies (13) have been studied as heparin neutralising agents, but up to now, protamine, in spite of its well known side effects, remains clinically the most extensively employed heparin antagonist. In addition, there is a need for antidotes for the newer coagulants such as danaparoid, fondaparinux and idraparinux (14). Specific reversal agents would be very valuable. The potential of non-polymeric, small organic molecules as heparin inhibitors is poorly explored. To our knowledge, only a polycationic calixarene derivative was reported to effectively neutralise UFH and LMWH (15).

### Design of fluorescent ruthenium complexes

There is a single literature report on the use of a ruthenium complex  $[\text{Ru}(2,2'\text{-bipyridine})_3]^{2+}$  for the detection of heparin in gels by fluorescent staining (after electrophoresis, isolation, incubation and washing) (16). In buffered solution or serum containing samples, this complex does not respond to heparin.

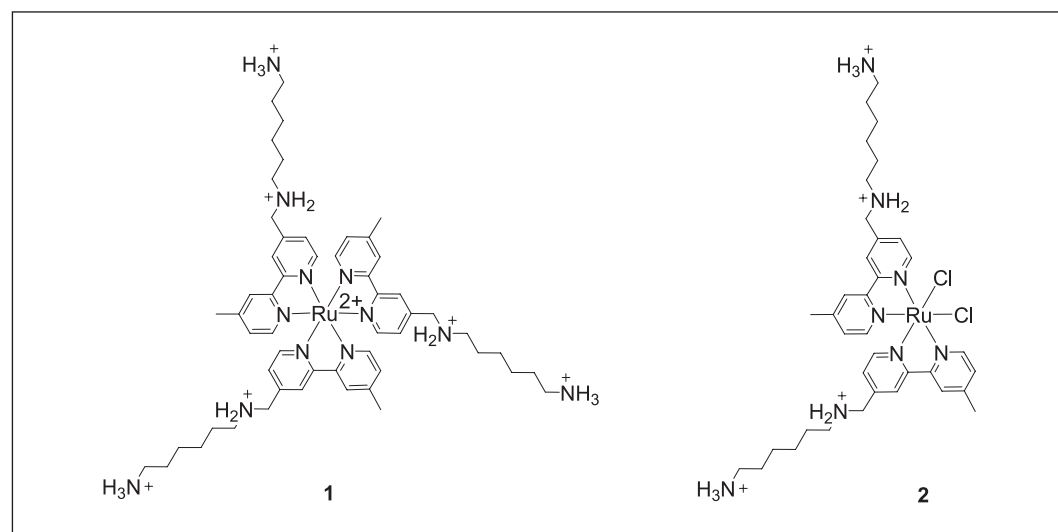
Attachment of cationic residues to the bipyridine ligands is essential to make this type of complex a suitable probe for heparin detection in solution. Presumably, the substituents increase the affinity to polyanionic heparin for electrostatic reasons. An advantage of the ruthenium complexes is the versatile structural design and the ease of multiple functionalisation. For example, three diamine residues are readily introduced by ruthenium-centered assembly of three diamine-modified bipyridines.

In addition, fluorescence emission of tris(2,2'-bipyridyl) ruthenium(II) complexes in the red part of the visible spectrum increases signal to background ratio in serum and plasma samples since autofluorescence in this medium is low at  $> 600$  nm.

