Surfactants are potent Stabilizers for Proteins against Interfacial Stresses

- “Interfacial stresses” are encountered during many stages of production, shipment and use e.g.
  - Air/liquid interfaces (e.g. shaking)
  - Ice/Liquid interfaces (e.g. freezing and thawing)
  - Surface interactions (e.g. manufacturing equipment)

- Interfacial stresses may lead to “protein instabilities” such as adsorption, aggregation or precipitation (“particle formation”) Kiese et al, J. Pharm Sci. 2008, 2009

- Surfactants (such as the non-ionic Polysorbate 20 or 80) effectively protect Proteins against aggregation caused by interface-induced stresses and adsorption
Polysorbate 20 and 80 are the most widely used Surfactants in Protein Formulations

Polysorbate 80

1,4 anhydro-sorbitol

Poly-oxo-ethylene

Fatty acid ester

Polysorbate 20

70% of marketed antibodies are formulated with Polysorbates
Typical concentrations of PS20/PS80 range from 0.001-0.1% (w/v)

How Polysorbates stabilize Proteins
Polysorbate stabilize (many) Proteins against shaking-induced aggregation/precipitation


Effect of mechanical stress on Mab T in the presence and absence of Polysorbate

<table>
<thead>
<tr>
<th>%</th>
<th>0 %</th>
<th>0.005 %</th>
<th>0.01 %</th>
<th>0.02 %</th>
<th>0.05 %</th>
<th>PS20</th>
</tr>
</thead>
</table>

• Note: the CMC of Polysorbates does not unequivocally correlate to the stabilization behaviour


Mechanism of Polysorbate Function(s)
Adsorption of polysorbate to interface: a surface tension treatment

Kishore Ravuri, yet unpublished results

Stronger adsorption of polysorbate 20 observed in surface tension isotherm demonstrating that the stabilizing ability of the surfactant is surface energy driven.

Langmuir adsorption isotherm

\[ \Gamma = \frac{G}{\Gamma_m - G} \]

adsorption equation

\[ \Pi_g = k_g T \gamma_m \ln \left( \frac{G_m}{G_m - G} \right) \]

Interaction of non-ionic surfactants with proteins: Studies with ITC

Kishore Ravuri, yet unpublished results

ITC titration of a non-ionic surfactant into a 10mg/mL mAb formulation, 20mM His/HCl, pH6, 25°C

- Low binding constants (K = 10^2-10^3 M^-1)
- Comparable to literature e.g. Garidel et al.
- The above results confirms that interactions between surfactant and protein are non specific.
- Stabilizing effect of non-ionic surfactants is NOT interaction driven.
Synthesis of Polysorbates and Polysorbate Heterogeneity

Industrial Synthesis of Polysorbates

Side Products in Synthesis of Polysorbates

PEG, Fatty acid/salts

• High batch to batch & supplier variability

Some challenges in the context of the use of Polysorbates in Protein Formulations

1. Adsorption of Polysorbate
Polysorbates can significantly adsorb to some filters

- Dilution effects in filters (e.g. due to residual water), observed via parallel change of protein concentration and conductivity
- Adsorption of Polysorbate 80 to filters (>80% recovery after 3L pre-rinse with Product)

Some challenges in the context of the use of Polysorbates in Protein Formulations

2. Polysorbates in Formulations used for preclinical animal (Dog) studies
Pseudoallergenic reaction in dogs after administration of Polysorbate

• Polysorbate 20 (Tween 20) used for i.v. formulation of protein (stabilizer)

• A tox study was performed with rats and dogs: also placebo vehicle showed a pseudoallergic reaction in the dogs (well tolerated in rats and monkeys – as well as humans)

⇒ dogs to be excluded for tox studies with formulations containing polysorbates because hypersensitive compared to other species

• Potential mechanisms:
  – Histamine-releasing properties of polysorbates Masini et al. (1985)
    10 mg/kg of Polysorbate 80* caused severe hypotension after first administration and increase in plasma histamine
  – Haemolytic properties of polysorbates Krantz et al. (1948)
    haemolysis of red blood cells in vitro due to the solubilizing activity of Tween 20 at concentrations > 100 mg/100 ml

Some challenges in the context of the use of Polysorbates in Protein Formulations

3. Degradation of Polysorbates in Bulk and/or aqueous formulation
Polysorbates Can Undergo Degradation

Temperature  Auto-oxidation  Hydrolysis  pH, Temperature
Light, O₂, metals  →  Peroxides, formic and acetic acid  →  Fatty acids

Mechanistic picture

Findings from investigations:
Degradants isolated from placebo formulations stored at 25°C for 20 months
Dominant Mechanisms In Polysorbate Degradation

**Ester hydrolysis**

Profile of NMR signals of fatty acid in PS20

![Ester hydrolysis profile](image)

