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Affinity capillary electrophoresis employing immobilized glycosaminoglycan to resolve heparin-binding peptides

A new capillary electrophoresis technique has been developed for the affinity resolution of synthetic heparin-binding peptides using an immobilized glycosaminoglycan. Heparin and heparan sulfate were immobilized onto fused silica capillaries using biotin-neutravidin conjugation. These capillaries exhibited markedly reduced electroosmotic flow and were able to distinguish peptides based on the heparin binding domain of acidic fibroblast growth factor (residues 125–144, GLKKNGSCKRGPRTHYGQKA) that differed only in the stereochemistry of the proline amino acid residue. The peptide based on the native sequence was retarded compared to the peptide having unnatural stereochemistry, consistent with its stronger interaction for immobilized glycosaminoglycan. Improved resolution is also obtained for additional arginine and lysine containing heparin-binding peptides.

1 Introduction

The glycosaminoglycans (GAGs) heparan sulfate and heparin are related, linear, polydisperse, micro-heterogeneous polysaccharides. GAGs exert their biological activities through the activation or inactivation of interacting proteins [1]. These interactions play important roles in the normal physiology of animals [2] and are also involved in certain pathologic processes [3]. Heparan sulfate plays a major role in cell-cell and cell-protein interaction [1]. Heparan sulfate is also believed to be an endogenous receptor for circulating growth factors and chemokines that regulate cell growth and migration [1]. Heparin and low molecular weight heparins are regularly used as an anticoagulant and as antithrombotic agents [4]. An understanding of how GAGs interact with proteins is needed to develop peptide-based therapeutics for the prevention and treatment of disease processes, including control of tumor cell replication, migration, invasion and vascularization [2, 3] that involve GAG-protein interactions.

Based on reports on the study of ligand-protein interaction using capillary electrophoresis (CE) [5, 6], we asked whether it would be possible to combine the low sample requirements and the high resolution of CE with the specificity and qualitative information available in heparin-affinity chromatography [7]. In a recent report [8], peptide-heparin interactions were characterized using conventional affinity capillary electrophoresis (ACE), where the peptide was monitored in the presence of differing amounts of heparin added to the electrophoresis buffer. While this new approach represents an important development, it has potential drawbacks in the analysis of heparin-protein/peptide interactions: (i) the possibility of varying electroosmotic flow (EOF) at different concentrations of highly charged ligands (*i.e.*, heparin) and (ii) the need for a large excess of one of the binding pairs. Indeed, despite extensive efforts in our laboratory we have been unable to separate certain heparin binding proteins/peptides by conventional ACE. An alternative approach is to immobilize heparin or heparan sulfate onto the interior wall or a standard capillary in much the same manner as traditional affinity matrices. This leads to a fixed concentration of heparin or heparan sulfate ligand and a constant EOF. Modified capillaries, with a covalently bonded polymer or protein [7] coatings, have been used almost exclusively either to reduce electroosmotic flow [9] or to reduce protein interactions with the capillary [10]. Immobilization of biological species onto capillary walls has only recently been reported. Biosensor devices have used immobilized enzyme at one end of a capillary to quantify glutamate through the detection of fluorescent NADH [11], and β-galactosidase, immobilized on the capillary surface, has been used to study enzyme kinetics [9]. This communication reports the first example of an affinity ligand being immobilized onto a capillary for use during electrophoresis. Here we employ this new approach for the separation of heparin-binding peptides.

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Abbreviations: aFGF, acidic fibroblast growth factor; **APTES,** 3-aminopropyltriethoxysilane; **GAG,** glycosaminoglycan; **HABA,** hydroxyazobenzoic acid; **NHS-LC,** *N*-hydroxyl succinimide-long chain

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2 Materials and methods

2.1 Materials

Heparin and heparan sulfate (USP), sodium salts, from porcine intestinal mucosa, were from Celsus Laboratories (Cincinnati, OH). 3-Aminopropyltriethoxysilane (APTES) was from Aldrich Chemical Co. (Milwaukee, WI). Neutravidin, succinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin), biotin hydrazide, 1,ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and 2-(4′-hydroxyazobenzene) benzoic acid (HABA) were from Pierce (Rockford, IL). Blocked amino acids, *N*-termini acetylated and *C*-termini amidated arginine and lysine were from BaChem (King of Prussia, PA). Peptides, *N*-acetylated and *C*-amidated, were purchased from PeptidoGenic (Livermore, CA). Dialysis membrane was from Spectrum (Houston, TX). Fused silica capillary, 75 μm ID, 363 μm was from Dionex (Sunnyvale, CA) and the neutral coated capillary was from Supelco (Bellefonte, PA). All other reagents used were analytical grade.

