Lyophilization of Proteins –
FTIR-Guided Formulation Development

Dr. Heiko A. Schiffter
heiko.schiffter@gmail.com
http://www.ibme.ox.ac.uk (until 30th April 2012)
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- MPM and FD Microscopy Setup
- 3D Imaging of FD stage samples using MPM
- 3D Imaging of Phase Separation using MPM
- In-situ Study of Phase Separation using MPM

Summary and Conclusions
Part I:

FTIR Microscopy and Chemical Imaging

• Idea and Objective
• FTIR protein spectrum and analysis
• Multivariate data analysis
• In situ freeze-drying formulation development
• In situ study of phase separation
The Background Idea

- **Spray-drying** is a complex drying process in regards to the drying kinetics and product morphology.
- **Single Droplet Drying** of proteins and protein formulations via *acoustic levitation* for the determination of drying kinetics and product characteristics in spray-drying.
- What is acoustic levitation?
The Background Idea

- Single Droplet Drying using acoustic levitation
  - for drying kinetics analysis
  - A better understanding for the product at hand
Formulation of Biopharmaceuticals

McNally, E.J. and Lockwood, C.E., The importance of a thorough preformulation study (illustrated by Leigh Rodano, BI Pharmaceuticals, Inc.)
Part 1: FTIR spectroscopy

Freeze-drying basics

• Freeze-drying is a complex multistage process

Risk of product instability during each process step!
(Freeze-concentration, phase separation, interfacial adsorption, dehydration)

• Objective:
  To develop a tool for the in-situ study of product stability and excipient behaviour at the small scale during freeze-drying
FPA-FTIR freeze-drying microscopy system

- FTIR microscope with focal plane array (FPA, 64 x 64) detector
- Integrated miniature freeze-drying system
Part 1: FTIR spectroscopy

FTIR protein spectrum

Qualitative and quantitative information about the protein secondary structure is obtained from the Amide bands of the infrared spectrum.

\[ \text{Absorbance} \]

\[ \text{Wavenumber [cm}^{-1}\text{]} \]

\[ \alpha\text{-helix} \]

\[ \beta\text{-sheet} \]

Amide I

Amide II

Amide III + further vibrations

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FTIR protein spectrum analysis

Analysis methods:

- Subtraction spectrum
- Area of overlap
- 2nd derivative spectrum
- Peak fitting (Gaussian curve fitting)

No quantitative secondary structure data

Time intensive and highly subjective

<table>
<thead>
<tr>
<th>Structure</th>
<th>Wavenumber [cm⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helix</td>
<td>1648 – 1657</td>
</tr>
<tr>
<td>β-sheet</td>
<td>1623 – 1641</td>
</tr>
<tr>
<td></td>
<td>1674 – 1695</td>
</tr>
<tr>
<td>unordered</td>
<td>1642 – 1657</td>
</tr>
<tr>
<td>turn</td>
<td>1662 – 1686</td>
</tr>
</tbody>
</table>
FTIR- spectra evaluations using multivariate data analysis

- Objective and fast approach towards spectra evaluation by **multivariate data analysis using a partial least square (PLS) algorithm**
  - Relates a known concentration (here amount of secondary structure) to absorbencies to generate a calibration curve
  - Quantitative decomposition technique
  - Decomposes spectral data and structural content into two sets of vectors (variance spectra – eigenvectors) and scores (critical shapes – loading factors)
  - As spectral information and secondary structure are connected, the two sets of scores can be related to each other by regression and a calibration model can be constructed.
  - Spectral decomposition and regression are performed in one step
Part 1: FTIR spectroscopy

Multivariate data analysis - pure component spectra

\( \alpha \)-helix (1660 – 1650 cm\(^{-1}\))

Intramolecular \( \beta \)-sheet (1695 – 1683 cm\(^{-1}\) and 1644 – 1620 cm\(^{-1}\))

