JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Mallard Blue: A High-Affinity Selective Heparin Sensor That Operates in Highly Competitive Media

Stephen M. Bromfield,[†] Anna Barnard,[†] Paola Posocco,[‡] Maurizio Fermeglia,[‡] Sabrina Pricl,[‡,§] and David K. Smith*,[†]

[†]Department of Chemistry, University of York, Heslington, York, YO10 5DD, U.K.

[‡]Molecular Simulation Engineering (MOSE) Laboratory, Department of Industrial Engineering and Information Technology (DI3), University of Trieste, 34127 Trieste, Italy

[§]National Interuniversity Consortium for Material Science and Technology (INSTM), Research Unit MOSE-DEA, University of Trieste, Trieste, Italy

Supporting Information

ABSTRACT: We report the simple synthesis and full investigation of a novel heparin binding dye, mallard blue, an arginine-functionalized thionine. This dye binds heparin in highly competitive media, including water with high levels of competitive electrolyte, buffered aqueous solution and human serum. The dye reports on heparin levels by a significant change in its UV−vis spectroscopic profile. Molecular dynamics modeling provides detailed insight into the binding mode. Heparin binding is shown to be selective over other glycosaminoglycans, such as hyaluronic acid and chondroitin sulfate. Importantly, we demonstrate that, in the most competitive conditions, mallard blue outperforms standard dyes used for heparin sensing such as azure A.

Heparin is the most charge dense naturally occurring polyanion in biological systems (Figure 1),[1] and is used as an anticoagulant drug, for example, during surgery. As a result of its clinical importance, there has been a surge of interest in developing heparin sensors which operate under biologically relevant conditions and in highly competitive media. Perhaps the leading strategy has been the development of cationic indicator dyes which can report on heparin spectroscopically. Simple cationic dyes such as methylene blue[2] and azure A[3] exhibit a UV response to heparin in noncompetitive conditions but lose efficacy as electrolytic competition increases. Examples of heparin sensors able to detect heparin in more competitive media include the landmark boronic acid functionalized tricationic heparin binding scaffold from Anslyn and co-workers which worked in serum.[4] A range of other heparin sensors have also been recently reported based on a variety of different chemistries.[5] To a greater or lesser extent, these sensors indicate the presence, or sometimes the quantity/concentration, of heparin in a given sample within a variety of media although not always within the clinically relevant concentration range. Two of the most impressive heparin sensors to date, heparin blue and heparin orange, were discovered by Wang and Chang and operate in biological media.[6] However, one of the factors limiting the widespread use of many heparin sensors is the multistep syntheses which are required. As such, much attention still focuses on commercially available systems such as methylene blue[2] and azure A.[3] Here, we report a novel heparin sensor, which operates in human serum, and can be made in two facile synthetic steps from commercial starting materials.

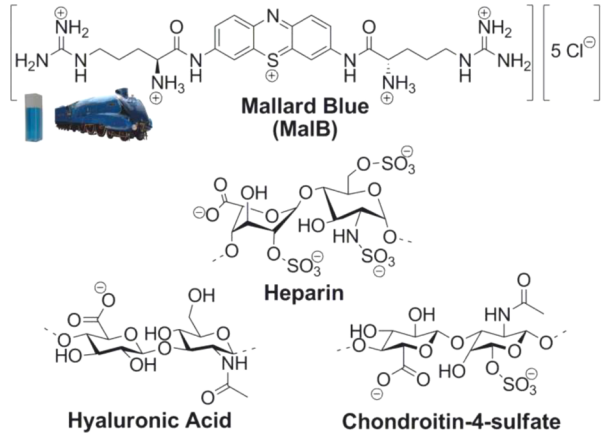

Figure 1. Three prevalent glycosaminoglycans (GAGs) tested in this study: heparin, hyaluronic acid, and chondroitin-4-sulfate. The structures represent the predominant "average" repeat unit present in these GAGs, which actually have random sequences, with biological activity being associated with well-defined and specific saccharide sequences (a pentasaccharide in the case of heparin).