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2.2 Buffers

Carbonate buffer, 50 mM sodium carbonate, pH 8.3; phosphate buffer, 100 mM sodium phosphate, 150 mM NaCl, pH 8.5; CE running buffer, 50 mM sodium phosphate buffer, pH 7.4; carbodiimide buffer, 0.1 M MES, pH 4.9; heparin lyase buffer, 50 mM sodium phosphate buffer, pH 7.6, were used.

2.3 Biotinylation of heparin and heparan sulfate via primary amino groups

The *N*-succinimidal ester of biotin was reacted with the free amine groups of heparin or heparan sulfate as follows. In separate glass vials, 10 mg (0.7 μmole) of heparin and heparan sulfate were dissolved in 0.4 mL distilled water and mixed with 20 μL (4.4 μmole) of NHS-LC-biotin from a freshly prepared 100 mg/mL solution in dry *N,N'*-dimethylformamide. The reactions were carried out at room temperature with constant stirring. After 2 h, an additional 20 μL of NHS-LC-biotin was added from a 100 mg/mL solution in dry-*N,N'*-dimethylformamide. The reactions were terminated after 4 h by methanol precipitation of the glycosaminoglycans. Methanol was added to 80% (v/v) and the reaction vials were stored for 12 h at 4°C. Heparin and heparan sulfate were recovered by centrifugation (8000 $\times$ *g*, 10 min, 20°C). The reaction mixtures were dissolved in 1 mL distilled water and exhaustively dialyzed using 3500 molecular weight cut-off membranes against 3 $\times$ 4 L distilled water over 36 h to remove unreacted biotin. Following dialysis, the samples were freeze-dried and stored at -20°C until used in the capillary modification. The degree of biotinylation was determined using the commercially available HABA assay but using 0.1 M sodium bicarbonate buffer to eliminate the interference in this assay by heparin [12] and heparan sulfate.

2.4 Biotinylation of heparin *via* carboxy groups

Heparin that had been acetylated to remove any residual primary amines [13] (20 mg, 1.4 μg) was dissolved in 1 mL of carbodiimide buffer. Biotin hydrazide (4 molar equivalents in 1 mL of carbodiimide buffer) was added, followed by EDC (10 molar equivalents in 0.5 mL of carbodiimide buffer). The mixture was stirred at room temperature for 2 h. The mixture was then exhaustively dialyzed, freeze-dried and assayed for degree of biotinylation as previously described.

2.5 Capillary modification

A modification of the immobilization method of Olson Cosford and Kuhr was used [11]. Fused silica capillary (75 μm ID, 363 μm ID) was installed into a capillary cartridge for automation of the various washing and incubation steps. The total capillary length was 77 cm with an effective separation length (injection end to detector) of 70 cm. The capillary was rinsed using high pressure for 30 min each with 0.1 M NaOH, distilled water, methanol and dry acetone. Surface silanol groups were converted to primary amines by treatment with APTES (0.5 mL volume of 2% v/v APTES in dry acetone). The capillary was cured by incubation at 45°C for 12 h. Residual APTES was removed by rinsing the capillary with carbonate buffer using a high-pressure rinse for 60 min (20 p.s.i.). The capillary was biotinylated by treatment with NHS-LC-biotin. Using low pressure (0.5 p.s.i.), the capillary was flushed for 30 min with NHS-LC-biotin prepared by diluting a 50 mg/mL solution of NHS-LC-biotin in *N,N'*-dimethylformamide into carbonate buffer to a final concentration of 5 mg/mL. The capillary was flushed with a second freshly prepared solution of NHS-LC-biotin and incubated at 4°C for 20 min. After a 15 min high-pressure rinse with phosphate buffer, the capillary was flushed with 0.1 mg/mL neutravidin in phosphate buffer for 30 min using low pressure followed by 1 h incubation at room temperature. A 2 min high-pressure rinse was performed with distilled water followed by phosphate buffer. The affinity layer was introduced by a 1 h low-pressure rinse with biotinylated heparin or heparan sulfate (2.5 mg/mL in phosphate buffer) followed by a 12 h incubation at 4°C. A final 10 min high-pressure phosphate buffer rinse removed noninteracting glycosaminoglycans.

