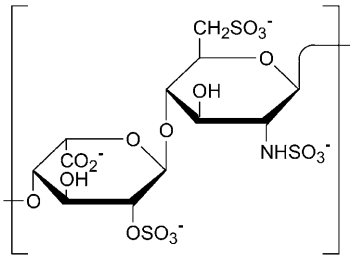
DOI: 10.1002/cbic.200800420

# Nanomolar Heparin Detection with an Artificial Enzyme

Laurent Vial and Pascal Dumy*[a]

Heparin is an anionic and polymeric glycosaminoglycan that is clinically used as an anticoagulant (Scheme 1).[1] Its activity




**Scheme 1.** Major unit of heparin.

occurs by binding to the protease inhibitor antithrombin III (AT-III). After heparin binding, AT-III undergoes a conformational change and is converted into a more potent inhibitor that inactivates proteases involved in blood clotting, most notably thrombin and factor Xa. Heparin is administered at dosing levels as low as 2 U mL$^{-1}$ (800 nM) during surgery and emergency deep veinous thrombosis (DVT), and 0.2 U mL$^{-1}$ (80 nM) in post-operative and long-term DVT prophylaxis. Heparin levels must be closely monitored to prevent complications (for example, haemorrhaging).[2] Methods for heparin quantification include the measurement of the activated clotting time, activated partial thromboplastin time, chromogenic antifactor Xa assays, electrochemical assays, and complexation with protamine.[3] However, these methods are generally imperfect due to their inaccuracy, cost or difficulties in implementation in a clinical environment.[4] Recently, the use of designed synthetic receptors for heparin that incorporate a fluorescent reporter of the binding event has emerged as an interesting alternative for heparin sensing.[5]

We recently reported that the cationic miniature esterase **1** (Figure 1) is able to catalyse the hydrolysis of an anionic fluorogenic ester (**2**) to give the fluorescent alcohol **3** (Scheme 2).[6,7] The catalytic activity was credited to either nucleophilic (or general-base) catalysis, which was assisted by electrostatic molecular recognition of the substrate. It was shown that the appearance of the fluorescent signal during the course of the reaction could be selectively modulated in the presence of anionic metabolites, which act as inhibitors. We decided to apply this original detection strategy, which is based on the inhibition of an artificial enzyme, to the sensing of heparin.

[a] *Dr. L. Vial, Prof. P. Dumy*
*Département de Chimie Moléculaire, UMR CNRS 5250*
*ICMG FR-2607, Université Joseph Fourier*
*301 rue de la chimie, BP53, 38041 Grenoble Cedex 9 (France)*
*Fax: (+33) 4-7651-4946*
*E-mail: pascal.dumy@ujf-grenoble.fr*

Because short cyclic or linear peptides that incorporate several lysine/arginine residues are known to efficiently bind heparin through electrostatic interactions,[8] we initially imagined that the highly anionic nature of heparin would make it an efficient inhibitor for our cationic enzyme mimic **1**, thus allowing its detection and quantification. However, the kinetic study of the hydrolysis of **2** in the presence of **1** at pH 7.4 revealed a modest substrate binding value of $K_M = 0.14$ mM.[6] We thus realized that **1** would probably not be able to bind and therefore to catalyse the hydrolysis of **2** in the low-concentration conditions that are necessary for the detection of heparin in clinical settings (vide supra). For this reason, we designed cyclic peptide **4** (Figure 1), which was built upon the success of miniature esterase **1**. Like peptide **1**, peptide **4** incorporates a histidine amino acid as a nucleophilic/basic centre, and a larger number of cationic lysine residues (that is, seven vs. three in peptide **1**). We expected that these additional positive charges would lead to a much more efficient binding of: 1) ester **2**, and as a consequence would allow our peptide to catalyse its

