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Capillary Electrophoresis with Stationary Nanogel Zones of Galactosidase and *Erythrina Cristagalli* Lectin for the Determination of $\beta(1-3)$ -Linked Galactose in Glycans

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Abstract

A thermally responsive nanogel is used to create stationary zones of enzyme and lectin in a separation capillary. Once patterned in the capillary, analyte is driven through the zone, where it is converted to a specific product if an enzyme is used or captured if a lectin is used. These stationary zones are easily expelled after the analysis and then re-patterned in the capillary. The nanogel is compatible with enzymes and lectins and improves the stability of galactosidase, enabling more cost-effective use of biological reagents that provide insight into glycan structure. A feature of using stationary zones is that the reaction time can be controlled by the length of the zone, the applied field controlling the analyte mobility, or the use of electrophoretic mixing by switching the polarity of the applied voltage while the analyte is located in the zone. The temperature, applied voltage, and length of the stationary zone, which are factors that enhance the performance of the enzyme, are characterized. The combined use of enzymes and lectins in capillary electrophoresis is a new strategy to advance rapid and automated analyses of glycans using nanoliter volumes of enzymes and lectins. The applicability of this use of stationary zones of enzyme and lectin in capillary electrophoresis is demonstrated with the identification of $\beta(1-3)$ -linked galactose in N-glycan.

1. Introduction

Protein glycosylation is a significant, but challenging post-translational modification that must be monitored.[1, 2] Characterizing glycosylation is difficult because these structures vary in their monomer sequence and monomer linkage. Different structural changes are relevant to different areas of biotechnology. For example, glycosylation affects the safety and efficacy of biological therapeutics and serves as a biomarker of disease.[3–5] The N-glycans that are associated with dysfunction arise from alterations of a predictable base structure that contains a mannose core attached to N-acetylglucosamine linked to galactose [6]. In glycoproteins found in animals, galactose residues are generally linked to an *N*-

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acetylglucosamine residue through a $\beta(1-4)$ linkage, (Gal $\beta(1-4)$ GlcNAc). Exceptions to this linkage specificity occur in protein modifications related to embryogenesis and in oncogenesis. The occurrence of a $\beta(1-3)$ linkage between galactose and *N*-acetylglucosamine (Gal $\beta(1-3)$ GlcNAc) is realized as a type-1 *N*-acetyl-D-lactosamine (LacNAc) modification to the N-glycan. This type-1 LacNAc modification has recently been reported in pluripotent cells[7] and as a potential biomarker of ovarian cancer.[8] Although type-1 LacNAc is recognized as an important structural feature, it is difficult to detect. Lectins and exoglycosidase enzymes hold potential to elucidate this and other structural features on glycans.

Lectins are harnessed to recognize the prevalence of general structural motifs and offer a simple method to screen glycosylation. However, lectins cannot easily be used to sequence glycan structure nor can they provide information about the linkages that compose the structure. Lectins are often used in an array format, and the level of detail obtained with an array scales with the size of the array itself. Arrays require surface printing, sample introduction, and processing. Expanding the size of the lectin array increases the amount of sample and lectin that is consumed. To alleviate the lack of structural specificity of microarrays, they can be combined with exoglycosidases that have a high specificity for a monomer and in some cases for a monomer linkage. This has recently been demonstrated with a microarray that combined 2 neuraminidases and 7 lectins to provide information about both the structural motifs present in glycoproteins and the sialic acid linkage.[9]

Exoglycosidases that cleave the terminal monomers on the non-reducing end of a glycan can have different degrees of monomer and linkage specificity. Exoglycosidases with high specificity can be used without lectins to probe the linkage and composition of terminal glycan monomers. The selectivity offered by these enzymes can be used in conjunction with capillary electrophoresis separations. Enzyme analyses of this type are performed off-line, with any change in the glycan size detected by a shift in the migration time obtained with capillary electrophoresis.[10–17] Offline enzyme reactions are conducted for incubation times of several hours and consume significant enzyme quantities. Although most off-line exoglycosidase analyses are performed on glycans derived from glycoproteins, analyses of intact proteins can be accomplished with capillary electrophoresis.[15, 18]

