Calcium phosphate nanocluster formation by phosphorylated proteins

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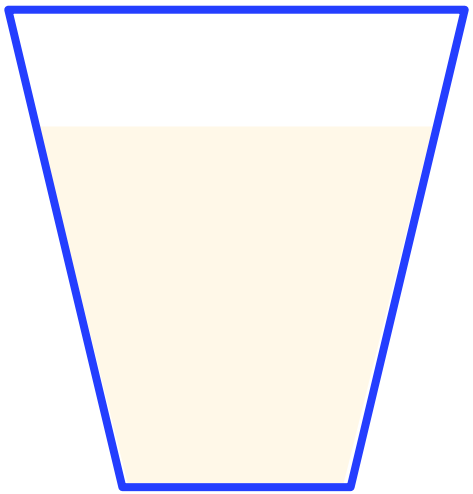
Neutrons for life sciences - Milk

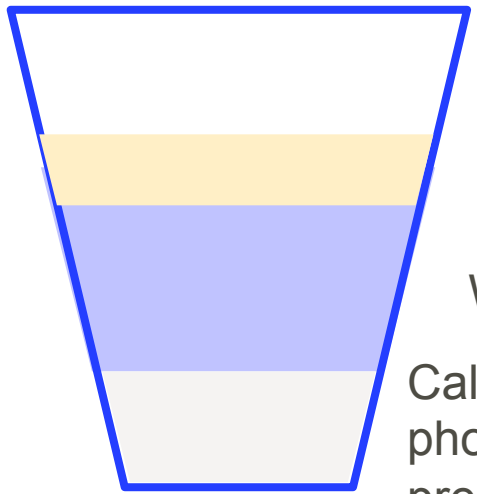
Milk – A colloidal and food chemists heaven



Stable milk

Milk is highly structured

- Milk fat globule – disperse the fat
- Casein micelle – stabilize the calcium phosphate
- Whey – solubilize free fatty acids and provide protein nutrition



Milk prepared by mixing the chemicals

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Much what we know about milk has is due to data from neutron and x-ray facilities

Milk gives strong legs!?!

The casein micelle keeps the high content of calcium phosphate dispersed in solution

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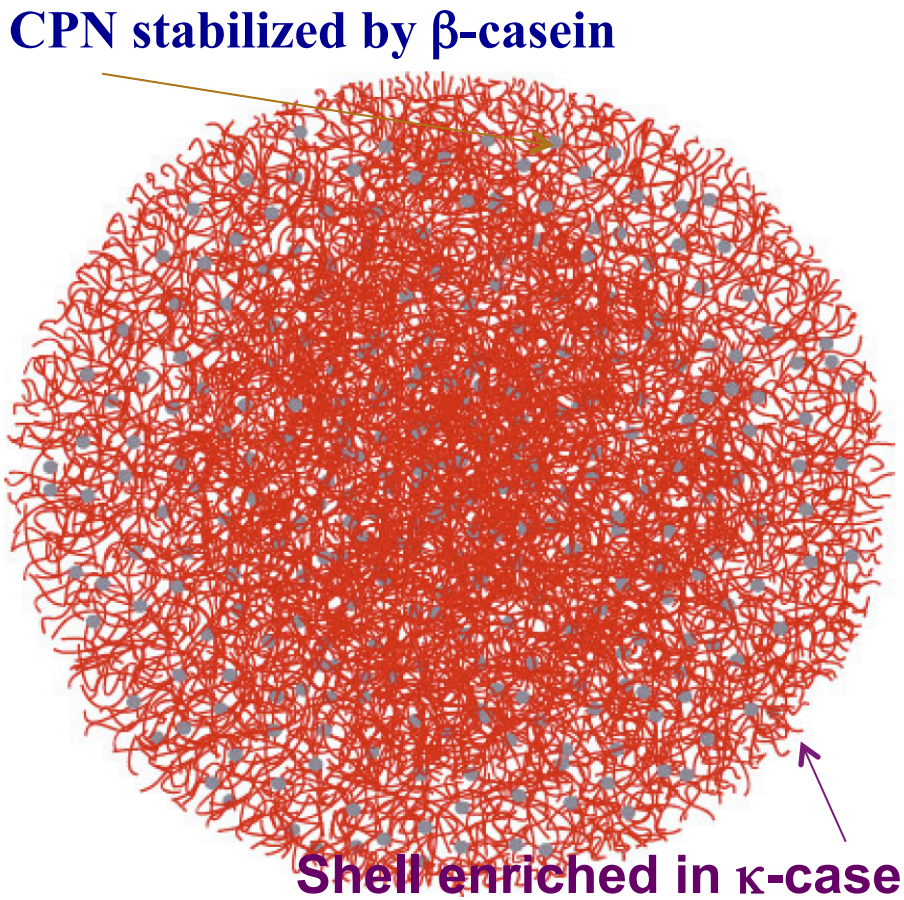
The “Holt” casein micelle model