We considered a range of different synthetic heparin binders,[7] and in our wish to employ a biomimetic approach, we looked to the way in which proteins bind heparin,[8] and thus chose arginine as an ideal binding component for a robust heparin sensor. As a dye unit, we chose thionine acetate, a structural analogue of commercially available heparin-binding dyes, methylene blue and azure A, and which contains two amines attached to a phenothiazine-like chromophoric core. As such, we considered our new dye might be a significantly enhanced member of this family of heparin-binding dyes. Thionine acetate was a suitable chromogenic building block (Figure 2) for the attachment of two arginine units. Tri-Boc-protected arginine was readily appended to the core in a TBTU-mediated peptide coupling reaction to

Received: December 4, 2012

   dx.doi.org/10.1021/ja311734d | J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

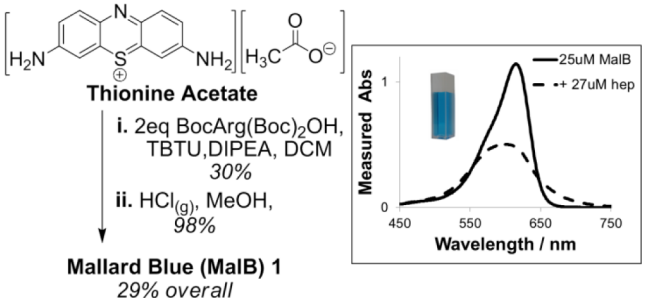

Figure 2. Synthetic scheme for new dye mallard blue (MalB, **1**) along with the UV−visible absorption band at 615 nm (25 μM, photo) before (solid line) and after (dashed line) binding to heparin.

afford, after Boc deprotection with HCl gas, the new dye **1**. The 29% yield, while rather low, is an improvement on previous attempts to functionalize thionine,[9] and the simple synthesis from commercial materials is highly attractive. We named this new dye mallard blue (MalB).[10]

Mallard blue has a UV−vis absorbance maximum at 615 nm and an optimized concentration of 25 μM in the presence of 150 mM NaCl, and 10 mM Tris-HCl gave a suitable absorbance intensity after incubation for 24 h at 50 °C to ensure the absence of aggregates. Titration of heparin into this solution reduces the absorbance intensity as the previously unbound MalB binds to the heparin, altering its optical properties (Figure 2). A 25 μM MalB solution was saturated by 27 μM of disaccharide,[11] indicating that ∼1 equiv of MalB binds to each disaccharide unit. This is equivalent to ∼5 cationic charges binding to 4 anionic charges, a ratio of ∼1.2:1.[12]

To better understand the interaction between MalB and heparin, we applied molecular dynamics (MD) modeling methods which allowed us to visualize an optimized binding mode as illustrated in Figure 3. Analysis of the MD trajectory

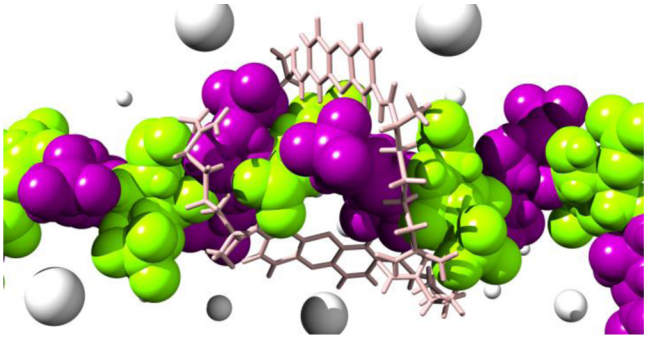

Figure 3. Equilibrated MD snapshot of the MalB-heparin system. MalB, rosy brown sticks; heparin (D-glucosamine units), dark magenta spheres; and CPK (L-iduronic acid units), chartreuse spheres. Some Cl^- and Na^+ ions are shown as large and small white spheres, respectively. Water omitted for clarity.

suggests that two MalB molecules can interact strongly with a tetrasaccharide sequence of heparin. This is in line with the experimental observations of stoichiometry, indicating a ∼1:1 ratio between cationic and anionic charges. Owing to the extensive charge complementarity between MalB and heparin, the binding is, as might be expected, dominated by electrostatics. The guanidinium cations anchor the dye molecules to heparin by closely interacting with the negatively charged $-COO^-$ and $-OSO_3^-$ groups of the two terminal tetrasaccharide sugars. The other charged and polar groups on MalB ($-NH_3^+$, S^+) and on the sugars ($-COO^-$, $-OSO_3^-$, -OH) are then brought in close contact. The limited flexibility of the polysaccharide chain means these groups assume their best configurations to maximize ion pairing and hydrogen bonding (also mediated by solution counterions) without incurring enthalpy and entropy penalties associated with poor organization.