The limitations of off-line enzyme processing have led to new integrated analyses of glycans with capillary electrophoresis. Methods of partial filling, plug-plug, and serial plug analyses have been used with either a lectin[19–21] or with an exoglycosidase.[20–23] Combinations of lectins and enzymes in capillary electrophoresis have not been fully exploited. Stationary zones of enzymes with nanogels have recently been described that offer new benefits to in-line analyses including the ability to increase the duration of the substrate incubation with electrophoretic mixing and to extend the lifetime of highly specific exoglycosidases, some of which are costly.[24] The performance of nanogel as a separation additive in capillary electrophoresis offers an additional advantage for separations of complex mixtures of glycans.[21, 25]

The success of in-capillary enzyme reactions is promising for the detection of $\beta(1-3)$ -linked galactose once a few complications involving the use of galactosidases are addressed. In

particular, the stability of galactosidase is a concern. The potential of some galactosidase enzymes to simultaneously function as a transgalactosidase as well as an exoglycosidase must also be considered.[26] Additionally, the specificity, catalytic rate, and conditions required for the enzyme reaction must be defined.[27] To expand research in glycan characterization, analytical technologies are required that are inexpensive, simple to implement and have potential for automated analysis.

A new approach is developed to enable the detection of galactose residues that contain a $\beta(1-3)$ -linkage by integrating enzyme and lectin in the separation capillary. An exoglycosidase enzyme specific for $\beta(1-4)$ -linked galactose is used in combination with a lectin that can bind to galactose that is $\beta(1-3,4)$ -linked to *N*-acetylglucosamine. Galactosidase and *Erythrina cristagalli* lectin are each prepared in nanogel to create stationary zones within a capillary electrophoresis separation. By combining the specificity of an exoglycosidase enzyme with the structural recognition of a lectin, $\beta(1-3)$ -linked galactose residues found in glycans are rapidly detected. The stability of neuraminidase in nanogels makes this method attractive.[24] In this report, it is demonstrated for the first time that nanogel stabilizes $\beta(1-4)$ -galactosidase. Moreover, nanogels can easily be patterned in-capillary and then used in an analysis, expelled, and replaced repeatedly. This innovation increases the analytical throughput because the approach does not require printing or covalent immobilization of the enzyme or lectin. The implementation of this method and practical factors that impact the conversion, selection, and separation of glycans that contain $\beta(1-3)$ -linked galactose are discussed in this report. The benefits of this strategy are demonstrated with fetuin glycosylation.

2. Materials and Methods

2.1 Chemicals and reagents

The asialo-, agalacto-, biantennary complex N-glycan, herein referred to as agalactosylated biantennary N-glycan (GKC-004300), the asialo-, monogalactosylated biantennary complex N-glycan, herein referred to as monogalactosylated biantennary N-glycan (GKC-014300), the asialo-, galactosylated biantennary complex N-glycan, herein referred to as galactosylated biantennary N-glycan (GKC-024300), and the $\beta(1-4)$ -galactosidase (GKX-5014) were from Prozyme (Hayward, CA). Acetic acid was from Fisher Scientific (Pittsburgh, PA). Sodium phosphate, calcium chloride, 3-(*N*-morpholino)- propanesulfonic acid, 8-aminopyrene-1,3,6-trisulfonic acid, triethylamine, acetonitrile, asialofetuin and sodium cyanoborohydride (dissolved in tetrahydrofuran) were from Sigma-Aldrich (St. Louis, MO). Rapid peptide N-glycosidase (PNGase F) was obtained from New England Biolabs (Ipswich, MA). *Erythrina cristagalli* lectin was from Vector Labs (Burlingame, CA). Deionized water was from an Elga Purelab ultra water system (Lowell, MA). The phospholipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), were from Avanti Polar Lipids (Alabaster, AL). Phospholipid nanogels were prepared as described previously, aliquoted, and stored at -20°C . [21, 23, 25] The nanogel preparation was composed of $[\text{DMPC}]/[\text{DHPC}] = 2.5$, with a final concentration of 10% lipid by weight.

2.2 Preparation and derivatization of standards

The glycans were labeled with a fluorescent chromophore as previously described.[24] Glycan samples were purified, removing excess APTS and APTS labeled by-products with DPA-6S stationary phase extraction cartridges as described previously.[21] Once purified, the samples were dried in a SpeedVac concentrator and then reconstituted in water and stored at -20°C . For further analysis glycan samples were diluted at least 50-fold in 50 mM sodium phosphate buffered to pH 6.

