Supercharging Proteins with Small Polyvalent Anions to Offset Aggregation

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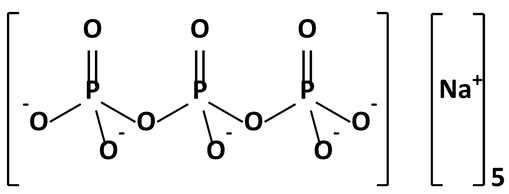
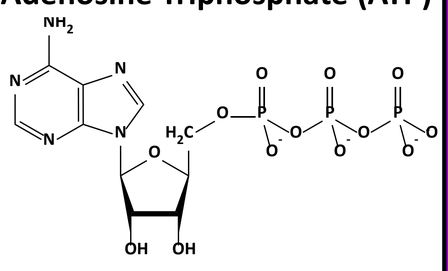
Introduction

- Controlling protein phase-behaviour is critical for the biopharmaceutical industry which aims to employ proteins as therapeutics and a number of biological processes that take place in the cell.[1,2]
- Protein phase misregulation often leads to protein aggregation, precipitation and unfolding which reduce therapeutic effectiveness and can lead to the development of neurodegenerative diseases.[1,2]
- Previously, positively and negatively supercharged variants of proteins were developed by mutating solvent exposed residues to either positive or negative residues. All supercharged variants were resistant to heat-induced aggregation and regained their original fold when cooled.[3]
- Recent studies have proposed new biological roles for adenosine triphosphate (ATP) beyond an energy source in which it regulates membraneless organelle formation and acts as a hydrotrope to prevent protein aggregation *in vitro* and *in vivo*.[4] The mechanism by which ATP prevents aggregation is unknown.

Sodium Tripolyphosphate (STPP)



Adenosine Triphosphate (ATP)

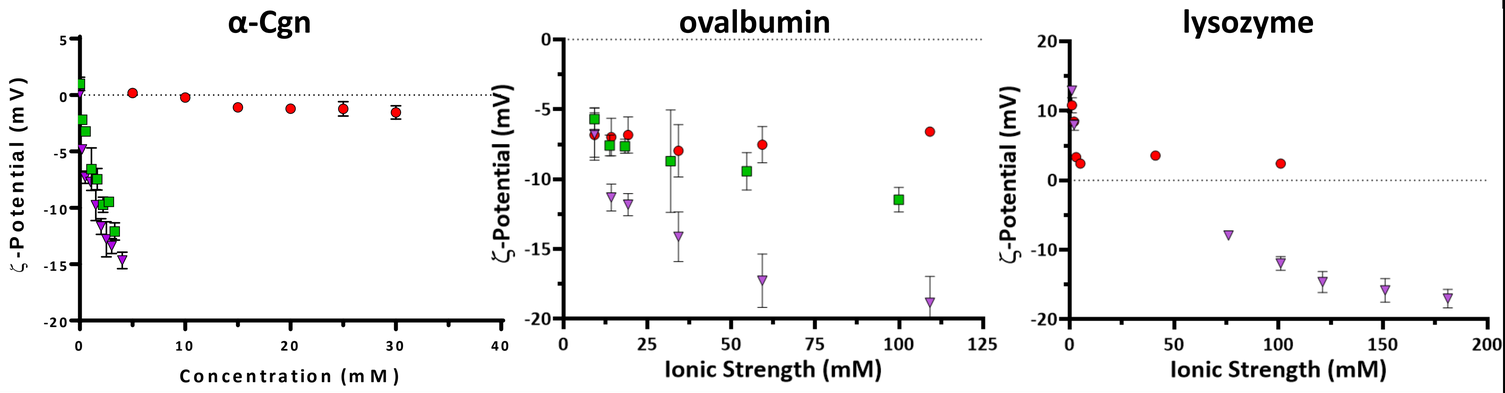


Research Aims

- We aim to investigate how STPP and ATP influence
  - Protein net charge by measuring ζ-potentials.
  - Protein-protein interactions with static light scattering (SLS).
  - Onset of protein aggregation temperature (T_{agg}) with dynamic light scattering (DLS).
- Can polyvalent anions be used as co-solvents to supercharge proteins and increase their resistance to aggregation?

