

Sipley, J., et al. *Ann. N. Y. Acad. Sci.* 878:
372-87, 1999

- 19) Kobayashi, J., Yamada, S., and Kawasaki, H.
Hepatology 20: 1412-7, 1994
- 20) Inuzuka, S., Ueno, T., Torimura, T., et al.
Hepatology 15: 629-36, 1992

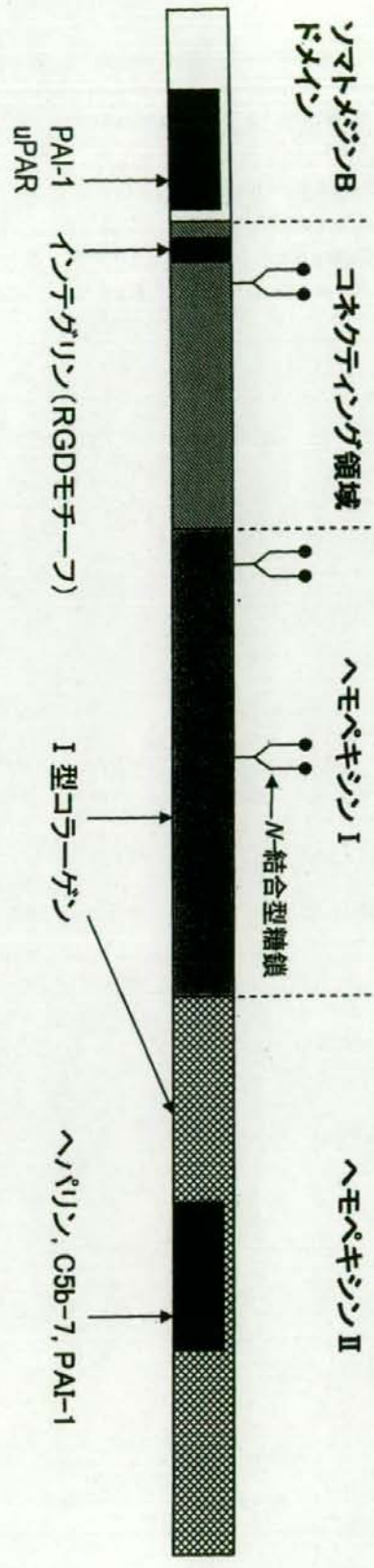


図1 ビトロネクチンの一次構造

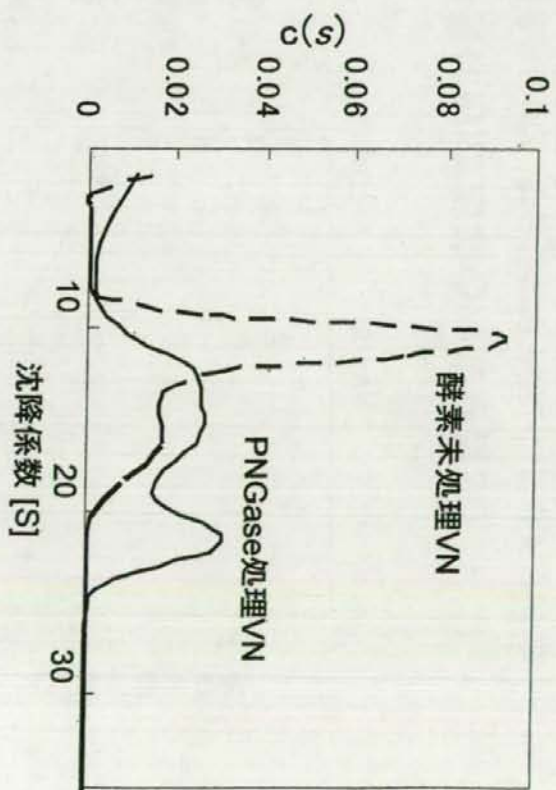
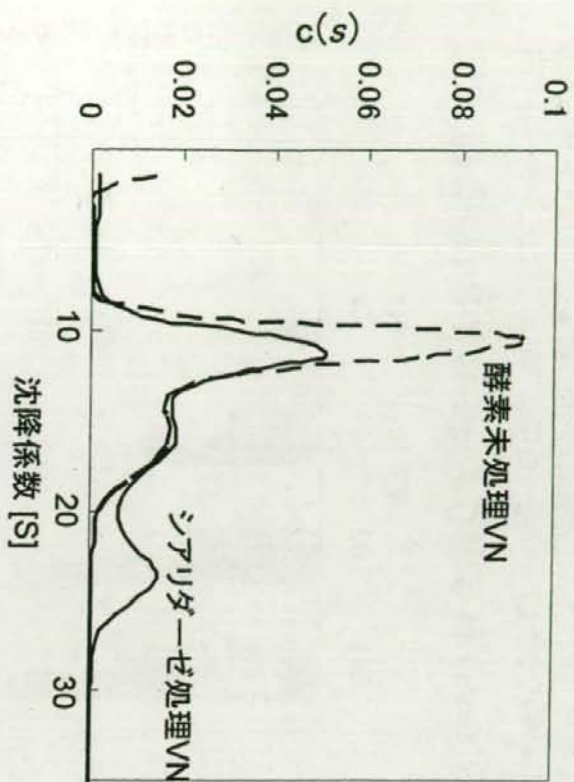


図2 糖鎖除去VNの多量体形成能の増強

横軸に沈降係数(∞ 多量体の分子量)、縦軸に分布を示す。糖鎖末端のシアル酸を除去するシアリダーゼ処理(左図)、またはN-結合型糖鎖を除去するPNGase F処理(右図)を行ったVNは、未処理VN(破線)より高分子量の多量体を形成する。

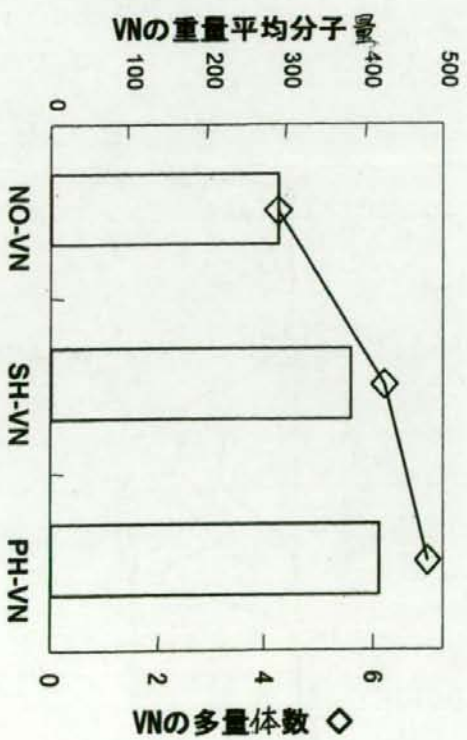
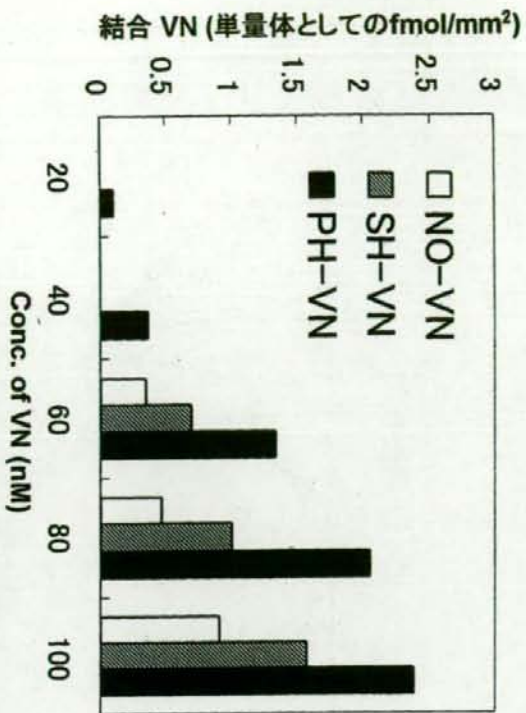
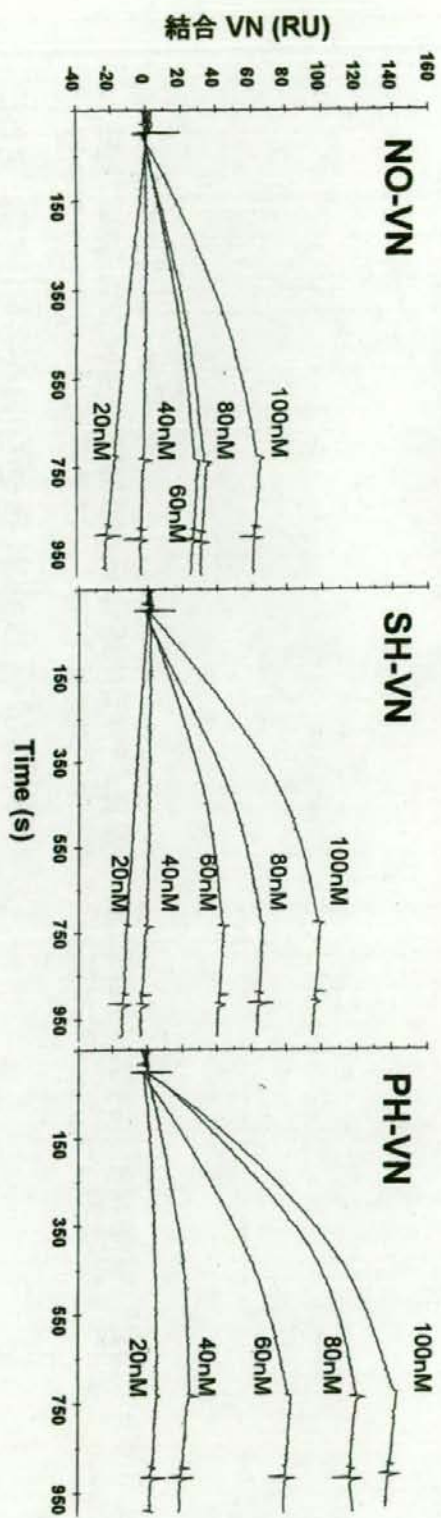


