A fluorescent probe for the quantification of heparin in clinical samples with minimal matrix interference†

Helga Szelke,[a] Sarah Schübel,[a] Job Harenberg[b] and Roland Krämer*[a]

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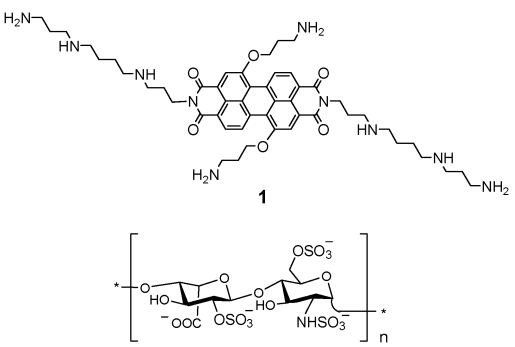
A red-fluorescent perylene diimide probe allows sensitive quantification of heparin, a widely used antithrombotic drug, in plasma and serum samples with minimal interference from matrix components.

Heparins are major clinical anticoagulants, with about half a billion doses applied annually.[1] Heparin is a linear, polydisperse polysaccharide that consists predominantly (>70%) of a trisulfated disaccharide repeating unit (Scheme 1) and has a high negative charge density. Unfractionated heparin (UFH, mean molecular weight between 13 000 and 15 000), isolated from natural sources, has been clinically applied since the 1930s. By many indications, there has been a trend[2] towards the use of fractionated low molecular weight heparins (LMWH, mean molecular weight between 3000 and 5000), manufactured by partial depolymerisation of UFH. LMWHs have lower side effects and a more favourable pharmacokinetics. It is often critical to maintain heparin levels that are sufficient to prevent thrombosis but on the other hand avoid risks of bleeding. Monitoring of heparins is recommended[2] for a variety of patient subgroups, in anticoagulation reversal during acute bleeding and routinely for therapeutic UFH application. Despite their recognized limitations,[2b,3] measurement of activated partial thrombine time APTT (the ability to delay clotting) and *anti*-Xa assay (inhibition of a specific blood coagulation factor), remain the currently accepted practice for laboratory monitoring of UFH and LMWH, respectively, in patients' blood. There are no established methods for the reliable and convenient point-of-care monitoring of LMWH.[4] These limitations have stimulated interest in alternative detection schemes,[5] with a particular emphasis on methods that would facilitate the detection of heparin by simple means in clinical samples.

Detection of heparin by molecular indicators goes back to toluidine blue staining, and interest in simple, colorimetric or fluorimetric assays has been greatly renewed in recent years.[6] Very few small-molecule probes, however, are capable of the sensitive fluorescence detection of heparin in clinically relevant plasma (cell-free blood) or serum (plasma without clotting factors) samples. Such probes were pioneered by Anslyn[7] and also described by Chang[8] and Zhang and Zhu.[9] Heparin binding in solution is accompanied by a decrease of UV[7] or increase of visible fluorescence.[8,9] Problems related to matrix interference have not yet been fully overcome by the currently available probes. Compared with buffered aqueous solutions, higher concentrations of heparin are required to trigger a comparable fluorescence response in plasma or serum containing samples.[7,8] In two of the above-mentioned assays, the volume of serum added to the detection solutions had to be kept rather low (2 vol%[7] or 0.2 vol%[9]), so that heparin levels above the clinically relevant range might be required in the original serum sample. Probe–heparin interaction can be affected by competitive binding of either heparin or the probe (or both). Matrix interference is generally of great concern in serum and plasma diagnostics and drug monitoring[10] since it may lead to inaccurate and poorly reproducible data. Calibration considers general matrix effects, but specific interferences involve the risk that the response of the probe depends on medium composition, considering that about 30 more abundant (*i.e.* $c \geq 1$ μM) plasma proteins vary 2–30 fold in their individual concentration from person to person, or depending on the physiological state.[11] For laboratory and point-of-care monitoring of heparin and for pharmacokinetic studies,[12] a probe that is less sensitive to medium interference would be more desirable.

Encouraged by a study on polyammonium-modified calixarenes which bind heparin very strongly,[13] we have recently linked multiple ammonium groups to a fluorescent ruthenium(II)-*tris*(2,2′-bipyridine) complex in order to strengthen electrostatic probe–target interaction.[14] However, the problem of altered emission response to heparin in serum samples persisted. A number of studies, including those in our own laboratory, on the targeting of polyanionic DNA by cationic



Scheme 1 Structure of **1** (above, major 1,7-isomer‡) and of the major repeating disaccharide unit of heparin (below).