- A micelle with radius of 100 nm comprises about 15000 polypeptide chains and about 800 calcium phosphate nano clusters (CPN).
- 75% of the micelle volume is water
- Protein matrix has an uneven peptide segment distribution

Kruif CGd & Holt C (2003) Casein Micelle structure, functions and interactions. In Advanced Dairy Chemistry (Fox PF & McSweeney PLH, eds), pp. 675-698. Kluwer Academic/Plenum, New York.

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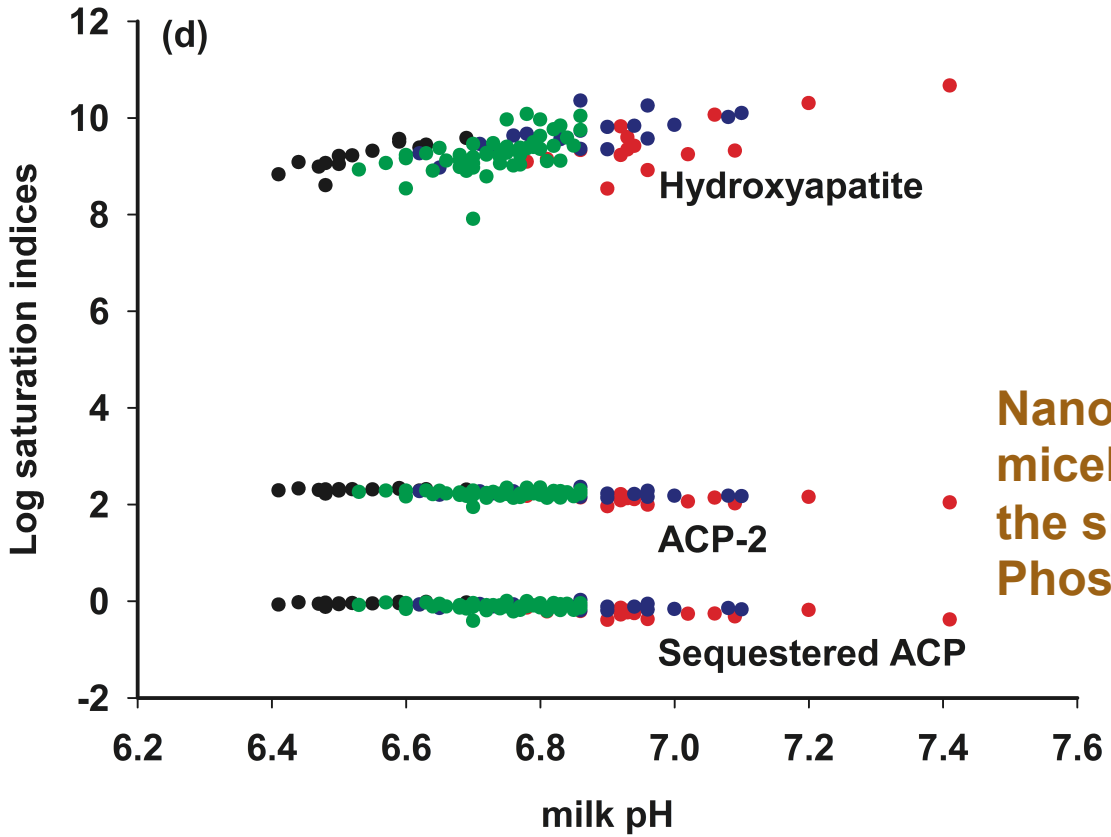
Saturation indices (*S*)