Although the primary binding mode is electrostatic, MalB is particularly well optimized for heparin binding; indeed as seen below, it is highly tolerant of very large quantities of electrolyte. Furthermore, detailed modeling indicates that the binding configuration is oriented and organized by a number of neutral hydrogen bonds. It was also noted from the modeling that all of the side chains of MalB are directly involved in heparin binding, with the terminal groups playing a key role in the initial anchoring and alignment of the MalB molecules to the biomacromolecule (see Supporting Information (SI) for more detail). Experimentally, the particular optimization of MalB for heparin binding, and the fact that simple charge−charge electrostatic interactions are not the only factor which enable effective binding, were demonstrated by the observation that an equimolar amount of an similarly charged cationic species (e.g., tetra-cationic generation zero PAMAM dendrimer) was effectively unable to sigificantly displace MalB from its complex with heparin (see SI).

To experimentally probe the tolerance of MalB binding to heparin to highly competitive conditions, the effect of increasing electrolyte/buffer concentration was determined. A solution of heparin-saturated MalB (25 μM MalB, 27 μM disaccharide) was titrated with increasing amounts of NaCl or Tris-HCl up to a final concentration of 1 M. Figure 4 presents the percentage

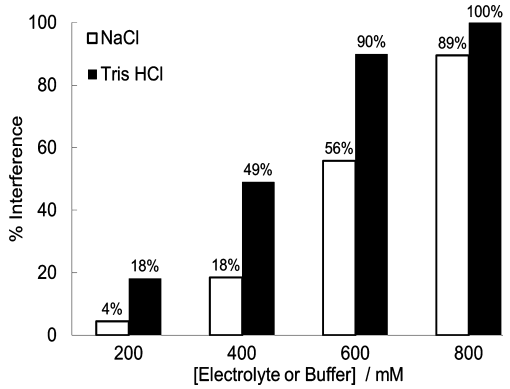

Figure 4. Extent to which electrolyte (open bars) or buffer (filled bars) disrupts the MalB-heparin interaction compared to binding in pure water as quantified by the absorption from UV−vis spectroscopy at 615 nm.

interference with binding caused by the addition of either electrolyte or Tris-HCl buffer. Notably, at 200 mM NaCl, there is very little perturbation of the binding, even though sodium cations are present in a 1600-fold excess compared with the charge on MalB itself. In general, the buffer causes slightly greater interference than sodium chloride, but the interaction between MalB and heparin remained very significant and easily measurable with excellent dynamic range up to concentrations of 400 mM NaCl and 200 mM Tris-HCl. Detectable binding was still observed even under much more competitive conditions. This indicates that the MalB-heparin interaction is highly

dx.doi.org/10.1021/ja311734d | J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

tolerant of both electrolyte and buffer, and can potentially be useful under very competitive conditions.

One of the greatest challenges for any heparin sensor operating in biological media is selectivity. In addition to heparin, many other electrolytes and polyanions are potential competitors for binding. In particular, other glycosaminoglycans (GAGs) are likely to be targeted by any heparin binder due to their similar structures.[13] In addition to heparin, there are five other structurally related GAGs: heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate (CS), keratin sulfate (KS), and hyaluronic acid (HA). For this work, however, in line with benchmark heparin binding studies,[4b] HA and CS were selected for titration into MalB to probe their binding interactions relative to heparin and hence determine the degree of selectivity. In turn, each GAG was titrated into a solution of MalB (25 μM, 150 mM NaCl, 10 mM Tris-HCl) and the resulting absorbance at 615 nm was plotted (Figure 5). Neither HA nor CS was able to perturb

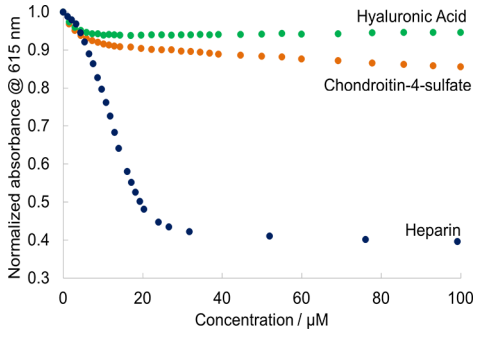

Figure 5. Normalized response of mallard blue absorbance at 615 nm with increasing GAG concentrations.

the UV−vis spectrum of MalB to any significant extent; heparin induced a much larger response. Of HA and CS, MalB interacts more significantly with CS. This may be either a consequence of CS containing a sulfate group or, more likely, the overall greater anionic charge density of CS compared with HA. In any case, this study indicates that MalB acts as a selective sensor for heparin over CS and HA, potentially competitive GAGs.

