ous analysis of bovine κ-casein and BLAD alleles by multiplex PCR followed by parallel digestion with two restriction enzymes. Anim. Genet. *27*:207-209.

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Attila Zsolnai and László Fésüs
Research Institute for Animal Breeding and Nutrition
Herceghalom, Hungary

Directional Immobilization of Heparin onto the Nonporous Surface of Polystyrene Microplates

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Heparin, a sulfated polysaccharide, exhibits anticoagulant activity mediated through the binding of a specific pentasaccharide sequence in its structure to the protein antithrombin III, a coagulation protease inhibitor (7). Heparin also binds to a large number of other biologically important proteins including growth factors, cell adhesion proteins and enzymes (7).

Heparinized supports have been prepared for the affinity chromatographic separation of heparin-binding proteins using both noncovalent (4) and covalent coupling chemistries (1). Directional covalent coupling chemistries at the aldehyde functionality on the reducing end of heparin include reductive amination to amine-functionalized matrices (5,9) and reaction with surfaces containing hydrazido groups (5,8,9). Covalent immobilization through heparin's reducing end mimics the orientation of the naturally occurring proteoglycan, reduces steric interference between the matrix and heparin-binding proteins, provides identical orientation of heparin chains and exposes all of heparin's binding sites (9). While this directional coupling chemistry has been used to immobilize heparin to porous matrices for affinity chromatography (9), the marked reduction in the surface area of the nonporous surface of polystyrene microplates makes it difficult to demonstrate that covalent immobilization has taken place. The directional immobilization of heparin on microplates should facilitate the high-throughput screening of biological samples for the presence of heparin-binding proteins (7).

Immobilization to Hydrazido Polystyrene

Hydrazido polystyrene plates (Corning Costar, Cambridge, MA, USA) were washed with water, dried and treated with 200 µL of 50-mg/mL heparin (Celsus Laboratories, Cincinnati, OH, USA) in dry formamide (or 2–20 µL of labeled heparin [0.5 mg/mL in dry formamide]) per well. The plates were shaken at 50°C for 24 h. The wells were then washed with 16% NaCl followed by water to remove all the unbound heparin. The excess binding sites were then blocked using 10 µL of acetic anhydride in 200 µL of 0.1 M sodium acetate. The wells were incubated at room temperature for 2 h and the excess acetic acid washed away with water.

Immobilization to Amino Polystyrene

Amino polystyrene plates (Corning Costar) were washed with water, dried and treated with 2–100 µL of heparin (0.5 mg/mL in dry formamide) per well. Formamide was added to bring the final volume in each well to 100 µL. The plates were shaken at 100 rpm at 50°C for 2 h. Sodium cyanoborohydride (1 mg) was added to each well, and the plates were maintained at 100 rpm at 50°C for 24 h. The wells were then washed and blocked as described above. In preparing the control plates, no heparin was added to the wells.

Detection of Immobilized Heparin by Amidolytic Assay

Heparin, immobilized onto hydrazido polystyrene microplates, was analyzed by factor Xa amidolytic assay (Pharmacia Biotech, Uppsala, Sweden). Antithrombin (10 µL) was incubated in each well for 3 min at 37°C, and factor Xa (25 µL) was added. After 30 s, chromogenic substrate (50 µL Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide) was added, and the mixture was held at 37°C for 3 min and quenched by adding 75 µL of 2% citric acid. A_{405} was measured using a microplate reader. A standard curve, constructed using 0.1–0.7 U/mL of soluble heparin, was used to calculate the amount of heparin immobilized. This assay demonstrated that the yield of the immobilization reaction, based on heparin added, was approximately 0.1%. The quantity of heparin immobilized on the hydrazido polystyrene plates and amino polystyrene plates was 6–12 µg/cm^2 and 3–9 µg/cm^2, respectively. Since this method was operating near its limit of sensitivity, labeled heparin

was prepared for more highly sensitive detection.

