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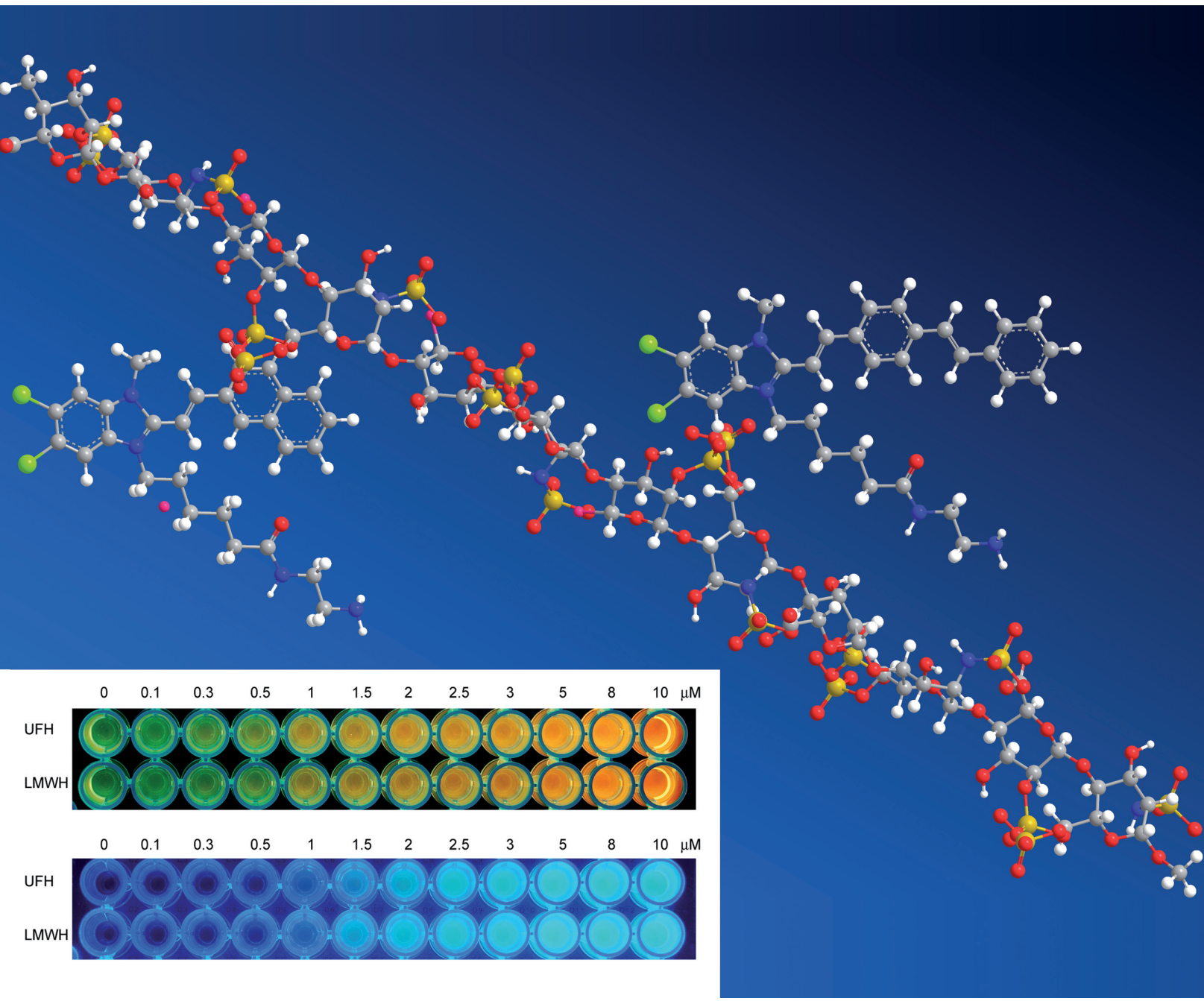


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S. Wang and Y.-T. Chang

Discovery of heparin chemosensors through diversity orientated fluorescence library approach

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# Discovery of heparin chemosensors through diversity oriented fluorescence library approach†

