Supramolecular Probes

DOI: 10.1002/anie.200805238

Pattern-Based Recognition of Heparin Contaminants by an Array of Self-Assembling Fluorescent Receptors**

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During late 2007 and early 2008, 81 patients in the USA and Germany died and hundreds were seriously injured after being administered contaminated unfractionated heparin sulfate (UFH) while undergoing anticoagulation therapy.[1] Multiple orthogonal analytical techniques, including extensive high-field NMR spectroscopy, HPLC, and capillary electrophoresis, were needed to identify the contaminant as the semisynthetic glycosaminoglycan, oversulfated chondroitin sulfate (OSCS).[2] Even though some batches of heparin were found to contain up to a third of this non-natural form of chondroitin sulfate, its presence was masked in standard quality-control assays owing to the inherent anticoagulant activity of OSCS.[3] The development of quick and reliable tests for heparin contaminants is currently of great interest.[4] Herein we report the design and evaluation of a fluorescent receptor array that is able to assess the quality of a heparin sample by quickly differentiating UFH from OSCS and other commonly encountered negatively charged polymers.

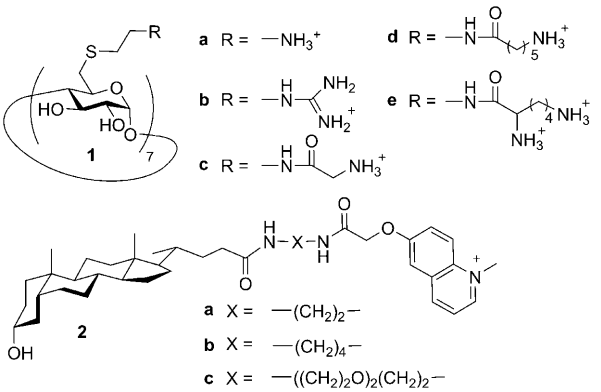
The design of selective receptors for biological macromolecules, such as UFH, poses a significant challenge.[5] As an alternative, chemists have turned towards differential arrays which do not rely on receptors that are specific for a particular molecule, but on a unique diagnostic pattern that is derived from an array of receptors with broad specificity.[6] Many of the most successful examples of solution-phase differential receptor arrays are based on indicator-displacement assays (IDAs).[7] The power of IDAs lies in their modular nature, which enables many unique receptors to be constructed rapidly with a minimum number of synthetic steps. Herein we describe an alternative modular receptor array that does not rely on dye displacement, but on the binding of an environmentally sensitive fluorophore proximal to the analyte-recognition site.

A wide variety of different colorimetric and fluorescent heparin indicators have been reported with mechanisms of heparin sensing based on boronic acids,[8] heparin-specific peptides,[9] labeled heparin-binding proteins,[10] changes in polymer conformation,[11] and fluorophore aggregation.[12] However, none of these strategies provide a modular design that enables the facile generation, and optimization, of a receptor array capable of differentiating between negatively charged polymers.

To design a modular receptor with a high propensity for binding negatively charged biopolymers, we employed the cyclodextrin (CD) **1a** as a scaffold.[13] The primary amine groups of **1a** were functionalized to provide the amide and guanidino derivatives **1b–e** as a small collection of polycationic receptors (Scheme 1). The different modes of electrostatic recognition of CDs **1a–e** towards the polyanionic biopolymers provides the diversity that is needed to generate specific response patterns in the receptor array for each analyte.

Conveniently, **1a** also forms a remarkably stable inclusion complex ($K_D = 18$ nM) with lithocholic acid (LCA).[14] The stability of LCA–β-cyclodextrin complexes has previously enabled the innovative application of this complex in directing protein–protein interactions.[15] According to previous NMR spectroscopic studies, LCA binds to **1a** with the carboxylic acid oriented through the primary rim of the CD.[13,16] This binding orientation provides a simple and modular way to position a fluorophore near the positively charged analyte-binding site of CDs **1a–e** (Figure 1). The quinolinium fluorophore employed is an efficient fluorescent reporter of heparin-binding events driven by electrostatic interactions in aqueous solution.[9a] Thus, the synthesis of a quinolinium fluorophore tethered through a variable spacer to LCA (to give **2a–c**), followed by complexation with CDs **1a–e**, rapidly generated fifteen potential fluorescent receptors for the desired analytes.

An initial evaluation of complexes **1a·2a–c** was carried out with UFH as the analyte. The optimum conditions with respect to both fluorescence response and binding affinity for



Scheme 1. Modular self-assembling fluorescent receptors for polyanionic biopolymers. The receptors consist of a polycationic cyclodextrin host (**1a–e**) and a fluorescent reporter tethered to a lithocholic acid guest (**2a–c**).

