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A Colorimetric Sensing Ensemble for Heparin

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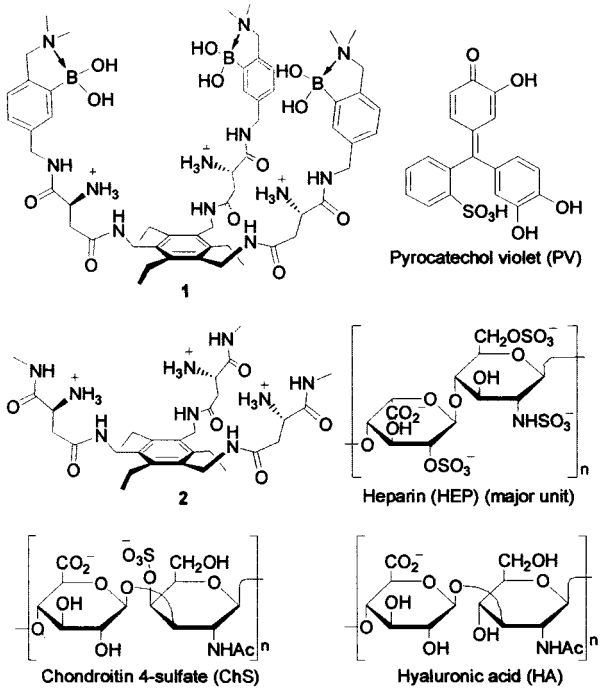
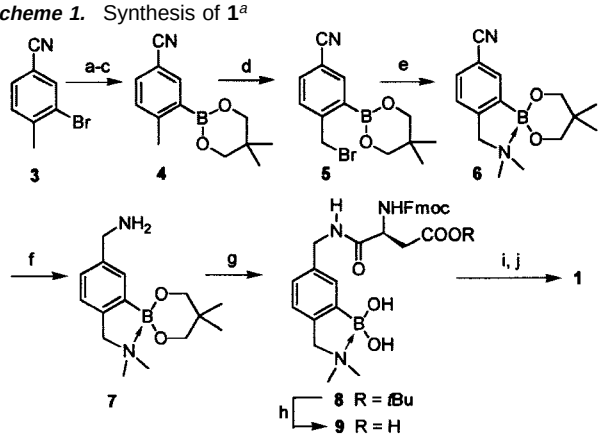
Anticoagulation therapy is used during cardiopulmonary bypass surgery to prevent thrombosis of the extracorporeal circuit and to mediate activation of the hemostatic system.[1] Heparin is the agent of choice because it is reversible, well-tolerated, inexpensive, and very effective. During surgery, it is commonly monitored using a process which entails determining the activated clotting time (ACT).[2] Typically, the ACT is measured by a physical means, such as mass deposition, plunger movement, or viscokinetic changes. More recently, some chemical-based sensing schemes have emerged. One measures heparin indirectly by monitoring its inhibition of thrombin activity on a flourogenic substrate,[3] while another uses a potentiometric signal generated from deposition of heparin into a membrane.[4] Although these assays are already in practice or undergoing clinical testing, a simple single-step colorimetric method could be useful and competitive with these other methods. Herein, we report the use of a designed receptor, possessing a novel amino acid with a boronic acid side chain, that when used in an indicator displacement assay[5] shows strong binding to heparin with good selectivity over glycosaminoglycans with lower anionic charge density.



Heparin is a highly negatively charged oligosaccharide mainly composed of repeating disaccharide units of 1→4 linked sulfated iduronic acid and sulfated glucosamine.[6] Anion−cation interaction plays a dominant role in heparin−protein interactions.[7] Boronic acids rapidly and reversibly form cyclic esters with diols in aqueous media and are widely used for binding and sensing of saccharides.[8] A peptide possessing both cationic and boronic acid binding sites should be a potential receptor for heparin and other oligomeric anionic saccharides. Thus, we designed and synthesized **1**, which contains ammoniums and an amino acid with a boronic acid side chain (Scheme 1). The hexasubstitution of the benzene in **1** enforces adjacent groups alternating up and down,[9] creating a preorganized cavity for binding interactions.[10]

Scheme 1. Synthesis of **1**[a]



