Heparin sensing based on multisite-binding induced highly ordered perylene nanoaggregates†

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Highly ordered perylene nanoaggregates with ultra-low fluorescence were employed for the selective and sensitive fluorescence sensing of heparin. A supramolecular host–guest complex was used as a displacement probe to improve the sensitivity.

The design of new chemical and biological sensors for biologically important molecules has received enormous attention in recent years.1 Heparin is known as the foremost clinical anticoagulant and a life-saving drug with more than 500 million doses prescribed worldwide annually.2 Heparin is a heterogeneous mixture of highly negatively charged linear biopolymers with repeating trisulfated disaccharide units and has an average molecular weight of 13 000 to 15 000 Daltons.3 It is critical to maintain therapeutic levels of heparin to prevent thrombosis, while minimizing the risks of bleeding. The most widely used laboratory assay for monitoring heparin is activated partial thromboplastin time (aPTT) or activated clotting time assay (aCTA).4 However, these methods are inaccurate, expensive, and time-consuming. Thus, simple, rapid, and inexpensive methods for monitoring heparin levels are highly desired.

Fluorescent displacement probes based on host–guest complexes of macrocycles (e.g. cyclodextrins, calixarenes, and cucurbit[8]urils [CB[8]]) and fluorescent dyes have recently attracted attention due to their notable advantages.5 For instance, these probes utilize self-assembly of host and guest molecules through multiple non-covalent interactions without complicated and expensive covalent labeling procedures. In addition, the fluorescence signal from these probes is reversible in response to certain target molecules, allowing them to sense many biologically and environmentally important analytes.6 The sensitivity of fluorescent displacement probes is highly dependent on the binding affinity of the complexes as well as the fluorescence changes in response to a target. Different strategies have been applied in designing new fluorescent displacement probes for the sensitive detection of different targets of interest, including structural modifications of guest molecules to enhance their binding affinities.7 In this work, a new perylene derivative PDI1 containing two rigid and cationic pyridinium side chains (Fig. 1) was prepared. Based on our previous work,7 we felt that the formation of strong host–guest CB[8]:PDI1 complexes would enhance the initial fluorescence of the probe (up to 600% increase), resulting in improved sensitivity. It was hypothesized that the multisite-binding of

Fig. 1 (a) Schematic illustration of the formation of host–guest CB[8]:PDI1 complex and heparin induced highly ordered aggregates. (b) The chemical structures of PDI1, CB[8], and heparin.
heparin (HEP) with PD11 via strong electrostatic and π–π interactions would lead to highly ordered HEP:PD11 nanoaggregates, resulting in significant self-quenching of fluorescence. Herein we demonstrate that the CB[8]:PD11 complex can serve as a fluorescent displacement probe for the highly sensitive detection of HEP through the competitive multisite-binding of HEP to PD11 over CB[8]. A detection limit of 2.4 ng mL⁻¹ (~0.13 nM) for HEP was achieved, which is comparable to that of most reported methods.⁹

PD11 was prepared in three reaction steps (imidization, quaternization, and anion exchange) from a perylene dihydride [Scheme S1, ESI†]. The detailed synthetic procedure and characterization are provided in the ESI.⁷ PD11 is very soluble in common polar solvents (e.g. water, MeOH, CH₃CN, and DMSO) due to its hydrophilic pyridinium side-chains with permanent positive charges. In methanol, PD11 (10 μM) exhibits two absorption peaks with λmax at ~490 and ~525 nm and emits strongly in the range of 500–600 nm with a max yield of PDI1 because of the pyridium effect.⁹ Deaggregation of PD11 was reached with 3.0 equivalents of CB[8], suggesting efficient formation of CB[8] : host–guest complex for PDI1 at a higher concentration. The absorption of PD11 in different concentrations in water indicates that the ratio of the absorption at ~503 nm and ~540 increases as the concentration increases from 1 × 10⁻⁶ M to 1.0 × 10⁻³ M (Fig. 2b), suggesting an increased H-aggregation at a higher concentration.¹¹