- Saturation indices (*S*) were obtained by dividing the ion activity product by the corresponding thermodynamic solubility constant
- Undersaturated solutions: $S < 1$
- Saturated solutions: $S = 1$
- Supersaturated solutions: $S > 1$

C. Holt, S. Lenton, T. Nylander, E. S. Sørensen, S. C.M. Teixeira: Mineralisation of soft and hard tissues and the stability of biofluids. Journal of Structural Biology, 2014, 185, 383-396

Milk contains more Ca Phosphate than can be solubilized, i.e the solution should be supersaturated



Nanocluster model of casein micelle formation take care of the supersaturated Ca Phosphate in milk

ACP = Amorphous Calcium Phosphate

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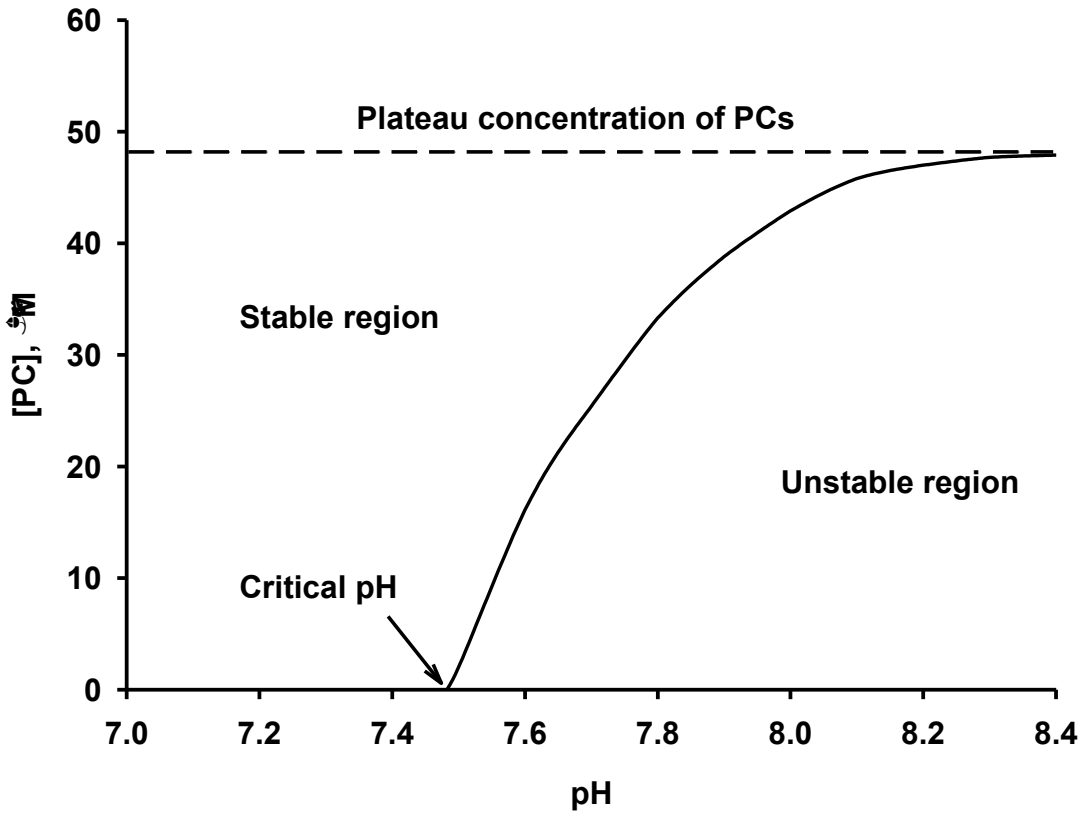
Stability diagram for a solution of salts and peptides containing a phosphate centre



