USP Biologics Open Forum:
April 28, 2021, 11am - 12pm EDT

Shaping Tomorrow’s Solutions to Today’s Biologics Quality Challenges:

Update on USP’s Work Supporting Multi-Attribute Methods for Biologics
Update on MAM Laboratory Studies and Future Plans

Diane McCarthy, Ph.D.
Director, Biologics Pipeline Development
USP

200usp
The standard of trust
Overview of MAM

Examples of Quality Attributes Assessed with MAM

<table>
<thead>
<tr>
<th>Quality Attribute</th>
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<tbody>
<tr>
<td>Deamidation</td>
</tr>
<tr>
<td>Oxidation</td>
</tr>
<tr>
<td>N- and C-terminal clipping</td>
</tr>
<tr>
<td>Pyroglutamate</td>
</tr>
<tr>
<td>Glycosylation</td>
</tr>
<tr>
<td>Glycation</td>
</tr>
<tr>
<td>Phosphorylation</td>
</tr>
<tr>
<td>Sulfation</td>
</tr>
<tr>
<td>Methylation</td>
</tr>
<tr>
<td>Acetylation</td>
</tr>
<tr>
<td>Hydroxylation</td>
</tr>
<tr>
<td>and more…</td>
</tr>
</tbody>
</table>

USP activities related to MAM

- Stakeholder input identified MAM as an area of interest
  - Roundtable on monoclonal antibodies identified post-translational modifications such as deamidation and oxidation as challenges and opportunities for standards
  - 2020 Stakeholder Forum identified a need for both documentary and physical standards to support MAM
  - New USP Expert Panel was created to draft a general chapter on MAM and to advise on additional standard development

- USP initiated work on MAM in 2019 as a method to provide more efficient and comprehensive protein characterization of USP Reference Standards
  - Surveys of stakeholders to understand current practices for MAM/peptide mapping
  - Collaborations to develop MAM methods and utilize them to evaluate 3 USP mAb reference standards
    - Evaluate mAbs to identify materials and attributes that would be useful for physical standards
    - Focus on sample preparation, which has a big impact on deamidation
Compared data obtained from multiple labs and using different digestion methods.

Most results were consistent across labs and conditions:
- Lysine clipping
- Pyroglutamate
- Glycosylation
- Oxidation
Deamidation levels varied across labs and methods

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Modification</th>
<th>Relative % of Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lab 1 Method 1</td>
</tr>
<tr>
<td>Peptide 1</td>
<td>Oxidation</td>
<td>---</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>Deamidation</td>
<td>14.50%</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>ND*</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>Deamidation</td>
<td>41.80%</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>---</td>
</tr>
<tr>
<td>Peptide 4</td>
<td>Deamidation</td>
<td>ND</td>
</tr>
<tr>
<td>Peptide 5</td>
<td>Deamidation</td>
<td>36.20%</td>
</tr>
<tr>
<td>Peptide 6</td>
<td>Deamidation</td>
<td>9.40%</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>ND</td>
</tr>
</tbody>
</table>

Major differences observed in percentage of deamidation

- Ranged from undetectable to over 40% depending on reduction/alkylation and digestion conditions
Multiphase study to assess factors that contribute to variable deamidation results

- **First Step: Identified variables**
  - Reviewed MAM and peptide mapping methods in over 15 publications
  - Talked with MAM experts to get their input on parameters to test

- **Designed two phase study to assess impact of variables**
  - Assessment of three different mAbs provided insight into molecule-based variability

- **Criteria for comparison included:**
  - Sequence Coverage
  - Missed Cleavages
  - % of Trypsin Peptides
  - % of Deamidated Products
  - % Oxidated Products
  - % Non-specific cleavages

- **Phase 1**
  - Varied reduction/alkylation conditions
  - Fixed digestion condition

- **Phase 2**
  - Varied digestion conditions
  - Fixed reduction/alkylation conditions
Focused on reduction and alkylation step

Key variables included

- pH
  - 7.4
  - 8.3
- Temperature
  - 4 °C
  - Room temperature
  - 37 °C
  - 60 °C

Denaturation
7.5 M Guanidine HCl, 100mM Tris, 0.1mM EDTA

Reduction
2 pHes x 4 temperatures
- pH 7.4
- pH 8.3
- 4 °C
- RT
- 37 °C
- 60 °C

Alkylation
15mM iodoacetamide
30 min RT

Buffer Exchange
10kDa filter
3 times 3x sample volume

Trypsin Digestion
100 mM Tris, pH 7.4
1:10 Sequencing Grade Trypsin
4 hr at 37°C
Stop with 0.1% formic acid
Phase 1 results: influence of pH and temperature

- Deamidation increased with
  - Increased temperature
  - Increased pH

- Changes in deamidation were highly peptide specific

- Changes in pH and temperature did not have a significant effect on overall digest as measured by
  - Coverage
  - Missed or nonspecific cleavages
  - Missing carboxyamidomethylation
Based on Phase 1 Study, selected pH 7.4 and room temperature for reduction and alkylation in Phase 2 study.

Phase 2 study varied:
- Trypsin source
- Enzyme: substrate ratio
- Digestion time

<table>
<thead>
<tr>
<th>pH and Temperature</th>
<th>Enzyme: Substrate Ratio</th>
<th>Trypsin Source</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4 37 °C</td>
<td>1:50</td>
<td>Trypsin Gold</td>
<td>1, 2, 4, 6, 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trypsin Gold</td>
<td>1, 2, 4, 6, 16</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>Sequencing Grade</td>
<td>1, 2, 4, 6, 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Roche Trypsin</td>
<td>1, 2, 4, 6, 16</td>
</tr>
</tbody>
</table>
Phase 2 results

- All digestion conditions yielded good coverage
- Digestion time had the greatest impact on deamidation
- Trypsin source had little impact
Deamidation rates were highly context specific

Study identified several key factors that can lead to sample preparation induced modifications, with a focus on deamidation

- Lower temperatures and pH during the reduction and alkylation step reduced deamidation
- Digestion time had the greatest impact on deamidation in the digestion step

Outcomes and next steps

- Method developed in this study will be used to replicate the results in an independent lab
- Work also provided the foundation for developing pre-digested mAb reference standards
  - Pre-digested mAb standards, coupled with the existing USP mAbs, will provide users the ability to assess both the instrumentation and assay itself (including sample preparation) for peptide mapping and MAM applications.
Acknowledgements

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- Parastoo Azadi
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USP Stakeholders
- Working Group Members
- Expert Volunteers
- Other Stakeholders

USP
- Tim Guo
- Hua Wang
- Niomi Peckham
Evolution of MAM at USP

**Stakeholder Engagement**

- **2017/18**
  - Roundtables on monoclonal antibodies identified analysis of PTMs as a challenge

- **2019**
  - Initiated characterization of USP mAbs
  - Conducted surveys of stakeholders to understand current practices
  - Solicited feedback from Mass Spec Peptides working group and other stakeholders on physical standards

- **2020**
  - Stakeholder Forum on MAM and additional survey on best practices
  - Recruited and initiated MAM Expert Panel
  - Published MAM article in GEN
  - Explored new collaborations to support standard development

- **2021**
  - Established collaboration to expand MAM work in independent laboratory
  - Initiated work to produce pre-digested mAb standards
Thank You

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Diane McCarthy | diane.mccarthy@USP.org | USPbiologics@usp.org | https://www.usp.org/biologics | @USPharmacopeia

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