## Materials and methods

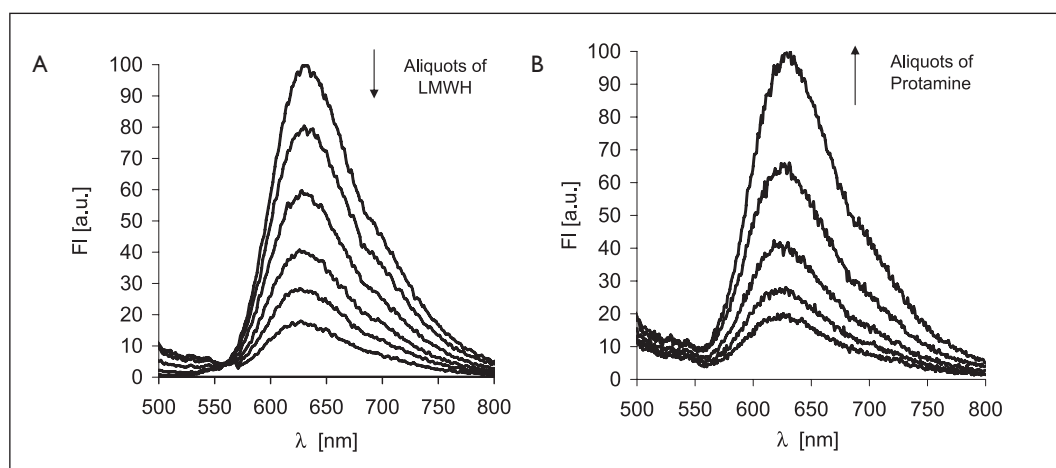
### Reagents, plasma and serum samples

Details of the synthesis, purification and characterisation of compounds 1 and 2 will be described elsewhere. Spiking experiments were performed using a pool of plasma and serum derived from subjects with normal coagulation profiles. Venous blood samples were obtained by clean vein puncture into serum separator tubes for serum collection or plastic vials containing 3.8% sodium citrate (1/9, v/v, citrate/blood) for plasma collection. Blood samples for serum preparation were allowed to clot for 30 minutes (min), then centrifuged at  $1,800 \times g$  for 15 min. Samples for plasma preparation were centrifuged immediately at  $1800 \times g$  for 10 min. Both sample types were shock frozen and stored at  $-25^\circ\text{C}$ . Stock solutions of LMWH (dalteparin sodium, Pfizer, Berlin, Germany), UFH (heparin sodium, Hoffmann-La Roche, Grenzach-Wyhlen, Germany), fondaparinux sodium (Sanofi-Synthelabo, Berlin, Germany), dermatan sulfate (sodium salt from porcine intestinal mucosa,  $>90\%$ , Sigma-Aldrich, Taufkirchen, Germany), chondroitin sulfate A (sodium salt from bovine trachea,  $\sim 70\%$ , Sigma-Aldrich), hyaluronic acid (potassium salt from human umbilical cord, Sigma-Aldrich) and protamine sulfate (from salmon, Grade X, Sigma-Aldrich) were freshly prepared.  $\text{SiO}_2$  microbeads ( $4 \mu\text{m}$ ) coated with an epoxy-functionalised polymer were obtained from G. Kisker GbR (Stein-



**Scheme 1: Structures of ruthenium compounds 1 and 2.** (Cl<sup>-</sup> counterions not shown)

**Figure 1: Response of 1 to LMWH (A) and reversibility of binding upon titration with protamine (B).** A) 10  $\mu$ M 1, 10  $\mu$ M 2 in aqueous buffer (10 mM MOPS, pH 7.0, 50 mM NaCl) doped with 0–2 IU/ml LMWH. B) A preformed 1-LMWH complex (10  $\mu$ M 1, 10  $\mu$ M 2, 2 IU/ml LMWH) was titrated with protamine (ca. 0–1.3 nM) in aqueous buffer (10 mM MOPS, pH 7.0, 50 mM NaCl).



furt, Germany). Beads were incubated at room temperature with an excess of 1+2 (1:1) mixture in Tris buffer (0.1 M, pH 9.0) for 1 h and washed with water.

### Fluorescence measurements

Screening and optimisation of the assay for LMWH in serum samples was performed on a Fluoroskan Ascent FL (ThermoFischer Scientific, Langensfeld, Germany) fluorescence microplate reader ( $\lambda_{\text{ex}}$  460 nm,  $\lambda_{\text{em}}$  635 nm). Calibrations and results shown in Figures 1–3 were measured in disposable 3 ml plastic cuvettes using a highly sensitive Cary Eclipse fluorimeter (Varian, Darmstadt, Germany). Detector sensitivity was adjusted to the fluorescence intensity of the sample. Units are arbitrary.