Intermolecular \( \beta \)-sheet (1620 – 1595 cm\(^{-1}\))
<table>
<thead>
<tr>
<th>Protein</th>
<th>α-helix (actual)</th>
<th>α-helix (calculated)</th>
<th>intra β-sheet (actual)</th>
<th>intra β-sheet (calculated)</th>
<th>inter β-sheet (actual)</th>
<th>inter β-sheet (calculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>alkaline phosphatase</td>
<td>36.21%</td>
<td>37.68%</td>
<td>34.49%</td>
<td>35.15%</td>
<td>4.26%</td>
<td>4.66%</td>
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<tr>
<td>bovine serum albumin</td>
<td>69.64%</td>
<td>71.19%</td>
<td>17.39%</td>
<td>13.95%</td>
<td>1.78%</td>
<td>2.28%</td>
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<tr>
<td>carbonic anhydrase</td>
<td>14.05%</td>
<td>15.99%</td>
<td>46.29%</td>
<td>47.59%</td>
<td>7.15%</td>
<td>6.44%</td>
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<tr>
<td>carboxypeptidase A</td>
<td>35.04%</td>
<td>37.45%</td>
<td>28.18%</td>
<td>27.74%</td>
<td>6.56%</td>
<td>6.03%</td>
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<td>catalase</td>
<td>35.86%</td>
<td>32.32%</td>
<td>38.91%</td>
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<td>6.64%</td>
<td>6.69%</td>
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<tr>
<td>concanavalin A</td>
<td>0.00%</td>
<td>0.00%</td>
<td>60.45%</td>
<td>63.89%</td>
<td>9.45%</td>
<td>9.70%</td>
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<tr>
<td>α-chymotrypsin</td>
<td>10.00%</td>
<td>13.61%</td>
<td>49.00%</td>
<td>52.47%</td>
<td>n/a</td>
<td>5.59%</td>
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<td>β-galactosidase</td>
<td>42.78%</td>
<td>39.51%</td>
<td>36.05%</td>
<td>35.47%</td>
<td>3.63%</td>
<td>4.72%</td>
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<tr>
<td>glucagon</td>
<td>59.12%</td>
<td>57.15%</td>
<td>15.88%</td>
<td>17.05%</td>
<td>0.42%</td>
<td>0.23%</td>
</tr>
<tr>
<td>haemoglobin</td>
<td>74.68%</td>
<td>79.59%</td>
<td>10.63%</td>
<td>10.64%</td>
<td>2.79%</td>
<td>2.09%</td>
</tr>
<tr>
<td>human serum albumin</td>
<td>74.48%</td>
<td>77.62%</td>
<td>13.81%</td>
<td>10.76%</td>
<td>0.57%</td>
<td>1.00%</td>
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<tr>
<td>insulin</td>
<td>56.14%</td>
<td>51.23%</td>
<td>23.92%</td>
<td>22.46%</td>
<td>5.00%</td>
<td>4.79%</td>
</tr>
<tr>
<td>lactate dehydrogenase</td>
<td>56.81%</td>
<td>61.39%</td>
<td>25.32%</td>
<td>23.46%</td>
<td>2.92%</td>
<td>1.94%</td>
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<tr>
<td>lysozyme</td>
<td>51.92%</td>
<td>49.57%</td>
<td>21.84%</td>
<td>21.84%</td>
<td>4.17%</td>
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<tr>
<td>myoglobin</td>
<td>80.71%</td>
<td>78.04%</td>
<td>9.68%</td>
<td>12.56%</td>
<td>2.25%</td>
<td>2.28%</td>
</tr>
<tr>
<td>ribonuclease A</td>
<td>24.78%</td>
<td>23.39%</td>
<td>40.26%</td>
<td>42.09%</td>
<td>2.92%</td>
<td>3.65%</td>
</tr>
</tbody>
</table>
Multivariate data analysis - FTIR calibration curves

- **α-helix**
  - Calculated [%] vs. Actual [%]
  - Correlation coefficient: \( r = 0.992 \)

- **Intramolecular β-sheet**
  - Calculated [%] vs. Actual [%]
  - Correlation coefficient: \( r = 0.992 \)

- **Intermolecular β-sheet**
  - Calculated [%] vs. Actual [%]
  - Correlation coefficient: \( r = 0.979 \)
In-situ freeze-drying formulation development

