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## A Functional Assay for Heparin in Serum Using a Designed Synthetic Receptor\*\*

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A common objective when developing synthetic receptors is high binding affinity and specificity for an analyte. This aim is challenging when targeting a complex analyte in a competitive crude medium such as serum, urine, or saliva.<sup>[1]</sup> We report herein the development of a synthetic receptor with high affinity and selectivity in human and equine serum for the clinical anticoagulant heparin.<sup>[2]</sup>

Two forms of heparin are in clinical use: unfractionated heparin (UFH) with a molecular weight range from 3000-30000 Da, and low-molecular-weight heparin (LMWH) with a mean molecular weight of 5000 Da. Heparin concentration and activity is monitored during surgery, and in postoperative therapy, to prevent complications such as hemorrhaging.<sup>[3]</sup> Current methods for heparin quantification employ the activated clotting time (ACT), activated partial thromboplastin time (aPTT), chromogenic antifactor Xa assay, electrochemical and piezoelectric assays, and complexation with protamine.<sup>[4]</sup> Although these are proven methods, they are often arduous, inaccurate, costly, and not always amenable to clinical settings.<sup>[5]</sup> Recently, an engineered GST fusion protein containing three hyaluronan binding domains from a heparin binding protein was used to accurately measure heparin in plasma at clinically relevant values, but it has not been employed clinically.<sup>[6]</sup>

Heparin cannot be expressed exactly using a conventional chemical formula. It is a heterogeneous mixture of diverse chain lengths consisting of repeating copolymers of  $1\rightarrow 4$ -linked iduronic acid and glucosamine residues in a semi-random order (Figure 1b). Heparin has the highest anionic charge to mass ratio of any biopolymer, as a result of numerous sulfate and carboxylate functionalities in the biopolymer chain. Its activity occurs by binding to anti-thrombin III, a naturally occurring protease inhibitor, which accelerates the rate of inhibition of coagulation proteases factor Xa and thrombin by antithrombin III.<sup>[7]</sup> Clinically administered heparin binds to its natural substrate anti-thrombin III primarily through cationic ion-pairing interactions with the sulfate and carboxylate groups.<sup>[8]</sup> Similarly, the

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cationic protein protamine is an excellent ligand for heparin and is often used for the calibration of aPTT reagents. The highly anionic nature of heparin makes it an ideal target for synthetic receptors that incorporate functional groups for molecular recognition which participate in ion-pairing or other anion-binding interactions.

The design of heparin receptor **1** (Figure 1 a) builds upon the successes of synthetic receptors reported previously. For



Figure 1. a) Heparin receptor 1. b) Major unit of heparin.

example, we recently created receptor **2** (Figure 2), which incorporates phenylboronic acids and ammonium groups, for the complexation of anionic polysaccharides such as heparin, hyaluronic acid, and chondroitin-4-sulfate.<sup>[9]</sup> Phenylboronic acids with an *ortho*-aminomethyl group are known to form boronate esters with alcohols in aqueous media, and have been used extensively for the molecular recognition of saccharides.<sup>[10]</sup> It has been found that these boronate esters have particularly high affinity for anions with closely appended hydroxy groups.<sup>[11]</sup> Furthermore, ion pairing is anticipated between the ammonium functionalities and the carboxylate and sulfate groups. Receptor **2** had good selectivity for heparin relative to the other anionic polysaccharides. However, because heparin does not consist of a single entity, its concentration must be defined by considering



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*Figure 2.* Previously reported receptor that had insufficient sensitivity to be active and accurate in a biological medium.

various subunits so that a binding constant can be measured. A binding affinity of  $3.8 \times 10^4 \,\mathrm{m^{-1}}$  was calculated when using a heparin concentration that is defined by the concentration of each disaccharide unit. This affinity was insufficient to allow quantification of heparin in serum at physiologically relevant concentrations. Moreover, although an indicator-displacement assay with **2** gave a dramatic yellow to purple color change in response to heparin,<sup>[12]</sup> we found that the indicator bound nonspecifically with proteins in crude serum, and thereby skewed the quantitative results.

From the lessons gleaned from the studies using 2, we designed a second-generation heparin receptor with two goals in mind, both of which are embodied in the structure of 1. First, the cavity was enlarged to allow the arms containing the boronic acid and ammonium groups to encompass a larger surface of the oligosaccharide, which we predicted would raise the affinity by cooperatively increasing the number of interactions. Second, a fluorescent scaffold was incorporated into the design of the receptor, thereby avoiding the use of an indicator-displacement assay. This new signaling technique should also increase the overall sensitivity of the system. We used 1,3,5-triphenylethynylbenzene as the core unit to satisfy both requirements. However, because of the excellent selectivity previously found for 2 with heparin, we retained the side arms containing the boronic acid and ammonium groups.