[a] *Universität Heidelberg, Anorganisch-Chemsiches Institut, Im Neuenheimer Feld 270, 69120 Heidelberg, Germany. E-mail: Roland.Kraemer@urz.uni-heidelberg.de; Fax: +49 62 21-54 85 99; Tel: +49 62 21-54 84 38*
[b] *Universität Heidelberg, Medizinische Fakultät Mannheim, Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Maybachstr. 14, 68169 Mannheim, Germany. E-mail: job.harenberg@medma.uni-heidelberg.de; Fax: +49 621 383-9622; Tel: +49 62 383-9621*

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perylene diimide or naphthalene diimide derivatives,[15] often with modulation of the chromophores optical properties, provided a promising starting point for the design of alternative probes for heparin sensing.

Here we describe the novel probe **1**, a modified perylene-3,4:9,10-tetracarboxylic acid diimide which minimises medium-dependent interferences in heparin quantification and is at the same time sensitive enough to cover the clinically relevant concentration range.

1 was prepared in five reaction steps from commercially available compounds and isolated in the form of its ammonium salt $(H_8\mathbf{1})Cl_8$ as a water soluble violet powder.† Protonation of the amino groups at neutral pH is expected since the pK_a values of the protonated parent amines are 8.0–10.9 for spermine and 10.2 for 3-aminopropanol at I = 0.1 M.[16] As suggested for other water soluble perylene diimides,[17] strong fluorescence of **1** (broad emission at λ_{max} = 615 nm; Φ = 0.23 on excitation at 485 nm in 1 mM HCl, relative to rhodamine 6G in ethanol) is in accordance with a reduced tendency, due to good solubilisation and electrostatic repulsion, to form nonfluorescent aggregates, held together by hydrophobic interactions between the perylene cores. The O-substituents at the perylene skeleton shift the fluorescence to longer wavelength compared to the unsubstituted analog (λ_{max} = 548 nm). A red fluorescence signal improves the signal-to-background ratio in serum and plasma samples, which on excitation at 485 nm have a significant autofluorescence in the range 490–560 nm. At longer wavelength, autofluorescence is reduced (to 25%, 11% and 3% at 615, 650 and 700 nm, respectively).

To study the response of **1** to heparin we focused on more widely applied LMWH, using a commercial LMWH sodium salt with average molecular weight 5000 (similar results were obtained with UFH but are not included in this communication). Fluorescence was recorded with a portable fluorimeter, equipped with a 485 nm excitation filter and a 665 nm cut off emission filter. The latter filter offers a good signal-to-background ratio since serum/plasma autofluorescence is very low at >665 nm. Fluorescence of **1** in buffered solution decreases approximately linearly (Fig. 1) with increasing heparin concentration (given as the concentration of the disaccharide in Scheme 1) with a calculated "loading" of one dye molecule/4.6 sugar moieties. We interpret fluorescence quenching as a consequence of the aggregation of **1** at the polyanionic heparin template. Metal-coordination induced aggregation was recently described as a signalling mechanism of a Hg^{2+} selective perylene diimide probe.[18] Response of **1** to LMWH in samples containing 20 vol% serum or plasma correlates well with the data of the control solution. Tosylate anion is added to counteract a more significant decrease of fluorescence in plasma/serum; presumably, the amphiphilic anion associates with **1** and disrupts weak interactions with plasma/serum components leading to fluorescence quenching. Note that, although heparin-free solutions have a somewhat lower fluorescence in the plasma/serum medium, response of the probe to heparin is comparable in buffer, serum and plasma. Equilibration is fast (within seconds) in all media. Binding is reversible since fluorescence is restored on addition of protamine, a strongly heparin-binding polypeptide.