図3 肝切除VNのコラーゲン結合性(SPR)と多量体形成(超遠心沈降平衡法)

センサーチップに固定化したコラーゲンへのVNの結合は濃度依存的で、部分肝切除手術24時間後のラット血漿VN(PH-VN)が最も高い結合性を示した。(NO: 非手術、SH: 偽手術、PH: 部分肝切除手術)

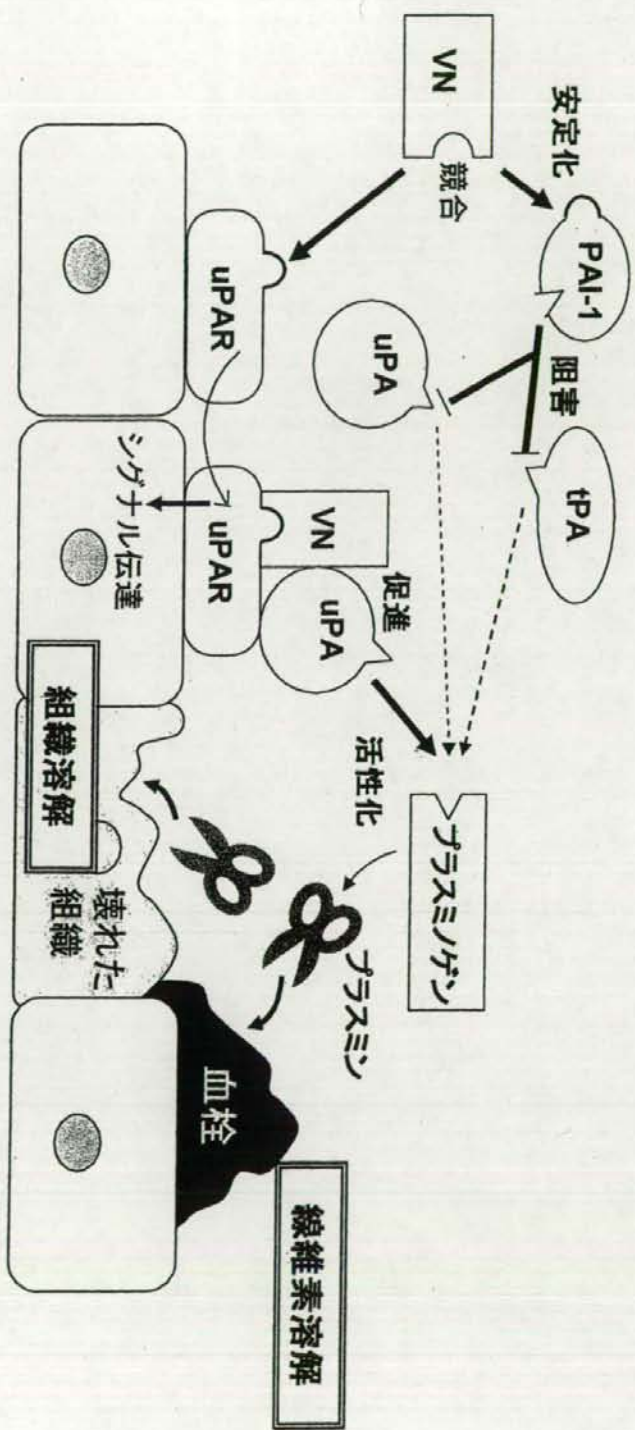


図4 線溶ならびに組織溶解系の調節機構

VNはPAI-1と結合し、その阻活性を安定化させることによってプラスミン産生を抑制する一方、uPA、UPARと三者複合体を形成することによって細胞表面の局所的溶解を促進させる。VNのPAI-1結合とUPAR結合は互いに競合する。このように線溶ならびに組織溶解反応は、複数の因子の発現量や結合性の変化によって複雑にコントロールされる。VNの糖鎖変化は→の相互作用に影響を及ぼす。

Development of Neoglycoconjugate Probes and 1 Detection of Lectins

Haruko Ogawa, Keiko Nakagawa

Keywords Neoproteoglycan, Neoglycoprotein, Pseudoproteoglycan, Hybrid glycoprotein, Lectin detection, Interaction analysis, Carbohydrate-binding

Introduction

Glycoconjugates are involved in essential biological processes, and interference with glycosylation is pathological and sometimes lethal in many organisms. Because the biological functions of glycans are exhibited primarily in their interactions with carbohydrate-binding proteins (lectins or receptors), which recognize individual glycoconjugates, we have developed various types of probes to detect lectins and analyze glycan interactions with high sensitivity. Commercially available glycoprobes are prepared by organic synthesis, a method that is suitable for large-scale production but requires large amounts of glycans and specialized synthesis steps. However, preparation of probes by glycobiochemists in the laboratory is technically feasible and requires only minute amounts of glycans or glycoconjugates, which can be purified from biological sources to provide a probe of adequate sensitivity for detection and to prepare affinity adsorbents. Our probes are prepared by derivatizing natural glycans or glycoconjugates by non-denaturing aqueous-phase reactions. In this report, we describe the development of neoglycoconjugate systems for sensitive lectin screening and interaction analyses. Examples of probes for lectin screening from animal, plant and fungal sources are shown (Chai et al. 2003; Matsumoto et al. 2001; Ogawa et al. 2003; Satoh et al. 2004).

Principles of Neoglycoconjugate Probe Preparation

Carbohydrate-binding proteins have conventionally been studied using probes that have glycan chains or glycoproteins labeled with a functional tag such as biotin, a fluorescent chromogen or an epitope for immunoenzymatic detection (Fig. 1a). Glycans are usually conjugated with a hydrophobic group of a protein or lipid to immobilize them on the solid phase, and these are called neoglycoproteins or neoglycolipids (Fig. 1b, c). For affinity adsorbents, glycosaminoglycans (GAGs) are directly immobilized onto gel beads (Fig. 1d).

Neoproteoglycans and Hybrid Glycoproteins

Neoproteoglycans (neoPGs) are developed by conjugating acidic or neutral glycans with proteins to immobilize GAGs or oligosaccharides effectively on solid substrates. NeoPGs

Graduate School of Humanities and Sciences and the Glycoscience Institute, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo, 112-8610 Japan
Tel: +3-5978-5343
Fax: +3-5978-5343
E-mail: hogawa@cc.ocha.ac.jp

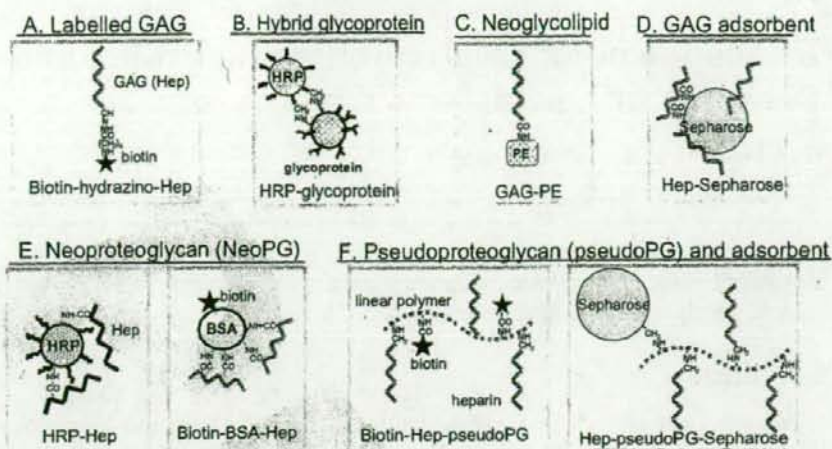


Fig. 1 ..

detect binding with high sensitivity; for example, heparin is coupled with bovine serum albumin (BSA) and labeled with biotin to obtain biotin-BSA-heparin to detect heparin-binding proteins (Fig. 1e, right; Chai et al. 2003). A series of neoPG probes containing colominic acid, fucoidin, heparin and pectic acid were used to screen lectins from extracts of 16 cultivable mushrooms in combination with various hybrid horseradish peroxidase (HRP)-conjugated glycoproteins having different glycan types (Fig. 1b). The specificities of the lectins in the mushroom extracts were classified according to the reaction pattern of the probe on screening by dot-blot assay, and one lectin was purified (Matsumoto et al. 2001). Using the neoPG probe prepared by coupling heparin with HRP (Fig. 1e, left), the interactions with *Psathyrella velutina* lectin or vitronectin are analyzed by solid-phase assays: ELISA, affinity chromatography, and Western blotting (Chai et al. 2003; Ogawa et al. 2003).