Chymotrypsin (unformulated) 20 mg/ml; freezing rate: 0.25°C/min and 1°C/min; Sample volume: 2.5 μl

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Stage</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>20°C 30mins</td>
<td>Freezing</td>
<td>1°C/min</td>
</tr>
<tr>
<td>-40°C 60mins 100mTorr</td>
<td>1st Drying</td>
<td>-27°C 60mins</td>
</tr>
<tr>
<td>1°C/min</td>
<td>2nd Drying</td>
<td>20°C 60mins 100mTorr</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>State</th>
<th>α-helix</th>
<th>Intramol β-sheet</th>
<th>Intermol β-sheet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprocessed</td>
<td>13.6%</td>
<td>52.5%</td>
<td>5.6%</td>
</tr>
<tr>
<td>Cooled</td>
<td>-0.2%</td>
<td>+0.3%</td>
<td>+/-0%</td>
</tr>
<tr>
<td>Frozen at (1°C/min)</td>
<td>-3.2%</td>
<td>-2.9%</td>
<td>+4.2%</td>
</tr>
<tr>
<td>Frozen at (0.25°C/min)</td>
<td>-4.8%</td>
<td>-3.0%</td>
<td>+5.9%</td>
</tr>
<tr>
<td>After freeze-thawing at 0.25°C/min</td>
<td>-1.9%</td>
<td>-1.1%</td>
<td>+1.9%</td>
</tr>
</tbody>
</table>

Absorbance [AU] vs. Wavelength [cm⁻¹]
**In-situ freeze-drying formulation development**

Chymotrypsin (unformulated) 20 mg/ml; freezing rate: 0.25°C/min; sample volume: 2.5 µl

![Absorbance graph](image)

<table>
<thead>
<tr>
<th></th>
<th>α-helix</th>
<th>Intramol β-sheet</th>
<th>Intermol β-sheet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprocessed</td>
<td>13.6%</td>
<td>52.5%</td>
<td>5.6%</td>
</tr>
<tr>
<td>Frozen at 0.25°C/min</td>
<td>−4.8%</td>
<td>−3.0%</td>
<td>+5.9%</td>
</tr>
<tr>
<td>Freeze-dried</td>
<td>−6.6%</td>
<td>−3.1%</td>
<td>+8.2%</td>
</tr>
<tr>
<td>Rehydrated</td>
<td>−3.2%</td>
<td>−2.1%</td>
<td>+4.1%</td>
</tr>
</tbody>
</table>
In-situ freeze-drying formulation development

Chymotrypsin (formulated) 20 mg/ml; concentration excipients: 30 mg/ml; freezing rate: 0.25°C/min and 1°C/min; sample volume: 2.5 μl

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature</th>
<th>Time</th>
<th>Rate</th>
<th>1st Drying</th>
<th>2nd Drying</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing</td>
<td>-40°C</td>
<td>60mins</td>
<td>1°C/min</td>
<td>-27°C</td>
<td>60mins</td>
</tr>
<tr>
<td>Drying</td>
<td>20°C</td>
<td>60mins</td>
<td>100mTorr</td>
<td></td>
<td>20°C</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>α-helix</th>
<th>Intramol β-sheet</th>
<th>Intermol β-sheet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprocessed</td>
<td>13.6%</td>
<td>52.5%</td>
<td>5.6%</td>
</tr>
<tr>
<td>Cooled</td>
<td>- 0.2%</td>
<td>+ 0.2%</td>
<td>+/- 0%</td>
</tr>
<tr>
<td>Frozen at 1°C/min</td>
<td>- 1.9%</td>
<td>+ 0.3%</td>
<td>+ 0.4%</td>
</tr>
<tr>
<td>Frozen at 0.25°C/min</td>
<td>- 2.9%</td>
<td>- 1.6%</td>
<td>+ 3.1%</td>
</tr>
<tr>
<td>After freeze-thawing at 0.25°C/min</td>
<td>- 0.5%</td>
<td>- 0.2%</td>
<td>+ 0.7%</td>
</tr>
</tbody>
</table>

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Presentation – 5th ISLFD Annual Meeting – 30th March 2012
**In-situ** freeze-drying formulation development