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[**] We gratefully acknowledge financial support from The Netherlands Organization for Scientific Research (NWO) and the Natural Sciences and Engineering Research Council of Canada (NSERC).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200805238.

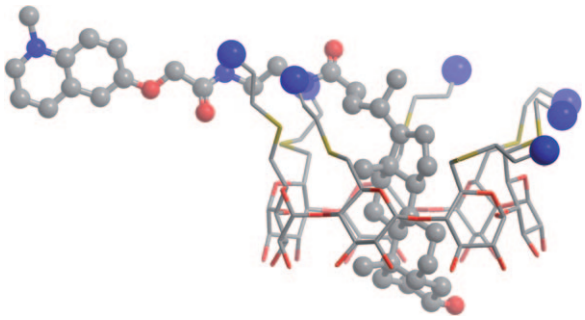

Figure 1. Model of CD **1a** (stick model) bound to the LCA–quinolinium conjugate **2a** (ball-and-stick model). The model shows the relative geometry and scale of the inclusion complex but is not the global-minimum-energy structure. Positively charged ammonium groups of the putative UFH-binding site are emphasized with enlarged spheres.

UFH were a 1:1 ratio of **1a** to the LCA–fluorophore conjugate **2** in phosphate-buffered saline (PBS; $NaPO_4$ 12 mM, NaCl 140 mM, pH 7.4). At a concentration of 7 μM, all three complexes **1a·2** displayed a linear increase in fluorescence upon titration with UFH (1–13 $\mu g\,mL^{-1}$; Figure 2). The relative increase in fluorescence (F/F_0) was greater with shorter LCA/quinolinium tethers X (Scheme 1), and the largest increase in fluorescence (sixfold) was observed for the **1a·2a** complex. Presumably, the shorter tethers bring the fluorophore closer to the polycationic binding site and thus maximize the change in environment experienced by the quinolinium ion upon analyte binding. Although control experiments with **2a–c** in the absence of a CD showed a fluorescence increase (2.5-fold) for **2a** when titrated with UFH, the presence of **1a** is essential for both the magnitude and linearity of the observed response.[17]

Assays were conducted with CDs **1a–e** and the LCA–fluorophore conjugate **2a** in 96-well plates to demonstrate the potential of the corresponding self-assembled fluorescent receptor complexes in pattern-based recognition. The following analytes were chosen to evaluate the discriminatory power of the array: unfractionated and low-molecular-weight

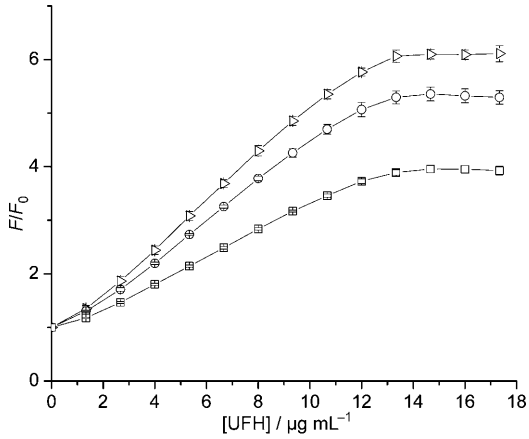

Figure 2. Fluorescence increase for **1a·2a** (△), **1a·2b** (○), and **1a·2c** (□) at a concentration of 7 μM in PBS when titrated with UFH (λ_{ex} = 320 nm, λ_{em} = 430 nm). Error bars indicate ± one standard deviation.

heparin (UFH and LMWH), heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate A (CS), oversulfated chondroitin sulfate (OSCS), polyglutamic acid (PGA), and polyacrylic acid (PAA).[17] Eight individual measurements of F/F_0 were made for each fluorescent-receptor/analyte combination at a concentration of 5 μM for the receptor complex and an analyte concentration of 6 $\mu g\,mL^{-1}$. The responses for each analyte are shown in Figure 3. Most notably, the signal

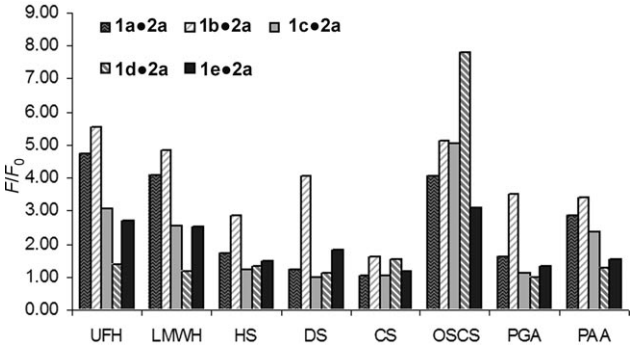

Figure 3. Distinctive diagnostic patterns for the analytes tested. The data represent the averages of eight individual measurements with a standard deviation ≤ 5%. All measurements were made in PBS.