Strongly dependent on pH and concentration of phosphate centers

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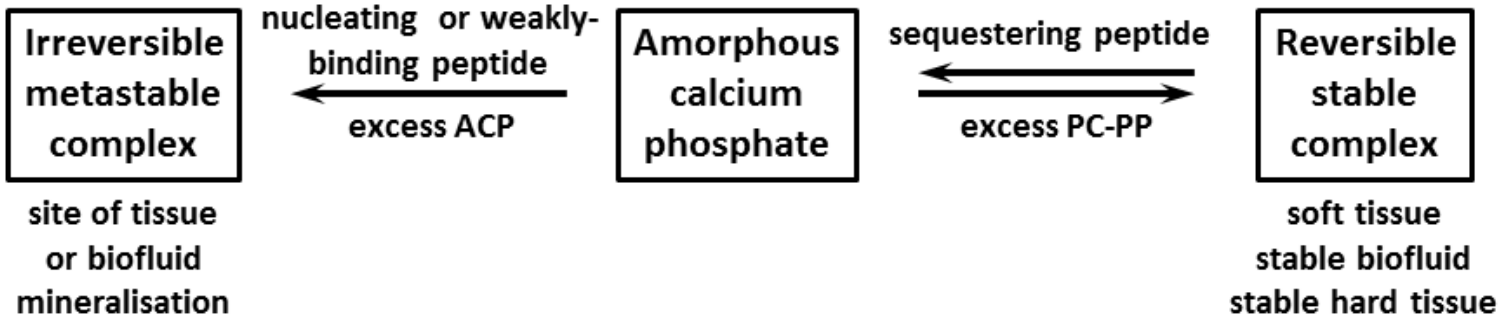
SEQUESTRATION OF AMORPHOUS CALCIUM PHOSPHATE BY PEPTIDES OR PROTEINS TO FORM NANOCLUSTER COMPLEXES

GENERAL IMPORTANCE IN THE CONTROL OF PHYSIOLOGICAL CALCIFICATION



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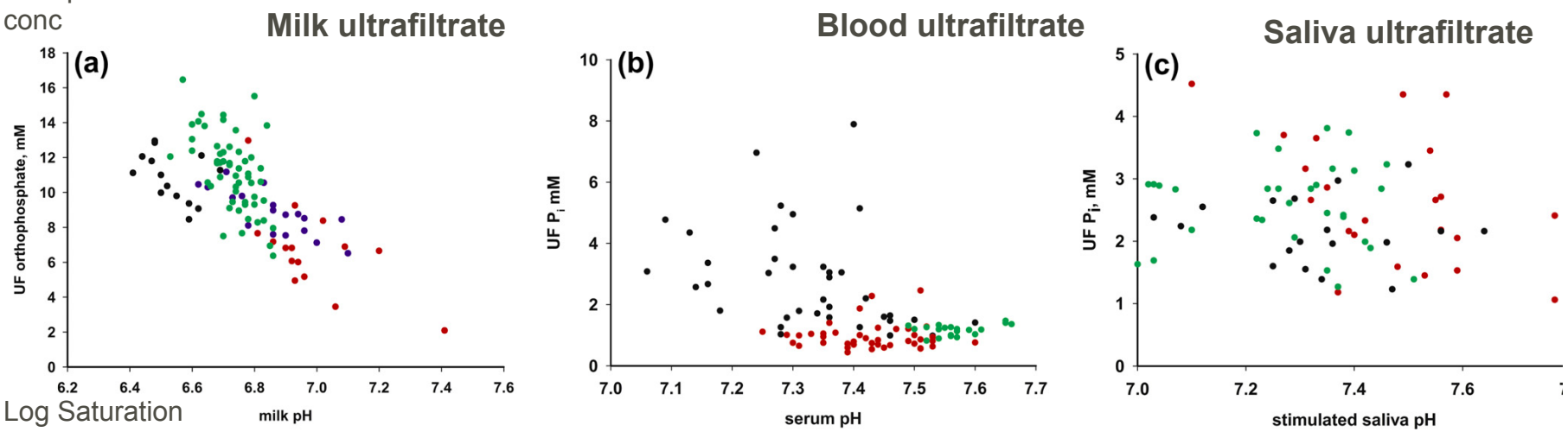
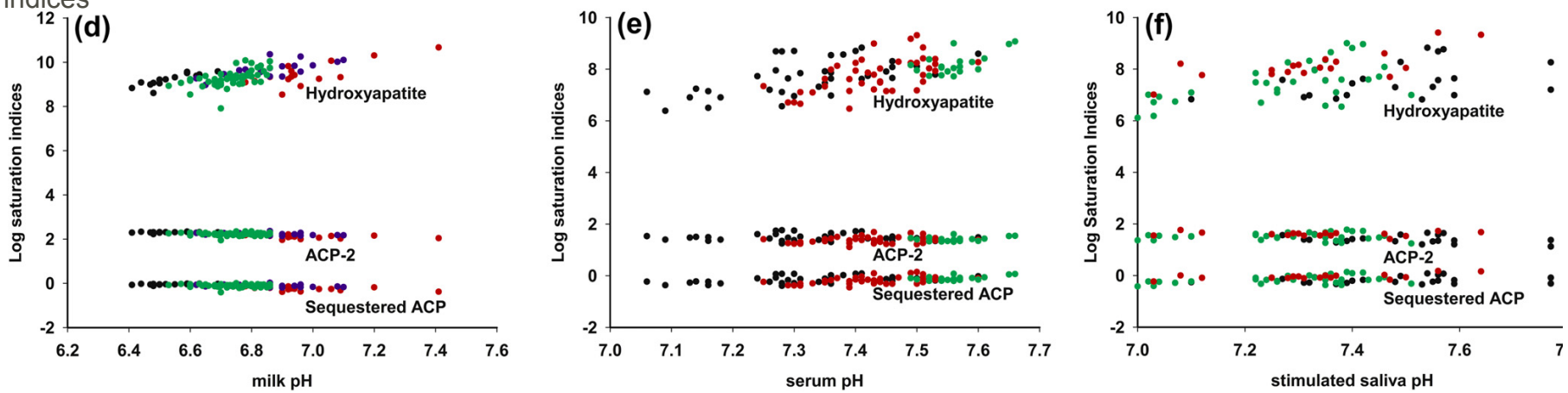
1. Considerable compositional variation
2. Biofluids are highly supersaturated with respect to hydroxyapatite.
3. Invariant saturation indices of ACP-2 (amorphous Ca phosphate) and sequestered ACP
4. Clear supersaturation in ACP-2, but not with respect to sequestered ACP

