Microarrays of heparin oligosaccharides obtained by nitrous acid depolymerization of isolated heparin†

Jose L. de Paz,[a] Dorothe Spillmann[b] and Peter H. Seeberger*[a]

Received (in Cambridge, UK) 12th April 2006, Accepted 1st June 2006
First published as an Advance Article on the web 14th June 2006
DOI: 10.1039/b605318a

Heparin oligosaccharides derived by nitrous acid depolymerization of heparin have been immobilized on amine-coated glass slides. The formation of a Schiff base creates heparin chips that are a suitable platform for the high-throughput analysis of carbohydrate–protein interactions.

Heparin is a highly sulfated, linear polymer that belongs to the family of glycosaminoglycans and participates in a plethora of biological processes by interaction with many proteins.[1] The chemical complexity and heterogeneity of this polysaccharide can explain the fact that, despite its widespread medical use as an anticoagulant drug, the structure–function relationship of defined heparin sequences is still poorly understood. The use of heparin oligosaccharide microarrays[2,3] holds the potential to substantially improve the understanding of heparin–protein interactions.

Herein, we disclose the preparation of microarrays containing heparin fragments obtained by nitrous acid depolymerization. The utility of these chips for the high-throughput analysis of heparin–protein interactions is illustrated by incubation with the proteins antithrombin III (AT-III), acidic, and basic fibroblast growth factors (FGF-1 and FGF-2).

Several enzymatic and chemical methods for the depolymerization and sequence analysis of heparin exist.[4] The most successful chemical method is depolymerization with nitrous acid[5] that cleaves heparin chains at either *N*-unsubstituted or *N*-sulfated glucosamine residues, to produce oligosaccharides containing a 2,5-anhydromannose unit at the reducing end. The aldehyde group at position 1 of the 2,5-anhydromannose unit is more reactive than aldehyde groups of reducing sugars since it is not in equilibrium with unreactive closed ring forms.[6] This increased reactivity of the reducing end allows for the attachment of deaminated heparin fragments to surfaces by either formation of a Schiff base (Fig. 1) or *via* reductive amination.

Initially, we employed fluorescein isothiocyanate (FITC) labelled deaminated heparin (average molecular weight 5 KDa) to optimize the immobilization procedure. FITC-labelled heparin in sodium bicarbonate buffer[7] (100 mM, pH 9.0) was printed on GAPS (Gamma Amino Propyl Silane) amine-coated slides using a robotic arrayer (Fig. 2). After printing, the remaining amine

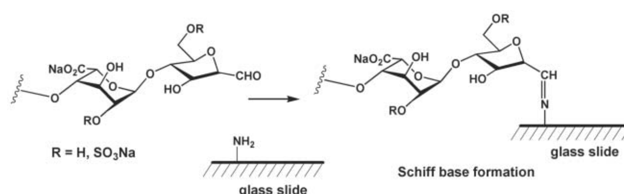


Fig. 1 Immobilization of deaminated heparin oligosaccharides on amine-coated glass slides.

groups were quenched by treatment with a carboxybenzaldehyde-containing solution. Two-step quenching with disuccinimidyl carbonate followed by ethanolamine yielded higher background signals. After extensive washing, the slides were scanned and the fluorescence intensity was plotted against the heparin concentration (Fig. 2). The retained fluorescence signal demonstrated that deaminated heparin was immobilized on GAPS slides. This experiment also showed that as little as 63 μg mL^{-1} (~12 picomoles per spot) was sufficient to obtain a signal above background. The stability of the attached heparin was demonstrated by incubation in hybridization buffer (PBS containing 1% BSA).† Alternatively, FITC-labelled heparin was also immobilized on GAPS slides by using reductive amination conditions.[8]‡

With a straightforward method to attach deaminated heparin to amine-coated glass slides at hand, the next step was the preparation of microarrays containing heparin fragments obtained

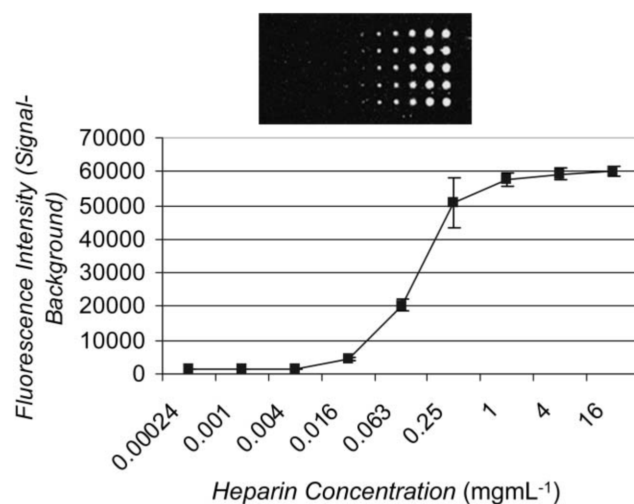


Fig. 2 Top: Image of a microarray containing FITC-labeled heparin. Bottom: Quantitative illustration relating fluorescence intensity to the concentration of printed heparin.