The host–guest complexation between CB[8] and PD11 was investigated in detail using UV-vis and fluorescence spectroscopy. As shown in Fig. 2c, the addition of CB[8] to PD11 (10 μM) caused a gradual increase in the absorption at 540 nm with increased A₅₄₀/A₅₀₃ ratios. The fluorescence intensity of PD11 at λmax increased gradually as the concentration of CB[8] increased and a maximum enhancement (~600%) was reached with 3.0 equivalents of CB[8], suggesting efficient deaggregation of PD11 aggregates upon encapsulation with CB[8] through hydrophobic and electrostatic interactions (Fig. 2d).⁹ The Job plot indicates a maximum at a molar fraction of the guest equal to 0.5, indicating the formation of a 1:1 host–guest complex for PD11 with CB8 (Fig. S1, ESI†). The formation of CB[8]:PD11 1:1 complex was further confirmed using mass spectroscopy ([CB[8]:PD11 + 3H]: observed mass 1936.84 Da, calculated mass 1936.29 Da, Fig. S2, ESI†). The binding affinity (Kₒ) of the CB[8]:PD11 complex in water as determined using fluorescence titration was calculated to be ~2.6 × 10⁵ M⁻¹ using a 1:1 binding model with the Origin program (Fig. S3, ESI†).¹² It is about 2.6 times the binding affinity of a previously reported CB[8]:PD11 complex, probably because of the pyridium effect.⁹

The multisite-binding of HEP with PD11 in water was investigated using UV-vis and fluorescence spectroscopy. The size and surface properties of HEP:PD11 aggregates were investigated using cryo-electron microscopy (cryo-EM) and dynamic light scattering (DLS). As shown in Fig. 2e, the addition of HEP to PD11 (10 μM) caused a gradual decrease of the absorption at λmax (503 nm) and the peak at λmax decreased about 50% in the presence of 9.8 μg mL⁻¹ (or ~0.54 μM, ~38 μM negative charges) of HEP.¹³ The fluorescence intensity of PD11 at λmax (550 nm) decreased gradually as the concentration of HEP increased and the intensity dropped to about 0.45% of the initial intensity in the presence of 9.8 μg mL⁻¹ of HEP (Fig. 2f). The QY was calculated to be only 0.04%, which is extremely low. The ratio of the fluorescence intensity of PD11 vs. HEP:PD11 and CB[8]:PD11 vs. HEP:PD11 is greater than 200 and 1000, respectively. These results suggest possible formation of highly ordered non-fluorescent HEP:PD11 aggregates. The cryo-EM imaging of a HEP:PD11 solution revealed the formation of nanobelt-shaped aggregates (~40 nm in width) with uniformly aligned interior alternating strips. The light (~2.5 nm) and

![Fig. 2](image-url)
dark stems are from the hydrophobic aromatic cores with higher electron density and the charged backbones with lower electron density, respectively (Fig. 3 and Fig. S4, ESI†). These observations support the proposed structure of highly ordered HEP:PDI1 aggregates (Fig. 3e). To our knowledge, this is the first time perylene H-aggregates formed in a diluted aqueous solution have been visualized. The DLS analysis revealed the presence of HEP:PDI1 nano-aggregates with a size distribution peak (by volume) at ~40 nm and a zeta potential distribution peak at ~55 mV (Fig. S5 and S6, ESI†), suggesting high stability of negatively charged nano-aggregates in water. In comparison, cryo-EM images of PDI1 only in water showed long filaments (Fig. S7, ESI†) and the DLS analysis revealed two size distributions, one at ~150 nm and another at ~700 nm with a high polydispersity index (PDI) of 1.0 compared to HEP:PDI1 nano-aggregates with a PDI of 0.37. This suggests that the PDI1 sample is polydisperse. In addition, the mean surface zeta potential was measured to be +47 mV, indicating positively charged PDI1 aggregates (Fig. S6, ESI†). The stability of HEP:PDI1 nano-aggregates was further investigated using a temperature study. As shown in Fig. S8 (ESI†), no significant change was observed when the sample was heated to 65 °C, suggesting high stability of HEP:PDI1 aggregates at high temperature, presumably due to the strong electrostatic interactions between highly negatively charged HEP and positively charged PDI1 aggregates. In the control experiment with PDI1 only, the monomer peak at 540 nm increased as the temperature increased from 25 to 65 °C, suggesting gradual deaggregation with increasing temperatures.9

