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Pyrene-based heparin sensors in competitive aqueous media – the role of self-assembled multivalency (SAMul)

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Ching W. Chan[a] and David K. Smith[a,*]

Amine-functionalised pyrene derivatives are reported and their ability to detect heparin via a fluorescent response determined – different responses are observed dependent on whether self-assembled multivalent binding between sensor and analyte takes place, and ratiometric heparin sensors which can detect this surgically-relevant polyanion in competitive media are reported.

Heparin is the most charge-dense naturally-occurring polyanion in biological systems.[1] Clinically, it is used as an anti-coagulant during cardiopulmonary surgery and to treat emergency deep venous thrombosis (DVT).[2] The quantification of heparin levels in blood is typically achieved via an activated clotting time assay,[3] which has the drawback of being relatively slow, and unable to be performed on a patient *in situ*.[4] Therefore, the development of heparin sensors which operate in biologically-relevant conditions is of considerable interest.[5] In recent years, a variety of heparin sensors have been reported – for example using electrochemical[6] or colorimetric[7] methods. Fluorescent heparin sensors are of particular interest,[8] however, some of those reported have limitations such as poor selectivity, short emission wavelengths, single-wavelength detection (necessitating external calibration), or low-affinity in competitive media. It is worth noting that a number of sensors which report binding 'in serum' actually achieve this by adding a small amount of heparinised serum to the sensor in buffer – in the assay itself, levels of serum are relatively low (<10%). The development of robust, ratiometric dual-wavelength heparin sensors which operate in highly-competitive media remains a desirable goal.

In sensor technology, pyrene is a useful fluorophore for ratiometric sensors, as it can form an excimer when excited-state and ground-state molecules are brought into close proximity.[9] Self-assembled pyrene derivatives have been used to intervene in biological processes.[10] Several pyrene derivatives have been reported as heparin sensors.[11] In some cases, low affinities necessitated the use of co-solvents during heparin-binding assays, although sophisticated peptide-functionalised pyrene derivatives have been reported to achieve ultra-sensitive heparin detection.[11e]

Multivalency, in which multiple interactions form between binder and target, is an effective way of achieving high-affinity binding to nanoscale surfaces, such as that of polyanionic heparin,[5] in competitive media.[12] Self-assembled multivalency (SAMul), in which non-covalent self-assembly is used to organise multiple ligands, which as a result show high-affinity binding to a target, has emerged as a strategy of interest.[13] We have employed this approach to develop heparin binders.[14] In this new research, we reasoned that pyrene could act as a both a fluorescent unit to detect heparin ratiometrically and also provide the driving force for self-assembly. This could offer a two-in-one approach to high-affinity binding and ratiometric sensing.

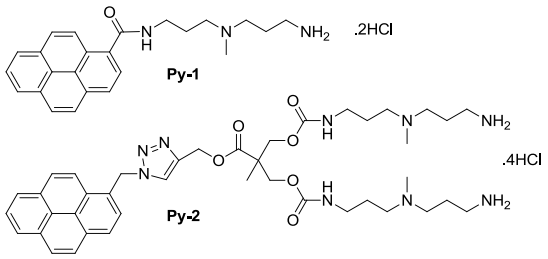

Figure 1. Structures of **Py-1** and **Py-2** – fluorescent heparin sensors.

We targeted relatively simple pyrene-derived sensors – synthetic simplicity is a desirable feature for sensors with potential clinical applications. Two pyrene derivatives were synthesised (**Py-1** and **Py-2**, Fig. 1), each functionalised with the same amine ligand (di-3-aminopropylmethylamine, DAPMA), in order to explore the effects of ligand organisation on self-assembly, multivalent heparin binding and sensing. The synthesis of **Py-1** was trivially achieved by coupling pyrene carboxylic acid to mono-Boc-protected DAPMA, followed by deprotection with HCl. The synthesis of **Py-2** employed click chemistry to couple a divalent DAPMA ligand, developed by us

[a] *Department of Chemistry, University of York, Heslington, York, YO10 5DD, UK. Email: david.smith@york.ac.uk*

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elsewhere,[14] to the pyrene. After optimisation, these syntheses worked in good yields (for full details, see ESI).

The ability of **Py-1** and **Py-2** to self-assemble in buffered water was examined by fluorescence spectroscopy. The pyrene unit was excited at 363 nm and emission intensity recorded at 495 nm (Fig. 2). The concentration at which this excimer emission band significantly increased in intensity was taken to represent the critical aggregation concentration (CAC). Under these micromolar conditions, **Py-1** did not self-assemble, with the fluorescence intensity remaining low, and increasingly linearly across the whole concentration range. However, **Py-2** showed a discontinuous increase in fluorescence intensity on increasing concentration, and the CAC was estimated as 21 ± 4 μM in PBS buffer. We suggest that the more flexible structure of **Py-2** and the different balance of hydrophilic/hydrophobic groups compared to **Py-1**, give it greater propensity to self-assemble.

