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Interaction of heparin with cationic molecular probes: Probe charge is a major determinant of binding stoichiometry and affinity

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ABSTRACT

Fluorescent perylenediimide probes modified with 2, 4, 6, or 8 ammonium groups were synthesized and their binding to the antithrombotic drug heparin was studied by fluorescence spectroscopy in solution. The polyanionic polysaccharide strands of heparin bind more probe molecules per sugar unit when the charge of the latter is low, and stability of the probe-heparin complex increases with increasing probe charge.

Heparins are linear, polydisperse polysaccharides that consist predominantly (>70%) of a trisulfated disaccharide repeating unit (Scheme 1) and have a high negative charge density. With about half a billion doses applied annually, heparins are among the most widely used anticoagulants.[1] Unfractionated heparin (UFH) with a mean molecular weight between 13,000 and 15,000 is clinically applied since the 1930s but has been widely replaced[2] by low molecular weight heparins (LMWH) with mean molecular weight between 3000 and 5000, manufactured by partial depolymerisation of UFH. 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. There have been intensive efforts to develop simple and reliable assays and sensor systems that could detect heparin levels in blood, plasma or serum samples.[3] Measurement of activated partial thrombin time APTT (the ability to delay clotting) and anti-Xa assay (inhibition of a specific blood coagulation factor), are the currently accepted practice for laboratory monitoring of UFH and LMWH, respectively, in patients' blood.

The recognized limitations[4,2b] of these assays have stimulated the development of alternative detection schemes for heparin, including simple colorimetric and fluorimetric detection by molecular probes.[5] The latter have a strong potential for rapid point-of-care testing of heparin, for the quality control of heparin samples,[5i] and as small-molecule labels or antagonists of cell surface glycosaminoglycans[6] which regulate a wide variety of biological activities. Target specifity, in particular in complex biological matrices, is challenging problem in such applications, and the rational design of probes with optimal application dependent performance requires a better understanding of the structural features that determine probe-target interaction. In nearly all of the reported[5] molecular probes, electrostatic interaction between one or several ammonium groups of the probe and the sulfates of heparin (or other glycosaminoglycans) appears to favour probe-target binding. To our knowledge, the influence of probe charge on the interaction with heparin has not yet been systematically explored.

We have recently described[7] a fluorescent perylene diimide derivative $(H_8\mathbf{1})Cl_8$ with eight ammonium groups that binds heparin with high affinity and selectivity and allows quantification of heparin in blood serum and plasma with minimal matrix interference. Here we compare **1** with newly synthesized perylene diimides **2, 3** and **4** which in their protonated forms carry six, four and two ammonium groups, respectively (Scheme 1). We analyze the stoichiometry of their complexes with heparin and rank their binding affinities.

Details on the syntheses of compounds **2**–**4** are given in the Supplementary data. As observed for **1**, compounds **2**–**4** are expected to be isolated as a mixture of two isomers with respect to the position of the two substituents at the perylene core: A major 1,7 and a minor 1,6-isomer (only the 1,7-isomer is shown in Scheme 1). In the case of **1**, we were able to isolate the isomers and found that they have very similar photophysical properties, and that their fluorescence response to heparin is indistinguishable.[7] We assume a comparable behaviour of probes **2**–**4** and have therefore not attempted the laborious isolation of the 1,7 and 1,6-isomers. The probes were used as obtained from synthesis.

Absorbance and fluorescence spectra of **1**–**4** (Table 1) are influenced by the substituents at the perylene core. Emission spectra of

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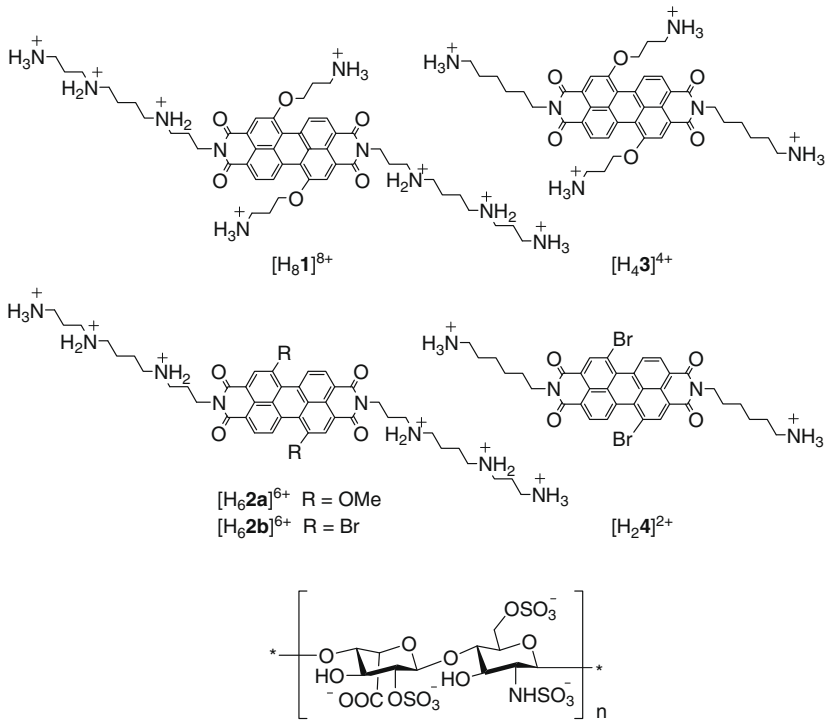

