New Developments in Capillary Electrophoresis with focus on Bioanalysis

Lecture 4

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Lecture Today

- Focus on analysis of protein isoforms
- Special focus on carbohydrate analysis
- Applications in
  - Pharmaceutical industry
  - Diagnostics

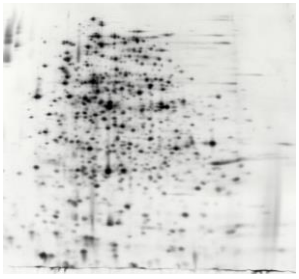
CE of proteins

- Proteins are modified after formation
  - Post translational modifications (PTMs)
- The proteins are modified during there lifetime
- A protein often consist of many closely related isoforms
  - Heterogeneous mixture due to differential PTMs

CE of proteins

- 2 dimensional SDS-PAGE in combination with mass spectrometry is a common tool in proteomics
- Separation techniques with high peak capacity is needed due to the huge amount of proteins.
  - High amount of proteins
  - Protein isoforms increase the complexity

2D Electrophoresis



Usually isoelectric point (IEF) and molecular weight (SDS-PAGE) is the basis of separation

Robots can be used to isolate protein spots for mass spectrometry analysis

CE of proteins

- One dimensional separation not enough for more complicated separation tasks.
- Two ortogonal techniques increase the peak capacity.

CE of proteins

- High resolution mass spectrometry important for identification of proteins.
  - High resolution MS (for example Orbitrap)
  - Identification of complex molecules (for example intact glycoproteins)
- MS can be combined with on capillary detection for quantification

Post translational modifications

- Not coded in the DNA.
- Instead the state of the cell determines how the proteins are modified
  - Presence of enzymes
  - Presence of reactants.

Post translational modification

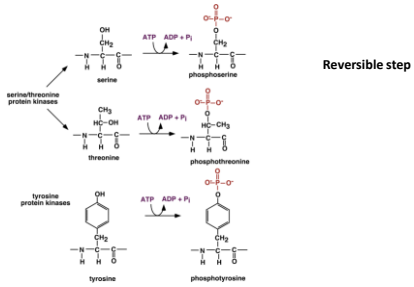
For example:

- Phosphorylation
- Glycosylation
- Ubiquitination

Protein phosphorylation

- Regulation of kinases and phosphatases.
  - Regulate phosphorylation
  - Important for many signalling pathways
- Phosphorylation can activate or deactivate enzymes
- Important for regulation of the metabolism

Protein phosphorylation



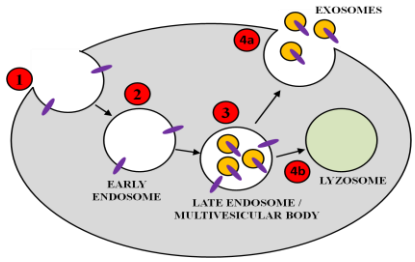
Reversible step

Ubiquitination

- Important for degragration of proteins
- Important for sorting in the endosomes

Importance of PTMs

Sorting of proteins in the endosomes



Exosomes

- 40-100 nm vesicles
- Exosomes are released when multivesicular bodies fuse with the plasma membrane
- Contain
  - Proteins
  - miRNA
  - mRNA

Exosomes

- The composition is dependent of
  - Type of Cell of origin
  - State of cell of origin
- The content of the exosomes can be transported between cells
  - The content is active in the recieving cell

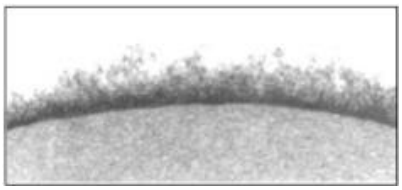
Glycosylation of proteins

- Significant amount of the mass of a glycoprotein
- Most abundant form of PTM
- Important for cell-cell communication

Protein glycosylation

- Alternation in glycosylation can change the biological activity of a glycoprotein
- Glycosylation of insulin – might be important in diabetes

Glycosylation



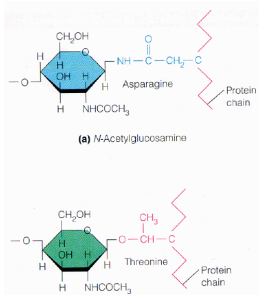
Electron micrograph of a glycocalyx

Thick carbohydrate coating that surround virtually all cells.
Compsed of protein- and lipid-bound oligosaccharides.

