Characterization of PEGylated Erythropoietin by CZE (UV/MS)
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Amazing where you can go

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Characterization of PEGylated proteins

**Purpose**
- Characterization/identification in general
- Quantification of PEGylation degree
- Localisation of PEGylation site (random PEGylation)
- Analysis of charge variants (e.g. deamidation)
- Final: test method suitable for routine analysis

**Challenge**
- Characterization of PEGylated proteins is demanding by standard methods as the polydispersity and impurities from PEGs complicate matters
- PEGylation imparts charge and size heterogeneity, especially if a 30 kDa PEG is attached to a few potential PEGylation sites
- In EPO complexity is additionally increased by glycosylation

**Approach:**
- Reducing complexity of PEG-Erythropoietin by
  1. desialylation or deglycosylation
  2. followed by Lys-C digest and
  3. CZE-UV peptide mapping (identification: CZE-MS)
Analytical tools for the characterization of PEGylated proteins

<table>
<thead>
<tr>
<th>Method</th>
<th>Measurement/Property</th>
<th>Notes</th>
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</table>
| HPLC/IEX/SEC* (MALLS) | hydrodynamic size 1-50 nm (molar mass, size kDa - MDa (10 - 500 nm)) | 1. Molecular weight distribution  
2. Ratio of pegylated and non pegylated protein of the intact protein |
| CZE/CZE-SDS*    | charge/hydrodynamic size hydrodynamic size kDa - MDa | 2. Pegylation site and individual degree of pegylation of digested protein |
| AF4             | hydrodynamic size 1 nm - few μm                  |                                                                      |
| AUC             | molecular weight and shape (0.1 nm - 0.1 μm)     |                                                                      |
| MALDI/HPLC-MS   | absolute mass 1 - 400 kDa                        |                                                                      |
| MALDI/HPLC-MS   | ionization/hydrophobicity/absolute mass 1 - 1 000 000 m/z (TOF) |                                                                      |
| CZE*- (MS)      | charge/hydrodynamic size Da - MDa                |                                                                      |

* QC test method
The concept: CZE (Lys-C) peptide mapping of PEGylated protein

- disappearing/decreasing signals (in peptide map of pegP) indicates that the considered peptide is PEGylated completely/partially
- the number of new signals correlates with the number of PEGylation sites
Characteristics of EPO/pegEPO

Erythropoietin (EPO, beta)
- 165 amino acids
- 3 N-glycans (sialylated)
- 1 O-glycans (sialylated)
- 2 disulfide bridges (Cys 7-161, 29-33)

PEG-Erythropoietin (pegEPO, beta)
- approx. 1 PEG molecule attached
- 9 potential PEGylation sites (K)
- Preferred: K45 and K52

Molecular weight:
- EPO: approx. 30 kDa
- pegEPO: approx. 60 kDa (30 kDa polyethylene glycol)
Expected peptides resulting from Lys-C digest

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Mass [Da]</th>
<th>pI</th>
</tr>
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<tbody>
<tr>
<td>APPRLICDSRVLERYLLEAK</td>
<td>2343</td>
<td>8.2</td>
</tr>
<tr>
<td>EAENITTGCAEHCSLNENITVPDTK</td>
<td>2690</td>
<td>4.3</td>
</tr>
<tr>
<td>VNHYAWK</td>
<td>927</td>
<td>8.6</td>
</tr>
<tr>
<td>RMEVGQQAVEVWQGLALLSEAVL</td>
<td>5025</td>
<td>4.9</td>
</tr>
<tr>
<td>AVSGLRLTLPPRALGAK</td>
<td>1955</td>
<td>12.0</td>
</tr>
<tr>
<td>EAISPPDAASAAPLRTITADTFRK</td>
<td>2499</td>
<td>6.2</td>
</tr>
<tr>
<td>LFRVYSNFLRGK</td>
<td>1499</td>
<td>11.0</td>
</tr>
<tr>
<td>LK</td>
<td>259</td>
<td>8.8</td>
</tr>
<tr>
<td>LYTGEACRTGD</td>
<td>1185</td>
<td>4.4</td>
</tr>
</tbody>
</table>

- **Peptides including glycans**
- **Most probable pegylation site**
Expected peptides resulting from Lys-C digest

Reduced complexity

Reduced charge heterogeneity

Reduced size heterogeneity
CZE and GCE of intact protein conjugate

Charge/size variants
CZE, EACA, pH=4.5

Charge/size variants
CZE, AcA, pH=2.5

Charge variants

- Size and charge variants of the glycans
- Size variants PEG
- Charge variants of the protein backbone

Size variants

SDS-GCE

EPO

pegEPO

ca. 40 kDa

7 min
CZE peptide map (Lys-C)
EPO/PEGylatedEPO

1. Changed A%?
2. New signals?
3. Stable digestion
4. Internal reference
5. Identification
6. Further reduction of complexity

glycosylated, non-glycosylated
CZE peptide map (Lys-C) of EPO
desialylated (ds), deglycosylated (dg)

glycosylated, desialylated, deglycosylated
CZE peptide map (Lys-C) of pegEPO
desialylated (ds), deglycosylated (dg)

\[ \frac{q_1}{r h_1} \gg \frac{q_2}{r h_2} \]

pegEPO (ds)
pegEPO (dg)
PEGp1?
PEGp3/4?
PEGp2/3?

glycosylated, desialylated, deglycosylated, PEGylated
CZE peptide map (Lys-C)

Analytical result

Reduction of signal, RF ($A\%_{depeg}, MW$)

- $p_1: 0.4$ (11%, 2340)
- $p_2: 0.3$ (8%, 2690)
- $p_3: 0.2$ (13%, 900)
- $p_4: 0.4$ (32%, 5000)

$$\sum_{n=1}^{x} (A\%_{depeg}RF) = \sum_{n=1}^{x} (A\%_{peg})$$

EPO(dg)

PEGp1?

PEGp2/3?

PEGp3/4

pegEPO(dg)

E.g. a signal of 15% and a PEGylation probability of 10% leads to a reduction of 10%
Conclusion

- The separation of both pegylated and non-pegylated peptides can be achieved within one CE run ranging from **500 to 32'000 Da**
- 3 (main) pegylated peptides have been found directly (does not correspond to the number of pegylation sites)
- It is unclear if Lys-C is **sterically hindered** due to pegylation, pegylated single and twin peptides can be expected
- So far, the **determination of pegylation site and individual pegylation degree** can only be determined imprecisely; reasons are very slight changes in the profile of non pegylated peptides and low pegylation degree in the presence of 4-5 potential pegylation sites
- The **identification of pegylated peptides** is indispensable for the calculation of a precise pegylation degree of the individual pegylation sites
- The A% analysis of pegylated peptides (sum or individual) allows a **quick estimation of the overall pegylation state** (correlates to the pegylation degree), possibly suitable as a QC test method
- **Rough characterization of glycans** in terms of number of sialic acids and antennary is feasible
- **Further investigation** will be needed in order to precise the individual pegylation sites/degree
Thank you for your interest and attention!

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