[a] Conditions: (a) *n*-BuLi, THF, −98 °C, (b) $B(OMe)_3$, −98 °C to rt, (c) neopentyl glycol, toluene, reflux, 88%, (d) NBS, AIBN, CCl_4, reflux, (e) dimethylamine, ether, 78% (two steps), (f) 500 psi H_2, Raney-Ni, MeOH/NH_3, quantitative, (g) *N*-Fmoc-Asp(*O*-*t*-Bu)-OH, DCC/HOBt, CH_2Cl_2, 77%, (h) TFA, CH_2Cl_2/thioanisole, 93%, (i) 1,3,5-triaminomethyl-2,4,6-triethylbenzene, PyBOP, DIEA, DMF, (j) piperidine, DMF, 68%.

Indicator displacement assays are used to convert synthetic receptors into sensors without requiring the covalent introduction of a reporter moiety.[5] With these systems, analyte binding leads to indicator displacement from the binding cavity, which in turn yields an optical signal modulation. By using pyrocatechol violet as an indicator, the binding and sensing ability of **1** with heparin sodium salt (HEP), chondroitin 4-sulfate sodium salt (ChS), hyaluronic acid sodium salt (HA), and heparin disaccharide I-S was studied using UV−vis spectroscopy in a 1:1 (v/v) water/methanol solution buffered with 10 mM HEPES at pH 7.4. The binding of pyrocatechol violet with **1** causes a decrease around 430 nm and an increase around 526 nm in its absorbance spectra with an isosbestic point at 472 nm, and a color change from yellow to grayish purple. Computer fitting of the titration data with a typical 1:1 binding algorithm gave a binding constant of 7.1×10^3 M^{-1}.[11] As shown in Figure 1A, addition of heparin to a solution of **1** and the indicator causes an absorbance increase around 430 nm and a decrease around 526 nm, with a color change back to yellow, revealing that the indicator was displaced from the binding cavity of host **1** by the analyte. A control experiment showed that direct addition of heparin

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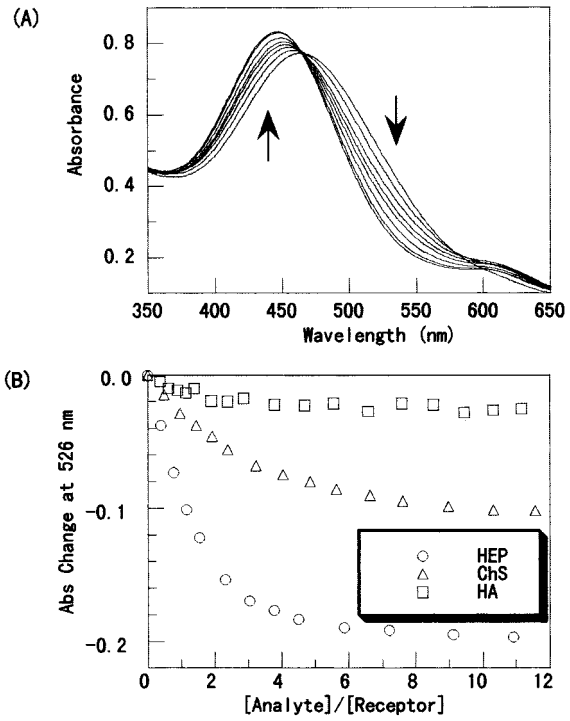

Figure 1. (A) Absorbance spectra of a 0.054 mM PV and 0.16 mM **1** solution in 1:1 H_2O/MeOH buffered with 10 mM HEPES at pH 7.4 in the presence of 0−1.75 mM HEP. (B) Absorbance change at 526 nm of the PV and **1** solution upon addition of different analytes.