Glycosylation of proteins

- N-linked – via the amines of asparagines
- O-linked – via the hydroxyl group of serines and threonines
  - Blood groups – O-linked glycans

Glycosylation of proteins



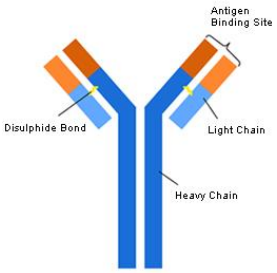
Glycoproteins as recombinant drugs

- Glycosylation patterns important for function
- Important to control production procedure
- Glycosylation profiles may vary based on the host system used for production
- Host system not the same cell machinery as humans.
- Analytical methods to analyze differences in glycosylation patterns

Glycoproteins as recombinant drugs

- Cell culture conditions may have a significant effect on glycosylation
- Batch-to-batch consistency

Monoclonal antibodies



SDS-CGE

- For recombinant monoclonal antibodies
- Addition of N-linked carbohydrate chain to the heavy chain of IgG.
- Reduced fragments of IgG can be analyzed to decrease complexity

Recombinant monoclonal antibodies

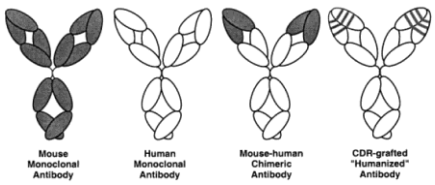
- Growing industry
- High specificity makes antibodies excellent for human therapy
- Immune system to fight diseases
- Recombinant production is an alternative to production in mice

Humanized mAb

- The complementary regions, which are the responsible for antigen binding within the variable regions, have been transferred to human frameworks creating ‘‘humanized’’ antibodies. This is, in essence a human Ab with small segments containing mouse Ab genes.

Monoclonal antibodies

- Humanize monoclonal antibodies



Applications of MABs

- Diagnostic tests
  - Detect the presence of a substance
  - Useful for detecting a antigen in tissue section
- Therapy
  - Specific binding to target cells or proteins
  - Stimulate the immune system to attack those targets.

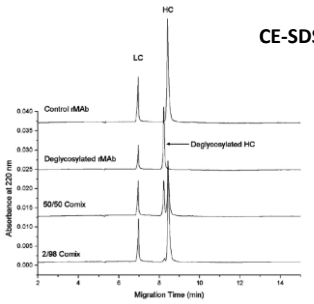
Applications of MABs

- Treatment of cancer
  - MABs that bind to cancer cell-specific antigens and induce an immunological response against target cancer cells.
- Treatment of autoimmune diseases

Therapeutic proteins

- During production and shelf life of therapeutic proteins, several PTMs can occur:
  - for example deamidation, oxidation and proteolytic cleavages

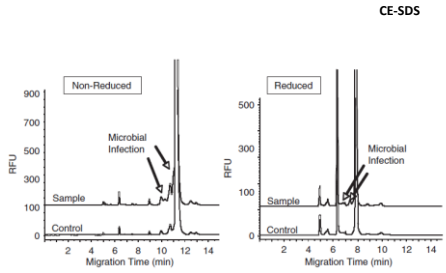
Recombinant monoclonal antibodies



Glycosylation relatively large in size.
Imporatant to detect glycosylation at low levels.

Recombinant monoclonal antibodies

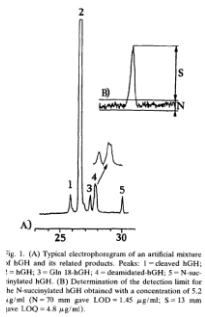
Detecting impurities



CE-SDS

- SDS can also be used to evaluate heterogenity, purity and manufacturing consistency
- Linear or slightly branched polymers: polyacrylamide, polyethylene oxide, polyethylene glycol, dextran.
- Add flexibility, water soluble, replaceable after each analysis
- CE-SDS with fluorescence detection, to replace silver staining
- However, less compatible with MS

Separation of variants of human growth hormone



ig. 1. (A) Typical electropherogram of an artificial mixture f hGH and its related products. Peaks: 1 = cleaved hGH; = hGH; 3 = Glu 18-hGH; 4 = deamidated-hGH; 5 = N-suc- inylated hGH. (B) Determination of the detection limit for he N-succinylated hGH obtained with a concentration of 5.2 g/ml. (N = 70 mm gave LOD = 1.45 μg/ml; S = 13 mm gave LOQ = 4.8 μg/ml).

