PEG-Protein Interactions for Stable Formulations Studied by CG-MALS

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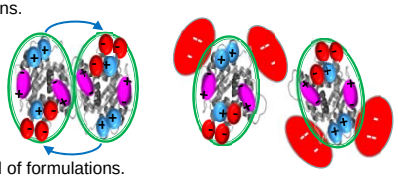
1. Background

Many therapeutic proteins such as antibodies exhibit non-specific self-association at concentrations > 10 mg/mL typical of final drug-product formulations.

Protein self-attraction can arise from a global dipole moment, local dipoles, van der Waals forces, hydrophobic forces and salt bridges.

This behavior is detrimental to solubility, stability and viscosity. One mechanism for reducing protein-protein attraction is to stabilize monomers or low-order oligomers by adding an excipient that binds more favorably to the protein than the protein does to itself, surrounding it with a 'shield' and so excluding other proteins.

We examine the potential role of a model excipient (PEG) in stabilizing a model non-specific, self-attractive protein (lysozyme) at concentrations typical of formulations.



2. Analyzing Self- and Hetero-interactions in Non-Ideal Solutions

1. Non-specific interactions are typically analyzed in terms of the osmotic virial coefficients: A_2 (a.k.a. B_{22}) and A_{11} (a.k.a. B_{23}).

$$\frac{R}{K} = \frac{M_A c_A \left(dn/dc_A\right)^2}{1+2A_2^A M c_A + \ldots} + \frac{M_B c_B \left(dn/dc_B\right)^2}{1+2A_2^B M c_B + \ldots} - A_{11} M_A M_B c_A c_B$$

2. Measured virial coefficients are referenced to the 'hard core' interaction (excluded volume) calculated from the hydrodynamic radius r_H: $A_2^{exc} = 16\pi r_H^3/3M^2$.

3. Attractive interactions can also be modeled as pseudo-specific binding, assigning repulsive effects to A_2^{rep}. This provides a more intuitive interpretation of the data: stoichiometry and K_D's of self + hetero-complexes.

$$\frac{R}{K} = \frac{M_A c_A^{free} \left(dn/dc_A\right)^2 + M_B c_B^{free} \left(dn/dc_B\right)^2 + \sum_{i,j}\left[iM_A \left(dn/dc_A\right)^2 + jM_B \left(dn/dc_B\right)^2\right] c_{ij}}{1+8\varphi_A + 30\varphi_A^2 + \ldots + 8\varphi_B + 30\varphi_B^2 + \ldots}$$

$$\varphi_A = \frac{V_A^{eff}}{M_A} c_A^{total} = \frac{A_2^{A,rep}}{4M_A^2}; \qquad \varphi_B = \frac{V_B^{eff}}{M_B} c_B^{total} = \frac{A_2^{B,rep}}{4M_B^2}$$

3. Methods

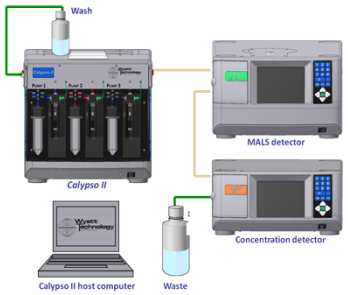
Hen egg white lysozyme exhibits self-attraction at physiological pH and salt concentration, forming quasi-specific, reversible dimers at concentrations up to 40 mg/mL. PEG is believed to interact hydrophobically with lysozyme at the cleft site (Furness et al., Biomaterials (1998) **15**: 1361-9), but larger PEG polymers in PBS also carry an effective net charge Z*, measured by electrophoretic light scattering (Wyatt Möbiuζ™). .

CG-MALS was applied to measure self- and cross-interactions of lysozyme and 8 kDa PEG in Dulbecco's PBS.

4. Composition-Gradient Multi-Angle, Static Light Scattering

Apparatus:

- ❖Wyatt DAWN® HELEOS™ multi-angle, static light scattering detector
- ❖Wyatt Calypso® II composition gradient system & software
- ❖Optilab® T-rEX ™ dRI concentration detector



CG-MALS Measures:

Specific Interactions:

- ✓ Label-free and immobilization-free
- ✓ Absolute stoichiometry and equilibrium association constants K_D from sub-nM to mM
- ✓ Simultaneous self- *and* hetero-association
- ✓ Multivalent complexes
- ✓ Multi-protein, bispecific and chimeras

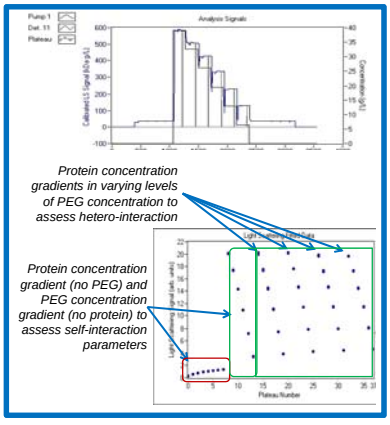
Non-Specific Interactions: virial coefficients = general intermolecular attraction/repulsion

