CAPILLARY ELECTROPHORESIS OF GLYCANS
Biotechnology and biomedical applications

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Significance of protein glycosylation

- There has been a rapid recent progress in glycomics research to understand the biological role of carbohydrate moieties of glycoproteins.
- Alterations in protein glycosylation with variable site occupancy or changes in oligosaccharide structures (microheterogeneity) can lead to biological activity changes.
- Glycosylation types: N-linked glycosylation (through the amide nitrogen of asparagine side chains) and O-linked glycosylation (through the hydroxy oxygen of serine and threonine side chains).
For N-linked oligosaccharides, a 14-sugar precursor is first added to the asparagine in the polypeptide chain of the target protein. The structure of this precursor is common to most eukaryotes, and contains 3 glucose, 9 mannose, and 2 N-acetylglucosamine molecules. A complex set of reactions attaches this branched chain to a carrier molecule (dolichol), and then it is transferred to the appropriate point on the polypeptide chain as it is translocated into the ER lumen.

Types of N-linked glycosylation:
- High-mannose type: contains just two N-acetylglucosamines with many mannose residues.
- Complex type: can contain almost any number of the other types of sugars, including more than the original two N-acetylglucosamines.
- Hybrid type: combination of the two above.
The increased size of N-glycans that occurs upon malignant transformation can be explained by an elevation in GlcNAc transferase-V (GNT-V) activity.
Malignant cells release glycoproteins carrying disease-related glycans into the interstitial space, where they can reach the circulation.

Glycan analysis options

**Challenge:** complex, diversified structures; no chromophore / fluorophore groups; mostly not charged

Advantages of CGE over other analytical methods

- **GC** requires derivatization resulting in stereoisomers and complex separation patterns.
- **HPLC**: - HPAE/PAD: limited separation efficiency, non-specific detector response
  - Normal phase and HILIC: time consuming, low efficiency,
  - UPLC: no current informatics solution
  - Graphitized carbon: excessive retention of sialylated structures
- **Structural characterization** – MS (limited information on anomeric configuration and other isoforms), NMR (high quantity and purity requirements).
- **PAGE** – slow, labor intensive and not quantitative.
- **CE** – fast, efficient, automated, very sensitive, small sample volume requirements, easy multiplexing, different modes of separation; however, requires charged UV/fluorophore tags to assure electromigration and UV/LIF detectability. Informatics database is being built.
High separation efficiency
Small sample volume (1-10 μl)
Fast separation (min - sec)
Predictable selectivity
Full automation
Quantification/good dynamic range
Reproducibility/robustness
Multi-capillary option (CAE)
Multi-spectral imaging
Coupling to mass spectrometer
SEPARATION MODES IN CAPILLARY ELECTROPHORESIS

- Capillary zone electrophoresis (CZE)
- Micellar electrokinetic chromatography (MEKC)
- Capillary gel electrophoresis (CGE)
- Capillary isoelectric focusing (CIEF)
- Capillary affinity electrophoresis (CAE)
- Capillary isotachophoresis (ITP)
- Capillary electrochromatography (CEC)

- Micropreparative applications
CGE: From the first oligonucleotide separation to sequencing the Human Genome


Separation of APTS labeled high-mannose type glycans released from bovine ribonuclease B

Inset: Structural representation of the high-mannose type N-linked oligosaccharides.
Migration time normalization for CAE operation

Sample preparation for CGE based N-linked glycan analysis

1. Release of N-linked glycan structures by Peptide N-glycosidase F (PNGaseF) digestion
2. Removal of the deglycosylated proteins
   - ice-cold ethanol precipitation/centrifugation
   - membrane filtration
3. Labeling of the released sugar structures by reductive amination using l-aminopyrene-3,6,8-trisulfonic acid (APTS)
Methods to Accelerate Enzyme Catalyzed N-deglycosylation of Glycoproteins