Phosphate conc



Log Saturation indices

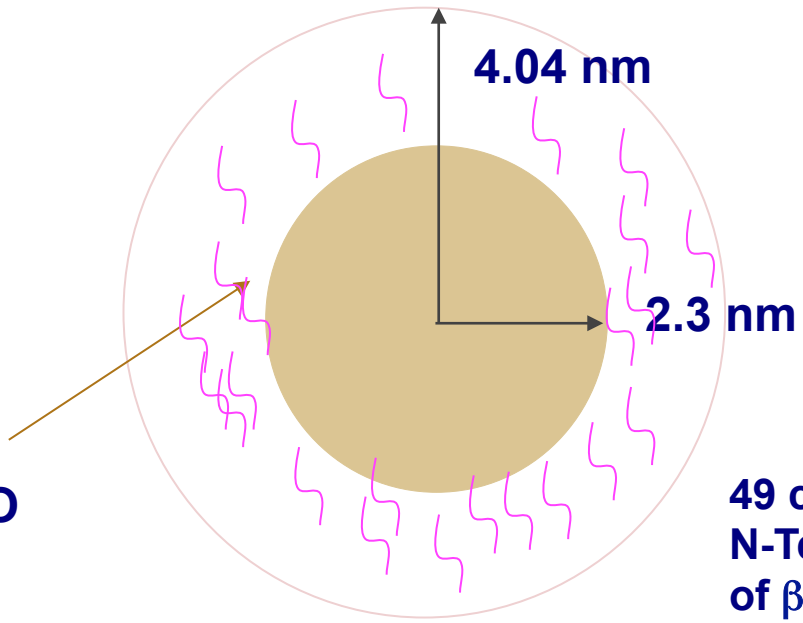


Log Saturation = 0 corresponds to saturated solution and higher values means supersaturation

β-Casein

- Flexible, major casein in milk
- None or little ordered secondary structure
- Amphiphilic - self-assemble into micellar type of aggregates (cmc ≈0.5 mg/ml)
- Calciumbinding protein (five phosphorylated serine) in one cluster
- Biological role is to stabilise calcium phosphate nano-clusters