For the stability study, the $\beta(1-4)$ -galactosidase was reconstituted to a final concentration of 200 $\mu\text{Unit}/\mu\text{L}$ in two separate solutions that differed only by the presence of the self-assembled phospholipids that form nanogel. In one preparation the enzyme was suspended in 50 mM sodium phosphate with pH adjusted to 6. In the second preparation the enzyme was suspended in 10% nanogel that was made using 50 mM sodium phosphate with pH adjusted to 6. To create the $\beta(1-4)$ -galactosidase in nanogel a 20% nanogel preparation was made in 50 mM sodium phosphate buffered to pH 6. The $\beta(1-4)$ -galactosidase was reconstituted with aqueous buffer and then mixed 1:1 with a 20% nanogel preparation. This 1:1 dilution of nanogel ensured that the concentration was precisely 10%, which was important because the viscosity of the non-Newtonian nanogel was dependent upon the concentration of the preparation.[28] For $\beta(1-4)$ -galactosidase enzyme, 1 Unit was defined as the amount of enzyme required to hydrolyze 1 μmole 2-nitrophenyl β -D-galactopyranoside per min at pH 6.0 and 37°C . [29]

Erythrina cristagalli lectin comes from the manufacturer with lactose added as a stabilizer. The lactose was removed using a 10 kDa molecular weight cut off filter (28-9322-47, Pall Corporation, Ann Arbor, MI) to avoid competition with the glycan binding. The lectin was rinsed with a solution of 0.1 mM calcium chloride, 100 mM 3-(N-morpholino)-propanesulfonic acid buffered to pH 7. The *Erythrina cristagalli* lectin was then collected from the molecular weight cutoff filter. The volume of this collected fraction, which is less than or equal to 20 μL , was measured with a pipette. A 10 μL volume of 50 mM sodium phosphate buffered to pH 6.4 and containing 0.1 mM calcium chloride was then added to the upper compartment of the molecular weight cutoff filter, mixed, removed, and combined with the fraction containing the protein. This lectin sample was then mixed 1:1 with a 20% nanogel preparation that contained 50 mM sodium phosphate buffered to pH 6.4. The final concentration of the *Erythrina cristagalli* lectin preparation was 130 μM lectin in 10% nanogel.

2.3 Capillary Electrophoresis

Analyses were performed using a P/ACE MDQ or MDQ Plus (Sciex, Redwood City, CA) configured by the manufacturer with laser induced fluorescence detection (3 mW air cooled argon ion laser or 3 mW solid state laser, with $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$). A 25 μm internal diameter, 360 μm outer diameter fused silica capillary (Polymicro Technologies, Phoenix, AZ) was used for separation. Each day capillaries were prepared as described in the supporting information. Briefly, a semi-permanent lipid coating composed of a 5% solution of phospholipid with [DMPC]/[DHPC] equal to 0.5 that also contained 12.5 mM calcium was applied to the separation capillary at the beginning of each day and between

each run. This semi-permanent coating suppressed the electroosmotic flow. The electrophoresis was performed using reversed polarity to separate the negatively-charged APTS-labeled glycans (anodic reservoir near detector, cathodic reservoir near the site of injection). Unless otherwise noted, the background electrolyte was sodium phosphate buffered to pH 6. Ambient thermal control of the room and instrument was maintained using a portable air conditioner as was described previously.[25] The lipid-based nanogel was easily introduced in the capillary at a temperature of 19 °C. Prior to each run the capillary was held at 19 °C. Data collection and analyses were performed using 32 Karat Software version 7.0 or 10.2.

For studies related to enzyme characterization and optimization, the stationary enzyme zone was introduced at 69 kPa (10 psi) for 7 s followed by injection of background electrolyte at 69 kPa (10 psi) for 21 s. The purpose of the 21 s introduction of background electrolyte was to push the enzyme to the thermostable region of the capillary. To determine the terminal residue in asialofetuin glycans the stationary lectin zone was introduced at 69 kPa (10 psi) for 7 s, followed by injection of background electrolyte at 69 kPa (10 psi) for 7 s, and then the stationary enzyme zone was introduced at 69 kPa (10 psi) for 14 s. After the capillary was filled, the temperature of the separation was increased to 35 °C for the sample injection, incubation, and separation. Injections included a background electrolyte pre-plug of 6.9 kPa (1.0 psi) for 7 s and a background electrolyte post-plug of 3.4 kPa (0.5 psi) for 5 s. After a run was complete, a 172 kPa (25 psi) background electrolyte flush for 5 min was applied in the reverse direction to push out any remaining protein toward the site of injection.