### Heptest coagulation assay

Clotting time was measured on a KC 10a micro device (Amelung Co., Lemgo, Germany). Reagents bovine FXa and Recalmix were purchased from Kappes Laborservice, Augsburg, Germany (originally from Haemachem, St. Louis, MO, USA). Forty microliters of plasma, 5  $\mu$ l of LMWH (10 IU/ml), 5  $\mu$ l of compound 1 and 50  $\mu$ l of FXa were incubated at 37°C for 2 min (micromethod). Clotting was started by the addition of 50  $\mu$ l of Recalmix (17).

### Chromogenic factor Xa assay

Factor Xa activity was determined using the S2222 chromogenic assay (Chromogenix, Milano, Italy) in combination with a Dynatech MR 7000 Spectrophotometer. Assays were calibrated following addition of 2<sup>nd</sup> WHO International Standard for LMWH to a final concentration of 1.0 IU/ml anti Xa activity (17).

## Results and discussion

### Ruthenium compounds 1 and 2

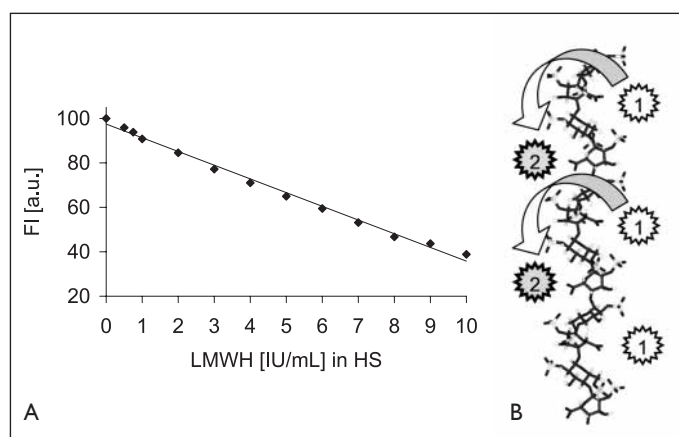
The fluorescent orange-red ruthenium complex 1 and non-fluorescent purple complex 2 are polycations with a maximum positive charge of 8 for 1 and 4 for 2 in the fully protonated state. The terminal amino groups are expected to be strongly basic and thus protonated at neutral pH. The secondary amino groups are less basic due to the electron withdrawing effect of the bipyridyl unit.

Taking into account electrostatic repulsion of multiple ammonium groups, only partial protonation of the secondary amino groups is assumed at pH 7.

### Direct detection of LMWH by fluorescence assay

We focused on detection of LMWH, meanwhile the most widely used heparin subtype. LMWHs have, depending on the manufacturing method, a mean molecular weight between 4,000 and 6,000 (heparin cannot be expressed exactly using a conventional chemical formula). 1 exhibits the typical red fluorescence ( $\lambda_{\text{max}}$  630 nm) of tris(2,2'-bipyridyl) ruthenium(II) complexes on irradiation at the absorbance maximum at 460 nm. However, purified samples of 1 exhibit only minor changes of fluorescence on addition of heparin. Interestingly, a 1:1 mixture of 1 and 2 is a much better indicator of heparin, with a strong decrease of fluorescence at increasing heparin concentration (Fig. 1a). We suggest that the quenching process is triggered by co-assembly of the complexes on the heparin template, (Fig. 2b) resulting in sufficient quenching of the fluorescence of 1 by 2. Energy transfer from electronically excited 1 (absorbance maximum at 460 nm) to nonfluorescent 2 (absorbance maximum at 500 nm) is a plausible mechanism of fluorescence quenching, although electron transfer processes have also been reported for pairs of related complexes (18). Target binding is anticipated by ion pairing of polycations 1 and 2 to polyanionic heparin which has the highest charge to mass ratio of any biopolymer. A hypothetical assembly of 1 and 2 with a heparin sequence of regular sulfation degree is shown in Figure 2b. Fluorescence response of the probe does not allow a discrimination of different sulfation patterns which are unevenly distributed along the heparin chain (19).