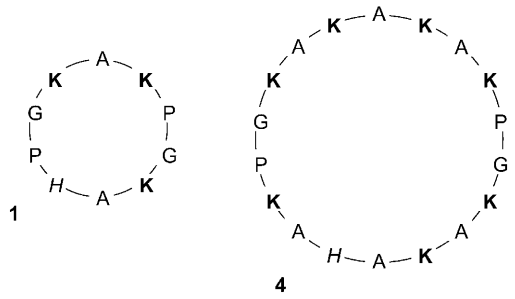
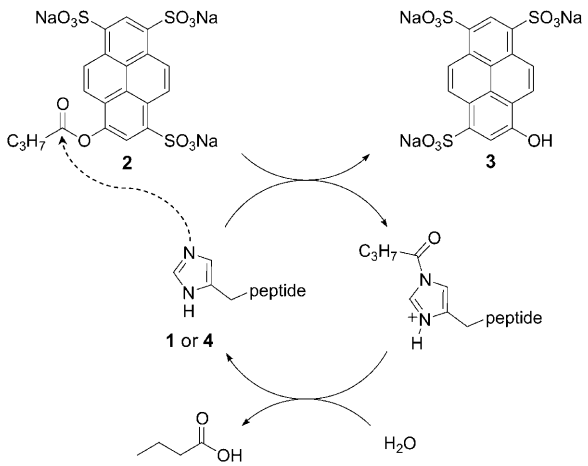



**Figure 1.** Catalytic peptides **1** and **4**. Nucleophilic/basic and cationic amino acids are highlighted in italic and bold respectively.




**Scheme 2.** Miniature esterase-catalysed ester hydrolysis.

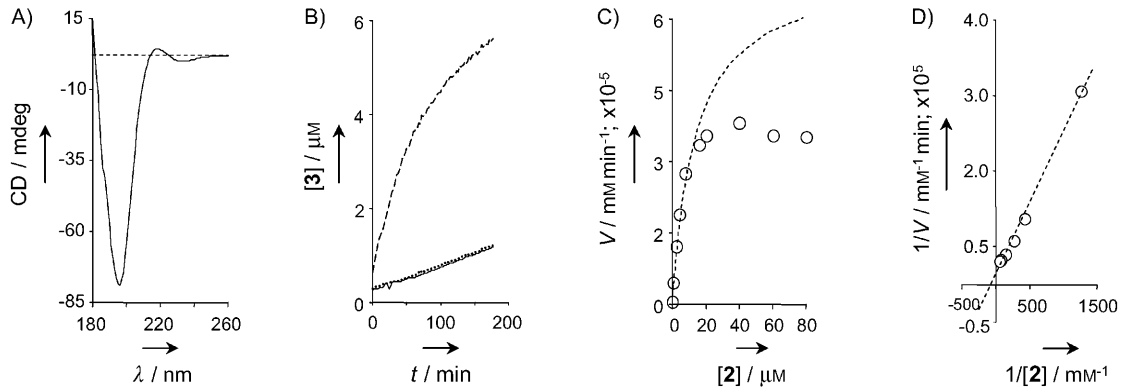



**Figure 2.** A) Far-UV circular dichroism spectrum of **4** in $H_2O$ (0.5 mg mL$^{-1}$). B) Time plot formation of **3** from hydrolysis of ester **2** (20 μM) in the presence of catalytic peptide **4** (-----) or 4-methyl-imidazole (····), and background reaction (——). C) Substrate inhibition of peptide **4**-catalysed hydrolysis of ester **2**. Dotted line: hyperbolic curve fitted with the Michaelis–Menten model after truncating the inhibited rates at high substrate concentrations. D) Lineweaver–Burk plot of the hydrolysis of ester **2** with catalytic peptide **4**. General conditions: 0.5 μM catalyst, 25 °C, 20 mM Tris buffer pH 7.4.

hydrolysis at very low concentration, and 2) heparin, therefore increasing the ability of this one to inhibit the hydrolysis reaction.

