The Effect of Polyvalent anionic Excipients on Protein-Protein Interactions

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Protein-Protein Interactions

- **Understanding protein-protein interactions is important for understanding multiple processes such as**
  - **Protein aggregation**
  - **Protein phase behaviour**
  - **Protein crystallisation**
  - **Protein purification**
- **Protein-protein interactions can be modulated by the addition of a single solute or a mixture of solutes including salts, osmolytes and amino acids.**
- **The mechanism by which solutes interact with proteins and influence protein-protein interactions is not fully understood.**
- **Understanding of how protein-solute interactions modulate protein-protein interactions could provide researchers with a toolset on how to design better formulations to limit protein aggregation and develop novel compounds.**

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Research Aims

- **To determine how ions with different charge states influence protein-protein interactions by measuring B_{22} and k_D values.**
- **Can polyvalent anions increase protein-protein repulsion and protein solubility by inverting the net charge of the protein charge or overcharging the protein surface?**
- **Can polyvalent anions be used to increase protein resistance to aggregation?**

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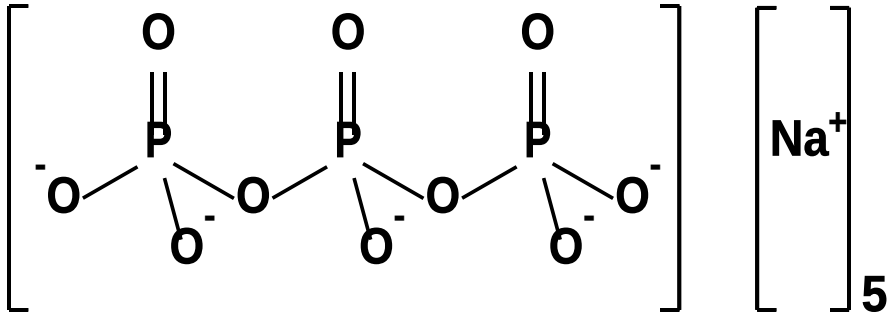
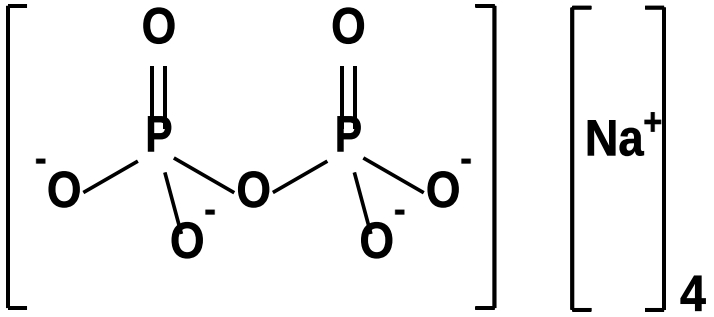
Polyvalent Anions

Sodium Tripolyphosphate (STPP)



pKa_1: 1 pKa_2: 2.2 pKa_3: 2.3 pKa_4: 5.7 pKa_5: 8.5

Sodium Pyrophosphate (SPP)



pKa_1: 0.91 pKa_2: 2.1 pKa_3: 6.7 pKa_4: 9.32

- **The results in this study suggest that STPP and SPP could be used as an alternative salts to reduce the strength of protein-protein interactions.**
- **Both STPP and SPP are "Generally recognised as safe" by the FDA and already found in a number of cosmetic and food products.**

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Reentrant Condensation

- Both STPP and SPP were found to influence lysozyme phase behaviour.
- This behaviour has been termed reentrant condensation and previously observed for polyvalent cations and acidic proteins
- Lysozyme-buffer solutions are prepared and the polyvalent anion is added to the protein-buffer solution to give the desired protein and anion concentrations.
- Solutions are allowed to equilibrate for 1 hour.
- Samples are then centrifuged at 10,000 rpm for 2 minutes, allowed to equilibrate for 1 hour and centrifuged again.
- The supernatant is removed and its 280 nm absorbance measured.

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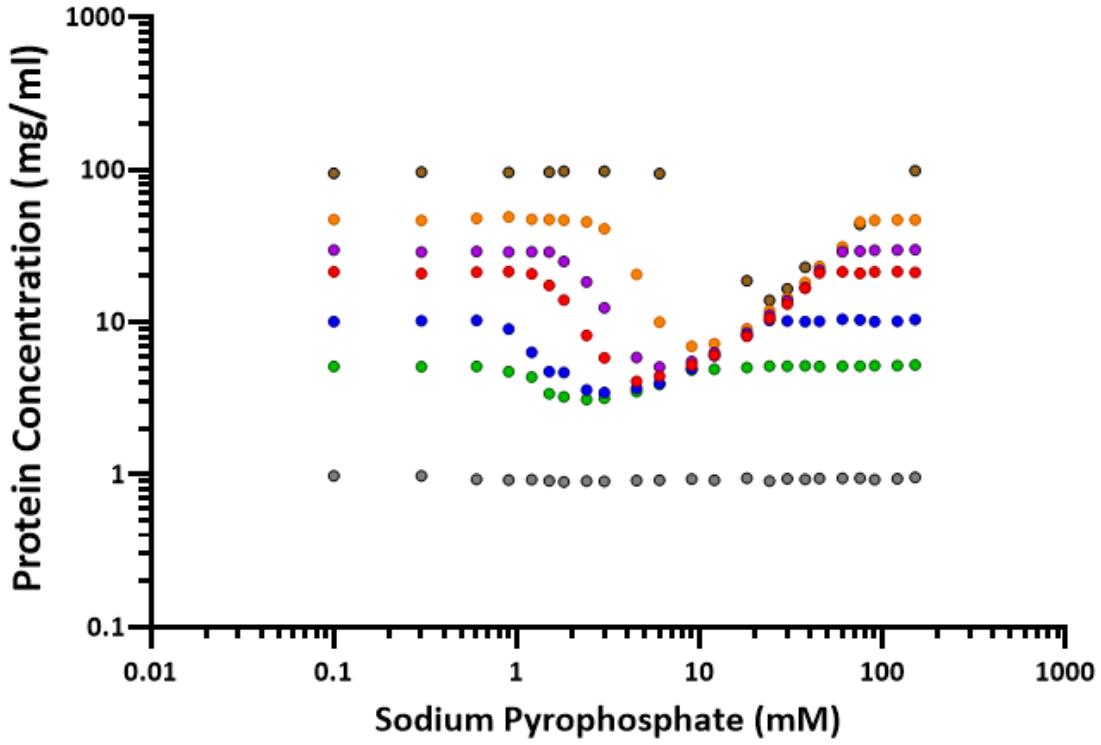
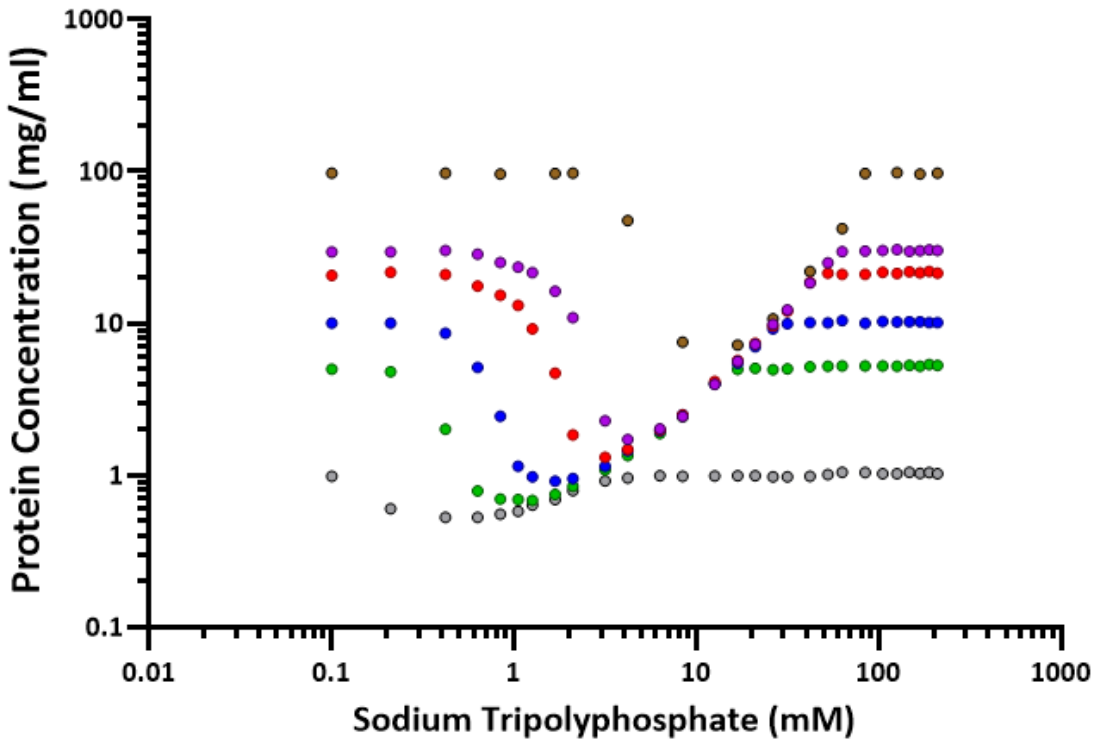
Reentrant Condensation Experiments