3. Results and Discussion

A method to identify the presence of the β (1-3)-linked galactose residues associated with type-1 LacNAc modifications to glycoproteins was developed using dual stationary zones of β (1-4)-galactosidase and *Erythrina cristagalli* lectin. Lectins and enzymes were harnessed differently in capillary electrophoresis. When the galactosidase enzyme matched a terminal residue in a glycan it was cleaved as depicted in Figure 1, leading to the appearance of the product at a migration time different from that of the substrate. When the specificity of the *Erythrina cristagalli* lectin for galactose residues matched the structural features of the glycan, they were bound to the lectin, removing the glycan peak from the electropherogram. The conditions for interaction with lectins were straightforward; however, the β (1-4)-galactosidase required some optimization.

3.1 Creating a stationary enzyme zone in-capillary

A zone of enzyme was created in the capillary with nanogel as depicted in Figure 1. This approach, previously reported with the enzyme neuraminidase,[24] was possible because nanogel is a fluid with a thermally switchable viscosity.[28, 30] The nanogel was introduced into the capillary as a low viscosity fluid at 19 °C and then held in place by transforming it into a high viscosity material at temperatures ranging from 25 to 35 °C. The surface was passivated with a semi-permanent phospholipid coating, which suppressed the electroosmotic flow. The galactosidase was essentially stationary in the capillary in the absence of electroosmotic flow, in the high viscosity matrix, and as the pH of the nanogel

was close to the predicted isoelectric point of 5.68 (personal communication Prozyme, Inc. Tech Support). Separations were performed using reversed polarity. The negatively-charged substrate was electrokinetically driven into the enzyme zone, where it was converted to product. Following enzyme treatment the product was then separated in the remaining portion of the capillary. In prior reports the capillary was filled with nanogel to improve the separation;[21, 24, 25, 31, 32] however, when higher resolution was not required the stationary zone of enzyme functioned equally well when it was introduced into a capillary filled with aqueous background electrolyte instead of nanogel.[24]

3.2 Effect of nanogel on $\beta(1-4)$ -galactosidase activity and stability

Previously, the rate of neuraminidase catalysis was higher and the stability of neuraminidase was longer when the enzyme was reconstituted in nanogel as compared to aqueous solution. [24] There is evidence in the literature that phospholipid monolayers improve the performance of exoglycosidase enzymes.[33–36] The effect of nanogel on the activity and stability of $\beta(1-4)$ -galactosidase was evaluated by monitoring the extent of galactose removal for enzyme reconstituted in aqueous buffer as compared to nanogel. Therefore, the 200 $\mu\text{Unit}/\mu\text{L}$ enzyme preparation was composed of sodium phosphate buffered to a pH of 6 or in nanogel that also contained sodium phosphate buffered to a pH of 6. The enzyme was mixed outside of the capillary with either buffer or with nanogel, immediately placed in the instrument, and the first run was initiated. The time points were measured from the moment the first programmed run began. A 100 μL aliquot of each 200 $\mu\text{Unit}/\mu\text{L}$ enzyme preparation (aqueous or nanogel) was stored in a separate vial in the sample bay of the instrument, which was maintained at 4 °C. The 200 $\mu\text{Unit}/\mu\text{L}$ preparations of $\beta(1-4)$ -galactosidase were assessed by quantifying the rate of conversion of monogalactosylated biantennary N-glycan substrate to agalactosylated biantennary N-glycan. For each enzyme preparation, the stationary enzyme zone was introduced at 69 kPa (10 psi) for 7 s and then pushed into the thermostated region of the capillary by injection of background electrolyte at 69 kPa (10 psi) for 21 s. As the isoelectric point of galactosidase (5.68) was close to the pH of both the aqueous and nanogel preparations, the enzyme zones remained stationary in the separation capillary. At the end of each determination the enzyme (~ 6 nL) was expelled and the capillary flushed. A new stationary zone was created in the capillary for each determination of enzyme by drawing a fresh sample from the appropriate vial stored in the refrigerated sample bay.