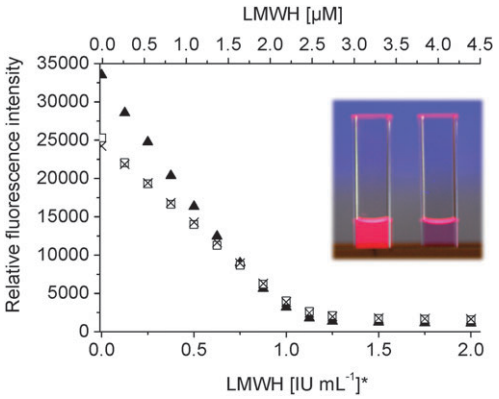

Fig. 1 Fluorescence intensity (arbitrary values) of solutions containing **1** (1 μM) and varying LMWH levels in different media: ▲ Control (buffered H_2O), □ 20 vol% human blood serum, × 20 vol% human blood plasma. T = 20 °C, pH 7.0, buffer 60 mM 3-(*N*-morpholino) propanesulfonic acid, $I \approx$ 0.2 M (200 mM sodium tosylate). Excitation at 485 nm, emission >665 nm. All data points are averages of three measurements. The top LMWH scale indicates actual LMWH concentrations (using a molar mass 665.5 of the disaccharide tetrasodium salt in Scheme 1) in assay solutions. *The bottom scale relates to the LMWH activity IU/mL which a corresponding clinical plasma or serum (or a control) sample would have prior to addition to the assay solution. Actual LMWH activity levels in the assay solutions are five times lower due to dilution. Inset: red fluorescent (left cuvette) and heparin-quenched (right cuvette) solutions of **1**.

Apparently, **1** associates with heparin in a highly selective manner, with minimal interference by serum or plasma components.

In a clinical context, LMWH is quantified by its *anti*-Xa activity in IU mL^{-1} of a patients plasma sample. For the LMWH sample used in this study, we determined the *anti*-Xa activity 140 IU mg^{-1}. The bottom scale of Fig. 1 relates to the heparin activity level in the plasma or serum sample that would be added to the assay solution in a routine diagnostic setting. At 1 μM **1**, the probe covers both the generally recommended[19] therapeutic (0.5–1.0 IU mL^{-1}) and prophylactic (0.2–0.4 IU mL^{-1}) range of LMWH in plasma. If necessary, concentration of **1** can be adjusted for a more precise monitoring at both higher or lower levels. For example, when the concentration of **1** is reduced to 0.5 μM, a 50% and 20% fluorescence decrease is triggered by 0.25 IU mL^{-1} and 0.1 IU mL^{-1}, respectively, at a signal-to-background ratio >4 (data not shown).

In a preliminary evaluation of inter-individual variations, we analysed the response of **1** to heparin-spiked serum samples of five randomly selected healthy people. For clarity, data in Fig. 2 are limited to the fluorescence intensity readout for selected heparin levels. The coefficient of variation CV = (standard deviation/mean × 100) for the five patients is 8% at 0.25 and 0.5 IU mL^{-1}. Note that in a validation protocol of *anti*-Xa assay,[19] the established clinical method for LMWH quantification, the CV even for within-run precision (*same* sample measured repeatedly) is, depending on instrumentation, in the range 5–25% for comparable heparin levels.

In conclusion, we designed a fluorescent perylene diimide probe **1** for the detection of heparin with high sensitivity and

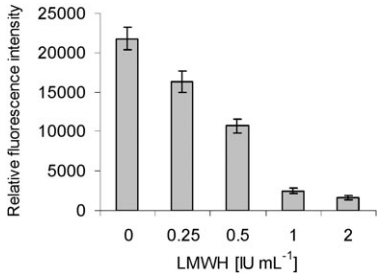

Fig. 2 Fluorescence intensity (arbitrary values) of aqueous solutions containing **1** (1 μM) after addition of 20 vol% LMWH-spiked (0, 0.25, 0.5, 1 and 2 U/mL) human blood serum of five different individuals. Error bars indicate the inter-individual standard deviation. pH 7.0, 60 mM 3-(*N*-morpholino) propanesulfonic acid; $T = 20$ °C, $I \approx 0.2$ M (200 mM sodium tosylate). Excitation at 485 nm, emission >665 nm.

selectivity in clinical samples. While the alkoxy-substituents at the perylene core shift fluorescence to wavelengths >600 nm to provide a high signal-to-background ratio, the multiple ammonium groups attached to the N-imide sites favour selective association with the polyanionic heparin target. **1** is a rare example of a small-molecule fluorescent probe that quantifies a molecular target with minimal interference from highly competitive serum and plasma components. Ease of application combined with matrix-independent response make this heparin probe attractive for both routine laboratory and fast point-of-care testing as well as high-throughput pharmacokinetic studies, ideally without the need for patient-specific calibration.

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Notes and references

‡ **1** is isolated as a 2 : 1 mixture of two isomers with respect to the position (1,7 or 1,6) of the –OR substituents. Only the major 1,7-isomer is shown in Fig. 1. Small quantities of the isomers could be isolated by HPLC and were shown to have both very similar fluorescence emission profiles and response to LMWH in serum samples.