Decrease of fluorescence intensity of 1 at 630 nm with increasing heparin concentration was first monitored in buffered aqueous solution (Fig. 1a). Response of the probe to heparin is instantaneous and binding is reversible, as demonstrated by rapid fluorescence recovery on addition of protamine, a polycationic polypeptide and high-affinity heparin binder (Fig. 1b). Next, the fluorescence emission was used to generate calibration curves for LMWH in serum. A buffered solution of 1+2 containing 10% human blood serum was titrated with LMWH (Fig. 2a). For LMWH serum concentrations in the range 0–10 IU/ml, depen-



**Figure 2: Detection of LMWH by fluorescence assay.** A) Calibration curve for fluorescence detection of LMWH by 1+2 mixture in the presence of human serum. Human serum is diluted 10-fold with reagent solution (5  $\mu$ M 1, 5  $\mu$ M 2, 10 mM MOPS, pH 7.0, 50 mM NaCl) and LMWH added (0–10 IU/ml). Fluorescence emission at 630 nm ( $FI$ ,  $\lambda_{ex}$  460 nm) is recorded immediately after mixing. B) Proposed co-assembly of orange-red compound 1 (fluorophore) and purple compound 2 (fluorescence quencher) to heparin, leading to effective quenching of the fluorescence of 1 by energy transfer (symbolised by arrow) or electron transfer. The structure of the heparin strand derived from NMR and molecular-modelling studies and is a representative of a “regular” sequence (20). (Heparin chains also include under- and oversulfated sequences.)

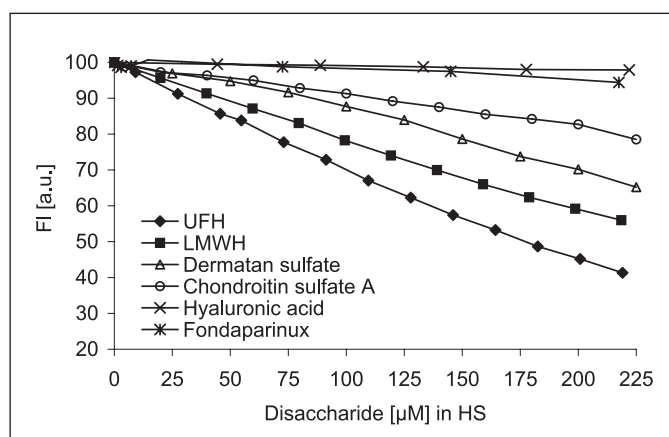
dence of fluorescence intensity on LMWH concentration is nearly linear. When samples of human serum were preincubated with LMWH at four representative concentrations (1, 3, 5 and 7 IU/ml) for 15 min and then diluted 10-fold with aqueous buffer containing 1, emission intensities compare well (within 2%) with the established calibration curve. It should be noted that all experiments were performed with the same batch of Dalteparin sodium (Pfizer); somewhat different analytical responses might be obtained with LMWH samples recommended as reference standards.

In plasma samples, fluorescence response of the probe is somewhat worse than in serum (data not shown). This might be a consequence of competitive binding of LMWH by plasma proteins such as fibrinogen (21).

Lower LMWH concentrations (0.2–2 IU/ml) in serum samples can be monitored using lower probe concentration (ca. 2  $\mu$ M). However, fluorescence is no longer linearly dependent on LMWH concentration, indicating that binding of the probe to heparin at low concentration is not quantitative. This problem might be overcome by structural optimisation of the probe, to achieve strong binding to heparin even at low concentrations.

### Selectivity of fluorescence assay

Response of the probe was also studied with other glycosaminoglycans and related compounds (chondroitin sulfate A, dermatan sulfate and hyaluronic acid) which have a lower sulfation degree than LMWH. Figure 3 shows that the probes are selective but not specific for LMWH. Selectivity decreases with degree of sulfation and may depend on both charge density and/or molecular weight (length) of the glycosaminoglycan. Fluorescence re-



**Figure 3: Selectivity of the fluorescence assay.** Human serum diluted 10-fold with reagent solution (10  $\mu$ M 1, 10  $\mu$ M 2, 10 mM MOPS, pH 7.0, 50 mM NaCl) and the corresponding polysaccharide added. Fluorescence emission at 630 nm ( $FI$ ,  $\lambda_{ex}$  460 nm) is recorded immediately after mixing.

sponse of the probe to unfractionated heparin (UFH) is slightly better than for LMWH. Interestingly, only negligible response of a 1+2 mixture is observed toward fondaparinux, a synthetic pentasaccharide corresponding to the highly sulfated antithrombin binding sequence of heparin. This is interpreted by small target size (chain length) which does not allow effective co-assembly of 1 and 2 (compare Fig. 2b).