The results of this study are summarized in Table 1. The analyses of enzyme conversion demonstrated the reproducibility of the method when the nanogel-enzyme zone was introduced into the capillary. For the 6 nanogel-enzyme zones analyzed (time points: 1, 5, 9, 13, 17, 21 h), the combined area of the glycan substrate and glycan product peaks had a relative standard deviation of 8% ($n = 6$). The migration times of the substrate and product were also reproducible for these 6 nanogel-enzyme zones, generating relative standard deviation of 0.3% for substrate ($n = 6$), as well as product ($n = 6$). The percent conversion measured for freshly made enzyme preparations was more than 2 times higher in nanogel than aqueous buffer. Within 12 hours the $\beta(1-4)$ -galactosidase made in aqueous solution was inactive, but when reconstituted in nanogel it remained active (92 ± 4 % turnover, $n = 6$) during 21 hours of programmed analyses. The prior study revealed that nanogel preparations

of neuraminidase remained active for at least 30 days,[24] suggesting that the $\beta(1-4)$ -galactosidase would retain enzyme activity for an extended period as well.

3.3 Effect of the stationary nanogel position on enzyme cleavage

The temperature control of the separation capillary was enabled through a standard feature of the commercial instrument used in this study. The capillary electrophoresis instrument contained a cartridge, which interfaces the separation capillary to the programmable autosampling system. The capillary cartridge was connected to a liquid coolant system to circulate liquid coolant over the outside of the capillary. With this design an approximate length of 3.5 cm at each end of the capillary was not thermally regulated. Enzyme activity was temperature sensitive, which required that the stationary zone was positioned in the thermostated region of the capillary. Previously, this was accomplished for sialyllactose labeled with a UV-vis active chromophore in a capillary of 30 cm effective length and 40 cm total length by moving the stationary zone approximately 15 cm into the capillary and characterizing the position of the stationary nanogel zone.[24] In that prior study moving the enzyme, neuraminidase, to that position in the capillary was feasible because the catalytic rate of the enzyme was fast and because the mobilities of the sialyllactose substrate and lactose product were similar. When the same strategy was applied to $\beta(1-4)$ -galactosidase, the position of the stationary enzyme zone in the capillary adversely effected the interpretation of the electropherogram. This was due to the fact that the velocity of the monogalactosylated biantennary N-glycan substrate and agalactosylated biantennary N-glycan product differed. The separations of agalactosylated biantennary N-glycan and monogalactosylated biantennary N-glycan, as shown in Figures 2A and 2B, were driven through a stationary zone in the separation capillary as in the previous report.[24] The nanogel-enzyme zone was positioned within the thermostated region of the capillary by pushing the nanogel with a 45 s injection of aqueous buffer. In 2A the zone was composed of nanogel, while in Figure 2B the zone was composed of nanogel containing $\beta(1-4)$ -galactosidase. Two product peaks were present in trace 2B because the agalactosylated biantennary N-glycan standard passed through the zone before the monogalactosylated biantennary N-glycan substrate reached the zone and was converted to agalactosylated biantennary N-glycan product.

This was corrected by using a special reservoir developed and reported earlier [37] that provided thermal regulation at the inlet end of the separation capillary. Briefly, to thermostat the inlet end of the capillary a P/ACE MDQ large-volume reservoir tray was modified to accept recirculating coolant from a water bath (see Figure S-1 in the supporting information), which allowed for more options for placement of the enzyme nanogel zone. The temperature of the buffer vial was equilibrated to the temperature of the recirculating bath. During enzyme hydrolysis the capillary was maintained to the specified temperature; however, when the incubation was completed the capillary was moved to a vial that was not thermostated. This prevented the accumulation of condensation near the top of the vial, which could potentially lead to electrical arcing. With this modification, the stationary nanogel zone was positioned at the inlet end of the capillary, and was used to obtain the separations shown in Figures 2C and 2D. In Figure 2C the zone was composed of nanogel, while in Figure 2D the zone was composed of nanogel containing $\beta(1-4)$ -galactosidase. A

single product peak was obtained in trace 2D because the agalactosylated biantennary N-glycan standard and monogalactosylated biantennary N-glycan substrate reached the enzyme zone at the same time. The agalactosylated biantennary N-glycan product formed in the enzyme zone migrated from the same location as the agalactosylated biantennary N-glycan standard added to the sample.