Pseudoproteoglycans (PseudoPGs)

A proteoglycan (PG) is a supermolecule consisting of one or more GAG chains attached to a core protein that binds link proteins and hyaluronans. PGs have signaling roles and cell modulatory functions in the extracellular matrix and at the cell surface. Some conventional probes, such as biotin-hydrazino-Hep and neoPGs, react with biological ligands other than those of PGs because these probes are dissimilar from the higher-order structure of natural PGs. Pseudoproteoglycans (pseudoPGs) simulate the structure of a PG monomer with more than one GAG side chain attached to a linear polymer strand, such as poly-L-lysine or linear polyacrylamide, and are biotinylated using NHS-biotin. Then, the remaining amino groups are blocked by *N*-acetylation to obtain biotinyl GAG-pseudoPG probes for use as probes and affinity adsorbents to search for and locate PG-binding substances in biological materials (Fig. 1f; Satoh et al. 2004). Binding studies indicate that heparin-binding proteins react differently to biotinyl pseudoPG and biotin hydrazine-Hep on Western blotting and affinity chromatography. Remarkably, several proteins in rat brain extracts bound specifically to either, but not both, of the probes,

indicating that the Hep-binding proteins recognize a higher-order structure of PG. Surface plasmon resonance studies of known heparin-binding proteins with a pseudoPG probe bound to an avidin-immobilized chip showed that the biotinyl Hep-pseudoPG probe had an affinity higher by one order to several protein ligands than to biotin-hydrazino-Hep. The effect of the pseudoPG structure on the binding differed depending on the heparin-binding protein, and some of them exhibited recognition for a higher-order structure of PG.

PseudoPG probes prepared with lacto-*N*-neotetraose (LNnT) detected the LNnT-binding lectin in extracts of *Pleurocybella porrigens* with greater sensitivity than a commercial sugar-biotinyl polymer probe that has an affinity 10^2 – 10^5 higher than that of free sugars. The pseudoPG probe demonstrates the biological significance of the proteoglycan structure in carbohydrate recognition and can be used for the detection and separation of proteins and recognition of events that involve higher-order PG structures.

To reveal glycan functions, it is important to identify a recognizable molecule as a glycoconjugate rather than as an oligosaccharide. A hybrid glycoprotein that is prepared by using the NH_2 or COOH group of a glycoprotein will be as useful for this purpose as neoPG and pseudoPG probes. The preparation of probes and procedures for lectin screening are easily applied to probing glycoconjugates to search for biological ligands. We are currently developing a new method of probing glycoconjugates and will use it for screening receptors and characterizing lectins that have specificities useful for studying pathological states.

Procedures

Biotin-neoPG Probe Preparation

1. Dissolve GAG (3 mg) in 0.4 ml of distilled water in a 1.5 ml plastic tube.
2. Dissolve *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (3 mg) in 0.6 ml of ethanol and add it to the tube.
3. Incubate the mixture at room temperature for 2 h with shaking.
4. Add BSA or HRP solution (6 mg/0.5 ml of distilled water) and incubate at 4°C for 2 days with gentle shaking.
5. Check the formation of neoPG by observing the change in the migration position of BSA or HRP on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*.
6. For biotinylation of BSA-neoPG, add 222 μl of 0.1% *N*-hydroxysuccinimidylbiotin (NHS-biotin) to 3 ml of the sample in 50 mM sodium bicarbonate buffer (pH 8.5) and stand for 2 h in ice.
7. Ultrafiltrate the reaction mixture with Tris-buffered saline, pH 7.5, to remove excess reagent by using a membrane of exclusion molecular weight 50,000 or 100,000.
8. Choose the suitable concentration of the neoPG probe with a spot test and color development**.

Comments

*The coupling of GAG with protein is detected by staining the electrotransferred protein band on a PVDF membrane after SDS-PAGE with 0.5% toluidine blue and destaining with water.

**We typically use 10 $\mu\text{g}/\text{ml}$ as a soluble probe for ELISA.

References

- Chai W, Stoll MS, Galustian C, Lawson AM, Feizi T (2003) Neoglycolipid technology: deciphering information content of glycome. *Methods Enzymol* 362:160–195
- Matsumoto H, Natsume A, Ueda H, Saito T, Ogawa H (2001) Screening of a unique lectin from 16 cultivable mushrooms with hybrid glycoprotein and neoproteoglycan probes and purification of a novel *N*-acetylglucosamine-specific lectin from *Oudemansiella platyphylla* fruiting body. *Biochim Biophys Acta* 1526:37–43
- Ogawa H, Ueda H, Natsume A, and Suzuki R (2003) Preparation and utility of neoproteoglycan probes in the interaction analyses with glycosaminoglycan-binding proteins. *Methods Enzymol* 362:196–209
- Sato K, Yamagami M, Ogawa H (2004) Utility of pseudoproteoglycan (pseudoPG) probes that simulate PG supramolecular structure. *Glycobiology* 14:1095–1096
- Ueda H, Kojima K, Saitoh T, Ogawa H (1999) Multispecificities of a *Psathyrella velutina* mushroom lectin: binding toward heparin/pectin is exhibited by a site different from its *N*-acetylglucosamine/*N*-acetylneuraminic acid-specific site. *J Biochem Tokyo* 126:530–537

3



Note

Glycosylation and ligand-binding activities of rat plasma fibronectin during liver regeneration after partial hepatectomy

Kotone Sano,^a Miho Asahi,^a Maiko Yanagibashi,^a Noritaka Hashii,^c
Satsuki Itoh,^c Nana Kawasaki^c and Haruko Ogawa^{a,b,*}^aGraduate School of Humanities and Sciences, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan^bGlycoscience Institute, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan^cDivision of Biological Chemistry and Biologicals, National Institute of Health Sciences, Tokyo 158-8501, Japan

Received 10 September 2007; received in revised form 18 February 2008; accepted 19 March 2008

Abstract—Fibronectin (FN) is a multifunctional glycoprotein present in the extracellular matrix (ECM) and plasma. We previously reported that the glycosylation and ligand-binding of vitronectin (VN) change markedly after partial hepatectomy (PH). Here we show the changes of FN during liver regeneration. The yields of purified sham-operated (SH-) and PH-FN were higher than that of non-operated (NO)-FN, while binding activities of FNs to ECM ligands were changed only slightly by hepatectomy. The carbohydrate concentration of PH-FN decreased to 66% of that of NO- and SH-FN. By using LC/MSⁿ, eight kinds of complex-type N-glycan structures were found to be present in all FNs, and bi- and trisialobiantennary glycans were the major structures. Fucosylation was markedly increased, while O-acetylation of sialic acid was found to be decreased in PH-FN. The alterations in glycosylation and biological activities of FN after PH are different from those of VN, suggesting that these glycoproteins play different biological functions in tissue remodeling.

© 2008 Elsevier Ltd. All rights reserved.

Keywords: Fibronectin; Glycosylation; Liver regeneration; Vitronectin; Partial hepatectomy

Numerous biological phenomena are mediated by the recognition of specific oligosaccharide signals. Among the functions of protein glycosylation are the stabilizing active conformations, protecting against proteolysis, and affording solubility to proteins.¹ Clarification of the molecular mechanisms by which glycosylation plays these roles would enable the use of glycosylation in molecular engineering for therapeutic purposes.

Partial (70%) hepatectomy is often used to study liver regeneration mechanisms. The remaining liver recovers its former weight within about two weeks in humans or 10 days in the rat.² Matrix degradation occurs in the early stage of this process, followed by biosynthesis of the matrix, cell proliferation, and cell differentiation.

Previously, collagen-binding activities of VN were found to be significantly affected by alterations in N-glycosylation.³ A marked increase in VN was found to be controlled by the increased multimerization induced by glycosylation changes during liver regeneration after PH of rats.⁴

FN is a typical ECM glycoprotein synthesized by various cells, including hepatocytes, that plays critical roles in many biological and pathological processes including embryogenesis, wound healing, metastasis, fibrosis, and thrombosis.⁵ It exists primarily as a soluble dimer in body fluids such as plasma at high concentrations (0.3 mg/mL in humans)⁶ that is assembled into insoluble fibrils with a fibrillar polymeric matrix during matrix assembly.⁷ In addition to regulation of the biological activities of FN by alternative splicing of the III-type modules or V segment,^{8,9} N- and O-glycosylation of human FN affects the interaction with biological ligands including cell receptors,¹⁰ suggesting that glycosylation

* Corresponding author. Address: Graduate School of Humanities and Sciences, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan. Tel./fax: +81 3 5978 5343; e-mail: ogawa.haruko@ocha.ac.jp

modulates the physiological functions of FN. The concentration of FN has been reported to triple at the protein and mRNA levels during liver regeneration,¹¹ but a change in glycosylation and biological activity has not been demonstrated yet. In this study, we focused on the glycan structure and ligand-binding activities of rat FN during liver regeneration and found differential changes in ECM glycoproteins to regulate biological activities. This research is the first to elucidate the details of the glycan structure of a particular glycoprotein synthesized in the early stage of liver regeneration.

The protein concentration in SH-plasma increased slightly 6–24 h after operation, while in PH-plasma it had decreased to about 60% of the level in NO-plasma at 24 h after PH; then it gradually returned to a normal level (data not shown).³ In contrast, the FN concentration in the plasma increased by 10% between 24 and 120 h after PH compared with NO, while after SH it was increased by 25% at 6 h and had returned to a normal level in 7 days (data not shown). The relative increase of FN in plasma proteins after PH and SH indicates that FN is an acute phase reactant.