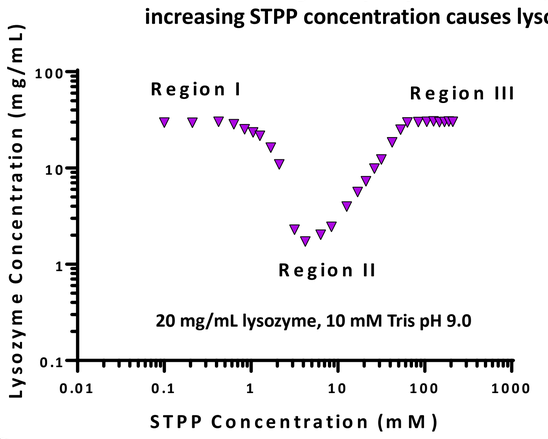
Effect of Polyvalent Anions on Protein Net Charge

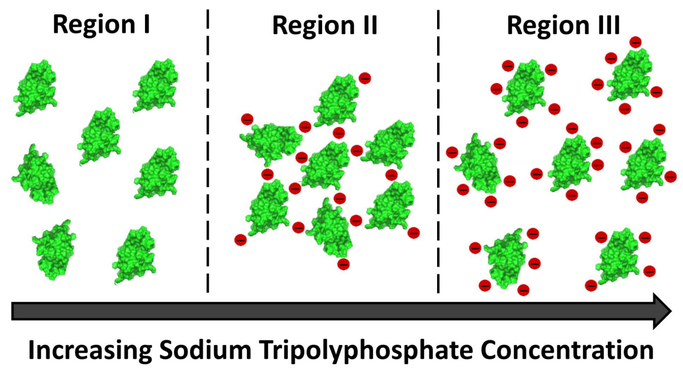
- For neutrally charged α-chymotrypsinogen (α-Cgn) and negatively charged ovalbumin addition of NaCl has little effect on protein net charge, whereas addition of more highly charged anions such as STPP and ATP leads to protein supercharging.
- More complex behaviour is observed for the positive protein lysozyme. NaCl neutralises the positive net charge of lysozyme whereas the polyvalent anion STPP inverts the net charge of lysozyme from positive to negative.
  - Low concentrations of STPP induces lysozyme phase-separation and further increasing STPP concentration causes the phase-separated lysozyme to resolubilise (lower left graph). This effect is termed reentrant condensation (RC), ATP also induces RC.
  - It should be noted that other polyanions such as sulphate and citrate invert lysozyme net charge but do not induce lysozyme phase-separation.
- The ζ-potentials show that polyvalent anions consistently supercharge negative proteins and invert the net charge of positive proteins.



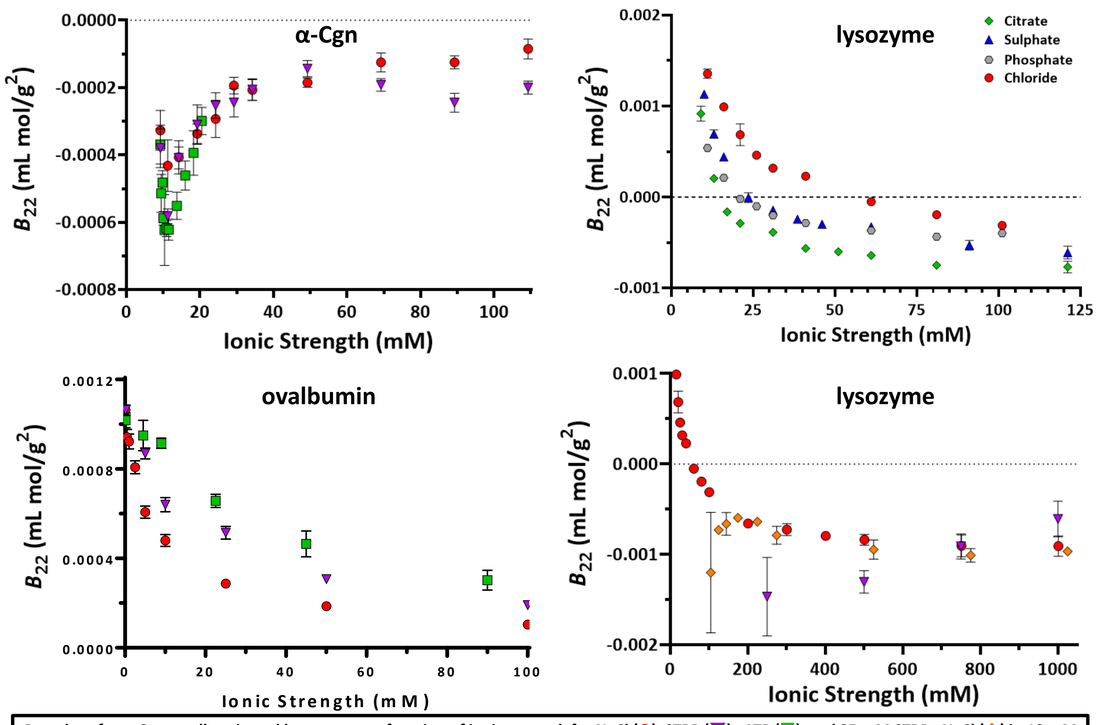
ζ-potentials were all determined at 1 mg/mL protein in 10 mM tris buffer, α-Cgn and ovalbumin were tested at pH 7.0 and lysozyme was tested at pH 9.0. chloride (●), STPP (▼) and ATP (■).