Chymotrypsin (formulated) 20 mg/ml; concentration excipients: 30 mg/ml; freezing rate: **0.25°C/min**; sample volume: 2.5 μl

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st Freezing</td>
<td>-40°C</td>
<td>60 mins</td>
<td>1°C/min</td>
</tr>
<tr>
<td>1st Drying</td>
<td>-27°C</td>
<td>60 mins</td>
<td>100 mTorr</td>
</tr>
<tr>
<td>2nd Drying</td>
<td>20°C</td>
<td>60 mins</td>
<td>100 mTorr</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>α-helix</th>
<th>Intramol β-sheet</th>
<th>Intermol β-sheet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprocessed</td>
<td>13.6%</td>
<td>52.5%</td>
<td>5.6%</td>
</tr>
<tr>
<td>Frozen at 0.25°C/min</td>
<td>-2.9%</td>
<td>-1.6%</td>
<td>+3.1%</td>
</tr>
<tr>
<td>Freeze-dried</td>
<td>-3.5%</td>
<td>-2.1%</td>
<td>+4.5%</td>
</tr>
<tr>
<td>Rehydrated</td>
<td>-1.1%</td>
<td>-0.4%</td>
<td>+1.0%</td>
</tr>
</tbody>
</table>

![Absorbance vs. Wavelength](chart.png)

**Presentation – 5th ISLFD Annual Meeting – 30th March 2012**

Dr. Heiko A. Schiffter – IBME Oxford
**In-situ freeze-drying formulation development**

20mg/ml rh albumin in 145 mM NaCl solution at pH 7.1, with 30mM Octanoate, 15µg/L Polysorbate 80

![Graph showing Structure Change Compared with Solution at 20 Degree C (%) for different physical states: Frozen at -40 Degree C and Dried at 20 Degree C.](Image)

Part 1: FTIR spectroscopy
Comparison of formulations before & after FD

Comparison of defatted rh albumin at 20 mg/ml, 50 mg/ml, 50 mg/ml with 5% (w/v) sucrose, and 50 mg/ml with 5% (w/v) trehalose, (alpha-helix, fluorescence emission and aggregate, before and after freeze-drying)

Comparison of formulated rhA at 20 mg/ml, 50 mg/ml, 50 mg/ml with 5% (w/v) sucrose, and 50 mg/ml with 5% (w/v) trehalose, (alpha-helix, fluorescence emission and aggregate, before and after freeze-drying)
Tc and Tg’ Determination

Example:
50 mg/ml rh albumin formulated with 50 mg/ml trehalose

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In-situ study of phase separation during lyophilization

20°C 30mins  | Freezing 1°C/min  | -40°C 60mins | 1°C/мин  | 1st Drying -27°C 60mins 100mTorr | 1°C/мин  | 2nd Drying 20°C 60mins 100mTorr

5% (w/w) PEG 4000 and 5% (w/w) Dextran 500 kDa in 145 mM NaCl solution at pH 7.1

Visible light image of freeze-dried mixture of PEG 4000 and Dextran 500
FPA-FTIR image of Dextran 500 distribution within the freeze-dried mixture
FPA-FTIR image of PEG 4000 distribution within freeze-dried mixture
**In-situ** study of phase separation during lyophilization

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Process</th>
<th>Duration</th>
<th>Comments</th>
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<tbody>
<tr>
<td>20°C</td>
<td>Freezing</td>
<td>1°C/min</td>
<td>1°C/min</td>
</tr>
<tr>
<td>-40°C</td>
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<td>60mins</td>
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<tr>
<td>1°C/min</td>
<td>1st Drying</td>
<td></td>
<td>-27°C 60mins 100mTorr</td>
</tr>
<tr>
<td>1°C/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20°C</td>
<td>2nd Drying</td>
<td></td>
<td>60mins 100mTorr</td>
</tr>
</tbody>
</table>

20 mg/ml rh albumin in 145 mM NaCl solution at pH 7.1, 5% (w/w) PEG 4000 and 5% (w/w) Dextran 500 kDa