Further studies were performed to explore HEP sensing using the CB[8]:PDI1 complex as the displacement probe. As shown in Fig. 4a, the absorption of the CB[8]:PDI1 complex (3 : 1, 10 μM PDI1) decreased gradually with increased A_{530}/A_{450} ratio as the concentration of HEP increased from 0 to 9.8 μg mL⁻¹. The fluorescence intensity of the complex decreased gradually as the concentration of HEP increased and dropped about 99.7% in the presence of 9.8 μg mL⁻¹ of HEP, suggesting a complete displacement of CB8 by HEP through the formation of non-fluorescent HEP:PDI1 nano-aggregates (Fig. 4b). The selectivity of the displacement probe for HEP over other common anions and molecules was further investigated. As shown Fig. 5a and b, the addition of excess sulfate, phosphate, L-ascorbic acid, glucose, BSA, or HSA did not significantly affect the displacement of CB8 by HEP. (c) The fluorescence changes (A/F) of PDI1 nano-aggregates in the presence of 0, 2.5, 4.9, and 9.8 μg mL⁻¹ of HEP. (d) The Normalized emission intensity (at λ_{max}) of CB[8]:PDI1 (3 : 1, 10 μM PDI1) in the presence of 0, 2.5, 4.9, and 9.8 μg mL⁻¹ of HEP in Tris buffer (pH 7.4) with 1% human serum.
not cause a significant change on either the UV-vis absorption or the fluorescence emission. In contrast, the addition of 9.8 μg mL⁻¹ of HEP caused a 50% drop of the absorption and a 99.7% drop of the fluorescence emission. This study suggested the good selectivity of the probe to HEP. The sensitivity of the displacement probe for HEP was performed using a low concentration of PDI1 (100 nM) with excess CB[8] (100 equiv., 10 μM) in a Tris-buffer (pH 7.4). The fluorescence titration curve shows a linear relation between the fluorescence change and the concentration of HEP in the range of 0 to 49 ng mL⁻¹ with a correlation coefficient of 0.98 (Fig. 5c and Fig. S9, ESI†). The detection limit (LOD) was calculated to be 2.4 ng mL⁻¹ (or ~0.13 nM or 0.5 μM L⁻¹) (LOD = 3σ/K), which is 4000 times lower than a recommended clinical dose amount (10 000 units/68 kg patient or 2.0 units per mL in blood) and superior to most HEP detection methods reported previously (Table S1, ESI†). The detection limit (LOD) was calculated to be 2.4 ng mL⁻¹ (or ~0.13 nM or 0.5 μM L⁻¹) (LOD = 3σ/K), which is 4000 times lower than a recommended clinical dose amount (10 000 units/68 kg patient or 2.0 units per mL in blood) and superior to most HEP detection methods reported previously (Table S1, ESI†).

In summary, we have designed and synthesized a new functionalized pyrene derivative PDI1 with pyridium side chains. CB[8] binding with PDI1 leads to highly fluorescent host–guest CB[8]:PDI1 complexes through hydrophobic and electrostatic interactions. HEP multisite-binding with PDI1 leads to highly ordered HEP:PDI1 nano-aggregates with ultra-low fluorescence (QY: 0.04%). More interestingly, the morphology of these highly ordered HEP:PDI1 nano-aggregates in a dilute solution was directly visualized using cryo-EM for the first time. We further demonstrated the application of the CB[8]:PDI1 complex as a fluorescence displacement probe for monitoring HEP in human serum samples.

Potential clinical application of the CB[8]:PDI1 complex as a fluorescent displacement probe for monitoring HEP in human serum samples is suggested. The potential application of the CB[8]:PDI1 complexes as a simple, rapid, and inexpensive probe for the sensitive detection of HEP. Beyond sensing applications, the general supramolecular approach for highly ordered nano-aggregates using the multisite-binding strategy via non-covalent interactions is expected to be valuable in the development of new highly organized materials.

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Conflicts of interest
There are no conflicts to declare.