FN from rat plasma at 24 h after PH, SH, and NO was purified by affinity chromatography on a gelatin-Sepharose column. FN was scarcely eluted at pH 5.2, but was completely eluted with 6 M urea.^{12,13} Because urea-eluted FN recovered biological activities after removal of the urea,¹² it was used for the following experiments. As shown in Figure 1A, each FN produced major double bands at the migration position around 230 kDa on SDS-PAGE. Compared with NO-FN, the amounts of purified FN were increased 1.6–2 times after SH and 1.2–1.4 times after PH (Fig. 1B). The increased yields of urea-purified PH- and SH-FN coincided with the elevated FN concentrations in plasma. The ratio of purified FN to total FN in plasma was also increased about 1.7 and 1.3 times after SH and PH, respectively (data not shown), suggesting that the gelatin-binding activity of FN was enhanced after surgery.

Ligand-binding activities of FN: FN binding to gelatin was slightly increased in SH-FN in plasma (Fig. 2A) in accordance with the high yield of SH-FN in gelatin affinity chromatography (Fig. 1B). The binding to other ligands, strongly to collagen types I–III and heparin-BSA but weakly to collagen types IV and V, hardly changed after surgery (data not shown for collagen types II, III, and V, and heparin-BSA), although both the concentrations in plasma and the yields of purified FN were increased in SH and PH. This is probably because other factors in plasma interfered with FN binding to the ligands.

As shown in Figure 2B, slightly more purified PH-FN than SH- and NO-FN bound to collagen type I, and more PH- and SH-FN than NO-FN bound to collagen type IV, indicating that surgery only increased collagen binding activities slightly. The very small alterations in collagen-

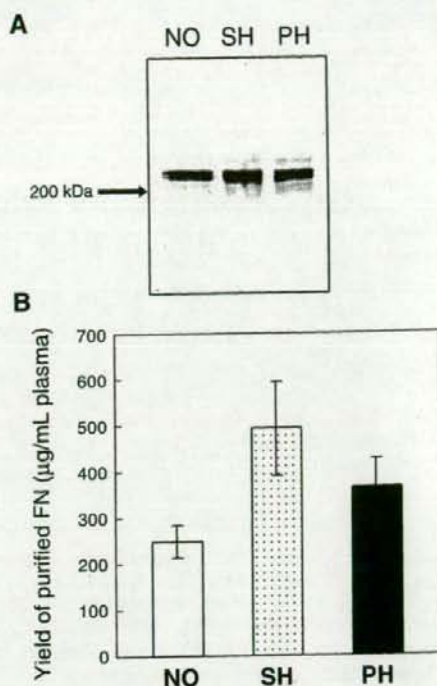


Figure 1. Purification of FN from NO, SH, and PH plasma at 24 h after operations. (A) SDS-PAGE of purified FNs using 6% polyacrylamide gels under reducing condition. (B) Yield of purified FN from each plasma. Error bars were obtained from three experiments. Open bar, NO; dotted bar, SH; solid bar, PH.

binding activities of FN by PH compared to those of VN³ indicate that FN and VN contribute differently to matrix remodeling during liver regeneration. In contrast, the binding activities of purified FNs to gelatin and collagen types IV and V were significantly higher than those of plasma FN before purification (Fig. 2A), indicating that urea-denaturation of FN activates the binding activities to some ligands. Consistent with our results, urea-denaturation of FN has been reported to induce the sequential unfolding and exposure of the type III domain to regulate the interaction with the ECM proteins.¹⁴

As shown in Figure 3, the ligand-binding activities of de-N-glycosylated FN were significantly increased, in contrast to the only slight increase in the binding activities after surgery, showing that glycosylation affects the ligand-binding of FN (Fig. 2). The 2.9-fold increase in binding of VN to type I collagen after de-N-glycosylation^{3,4} is similar.

Changes in glycosylation of FN during liver regeneration: As summarized in Table 1, the most conspicuous change after PH was the increase in FN reactivity to *Aleuria aurantia* lectin (AAL), suggesting an increase of fucosylation in PH-FN. The reactivity to peanut

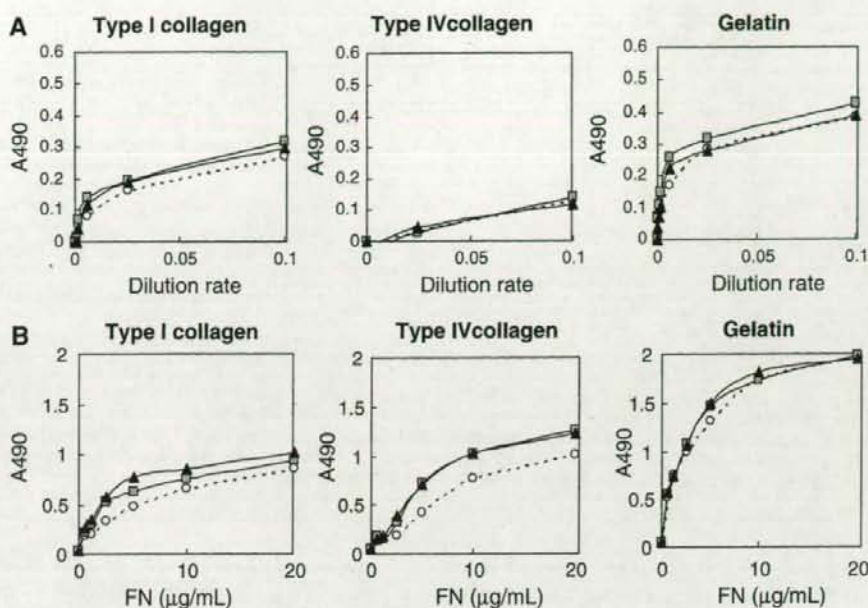


Figure 2. Binding activity of FNs in NO, SH, and PH plasma (A) and purified FNs (B) to collagen types I, and IV and gelatin by ELISA. (A) Binding of FNs in plasma. (B) Binding of purified FNs. Symbols used are circle, NO-FN; square, SH-FN; solid triangle, PH-FN.

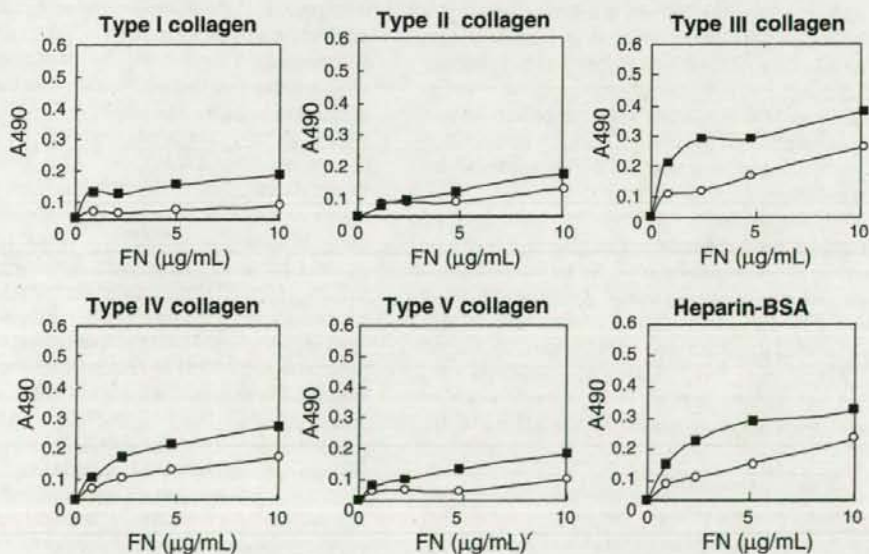


Figure 3. Effect of de-N-glycosylation on the binding activity of FN to collagen types I–V and heparin-BSA by ELISA. Symbols used are circle, control human FN treated without PNGase F; solid square, de-N-glycosylated human FN by PNGase F treatment.

lectin (PNA) after mild acid desialylation of FNs clearly indicated the presence of O-type glycans. The reactivity to *Psathyrella velutina* lectin (PVL) was increased in PH-

FN but disappeared in all FNs after desialylation, indicating that sialylated glycans having more than trisialyl residues¹⁵ increased after PH.

Table 1. Reactivity of various lectins with FNs

	NO-FN	SH-FN	PH-FN	Specificity
Con A	++	++	++	N-Linked biantennary or high Man-type
SNA	++	++	++	Sialyl α -2-6 Gal
MAM	+	+	++	Sialyl α -2-3 Gal
L-PHA	+++	+++	+++	N-Linked tri- or tetraantennary
E-PHA	++	++	+++	N-Linked bi- or triantennary
	(+++)	(++)	(+)	
AAL	++	+	+++	Core or outer fucosylated
LCA	++	++	++	N-Linked, core-fucosylated
PNA	-	-	-	O-Linked, unsialylated Gal β 1-3GalNAc
	[++]	[+++]	[+++]	
PVL	++	++	+++	Non-reducing terminal GlcNAc or tri- or tetra sialyl
	[-]	[-]	[-]	
RCA	+++	+++	+++	Non-reducing terminal Gal β
WGA	++	++	++	GlcNAc

Reactivity of FNs with biotinyl lectins by dot blotting is expressed as staining intensity. +, indicates positive staining; -, negative staining; Reactivity after digestion with hexosaminidase is indicated in parentheses, and reactivity after mild acid desialylation is indicated in brackets. Abbreviations used are: Con A, Concanavalin A; SNA, *Sambucus nigra* agglutinin; MAM, *Maackia amurensis* mitogen; L-PHA, *Phaseolus vulgaris* leukoagglutinin; E-PHA, *Phaseolus vulgaris* erythroagglutinin; AAL, *Aleuria aurantia* lectin; LCA, *Lens culinaris* agglutinin; PNA, peanut agglutinin; PVL, *Psathyrella velutina* lectin; RCA, *Ricinus communis* agglutinin; WGA, wheat germ agglutinin.