- Microwave assisted deglycosylation of N-linked glycans
- Immobilized PNGase F enzyme reactors in capillary columns
- Integrated microfluidic chip for rapid deglycosylation
- Pressure cycling technology (PCT)
PCT-enhanced enzyme reactions

- Kinetic advantage: pressure promotes water dissociation
- Many hydrolytic reactions are accelerated
- Substrate binding – pressure reversibly denatures substrate protein, revealing hindered cleavage sites
- PCT accelerates and improves reduction/alkylation
- Enzymes: Trypsin, Chymotrypsin, Pepsin, Lys-C, Glu-C, Asp-N, Proteinase K, PNGase F tested to date – all positive
- Both in-solution and in-gel digestion protocols benefit from PCT
Comparative CE Analysis of APTS Labeled Released Glycans from Polyclonal Human IgG Using PCT and Atmospheric N-deglycosylation with 1:2500 Enzyme:Substrate Molar Ratio.

A) APTS labeled maltooligosaccharide ladder
B) PCT: 30 kPsi, 5 min. 37°C
C) Atmospheric pressure, 3 hours, 37°C
D) Atmospheric pressure, 5 min, 37°C
IS2: maltose - APTS.

Z.Szabo, A.Guttman, B.L.Karger, Rapid release of N-linked glycans from glycoproteins by pressure cycling technology, Analytical Chemistry 82 (2010) 2588-2593.
Advantages of Pressure Cycling Technology (PCT) Assisted Enzymatic N-deglycosylation

- The high pressure facilitates conformation changes of the target glycoprotein, increasing the accessibility of the endoglycosidase to the cleavage sites.

- 1:2500 enzyme : substrate molar ratio at 30 kPsi and 37°C quantitatively released the asparagine linked glycans in minutes.

- Pressure cycling apparently did not lead to any loss of sialic acid residues.

- The microliter scale reaction volume alleviated possible precipitation related issues.

- PCT offers simultaneous processing of 12 samples.
Sugar labeling by APTS

Purpose:
Introduction of label and charge

- Reductive amination
- Sugar reducing ends only
- ex 488 nm / em 520 nm LIF, excellent sensitivity
- Simple, one step reaction
- Great efficiency (over 90%) under optimized conditions
  (reagent concentration, time, temperature, pH, solvent)
- Non-selective: uniform labeling for most structures
- Easy quantification: one fluorophore per sugar molecule
Derivatization yield and desialylation kinetics

Trisialylated triantennary \( (2 \times \alpha 2,3) \) oligosaccharide, derivatization time: 2 hours at all temperatures.

Time Dependence of APTS Derivatization of 5 nmol Maltoheptaose

Solid line: 10x excess of APTS, 0.6 M citric acid catalyst
Dotted line: 100x excess of APTS, 7.5% acetic acid catalyst
Reaction temperature: 55°C

Sample purification options for excess APTS removal

1) Size exclusion chromatography using 96 well filter plate filled with 100 ul Sephadex G10 resin

2) G10 bead filled pipette tips
   - 200 ul pipette tips filled with 160 ul G10 resin
   - conditioning and elution with 50 % acetonitrile

3) Normal phase bead filled pipette tips
   - 1000 ul pipette tips filled with 10 ul DPA-6S normal phase polyamide resin
   - washing: 95% acetonitrile / 5% water
   - elution: 20% acetonitrile / 80% water
Sample purification results

Monosaccharide composition analysis by CE

1=AMAC (9-aminoacridone, eof marker)
2=Neu5Ac-AMAC; 3=GalNAc-APTS; 4=GlcNAc-APTS; 5=Man-APTS; 6=Glc-APTS; 7=Fuc-APTS; 8=Gal-APTS; 9=APTS

Flowchart of N-linked oligosaccharide sequencing

Purified Glycoprotein

PNGase F Digestion 2 hours

Free N-linked Oligosaccharides

Labeling with APTS 90 min at 55°C

Labeled Oligosaccharides

Exoglycosidase Matrix Digestion 16 hours

Sequencing Digests

Combine Digests 5 min

Capillary Gel Electrophoresis

Computerized Data Analysis 10 min

Oligosaccharide Sequence
Boronic acid – Lectin Affinity Chromatography (BLAC) enrichment of glycoproteins

Boronic acid
\[
\text{R} - \overset{\text{OH}}{\text{B}} - \overset{\text{OH}}{\text{O}}
\]

Lectin affinity
- Concavalin A
- Wheat Germ Agglutinin
- Jacalin
...