Synthesis of peptide **4** was carried out as reported previously for its analogue **1** (see the Experimental Section).[6] Qualitative indication of the secondary structure of **4** was provided by its far-UV circular dichroism spectrum in water (Figure 2A). Peptide **4** exhibited an unexpected spectrum with minima at $\lambda = 195$ nm, which is indicative of a random-coil conformation in solution. Such a result was surprising with regard to the existence of two type II β-turn inducers within the peptide sequence, which usually constrain peptides into an antiparallel β-sheet conformation.[9] This behaviour was attributed to the cationic lysine residues that probably destabilize a potential β-sheet conformation through charge repulsion between the strands.

Hydrolysis of **2** in water at pH 7.4 was followed by fluorescence, and enzyme-like kinetics were observed in the presence of **4** with multiple-turnover activity (Figure 2B). Kinetic data analysis showed that the velocity of the reaction drops at high substrate concentration (Figure 2C). This is indicative of substrate inhibition when two substrate molecules bind to the active site at the same time.[10] This behaviour is good agreement with the relative number of positive charges in peptide **4** (i.e., seven) and negative charges in ester **2** (i.e., three). Only the straight line section of the Lineweaver–Burk plot (Figure 2D) was used to determine, pseudo-first-order rate constant of $k_{cat} = 0.107$ min$^{-1}$, and rate enhancement of $k_{cat}/k_{uncat} = 1130$. We measured an efficient substrate binding value of $K_M = 9.8$ μM, which is one order of magnitude below the value obtained with **1**. This result validated our catalyst design. Under the same conditions, catalysis by 4(5)-methylimidazole as a reference catalyst was extremely inefficient (Figure 2B). Catalytic peptide **4** is 992-fold more active that 4(5)-methylimidazole as measured by the ratio of the specificity constant $k_{cat}/K_M$ to the second-order rate constant for 4(5)-methylimidazole catalysis $k_{MeIm}$.

Inhibition studies have shown that heparin was able to efficiently modulate the appearance of the fluorescence when **4** was used as catalyst (Figure 3A).[11] In conditions where peptide **2** and ester **4** were used at sufficiently low concentration (i.e., 0.5 and 12 μM respectively), heparin could be detected with a remarkable 13 nM sensitivity; that is among the lowest detection limits reported so far for fluorescent heparin sensors and is markedly below the lowest administered doses of heparin.[5] Such a sensitivity is a clear advantage for sensing experiments because it allows the preliminary treatment of complex media like biological fluids and, as a result, their dilution (vide infra). A linear response could be observed in the concentration range from 13 to 200 nM heparin, permitting therefore the generation of a calibration curve for the quantitative meas-

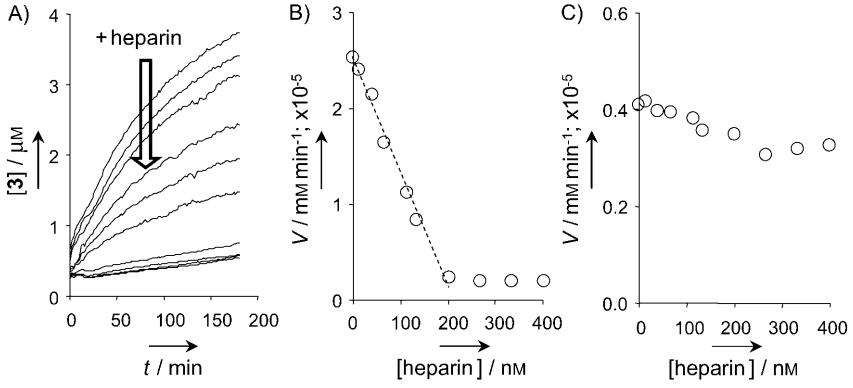



**Figure 3.** A) Time plot formation of **3** in presence of catalytic peptide **4** and increasing amount of heparin (0–400 nM). B) Initial rate of production of **3** vs. heparin concentration plot in the presence of catalytic peptide **4**. C) Initial rate of production of **3** vs. heparin concentration plot in the presence of catalytic peptide **1**. General conditions: 0.5 μM peptide **1** or **4**, 12 μM ester **2**, 25 °C, 20 mM Tris buffer pH 7.4.

urements of heparin levels (Figure 3B). Interestingly, less than a stoichiometric equivalent (i.e., 0.4 equiv) of heparin was required to completely turn off the catalytic activity of **4**. This suggests a 1:2.5 binding ratio between heparin and peptide **4**, which is consistent with their relative number of opposite charges.[12] As anticipated, no catalysis and consequently no inhibition with heparin could be observed with peptide **1** in the same conditions (Figure 3C).