profile for OSCS was dramatically different from that observed for any of the other polyanions. The use of **1d·2a** produced a large response with OSCS, whereas little or no binding was observed with the other analytes and this receptor complex. As expected, the two forms of heparin (UFH and LMWH) gave nearly identical response patterns. The difference in overall magnitude of the signals with UFH and LMWH may be caused by the smaller number of high-affinity binding sites in LMWH than in UFH. The less highly charged analytes HS, DS, and PGA gave similar signal profiles. The most similar response to that of heparin with receptors **1a·2a–c** was observed for PAA; however, the highly charged receptor complex **1a·2e** was able to distinguish these analytes.

The statistics program SYSTAT[18] was used to perform a linear discriminant analysis (LDA)[19] of the fluorescence array data. A graphical representation of this analysis in the form of a two-dimensional score plot is given in Figure 4. The dispersion between the groups of analytes in the score plot is indicative of the analytical power of the receptor array in discriminating between analytes. "Jackknifed" classification matrices[20] were taken to evaluate the LDA results, and all analytes were discriminated with 100% accuracy.[21] In a second set of experiments, in which UFH, DS, and OSCS were investigated at concentrations of 2, 4, 6, and 8 $\mu g\,mL^{-1}$, both the analytes and their concentrations could be distinguished readily.[17]

We were interested in whether our receptor array would also be able to accurately distinguish a pure sample of UFH from one containing a natural contaminant, such as DS, or the potentially deadly contaminant OSCS. Mixtures of UFH containing 10, 25, 50, and 75% OSCS and DS were submitted to our receptor array at a total concentration of 6 $\mu g\,mL^{-1}$.

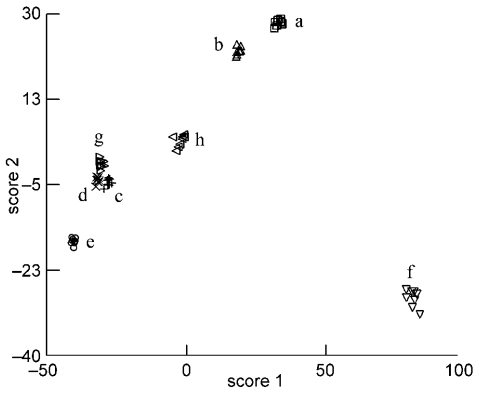

Figure 4. Two-dimensional LDA score plot for the analysis of UFH (a), LMWH (b), HS (c), DS (d), CS (e), OSCS (f), PGA (g), and PAA (h). Eight separate measurements were made for each analyte.

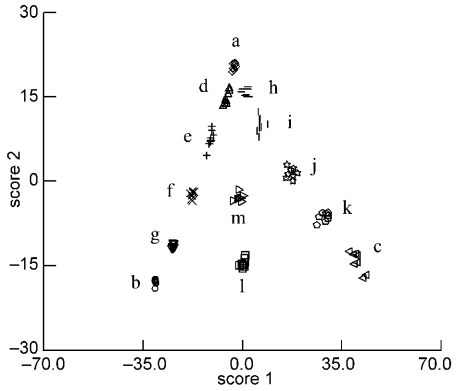

Figure 5. Two-dimensional LDA score plot for the analysis of UFH (a) and the contaminants DS (b) and OSCS (c). Mixtures of UFH with 10, 25, 50, and 75% DS (d–g) or OSCS (h–k) were analyzed, as well as 1:1 DS/OSCS (l) and 1:1:1 DS/OSCS/UFH (m). Eight separate measurements were made for each analyte and mixture.

Furthermore, a 1:1 mixture of OSCS and DS and a 1:1:1 mixture of all three analytes were tested. Remarkably, the resultant LDA score plot (Figure 5) enables the identification of both the ratio of the mixture and the identity of its components.[20] Discrimination with 100% accuracy was possible for samples with as little as 10% contamination; thus, this type of differential array has great potential for the quality control of heparin.

In summary, we have described a novel strategy for the rapid assembly of an array of supramolecular fluorescent receptors based on a modified cyclodextrin host–guest complex. The analytical power of these differential arrays was demonstrated by accurate discrimination between heparin and common potential contaminants of this important drug. As both **1a** and LCA are relatively easy to functionalize, other self-assembling fluorescent receptors based on this scaffold can be envisioned for a much broader variety of analytes.

Received: October 26, 2008
Published online: February 4, 2009

Keywords: fluorescence · heparin · molecular recognition · receptors · self-assembly

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[21] The predictive ability of the receptor array was determined by a cross-validation routine, in which 50% of the dataset was taken out randomly and used as a training set for the LDA, and the omitted remainder was reclassified.