**Shenliang Wang[a] and Young-Tae Chang*[abcd]**

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**Two novel heparin sensors, Heparin Orange and Heparin Blue, were developed by a diversity oriented fluorescence library approach (DOFLA) from a benzimidazolium library; the discovered compounds showed remarkable properties and have the potential to be applied to monitoring heparin levels in clinical plasma samples for point-of-care detection.**

Heparin is a naturally occurring highly sulfated polysaccharide.[1] It has been used clinically as a major anticoagulant to prevent thrombosis during surgery and to treat thrombotic disease since the early part of the 20th century. There are two major forms of heparin in clinical use, known as unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH), both of which are largely accounted for by repeating sequences of the trisulfated disaccharide (Fig. 1a), with UFH having a molecular weight range from 3000 to 30 000 Da and LMWH having an average molecular weight of 4000 to 5000 Da.[1b] Despite the long history and widespread use of heparin, close monitoring and control of the heparin blood level during the application of UFH and LMWH is of crucial importance due to the risk of adverse effects such as hemorrhages and heparin-induced thrombocytopenia (HIT) resulting from heparin overdoses.[2] Various assays have been established to monitor the heparin concentration, including the most commonly used activated partial thromboplastin time (aPTT), anti-Xa and activated clotting time (ACT) assays.[3] Although methods for monitoring heparin have been improving through the decades, the ideal method remains controversial.[4]

There have been continuous advances in fluorescent chemosensors throughout the decades and various chemosensors have been successfully utilized in diverse chemical, biological and medical applications.[5] The development of chemosensors for bio-relevant analytes is an important field of recent interest and the development of chemosensors for heparin assays was marked by the pioneering work of Anslyn *et al.*[6] A tripodal boronic acid based small molecule was designed and synthesized for heparin assays and was demonstrated as a fluorescence quenching sensor.[6a] While this was the first successful fluorescence based heparin sensor demonstrated in a serum environment, the use of a short emission wavelength (355 nm) might suffer from the auto-fluorescence in a more complex biological sample. More recently, a peptide based sensor was developed with a fluorescence emission increase at 440 nm.[7] However, as stated in the article, the detection limit of the reported sensor is outside the clinical usage range. An ideal fluorescent heparin sensor for clinical use should emit fluorescence at a longer wavelength than those of auto-fluorescence in a biological sample and should respond selectively to heparin without any strong interference from other components of plasma. As an alternative to the rational design of a fluorescent sensor, we have developed a diversity oriented fluorescence library approach (DOFLA) providing selective sensors for DNA, RNA, amyloid protein, serum albumin, GTP and glutathione.[8] By applying the DOFLA, herein we report the discovery of novel and clinically practical heparin sensors with visible light range detection from a benzimidazolium dye library (Fig. 1b).[8a]

Due to the complexity of heparin, in this paper we use the repeating sugar dimer as the unit of the molecular weight of heparin. A primary screening of the dye library was performed with four different concentrations of heparin (2, 5, 10 and 20 μM) in 10 mM HEPES buffer (pH = 7.4) in 96-well microplates using a fluorescence plate reader. To check their

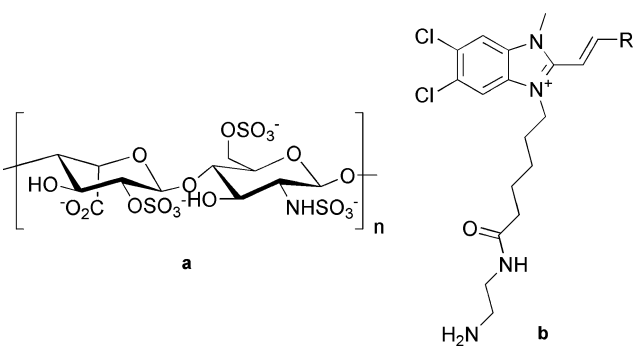


**Fig. 1** Structure of repeating unit of heparin and benzimidazolium dye library.

[a] *Department of Chemistry, New York University, New York, New York 10003, USA*
[b] *Department of Chemistry, National University of Singapore, Singapore 117543*
[c] *NUS MedChem Program of the Office of Life Sciences, National University of Singapore, Singapore 117543*
[d] *Laboratory of Bioimaging Probe Development, Singapore Bioimaging Consortium, Singapore 138667. E-mail: chmcyt@nus.edu.sg; Fax: +65 6779 1691; Tel: +65 6516 6774*

