Protein instability and stability, a formulation challenge

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Protein formulation

→ Develop a formulation and manufacturing process that will maintain the integrity of a protein drug during
  – routine pharmaceutical processing
  – storage
  – handling and delivery to the patient
→ knowing that proteins typically raise more stability issues than small molecules
Factor influencing protein stability

- Manufacturing procedure (e.g. Bacteria contamination, chaotropic/denaturating agents)
- **Drug formulation**
- Heterogeneity and secondary/tertiary structure (e.g. posttranslational modifications)
- Bioassay/activity
- Clinical efficacy (e.g. formulation prior to administration, In-use stability)
- Stability (freeze/thaw cycle)
- Physicochemical properties (e.g. pH, salt concentration, protein concentration)
- Impurity profile (e.g. oxidation, deamidation)
- Immunogenicity (e.g. HCP proteins, adducts, aggregates)
- Extractables / leachables (e.g. rubber sealings, container, W metal needle)
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<td>- Oxidation</td>
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Model proteins

*rHuG-CSF structure*

175 amino acids, average mass 18 800 Da

α-helical structure

2 disulfide bonds:
  Cys37 - Cys43
  Cys65 - Cys75

non-glycosylated

expressed in *Escherichia coli*

- treatment of neutropenia caused by chemotherapy for cancer treatment
- stimulates bone marrow precursors to form granulocytic colonies (neutrophils)
**Why cytokine model?**

- Recombinant methionyl human granulocyte colony stimulating factor (rHuG-CSF) macromolecule is extremely sensitive to agitation, pH, ionic strength, temperature, light, freezing, etc. thus making the protein macromolecule almost ideal for a PPSS study (Herman et al., 1996 *Plenum Press, New York*).

- Cytokine macromolecule rHuG-CSF belongs to a distinct structural class of growth factors and folds into a four-helical bundle with a left handed twist and overall dimensions of 45 Å x 26 Å x 26 Å (Hill et al., *Proc. Natl. Acad. Sci. U. S. A.* 1993).

- The structure of rHuG-CSF is stabilized by two disulfide bonds, both of which are required for activity.

- The molecule also contains one free cysteine residue (Ricci et al., *Prot. Sci.* 2003). When dissolved only in water, rHuG-CSF produced in mammalian cell (one O-linked glycan on Thr133 glycosylated, known as Lenogastrim) and *E. Coli* (non-glycosylated, known as Filgrastim) share the same extreme instability relative to formulated pharmaceutical product.
Measuring protein stability, 
H/D exchange experiment, tertiary structure stability

Linear mode MALDI-TOF MS spectra of properly stored *rHuINF α*-2b obtained after 0, 30, 470 and 2670 minutes of H/D exchange. Samples were diluted and incubated in D2O at 25°C, except the sample denoted as 0 min, dissolved in water.

The kinetics of deuterium incorporation into *rHuINF α*-2b at 25°C. Samples were stored at -80°C for one year (unfrozen once and frozen and unfrozen three times) and -20°C for one and two years.
Assignments based on deuterium incorporation after 30 minutes were correct in all cases, suggesting that ESI-MS in combination with H/D exchange is a reproducible and reliable method for distinguishing r-Human insulin from LysPro insulin and for evaluating the consistency of different batches of different insulin types.

After 12 hours of incubation, the thermaly denatured bovine insulin exchanged 15 protons more than the native bovine insulin.

The results show that one can determine reliably whether an insulin sample is r-Human or isomeric LysPro by measuring the extent of deuterium incorporation after the exchange had become relatively constant (e.g., after 15 minutes).
Measuring protein stability, circular dichroism, secondary structure stability

Temperature dependency of ellipticity at 222 nm ($\Theta_{222}$) of rHuG-CSF in non stabilized sample (acetate buffer pH 4.0). The $Tm$ value is determined as the point of inflection of this curve.
Thermodynamic stability

Native \xleftrightarrow{K_D} \text{Denaturated}

\[ \Delta G_D^0 = -RT \ln K_D \]

Native \xrightleftharpoons{k_B/k_N} \text{Denatured} \xrightarrow{k_i} \text{Irreversibly denatured state}
Measuring protein stability, circular dichroism, secondary structure stability $T_m$ vs. pH

$T_m$ (denaturation temperature) calculated from circular dichroism spectra vs. pH for Filgrastim solution (0.5 mg/ml)