1 N. Mackman, *Nature*, 2008, **451**, 914; S. J. Williams and G. J. Davies, *Trends Biotechnol.*, 2001, **19**, 356.
2 (*a*) J. Harenberg, *Semin. Thromb. Hemostasis*, 2008, **34**, 779; (*b*) E. Gray, B. Mulloy and T. W. Barrowcliffe, *Thromb. Haemost.*, 2008, **99**, 807.
3 T. Baglin, T. W. Barrowcliffe, A. Cohen and M. Greaves, *Br. J. Haematol.*, 2006, **133**, 19.
4 S. A. Spinler, A. K. Wittkowsky, E. A. Nutescu and M. A. Smythe, *Ann. Pharmacother.*, 2005, **39**, 1275.
5 R. Jelinek and S. Kolusheva, *Chem. Rev.*, 2004, **104**, 5987.
6 (*a*) Zh. Zhong and E. V. Anslyn, *J. Am. Chem. Soc.*, 2002, **124**, 9014; (*b*) W. Sun, H. Bandmann and T. Schrader, *Chem.–Eur. J.*, 2007, **13**, 7701; (*c*) J. C. Sauceda, R. M. Duke and M. Nitz, *ChemBioChem*, 2007, **8**, 391; (*d*) K.-Y. Pu and B. Liu, *Macromolecules*, 2008, **41**, 6636; (*e*) T. Briza, Z. KejikI. Cisarova, J. Kralova, P. Martasek and V. Kral, *Chem. Commun.*, 2008, 1901; (*f*) R. B. C. Jagt, R. F. Gomez-Biagi and M. Nitz, *Angew. Chem., Int. Ed.*, 2009, **48**, 1995.
7 A. T. Wright, Z. L. Zhong and E. V. Anslyn, *Angew. Chem., Int. Ed.*, 2005, **44**, 5679.
8 S. Wang and Y.-T. Chang, *Chem. Commun.*, 2008, 1173.
9 M. Wang, D. Zhang, G. Zhang and D. Zhu, *Chem. Commun.*, 2008, 4469.
10 (*a*) B. Maibohn, *Pharmacokinetics and Pharmacodynamics of Biotech Drugs*, Wiley-VCH, 2006, p. 159; (*b*) S. Rochat, Z. Grote and K. Severin, *Org. Biomol. Chem.*, 2009, **7**, 1147.
11 N. L. Anderson and N. G. Anderson, *Mol. Cell. Proteomics*, 2002, **1**, 845.
12 F. Zhang, P. Sun, E. Munoz, L. Chi, S. Sakai, T. Toida, H. Zhang, S. Mousa and R. J. Linhardt, *Anal. Biochem.*, 2006, **353**, 284.
13 T. Mecca, G. M. L. Consoli, C. Geraci, R. La Spina and F. Cunsolo, *Org. Biomol. Chem.*, 2006, **4**, 3763.
14 H. Szelke, J. Harenberg and R. Krämer, *Thromb. Haemost.*, 2009, **102**, 859.
15 Selected examples: (*a*) O. Y. Fedoroff, M. Salazar, H. Han, V. V. Chemeris, S. M. Kerwin and L. H. Hurley, *Biochemistry*, 1998, **37**, 12367; (*b*) J. T. Kern, P. W. Thomas and S. M. Kerwin, *Biochemistry*, 2002, **41**, 11379; (*c*) L. Rossetti, M. Franceschin, A. Bianco, G. Ortaggi and M. Savino, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 2527; (*d*) A. Mokhir and R. Krämer, *Bioconjugate Chem.*, 2003, **14**, 877; (*e*) A. Mokhir, H. Wolf and R. Krämer, *J. Am. Chem. Soc.*, 2004, **126**, 6208; (*f*) S. Krauß, M. Lysetska and F. Würthner, *Lett. Org. Chem.*, 2005, **2**, 349.
16 (*a*) D. Templeton and B. Sarkar, *Can. J. Chem.*, 1985, **63**, 3122; (*b*) J. Bunting and D. Stefanidis, *J. Am. Chem. Soc.*, 1990, **112**, 779.
17 Ch. Kohl, T. Weil, J. Qu and K. Muellen, *Chem.–Eur. J.*, 2004, **10**, 5297.
18 Y. Che, X. Yang and L. Zang, *Chem. Commun.*, 2008, 1413.
19 L. Houbouyan, B. Boutiere, G. Contant, M. D. Dautzenberg, P. Fievet, G. Potrn, A. Vassault and Y. Gourmelin, *Clin. Chem.*, 1996, **42**, 1223.