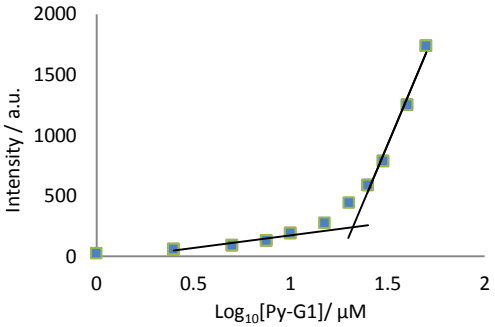

Figure 2 Fluorescence intensity associated with the excimer band of **Py-2** at 495 nm (excitation at 363 nm) as recorded for increasing amounts of **Py-2** in PBS Buffer. The discontinuity is taken as an approximation to the critical aggregation concentration.

The self-assembled nanostructures formed by **Py-2** were then visualised using transmission electron microscopy (TEM). The system appeared to form relatively polydisperse spherical assemblies with approximate diameters ranging from ca. 10 to 40 nm (Fig. 3) – given the (much smaller) molecular size (2-3 nm), these could be vesicular objects or clusters of micellar assemblies which aggregate on drying.

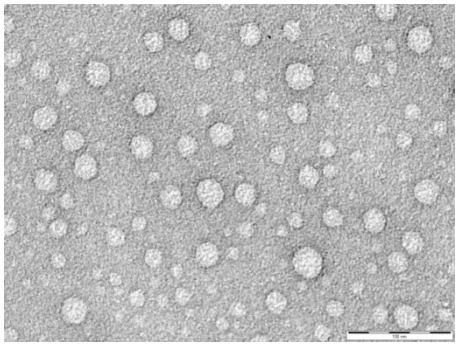

Figure 3. TEM image of **Py-2** dried from aqueous solution (1 mg/mL). Scale bar = 100 nm.

Dynamic light scattering (DLS), which investigates self-assembled objects in solution (rather than deposited on a surface as in TEM) supported the formation of self-assembled nanostructures (12.3 ± 3.7 nm, Fig. S1) for **Py-2**. As expected, the zeta potential of these self-assembled nanostructures was positive (+27.9 ± 1.4 mV) as a result of protonation of the DAPMA ligands at physiological pH. As such, these self-assembled nanostructures might be expected to show high affinity towards polyanionic heparin. It should be noted that DLS is recorded at relatively high (1 mg/ml) concentration (2.2 mM for **Py-1** and 1.1 mM for **Py-2**). Under these conditions, **Py-1** also self-assembled (Fig. S2). Aggregation at elevated concentrations was also supported by TEM (Fig. S3), in which samples were dried from millimolar aqueous solutions. This demonstrates some potential of **Py-1** to assemble, but not at the concentrations used for heparin binding (60 μM) as described in the next section.

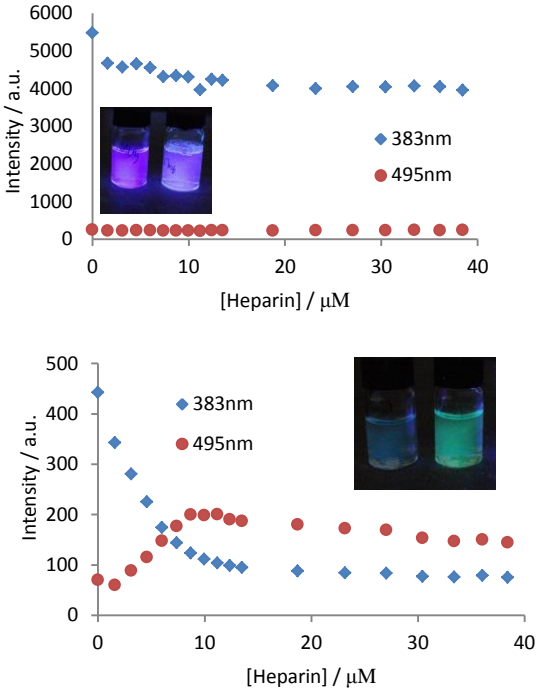

Figure 4. Fluorescence intensities extracted at 383 nm and 495 nm for the titration of heparin into **Py-1** (top) and **Py-2** (bottom). Images are included (under UV lamp irradiation) of samples in the absence (left) and presence (right) of heparin.