3.4 Other factors that influence $\beta(1-4)$ -galactosidase performance

The effects of temperature, analyte velocity, and length of the enzyme zone were characterized. The effect of turnover at 3 temperatures (25, 30, 35 °C) demonstrated only nominal improvement in percent turnover above 25 °C (see Table 2). The velocity of the substrate through the stationary enzyme zone impacted the rate of turnover as well. Slower transit time through the zone resulted in longer periods of incubation. As summarized in Table 3, higher turnover was achieved by driving the substrate through the zone at a lower applied voltage. Electrophoretic mixing, achieved by cycling the polarity of the applied electric field, was previously used to control the incubation time.[24]

The length of the stationary enzyme zone dictated the percent turnover, as summarized in Table 4. Flow injection analyses were performed to convert the pressure and time used to introduce the nanogel into a length. Nanogel zones introduced with a pressure of 69 kPa for 14, 10.5, and 7 s were estimated to be 2.6, 2.0 and 1.3 cm long, respectively. Nanogel-enzyme zones of different length provided more flexibility in the method. Longer zones of enzyme led to more interactions between enzyme and substrate. This was also true for the lectin. The lengths of the nanogel-enzyme and nanogel-lectin zones used for these studies were 14 s and 7 s, respectively. A 7 s lectin plug captured glycan that contained terminal galactose at 400 V/cm; whereas, a 14 s enzyme plug was used to provide better control of the glycan migration through the fixed stationary zone during mixing with polarity cycling. In addition, the 14 s enzyme plug enabled the use of lower enzyme concentrations, which was more cost-effective and advantageous because high concentrations of enzyme could lead to substrate contamination.

The results in Table 4 demonstrated that different lengths of nanogel-enzyme zone did not affect the reproducibility of the method. The reproducibility of the rate of enzyme conversion was 3, 2, and 5% ($n = 3$) for lengths estimated to be 2.6, 2.0, and 1.3 cm, respectively. The combined area of the glycan substrate and glycan product peaks had a relative standard deviation of 3, 7, and 9% for lengths of 2.6, 2.0, and 1.3 cm, respectively. The migration times of the substrate and product were also reproducible for each length of nanogel used. The substrate peak had a relative standard deviation of 1, 0.2, and 0.5% for lengths of 2.6, 2.0, and 1.3 cm, respectively. The product peak had a relative standard deviation of 1, 0.3, and 0.5% for lengths of 2.6, 2.0, and 1.3 cm, respectively.

3.5 Identification of $\beta(1-3)$ -linked galactose using $\beta(1-4)$ -galactosidase and *Erythrina cristagalli* lectin

With enzyme optimization complete, the enzyme and lectin were combined to demonstrate the presence of any $\beta(1-3)$ -linked galactose. Glycans were removed from asialofetuin, labeled, and subject to analyses with enzyme and lectin. Agalactosylated biantennary N-

glycan was added to the glycan sample to serve as an internal standard because it does not respond to either the $\beta(1-4)$ -galactosidase or the *Erythrina cristagalli* lectin. The trace in Figure 3A was obtained with stationary nanogel zones devoid of enzyme and of lectin. The trace in Figure 3B was obtained with a stationary nanogel zone of *Erythrina cristagalli* lectin, which demonstrated the specificity for the presence of terminal galactose residues. The galactosylated biantennary N-glycan and galactosylated triantennary N-glycans were missing from the electropherogram because they were captured by the lectin; whereas, the internal standard, which did not contain galactose residues, migrated through the lectin zone. The trace in Figure 3C was obtained by driving the glycans through the $\beta(1-4)$ -galactosidase and mixing with polarity cycling (7-pass) to remove only $\beta(1-4)$ -linked galactose residues. This produced an electropherogram with only 3 peaks: the agalactosylated biantennary N-glycan, the agalactosylated triantennary N-glycan, and the $\beta(1-3)$ -linked, monogalactosylated triantennary N-glycan known to be present in asialofetuin. The trace in Figure 3D was obtained by driving the glycans first through a stationary nanogel zone of $\beta(1-4)$ -galactosidase, mixing with polarity cycling (7-pass), and then driving the glycans through a stationary nanogel zone of *Erythrina cristagalli* lectin. The resulting electropherogram showed that the $\beta(1-3)$ -linked monogalactosylated triantennary N-glycan was bound to the lectin and thus retained in the capillary.