Repetition of the above inhibition experiments in presence of various amount of human plasma in the Tris buffer (10–100%) revealed that plasma is actually able to catalyse the hydrolysis of ester **2** more efficiently than peptide **4** does (data not shown). The large number of proteins in plasma, and as a consequence the potential high concentration of histidine residues that can act as nucleophilic/basic centres, might explain this result.[13] Thus, it was evident that the use of miniature esterase **4** is only suitable for the sensing of heparin in "protein-free" solutions. Because it is possible to isolate heparin from whole blood or tissues by simple extraction procedures,[14] we believe that our sensor might find applications in biological or medical research. We also envisioned that heparin might be extracted directly from biological media by using peptide **4** immobilized on beads.[15–16] Subsequently, the measurement of the catalytic activity after addition of the fluorogenic ester should allow the determination of the initial heparin concentration. Investigations in this direction are currently underway.

This study highlights the convenience of using enzyme mimics in biosensors because they are easily accessible and can be fine-tuned to achieve the required properties. Given that we have met with promising degrees of success in our two first attempts to use artificial enzymes as sensors, we are optimistic of the potential of this original approach for new sensor discovery.

## Experimental Section

**Materials and reagents**: Protected amino acids and ChloroTrityl™ resin were obtained from Advanced ChemTech Europe (Brussels, Belgium) and Bachem (Voisins-le-Bretonneux, France). All amino acids were of the l configuration. Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) was purchased from France Biochem (Meudon, France) and other reagents from Aldrich, Acros and EMD Biosciences (Fontenay sous Bois, France). RP-HPLC experiments were performed on Waters equipment, which consisted of a Waters 600 controller, a Waters 2487 Dual Absorbance Detector and a Waters In-Line Degasser. The analytical column (Nucleosil 100 Å 5 μm C18 particles, 250×4.6 mm) was operated at 1.3 mL min$^{-1}$ and the preparative column (Delta-Pak™ 300 Å 15 μm C18 particles, 200×25 mm) at 22 mL min$^{-1}$, with UV monitoring at 214 nm and 250 nm. Solvent A was water that contained 0.1% TFA (*w/v*) and solvent B was a mixture of acetonitrile/water (9:1) that contained 0.1% TFA (*w/v*). Mass spectra were obtained by electron spray ionization (ES-MS) on a VG Platform II (Micromass). Fluorescence measurements were carried out with a SpectraMax fluorescence detector.