Table 1. Binding Constants between **1** and Anionic Analytes[a,b]

analyte (as sodium salts)	binding constant (M^{-1})
heparin	3.8×10^4
chondroitin 4-sulfate	6.4×10^3
hyaluronic acid	*c*
heparin disaccharide I−S	6.2×10^3

[a] Measured by competitive spectrophotometry[11] in 1:1 (v/v) water/methanol buffered with 10 mM HEPES at pH 7.4. [b] Concentration of glycosaminoglycans refers to their disaccharide units. [c] Spectral change was too small to estimate a binding constant.

to the indicator does not change the absorbance spectrum at all. The binding constant (3.8×10^4 M^{-1}) between **1** and heparin was measured by competitive spectrophotometric method.[11] Addition of chondroitin or hyaluronic acid also decreases the absorbance around 526 nm, but the saturated absorbance changes are only about 60 and 10%, respectively, of that in the case of heparin (Figure 1B), and the binding constants are lower (Table 1). These binding constants are not for a discrete 1:1 stoichiometry, since undoubtedly more than 1 equiv of **1** can associate with each heparin strand. To measure a binding constant relevant to a 1:1 stoichiometry, we examined heparin disaccharide I-S. The disaccharide has a high affinity also, indicating that there is only a modest enhancement of the affinity due to polymerization.

The selectivity shown in Table 1 is related to the anionic charge density of the glycosaminoglycan analytes, that is, HEP > ChS > HA, suggesting that electrostatic interactions play a dominant role in the binding. To prove that the boronic acid groups in receptor **1** do play a role in the binding, compound **2** lacking the boronic acid groups was synthesized and used as a control. No significant change was observed on the spectrum of pyrocatechol violet upon the addition of **2**. Instead, alizarin complexone was used as an indicator, which gave a small change on the absorbance spectrum upon binding with **2**. However, no displacement took place when heparin was added to a solution of **2** and alizarin complexone. Therefore, **2** has far lower affinity than receptor **1** toward heparin.

In conclusion, a designed receptor possessing ammoniums and a novel boronic acid-containing amino acid shows good affinity and selectivity for heparin over similar polysaccharides possessing lower anionic charge density. The affinity for heparin is similar to that for a heparin disaccharide, indicating that disaccharidic units are the likely sites for binding of **1**. We are currently exploring the use of the colorimetric indicator displacement method in serum.