An assay for UFH or LMWH in serum requires a binding interaction between 1 and heparin in the nM range. Titrations of 1 with UFH and LMWH in water buffered with 10 mm 2-[4-(2-hydroxyethyl]-1-piperazinyl]ethanesulfonic acid (HEPES) at pH 7.4 were monitored by fluorescence spectroscopy to determine the affinity of 1 for UFH and LMWH. The binding of UFH and LMWH with 1 caused a decrease in the emission intensity, which resulted in a near complete quenching of the receptor's emission. Presumably, the interaction of heparin with 1 leads to conformational restriction of the receptor "arms", thereby modulating the fluorescence-a technique used routinely by Finney and co-workers for creating chemosensors.<sup>[13]</sup> Titration data at 357 nm was used to generate a binding isotherm, which was analyzed by using a standard 1:1 binding algorithm (Figure 3). As discussed above, the heterogeneous structure of heparin means that one must define a



**Figure 3.** 1:1 binding isotherm for 1 and UFH. The  $K_a$  value obtained from the curve was  $1.4 \times 10^8 \, \text{m}^{-1}$ .

repeating unit with which the receptor interacts. The binding isotherm shown in Figure 3 was achieved by defining the concentration of heparin to be that of four saccharide units (an integral number of saccharides is not required to fit the binding isotherm). The number four supports a stoichiometry where each receptor on average spans four saccharide units along the heparin biopolymer. The calculated association constant between 1 and UFH is  $1.4 \times 10^8 \text{ m}^{-1}$ . This value corresponds to an increase in affinity of nearly  $10^4$  for 1 over 2, which was gained by increasing the size of the scaffold. It should be noted that the glycosaminoglycuronans hyaluronic acid and chondroitin-4-sulfate did not bind to 1 at low  $\mu m$ concentrations. This result is further evidence for the high selectivity of 1 for UFH and LMWH.

As noted above, several clinical methods (such as aPTT) are calibrated by titration with protamine. Protamine sequesters heparin, thereby lowering its bioavailability to bind antithrombin III. Therefore, if there is a specific binding interaction between heparin and 1 as postulated above, protamine should strip heparin from receptor 1, thereby restoring the fluorescence. Indeed, fluorescence could be fully reestablished by titration of the complex formed between receptor 1 and either UFH or LMWH with protamine (Figure 4). This observation illustrates that the binding between 1 and heparin is reversible, and acts in an analogous way to that between heparin and antithrombin III.

Our last study targeted the creation of calibration curves for monitoring UFH and LMWH in serum. Heparin is administered intravenously or subcutaneously at therapeutic dosing levels of  $2-8 \text{ UmL}^{-1}$  (0.8–3.2 µM) during cardiopulmonary surgery and emergency deep venous thrombosis (DVT) conditions to prevent excessive clotting. However, patients are treated at therapeutic dosing levels of 0.2- $2 \text{ UmL}^{-1}$  (0.08 µm–0.8 µm) in post-operative and long-term anticoagulant care of DVT. Human and equine serums were doped with UFH and LMWH at these dosing levels to simulate monitoring conditions in a clinical setting. A serum sample (32 µL) doped with UFH or LMWH was added to a fluorimeter cell containing a total volume of 1.5 mL HEPES (10 mM) in deionized water. To this was added  $2 \mu L$  of 1  $(2.24 \times 10^{-3} \,\mathrm{M}^{-1})$ . The fluorescence stabilized over a period of 18 minutes, which contrasts the instantaneous response found



Figure 4. Reversibility of LMWH:1 binding upon titration with protamine. a) Emission intensity at 357 nm. Dilution was carried out with aliquots of buffered water (HEPES, pH 7.4). First 11 aliquots of heparin were added (▲), and then 12 aliquots of protamine were added (●). The fluorescent emission was reestablished upon titration. b) Emission spectrum of 1 upon addition of LMWH. The addition of protamine reverses the spectrum.

in buffered water, and indicates that the formation of the complex with heparin in serum was slow on the laboratory timescale. This result is potentially a consequence of the kinetics of release of heparin from natural receptors in the sera. Emission spectra were recorded after 18 minutes for each of nine samples with various heparin concentrations to generate calibration curves (355 nm, Figure 5). Increased levels of heparin in serum correlated linearly with lower emission responses for both UFH and LMWH within the range of clinically relevant concentrations, as was observed for the fluorimetric titrations using pure heparin in buffered water. Furthermore, the method worked in both equine and human samples, thus illustrating that the affinity of the synthetic receptor for heparin is independent of the mammalian source, and could potentially be used for either human or veterinary applications.

In summary, we have demonstrated a functional synthetic fluorescent assay for the clinically administered anticoagulant heparin. The synthetic receptor highlighted herein showed remarkable selectivity and affinity for heparin, even in crude serum. The fluorescence emission was used to generate calibration curves for UFH and LMWH in serum at clinically relevant dosing levels  $(0.2-8.0 \text{ UmL}^{-1})$ . These calibration curves allow the heparin concentration in an unknown sample to be determined by comparative analysis of the fluorescence emission. This study demonstrates that synthetic receptors



**Figure 5.** Calibration curves for a) LMWH in human serum and b) UFH in human serum. Conformational changes to 1 upon binding to heparin result in a diminished fluorescence emission. Greater concentrations of UFH and LMWH result in increased fluorescent quenching. The range of detection for both UFH and LMWH was 0–9 UmL<sup>-1</sup> (0–3.6  $\mu$ M).

can be created to target very complex bioanalytes, and function successfully in physiological settings.

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