**Peptide 4 synthesis**: ChloroTrityl™ resin (0.414 g) was preswolle in dry $CH_2Cl_2$ (5 mL). 9-fluorenylmethoxycarbonyl (Fmoc)-Gly-OH was added, followed by $iPr_2EtN$ (4 equiv). The suspension was shaken for 90 min at room temperature. The supernatant was removed by suction and then a $CH_2Cl_2$/MeOH/$iPr_2EtN$ (17:2:1) mixture was added to cap the residual chlorotrityl groups on the resin. The loading of the derivatized resin (0.082 mmol g$^{-1}$) was determined by Fmoc quantification from UV absorption at 299 nm. Assembly of the linear-protected peptide Lys(boc)-Ala-Lys(boc)-Ala-His(tr)-Ala-Lys(boc)-Pro-Gly-Lys(boc)-Ala-Lys(boc)-Ala-Lys(boc)-Ala-Lys(boc)-Pro-Gly-OH was carried out by using an automatic peptide synthesizer (model 433A from Applied Biosystems) on ChloroTrityl™ resin by using PyBOP/Fmoc chemistry. The linear, protected peptide was recovered directly upon acid cleavage with hexafluoroisopropanol (HFIP)/$CH_2Cl_2$ (4:1). The residue was dissolved in $H_2O$/acetonitrile, and then purified by RP-HPLC (elution with A/B (95:5) to 100% B in 15 min, $t_R$ = 11.9 min) to afford the desired compound as a white solid after lyophilization. The previous solid was dissolved in DMF (0.5 mM), and the pH was adjusted to 8–9 by addition of $iPr_2EtN$. PyBOP (1.2 equiv) was added, and the solution was stirred at room temperature for 2–3 h. DMF was evaporated under reduced pressure. Removal of the side-chain protecting groups was performed in TFA/triisopropylsilane (TIS)/$H_2O$ (95:2.5:2.5). After 12 h of stirring, the solution was evaporated to yield an oily residue, which was purified by RP-HPLC (elution with A/B (95:5) to (85:15) in 20 min, $t_R$ = 7.3 min) to afford pure **4** as an octahydrotriflate salt (6 mg, 15% overall yield). ESI-MS (positive mode) for $C_{80}H_{141}N_{27}O_{18}$ (calcd $M_W$ = 1769.2 g mol$^{-1}$): *m/z* 885.1 $[M+2H]^{2+}$, 590.6 $[M+3H]^{3+}$, 443.2 $[M+4H]^{4+}$, 354.8 $[M+5H]^{5+}$.

**Kinetic studies**: The kinetic measurements were carried out by using a SpectraMax fluorescence plate reader ($\lambda_{ex}$ = 460 nm, $\lambda_{em}$ = 530 nm) at 25 °C. Assays were followed in individual wells of round-bottom polystyrene 96-well plates. Kinetic experiments were typically followed for 2–12 h. Tris buffer (20 mM, pH 7.4) was freshly prepared by using MilliQ deionized $H_2O$, the pH was adjusted with aq. 1 N NaOH and aq.1 N HCl solutions.

In a typical experiment, Tris buffer (210 μL) was first added in a well, then a solution of peptide **4** in Tris buffer (25 μL, 0.005 mM, final concentration in the well: 0.5 μM), and last a solution of **2** in $H_2O$ (15 μL, 0.2 mM, final concentration in the well: 12 μM). Fluorescence data were converted to product concentration by means of a calibration curve with pure product **3**, which was linear in the concentration range that was used. Michaelis–Menten parameters were obtained from the linear double reciprocal plot of 1 V vs. 1/[**2**] that was measured with (final concentrations) 0.5 μM peptide **4** or no peptide in Tris buffer. The catalytic rate constant $k_{cat}$ for the hydrolysis was given by $k_{cat} = V_{max}/[\mathbf{4}]$. The inhibition experiments were performed similarly with 0.5 μM peptide, 12 μM substrate and 13, 40, 67, 114, 133, 200, 266, 333 and 400 nM inhibitor in Tris buffer. The reaction rate with 4(5)-methylimidazole (MeIm) was obtained under the same previous conditions with 24, 48, 64, 96, 200, 400, 600 and 800 μM MeIm and 200 μM **2** in Tris buffer. The second-order rate constant $k_{MeIm}$ was calculated from linear regression of the experimentally measured pseudo-first-order rate constants as a function of MeIm concentrations.

## Acknowledgements

*This work was supported by the Centre National de la Recherche Scientifique (CNRS), the Université Joseph Fourier de Grenoble (U.J.F.), and the Pôle d'Innovation NanoBio. The authors are greatly indebted to the anonymous referees for valuable suggestions and comments about this work. We also thank Prof. B. Pollack from the Hospital of Grenoble for providing us with human plasma.*

**Keywords:** electrostatic interactions · esterases · fluorescent probes · heparin · peptides


[1] I. Capila, R. J. Linhardt, *Angew. Chem.* **2002**, *114*, 426–450; *Angew. Chem. Int. Ed.* **2002**, *41*, 390–412.

[2] a) G. J. Despotis, G. Gravlee, K. Filos, J. Levy, *Anesthesiology* **1999**, *91*, 1122–1151; b) J. Hirsh, J. E. Dalen, D. Deykin, L. Poller, *Chest* **1992**, *102*, 337S–351S.