Calcium phosphate nanocluster (CPN) Core-shell model according to Holt et al.[1]



355 $CaHPO_4 \cdot 2H_2O$

SLD = $2.7 \times 10^{-6} Å^{-2}$
Concentration 0.1 mg/ml

49 chains of25-aminoacid N-Terminal phosphopeptide of β-casein

[1]Holt, Timmins, Errington, Leaver Eur. J. Biochem. 252 (1998) 73-78

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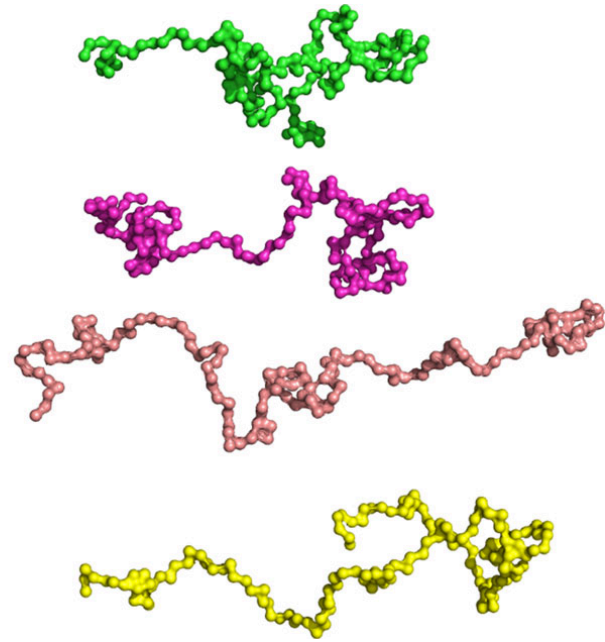
Osteopontin (OPN)

A molecule for all seasons with a number of different roles including:

- cell adhesion
- signalling
- migration and survival in many cell types

Main roles

- Biomineralization
- Immuno-modulation



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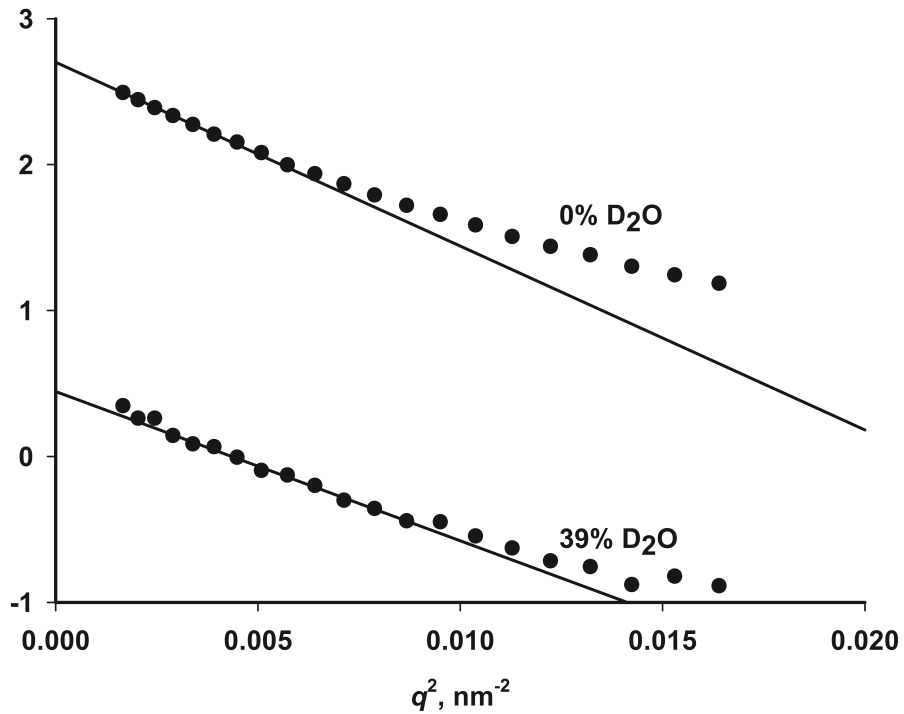
Guinier plots of the small-angle neutron scattering of OPNmix (osteopontin fraction from milk) nanoclusters in 0% and 39% D_2O