Obviously, the data in Figure 5 could be used to calculate effective binding constants, however, given that heparin is a polydisperse saccharide, with variable repeat units, we opted not to do this. It is hard to define meaningful concentrations, or to be certain about the relative binding of MalB to different parts of the heparin polysaccharide structure and as such, the resulting binding affinity is ambiguous. We reason that the polydisperse nature of heparin also explains the slightly sigmoidal nature of the binding curve between MalB and heparin, as there is clearly not going to be a single binding mode or well-defined stoichiometry that can be applied to every part of the polysaccharide.

Given the selectivity and high apparent affinity of the MalB-heparin interaction, we decided to probe the ability of this dye to operate in highly competitive human serum. Typically human serum contains competitor electrolytes including, most significantly, 150 mM Na^+, 110 mM Cl^- and 25 mM HCO_3^-, in addition to significant levels of proteins such as serum albumins, many of which are charged.[14] In this experiment, a sample of 100% human serum (0.5 mL) containing heparin of a known concentration was added to a buffered aqueous solution of MalB (1.5 mL, 25 μM in Tris-HCl, 20 mM). The response of the visible band of MalB at 615 nm was used to detect heparin. Figure 6 shows that under these conditions, MalB could detect heparin at

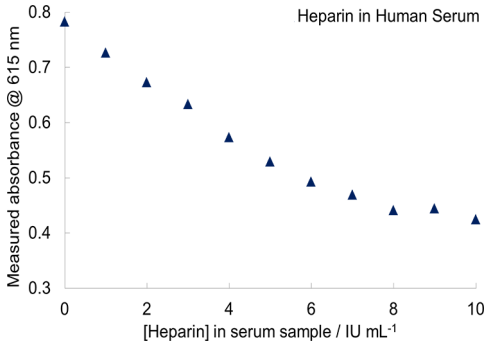

Figure 6. Detection of heparin in 100% serum added to a sample of MalB, as monitored by the UV−vis absorbance band of MalB at 615 nm.

concentrations as low as 1 IU/mL.[15] By changing the precise assay conditions (concentrations of MalB, etc.), the detection range can easily be tuned to detect (e.g.) higher serum levels of heparin. In addition to operating in human serum, we demonstrated that MalB detects heparin at an equivalent concentration range in horse serum (data not shown).

We then decided to benchmark the performance of our dye against a literature standard commercially available system. Azure A (AA) has been reported to detect heparin in human plasma.[3a] However, we were somewhat surprised that this relatively low-charged dye (when compared with MalB) could apparently still operate under such competitive conditions. Interestingly, the binding of AA to hyaluronic acid[16] and heparin[3b] has been probed previously, with studies showing increasing electrolyte concentration disrupted the AA-substrate interaction. pH was also demonstrated to affect the response of AA regardless of which absorption maximum was monitored. Notably, the previous work of Klein et al.[3a] "in plasma" was conducted exclusively by adding plasma to the dye in unbuffered distilled water, which could lead to significant pH change-induced problems.

The performance of MalB was compared to AA in like-for-like buffered conditions of our assay: namely, 25 μM AA, 20 mM Tris-HCl and heparin dissolved in 100% human serum. As shown in Figure 7, AA exhibited no change upon titration of serum containing differing amounts of heparin. This remains the case irrespective of the wavelength chosen to monitor the dye. Even in the absence of buffer, AA still does not respond to heparin under these conditions. We therefore reason that, under these challenging conditions, MalB is able to outperform "standard" dyes in this class such as AA. We conclude that detection

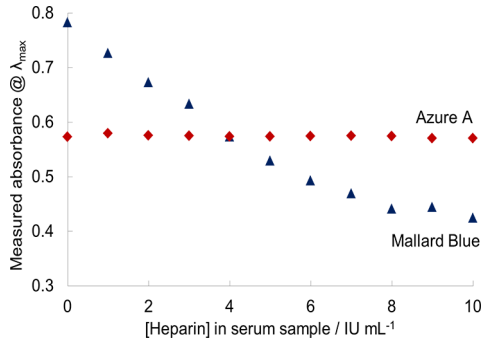

Figure 7. Graph showing measured UV absorbance at λ_{max} for mallard blue (λ_{max} 615 nm) and azure A (λ_{max} 634 nm) upon titration of heparin-containing serum.

dx.doi.org/10.1021/ja311734d | J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

therefore occurs in the clinically relevant range, it is the best-in-class for this family of dyes, and is comparable with the best previously reported synthetic heparin sensors.