As shown in Table 2, the total carbohydrate concentration of PH-FN was markedly decreased compared with those of NO- and SH-FN. The decrease in the ratio of the concentrations of GalNAc to total carbohydrates was notable in PH-FN, indicating that FN is highly O-glycosylated, but that the ratio is lowered in PH-FN. The Fuc concentration increased to about twice that in NO- and SH-FN, in contrast to plasma FN, in which the relative molar ratio of fucose was not changed by PH, although both of these glycoproteins are synthesized in the liver. The isoelectric points of NO-, SH-, and PH-FN, as determined by 2D-PAGE, showed no difference ($pI = 5.7$), suggesting that these FNs are sialylated to the same degree (data not shown).

The N-glycan structures of FN were determined by LC/MS to be the sugar alcohols. Rat plasma FN has seven potential N-glycosylation sites. As shown in Table 3 and Figure 4A, the most frequent N-glycan structures of rat plasma FN were biantennary complex type disialoglycans (BiNA(2)), which are very similar to human plasma FN,¹⁶ and then biantennary trisialoglycans (BiNA(3)). As summarized in Table 3, eight kinds of N-glycan backbones were found. If variations of the

number of O-acetylations of sialic acid residues were included, the varieties of structures would total more than 15 (Fig. 4). The increased ratios of fucosylation in PH-FN were in accordance with the carbohydrate composition results and reactivity with AAL. Because fucosylated glycans are known to participate in embryonic growth, differentiation, cell recognition, cancer formation, and inflammation,¹⁷ the glycans of FN synthesized during liver regeneration may regulate tissue remodeling by increasing fucosylation through a change in binding to other ligands.

The glycans contained various concentrations of O-acetylated Neu5NAc (Fig. 4B). The ratio of non-O-acetylated glycans in each backbone structure was higher in PH-FN than that in NO- and SH-FN. For example, Ac(0) in BiNA(2), a major glycan, was 70% in PH-FN, much higher than those of NO- (52%) and SH-FN (57%). This indicates that the O-acetylation of neuraminic acid is lowered after PH. Sialic acids from total membranes of rat liver have surprisingly high levels (approximately 20%) of O-acetylation at the 7- or 9-position.¹⁸ These modifications modulate many biological interactions,¹⁹⁻²¹ such as that the binding of Siglec-1 (sialoadhesin) and Siglec-2 (CD22) to glycoconjugates, which are hindered by 9-O-acetylation.^{22,23} In the early stage of liver regeneration, the immune system may be regulated by the interaction of FN glycans with Siglecs via the change in O-acetylation.

These results indicate that the alterations in ligand-binding and glycosylation of FN and VN were significantly different in the early stage of liver regeneration and demonstrate that these glycoproteins play different biological roles in the promotion of tissue remodeling processes. This study provides insight into the regulation of individual ECM glycoproteins by glycosylation.

Table 2. Carbohydrate composition of rat FNs

Carbohydrate	NO-FN	SH-FN	PH-FN
GalNAc	92 (9.3)	61 (5.3)	33 (4.1)
GlcNAc	33 (3.4)	37 (3.2)	21 (2.6)
Man	30 (3.0)	35 (3.0)	24 (3.0)
Fuc	1.9 (0.2)	2.5 (0.2)	4.5 (0.6)
Gal	37 (3.7)	57 (5.0)	44 (5.5)
Total	194	193	127

Values are expressed as mol/mol FN. Relative molar ratios of each carbohydrate are expressed in parentheses by taking the value of Man as 3.0.

Table 3. Oligosaccharide structures of rat FN

Glycan structures	Abbreviation
	BiNA(1)
	BiNA(1)F
	BiNA(2)
	BiNA(2)F
	BiNA(3)
	BiNA(3)F
	TiNA(3)
	TriNA(3)F

Abbreviations used for glycan backbone structures are: BiNA(1), biantennary glycan with one *N*-acetylneuraminic acid; BiNA(1)F, biantennary glycan with one *N*-acetylneuraminic acid and a fucose linked to a penultimate GlcNAc; BiNA(2), biantennary glycan with two *N*-acetylneuraminic acids; BiNA(2)F, biantennary glycan with two *N*-acetylneuraminic acids and a fucose linked to a penultimate GlcNAc; BiNA(3), biantennary glycan with three *N*-acetylneuraminic acids; BiNA(3)F, biantennary glycan with three *N*-acetylneuraminic acids and a fucose linked to a penultimate GlcNAc; TiNA(3), triantennary glycan with three *N*-acetylneuraminic acids; and TiNA(3)F, triantennary glycan with three *N*-acetylneuraminic acids and a fucose linked to a penultimate GlcNAc.

1. Experimental

1.1. Animals and purification of rat FN from plasma

Male Wistar rats aged 5 weeks (weighing about 110 g; Nihon Clea, Tokyo, Japan) were subjected to two-thirds PH or SH as described previously.^{3,24} Plasma was collected from NO rats, or 6, 24, 48, 120, and 168 h after SH and PH and stored at -80°C until use. FN was purified from PH-, SH-, or NO-plasma using gelatin-Sepharose as described previously.²⁵ The purity of FNs was checked by SDS-PAGE.¹⁰

1.2. Determination of FN concentration in plasma (sandwich ELISA)

Wells of microtiter plates (Immulon 1, Dynatech Laboratories Inc., Chantilly, VA) were coated with rabbit anti-human FN IgGs (50 μL in 0.5% BSA/PBS) at 4°C overnight. The wells were washed with TBS, and various concentrations of rat plasma or purified FN (50 μL) were added and incubated for 2 h at room temperature. After washing, the wells were blocked with 0.5% BSA/PBS for 1 h, then bound FN was detected with horseradish peroxidase (HRP)-anti-FN IgGs and visualized with *o*-phenylenediamine/0.007% H_2O_2 . After stopping the reaction with 4 M H_2SO_4 , absorbance was measured at 490 nm using a microplate reader.

1.3. Assays of FN binding to immobilized ligands (ELISA)

Wells of microtiter plates were coated with gelatin, heparin-BSA, or collagen types I–V (10 $\mu\text{g}/\text{mL}$, 50 μL) in 0.1 M carbonate buffer (pH 9.0), and reacted with various concentrations of plasma or FN. The bound FN was detected by ELISA.^{26,27}

1.4. Carbohydrate analyses of FNs

For lectin reactivity, FNs (0.5 μg) were dot-blotted onto a PVDF membrane and reacted with HRP-lectins as previously described.²⁸ The staining intensities were measured by the software program Scion Image. To determine carbohydrate composition, FNs (4.5 μg) were hydrolyzed, and the carbohydrates were analyzed according to the previously described method.³

1.5. Analysis of *N*-glycan structures by mass spectrometry (LC/MSⁿ)

FN (30 $\mu\text{g}/\text{about}$ 200 μL) was reduced by boiling with 2% of 2-mercaptoethanol for 1 min. *N*-Linked oligosaccharides were released from the FN with *N*-glycosidase F (4.8 U) and reduced with 0.25 M NaBH_4 . The oligosaccharides were desalted with ENVI-Carb C (Supelco Co., Ltd, Bellefonte, PA, USA) and subjected to LC/MSⁿ. The eluents were 2% acetonitrile/5 mM ammonium acetate (pH 9.6) (pump A), and 80% acetonitrile/5 mM ammonium acetate (pH 9.6) (pump B). The oligosaccharides were separated on a Hypercarb column (0.2 \times 150 mm, Thermo Fisher Scientific, Waltham, MA, USA) with a 5–30% linear gradient of the pump B eluent over 60 min at a flow rate of 2 $\mu\text{L}/\text{min}$. The MS spectra were acquired by using an LTQ-FT system (Thermo Fisher Scientific) with a single mass scan (m/z 7000–2000) on a Fourier transformation ion cyclotron resonance mass spectrometer, and data-dependent