Scheme 1. Top: Structures of perylene diimide probes **1**–**4** in their protonated form. Bottom: Structure of the major repeating disaccharide unit of heparin.

probes **1**, **2a** and **3** which have alkoxy substituents at the perylene core are readily distinguished from those of the other probes by a significant redshift, λ_{max}, ≈ 615 nm for **1**, **2a** and **3** versus ≈565 nm for **2b** and **4**. Importantly, all probes have in water at pH 7 a sufficiently intense fluorescence to accurately explore their response to heparin at 0.1 μM probe concentration.

Determination of the pK_a values of the probes pH titration was complicated by their limited solubility in water, or the tendency to precipitate at higher concentrations during pH titration. We therefore applied a previously reported method for the determination of the pK_a values. Briefly, the presence of a free amino group is indicated by fluorescence quenching due to photoinduced electron transfer.[8] Very low dye concentrations (0.1 μM) were used to minimize by aggregation (see Fig. S1, Supplementary data). By monitoring the fluorescence decrease with increasing pH (Supplementary data), we have determined approximate pK_a values 8.6 (**1**), 8.7 (**2a**), 8.9 (**2b**), 8.8 (**3**) and 9.2 (**4**) at 0.1 M ion strength. These values are plausible, considering a reported first pK_a value 8.2 for protonated *N*1-acetylspermine,[9,10] pK_a 9.9 for protonated *N*,*N*'-bis(aminoethyl)perylenediimide,[8] and pK_a 10.2 for protonated 3-aminopropanol.[11] The pK_a of **4**, lower than the pK_a of 1,6-diaminohexane (9.9 and 10.6),[12] might be affected by aggregation at alkaline pH of **4**, which is the most hydrophobic molecule of **1**–**4**. Validation of the protonation of compounds **1**–**4** at pH 7 is important for the interpretation of their interaction with polyanionic heparin.

To study response of the compounds to heparin we focused on more widely applied low molecular weight heparin, using a commercial LMWH sodium salt with an average molecular weight in of 5000. On addition of LMWH to a 0.1 μM solution of the probes, a strong quenching of probe fluorescence is observed in all cases. Decrease of probe fluorescence is linear with increasing heparin concentrations (see Fig. 1). We suggest that quenching is triggered by aggregation of the cationic probe molecules at the polyanionic heparin template.

Sharp end points of the fluorescence titration with heparin are observed. For each probe, a specific heparin concentration is required for complete fluorescence quenching, and a binding stoichiometry or 'loading' of the heparin strand with probe molecules can

Table 1
Visible absorbance maxima (with log of molar absorbance) and fluorescence emission maxima of probes **1**–**4** (10 μM) in aqueous solution at pH 7.0 (buffer 10 mM 3-(*N*-morpholino) propanesulfonic acid) and 20 °C

Compds	Abs λ_{max} (nm)	log ε ($M^{-1}cm^{-1}$)	Em λ_{max} (nm)
1	542	4.56	617
2a	543	4.42	620
2b	499	4.37	565
3	541	4.45	615
4	498	4.38	563

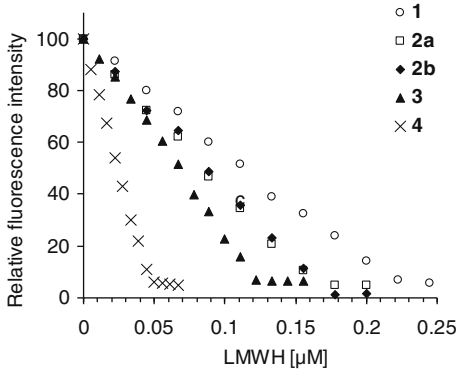

Figure 1. Fluorescence titration of probes **1**–**4** (0.1 μM) with LMWH. Initial fluorescence of the probe solutions is normalized to 100. Aqueous solution at pH 7.0 (buffer 10 mM 3-(*N*-morpholino) propanesulfonic acid), *T* = 20 °C. Excitation at the absorbance maximum of the probe, emission recorded at the emission maximum of the probe (Table 1). Concentration of LMWH is based on the molecular mass 644.2 of the major disaccharide unit (tetrasodium salt) shown in Scheme 1.