Measuring protein stability, circular dichroism, secondary structure stability, different stabilizers

Chemical structures of tested stabilizers: D(+)-Trehalose dihydrate, Xylitol, Cellobitol, D(+)-Turanose, D-(+)-Cellobiose, D-Leucrose, D-Lactitol monohydrate, D-Lyxose, or Sorbitol
Preparation of protein formulations

- Stabilizer concentration of 50 mg/ml

- pH of each formulation placebo was set at 4.0 using 10 mM sodium acetate buffer

- Polysorbate 80 was added to each sample to achieve final concentration of 0.04 mg/ml

- Protein concentration 2.86 mg/ml

- Two control samples: acetate buffer and sorbitol w/o polysorbate
The $T_m$ temperature determined by thermal denaturation studies for tested rHuG-CSF formulations
Measuring protein stability, degradation, oxidation, aggregation, deamidation... primary structure stability, different stabilizers

- Protein/peptide stabilizers pre-screening (PPSP) by peptide mapping LC/MS

- Protein aggregation monitoring by size exclusion chromatography (SEC HPLC, FFF)
  - thermal stress
  - agitation and aggregation

- Protein oxidation, deamidation and truncation monitoring by reverse phase chromatography (RP HPLC)

- Statistical data evaluation
Two-dimensional Coomassie-stained gel electrophoresis of rHuINF α-2b. Isoforms 1, 2, 3 and 4 were analyzed by MALDI-TOF mass spectrometry after in-gel trypsin digestion followed by peptide extraction.

Mass spectra (MALDI-TOF, reflectron) of tryptic peptides extracted from 2-D gel after in-gel digestion of isoform 2. The m/z values of fragments T13 and T18 (A), T3 (B), T19 and T7 (C), T8 (D) and T1-10 (E) revealed different extent of methionine oxidation.

Peptide stabilizers screening (PPSP) relies on idea of peptide degradation monitoring in different stabilizer solutions produced after proteolysis

- Smaller protein fragments are more amenable to analysis than large protein macromolecules which generally give limited amount of information after intact molecule analysis

- Depending on macromolecule size and the number of protein sites susceptible for degradation peptides were evaluated

- This concept remains unchanged in PPSP approach, protein fragments were quantified in time after 27, 40, and 54 hours

- Quantification was performed accurately by triple quadrupole mass spectrometer
**Protein/peptide stabilizers pre-screening (PPSP) by peptide mapping LC/MS**

*In silico*, theoretically calculated and observed monoisotopic masses of GCSF tryptic fragments T1-T10 (reduced disulfide bridges) used for stability estimation.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Amino acid sequence</th>
<th>m/z (theoretical monoisotopic)</th>
<th>Charge</th>
<th>m/z (observed)</th>
</tr>
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<tr>
<td>T3</td>
<td>K</td>
<td>147.1</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td>T7</td>
<td>R</td>
<td>175.1</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td>T9</td>
<td>VLR</td>
<td>387.3</td>
<td>1</td>
<td>387.3</td>
</tr>
<tr>
<td>T10</td>
<td>HLAQP</td>
<td>565.3</td>
<td>1</td>
<td>565.3</td>
</tr>
<tr>
<td>T5</td>
<td>LC*ATYK</td>
<td>698.4</td>
<td>1</td>
<td>698.3</td>
</tr>
<tr>
<td>T1</td>
<td>MTPLGPASSLPQSFLLK</td>
<td>894.0</td>
<td>2</td>
<td>893.9</td>
</tr>
<tr>
<td>T8</td>
<td>AGGVLVASHLQSFEVSYR</td>
<td>1017.0</td>
<td>2</td>
<td>1016.9</td>
</tr>
<tr>
<td>T4</td>
<td>IQGDGAALQEK</td>
<td>565.3</td>
<td>2</td>
<td>565.3</td>
</tr>
<tr>
<td>T2</td>
<td>CLEQVR</td>
<td>747.4</td>
<td>1</td>
<td>747.4</td>
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<tr>
<td>T6</td>
<td>LC<em>HPEELVLLGHSLGIPW APLSSC</em>PSQALQLAGC*LS QLSHGLFLYQGLLQALEG ISPELGPTLDTLQLDVADF ATTIWQQMEELGMAPAL QPTQGAMPAFASAFQR</td>
<td>1615.2</td>
<td>7</td>
<td>1615.3</td>
</tr>
</tbody>
</table>