† Electronic supplementary information (ESI) available: Complete experimental details; spectral data for Heparin Orange and Heparin Blue; fluorescence emission spectra of Heparin Orange and Heparin Blue upon addition of LMWH; fluorescent protamine titration of Heparin Orange and Heparin Blue; selectivity of Heparin Orange and Heparin Blue towards various biologically relevant analytes; picture of Heparin Orange and Heparin Blue with different concentrations of UFH and LMWH in pooled human plasma. See DOI: 10.1039/b717058k

practical application potential, the heparin responding dye compounds in buffer were further tested in the environment of 20% human plasma in HEPES buffer to see if their fluorescence response was still vivid. Throughout the whole procedure with various heparin concentrations, two compounds were found to possess the most distinct responses and were finally selected: G26 and G45, both from a benzimidazolium dye library.

Compound G26 (Fig. 2a) showed a strong red shift of the fluorescence from green (520 nm) to orange (595 nm) upon addition of either UFH or LMWH. The ratio of fluorescence intensities at 595 nm and 520 nm increased from 0.6 to 6.2 (Fig. 2c). Compound G45 (Fig. 2b) kept the same maximum emission wavelength while showing an around 150 fold increase of the fluorescence intensity in the blue range (480 nm) (Fig. 2d). The color changes of both compounds are so obvious that the results can be easily distinguished by the naked eye (Fig. 2g, 2h). From the visible color, we dubbed these compounds Heparin Orange and Heparin Blue, respectively. Both of the compounds were tested in another 37 conditions (biological anionic analytes, proteins, pH differences) and the high selectivity for heparin was confirmed (Fig. S9, S10†). Among the tested analytes, only chondroitin sulfate showed about 50% response to heparin in Heparin Blue, demonstrating that our dyes are selective for sulfated carbohydrate polymer. Dissociation constants obtained from titration data (Fig. 2e, 2f) lie in the same order of magnitude as micromolar range, with that of Heparin Blue ($K_d$ = 1.0 μM) being slightly higher than that of Heparin Orange ($K_d$ = 0.58 μM). Cationic competitor, protamine titration demonstrated that the binding of the dye to heparin is fully reversible in both cases (Fig. S11†). The detection range of both sensors in HEPES buffer is from ~0.1 to 10 μM.

For a real clinical application, we further tested the performance of Heparin Orange and Heparin Blue in human plasma. As serum is plasma from which clotting factors have been removed, plasma represents a less processed, more natural clinical sample. Heparin was mixed at various concentrations with human plasma and then diluted five times with 10 mM HEPES buffer (20% of plasma) before Heparin Orange or Heparin Blue was added. Due to the turbidity and higher auto-fluorescence background of plasma, the dye concentration was optimized as 20 μM instead of 10 μM in buffer. Heparin is administered at therapeutic dosing levels of 2-8 U $mL^{-1}$ (17–67 μM) during cardiovascular surgery and 0.2–1.2 U $mL^{-1}$ (1.7–10 μM) in post-operative and long-term care,[9,7] and the standard curves of heparin response of the two sensors lie well within the therapeutic range (Fig. 2i, 2j). The fluorescence response is so obvious that the color change can be distinguished by the naked eye (Fig. S12†). Considering the relative convenience of the clinical sample preparation and fluorescence detection in this assay, these two chemosensors have a strong potential to be developed into a point-of-care detection system for heparin.[10]

In conclusion, we have discovered two practically useful fluorescent chemosensors for clinically important heparin detection by DOFLA, and this demonstrates the power and general applicability of DOFLA in novel fluorescent chemosensor discovery. A broader assessment of Heparin Orange