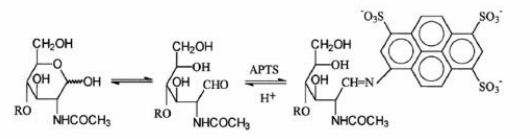
Carbonhydrate analysis by CE-LIF

- Glycosylation important for function of rMABs
- Consistant glycosylation required by legislation
- Chromophore introduced to carbonhydrate
  - Also add a charge

Carbonhydrate analysis by CE-LIF

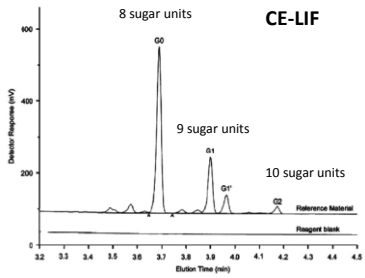
- Procedure – Oligosaccharides on rMABs
  - Enzymatic removal of oligosaccharides
  - Derivatisation with APTS
  - Analyze

Carbonhydrate analysis by CE-LIF



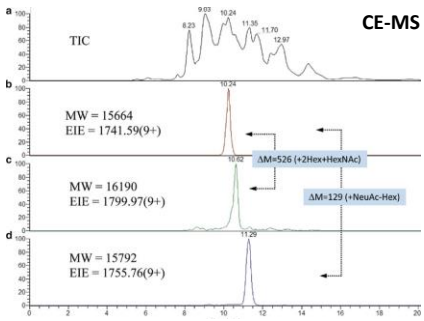
- Reductive amination
- Sugar reducing ends only
- ex 488 nm / em 520 nm LIF, excellent sensitivity
- Simple, one step reaction
- Great efficiency (over 90%) under optimized conditions (reagent concentration, time, temperature, pH, solvent)
- Non-selective: uniform labeling for most structures
- Easy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

Example of glycan analysis



Analysis of intact protein

Recombinant human chrionic gonadotropin (rHCG)



2D separation

- CE a high-speed / high-efficiency technique
- Ideal as the last dimension in a multidimension system
- Reversed phase LC frequently coupled to CZE
  - Orthogonal in separation mechanism
  - Solvents in HPLC and buffers in CE reasonable compatable.

2D-CE

- For separation of proteins and peptides
- Can be used for studies of single cells
- Fluorescent labeling of proteins/peptides

Capillary isoelectric focusing

- High resolution/peak capacity
- Make it suitable for analysis of complex protein mixtures.
- A good option as one dimension in 2D-CE

2D separation

Two-Dimensional (2D) Capillary Electrophoresis (CE) Systems

	2D mode	Detector	Interface	Sample
LC-CE	RPLC-CZE	MS	Valve-free hydrodynamic sampling device	Liver cancer tissue
	RPLC-MEKC	UV	Dynamic interface with pulse contact	Traditional Chinese medicine
	RPLC-CIEF	LIF	Fractionation	Yeast cell cytosol
	RPLC-chip CZE	LIF	Valve-free gating interface	BSA
	RPLC-CZE	LIF	Six-port valve interface	Ovalbumin
	SEC-CZE,	UV	Transverse flow-gated interface	Thyroglobulin, BSA, chicken egg albumin, myoglobin
	RPLC-CZE	LIF	Transverse flow-gated interface	A mixture of phenylalanine and glutamic
	RPLC-CZE-MS	ESI-MS	Transverse flow-gated interface	Glycosylated peptide mixtures
	RPLC-CZE SEC-RPLC-CZE	LIF	Optical-gated interface	Horse heart cytochrome c
	RPLC-CZE	LIF	Off-line	Cytochrome *c*, myoglobin
	SEC-CZE	UV	C18 trapping column	Enkephalins in cerebrospinal fluid
	CIEF-RPLC	UV	Microinjector	Soluble fraction of drosophila salivary glands
	GFC-CIEF	Column imaging	Microdialysis interface	Myoglobin, bovine serum albumin