2.6 Affinity capillary electrophoresis

All separations were performed using a Beckman P/ACE system 5500 equipped with a diode array detector. Data was collected from 190 to 300 nm at 1 nm increments using P/ACE Station software. Samples were applied using a 5 s low-pressure injection step. Separations were performed at 20°C and 20 kV in normal polarity (the sample was injected at the anode and detected at the cathode).

2.7 Quantitation of biotin loading of the capillary surface

Biotins immobilized to the capillary surface were titrated with HABA-neutravidin reagent prepared as described in the product literature. A standard immobilization procedure was halted at the biotin step. After extensive rinsing, the capillary (77 cm length) was split to form two 37 cm capillaries (for replicate determinations). The capillary was first filled with distilled water and then a 30 s injection of the HABA-neutravidin reagent was made. The sample plug was mobilized through the full length of the capillary by low pressure. The maximum absorbance at 500 nm was monitored during the sample plug. Injections were repeated until no additional increase in absorbance was observed. Absorbance was correlated to biotin concentration in the capillary by standard addition of biotin to the HABA-neutravidin stock. Absorbance correlated linearly with biotin concentration with a slope of -0.04 mAU μM^{-1} with $R^2 > 0.99$. This entire process was repeated on a second capillary.

3 Results and discussion

3.1 Immobilization of GAG

Immobilization of the GAG onto the surface of the capillary is illustrated in Fig. 1. Heparan sulfate was immobilized to the surface through biotin covalently

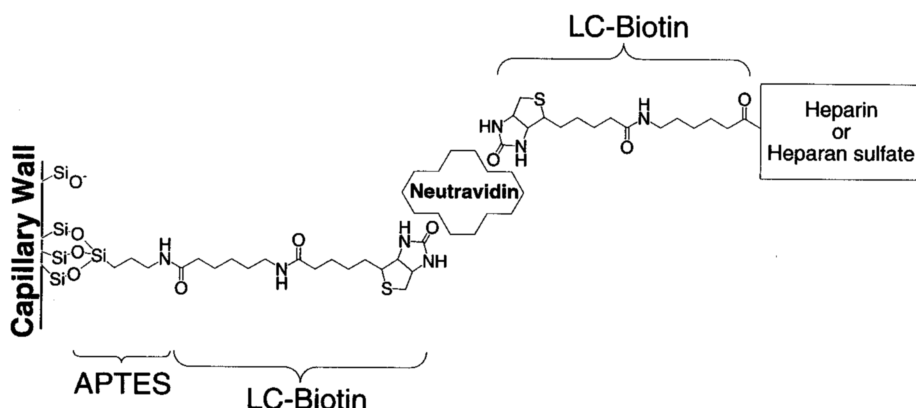

Figure 1. Schematic diagram showing the immobilization of GAG to the capillary surface.

attached (at a 2.5:1 ratio of biotin:heparan sulfate) through its primary amino group. Due to the small number of free amino groups present in heparin [14], the carboxyl groups were biotinylated (at a 1.8:1 ratio of biotin:heparin). Capillaries prepared using both biotinylation approaches performed comparably. Biotinylated GAG was bound tightly and noncovalently to the avidinylated capillary. The EOF in these modified capillaries was reduced (typically 0.2 mm/s linear velocity) compared to normal fused silica capillaries (1 mm/s) using the same running conditions.