Notes and references
1. (a) X. Sun and T. D. James, Chem. Rev., 2015, 115, 8001–8037; (b) J. Dong and M. Zhao, Trends Anal. Chem., 2016, 80, 190–203; (c) N. Mackman, Nature, 2008, 451, 914.
2. S. M. Bromfield, E. Wilde and D. K. Smith, Chem. Soc. Rev., 2013, 42, 9184–9195.
3. (a) S. L. Ni, X. Xiao, H. Cong, Q.-J. Zhu, S.-F. Xue and Z. Tao, Acc. Chem. Res., 2014, 47, 1386–1395; (b) L. C. Smith, D. G. Leach, B. E. Blaylock, O. A. Ali and A. R. Urbach, J. Am. Chem. Soc., 2015, 137, 3663–3669; (c) S. Sonzini, J. A. McCune, P. Ravn, O. A. Sherman and C. F. van der Walle, Chem. Commun., 2017, 53, 8842–8845; (d) M. Nilam, Ch Huang, S. Karmacharya, G. H. Aryal, L. Huang, W. S. Nau and K. I. Assaf, ChemComm, 2018, 5850–5854.
4. (a) G. Ghale and W. M. Nau, Acc. Chem. Res., 2014, 47, 2150–2159; (b) S. Sonzini, J. A. McCune, P. Ravn, O. A. Sherman and C. F. van der Walle, Chem. Commun., 2017, 53, 8842–8845; (c) G. H. Aryal, K. W. Hunter and L. Huang, Org. Biomol. Chem., 2018, 16, 7425–7429.
5. (a) G. H. Aryal, K. L. Assaf, K. W. Hunter, W. M. Nau and L. Huang, Chem. Commun., 2017, 53, 9242–9245; (b) G. H. Aryal, R. Vík, K. I. Assaf, K. W. Hunter, L. Huang, J. Jayawickramarajah and W. M. Nau, ChemComm, 2018, 3, 4699–4704; (c) G. H. Aryal, K. Lu, G. Chen, K. W. Hunter and L. Huang, Chem. Commun., 2019, 55, 13912–13915.
6. (a) N. H. Mudliar, P. M. Dongre and P. K. Singh, Sens. Actuators, B, 2019, 270, 809–814; (b) H. Liu, P. Song, R. Wei, K. Li and A. Tong, Talanta, 2014, 118, 348–352; (c) J. Zheng, T. Ye, J. Chen, L. Xu, X. Ji, C. Yang and Z. He, Biosens. Bioelectron., 2017, 90, 245–250; (d) L. Cai, R. Zhan, K. Y. Pu, X. Qi, H. Zhang, W. Huang and B. Liu, Anal. Chem., 2011, 83, 7849–7855; (e) S. Y. Hung and W. L. Tseng, Biosens. Bioelectron., 2014, 57, 186–191; (f) Z. Liu, Q. Ma, X. Wang, Z. Lin, H. Zhang, L. Xu and X. Su, Biosens. Bioelectron., 2014, 54, 617–622; (c) P. Guo, Y. Wang and Q. Zhuang, Sens. Actuators, B, 2019, 281, 126739.
7. F. Biedermann, E. Elmalen, I. Ghosh, W. M. Nau and O. A. Sherman, Angew. Chem., Int. Ed., 2012, 51, 7739–7743.
8. F. Wuthmer, C. R. Saha-Moller, B. Fimmel, S. Ogi, P. Leowannawat and D. Schmidt, Chem. Rev., 2016, 116, 962–1052.
9. (a) L. Huang, S.-W. Tam-Chang, W. Seo and K. Rove, Adv. Mater., 2007, 19, 4149–4152; (b) S.-W. Tam-Chang and L. Huang, Chem. Commun., 2008, 1957–1967.
10. (a) H. Bakirci, X. Zhang and W. M. Nau, J. Org. Chem., 2005, 70, 39–46; (b) W. M. Nau and X. Zhang, J. Am. Chem. Soc., 1999, 121, 8022–8032.
11. A. Shavarev and E. Bakker, J. Am. Chem. Soc., 2003, 125, 11192–11193.
12. (a) D. MacDougall and W. B. Crummett, Anal. Chem., 1980, 52, 2242–2249; (b) B. Zhang, L. Huang, M. Tang, K. W. Hunter, Y. Feng, Q. Sun, J. Wang and G. Chen, Microchem. Acta, 2018, 185, 385.