We then monitored the binding of both **Py-1** and **Py-2** to heparin. In particular, we initially wanted to determine if there was any difference in terms of sensing ability induced by the ability of **Py-2** to self-assemble in aqueous solution. We titrated heparin (in 10 mM Tris HCl buffer with 150mM NaCl) into a buffered solution of the binder (Fig. 4 and Figs. S4 and S7). Compound **Py-1**, which does not self-assemble, had a larger emission peak at 383 nm and a smaller ratio of I_{495}/I_{383}, reflecting its monomeric nature, while **Py-2** had a much larger I_{495}/I_{383} ratio, indicative of the fact it is self-assembled. On titrating **Py-1** with heparin, the emission at 383 nm decreased in intensity, however the excimer emission band at 495 nm was largely unaffected. In stark contrast, for **Py-2,** the fluorescence emission intensity at 383 nm decreased while the excimer emission band at 495 nm increased very significantly. This increased emission band at 495 nm is attributed to the formation of pyrene excimers being further induced by heparin binding. Clearly, the strong "switch-on" excimer emission can also be seen using the naked eye when the sample is under UV irradiation (Fig. 4 inset photograph), as the

fluorescence changes colour (and intensity) to be somewhat more 'green'. There was no such 'naked eye' change in the emission of **Py-1**.

These observations suggest that the large increase in excimer emission intensity is associated with the self-assembly of **Py-2**, and importantly, the reinforcement of this self-assembly event as a result of multivalent binding to polyanionic heparin. We have noted previously that self-assembly can induce multivalency, and also, conversely, that multivalent binding can induce/reinforce self-assembly.[14,15] Clearly, the fluorescence sensing mechanisms of **Py-1** and **Py-2** are significantly different under these conditions, with **Py-2** exhibiting what we define here for the first times as a self-assembled multivalent ('SAMul') sensing mechanism.

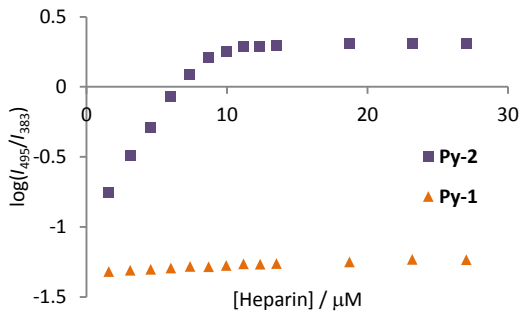

Figure 5, The changes of the fluorescence intensity ratio of **Py-G1** and **Py-G2** ($\log(I_{495}/I_{383})$) plotted against increasing heparin concentration in buffer.

By treating the data extracted at 383 nm and 495 nm, these compounds report on heparin concentrations in buffer solution through a ratiometric approach. The emission intensity ratio, I_{495}/I_{383}, increases as the concentration of heparin increases. To obtain a linear plot, $\log(I_{495}/I_{383})$ was plotted against heparin concentration (Fig. 5). Comparing the ratiometric response for **Py-1** and **Py-2**, it is evident that **Py-2** shows a much larger response. This is a result of the switching on of excimer emission by SAMul binding in **Py-2** leading to a much larger increase in I_{495} and hence giving more than one order of magnitude difference in I_{495}/I_{383}. Compound **Py-2** shows a linear response to heparin under these assay conditions from 1-10 μM – the clinically relevant range. Although **Py-1** shows a measurable response in its I_{383} band (Fig. 4) – this is relatively insignificant in the ratiometric approach (Fig. 5). Compound **Py-1** is less effective as a sensor under these conditions – although the response is somewhat linear, the dynamic range is very small. As such, under these conditions, the SAMul effect of **Py-2** makes it a much more effective and useful heparin sensor and the difference between SAMul and non-SAMul sensing is clear. We note that heparin is a polydisperse target with highly variable binding sites along the polymer chain – as such, we do not attempt to determine binding constants in this case.