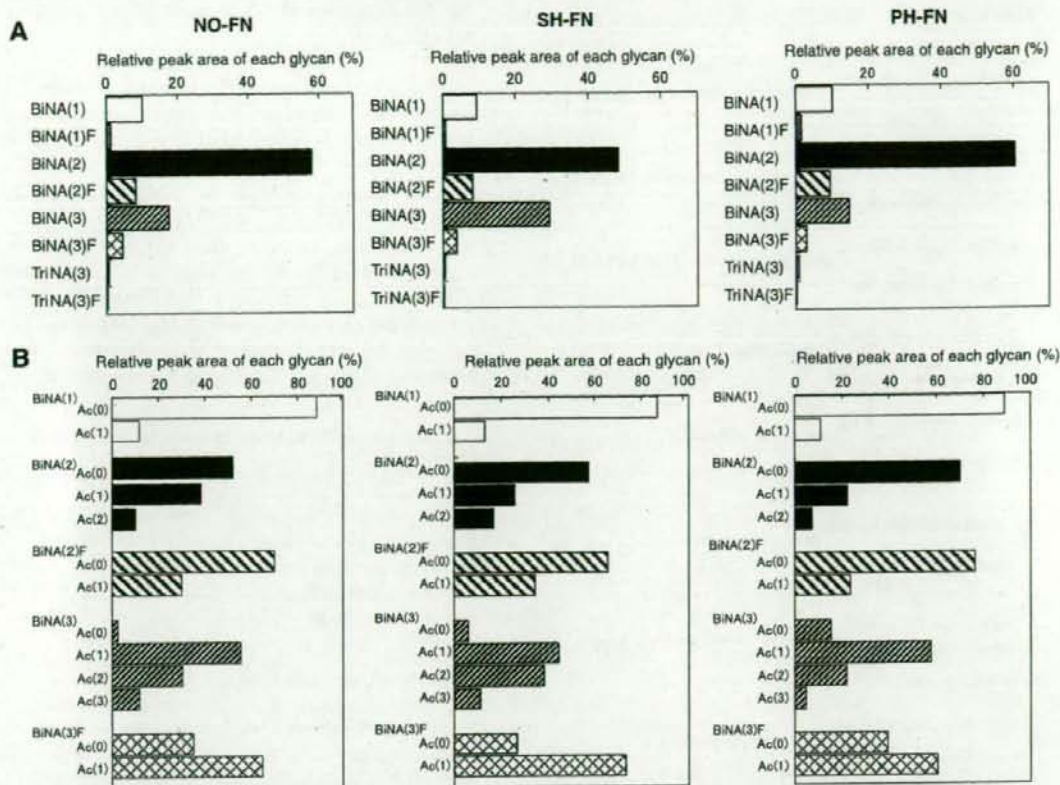


Figure 4. Ratios of each FN N-glycan. (A) The ratio of each glycan structure. (B) Ratios of glycans possessing various numbers of O-acetyl groups at the N-acetylneuraminic acid residues. The ratio of each glycan (the abbreviations are summarized in Table 3) was calculated from the relative peak area of mass chromatography signals in HPLC by taking the total peak area of each FN (A) or of each glycan (B) as 100%. Of the structures not expressed in (B), BiNA(1)F, TriNA(3), and TriNA(3)F, all without O-acetyl group (Ac(0)) were detected.

MS/MS and MS/MS/MS scans in both positive and negative ion modes.

Acknowledgments

This work was supported in part by a Grant from JSPS (12995 to KS) and a fellowship from Hayashi Memorial Foundation for Female Natural Scientists (KS) and Grants-in-aid for Scientific Research on Priority Areas 15040209 and 17046004 (HO) from the Ministry of Education, Culture, Sports, Science, and Technology. We thank our laboratory members for help in the operations and K. Ono for editing the English language of this article.

References

- Varki, A. *Glycobiology* **1993**, *3*, 97–130.
- Diehl, A. M.; Rai, R. M. *FASEB J.* **1996**, *10*, 215–227.
- Uchibori-Iwaki, H.; Yoneda, A.; Oda-Tamai, S.; Kato, S.; Akamatsu, N.; Otsuka, M.; Murase, K.; Kojima, K.; Suzuki, R.; Maeya, Y.; Tanabe, M.; Ogawa, H. *Glycobiology* **2000**, *10*, 865–874.
- Sano, K.; Asanuma-Date, K.; Arisaka, F.; Hattori, S.; Ogawa, H. *Glycobiology* **2007**, *17*, 784–794.
- Potts, J. R.; Campbell, I. D. *Matrix Biol.* **1996**, *15*, 313–320, Discussion 321.
- Dean, D. C.; Birkenmeier, T. M.; Rosen, G. D.; Weintraub, S. J. *Am. Rev. Respir. Dis.* **1991**, *144*, S25–S28.
- McDonald, J. A. *Annu. Rev. Cell Biol.* **1988**, *4*, 183–207.
- Chernousov, M. A.; Fogerty, F. J.; Koteliensky, V. E.; Mosher, D. F. *J. Biol. Chem.* **1991**, *266*, 10851–10858.
- Brown, L. F.; Dubin, D.; Lavigne, L.; Logan, B.; Dvorak, H. F.; Van de Water, L. *Am. J. Pathol.* **1993**, *142*, 793–801.
- Zheng, M.; Hakomori, S. *Arch. Biochem. Biophys.* **2000**, *374*, 93–99.
- Singh, P.; Reimer, C. L.; Peters, J. H.; Stepp, M. A.; Hynes, R. O.; Van De Water, L. *J. Invest. Dermatol.* **2004**, *123*, 1176–1181.
- Regnault, V.; Rivat, C.; Maugras, M.; Stoltz, J. F. *Rev. Fr. Transfus. Immunohematol.* **1988**, *31*, 19–34.
- Chifflet, S.; Bolatto, C.; Tolosa, S. J. *Biochem. Biophys. Methods* **2004**, *59*, 139–143.

14. Patel, S.; Chaffotte, A. F.; Goubard, F.; Pauthe, E. *Biochemistry* **2004**, *43*, 1724–1735.
15. Ueda, H.; Takahashi, N.; Ogawa, H. *J. Biol. Chem.* **2002**, *277*, 24916–24925.
16. Takasaki, S.; Yamashita, K.; Suzuki, K.; Kobata, A. *J. Biochem. (Tokyo)* **1980**, *88*, 1587–1594.
17. Poland, D. C.; Schalkwijk, C. G.; Stehouwer, C. D.; Koeleman, C. A.; van het Hof, B.; van Dijk, W. *Glycoconjugate J.* **2001**, *18*, 261–268.
18. Butor, C.; Diaz, S.; Varki, A. *J. Biol. Chem.* **1993**, *268*, 10197–10206.
19. Paulson, J. C. *Trends Biochem. Sci.* **1989**, *14*, 272–276.
20. Klein, A.; Roussel, P. *Biochimie* **1998**, *80*, 49–57.
21. Hakomori, S. *Curr. Opin. Immunol.* **1991**, *3*, 646–653.
22. Kelm, S.; Schauer, R.; Manuguerra, J. C.; Gross, H. J.; Crocker, P. R. *Glycoconjugate J.* **1994**, *11*, 576–585.
23. Sjöberg, E. R.; Powell, L. D.; Klein, A.; Varki, A. *J. Cell Biol.* **1994**, *126*, 549–562.
24. Higgins, G. M.; Anderson, R. M. *Arch. Pathol.* **1931**, *12*, 186–202.
25. Miekka, S. I.; Ingham, K. C.; Menache, D. *Thromb. Res.* **1982**, *27*, 1–14.
26. Gebb, C.; Hayman, E. G.; Engvall, E.; Ruoslahti, E. *J. Biol. Chem.* **1986**, *261*, 16698–16703.
27. Yoneda, A.; Ogawa, H.; Kojima, K.; Matsumoto, I. *Biochemistry* **1998**, *37*, 6351–6360.
28. Kitagaki-Ogawa, H.; Yatohgo, T.; Izumi, M.; Hayashi, M.; Kashiwagi, H.; Matsumoto, I.; Seno, N. *Biochim. Biophys. Acta* **1990**, *1033*, 49–56.

1 Glycoconj J
DOI 10.1007/s10719-008-9220-y

3
2

4 **Pseudoproteoglycan (pseudoPG) probes that simulate**
5 **PG macromolecular structure for screening and isolation**
6 **of PG-binding proteins**

7 Keiko Nakagawa · Kosuke Nakamura · Yuji Haishima ·
8 Makiko Yamagami · Kana Saito · Hiromi Sakagami ·
9 Haruko Ogawa

10 Received: 28 July 2008 / Revised: 30 September 2008 / Accepted: 8 December 2008
11 © Springer Science + Business Media, LLC 2008

12

13 **Abstract** A proteoglycan (PG) monomer is a macromole-
14 cule consisting of one or more glycosaminoglycan (GAG)
15 chains attached to a core protein. PGs have signaling roles
16 and modulatory functions in the extracellular matrix and at
17 the cell surface. To elucidate the functions of higher-order
18 PG structures, pseudoPGs that imitate the PG structure were
19 prepared to develop probes and affinity adsorbents. Poly-L-
20 lysine (PLL) or polyacrylamide (PAA) was coupled with
21 various GAGs, then biotinylated, and the remaining amino
22 groups were blocked to obtain the pseudoPG probes, biotinyl
23 PLL (BPL)- or PAA (BPA)-GAGs. Lactoferrin exhibited 30-
24 times higher affinity toward BPL-heparin than the conventional
25 single-strand probe, biotin-hydrazide-heparin. Heparin-PLL
26 was immobilized on a formyl-Sepharose and compared with
27 the Hep-Sepharose in which heparin was directly immobilized
28 to amino-Sepharose. Screening for ligands in normal rat brain
29 revealed several proteins that specifically bound to either of the
30 two adsorbents, indicating that the heparin-binding proteins
31 exhibit specific recognition depending on the higher-order
32 structure of the PG.