### Neutralisation of heparin by 1

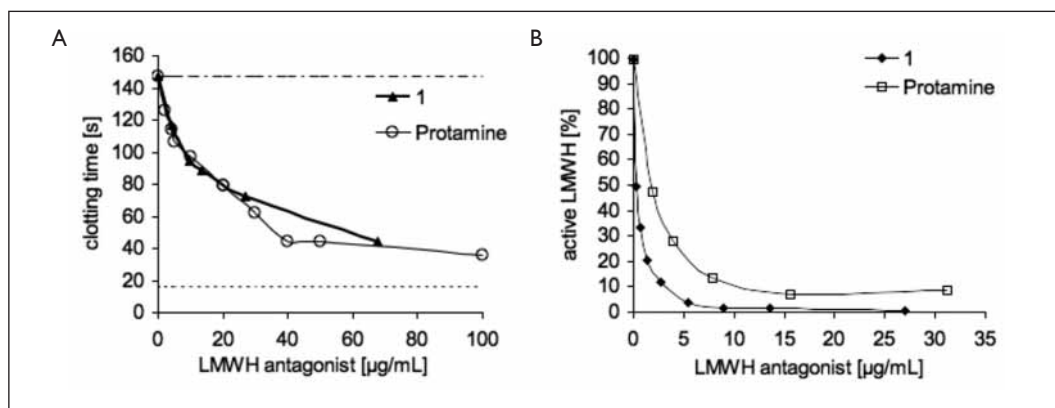
Neutralisation of anticoagulant activity of heparin by 1 was investigated using established heparin quantification methods. The clotting time (Heptest assay) of human plasma containing 1 IU/ml LMWH decreased with increasing concentrations of 1 (Fig. 4a). Heptest generally measures lower activity than other functional tests for heparin. The effect of 1 was found to be comparable with that of protamine. In contrast, compound 2 does not decrease the clotting time significantly. This observation confirms the importance of high positive charge density of the probe for effective heparin binding (maximum positive charge is 8 for 1 and 4 for 2).

The binding of reagent 1 to LMWH was also confirmed by a Chromogenic FXa assay. 1 inhibits the anti-Xa activity of heparin almost as effectively as protamine. Blocking 50% activity of 0.25 IU/ml LMWH requires a concentration of 0.4  $\mu$ g/ml of 1 vs. 1.9  $\mu$ g/ml of protamine (Fig. 4b).

### Immobilisation of heparin sensitive fluorescent probe

Fiber optic sensors (optodes) based on fluorescence quenching of polypyridyl Ru-complexes which are immobilised in a polymer or sol-gel matrix have been developed for the detection of dioxygen in gaseous or liquid media, including online monitoring of  $O_2$  in blood. Sensor devices of this type are commercially available, low-cost, portable and have low power requirements when blue LED is used for excitation. Thus, development of a fiber optic Ru-complex sensor for heparin monitoring in blood might profit from established sensor technology.

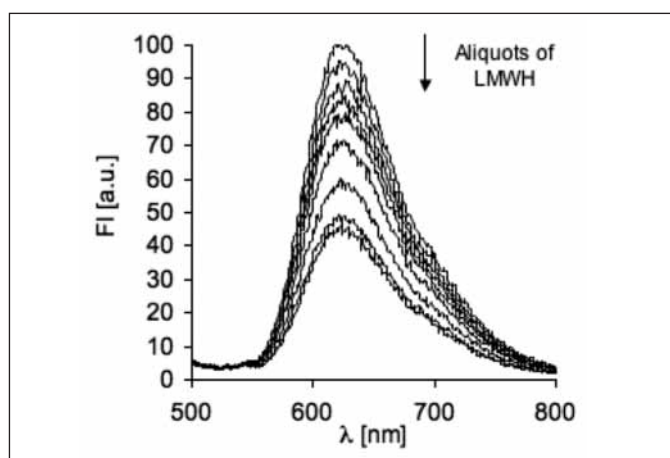
**Figure 4: Neutralisation of LMWH activity by 1 or protamine.** A) Measured by the Heptest clotting assay in the presence of 1 IU/ml LMWH in plasma. (40  $\mu$ l plasma, 5  $\mu$ l 1, 5  $\mu$ l LMWH (10 IU/ml). 1  $\mu$ M 1 corresponds to 1.3  $\mu$ g/ml. B) Measured with the chromogenic FXa S-2222 assay in diluted plasma by monitoring 405 nm absorbance. In the presence of 0.25 IU/ml LMWH, comparison of 1 with protamine.