- **Precipitation experiments showing how lysozyme solubility changes in the presence of increasing SPP and STPP concentration in 10 mM tris at pH 9.0.**

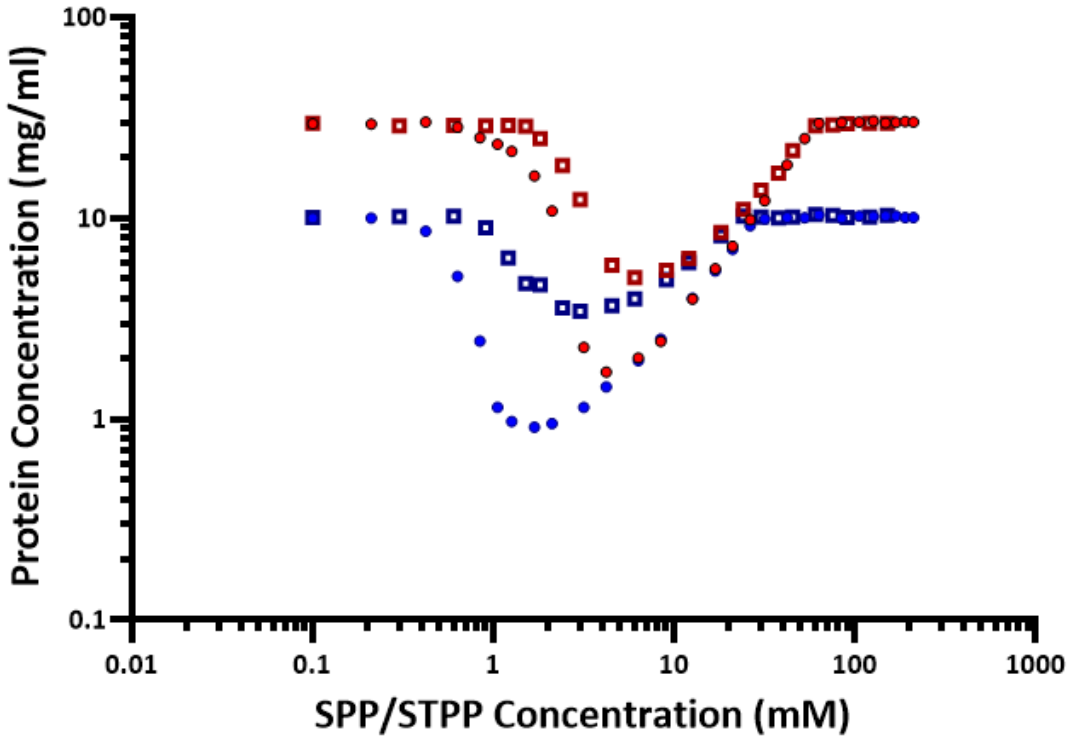


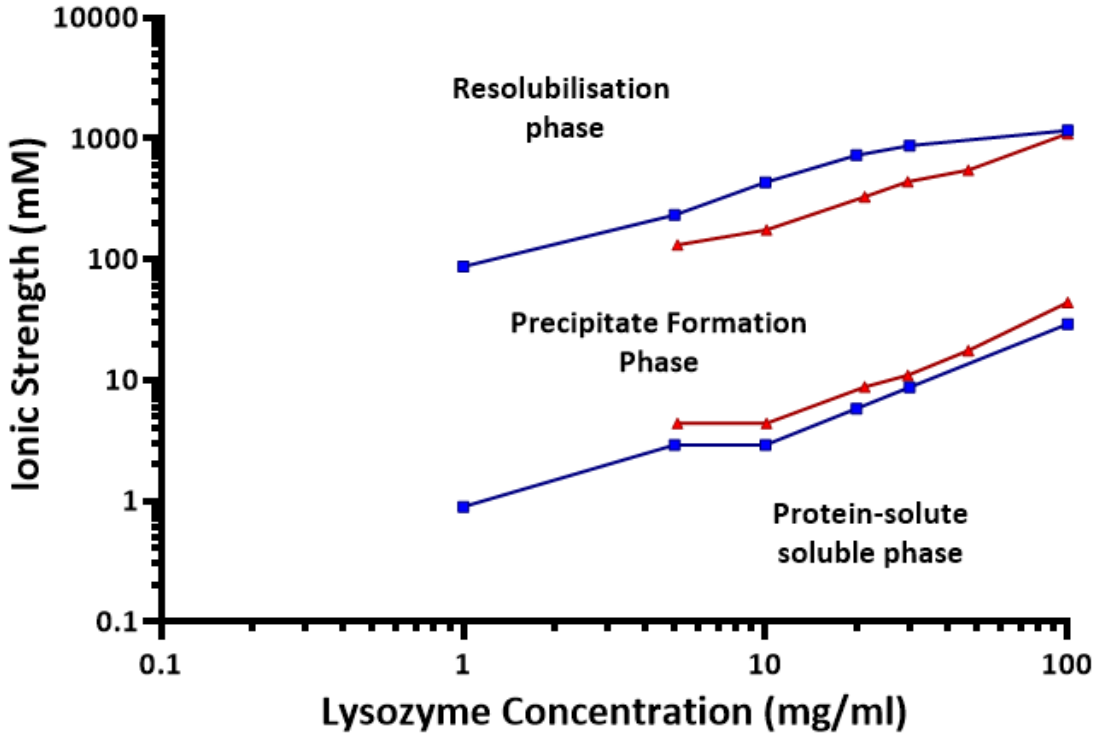


| = 1 mg/ml, | = 5 mg/ml, | = 10 mg/ml, | = 20 mg/ml, | = 30 mg/ml, | = 50 mg/ml, | = 100 mg/ml

Reentrant Condensation Experiments

- Tripolyphosphate was more effective at precipitating lysozyme than pyrophosphate at all protein concentrations.
- A phase diagram can be generated by plotting the concentrations at which precipitation and resolubilisation occur.





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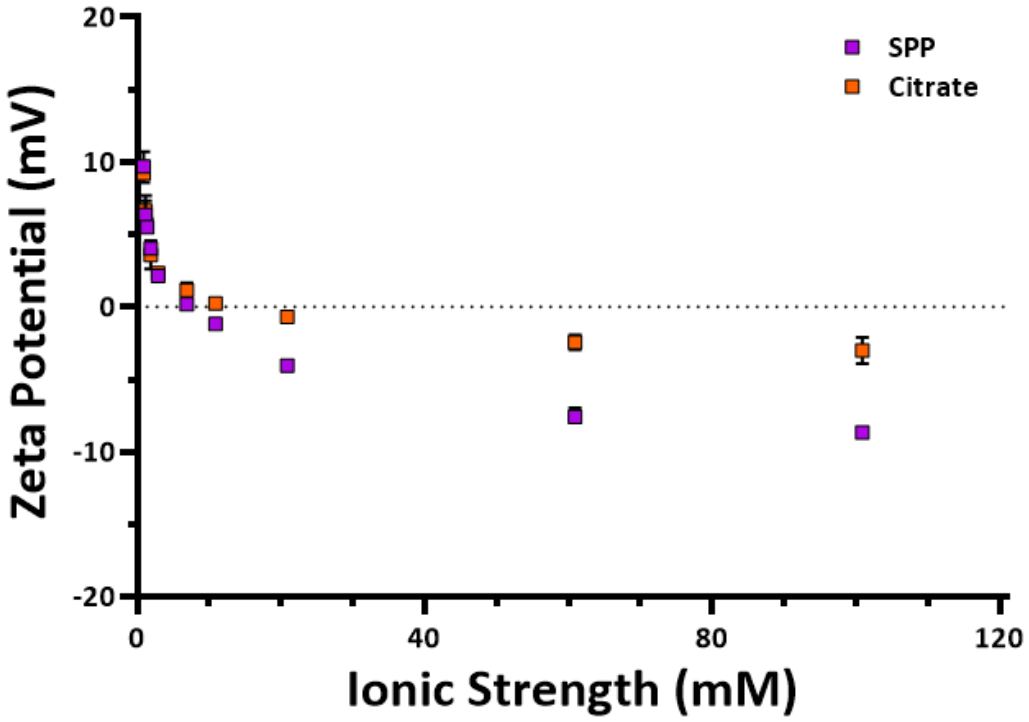
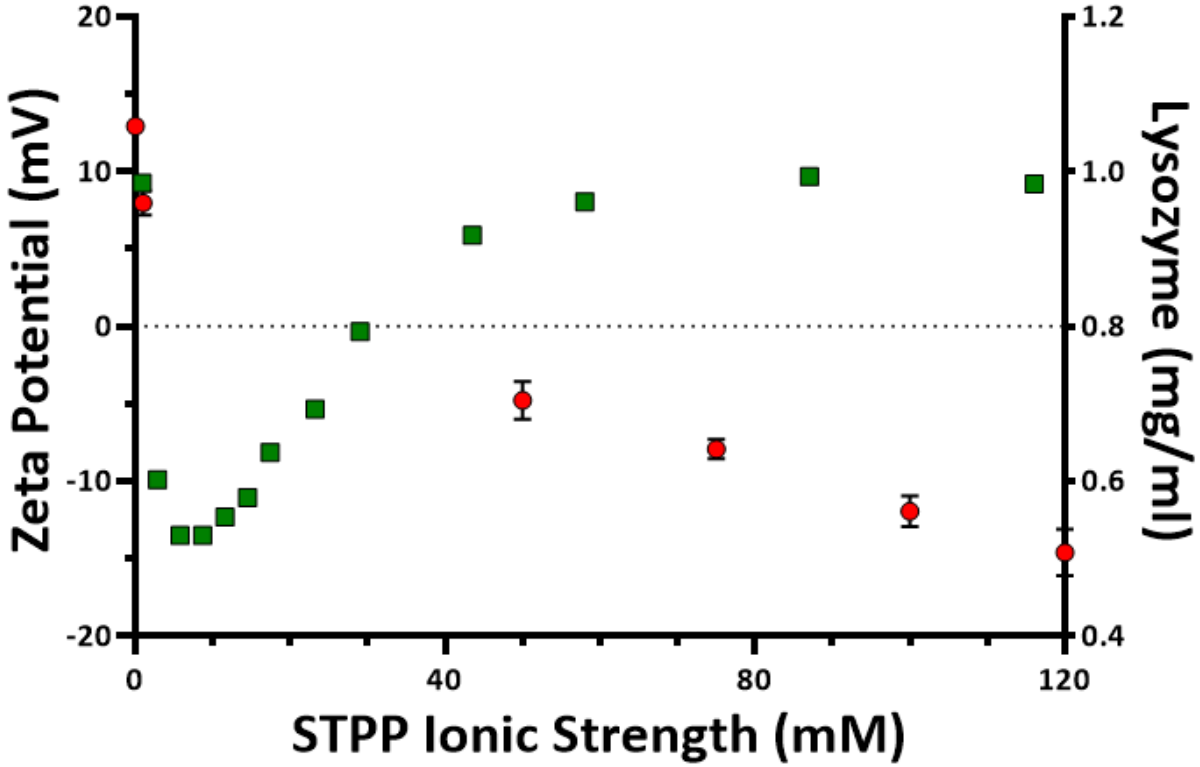
Zeta Potential Measurements