*free cystein residues after disulfide bridge reduction*
Stability of rHuG-CSF T1 and T2 fragments in tested samples at time points 27, 40 and 54 h determined by mass spectrometry
Stability of rHuG-CSF T4 and T5 fragment in tested samples at time points 27, 40 and 54 h determined by mass spectrometry
Stability of rHuG-CSF T6 and T8 fragments in tested samples at time points 27, 40 and 54 h determined by mass spectrometry.
Stability of rHuG-CSF T9 and T10 fragments in tested samples at time points 27, 40 and 54 h determined by mass spectrometry
Pre-screening data obtained by LC/MS peptide mapping experiment divided stabilizers in three groups:
Turanose, Trehalose, Lactitol, Lyxose, Leucrose, and Acetate buffer as top stabilizer candidates, Xylitol, Sorbitol and Sorbitol without polysorbate as border cases and Cellobiose and Cellobiitol as outliers.
Protein oxidation, deamidation and truncation monitoring by reverse phase chromatography (RP HPLC)

- Forced oxidation study of rHuG-CSF was performed by spiking the tested formulations with high concentration of hydrogen peroxide, incubating the sample at 25 °C and finally quenching the reaction by addition of L-methionine.

- Presence of two oxidized forms (Oxidized form 1 and Oxidized form 2) was observed. Oxidized form 1 and 2 both consist of monooxidized Met127 or Met138.

- Most probable oxidation sites are side-chains of methionines, Met1, Met122, Met127 and Met138.
Protein oxidation, deamidation and truncation monitoring
by reverse phase chromatography (RP HPLC)

Potential oxidation sites in *rHuG-CSF*. Any of four methionines present in *rHuG-CSF* potentially might be oxidized. However Met 127 and Met 138 are highly susceptible to oxidation.
Protein oxidation, deamidation and truncation monitoring by reverse phase chromatography (RP HPLC)

The amount of oxidized form 1 and oxidized forms 2, in tested rHuG-CSF samples subjected to forced oxidation conditions determined by RP HPLC
Protein aggregation monitoring by size exclusion chromatography (SEC HPLC, FFF)

Thermal stress

- rHuG-CSF aggregates are formed as a result of protein-protein interaction caused by short-term hydrophobic auto-association (approximate size 200 kDa) or disulfide bonds interactions (1000-4000 kDa)

The amount of dimers and aggregates in tested rHuG-CSF samples prior to exposure to stress conditions (start analysis) determined by SEC HPLC
The amount of dimers and aggregates in tested rHuG-CSF samples incubated at 40°C for one month determined by SEC HPLC.
Agitation and aggregation

Overlay of rHuG-CSF chromatograms analyzed by SEC, vortexing after 10 (blue), 30 (red), 120 (green), 270 (pink) i 420 seconds (brown)
The amount of dimers, aggregates, oligomers in tested rHuG-CSF samples subjected to agitation determined by SEC HPLC
Comprehensive screening results confirmed ranking of Turanose, Trehalose and Lactitol as top stabilizers and indicated two more: Cellobiitol and Xylitol. Sorbitol, Cellobiose and Acetate buffer remained border cases and Sorbitol without polysorbate, Lyxose and Leucrose showed little stabilizing effect.
What is an outlier?
Cumulative pre-screening and screening statistics

Turanose, D-Trehalose, Lactitol, Acetate buffer (non-stabilized sample), Xylitol, Cellobiitol, Sorbitol, D-Lyxose, Leucrose, Sorbitol without polysorbate, Cellobiose when looking at the overall performance
Conclusions:

- Comparison of intact protein stability and the protein excised pieces cannot be directly compared but PPSP can be statistically incorporated in overall data evaluation as any other stability indicative method.

- The method can pinpoint the weakest parts of the macromolecule and help us answering why some degradation process is more preferred than the other in chosen formulation (e.g. the least stable fragments T2, T5 and T6 contain cysteines which oxidation state can be directly correlated to stabilizer selection under certain pH conditions.

- There is no solid statistical connection found between stabilizers within the same structural group (cellobiitol and lactitol are disaccharide polyols, sorbitol and xylitol are monosaccharide polyols, lyxose is a monosaccharide sugar and cellobiose, trehalose, turanose and leucrose are disaccharide sugars).
- Since individual measures of protein stability are rarely equally important for the final utility of the product, one should consider performance under individual tests as well as the overall score so as to best meet the specific needs for a given product.