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Supporting Information Available: Experimental details for syntheses and characterization of **1** and **2**, absorbance spectral change of PV upon addition of **1**, and plots for determination of binding constants (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Despotis, G. J.; Gravlee, G.; Filos, K.; Levy, J. *Anesthesiology* **1999**, *91*, 1122−1151. (b) Hirsh, J.; Dalen, J.; Deykin, D.; Poller, L. *Chest* **1992**, *102*, 337S−351S.
(2) Gravlee, G. P.; Case, L. D.; Angert, K. C.; Rogers, A. T.; Miller, G. S. *Anesth. Analg.* **1988**, *67*, 469−472.
(3) (a) Mitchell, G. A.; Gargiulo, R. J.; Husbeby, R. M.; Lawson, D. E.; Pochron, S. P.; Schugner, J. A. *Thromb. Res.* **1978**, *13*, 47−52. (b) Umlas, J.; Taff, R. H.; Gauvin, G.; Sweirk, P. *Anesth. Analg.* **1983**, *62*, 1095−1099.
(4) (a) Mathison, S.; Bakker, E. *Anal. Chem.* **1999**, *71*, 4614−4621. (b) Ma, S.-C.; Yang, V. C.; Fu, B.; Meyerhoff, M. E. *Anal. Chem.* **1993**, *65*, 2078−2084.
(5) (a) Wiskur, S. L.; Ait-Haddou, H.; Lavigne, J. J.; Anslyn, E. V. *Acc. Chem. Res.* **2001**, *34*, 963−972. (b) Ait-Haddou, H.; Wiskur, S. L.; Lynch, V. M.; Anslyn, E. V. *J. Am. Chem. Soc.* **2001**, *123*, 11296−11297. (c) Wiskur, S. L.; Anslyn, E. V. *J. Am. Chem. Soc.* **2001**, *123*, 10109−10110. (d) Metzger, A.; Anslyn, E. V. *Angew. Chem., Int. Ed.* **1998**, *37*, 649−652. (e) Niikura, K.; Metzger, A.; Anslyn, E. V. *J. Am. Chem. Soc.* **1998**, *120*, 8533−8534.
(6) Casu, B. In *Advances in Carbohydrate Chemistry and Biochemistry*; Tipson, R. S., Horton, D., Eds.; Academic Press: Orlando, 1985; Vol. 43, pp 51−134.
(7) (a) Mulloy, B.; Linhard, R. J. *Curr. Opin. Struct. Biol.* **2001**, *11*, 623−628. (b) Hileman, R. E.; Fromm, J. R.; Weiler, J. M.; Linhardt, R. J. *Bioessays* **1998**, *20*, 156−167. (c) Sasisekharan, R.; Venkataraman, G. *Curr. Opin. Chem. Biol.* **2000**, *4*, 626−631.
(8) (a) James, T. D.; Sandanayake, K. R. A. S.; Shinkai, S. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1910−1922. (b) Sugasaki, A.; Sugiyasu, K.; Ikeda, M.; Takeuchi, M.; Shinkai, S. *J. Am. Chem. Soc.* **2001**, *123*, 10239−10244. (c) Cabell, L. A.; Monahan, M.-K.; Anslyn, E. V. *Tetrahedron Lett.* **1999**, *40*, 7753−7756. (d) Takeuchi, M.; Ikeda, M.; Sugasaki, A.; Shinkai, S. *Acc. Chem. Res.* **2001**, *34*, 865−873. (e) Mizuno, T.; Fukumatsu, T.; Takeuchi, M.; Shinkai, S. *J. Chem. Soc., Perkin Trans. 1* **2000**, 407−413. (f) Kobayashi, H.; Nakashima, K.; Ohshima, E.; Hisaeda, Y.; Hamachi, I.; Shinkai, S. *J. Chem. Soc., Perkin Trans. 2* **2000**, 997−1002. (g) Ikeda, M.; Shinkai, S.; Osuka, A. *Chem. Commun.* **2000**, 1047−1048. (h) James, T. D.; Shinkai, S. In *Topics in Current Chemistry*; Penadés, S., Ed.; Springer-Verlag: Berlin, Heidelberg, New York, 2002; Vol. 218, pp 159−200.
(9) (a) Iverson, D. J.; Hunter, G.; Blount, J. F.; Damewood, J. R.; Mislow, K. *J. Am. Chem. Soc.* **1981**, *103*, 6073−6083. (b) Kilway, K. V.; Siegel, J. S. *J. Am. Chem. Soc.* **1992**, *114*, 255−261. (c) Gottlieb, H. E.; Ben-Ary, C.; Hassner, A.; Marks, V. *Tetrahedron* **1999**, *55*, 4003−4014.
(10) (a) Jon, S. Y.; Kim, J.; Kim, M.; Park, S.-H.; Jeon, W. S.; Heo, J.; Kim, K. *Angew. Chem., Int. Ed.* **2001**, *40*, 2116−2119. (b) Kim, S.-G.; Ahn, K. H. *Chem. Eur. J.* **2000**, *6*, 3399−3403. (c) Chin, J.; Walsdorff, C.; Stranix, B.; Oh, J.; Chung, H. J.; Park, S.-M.; Kim, K. *Angew. Chem., Int. Ed.* **1999**, *38*, 2756−2758. (d) Tam-Chang, S.-W.; Stehouwer, J. S.; Hao, J. *J. Org. Chem.* **1999**, *64*, 334−335. (e) Hartshorn, C. M.; Steel, P. J. *Chem. Commun.* **1997**, 541−542. (f) Stack, T. D. P.; Hou, Z.; Raymond, K. N. *J. Am. Chem. Soc.* **1993**, *115*, 6466−6467. (g) Weizman, H.; Libman, J.; Shanzer, A. *J. Am. Chem. Soc.* **1998**, *120*, 2188−2189. (h) Rathore, R.; Lineman, S. V.; Kochi, J. K. *J. Am. Chem. Soc.* **1997**, *119*, 9393−9404. (i) Walsdorff, C.; Saak, W.; Pohl, S. *J. Chem. Soc., Dalton Trans.* **1997**, 1857−1861. (j) Szabo, T.; O'Leary, B. M.; Rebek, J., Jr. *Angew. Chem., Int. Ed.* **1998**, *37*, 3410−3413. (k) Hoskins, B. F.; Robson, R.; Slizys, D. A. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2752−2755.
(11) Conners, K. A. *Binding Constants, The Measurement of Molecular Complex Stability*; John Wiley and Sons: New York, 1987.

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