3.2 Separation of heparin-binding peptides

A recent study [15] in our laboratory explored the role of the conformation synthetic, heparin-binding peptides. The structure of these peptides was based on the heparin binding domain of acidic fibroblast growth factor (aFGF) having the sequence GLKKNGSCKRGPRTHYGQKA, residues 125–144. This study showed that basic amino acids positioned near a turn were important for heparin-binding affinity. A peptide in which L-proline (P) in the native peptide sequence was replaced with a D-proline showed reduced affinity for heparan sulfate. While both peptides interact with heparin and heparan sulfate, dissociation constants (K_d's) of 4.4 ± 0.4 μM and 7.5 ± 0.11 μM were observed for the heparan sulfate-interaction with native L-proline-containing peptide and D-proline-containing peptide, respectively. A racemic mixture of the D- and L-proline peptides (0.3 mg/mL, 130 μM) was injected (22 nL) onto the capillary. The two peptides, which differ only in the stereochemistry of a single amino acid, were resolved by a heparan sulfate coated capillary (Fig. 2). The two peaks were identified by spiking experiments using the D- and L-proline peptides. In affinity interactions, the more strongly interacting species are retarded more by the capillary and thus have a longer retention time (25.4 and 25.9 min for the D- and L-proline), suggesting that this separation is based on the different affinities of the two peptides. Results using the heparin-coated capillary were similar. No resolution was observed in a control experiment using a normal fused silica capillary (data not shown). An additional control experiment was carried out using a commercial capillary coated with a neutral, hydrophilic polymer that displays negligible EOF to rule out the possibility that the separation resulted from the reduced EOF in the modified capillary. Again, no resolution was observed (Fig. 2), again confirming that these results are consistent with a true affinity separation.

3.3 Determination of ligand density and capillary stability

An assay was developed to determine the number of biotins immobilized at the surface to verify the efficacy of the covalent modification steps and to provide an upper limit for the number of affinity ligands immobilized on the capillary surface. The change in the absorbance of elution buffer is shown for multiple injections of a HABA-neutravidin reagent onto a modified capillary (Fig. 3). All the available biotins are blocked by neutravidin after the fifth injection. A ligand density of 2.4 ± 0.2 × 10^{-11} mol of biotin in a 77 cm capillary length can be calculated based on the assumption that a single biotin on the surface will prevent one neutravidin from reaching the detector. Five 300 nL injections of a mixture of HABA (300 μM) and neutravidin (7 μM) were required to saturate the column. This corresponds to 3 × 10^{-13} mol/cm of biotin on the inner wall of the capillary. This number reflects the maximum number of affinity ligands that can be immobilized at the surface, assuming optimal conditions.

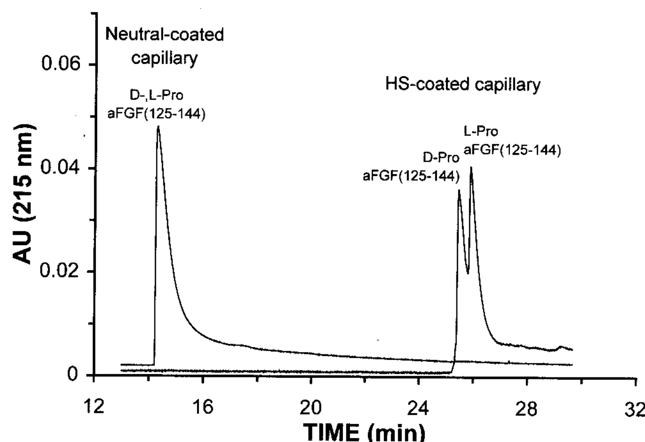

Figure 2. Separation of D- and L-proline aFGF peptides using neutral and heparan sulfate-coated capillaries. A racemic mixture of D- and L-proline containing peptides) 130 μM) were injected (22 nL) and subjected to electrophoresis using 50 mM sodium phosphate buffer, pH 7.4, 20°C and 20 kV.

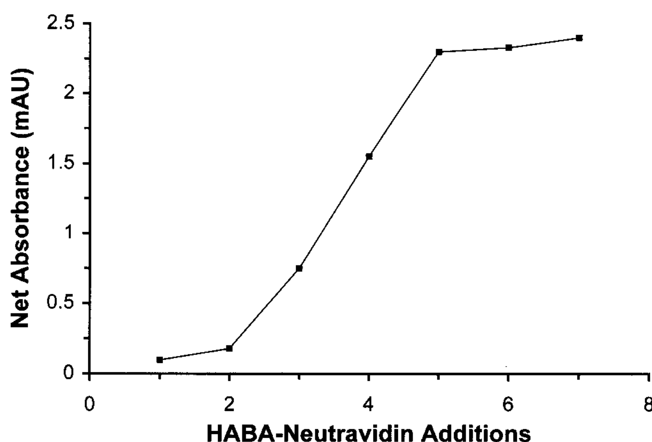

Figure 3. Quantitation of biotin loading of the capillary surface. Titration of biotins immobilized at the capillary surface using neutroavidin-HABA injections having absorbance at 500 nm.