- SANS data show an R_g=19.5±0.4 nm in 0% D_2O
- Close to the protein match point (39% D_2O), R_g= 17.5±0.5 nm
- => Core shell model with calcium phosphate core (amorphous) and protein shell

C. Holt, S. Lenton, T. Nylander, E. S. Sørensen, S. C.M. Teixeira: Mineralisation of soft and hard tissues and the stability of biofluids. Journal of Structural Biology, 2014, 185, 383-396

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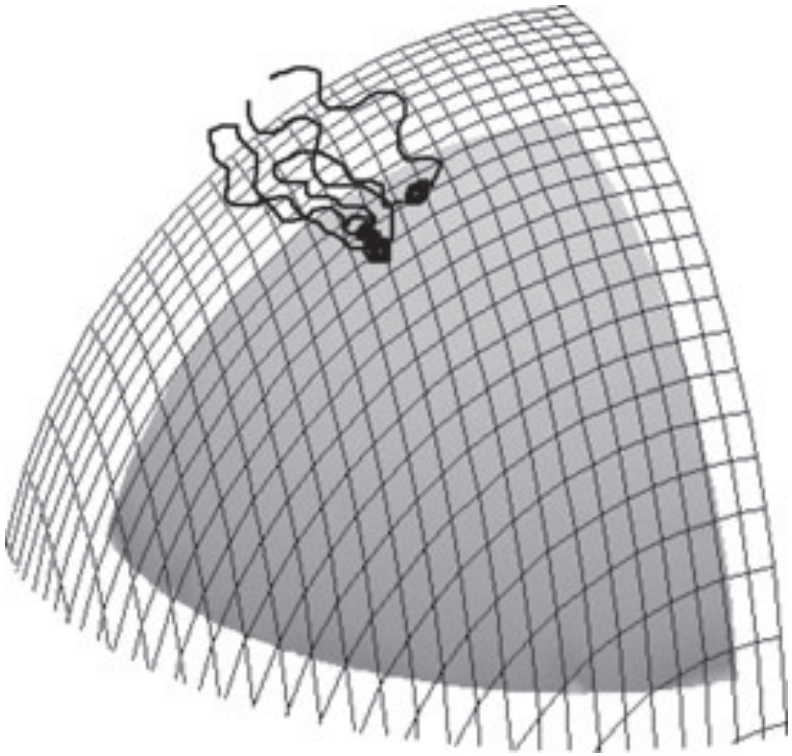
Representation of an eighth section of the OPN 1–149 nanocluster



The amorphous calcium phosphate core is surrounded by a shell of OPN 1–149 molecules, each anchored through its three phosphorylated clusters.

Holt, C., Sorensen, E.S., Clegg, R.A., 2009. Role of calcium phosphate nanoclusters in the control of calcification. FEBS J. 276, 2308–2323.

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The properties of the calcium sequestering peptide determine the size of the CPN

- β-Casein peptide -CPN significantly smaller than OPN – CPN
- It has been suggested that the number and density of phosphorylated centers on the peptide is determining the size. In fact the β-casein peptide has one phosphorylated center OPN has three!
- Other properties like the ability of the peptide to lower the interfacial tension between CPN and solution can contribute.
- The use of engineering mutant peptides with different number of phosphor centers can give important knowledge.

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Intrinsically disordered proteins (IDPs)

- IDPs are able to adapt to different environments
- Interact rapidly with one or more partners
- Lately received large attention- structure & dynamics
- Examples:
- Caseins
- Osteopontin

How does the dynamics affect calcium binding?

Elastic incoherent neutron scattering (EINS) probes mobility occurring on the nanosecond timescale

Lenton S, Seydel T, Nylander T, Holt C, Ha¨rtlein M, Teixeira S, Zaccai G. 2015 Dynamic footprint of sequestration in the molecular fluctuations of osteopontin. J. R. Soc. Interface 12: 20150506.