- **Zeta potential measurements of 1 mg/ml lysozyme with 0-300 mM citrate (orange) and SPP (purple) at pH 9 show that the charge of lysozyme inverts as the ionic strength of citrate and SPP is increased.**

- **It should be noted that SPP does not cause lysozyme precipitation at low lysozyme concentrations (≤ 1 mg/ml) and citrate does NOT cause lysozyme precipitation at any concentration even though it causes charge inversion.**





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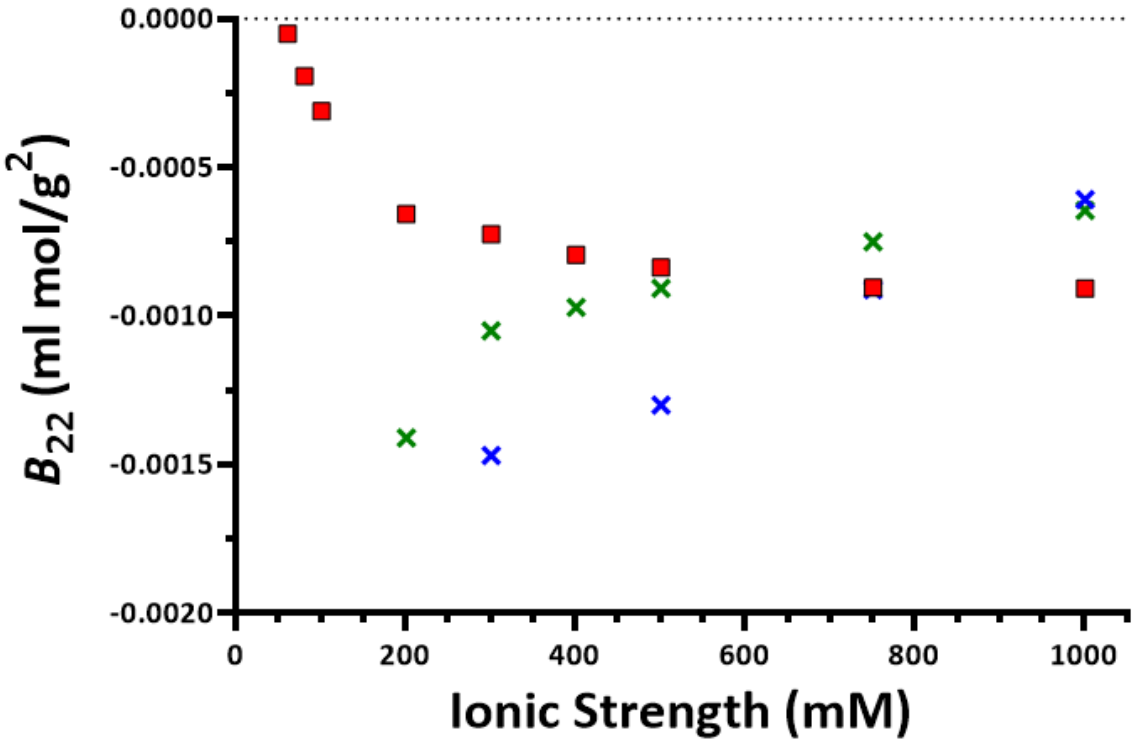
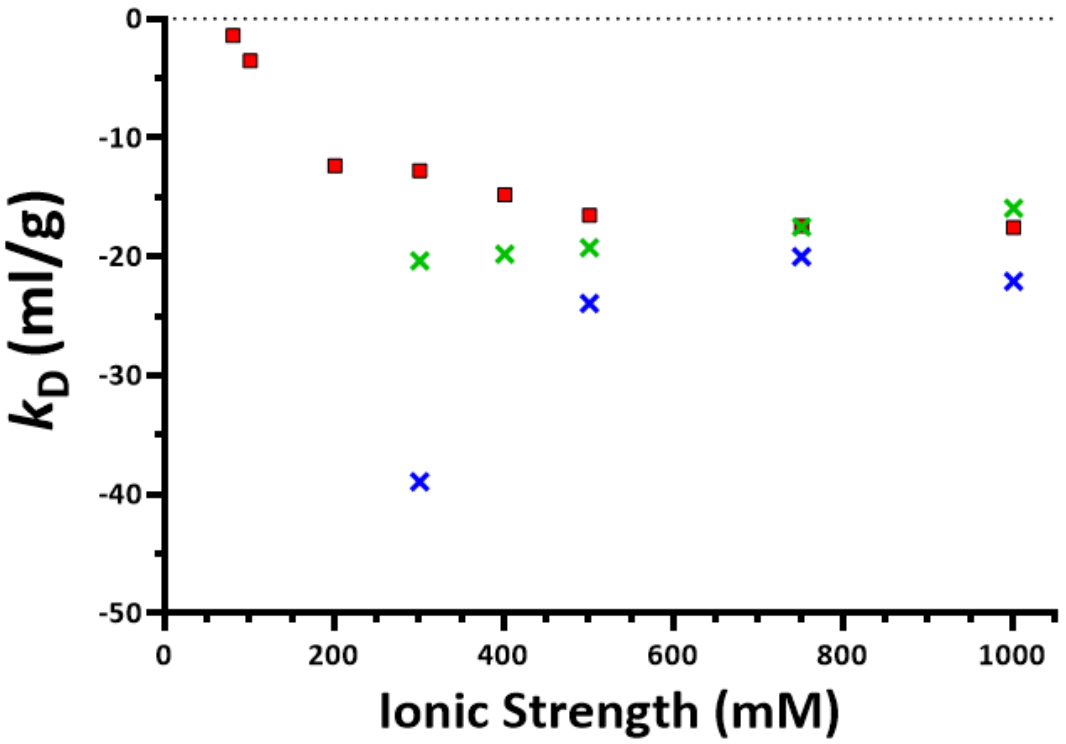
Effect of Salts on Lysozyme B_{22} and k_D Values

- B_{22} and k_D values could be determined in the soluble lysozyme:STPP/SPP fractions.
- B_{22} and k_D values did not show that the charge of the positive lysozyme molecules had been inverted.