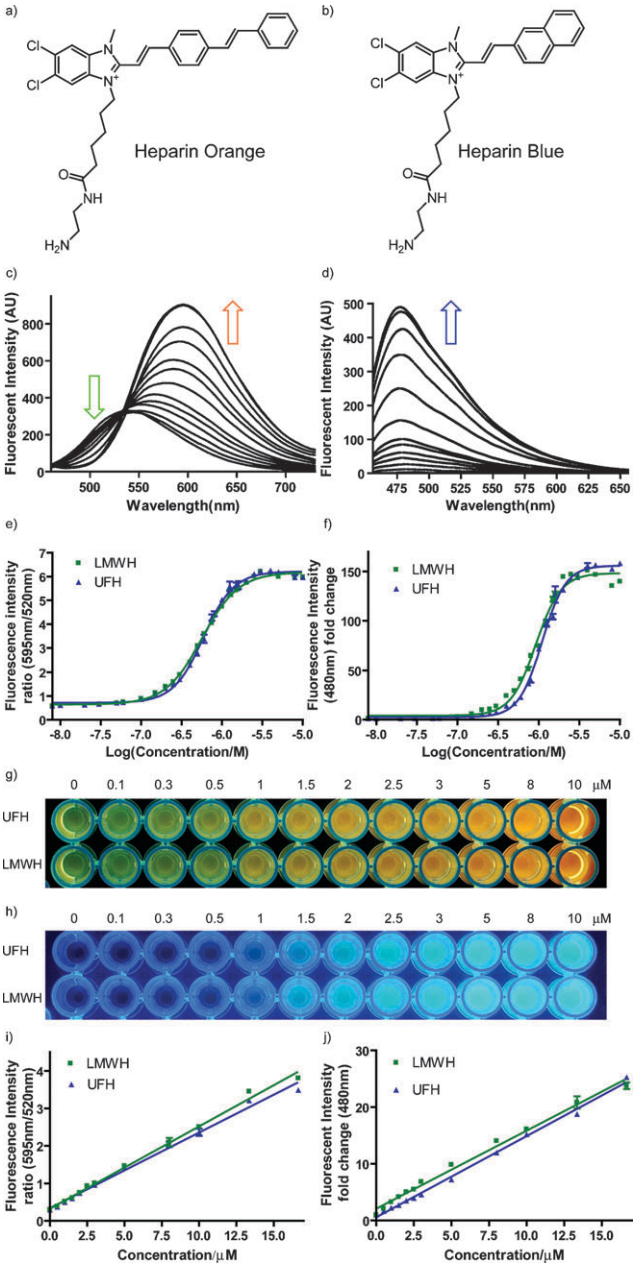


**Fig. 2** Structure of (a) Heparin Orange and (b) Heparin Blue; (c) fluorescence emission spectra (excitation: 380 nm, cutoff: 420 nm) of Heparin Orange (10 μM) and (d) fluorescence emission spectra (excitation: 420 nm, cutoff: 455 nm) of Heparin Blue (10 μM) with 0–10 μM UFH in 10 mM HEPES buffer (pH = 7.4); (e) Heparin Orange (10 μM) fluorescent emission ratio of 595 nm to 520 nm and (f) Heparin Blue (10 μM) fluorescent emission intensity fold change at 480 nm upon addition of LMWH (blue) and UFH (green) at indicated concentrations in 10 mM HEPES buffer (pH = 7.4), experiments were performed in tetraplicate; pictures of (g) Heparin Orange (10 μM) and (h) Heparin Blue (10 μM) with indicated concentrations of UFH and LMWH in 10 mM HEPES buffer (pH = 7.4) in a 96-well plate under 365 nm UV lamp light; (i) fluorescent emission ratio of Heparin Orange (20 μM) at 595 nm to 520 nm and (j) fluorescence emission intensity fold change of Heparin Blue (20 μM) at 480 nm upon addition of LMWH (blue) and UFH (green) at 0–16.7 μM in 20% human plasma with 10 mM HEPES buffer (pH = 7.4), experiments were performed in duplicate, concentrations indicate the final concentrations in the assay solutions, and the corresponding concentrations in original plasma should be multiplied by five.

and Heparin Blue for analyzing clinical samples will be undertaken in due course.

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