4. Conclusions and Future Directions

This report demonstrates the applicability of combining $\beta(1-4)$ -galactosidase and *Erythrina cristagalli* lectin. The linkage specificity could have been determined using combinations of $\beta(1-4)$ -galactosidase, $\beta(1-3)$ -galactosidase, and/or $\beta(1-3,4)$ -galactosidase. However, there are a few advantages to using lectins over multiple enzymes. Galactosidase enzymes have different optimum pH values and can contain excipients to stabilize them, including lactose, which is an inhibitor of enzyme activity. In addition, some galactosidases also function as a transgalactosidase enzyme, conjugating the liberated galactose residues back to a glycan or oligosaccharide terminus.[26] Lectins avoid confounding results that may be obtained using enzymes with different properties and from different sources.

The in-capillary method of enzyme and lectin processing is practical because the nanogel increases the enzyme lifetime. Nanoliter volumes of enzyme are consumed during each run, which decreases the overall cost of the enzymes used to facilitate the analyses. Furthermore, the low cost of the nanogel preparations, which are estimated at \$0.09 for 5 μ L,[38, 39] makes the method more affordable as well. Future efforts will center on adapting the method to different combinations of enzymes and lectins. Ultimately, this approach will be utilized for direct analyses of glycoproteins that contain signatures of type-1 LacNAc moieties, implicated in cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found in the on-line version.

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Highlights

- Fixed zones of enzyme or lectin were created in the capillary with nanogel
- $\beta(1-4)$ -galactosidase and *Erythrina cristagalli* lectin were used for hydrolysis and capture
- $\beta(1-4)$ -galactosidase maintained activity for much longer in nanogel than in aqueous buffer
- $\beta(1-3)$ -galactose linkages in glycans were identified
- Factors that enhance the enzyme performance and the separation are described c 2017 Published by Elsevier B.V.

Figure 1.

Conceptual illustration of electrophoretic migration of substrate in-capillary containing enzyme in a stationary zone. The asialo monogalactosylated biantennary N-glycan was converted to agalactosylated biantennary N-glycan following incubation. Note that the reducing end of the N-glycan was labeled with a fluorophore (e.g. 8-aminopyrene 1,3,6-trisulfonic acid).

Figure 2.

Depiction of electrophoretic migration of substrate in-capillary through a stationary zone of nanogel in two different positions. Injected sample was a mix of monogalactosylated biantennary N-glycan (substrate) and agalactosylated biantennary N-glycan (product, and migration time marker). The electropherogram in trace **A** was obtained in the presence of nanogel pushed into the capillary for 45 s at 69 kPa (10 psi). The trace in **B** was obtained under the same conditions but using a stationary zone of nanogel containing enzyme. The electropherogram in **C** was obtained with nanogel thermostated at the end of the capillary using the modified reservoir. The separation in trace **D** was obtained under the same conditions but using a stationary zone of nanogel containing enzyme. Sample was driven through nanogel using a pre-separation of 3kV. Separations were performed in a 25 μ m inner diameter capillary, at 35 $^{\circ}$ C with an effective length of 50 cm and $E = 400$ V/cm (reverse polarity). Other conditions are as described in section 2. Materials and Methods.

Figure 3.

Electropherograms of complex N-glycan obtained from asialofetuin incubated in $\beta(1-4)$ -galactosidase and *Erythrina cristagalli* lectin in different combinations. A stationary zone of nanogel/lectin and then nanogel/enzyme was loaded into the capillary at 69 kPa for 7 s and 14 s, respectively. Glycan standard agalactosylated biantennary N-glycan was added as a migration time marker. The sample was incubated in the first stationary zone using mixing with polarity cycling for 579 s at 3 kV for multiple passes as follows: 129 s forward then three repeats of 75 s reverse/75 s forward. The separation in trace **A** was obtained with no enzyme and no lectin. The asterisk in trace **A** denotes the position of Gal $\beta(1-3)$ GlcNAc or Gal $\beta(1-4)$ GlcNAc. The trace in **B** was obtained with a stationary zone of lectin selective for the terminal galactose. The trace in **C** was obtained with a stationary enzyme zone that was selective for $\beta 1-4$ linkages. The asterisk in trace **C** denotes the position of Gal $\beta(1-3)$ GlcNAc. The trace in **D** was obtained with a stationary enzyme zone and a stationary lectin zone. The data were obtained at 35°C with $\beta(1-4)$ -galactosidase (200 μ Unit/ μ L) and *Erythrina cristagalli* lectin (130 μ M) that is suspended in phospholipid nanogel. Separation conditions are the same as described in the Figure 2.