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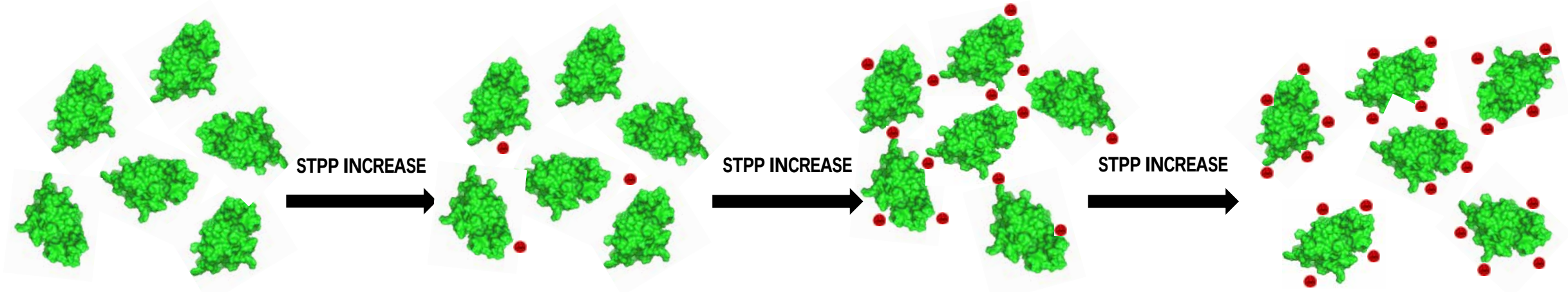
Possible Reentrant Condensation Mechanism



Lysozyme is soluble when no STPP/SPP is present

Lysozyme solubility reduces slightly when STPP/SPP concentration is increased

Lysozyme precipitates due to STPP/SPP electrostatically crosslinking proteins

Lysozyme charge is inverted and resolubilises occurs due to electrostatic repulsion

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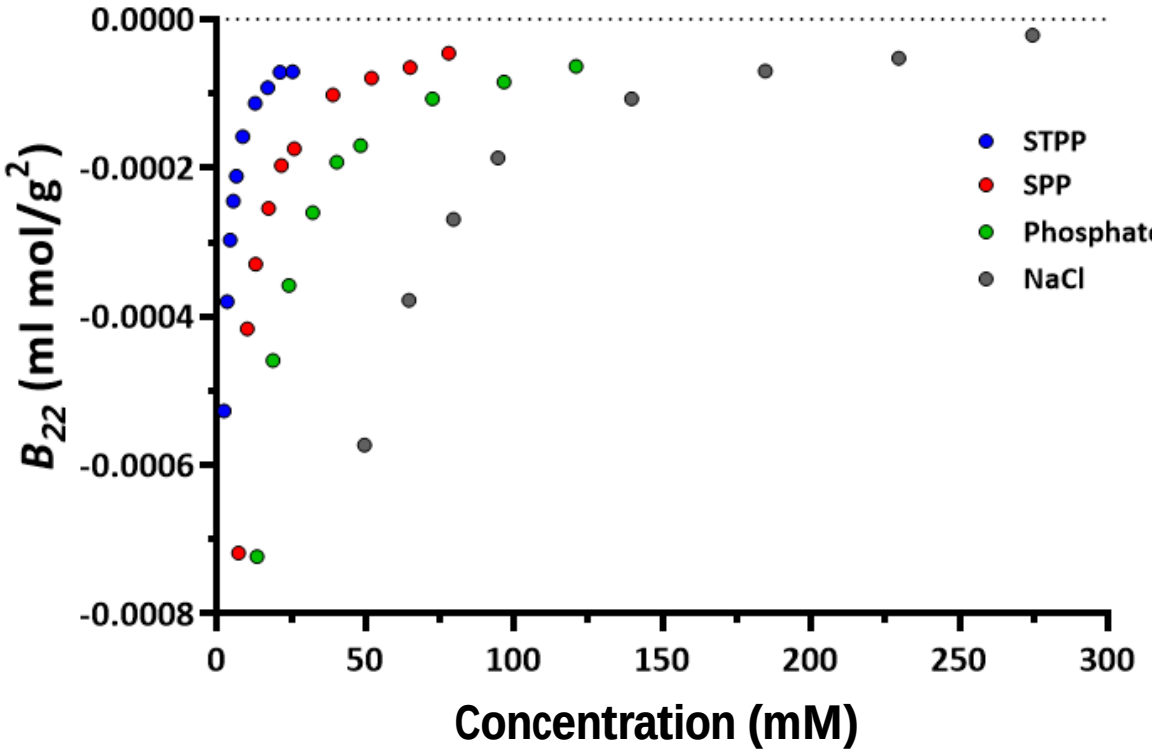
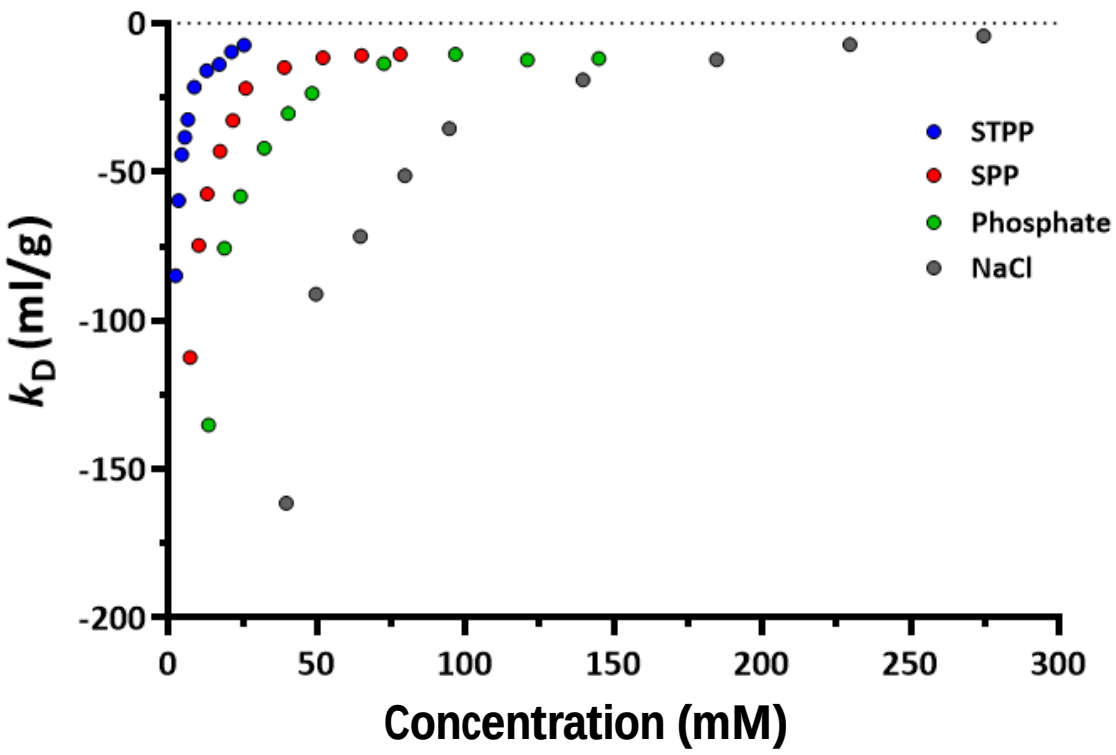
mAb1 B_{22} and k_D Values

- B_{22} and k_D values for mAb1 at different concentrations of four ions were determined in 10 mM Tris at pH 8.
- Anions with greater net charges such as the polyvalent anions STPP and SPP are better at preventing protein-protein interactions than chloride and phosphate.





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First Summary

- **The ability of anions with different charge states to modulate protein-protein interactions by SLS, DLS, zeta potentials and protein solubility studies have been measured.**

- **SLS and DLS measurements showed that anions decrease electrostatic repulsion between positively charged lysozyme molecules.**

    - **SLS and DLS measurements were unable to detect charge inversion.**
    - **Zeta potential measurements were able to detect charge inversion occurring when lysozyme was in the presence of increasing STPP concentrations.**

- **Polyvalent anions were more effective at reducing protein-protein attractive interactions between mAbs than monovalent ions.**

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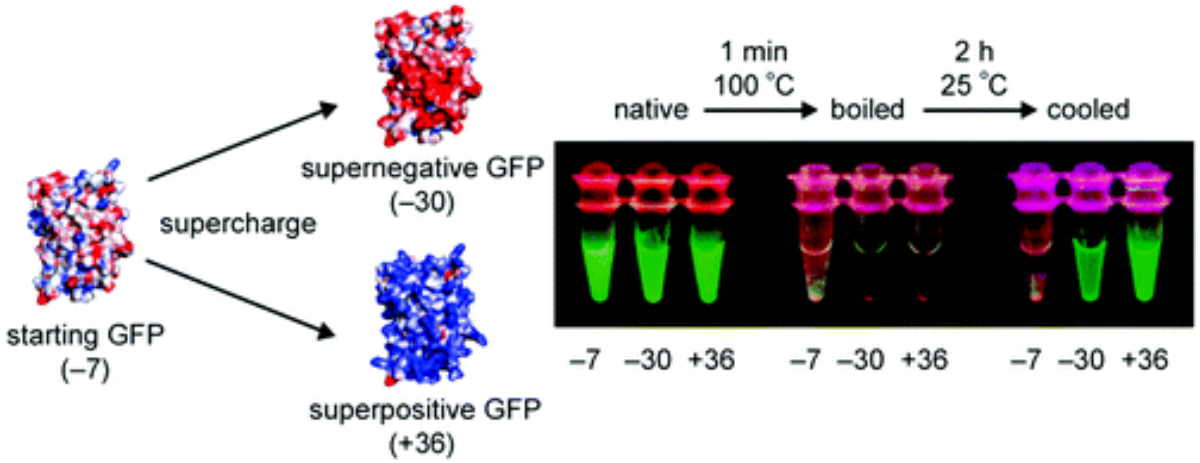
Supercharged Proteins

- Supercharged proteins are developed through extensive mutagenesis of solvent exposed residues to acidic and basic residues.

- Supercharged variants of carbonic anhydrase, green fluorescent protein (GFP) and streptavidin have been developed in which their activity, structure and stability have been conserved.



- All supercharged variants of the proteins were more resistant to aggregation and a greater percentage of protein refolded upon cooling compared to the wildtype.