We then monitored the ability of these sensors to detect heparin in 12.5% serum – this provides a serum-level typical of medicinal assays in which heparinised serum is added to a sample of sensor in buffer (Figs. S5 and S8). In this more competitive medium, the response of the sensor to heparin is somewhat more linear, and does not saturate in the same way – this would be reflective of the weaker binding expected in the more competitive medium. However, both sensors did show broadly linear responses across the clinically important range of 1-10 μM heparin (Fig. 6). Comparing **Py-1** and **Py-2** it is evident that in both cases, the ratio of peaks I_{495}/I_{383} increases with increasing amounts of heparin, suggesting an increasing excimer/monomer ratio. For **Py-2**, this ratio is significantly more in favour of the excimer than for **Py-1**, but in neither case does the excimer fully dominate, as it did for **Py-2** in buffer. As such, we suggest that self-assembled multivalency (SAMul) is somewhat disrupted by 12.5% serum. We propose this is due to components in serum, such as albumins, which can interact with the hydrophobic domains of the SAMul nanostructures and limit self-assembly.[16]

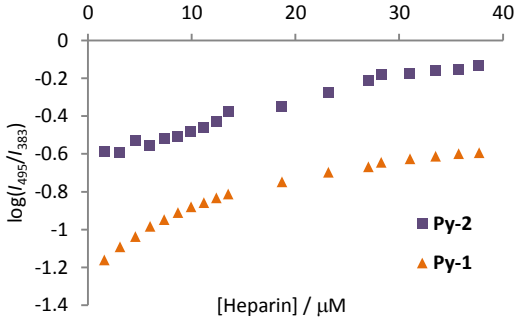

Figure 6, The changes of the fluorescence intensity ratio of **Py-G1** and **Py-G2** ($\log(I_{495}/I_{383})$) plotted against increasing heparin concentration in 12.5% serum..

The binding data can be explained by a model in which these sensors bind to heparin in serum, hence bringing pyrene units into close proximity and encouraging excimer emission, reflecting a less-ordered self-assembly process induced on the heparin backbone, rather than full self-assembled multivalency prior to, and reinforced by, heparin binding. Interestingly, **Py-1** operates quite well in 12.5% serum – demonstrating that very simple sensors can actually be quite efficient in clinically relevant conditions – perhaps **Py-1**, in which the pyrene is closer to the cationic ligands, is more readily brought into close proximity to other pyrenes once heparin binding occurs.

We then went on and tested both sensors in 100% serum. However, neither sensor showed a significant response under these highly competitive conditions (Figs. S6 and S9). In an attempt to detect a response, we therefore increased the concentration of the sensor ca. 10-fold. Under these conditions, we were able to detect the heparin – albeit at concentrations above clinically-relevant levels (Figs. S10 and S11). The mechanisms of response were similar to those seen in 12.5% serum, although in this case, the excimer band of **Py-2** became the dominant feature in the fluorescence spectrum – suggesting that even under highly competitive conditions, at these elevated concentrations the SAMul sensing mechanism can still operate to some extent – i.e. the elevated concentration means the self-assembled nanostructures are better able to resist the disruptive effect of serum components. It was clear from this assay that **Py-2** could still act as an effective heparin sensor under these conditions (Fig. S12). The study also indicates, once again, the surprising ability of even very simple **Py-1** to respond. Overall, it is

remarkable that a simple dication (**Py-1**) and tetracation (**Py-2**) can respond to heparin in such highly competitive media. We propose that the amphiphilic ligand design, and ability of pyrene to assemble *after* binding to heparin, play significant roles in assisting heparin sensing.

In summary, this paper demonstrates that two different pyrene derivatives can act as effective heparin sensors in competitive media using a ratiometric fluorescence sensing approach. In buffered aqueous solution, the assembly **Py-2** into pre-formed self-assembled multivalent (SAMul) nanostructures provides it with a significant (order of magnitude) advantage in terms of the dynamic range of sensory response and its ability to give a naked eye response (in comparison to non-assembling **Py-1**). This is the first example of which we are aware, in which SAMul-enhanced sensing has been reported and as such, we consider it a new mechanism for analyte detection. In the presence of serum, both ligands can still detect heparin ratiometrically – however, in this case, the SAMul sensing mechanism of **Py-2** is switched off (although at elevated concentrations, evidence for this mechanism can still be seen, even in 100% serum). Instead, sensing appears to occur due to a degree of assembly taking place once these cations have bound to heparin. We note that if SAMul-enhanced sensing could be more effectively maintained in serum (as it is in buffer), then this would be a very effective way of achieving heparin sensing, and would extend the detection of heparin in 100% serum down to lower concentrations. We suggest that future work in the field of SAMul sensors should aim to (i) target different analytes with high affinity and (ii) achieve enhanced serum stability. In this way, the unique SAMul sensory response can, as well as working in highly competitive buffer (where it may be of very significant use in its present form), also be applied in true highly competitive biological conditions or to samples present only at very low concentrations.

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Pyrene-based heparin sensors in competitive aqueous media – the role of self-assembled multivalency (SAMul)

Graphical Abstract

Simple functionalised pyrene derivatives can achieve ratiometric sensing of heparin with the precise sensing mechanism depending on whether the sensor self-assembles into a multivalent ligand display.