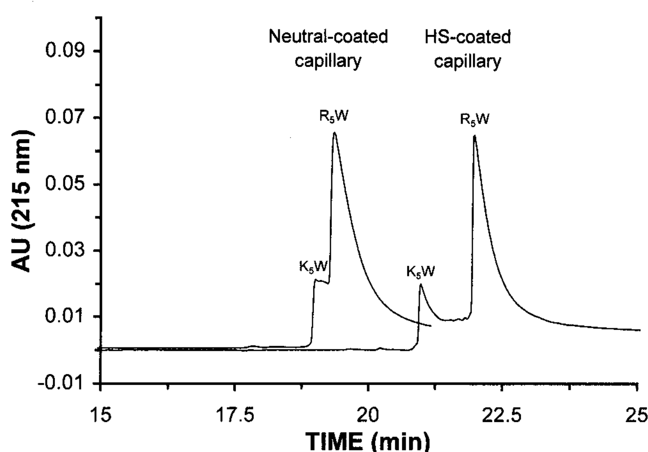

Figure 4. Separation of K_5W and R_5W peptides using neutral and heparan sulfate-coated capillaries. A mixture of K_5W and R_5W peptides (140 and 400 μM, respectively) were injected (22 nL) onto the capillaries and electrophoresis was performed as described in Fig. 2 legend.

Attempts to selectively remove the affinity ligand by treatment with 8 M guanidine hydrochloride and heparin lyase resulted only in partial loss of resolution of the D- and L-proline peptide mixture. Harsher treatment such as washing the capillary with 0.1 and 1 M sodium hydroxide resulted in a complete loss of resolution. However, EOF was essentially unchanged, indicating incomplete regeneration of the original silanol surface. These studies demonstrate the high level of surface stability in these affinity capillaries. A heparan sulfate-coated capillary stored for four months at 4°C gave comparable resolution to a freshly prepared capillary. More than 50 runs could be done on a single capillary without measurable loss in resolution.

Arginine-rich peptides bind heparin and heparan sulfate with several-fold higher affinity than do lysine-rich peptides [15]. Arginine and lysine polypeptides R_5W and K_5W (400 and 140 μm, respectively) were injected (22 nL) onto a neutral-coated capillary. These peptides differ sufficiently in their properties to be partially separated on this neutral-coated capillary (Fig. 4). Injection of the same sample mixture on a heparan sulfate-coated capillary resulted in markedly improved resolution. While both peptides are retained longer in the heparan sulfate-coated capillary relative to the neutral capillary as a result of their interaction with the affinity matrix, the higher affinity of R_5W for heparan sulfate slows its migration through the affinity capillary, increasing the observed separation.

4 Concluding remarks

We have demonstrated a new analytical technique that combines the low sample requirements and inherent high resolution of CE with the selectivity of affinity chromatography. This technique is well-suited to affinity interactions of any type, but may find its greatest utility for assessing weak binding species, which have typically been difficult to measure using affinity chromatography. Tightly binding species do not migrate under electrophoresis and may require the development of an elution step or modification of electrophoresis conditions, such as temperature and buffer composition. The use of an immobilized ligand offers the advantage of requiring smaller amounts of both ligand and ligate. This is especially important in studying heparan sulfate and heparin subfractions containing specific structural features, as these can generally only be prepared in limited quantities. There are, however, a number of drawbacks to the method described in this paper: it requires additional effort to accurately determine the concentration of the immobilized heparin ligand; the immobilized ligand might not be uniformly accessible for all interactions [12]; and there is a risk of altering the ligand characteristics through the derivatization chemistry. Despite these limitations, this technique could have important applications in the fields of biotechnology and pharmaceutical industry.

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