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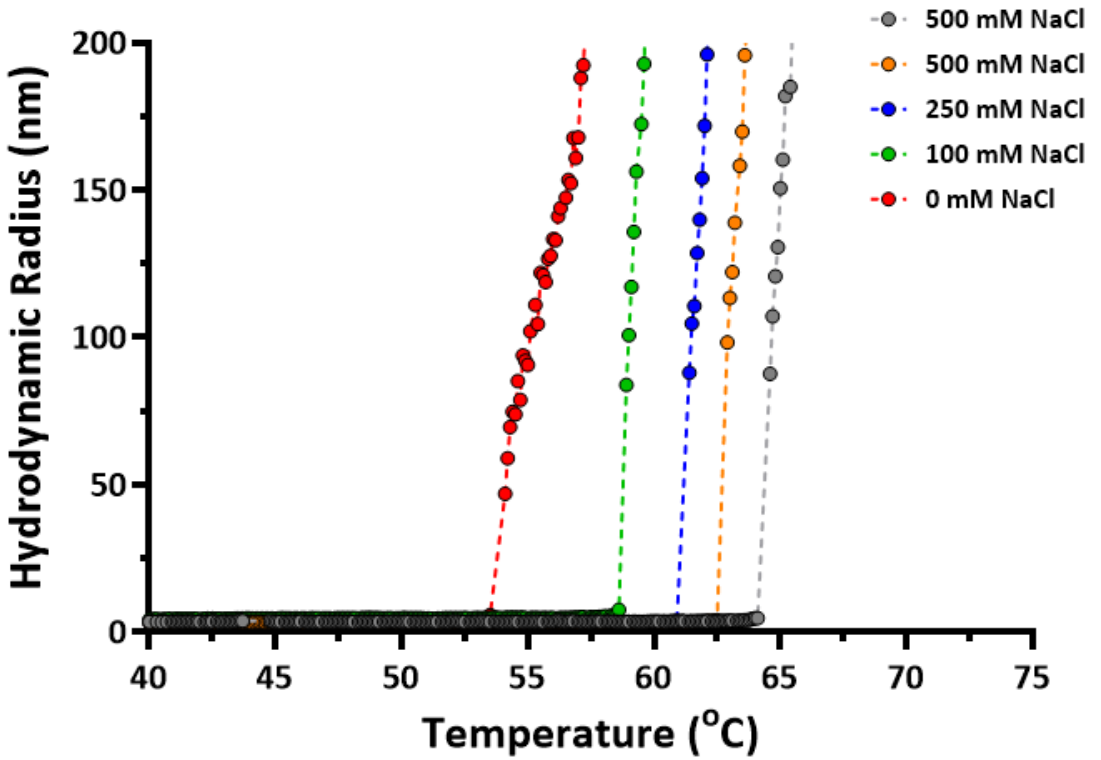
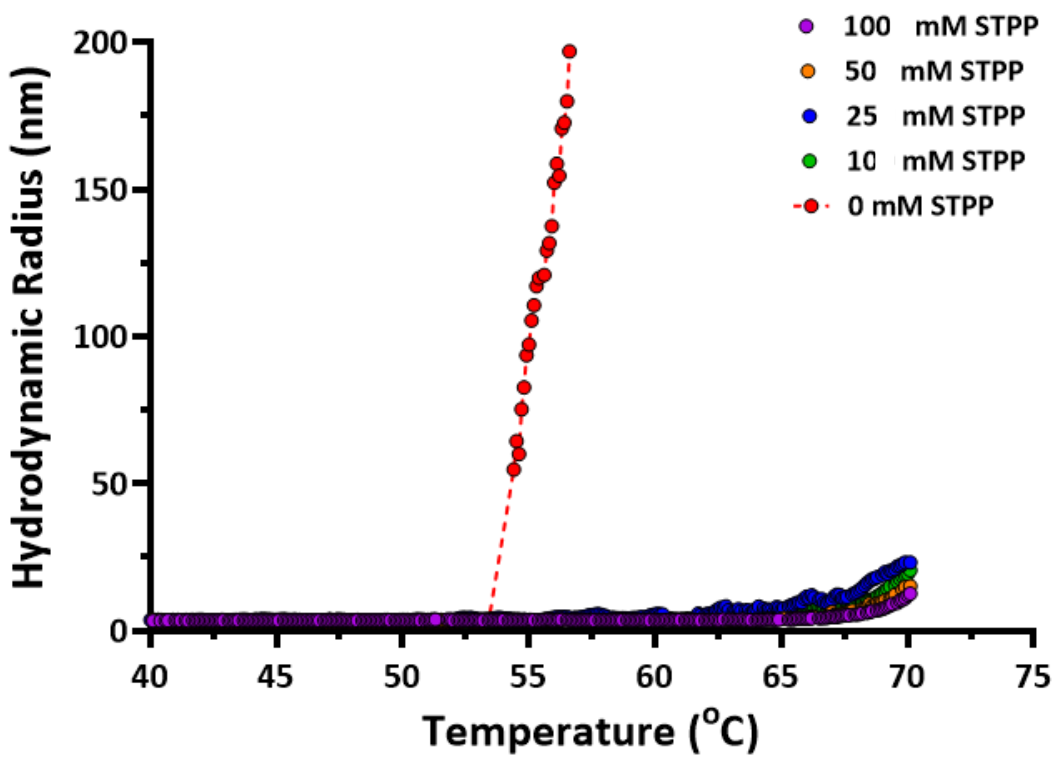
Effect of NaCl and STPP on Ovalbumin Aggregation

- Thermal ramp experiments were used to determine onset of aggregation temperature (T_{agg}) in the presence of NaCl and STPP at different concentrations.
- DLS was used to track the hydrodynamic radius of proteins as temperature is increased.





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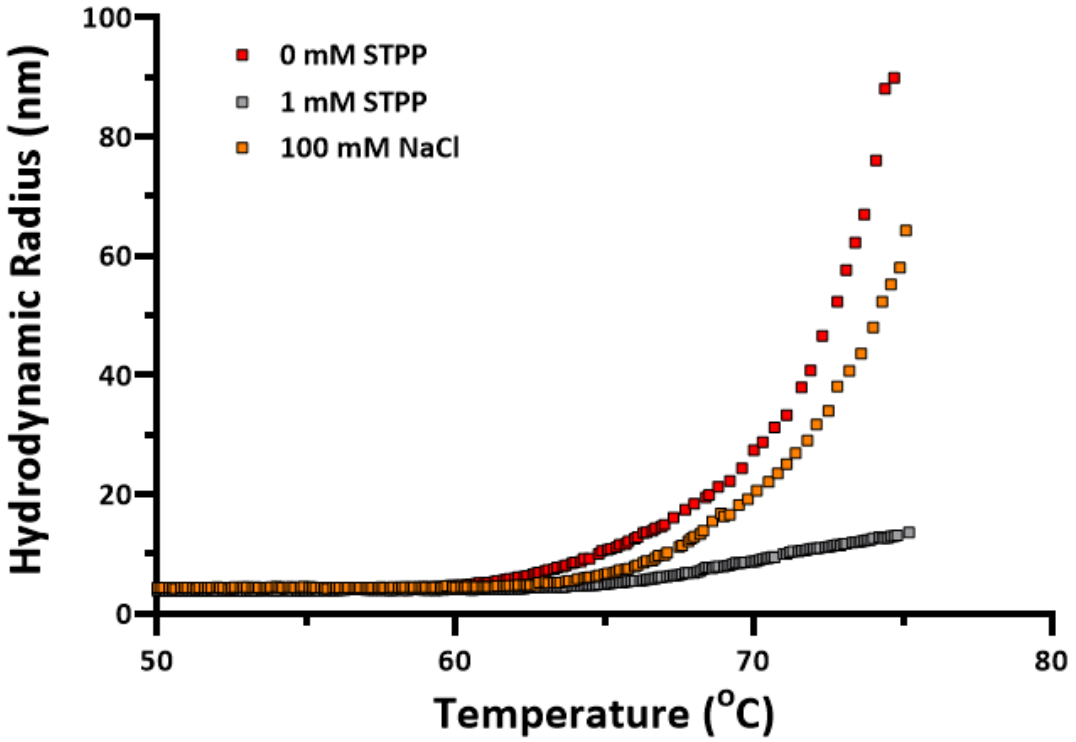
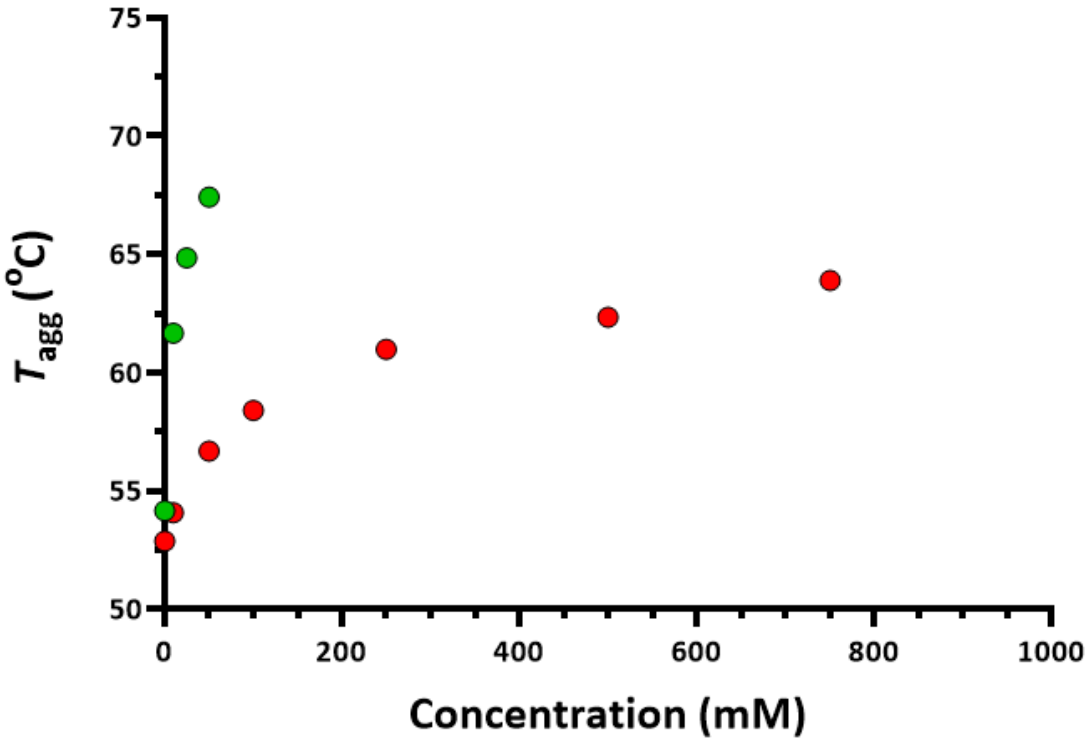
Effect of NaCl and STPP on Ovalbumin Aggregation