[a]Laboratory for Organic Chemistry, Swiss Federal Institute of Technology (ETH) Zürich, Wolfgang-Pauli-Str. 10, HCI F315, 8093, Zürich, Switzerland. E-mail: seeberger@org.chem.ethz.ch; Fax: +41 44 6331235; Tel: +41 44 6332103
[b]Department of Medical Biochemistry and Microbiology, Uppsala University, P. O. Box 582, SE-75123 Uppsala, Sweden
† Electronic supplementary information (ESI) available: experimental procedures for the preparation and use of heparin microarrays. See DOI: 10.1039/b605318a

 This journal is © The Royal Society of Chemistry 2006

by nitrous acid depolymerization and their use for the rapid analysis of heparin–protein interactions. Six different heparin sequences (octasaccharides **1–3** and decasaccharides **4–6**) were prepared using the following procedure. First, bovine intestinal heparin was partially deaminated at pH 1.5 and size fractionated on a BioGel P-10 column as described.[9] Size defined reducing octa- and decasaccharides were further fractionated on an antithrombin III-affinity column into pools with no affinity (octa **3**, deca **6**; eluted by 50 mM Tris–HCl pH 7.4, 0.15 M NaCl), low affinity (octa **2**, deca **5**; 50 mM Tris–HCl pH 7.4, 0.6 M NaCl) and high affinity (octa **1**, deca **4**, 50 mM Tris–HCl pH 7.4, 3 M NaCl) as described.[10] Thereafter, samples were desalted by gel chromatography on PD-10 columns and their disaccharide composition determined as described.[11] The low and high affinity samples contained species with a 3-*O*-sulfate group on the glucosamine unit (GlcN) that is essential for heparin–AT-III binding.[12] The difference between the low and high affinity fractions is the degree of 2-*O*-sulfation on iduronic acid (IdoA) and *N*- and 6-*O*-sulfation on GlcN units. Fractions **3** and **6** did not contain 3-*O*-sulfated disaccharide units.

Heparin oligosaccharides **1–6** were printed on amine-coated glass slides at concentrations ranging from 35 μM to 0.35 μM. The robotic arrayer delivered 1 nL of carbohydrate-containing solutions to create spots with an average diameter of 200 μm. All samples were printed in five replicates to generate an array of 90 spots (Fig. 3). After incubation and quenching of the unreacted amine groups, the slides were ready for hybridization experiments using AT-III, FGF-1 and FGF-2.

AT-III is a serine protease inhibitor of the blood coagulation cascade that requires heparin for full activation. A characteristic heparin pentasaccharide sequence,[12] containing the crucial $GlcNSO_3(3\text{-}OSO_3)$ unit, is responsible for the binding to AT-III. The incubation was carried out by using fluorescently-labelled AT-III.§ After removing unbound protein by washing, strongly fluorescent signals were observed for compounds **1**, **2**, **4** and **5** while no FITC–AT-III was bound to **3** and **6** (Fig. 3). These results were in agreement with those obtained by using affinity chromatography on AT-III as described above, and illustrate the utility of this strategy to attach deaminated oligosaccharides on glass slides while maintaining specificity of carbohydrate–protein interactions.

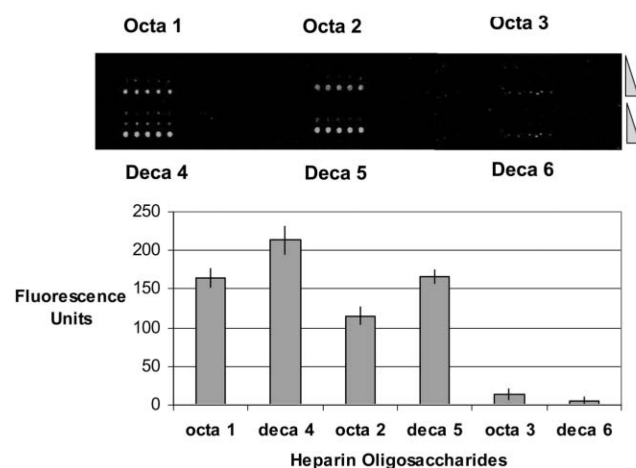


Fig. 3 Top: Microarray after incubation with AT-III. Bottom: Fluorescence signal observed for each arrayed carbohydrate binding to AT-III at 35 μM.

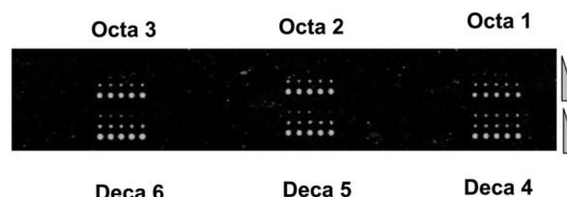


Fig. 4 Microarray after incubation with FGF-2.