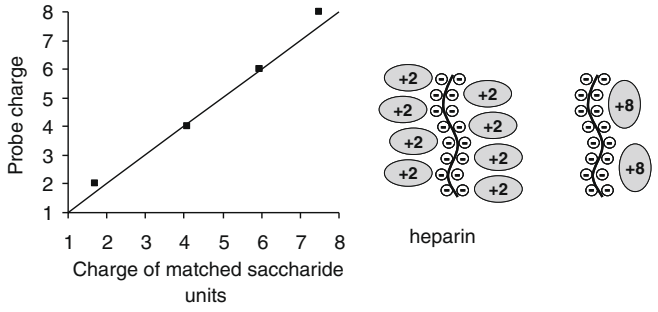

Figure 2. Left: Relation between charge of probes **1**–**4** (Scheme 1) and charge of 'matched' saccharide units on the heparin strand, as derived from the titration end points in Figure 1. The straight line indicates an idealized probe–heparin interaction with perfect charge compensation. Right: Illustration of binding stoichiometries of heparin-**1** and heparin-**4** complexes at the endpoint of fluorescence titration.

be calculated. The number of saccharide units per probe molecule is 1.3 for **4**, 2.4 for **3**, 3.2 for **2**, and 4.2 for **1**, that is, the number increases with increasing probe charge. Note that the nature of noncharged subsituents, –OMe in **2a** and –Br in **2b**, has no influence on the probe/saccharides ratio. We further analyzed the interaction in terms of probe charge and charge of the corresponding saccharide units, using the literature reported average charge −1.7 per monosaccharide of heparin[13] (Fig. 2). The observed correlation (Fig. 2) suggests that charge compensation is an important determinant of the stoichiometry of probe–heparin interaction.

Analysis of the binding affinities of the probes to heparin is complicated by the nature of the probe–heparin complexes, that is, the association of multiple probe molecules with a single heparin strand, but also by variations of saccharide sequence and sulfation pattern within a single heparin strand. We therefore have limited our efforts to the determination of relative binding strength of the probes to heparin. Attempts to discriminate binding affinities by addition of protamine, a polycationic protein and clinically applied heparin antagonist, failed due to very strong binding

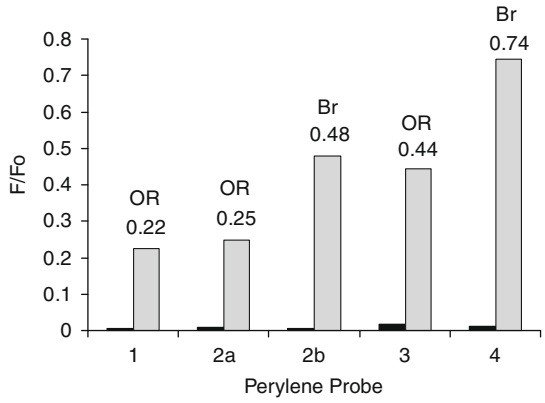

Figure 3. Fluorescence of probes **1**–**4** in the presence of heparin and hexadimethrine bromide. F_o is the fluorescence of the probe (1 μM) in the absence of heparin and hexadimethrine bromide. Probe–heparin complexes with low fluorescence were preformed by adding the LMWH quantities corresponding to the titration end points in Figure 1, to probes **1**–**4** (1 μM) (black bars). Aqueous solution at pH 7.0 (buffer 10 mM 3-(*N*-morpholino) propanesulfonic acid), $T = 20$ °C. Fluorescence F was recorded 60 min after the addition of hexadimethrine bromide (2 $\mu g mL^{-1}$, gray bars). Excitation at the absorbance maximum of the probe, emission recorded at the emission maximum of the probe (Table 1).

of protamine to heparin and displacement of the probes. In contrast, hexadimethrine bromide, a synthetic polymer (average mass 5000–10,000) with repeating [–$(CH_2)_6$–NH_2^+–] units and less effective heparin antagonist than protamine, allows a discrimination of probes **2**, **3** and **4** with respect to heparin binding affinity when used in low concentration (Fig. 3, gray bars). A qualitative ranking of the probes according to their binding affinity to heparin is: **1** ≈ **2a** > **2b** ≈ **3** > **4**.

There is an obvious trend of increasing stability of heparin–probe complex with increasing probe charge. However, probes **2a** and **2b** which have the same charge +6 and differ only in the nature of perylene substituents, –Br or –OMe, have significantly different affinities. Other structural features than probe charge might fine-tune the affinity to heparin. We suggest that the strength of hydrophobic interactions between the probe molecules after assembly on the heparin target contributes to the overall stability of the aggregates. Bulky –Br-substituents at the perylene core disfavour intermolecular π-stacking at short distances[14] and lower the energy gain from hydrophobic interactions compared with the –OMe substituted probe **2b**.

Another conclusion from Figure 3 is that both **1** and **2a** effectively compete with the polymeric antagonist for heparin binding, with only marginal gain of affinity when probe charge increases from +6 for **2a** and to +8 for in **1**.

In conclusion, the number of ammonium groups (2, 4, 6 or 8) attached to perylene molecular probes determines the stoichiometry and affinity of binding to heparin. Charge compensation is a predictor of binding stoichiometry, that is, the linear polycationic heparin strands bind more probe molecules when probe charge is small. Affinity of the probes increases with increasing probe charge.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.12.105.

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