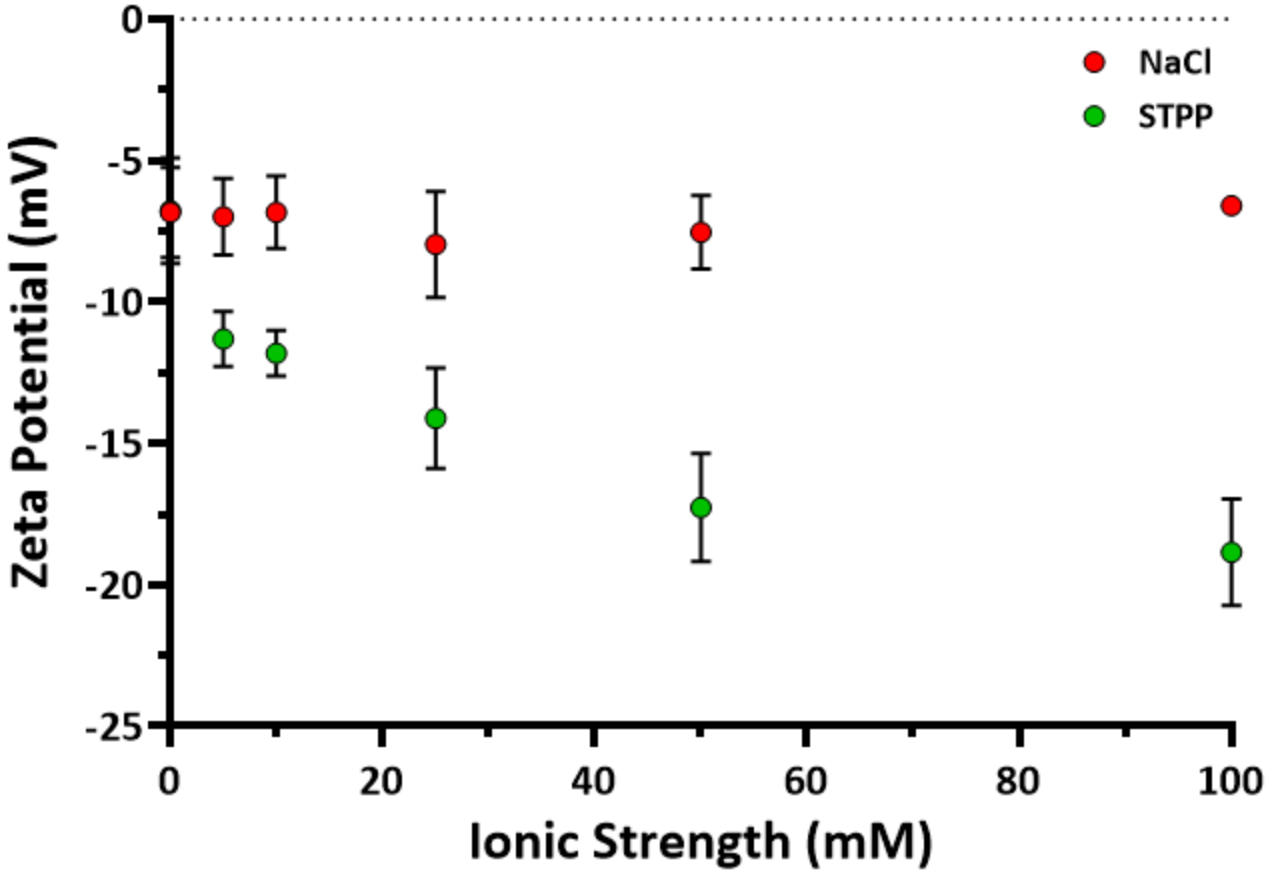
- These graphs show how T_{agg} temperatures change for ovalbumin in the presence of different concentrations of NaCl and STPP.
- Notice the how much more effective STPP is than NaCl at increasing T_{agg} when it is plotted as molar concentration.





Ovalbumin Zeta Potentials

- **Zeta potential measurements of 5 mg/ml ovalbumin with 0-100 mM NaCl and STPP at pH 7 show that STPP overcharges ovalbumin whereas NaCl has little effect on net charge.**



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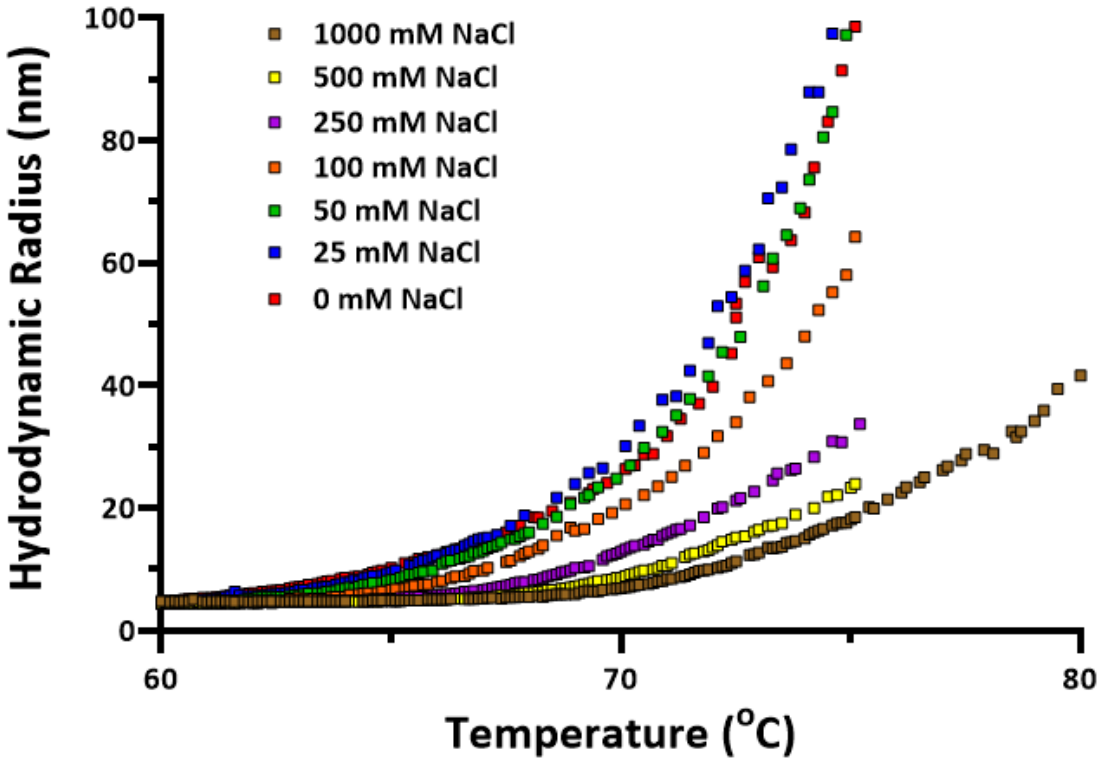
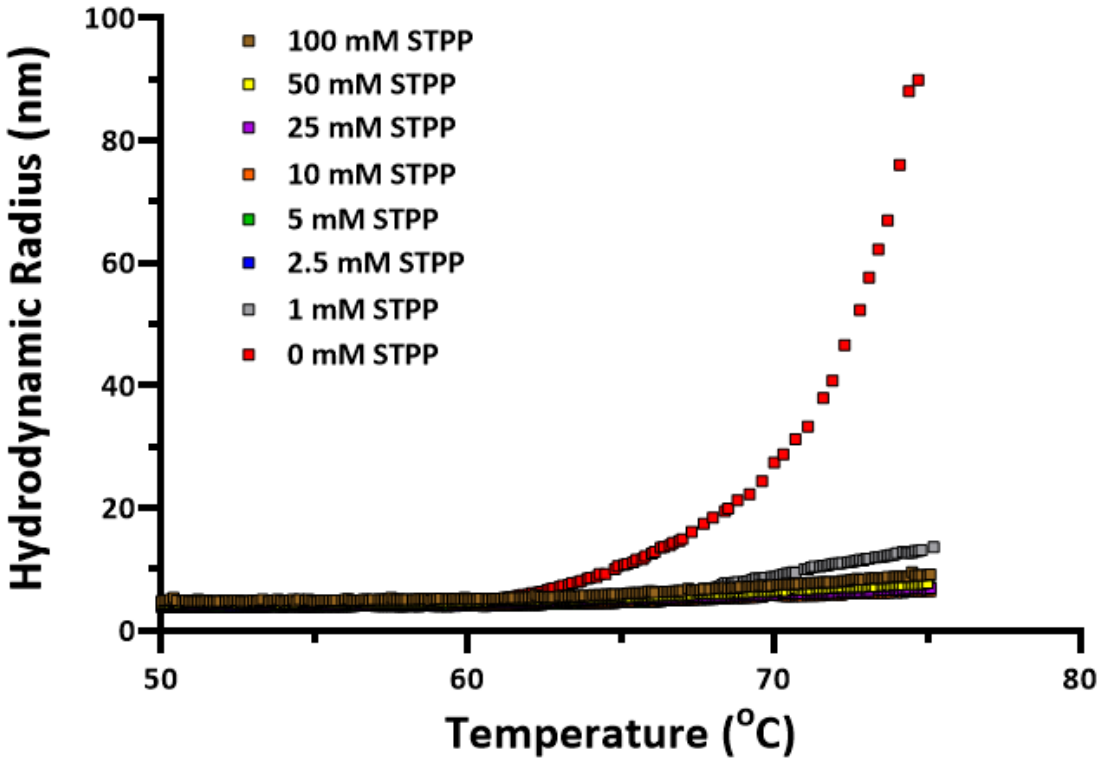
Effect of NaCl and STPP on BSA Aggregation