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>k (h⁻¹)</th>
<th>t₁/₂ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9e-07</td>
<td>5.50e05</td>
</tr>
<tr>
<td>25</td>
<td>5e-05</td>
<td>1.39e04</td>
</tr>
<tr>
<td>40</td>
<td>2e-04</td>
<td>3.47e03</td>
</tr>
<tr>
<td>PS80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4e-07</td>
<td>1.73e06</td>
</tr>
<tr>
<td>25</td>
<td>5e-05</td>
<td>1.39e04</td>
</tr>
</tbody>
</table>

- t₁/₂ of Polysorbate20 hydrolysis at 40°C was about 5 months
- t₁/₂ of Polysorbate20 hydrolysis at 5°C was negligible

**Auto-oxidation of PEG**

Buildup of acids and aldehydes

Mechanistic Picture

**Summary**

- Auto-oxidation plays a dominant role in degradation of polysorbates. The rate of **Hydrolysis is negligible** at pharmaceutically relevant conditions (drug product storage of 5 °C & 25 °C)
- Along with rupture of PEG chains, there also occurs rampant degradation at the **olefinic sites**.
- It is likely that the radical initiation occurs first at the olefin site and then spreads to the PEG chains.

![Peroxide formation in placebos containing 0.02% PS20 and PS80 at 40 °C](image1.png)

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Concentration (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>120</td>
</tr>
<tr>
<td>15</td>
<td>90</td>
</tr>
<tr>
<td>25</td>
<td>60</td>
</tr>
</tbody>
</table>

Changes in PS20 and PS80 concentration measurement by micelle method over 1 year at 25 and 5 °C

![Changes in PS20 and PS80 concentration measurement by micelle method over 1 year at 25 and 5 °C](image2.png)

Impact of Polysorbate degradation on protein formulations

**Overall considerations**

- Effect of decreased surfactant content
  - What is the impact of decreased surfactant content on the stability of the protein? Not enough stabilizer??

- Effect of insoluble degradants from hydrolysis
  - What do the degradants do to the protein?
  - Do degradants impact product quality other than interacting with protein?

- Effect of peroxides from auto-oxidation
  - What influence does auto-oxidation of PS have on the mAb?
Impact of Polysorbate degradation on protein formulations

End of shelf life shake stress

Impact of Polysorbate degradation on protein formulations

Surface pressure in aged formulations

- Degradation products of PS still show surface activity even after 60% loss of content by micelle assay
### Insoluble degradants

<table>
<thead>
<tr>
<th>FlowCam 10x</th>
<th>MFI 5x</th>
<th>MFI 14x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene size standards</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3µm</td>
<td>2µm</td>
<td>2µm</td>
</tr>
<tr>
<td>4µm</td>
<td>5µm</td>
<td>5µm</td>
</tr>
<tr>
<td>10µm</td>
<td>10µm</td>
<td>10µm</td>
</tr>
<tr>
<td>26µm</td>
<td>25µm</td>
<td>No adequate picture captured</td>
</tr>
</tbody>
</table>

| Excipient related particles | | |
| 3µm | 2µm | 2µm |
| 6µm | 6µm | 6µm |
| 12µm | 15µm | 12µm |
| 25µm | 26µm | |

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### Impact of Polysorbate degradation on protein formulations

**Oxidation**

<table>
<thead>
<tr>
<th>Surfactant content determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS20 + Exc.X</td>
</tr>
<tr>
<td><img src="image1" alt="Graph" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% Fc Methionine oxidation observed in protein at 25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS20 + Exc.X</td>
</tr>
<tr>
<td><img src="image3" alt="Graph" /></td>
</tr>
</tbody>
</table>
**Impact of Polysorbate degradation on protein formulations**

**Summary**

- Surface pressure experiments show comparable surface tension
- End of shelf life studies show effective protection even with decreased surfactant content

**Effect of decreased surfactant content**

- Fatty acids can appear as visible or subvisible particles
- Fatty acids may induce aggregation, above a threshold concentration

**Effect of insoluble degradants from hydrolysis**

- Degradation of polysorbates correlates to extent of oxidation in mAbs
- It is possible that peroxides generated from PS degradation causes mAb oxidation

**Effect of peroxides from auto-oxidation**

**Overall Summary and Conclusions**

- Polysorbates are a complex mixture of Sorbitan-POE-fattyacid esters
- Polysorbates stabilize (most) Proteins (against interfacial stress-induced aggregation) and can minimize protein adsorption to interfaces
- Polysorbates can adsorb to e.g. filters and other material
- Degradation may occur in bulk and/or pharmaceutical formulations via hydrolysis or by auto-oxidation (major pathway)
- Polysorbates can degrade and this requires sufficient attention during formulation development
Roche

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