In summary, this paper reports the facile synthesis of a cationic dye which exhibits high affinity for heparin, and is able to sense the presence of this anionic polysaccharide via simple UV−vis measurements at heparin concentrations as low as 1 IU/mL in 100% human serum. Computer MD simulations were employed to determine a plausible binding mode of MalB with heparin, and probe the underlying, stabilizing intermolecular interactions of the resulting complex, which demonstrate the key role of the guanidinium cations in anchoring the binding to polyanionic heparin, and confirm the experimentally observed stoichiometry. We have demonstrated that the dye shows a selective response to heparin, being largely unaffected by hyaluronic acid or chondroitin sulfate. Furthermore, it still operates when the heparin is added in 100% human or horse serum to a buffered solution of the dye. This is in contrast to some traditional heparin sensors such as azure A which would not work in our assay under the same conditions. As such, we suggest that MalB is one of the most easily synthesized, is the best-in-class for this family of dyes, and indeed is one of the highest-affinity heparin binders/sensors reported. Although MalB shows excellent performance, we anticipate that further work in the development of heparin sensors will include systems which have switch-on activity in the presence of heparin, rather than switch-off as reported for MalB, and those which can be used at multiple wavelengths in a ratiometric sense to allow quantitative studies in a dynamic setting.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic methods and characterization data for MalB; full details of the assay methods; details of MD methods and data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

david.smith@york.ac.uk

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was funded by BBSRC and supported in part by COST Network TD0802 (Dendrimers in Biomedicine). P.P., M.F., and S.P. acknowledge ESTECO for financial support (DDOS project). The HPC-Europa 2 Project (CINECA Supercomputing Center grant in the EU 7th Framework Program), and "MONALISA" (Iscra supercomputing grant to S.P.) is also gratefully acknowledged.

■ REFERENCES

(1) (a) Rabenstein, D. L. *Nat. Prod. Rep.* **2002**, *19*, 312. (b) Middeldorp, S. *Thromb. Res.* **2008**, *122*, 753. (c) Gandhi, N. S.; Mancera, R. L. *Drug Discovery Today* **2010**, *15*, 1058.

(2) Jiao, Q. C.; Liu, Q.; Sun, C.; He, H. *Talanta* **1999**, *48*, 1095.

(3) (a) Klein, M. D.; Drongowski, R. A.; Linhardt, R. J.; Langer, R. S. *Anal. Biochem.* **1982**, *124*, 59. (b) Jiao, Q.; Liu, Q. *Anal. Lett.* **1998**, *31*, 1311.

(4) (a) Zhong, Z.; Anslyn, E. V. *J. Am. Chem. Soc.* **2002**, *124*, 9014. (b) Wright, A. T.; Zhong, Z.; Anslyn, E. V. *Angew. Chem., Int. Ed.* **2005**, *44*, 5679.

(5) (a) Szelke, H.; Schubel, S.; Harenberg, J.; Kramer, R. *Chem. Commun.* **2010**, *46*, 1667. (b) Wang, M.; Zhang, D.; Zhang, G.; Zhu, D. *Chem. Commun.* **2008**, 4469. (c) Zeng, L.; Wang, P.; Zhang, H.; Zhuang, X.; Dai, Q.; Liu, W. *Org. Lett.* **2009**, *11*, 4294. (d) Pu, K.-Y.; Liu, B. *Macromolecules* **2008**, *41*, 6636. (e) Briza, T.; Kejik, Z.; Cisarova, I.; Kralova, J.; Martasek, P.; Kral, V. *Chem. Commun.* **2008**, 1901. (f) Cai, L.; Zhan, R.; Pu, K.-Y.; Qi, X.; Zhang, H.; Huang, W.; Liu, B. *Anal. Chem.* **2011**, *83*, 7849. (g) Cao, R.; Li, B. *Chem. Commun.* **2011**, *47*, 2865. (h) Gu, X.; Zhang, D.; Zhang, D. *Analyst* **2012**, *137*, 365. (i) Climent, E.; Calero, P.; Marcos, M. D.; Martinez-Manez, R.; Sancenon, R.; Soto, J. *Chem.—Eur. J.* **2009**, *15*, 1816. (j) Yeung, M. C.-L.; Yam, V. W.-W. *Chem.—Eur. J.* **2011**, *17*, 11987.