Table 1Effect of nanogel on enzyme performance¹

<u>Aqueous Enzyme</u>		<u>Nanogel Enzyme</u>	
Time point ²	%Turnover ³	Time point ²	%Turnover ³
0 h	37	1 h	93
4 h	41	5 h	100
8 h	10	9 h	89
12 h	0	13 h	93
16 h	0	17 h	91
20 h	0	21 h	87

¹Enzyme concentration was 200 µUnit/µL. The aqueous solution and nanogel (10%) were both buffered to pH 6. Substrate was 4 µM mono galactosylated biantennary N-glycan in 50 mM phosphate buffered to pH 6. Sample was injected (2kV 6 sec) followed by a pre-separation step (3kV for 16 min) to drive substrate through enzyme. Separations were performed with a background electrolyte of 50 mM sodium phosphate buffered to pH 6 in a 25 µm i.d. capillary with an effective length of 50 cm, E =400 V/cm (reverse polarity), and at 35 °C.

²The enzyme activities were monitored on the same instrument and separation capillary, alternating the enzyme preparation that was evaluated at 4 h intervals. Both enzyme preparations were mixed outside of the capillary with either buffer or with nanogel and then placed in the instrument. The time points were measured from the moment the first programmed run began. There was a delay from the start of the program to the point that substrate passed through the enzyme of approximately 18 minutes (0.3 hours).

³Enzyme turnover was calculated by dividing the product area by the total area of substrate and product. For the nanogel-enzyme preparation the RSD in area of total glycan (i.e. substrate and product combined) was 8% ($n = 6$ time points, 1, 5, 9, 13, 17, 21 h), the RSD of the substrate migration time was 0.3% ($n = 6$ time points, 1, 5, 9, 13, 17, 21 h), and the RSD of the product migration time was 0.3% ($n = 6$ time points, 1, 5, 9, 13, 17, 21 h). For the aqueous-enzyme preparation the RSD in area of total glycan (i.e. substrate and product combined) was 7% ($n = 6$ time points, 0, 4, 8, 12, 16, 20 h), the RSD of the substrate migration time was 0.3% ($n = 6$ time points, 1, 5, 9, 13, 17, 21 h).

Table 2Effect of temperature on turnover¹

<u>Temperature²</u>	<u>% Turnover²</u>
25°C	78
30°C	87
35°C	89

¹Enzyme (200 µUnit/µL) reconstituted in 10% nanogel, sample and separation conditions as described in Table 1.

²RSD in total glycan area < 8%, *n* = 3, and RSD in rate < 4%, *n* = 3.

Table 3Effect of Voltage on turnover¹

<u>Voltage</u>	<u>% Turnover²</u>
3 kV	58
12 kV	18
24 kV	12

¹ Enzyme (50 μ Unit/ μ L) reconstituted in 10% nanogel, sample, sample was injected (2kV 6 sec) and the separation then performed using the specified applied voltage, separation conditions as described in Table 1.

² RSD in total glycan area < 8%, $n = 3$, RSD in rate < 4%, $n = 3$.

Table 4Effect of plug length on turnover¹

<u>Estimated Plug Length²</u>	<u>% Turnover²</u>
2.6 cm	50
2.0 cm	68
1.3 cm	78

¹Enzyme (50 µUnit/µL) reconstituted in 10% nanogel, sample, separation conditions as described in Table 1

²Length was estimated with flow injection analyses. The enzyme-nanogel zone was introduced at 69 kPa for 14, 10.5, and 7 s for the 2.6, 2.0 and 1.3 cm plugs, respectively.

³RSD in total glycan area < 9% and RSD in rate < 3% for each length ($n = 3$ runs per each length). RSD in migration time 1% for substrate ($n = 3$ runs per length) and product ($n = 3$ runs per length).