At low concentrations of STPP in region I lysozyme is soluble, increasing STPP concentration causes lysozyme phase-separation in region II and further increasing STPP concentration causes lysozyme resolubilisation in region II as highlighted by the figures below.





Effect of Polyvalent Anions on Protein-Protein Interactions

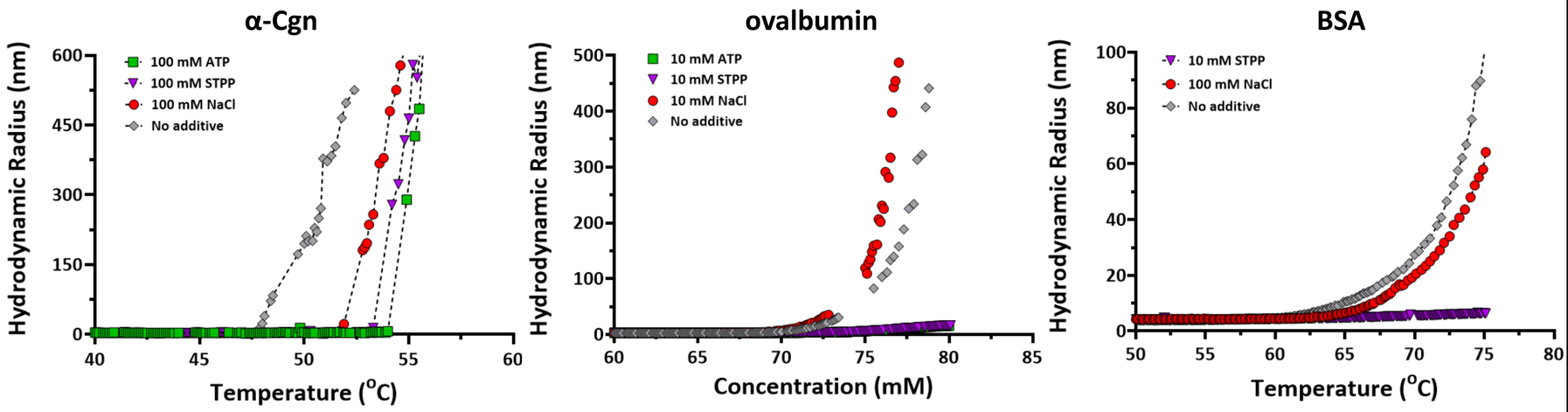


B_{22} values for α-Cgn, ovalbumin and lysozyme as a function of ionic strength for NaCl (●), STPP (▼), ATP (■) and 25 mM STPP+ NaCl (◆) in 10 mM tris buffer at pH 7.0 (pH 9.0 for lysozyme).