Ultrasenstive Detection of Immobilized Heparin

Heparin has an average of one unsubstituted amino group per chain, as demonstrated by fluorescence assay (10) and confirmed by proton nuclear magnetic resonance (NMR) (when acetylated with acetic anhydride, heparin shows an increase of one *N*-acetyl group per chain). Biotin and digoxigenin activated with *N*-hydroxysuccinimide (NHS) were used to label heparin at glucosamine residues containing unsubstituted amino groups. Heparin (5 mg) in 200 µL of 0.1 M $NaHCO_3$ was incubated with 2 mg of EZ-Link™ NHS-biotin (Pierce Chemical, Rockford, IL, USA) or NHS-digoxigenin (Boehringher Mannheim, Indianapolis, IN, USA) at 37°C for 6 h. The product was dialyzed (1000 MWCO Spectra/Por® tubing; Spectrum, Houston, TX, USA) and lyophilized. A strong anion-exchange column (Dowex-1; Sigma Chemical, St. Louis, MO, USA) (5 mL) was packed and washed with 10 mL of methanol followed by 10 mL of saturated NaCl. The sodium chloride was washed off and the column equilibrated with water. The partially purified product was dissolved in 500 µL of water and applied to the Dowex-1 column. The column was first eluted with 50 mL of water, and the heparin was released by washing with 15 mL of 16% NaCl, precipitated from 80 vol% methanol (1 vol 16% NaCl, 4 vol MeOH). The precipitated heparin was dissolved in water, desalted by dialysis and lyophilized. Twenty percent of the heparin chains were biotinylated as determined using the avidin-hydroxyazobenzoic acid (HABA) reagent for biotin detection (Pierce Chemical). Digoxigenin-labeled heparin gave a positive dot blot when tested using a digoxigenin detection kit (Boehringer Mannheim).

Heparin labeled with biotin or digoxigenin at an amino group in the center of the chain has a free reducing end. This reducing-end aldehyde was used to immobilize these compounds onto a functionalized polymer matrix (Figure 1) as described previously for nonlabeled heparin.

Next, the amount of immobilized heparin in each well was determined by an alkaline phosphatase-based assay for biotin and digoxigenin. The biotin-avidin and digoxigenin-antidigoxigenin assay systems are among the most sensitive, nonradioactive methods, capable of detecting as small as femtomole quantities of labeled sample (2,3). In the case of immobilized, biotinylated heparin, the wells were washed three times with 200 µL of Tris buffer (100 mM Tris-HCl, pH 7.5) and blocked with 200 µL of 0.2% nonfat dry milk for 30 min. The excess blocking reagent was removed, and the wells were washed three times with 200 µL of Tris-buffered saline (100 mM Tris-HCl, 50 mM NaCl, pH 9.5). The alkaline phosphatase assay was performed using the ABC Staining Kit (Pierce Chemical). Alkaline phosphatase-labeled streptavidin (10 µL) was diluted with 10 mL of Tris buffer, and 100 µL/well were added immediately to the wells and incubated at room temperature for 1 h. The wells were then washed three times with 200 µL of Tris-buffered saline followed by three washings with 200 µL of Tris buffer. The *p*-nitrophenylphosphate (PNPP)

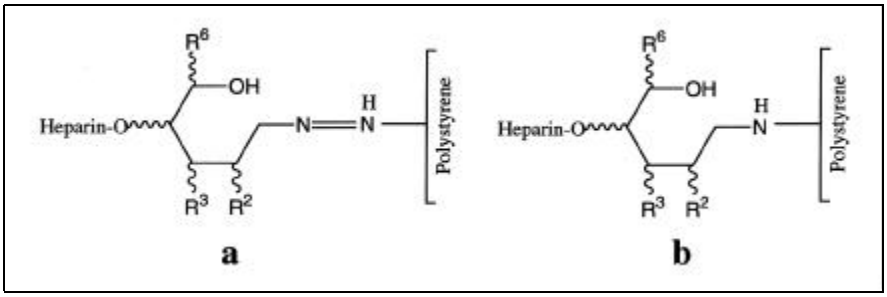

Figure 1. Heparin linked to (a) amino and (b) hydrazido polystyrene plates. The structure of the substituents at positions 2, 3 and 6, labeled R^2, R^3 and R^6, differ depending on the saccharide residue present at heparin's reducing end.

solution was prepared by dissolving one tablet of PNPP in 10 mL Tris buffer, and 100 µL of this solution were added to each well. The plate was incubated at room temperature for 3 h, and the reaction was quenched using 10 µL of 2 *N* NaOH. A_{405} was measured using a microplate reader. The immobilized digoxigenin heparin was determined using a digoxigenin detection kit. The wells were washed, blocked and washed as previously described. Alkaline phosphatase-labeled anti-digoxigenin antibodies (10 µL) were diluted with 10 mL of Tris buffer and added immediately to the wells (100 µL per well) and incubated at 4°C for 1 h. The wells were then washed three times with 200 µL of Tris-buffered saline followed by three washings with 200 µL of Tris buffer. The PNPP solution was prepared (as described above), and 100 µL of the solution were added to each well. The plate was incubated at room temperature for 30 min, and the reaction was quenched using 10 µL of 2 *N* NaOH. A_{405} was measured using a microplate reader (Figure 2).