33 **Keywords** Glycosaminoglycan · Heparin-binding proteins ·
34 Cyclophilin A · HSP90 · Amphoterin · Detection and separation

K. Nakagawa · K. Nakamura · M. Yamagami · K. Saito ·
H. Sakagami · H. Ogawa (✉)
Graduate School of Humanities and Sciences,
Ochanomizu University,
2-1-1 Otsuka, Bunkyo-ku,
Tokyo 112-8610, Japan
e-mail: ogawa.haruko@ocha.ac.jp

Y. Haishima
Division of Medical Devices,
National Institute of Health Sciences,
Tokyo, Japan

13 **Introduction**

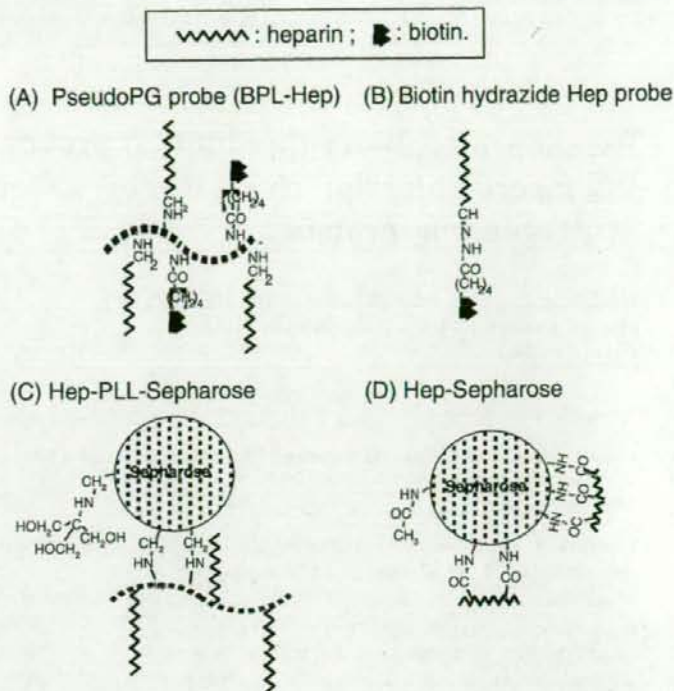
35

36 Proteoglycan (PG)s play signaling roles and modulate cell
37 functions in important cellular events such as cellular
38 proliferation, differentiation, adhesion and motility, mor-
39 phological formation, coagulation, and infection [1, 2]. The
40 structures and number of the glycosaminoglycan (GAG)
41 chains, in addition to the nature of the core protein, are
42 considered to determine the type of PG function [3].
43 However, information about the significance of the proteo-
44 glycan macromolecular structures still remains inadequate.
45 We prepared synthetic probes that simulate the structure of
46 PGs by attaching GAG side chains to a linear polymer
47 strand as the backbone to develop probes and affinity
48 adsorbents to search for and identify PG-binding substan-
49 ces. The new probes were named 'pseudoproteoglycans
50 (pseudoPGs)' (see Scheme 1). In this study, pseudoPGs
51 using five kinds of GAGs centering on heparin were
52 prepared, and they were applied to screen for and analyze
53 interactions with ligand proteins in extracts of rat brain.

54 Recently, significant roles for various proteoglycans in
55 the development, differentiation and morphogenesis of
56 neural tissues have been demonstrated using genetically
57 modified animals [4, 5]. The protein ligands that specifi-
58 cally recognize proteoglycans would be of importance to
59 understand the mechanisms of proteoglycan function, and
60 therefore, we tried to identify specific protein ligands in
61 brain extracts using pseudoPGs. The pseudoPGs exhibited
62 remarkable affinity and binding specificity toward several
63 heparin-binding proteins compared to the conventional
64 single-strand heparin probe. The utility of the pseudoPG
65 probes and the significance of the higher-order structure of
66 GAGs in the recognition of PG-binding proteins were
67 shown.

Q1

Scheme 1 Probes and adsorbents prepared in this study. **a** PseudoPG probe (BPL-Hep). **b** Biotin hydrazide Hep probe. **c** Hep-PLL-Sepharose. **d** Hep-Sepharose



68 **Materials and methods**

69 **Materials** Poly-L-lysine (PLL) with a Mw 15,000–30,000,
 70 bromide salt, and streptavidin-biotinylated horseradish
 71 peroxidase (HRP-ABC) complex were purchased from
 72 Sigma (St. Louis, MO, USA). Polyacrylamide (PAA) with
 73 an average Mw 10,000 was purchased from Aldrich
 74 (Sigma-Aldrich Japan, K.K., Tokyo, Japan), and PAAs of
 75 Mw 600,000–1,000,000 were from Polysciences Inc.
 76 (Warrington, PA, USA). Porcine intestinal mucosa heparin
 77 (Hep), *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
 78 (EEDQ), and unlabelled biotin were purchased from Wako
 79 Pure Chemicals (Osaka, Japan). *N*-Hydroxysuccinimide
 80 biotin and biotin-LC-hydrazide from Pierce (Rockford,
 81 IL, USA) were used for biotin labeling of the probes.
 82 Chondroitin sulfate A (CSA) and chondroitin sulfate B
 83 (CSB) were purchased from Seikagaku Kogyo (Tokyo,
 84 Japan). Colominic acid sodium salt was purchased from
 85 Nacalai Tesque Co. (Kyoto, Japan). D-Glucuro- γ -lactone
 86 was purchased from Merck Japan (Tokyo, Japan).
 87 A pullulan mixture (average Mr=5,900–788,000 Da) as
 88 a standard for size-exclusion HPLC was purchased
 89 from Showa Denko Co. (Tokyo, Japan). Rabbit anti-
 90 human cyclophilin A (CypA) antibodies were purchased
 91 from Upstate Group Inc. (Charlottesville, VA, USA),
 92 and HRP-labeled goat anti-rabbit IgG was from

Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, 93
 MD, USA). 94

Preparation of pseudoPG probes PLL (8 mg) was dis- 95
 solved in 1 ml of distilled water. To 250 μ l of the PLL 96
 solution, 80 μ l of 0.5 M phosphate-buffer (PB) and 6 mg of 97
 Hep or another GAG were added and mixed, then 210 μ l of 98
 4 M NaCl was added to dissolve the precipitate. NaBH₃CN 99
 solution (10 mg per ml of distilled water) was prepared, and 100
 20 μ l was added to the mixture and incubated at 40°C for 101
 9 days with shaking. After the reaction, the mixture was 102
 dialyzed against 0.1 M CH₃COONa to remove the reducing 103
 agent. If precipitation occurred, 2 M NaCl was added to the 104
 mixture to dissolve the precipitate. Then 200 μ l of 0.1% 105
 sulfo-*N*-hydroxysuccinimide (NHS-)biotin was added, and 106
 the mixture was incubated at room temperature for 1 h with 107
 shaking (PLL-GAG). For acetylation of the remaining 108
 amino groups, 100 μ l of acetic anhydride was added to the 109
 reaction mixture and incubated for 30 min on ice, then 110
 an additional 100 μ l was added, and incubated at room 111
 temperature for 30 min with shaking to obtain the 112
 pseudoPG biotinyl poly-L-lysine (BPL)-GAG probe 113
 (Scheme 1a). Then the mixture was dialyzed using a 114
 Spectra/Por CE dialysis tube (MWCO: 1,000, Spectrum, 115
 Gardena, CA, USA) against 1 M sodium phosphate buffer 116
 (pH 7.7). The coupling was monitored by the change in the 117

118 migration position on SDS-PAGE in which GAG and PLL
119 were detected by silver staining, or by cellulose acetate
120 membrane electrophoresis in which GAG was detected by
121 toluidine blue and PLL was detected by Coomassie brilliant
122 blue (CBB) staining [6]. A BPL-glucuronic acid probe was
123 prepared according to the same procedure by using D-
124 glucurono- γ -lactone that had been dissolved in 0.01 M
125 NaOH, then neutralized with dilute HCl to open the lactone
126 ring. As a control, a BPL probe was prepared without the
127 GAG coupling step.

128 *Size-exclusion HPLC* To measure molecular mass (Mr),
129 BPL-Hep was analyzed using an LC-10A HPLC system
130 (Shimadzu, Kyoto, Japan) equipped with a refractive index
131 (RI) detector, UV detector (abs. 220 nm), and multiangle
132 laserlight scattering (MALLS) by Dawn DSP (Wyatt
133 Technology Co., Ltd., Santa Barbara, CA, USA). TSKgel
134 α -6000 (7.8 \times 300 mm, Tosoh, Japan), Ultrahydrogel 500
135 and 250 (7.8 \times 300 mm, Waters, Milford, MA, USA), and
136 Protein KW802.5 (8 \times 300 mm, Shodex, Tokyo, Japan)
137 were selected as the gel filtration chromatography column,
138 and some of them were tandemly connected to improve
139 peak separation. The temperature of the column oven was
140 set at 40°C, and 0.067 M PB (pH 7.4) was used as the
141 mobile phase at a flow rate of 1 ml/min. The Mr was also
142 calculated from comparison with the retention time of the
143 pullulan standard mixture in the HPLC system.

144 *Preparation of biotin-hydrazide-Hep* A single-chain hepa-
145 rin probe, biotin-hydrazide-Hep (Bio-Hep) was prepared by
146 using biotin-LC-hydrazide as described for GlcNAc₅₋₆ [7].
147 Heparin (4 mg) was dissolved in 2 ml of 0.1 M sodium
148 acetate (pH 5.5) and mixed with one ninth vol. of biotin-
149 LC-hydrazide solution (4 mg in 216 μ l DMSO). The
150 mixture was incubated at room temperature for 4 days with
151 shaking (Scheme 1b). The Bio-Hep probe was dialyzed
152 against distilled water.