(6) Wang, S.; Chang, Y.-T. *Chem. Commun.* **2008**, 1173.

(7) (a) Wakefield, T. W.; Andrews, P. C.; Wrobleski, S. K.; Kadell, A. M.; Fazzalari, A.; Nichol, B. J.; Van der Kooi, T.; Stanley, J. C. *J. Surg. Res.* **1994**, *56*, 586. (b) Kikura, M.; Lee, M. K.; Levy, J. H. *Anesth. Analg.* **1996**, *83*, 223. (c) Choi, S.; Clements, D. J.; Pophristic, V.; Ivanov, I.; Vemparala, S.; Bennett, J. S.; Klein, J. L.; Winkler, J. E.; De Grado, W. E. *Angew. Chem., Int. Ed.* **2005**, *44*, 6685. (d) Mecca, T.; Consoli, G. M. L.; Geraci, C.; La Spina, R.; Cunsolo, F. *Org. Biomol. Chem.* **2006**, *4*, 3763. (e) Schuksz, M.; Fuster, M. M.; Brown, J. R.; Crawford, B. E.; Ditto, D. P.; Lawrence, R.; Glass, C. A.; Wang, L.; Tor, Y.; Esko, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 13075. (f) Udit, A. K.; Everett, C.; Gale, A. J.; Kyle, J. R.; Ozkan, M.; Finn, M. G. *ChemBioChem* **2009**, *10*, 503. (g) Gale, A. J.; Elias, D. J.; Averell, P. M.; Teirstein, P. S.; Buck, M.; Brown, S. D.; Polonskaya, Z.; Udit, A. K.; Finn, M. G. *Thromb. Res.* **2011**, *128*, E9−E13. (h) Rodrigo, A. C.; Barnard, A.; Cooper, J.; Smith, D. K. *Angew. Chem., Int. Ed.* **2011**, *50*, 4675. (i) Kalaska, B.; Sokolowska, E.; Kaminski, K.; Szczubialka, K.; Kramkowski, K.; Mogielnicki, A.; Nowakowska, M.; Buczko, W. *Eur. J. Pharmacol.* **2012**, *686*, 81.

(8) (a) Cardin, A. D.; Weintraub, H. J. R. *Arteriosclerosis* **1989**, *9*, 21. (b) Margalit, H.; Fischer, N.; Ben-Sasson, S. A. *J. Biol. Chem.* **1993**, *268*, 19228. (c) Gandhi, N. S.; Mancera, R. L. *Chem. Biol. Drug Des.* **2008**, *72*, 455.

(9) Williams, T. T.; Dohno, C.; Stemp, E. D. A.; Barton, J. K. *J. Am. Chem. Soc.* **2004**, *126*, 8148.

(10) The "Mallard" steam locomotive can be found in the National Railway Museum in York. This blue steam locomotive holds the world record for being the fastest steam-powered railway engine in the world.

(11) The reported heparin concentration is based on the amount of disaccharide. It should be noted that, as supplied, heparin only contains ∼30−40% of material with the active sequence of repeat units. However, all of the sample contains anionic saccharide units which can bind to MalB, even if they are in the wrong sequence. Hence, to best evaluate binding stoichiometries, we report the total concentration of the anionic disaccharide, irrespective of whether it is present in the active form of heparin or not. However, all presented data which refers to the "dose" of binders (in mg/units) refers to their ability to bind *only* the specific clinically active heparin.

(12) For clarity, during calculations, the molecular weight of the heparin disaccharide is assumed as that of the sodiated analogue of the heparin repeat unit shown in Figure 1: namely 665.40 g mol^{-1}.

(13) Barbucci, R.; Magnani, A.; Lamponi, S.; Albanese, A. *Polym. Adv. Technol.* **1996**, *7*, 675.

(14) Rhoades, R. A.; Bell, D. R. *Medical Physiology: Principles for Clinical Medicine*; Lippincott, Williams and Wilkins: Philadelphia , PA, 2008.

(15) IU is an international unit for heparin activity in a standard anticoagulation assay, and is the clinically relevant parameter when heparin is used *in vivo*.

(16) Chen, Q.; Li, X. L.; Liu, Q.; Jiao, Q. C.; Cao, W. G.; Wan, H. *Anal. Bioanal. Chem.* **2005**, *382*, 1513.