NaCl



STPP



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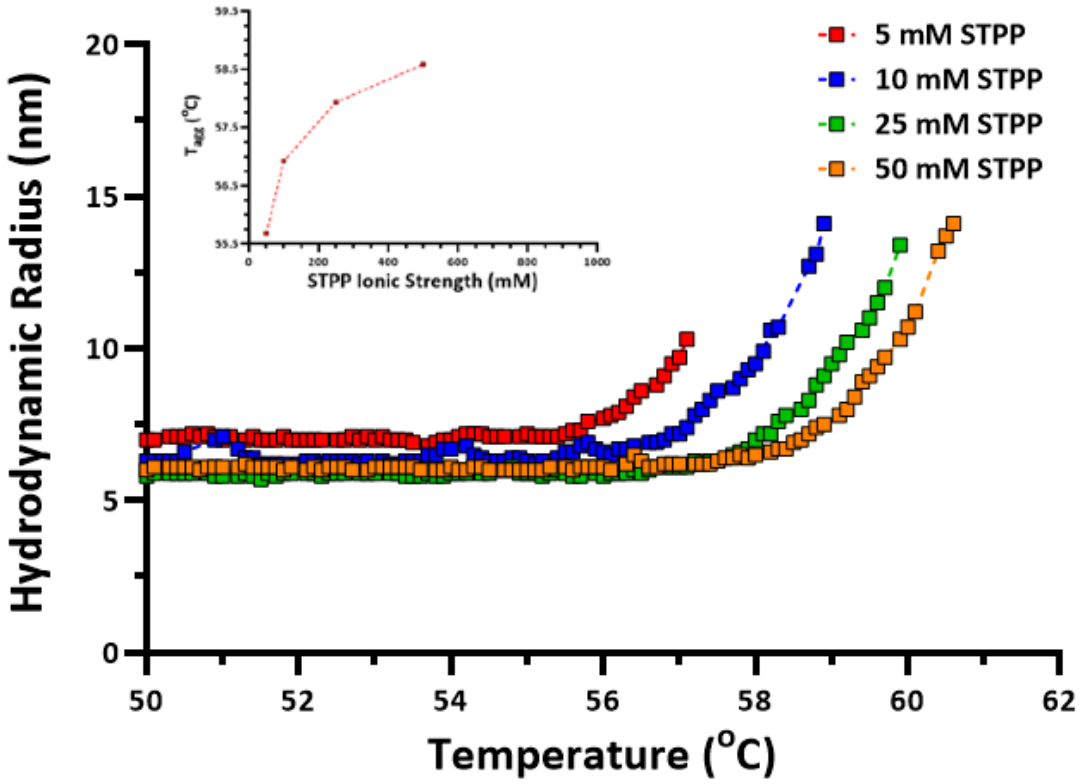
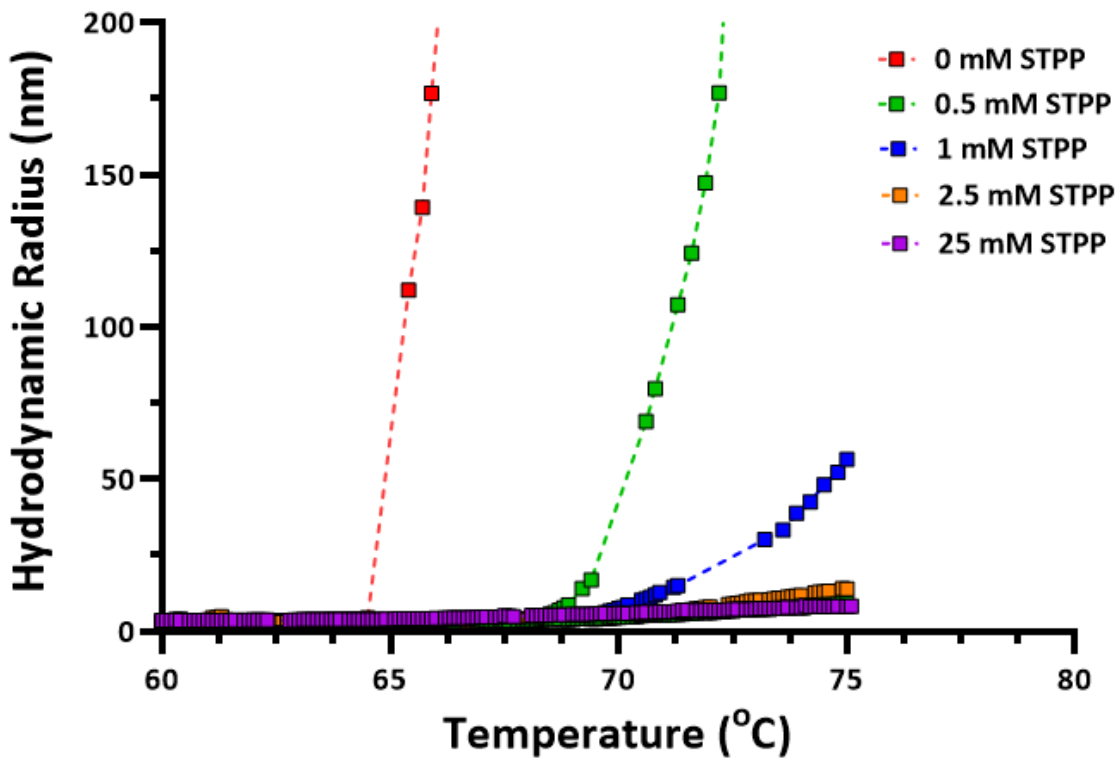
Effect of STPP on mAb1 and HSA Aggregation

mAb1



HSA



Second Summary

- **The polyvalent anion STPP (at much lower concentrations) has a larger effect on increasing BSA and ovalbumin resistance to aggregation compared to NaCl .**
- **STPP also increased HSA and mAb1 resistance to aggregation. However, the effect NaCl has on these proteins needs to be investigated.**
- **STPP appears to be more effective at reducing aggregate growth rate for proteins that are already negatively charged.**

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Future Work

- **Conduct more studies with the polyvalent anions and proteins/mAbs – is this effect universal?**
- **Can the polyvalent anions be used to phase-separate mAbs so that they can stored as a solid/gel to improve their stability?**
  - **Can you tune protein phase behavior with the polyvalent anions to make them liquid-liquid phase separate and store them in a stable form.**

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Acknowledgments

- **Dr Robin Curtis**
- **Kiah Murray**
- **Everyone in the Curtis group**
- **EPSRC for providing project funding**