- Next we studied how polyvalent anions influence protein-protein interactions between α-Cgn, ovalbumin and lysozyme by determining second virial coefficients (B_{22}) by SLS.
  - Positive B_{22} values indicate protein-protein repulsion and negative B_{22} values indicate protein-protein attraction.
- B_{22} values determined for α-Cgn, ovalbumin and lysozyme suggest that STPP influences protein-protein interactions in a protein a specific manner, which is in contrast to the universal supercharging effect observed for the ζ-potentials.
- α-Cgn exhibits anisotropic patch attraction at pH values close its pI at low ionic strengths; this is highlighted by the negative B_{22} values around 10 mM ionic strength. Increasing NaCl and STPP ionic strength causes B_{22} values to increase and plateau, indicating a decrease in the strength of protein-protein attraction and screening of electrostatics.
  - In the case of α-Cgn there was no difference between the effect monovalent and polyvalent anions had on B_{22} values.
- At low ionic strengths, interactions between ovalbumin molecules are repulsive. Increasing NaCl, STPP and ATP ionic strength causes B_{22} values to decrease and plateau, which indicates an increase in protein-protein attraction, with STPP and ATP being less effective at screening protein-protein repulsion than NaCl.
- Increasing ionic strength causes lysozyme B_{22} values to decrease, indicating a decrease in lysozyme-lysozyme repulsion. Higher valency anions are more effective at reducing B_{22} values (top right).
- Decreasing STPP ionic strength causes lysozyme B_{22} values to decrease, indicating attractive interactions between lysozyme molecules, at ionic strength <200 mM STPP induce lysozyme phase-separation (bottom right).

Can Supercharging Proteins with Polyvalent Anions Increase Aggregation Resistance?

- The figures show how the hydrodynamic radii of α-Cgn, ovalbumin and BSA as measured by DLS change with increasing temperature in the presence of NaCl, STPP and ATP.
- In the case of α-Cgn, STPP and ATP increase aggregation resistance compared to NaCl but have no effect on aggregate growth rate.
- For ovalbumin and BSA, STPP and ATP were also more effective than NaCl at improving aggregation resistance but were also able to significantly reduce aggregate growth rate.
- The results from these experiments combined with ζ-potentials show that polyvalent anions such as STPP and ATP could be incorporated into formulations to suppress aggregation and improve protein solubility by supercharging the protein surface.
- However, supercharging a protein does not guarantee that the aggregate growth rate will be slowed.



The figures above show how the hydrodynamic radii of α-Cgn, ovalbumin and BSA change as a function of temperature. All proteins were tested at concentrations of 5 mg/mL in 10 mM Tris at pH 7.0. ◆ = No salt, ● = NaCl, ▼ = STPP and ■ = ATP.

Future Work

- How do SPP and STPP effect the thermal stability and native structures of proteins they interact with?
- Can polyvalent anions be used to aid protein refolding?
- Can SPP and STPP be used to influence the phase behaviour of other basic proteins and can their effects be tuned to induce other phase-behaviours such as liquid-liquid phase separation and crystallisation?

Publications

1. Specific Ion and Buffer Effects on Protein-Protein Interactions of a Monoclonal Antibody. D. Roberts, R. Keeling, M. Tracka, C.F. van der Walle, S. Uddin, J. Warwicker and R. Curtis. *Molecular Pharmaceutics*, 2015, 12, 179-193.
2. Reentrant Condensation of Proteins in Solution Induced by Multivalent Counterions. F. Zhang, M. W. A. Skoda, R. M. J. Jacobs, S. Zorn, R. A. Martin, G. F. Clark, S. Weggler, A. Hildebrandt, O. Kohlbacher and F. Schreiber. *Physical Review Letters*, 2008, 101.
3. Supercharging Proteins Can Impart Unusual Resilience. M. S. Lawrence, K. J. Phillips and D. R. Liu. *Journal of the American Chemical Society*, 2007, 129, 10110-10112.
4. ATP as a Biological Hydrotrope. A. Patel, L. Malinovska, S. Saha, J. Wang, S. Alberti, Y. Krishnan and A. A. Hymam. *Science*, 2017, 356, 753-756.