Kinetics: binding, dissociation, aggregation

Applications:

- ✓ Receptor/ligand binding, inhibition
- ✓ Crowding effects, high-conc. formulations
- ✓ Optimization of buffers for stability, solubility, crystallization and purification

5a. Raw data

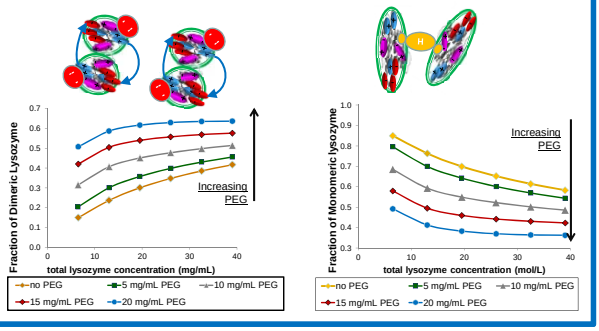


5c. Analysis of total protein oligomer vs PEG concentration

Increasing PEG leads to more overall dimer and less overall monomer

⇒ PEG-protein interaction is stronger than protein self interaction; PEG stabilizes lysozyme dimers.

⇒ Hydrophobic interaction does not adequately explain dominant 2:2 stoichiometry



5. Results

The self-interaction of lysozyme at high concentration is best characterized as a quasi-specific self-association forming homodimers with K_D=4.4 mM, subject to steric repulsion described by a positive A_2.

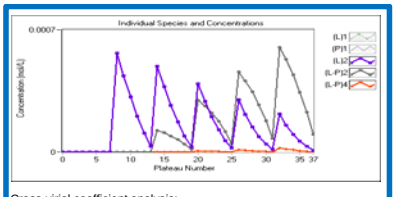
The self-interaction of PEG is repulsive and closely corresponds to the expected excluded volume A_2.

Cross-virial coefficient analysis gives $A_{11} = +1.6 \cdot 10^{-4}$ mol·mL/g^2, indicating that net PEG-protein interactions are attractive, since this value is well below the excluded volume A_{11}^{exc} of ~$2 \cdot 10^{-3}$ mol·mL/g^2. This correlates well to the effective opposite net charges: lysozyme – Z* = 1.25; PEG: Z* = -3.35.

Full interaction analysis of the CG-MALS data shows that PEG does not associate appreciably with monomeric lysozyme, only with dimers to form weakly bound 2:2, K_D=1 mM.

Apparently PEG below ~25 mg/mL stabilizes lysozyme dimers. Higher PEG concentrations induce rapid protein-PEG hetero-aggregation. The interaction appears to be electrostatic based on the stoichiometry.

5b. Global fit of binding affinity and stoichiometry



Cross-virial coefficient analysis:

A_2(A) = -4·10^{-4} mol·mL/g^2

A_{11} = 1.6·10^{-4} mol·mL/g^2 (compare excluded vol. A_{11}= 2·10^{-3} mol·mL/g^2)

A_2(PEG) = 1.6·10^{-3} mol·mL/g^2 (r_{ell}=2.4 nm)

Reversible association analysis:

A_2(A) = 2.6·10^{-4} mol·mL/g^2 (r_{ell}=1.75 nm)

K_D(AA) = 4.4 mM

K_D(AB) = 1.4 mM

Increasing PEG →

- ✓ *Less free monomer*
- ✓ *Less pure dimer*
- ✓ *More dimer-PEG complexes*
- ✓ *No monomer-PEG complexes*

5d. Additional protein-PEG interactions

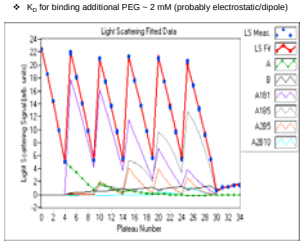
Lysozyme-PEG :

Too much PEG leads to rapid aggregation

BSA (A) - 8 kDa PEG (B) interaction:

best fit is AB, AB_5, A_2B_5, A_2B_{10}

- ❖ A_2(A) = 1·10^{-4} mol·mL/g^2 (r_{ell}=3.5 nm)
- ❖ No self-association of BSA
- ❖ K_D(AB) = 135 µM, possibly at known hydrophobic residues 209-218. (Rawat et al., Biochi. & Biophi. Res. Comm. 2010)
- ❖ K_D for binding additional PEG ~ 2 mM (probably electrostatic/dipole)



Conclusions

- PEG interacts favorably with proteins in concentrated solutions, but actually induces aggregation by binding to and enhancing protein oligomers.
- CG-MALS is an essential tool in identifying and quantifying the interactions present at highly concentrated formulations, in order to optimize excipients for colloidal stability.

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