The immobilized heparin labeled with biotin or digoxigenin both gave linear curves when the absorbance was plotted against the amount of heparin added to the well (Figure 1). Heparin lyase I breaks heparin down into disaccharide- to hexasaccharide-sized oligosaccharides (6). Each well containing immobilized heparin and each control well (containing no immobilized heparin) was treated with 10 mU of heparin lyase I (6) in 100 µL of 100 mM $NaPO_4$ and 50 mM NaCl, pH 7.1. The wells were maintained at 30°C for 12 h, after which the wells were washed with 16% NaCl and water and then air-dried. Heparin lyase I treatment of the wells removed most of the immobilized heparin by depolymerization (Figure 1). The color in the heparin lyase-treated wells was the same as in the blank wells containing no heparin. As the amount of immobilized heparin increased from 6 to 12 ng/well, the ability of the heparin lyase I to depolymerize the immobilized heparin decreased. Addition of more heparin lyase I did not increase the amount of heparin removed. The failure of heparin lyase I to act on heparin immobilized at high surface densities may be attributed to the steric factors preventing the lyase from reaching heparin chains that are very close to each other. Heparinized plates prepared using this method should be used immediately, because plates containing immobilized heparin, stored for several days at room temperature, show decreased heparin content. This instability may either result from the instability of a single covalent attachment site for the highly charged heparin chain to the hydrophobic polymer plate or an instability of the amino- or hydrazido-functionalized polystyrene surface.

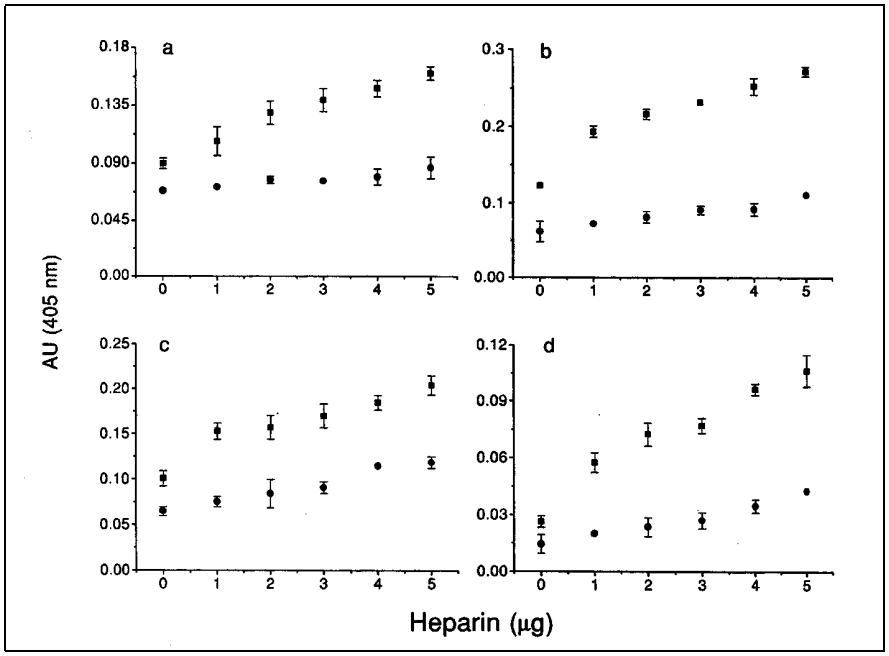

Figure 2. Heparin labeled with digoxigenin or biotin at an amino group in the center of the heparin chain, immobilized to the wells of a microplate, measured by absorbance using phosphatase color reaction. Plates before (■) and after (●) treatment with heparin lyase I: (a) digoxigenin-labeled heparin on hydrazido plates; (b) digoxigenin-labeled heparin on amino plates; (c) biotin-labeled heparin on hydrazido plates; (d) biotin-labeled heparin on amino plates. Each value is the average of 3 determinations. The standard deviation is shown on a value only when it is greater than the size of the symbol drawn. These experiments were performed on four different days using freshly prepared stock solutions of reagents. Thus, the absorbance observed (before heparin lyase I treatment) at 405 nm at each heparin concentration was different.