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mAb 1 B_{22} and k_D Values

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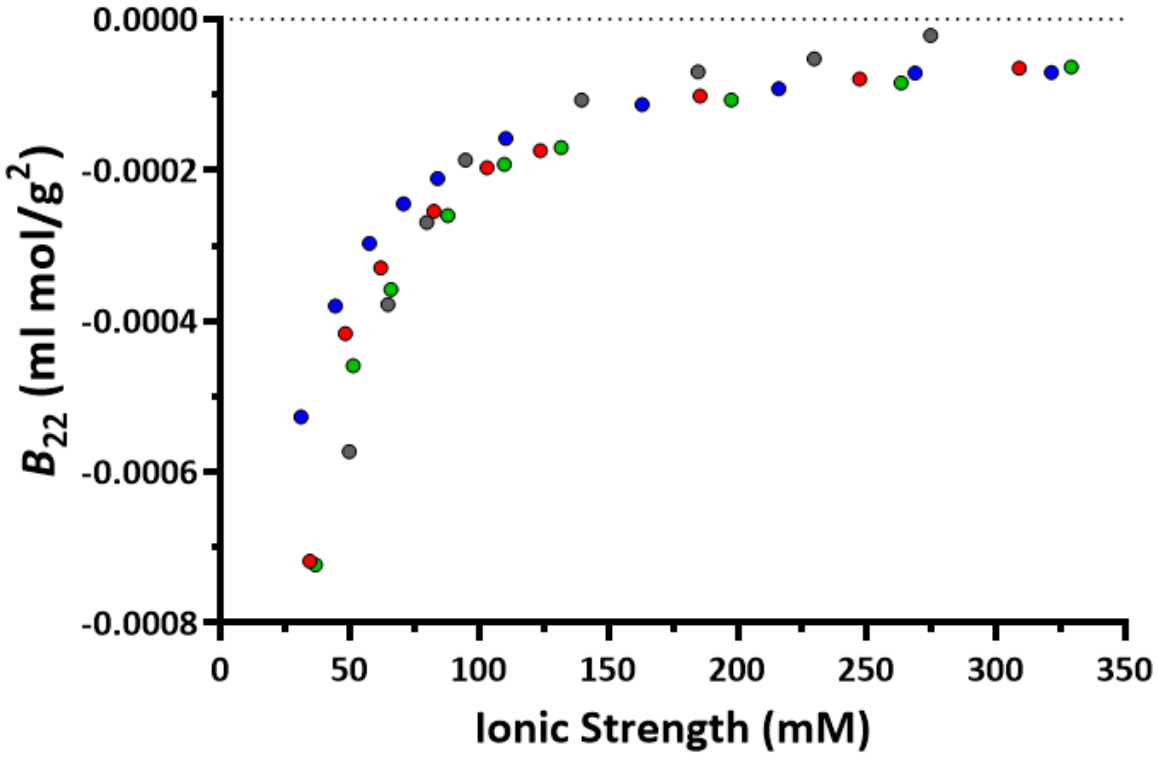

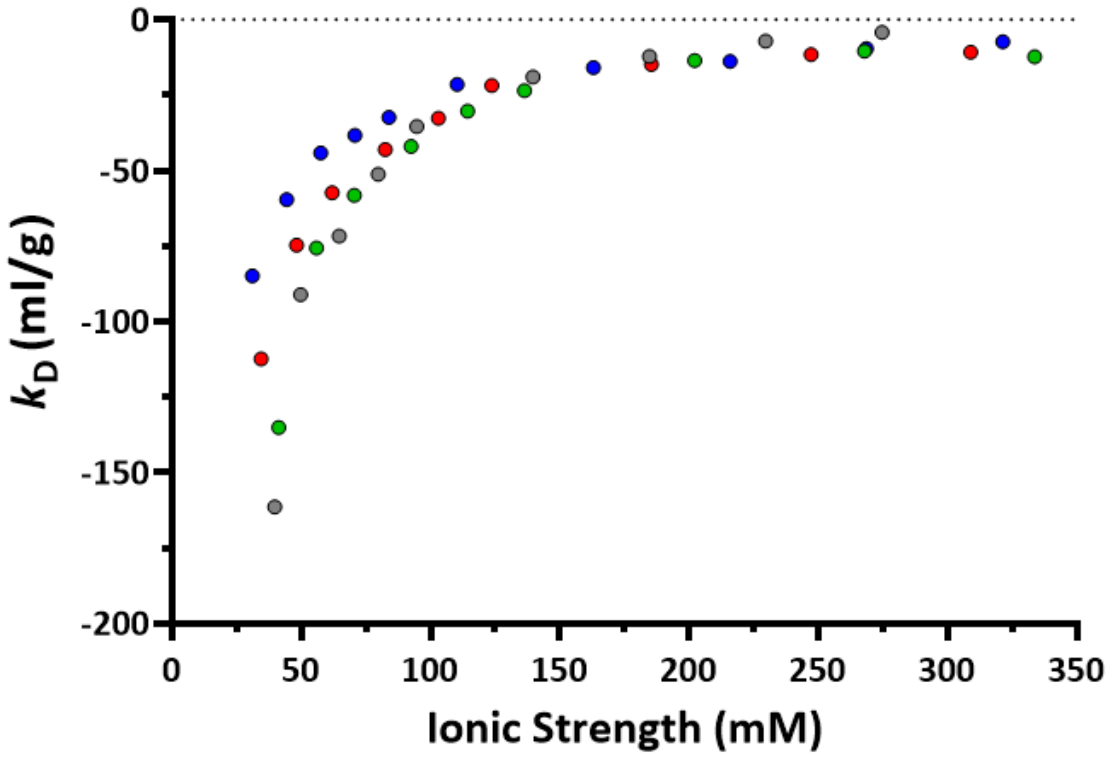

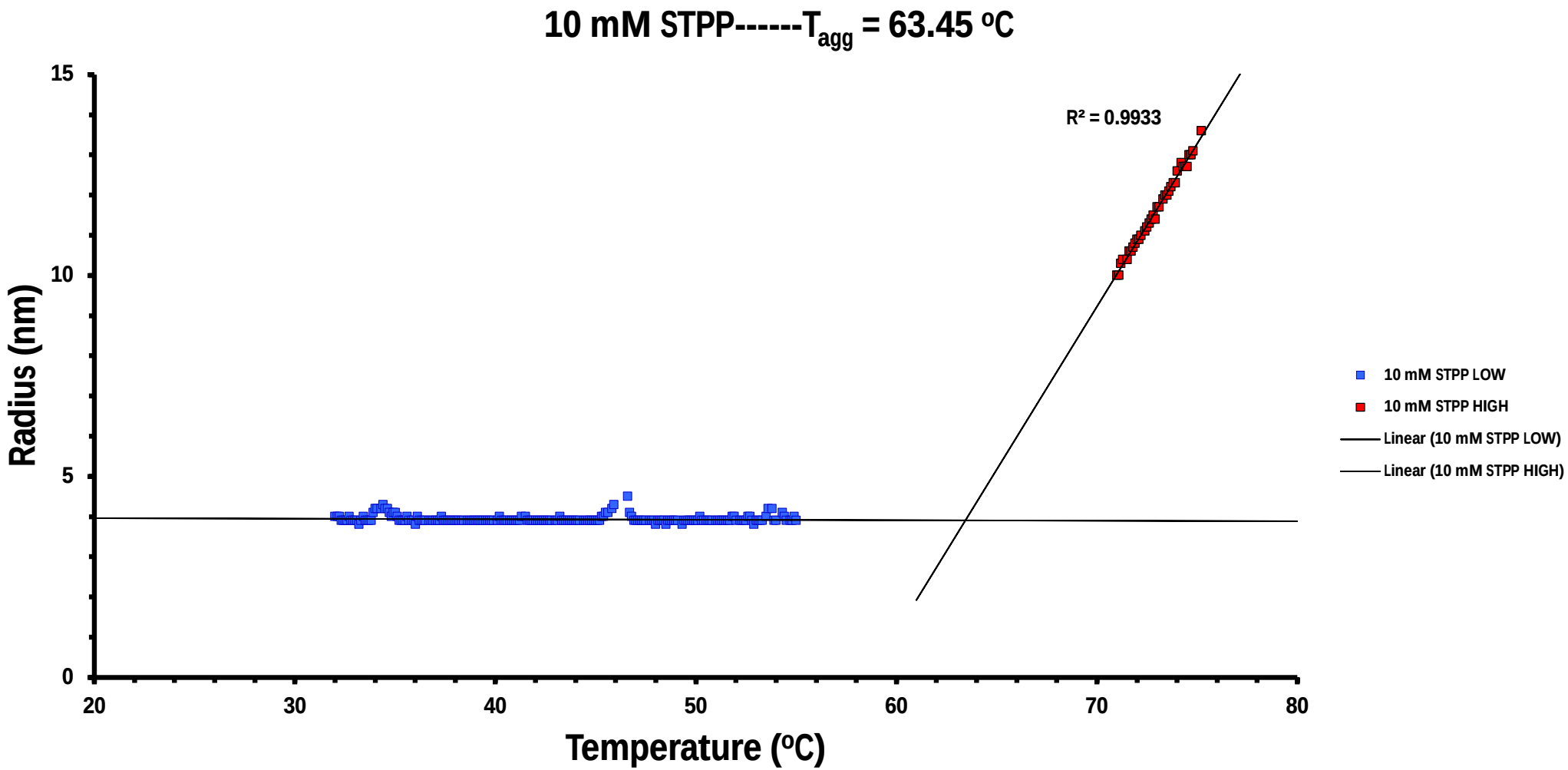
10 mM STPP------T_{agg} = 63.45 ºC
R² = 0.9933
Radius (nm)
15
10
5
0
20
30
40
50
60
70
80
Temperature (ºC)
10 mM STPP LOW
10 mM STPP HIGH
Linear (10 mM STPP LOW)
Linear (10 mM STPP HIGH)

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What is the Second Virial Coefficient (B_{22}) and Interaction Parameter (k_D)?

- The second virial coefficient (B_{22}) provides a direct measure of protein-protein interactions and are determined by SLS measurements.
- The interaction parameter (k_D) values are determined by DLS measurement and provide equivalent information to B_{22} values.