[3] a) T.-J. Cheng, T.-M. Lin, T.-H. Wu, H.-C. Chang, *Anal. Chim. Acta* **2001**, *432*, 101–111; b) S. Mathison, E. Bakker, *Anal. Chem.* **1999**, *71*, 4614–4621; c) N. Ramamurthy, N. Baliga, T. W. Wakefield, P. C. Andrews, V. C. Yang, M. E. Meyerhoff, *Anal. Biochem.* **1999**, *266*, 116–124; d) N. Ramamurthy, N. Baliga, J. A. Wahr, U. Schaller, V. C. Yang, M. E. Meyerhoff, *Clin. Chem.* **1998**, *44*, 606–631; e) J. M. Thomson, J. M. Poller in *Blood Coagulation and Haemostasis* (Ed.: J. M. Thomson), Churchill Livingstone, Edinburgh, **1989**, pp. 301–339; f) S. C. Ma, V. C. Yang, B. Fu, M. E. Meyerhoff, *Anal. Chem.* **1993**, *65*, 2078–2084; g) S. C. Ma, V. C. Yang, M. E. Meyerhoff, *Anal. Chem.* **1992**, *64*, 694–697; h) E. Homer, *Thromb. Haemostasis* **1985**, *54*, 29–31; i) G. A. Mitchell, R. J. Gargiulo, R. M. Huseby, D. E. Lawson, S. P. Pochron, J. A. Sehuanes, *Thromb. Res.* **1978**, *13*, 47–52.

[4] a) S. Kitchen, *Br. J. Haematol.* **2000**, *111*, 397–406; b) M. N. Levine, J. Hirsh, M. Gent, A. G. Turpie, M. Cruickshank, J. Weitz, D. Anderson, M. Johnson, *Arch. Int. Med. Exp.* **1994**, *154*, 49–56; c) L. Poller, J. M. Thomson, K. F. Yee, *Br. J. Haematol.* **1980**, *44*, 161–165.

[5] a) S. Wang, Y.-T. Chang, *Chem. Commun. (Cambridge)* **2008**, 1173–1175; b) W. Sun, H. Bandmann, T. Schrader, *Chem. Eur. J.* **2007**, *13*, 7701–7707; c) J. C. Sauceda, R. M. Duke, M. Nitz, *ChemBioChem* **2007**, *8*, 391–394; d) A. Wright, T. Z. Zhong, E. V. Anslyn, *Angew. Chem.* **2005**, *117*, 5825–5828; *Angew. Chem. Int. Ed.* **2005**, *44*, 5679–5682; e) Z. Zhong, E. V. Anslyn, *J. Am. Chem. Soc.* **2002**, *124*, 9014–9015.

[6] L. Vial, P. Dumy, *J. Am. Chem. Soc.* **2007**, *129*, 4884–4885.