**Images:**
- Visible light image of freeze-dried mixture of PEG4000 and Dextran 500
- FPA-FTIR image of distribution of PEG4000 within freeze-dried mixture
- FPA-FTIR image of distribution of Dextran within freeze-dried mixture
- FPA-FTIR image of distribution of recombinant within freeze-dried mixture

- PEG rich area
- Dextran rich area
- Mix area
In-situ study of phase separation during lyophilization

20 mg/ml rh albumin in 145 mM NaCl solution at pH 7.1, 5% (w/w) PEG 4000 and 5% (w/w) Dextran 500 kDa

Amide 1 region of rh albumin at different sample position
In-situ study of phase separation during lyophilization

20°C 10mins → Freezing 1°C/min → -40°C 60mins → 1°C/min → 1st Drying -27°C 60mins 10mTorr → 1°C/min → 2nd Drying 20°C 60mins 10mTorr

-20°C

Dextran
Protein
PEG
Protein 2nd Structural Change Distribution
Part II:

MPM Microscopy

- MPM and FD Microscopy Setup
- 3D Imaging of FD stage samples using MPM
- 3D Imaging of Phase Separation using MPM
- In-situ Study of Phase Separation using MPM
Multiphoton Microscopy & Freeze-Drying Microscopy System Set-up

- Laser Control System
- MPM Control System
- Cyrostage Control System
- Laser Adjust System
- LN2 Tank

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Part 2: Multiphoton Microscopy

3D Imaging of samples in a cryostage using MPM

3D MPM images of freeze-dried mixture of 10% w/v FITC-PEG5000 and 10% w/v Cascade Blue dextran (10 kDa)
(size 207 μm × 207 μm, resolution 0.2 μm × 0.2 μm × 0.5μm).
3D Imaging of Phase Separation during Freeze-Drying

Freeze-Dried Sample
10% w/v FITC-PEG5000 +
10% w/v TRITC-dextran155,000
(Size 621μm×621μm,
resolution 0.6 μm×0.6μm×1.5μm)

Frozen and annealed for 240mins at -40°C
10% w/v FITC-PEG5000 + 10% w/v Cascade
Blue dextran 10,000 + 20mg/ml Texas Red BSA
(Size 621μm×621μm,
resolution 0.6 μm×0.6μm×1.5μm)

Frozen and annealed for 240mins at -40°C
10% w/v FITC-PEG5000 + 10% w/v Cascade
Blue dextran 10,000 + 20mg/ml Texas Red BSA
(Size 148μm×148μm,
resolution 0.145μm×0.145μm)
In-situ Study of Phase Separation using MPM

Freeze-Drying Cycle with Annealing at -9°C for 240 mins
10% w/v FITC-PEG5000 + 10% w/v Cascade Blue dextran 10,000 + 20mg/ml Texas Red BSA

Visible Light Pictures 1000μm×1000μm (Resolution 1μm×1μm)
In-situ Study of Phase Separation using MPM

Freeze-Drying Cycle with Annealing at -9°C for 240 mins
10% w/v FITC-PEG5000 + 10% w/v Cascade Blue dextran 10,000 + 20mg/ml Texas Red BSA

3D MPM Images 621μm×621μm (Resolution 0.6 μm×0.6μm×1.5μm)  Zoom-In MPM Slice Images 148μm×148μm (Resolution 0.145μm×0.145μm)
Summary and conclusions

• FTIR spectroscopy can be used for a range of protein applications including the formulation development for freeze-drying

• Multivariate data analysis by PLS algorithm provides a rapid and objective way of quantitative evaluation of protein FTIR spectra

• The combination of FTIR and freeze-drying microscopy enables the in-situ study of protein stability during lyophilization in the small scale

• MPM enables to look into the pore formation and structure during freeze-drying

• Both, FTIR microscopy and MPM are expensive specialist techniques and relatively complicated data analysis techniques are necessary

• Only ultra small scale analysis possible and the question in regards to scalability is so far unanswered

• Final question: Will this ever be used wide spread?
Acknowledgements

- Dr. Renchen Liu
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- Prof. Geoffrey Lee
- Dr. Sebastian Vonhoff
- Mr Felix Wolf
- Phil Morton

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Division of Pharmaceutics, University of Erlangen

Thank you very much for your attention