153 *Immobilization of pseudoPG probe onto affinity adsorbent*
154 A pseudoPG adsorbent, Hep-PLL-Sepharose, was
155 prepared by immobilizing Hep-PLL to formyl-Sepharose.
156 Hep-PLL was reductively aminated by reacting 32.4 mg of
157 Hep and 10.8 mg of PLL as described above. The Hep-PLL
158 was dialyzed against 1 M sodium phosphate buffer and
159 immobilized on 10 g of formyl-Sepharose [8] by adding
160 60 mg NaBH₃CN at 4°C for 6 days. The water-washed gel
161 was incubated in 20 ml of 1 M Tris-HCl (pH 7.4), and
162 60 mg NaBH₃CN was added to block the remaining formyl
163 groups at room temperature for 1 h with shaking. After
164 washing, the gel was suspended in 5 ml of 0.2 M sodium
165 acetate, acetylated by adding 2.5 ml of acetic anhydride,
166 and incubated for 30 min on ice; then, an additional 2.5 ml
167 was added and incubated at room temperature for 30 min

with shaking to block the remaining amino groups 168
(Scheme 1c). The amount of heparin immobilized was 169
1.02 mg/g wet gel as calculated from the hexosamine 170
concentration after hydrolysis, which was measured accord- 171
ing to the method previously described [9]. For a 172
precolumn for Hep-PLL-Sepharose, PLL-Sepharose was 173
prepared by coupling PLL with formyl-Sepharose by 174
reductive amination at 4°C for 7 days by the same 175
procedure as described above for Hep-PLL. 176

Preparation of Hep-Sepharose Hep was immobilized via 177
its carboxyl groups to amino-Sepharose by a condensation 178
reaction with EEDQ. Hep (200 mg) was dissolved in 6 ml 179
of distilled water, and EEDQ (200 mg) was dissolved in 180
4 ml EtOH. The heparin and EEDQ solutions were added to 181
10 g amino-Sepharose [10], and the mixture was incubated 182
at 40°C for 3 days with shaking. Then the gel was washed 183
with water and the remaining amino groups were blocked 184
by *N*-acetylation using the same procedure as that for Hep- 185
PLL-Sepharose (Scheme 1d). The amount of heparin 186
immobilized was calculated to be 7.2 mg per g wet gel 187
according to the method described above [9]. Acetoamido- 188
Sepharose was prepared by *N*-acetylation of amino-Sepharose 189
without coupling to heparin. 190

Preparation of rat brain extract Whole rat brain tissues 191
were homogenized with a homogenizer in four volumes of 192
10 mM Na-phosphate buffer (pH 7.7) containing 0.13 M 193
NaCl, 0.02% NaN₃, 1 mM phenylmethylsulfonyl fluoride, 194
1 mM EDTA, and 20 mM ϵ -aminocaproic acid, and 195
extracted at 4°C overnight with shaking. The homogenate 196
was centrifuged at 20,500 \times g for 50 min at 4°C. The 197
supernatant thus obtained was used as the rat brain extract. 198

Affinity chromatography Rat brain extract (8 ml) was 199
applied to a Hep-PLL-Sepharose column equipped with a 200
precolumn of PLL-Sepharose or a Hep-Sepharose column 201
equipped with a precolumn of Sepharose that had been 202
equilibrated with 10 mM Na-phosphate buffer (pH 7.7) 203
-0.13 M NaCl. The bound proteins were eluted stepwise 204
with 10 mM Na-phosphate buffer (pH 7.7) containing 205
0.5 M NaCl, and then 2 M NaCl. The effluent was 206
monitored by the absorbance at 280 nm. 207

SDS-PAGE and identification of proteins SDS-PAGE was 208
carried out as described by Laemmli [11] under reducing or 209
nonreducing conditions. The electrophoresed proteins 210
(10 μ g/lane) were transferred to a polyvinylidene difluoride 211
(PVDF) membrane (Millipore, Bedford, MA, USA) in 212
100 mM Tris-HCl (pH 7.5) containing 192 mM glycine 213
and 20% MeOH at 100 mA for 100 min at room 214
temperature. The blotted membrane was stained with 215
CBB. Direct N-terminal sequencing of each protein on the 216

217 excised PVDF membrane was performed using a protein
218 sequencer, Procise cLC 492cLC (Applied Biosystems).
219 Proteins were identified by searching the N-terminal four
220 to ten amino acid sequences in the SWISSPROT database.
221 For immunodetection with anti-CypA antibodies, the
222 electroblotted PVDF membrane was blocked with 5%
223 bovine serum albumin-Tris buffered saline (BSA-TBS) at
224 4°C overnight, and reacted with rabbit anti-human CypA
225 antibody (1:5,000 dilution with TBS) for 1 h. After washing
226 three times with TBS containing 0.1% Tween 20, the
227 membrane was blocked with 5% BSA-TBS for 30 min and
228 reacted with HRP-labeled anti-rabbit IgG antibodies for 2 h.
229 After washing with TBS-Tween 20, the membrane was
230 reacted with HRP-ABC (1:1,000 dilution with TBS) for
231 2 hrs. After washing three times with TBS-Tween 20, the
232 color was developed with 0.02% 3,3'-diaminobenzidine
233 (DAB)-0.02% H₂O₂-TBS.

234 *Probe-binding assay on membrane* The blotted membrane
235 with proteins electrotransferred from polyacrylamide gels
236 was blocked with 5% BSA-TBS overnight and incubated
237 with each probe diluted to 24 µg/ml of GAG with 130 mM
238 sodium phosphate buffer for 1.5 h at 4°C. The membrane
239 was washed three times with TBS containing 0.1% Tween
240 20, then blocked with 5% BSA-TBS for 30 min, and
241 reacted with HRP-ABC (1:1,000 dil. with TBS) for 2 h.
242 Further washing and color development were performed as
243 described above for immunoblotting.

244 *Quantification of interactions between probes and various*
245 *heparin-binding proteins by SPR* Quantitative interaction
246 analyses were performed with a BIAcore2000 surface
247 plasmon resonance (SPR) biosensor. The SA sensorchip,
248 which is streptavidin covalently immobilized on a carbox-
249 ymethylated dextran matrix (GE Healthcare Bio-Science
250 Corp., Piscataway, USA), was equilibrated with 10 mM
251 HEPES-buffered saline (pH 7.4), 3 mM EDTA, and 0.005%
252 Surfactant P20 (HBS-EP), and then activated with three
253 consecutive 2-min injections of 40 µl activating buffer (1 M
254 NaCl and 50 mM NaOH) prior to the immobilization. The
255 flow cells were saturated with a 2-min injection of a
256 biotinylated probe, BPL-Hep or biotin-hydrazide-Hep (each
257 10 µg/ml in HBS-EP as heparin concentration), at a
258 constant flow rate of 10 µl/min at 25°C and were blocked
259 with sulfo-NHS-biotin (10 µg/ml). The reference flow cell
260 was prepared with BPL for BPL-Hep or biotin for biotin-
261 hydrazide-Hep as a ligand and used for correction of the
262 non-specific binding of analytes to the sample flow cell.

263 To measure the binding curves, three known heparin-
264 binding proteins, lactoferrin, thrombin, and anti-thrombin
265 III (AT-III), were separately injected onto the sensor chip at
266 various two-fold dilutions in HBS-EP for 120 s at a flow
267 rate of 20 µl/min at 25°C. After the injection of each HBP

concentration, binding surfaces were regenerated with two
consecutive 30-s injections of 10 mM HCl. Kinetic
parameters were calculated by global analysis using the
BIAevaluation software version 3.1.

Results

Preparation of probes The electrophoretic mobility of PLL
to the anode, which was detected with CBB, on the
cellulose acetate membrane was reduced by the coupling
of PLL with D-glucuronic acid by reductive amination.
Heparin migrated to a higher molecular weight position on
SDS-PAGE due to the coupling of PLL with heparin, as
detected with toluidine blue (data not shown). After N-
acetylation, the BPL-Hep was subjected to MALLS on
size-exclusion HPLC. As shown in Fig. 1a-d, as the molar
ratio of PLL to heparin increased, the area ratios of peak 1
to that of the heparin peak (peak 2) increased, indicating
that peak 1 corresponds to BPL-Hep and peak 2 to
unreacted heparin. Similar results were obtained for BPL-
CSA (data not shown). PLL before N-acetylation was
completely adsorbed onto the HPLC columns of Shodex
KW and TSK gel, while unconjugated but N-acetylated
PLL was detected at peak 2 with a smaller refractive index
(RI) signal than that of heparin. Using the four columns in
tandem, the void volume (V_0) and total volume (V_t) were
calculated according to the manufacturer's instructions to
be 24 and 46 ml, respectively.

Although an elevation of the molecular weight of BPL-
Hep compared to that of heparin was suggested by SDS-
PAGE, the yield of BPL-Hep was considered to be small
because the refractive index (RI) of peak 1 was scarcely
detected. Therefore, the absolute molecular weight of BPL-
Hep could not be determined by SEC-MALLS, but the
relative molecular mass was calculated from the elution
position of peak 1 using pullulan as a standard. As shown
in Fig. 1e, it was 160–230 kDa. The molecular mass of free
heparin was calculated by the elution position to be 24.5 +
1.3 kDa, and 17.8 kDa from MALLS and RI signals,
respectively, by using ASTRA software (Wyatt Technolo-
gies Inc.). Because the molecular mass of PLL was 15–
30 kDa according to the software results, five to 12 heparin
chains were immobilized on a PLL chain in BPL-Hep. The
reaction mixtures containing BPL-Hep or BPL were
dialyzed against the buffer and used for immobilization
onto the sensorchip by biotin-avidin interaction.

Interactions between pseudoPG probes and heparin-binding
proteins by SPR The total amounts of immobilized BPL-Hep,
BPL, and biotin-hydrazide-Hep corresponded to 350, 260,
and 130 Biacore resonance units (RU, 1000 RU=1 ng/mm²),