Miniaturization
Chromatographic pipette tip
Affinity resin
Handee spin column
Comparison of affinity micropartitioning using boronic acid (A), Con A (B) and BLAC/Con A (C) resin filled pipette tips.
Profiling of normal human serum glycans with (A) and without (B) BLAC/Con A affinity enrichment.
CGE profiling of human plasma samples

Without glucose removal

After glucose removal

Sample preparation and analysis of human serum glycoproteins by CE (glycan) and LC-MS (protein)
MAIN APPLICATIONS

- FOOD AND BEVERAGE INDUSTRY
- BIOTECHNOLOGY
- BIOPHARMACEUTICALS
- BIOMEDICAL
Capillary electrophoresis fingerprinting of oligosaccharides in beers

Ethanol from dry grind corn milling (current process)

- Starch in corn seed is enzymatically converted to sugar and fermented to ethanol
- Saccharification / Fermentation of Fiber would increase ethanol yields by 10%

(DDGS: distillers dried grains with solubles)
High throughput enzyme activity evaluation

Enzyme reaction monitoring: Celloooligomer standards
Cellohexaose substrate
9 different cellulases

The methylotrophic yeast *Pichia pastoris* is a preferred host for over-expression of recombinant proteins as it produces large quantities of properly folded proteins and capable of adding both N-linked and O-linked carbohydrate moieties to secreted proteins.

Phospholipase C (PLC) belongs to a class of enzymes that cleave phospholipids to produce diacylglycerol and a phosphorylated head group (e.g., inositol phosphate in the case of phosphoinositol-specific PLCs).

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SDS-PAGE analysis of intact and deglycosylated PLC

After SDS-PAGE, the bands corresponding to recombinant PLC were in-gel digested with chymotrypsin.

The resulting peptides were loaded onto a 10 cm C_{18} RP 100 μm i.d. capillary column and eluted into a QTOF mass spectrometer by means of a 45 minute gradient.
Glycopeptide analysis from recombinant PLC by μLC-MS
CE separation of high mannose type oligosaccharides

48 cm capillary column, I.D.: 50μm; LIF detection: Ex: 488nm, Em: 520 nm. Buffer: 25 mM acetate, pH 5; temp: 25°C; Pressure injection: 10 sec at 0.5 psi.

Digested PLC

Undigested (control) RNAse B

Digested RNAse B
Characterization of mAb N-linked Glycans

- IgG1 contains N-linked glycan structures (1 conserved Asn site).
- Variability depending on expression conditions
- Structural diversity, e.g., presence of galactose, sialic acid, or fucose.
- Glycosylation pattern determines biological activity: physico-chemical properties, cell-mediated effector functions (complement binding, activation, etc).
- Understanding the microheterogeneity of glycosylation is very important in clone selection and manufacturing of therapeutic recombinant mAbs.
CE – MS verification of the separated components

CE-MS extracted ion electropherograms of APTS-labeled sialylated biantennary glycans (panel A) and the MS traces of the corresponding major peaks (panel B)

N-linked glycan profiling of pooled healthy and prostate cancer patient sera after BLAC partitioning
The power of CGE in N-linked glycan profiling

F1=tetrasialo-triantennary-2xα2,6; F2=tetrasialo-triantennary-2xα2,3; F3=trisialo-triantennary-2xα2,6; F4=trisialo-triantennary-2xα2,3; M5-M9: Mannose 5 - Mannose 9.

The future of CE and CE-MS based carbohydrate analysis
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