[7] For examples of peptide-based esterase mimics, see: a) A. Esposito, E. Delort, D. Lagnoux, F. Djojo, J.-L. Reymond, *Angew. Chem.* **2003**, *115*, 1419–1421; *Angew. Chem. Int. Ed.* **2003**, *42*, 1381–1383; b) D. Lagnoux, E. Delort, C. Douat-Casassus, A. Esposito, J.-L. Reymond, *Chem. Eur. J.* **2004**, *10*, 1215–1226; c) C. Douat-Casassus, T. Darbre, J.-L. Reymond, *J. Am. Chem. Soc.* **2004**, *126*, 7817–7826; d) A. Clouet, T. Darbre, J.-L. Reymond, *Adv. Synth. Catal.* **2004**, *346*, 1195–1204; e) E. Delort, T. Darbre, J.-L. Reymond, *J. Am. Chem. Soc.* **2004**, *126*, 15642–15643; f) E. Delort, N.-Q. Nguyen-Trung, T. Darbre, J.-L. Reymond, *J. Org. Chem.* **2006**, *71*, 4468–4480; g) K. S. Broo, L. Brive, P. Ahlberg, L. Baltzer, *J. Am. Chem. Soc.* **1997**, *119*, 11362–11372; h) K. S. Broo, H. Nilsson, J. Nilsson, L. Baltzer, *J. Am. Chem. Soc.* **1998**, *120*, 10287–10295; i) L. Baltzer, K. S. Broo, H. Nilsson, J. Nilsson, *J. Bioorg. Med. Chem.* **1999**, *7*, 83–91; j) J. Nilsson, L. Baltzer, *Chem. Eur. J.* **2000**, *6*, 2214–2220; k) L. K. Andersson, M. Caspersson, L. Baltzer, *Chem. Eur. J.* **2002**, *8*, 3687–3689; l) D. L. Bolon, S. L. Mayo, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 14274–14279; m) Y. Wei, M. H. Hecht, *Protein Eng. Des. Sel.* **2004**, *17*, 67–75; n) A. J. Nicoll, R. K. Allemann, *Org. Biomol. Chem.* **2004**, *2*, 2175–2180; o) B. Baumeister, N. Sakai, S. Matile, *Org. Lett.* **2001**, *3*, 4229–4232; p) A. Som, N. Sakai, S. Matile, *Bioorg. Med. Chem.* **2003**, *11*, 1363–1369.

[8] a) M. P. Hoffman, J. A. Engbring, P. K. Nielsen, J. Vargas, Z. Steinberg, A. J. Karmand, M. Nomizu, Y. Yamada, H. K. Kleinman, *J. Biol. Chem.* **2001**, *276*, 22077–22085; b) J. R. Fromm, R. E. Hileman, J. M. Weiler, R. J. Linhardt, *Arch. Biochem. Biophys.* **1997**, *346*, 252–262.

[9] For a review on this family of decapeptidic scaffold, see: Y. Singh, G. T. Dolphin, J. Razkin, P. Dumy, *ChemBioChem* **2006**, *7*, 1298–1314.

[10] V. Leskovac in *Comprehensive Enzyme Kinetics*, Kluwer Academic/Plenum Publishers, New York, **2003**, p. 191.

[11] Low-molecular-weight heparin (Sigma, cat. H3400, average molecular weight of 3000 Da) was used for this study.

[12] Assuming seven positive charges for **4** and an average of nineteen negative charges for heparin. The average number of negative charges in heparin was calculated from an average molecular weight of the repeating disaccharide unit of 535 Da and an average of 2.4 sulfates and 1 carboxylate per disaccharide.

[13] The average plasma protein concentration is approximately 77 $g\,L^{-1}$. If only one fiftieth of the protein-constituting amino acids are histidine, the total histidine concentration in plasma is 11 mM. See: G. R. Wadsworth, C. J. Oliveiro, *Br. Med. J.* **1953**, *2*, 1138–1139.

[14] a) R. W. Wieanloz in *Methods in Carbohydrate Chemistry, Vol. 5*, Academic Press, New York, **1965**, p. 150; b) J. D. H. Homan, J. Lens, *Biochim. Biophys. Acta* **1948**, *2*, 333–336; c) A. F. Charles, D. A. Scott, *J. Biol. Chem.* **1933**, *102*, 425–429; d) B. Moazed, L. M. Hiebert, *J. Pharmacol. Exp. Ther.* **2007**, *322*, 299–305.

[15] For an example of a cyclic peptide immobilized on beads, see: O. Renaudet, P. Dumy, *Org. Biomol. Chem.* **2006**, *4*, 2628–2636.

[16] For recent examples of heparin removal systems, see: a) M. S. Varghese, D. Hildebrandt, D. Glasser, N. J. Crowther, D. M. Rubin, *Artif. Cells Blood Substitutes Biotechnol.* **2006**, *34*, 419–432; b) J. F. Liang, E. Emregul, V. C. Yang, *Blood Purif.* **2004**, *22*, 198–202.


Received: June 23, 2008
Published online on November 14, 2008