Application of 1 in a sensing device for optical online monitoring of heparin in blood would require immobilisation of the probe. In a preliminary investigation, we have immobilised a 1+2 (1:1) mixture by reaction with epoxide-functionalised  $\text{SiO}_2$  microbeads (4  $\mu$ m, Kisker GbR). At pH 9.0, the compounds readily attached to the beads, presumably by reaction of the amino groups with the epoxy function of the beads, with formation of a novel N-C-bond and a 1-amino-2-hydroxymoiety. The strong 630 nm emission of 1 is detected in a suspension of the beads in buffer by a standard fluorimeter. On addition of heparin, fluorescence decreases significantly (Fig. 5), but in contrast to the experiment in homogeneous buffered aqueous solution, response is slow and requires about 10 min for equilibration. When protamine is added to a suspension of the heparin-incubated microbeads, original fluorescence is readily restored. These observations indicate that fluorescence of immobilised 1 is strongly heparin-dependent. However, the slow on-rate requires an optimised or alternative immobilisation strategy.

## Conclusion

The potential of non-polymeric small molecules for the detection and neutralisation of heparin is poorly explored. We describe here a novel polycationic ruthenium compound 1 and its application as a fluorescent probe for the direct, simple and rapid detection of low molecular weight heparin in serum samples. 1 is red fluorescent at  $\lambda_{\text{max}}$  630 nm where autofluorescence of serum is low. While 1 alone displays only minor changes of fluorescence in the presence of heparin, a mixture of 1 and 2, a related bis(bipyridyl)dichloro ruthenium complex obtained as a by-product during synthesis of 1, responds to heparin by a nearly complete loss of fluorescence. This is interpreted by co-assembly of fluorescent compound 1 and fluorescence quencher 2 on the heparin template. In spiked serum samples, probe fluorescence is linearly dependent on heparin concentration for therapeutic heparin levels 2–8 IU/ml. Limitation of the method is its sensitivity towards other glycosaminoglycans currently being improved by structural modification of compound 1. Immobilisation of the probe would be essential for the development of ruthenium complex based fiber optic sensing device for heparin detection in biological samples. In a preliminary experiment, the probe was covalently immobilised on  $\text{SiO}_2$  microbeads. A strong



**Figure 5: Fluorescence of 1+2-functionalised  $\text{SiO}_2$  microbeads in dependence of LMWH concentration.** Emission spectra of 0.23 mg/ml beads in buffer (10 mM MOPS, pH 7.0, 50 mM NaCl). Equilibration time of 10 min required after LMWH addition.

decrease of microbead fluorescence is observed on addition of heparin, although with relatively long response time. Binding of 1 to heparin is confirmed by effective neutralisation of heparin activity in Heptest assay and chromogenic factor Xa assay. Effectiveness of 1 is comparable to that of protamine, a widely used heparin antagonist. In contrast, compound 2 which has a lower positive charge density than 1, is inefficient for heparin neutralisation.

The weaknesses of the methods are currently the low sensitivity and specificity to distinguish between UFH and LMWH, and fractions with low and high affinity for antithrombin, between catalytically active and inert heparin species, and the two varieties of heparin molecules in heparin and low molecular weight heparin preparations. At present there seems to be a relationship between probe response and both length and charge density of the glycosaminoglycans independent of their anticoagulant activity. The method is at present a measurement of total heparin and other biopolymers of high negative charge density.

Since protamine occasionally causes severe side effects and is ineffective toward newer synthetic heparinoids, a better under-

standing of the structural requirements for effective heparin binding of small molecules is of interest to the design of alternative antagonists for diagnostic purposes.

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