FGF-2 was also employed in hybridization experiments. Results showed that FGF-2 bound to all oligosaccharides (Fig. 4). This observation is in agreement with the fact that tetra- and hexasaccharides are sufficient to bind FGFs, and $GlcNSO_3(3\text{-}OSO_3)$ units are not essential for FGF–heparin interaction.[13] Similar results were obtained using FGF-1.†

In summary, we have prepared and used arrays displaying heparin oligosaccharides derived from natural sources by nitrous acid depolymerization. The aldehyde group of the 2,5-anhydromannose unit at the reducing end was used to attach the oligosaccharides to glass surfaces by formation of the corresponding Schiff base. The utility of this methodology was demonstrated by probing the carbohydrate affinity of three heparin-binding proteins, AT-III, FGF-1 and FGF-2. Additionally, only tiny amounts (∼picomoles) of isolated oligosaccharides are required for the preparation of these heparin chips. Therefore, the method described here can be applied to the high-throughput screening of heparin sequences obtained by chemical cleavage, providing precious information about the role of heparin in biological systems.

We thank Dr Jens Sobek (Functional Genomics Center, Zürich) for assistance in the construction of microarrays. We also thank ETH Zürich, the Swedish Foundation for Strategic Research (SSF), and the European Commission (Marie Curie Fellowship for J. L. P.) for financial support.

Notes and references

‡ The use of reductive amination conditions did not increase the retained fluorescence intensity. Attachment *via* the formation of a Schiff base was therefore used subsequently.

§ Alternatively, a sandwich procedure whereby the bound protein is detected with labelled secondary antibodies can also be used, as described in ref. 3.

1 (*a*) H. E. Conrad, *Heparin-Binding Proteins*, Academic Press: San Diego, CA, 1998; (*b*) I. Capila and R. J. Linhardt, *Angew. Chem., Int. Ed.*, 2002, **41**, 390–412.
2 For reviews, see: (*a*) I. Shin, S. Park and M. Lee, *Chem.–Eur. J.*, 2005, **11**, 2894–2901; (*b*) T. Feizi, F. Fazio, W. Chai and C.-H. Wong, *Curr. Opin. Struct. Biol.*, 2003, **13**, 637–645; (*c*) K. R. Love and P. H. Seeberger, *Angew. Chem., Int. Ed.*, 2002, **41**, 3583–3586.
3 J. L. De Paz, C. Noti and P. H. Seeberger, *J. Am. Chem. Soc.*, 2006, **128**, 2766–2767.
4 B. Casu and U. Lindahl, *Adv. Carbohydr. Chem. Biochem.*, 2001, **57**, 159–256.
5 D. Horton and K. D. Philips, *Carbohydr. Res.*, 1973, **30**, 367–374.
6 (*a*) E. A. Yates, M. O. Jones, C. E. Clarke, A. K. Powell, S. R. Johnson, A. Porch, P. P. Edwards and J. E. Turnbull, *J. Mater. Chem.*, 2003, **13**, 2061–2063; (*b*) A. K. Powell, E. A. Yates, D. G. Fernig and J. E. Turnbull, *Glycobiology*, 2004, **14**, 17R–30R.
7 V. Afanassiev, V. Hanemann and S. Wolfl, *Nucleic Acids Res.*, 2000, **28**, E66.

8 M. B. Biskup, J. U. Müller, R. Weingart and R. R. Schmidt, *ChemBioChem*, 2005, **6**, 1007–1015.
9 E. Feyzi, E. Trybala, T. Bergström, U. Lindahl and D. Spillmann, *J. Biol. Chem.*, 1997, **272**, 24850–24857.
10 U. Lindahl, G. Backstrom, M. Hook, L. Thunberg, L.-A. Fransson and A. Linker, *Proc. Nat. Acad. Sci. USA*, 1979, **76**, 3198–3202.
11 J. Ledin, W. Staatz, J.-P. Li, M. Götte, S. Selleck, L. Kjellén and D. Spillmann, *J. Biol. Chem.*, 2004, **279**, 42732–42741.
12 M. Petitou and C. A. A. van Boeckel, *Angew. Chem., Int. Ed.*, 2004, **43**, 3118–3133.
13 For a recent review, see: R. Raman, V. Sasisekharan and R. Sasisekharan, *Chem. Biol.*, 2005, **12**, 267–277.

Find a SOLUTION

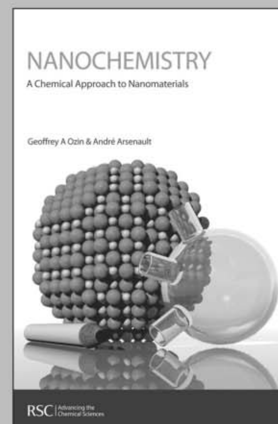
… with books from the RSC

Choose from exciting textbooks, research level books or reference books in a wide range of subject areas, including:

- Biological science
- Food and nutrition
- Materials and nanoscience
- Analytical and environmental sciences
- Organic, inorganic and physical chemistry

Look out for 3 new series coming soon …

- RSC Nanoscience & Nanotechnology Series
- Issues in Toxicology
- RSC Biomolecular Sciences Series



28040542

RSCPublishing

www.rsc.org/books

 This journal is © The Royal Society of Chemistry 2006