REFERENCES

1. **Farooqui, A.A. and L.A. Horrocks.** 1983. Heparin-sepharose affinity chromatography. Adv. Chromatogr. *23*:127-148.
2. **Hull, R. and A. Al-Hakim.** 1988. Chemically synthesized non-radioactive biotinylated long chain nucleic acid hybridization probes. Biochem. J. *251*:935-938.
3. **Kerkhof, L.** 1992. A comparison of substrates for quantifying the signal from a nonradiolabeled DNA probe. Anal. Biochem. *205*:359-364.
4. **Larm, O., R. Larsson and P. Olsson.** 1983. A new non-thrombogenic surface prepared by selective covalent binding of heparin via a modified reducing terminal residue. Biomater. Med. Devices Artif. Organs *11*:161-173.
5. **Linhardt, R.J.** 1992. Chemical and enzymatic methods for the depolymerization and modification of heparin, p. 385-401. *In* H. Ogura, A. Hasegawa and T. Suami (Eds.), Carbohydrates—Synthetic Methods and Applications in Medicinal Chemistry. Kodansha, Ltd., Tokyo.
6. **Linhardt, R.J.** 1994. Polysaccharide lyases, p. 17.13.17-17.13.32. *In* A. Varki (Ed.), Current Protocols in Molecular Biology, Analysis of Glycoconjugates. Wiley Interscience, Boston.
7. **Linhardt, R.J. and T. Toida.** 1997. Heparin analogs: development and applications, p. 277-341. *In* Z.B. Witczak, K.A. Nieforth (Eds.), Carbohydrates as Drugs. Marcell Dekker, New York.
8. **Liu, J., A. Pervin, C.M. Gallo, U.R. Desai, C.L. Van Gorp and R.J. Linhardt.** 1994. New approaches for the preparation of hydrophobic heparin derivatives. J. Pharm. Sci. *83*:1034-1039.
9. **Nadkarni, V.D., A. Pervin and R.J. Linhardt.** 1994. Directional immobilization of heparin to beaded supports. Anal. Biochem. *222*:59-67.
10. **Toida, T., H. Yoshida, H. Toyoda, T. Koshiishi, T. Imanari, R.E. Hileman, J.R. Fromm and R.J. Linhardt.** 1997. Structural differences and the presence of unsubstituted amino groups in heparin sulphates from different tissues and species. Biochem. J. *322*:499-506.

Address correspondence to Robert J. Linhardt, PHAR S328, College of Pharmacy, University of Iowa, Iowa City, IA 52242, USA. Internet: robert-linhardt@ uiowa.edu

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Varsha D. Nadkarni and Robert J. Linhardt
University of Iowa
Iowa City, IA, USA

Enhancement of Diaminobenzidine Colorimetric Signal in Immunoblotting

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Immunodetection methods such as immunohistochemistry and immunoblotting (Western blot analysis) are used to specifically and sensitively detect proteins of interest. In both immunohistochemical and immunoblotting techniques, a primary antibody binds to the protein of interest, and through the subsequent detection of this immunoglobulin with a conjugated secondary antibody, the presence of the protein of interest can be visualized using radioisotopic or enzymatic techniques. We report that the combination of 3,3′-diaminobenzidine (DAB) with imidazole and metal enhancement is a highly sensitive visualization technique for Western blot analysis. This procedure gives an intense brown- or blue/grey-colored reaction product, a high signal-to-noise ratio and greatly enhanced product detection compared to the standard DAB protocol.

Many visualization protocols use secondary antibodies conjugated directly to enzymes or linked to amplification systems that use various substrates to produce a detectable product. Enzymes most frequently used for these procedures include alkaline phosphatase and horseradish peroxidase (HRP). Studies have found that for light and electron immunohistochemistry, DAB is an exceptionally good substrate for HRP because, upon oxidation, DAB polymerizes to form an intense brown-colored precipitate that is insoluble in aqueous or organic solvents (3). Further studies have shown that for immunohistochemical staining, both the sensitivity and intensity of the standard HRP/DAB protocol of Graham and Karnovsky (3) can be increased by acidifying the buffer closer to the optimum pH for HRP enzymatic activity (13,14) or by the addition of imidazole to the reaction buffer (10,11,14). HRP catalyzes the transfer of electrons from DAB, resulting in its oxidation. The addition of imidazole has been suggested to induce formation of a third electron transfer site in HRP, thereby increasing its activity (10). Imidazole has been reported to increase HRP oxidation of DAB approximately fivefold (12). Finally, the addition of metals such as nickel, cobalt, copper and silver has been shown to improve DAB polymer formation and can change the color of the final reaction product (1,5,8). The nature of the DAB-metallic ion interaction is not definitively known but may be similar to the deposition of osmium in DAB polymers (5,9).

Immunoblotting, or Western blotting, is used to detect the amounts of specific protein in cell or tissue extracts after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretic transfer of the proteins onto a nitrocellulose or polyvinylidene difluoride (PVDF) filter (2,4). Following binding of a primary antibody and secondary antibody, both colorimetric and chemiluminescence-based detection of HRP or alkaline phosphatase are commonly used to generate a detectable signal. Chemiluminescence-based procedures are generally considered more sensitive than colorimetric detection (7).

In this study, Western blot analysis was performed, and the sensitivity and intensity of reaction product after visualization using the standard HRP/DAB oxidation protocol (3), with and without metals, was compared with imidazole-enhanced HRP/DAB with and without metals. Other HRP substrates (aminoethylcarbazole) or other intensification procedures (lower pH, silver or copper enhancement) were also tested and found to give a much lower signal-