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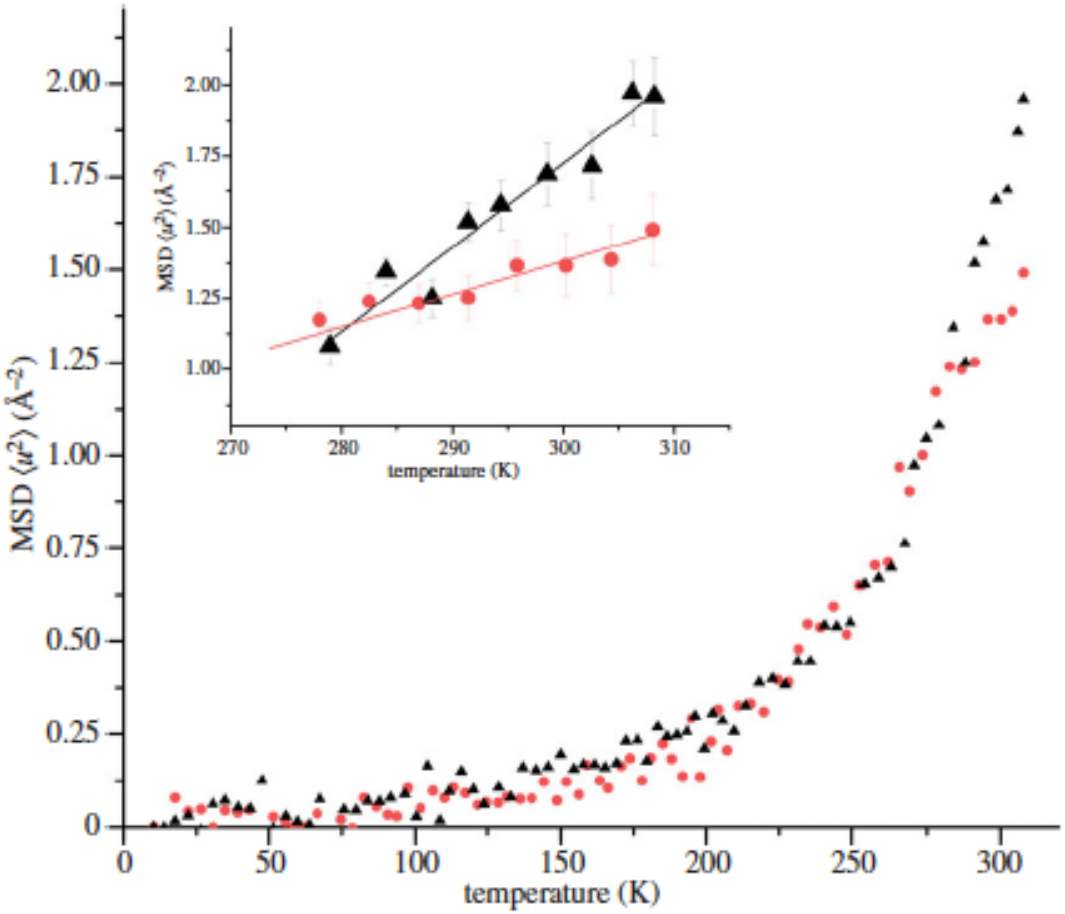
Elastic incoherent neutron scattering (EINS) data indicates a difference in protein flexibility when in free and nanocluster form.



MSDs as a function of temperature of OPN 1–149 (black triangles) and OPN CPN (dots), hydrated to 0.44 g D_2O per gram of protein, from IN16 neutron spectroscopy data

At higher temperatures, the MSD of OPN 1–149 is higher than that of OPN CPN, indicative of larger dynamics of free OPN 1–149.

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Conclusion on dynamics

- The dynamical behaviour observed for OPN 1–149 is in agreement with intrinsically disordered β-casein and Tau protein
- The high dynamics of OPN allows the rapid sequestration of amorphous calcium phosphate while maintaining a disordered state.
  - This enables peptide packing around the CPN core and may be of benefit for further CPN interactions.

Samuel Lenton, Tommy Nylander, Carl Holt, Lindsay Sawyer, Michael Härtlein, Harrald Müller, Susana C. M. Teixeira: Structural studies of hydrated samples of amorphous calcium phosphate and phosphoprotein nanoclusters. Eur. Biophys. J. 2016, DOI 10.1007/s00249-015-1109-7

ARE THE CALCIUM PHOSPHATE NANO CLUSTERS CRYSTALLINE OR AMORPHOUS?

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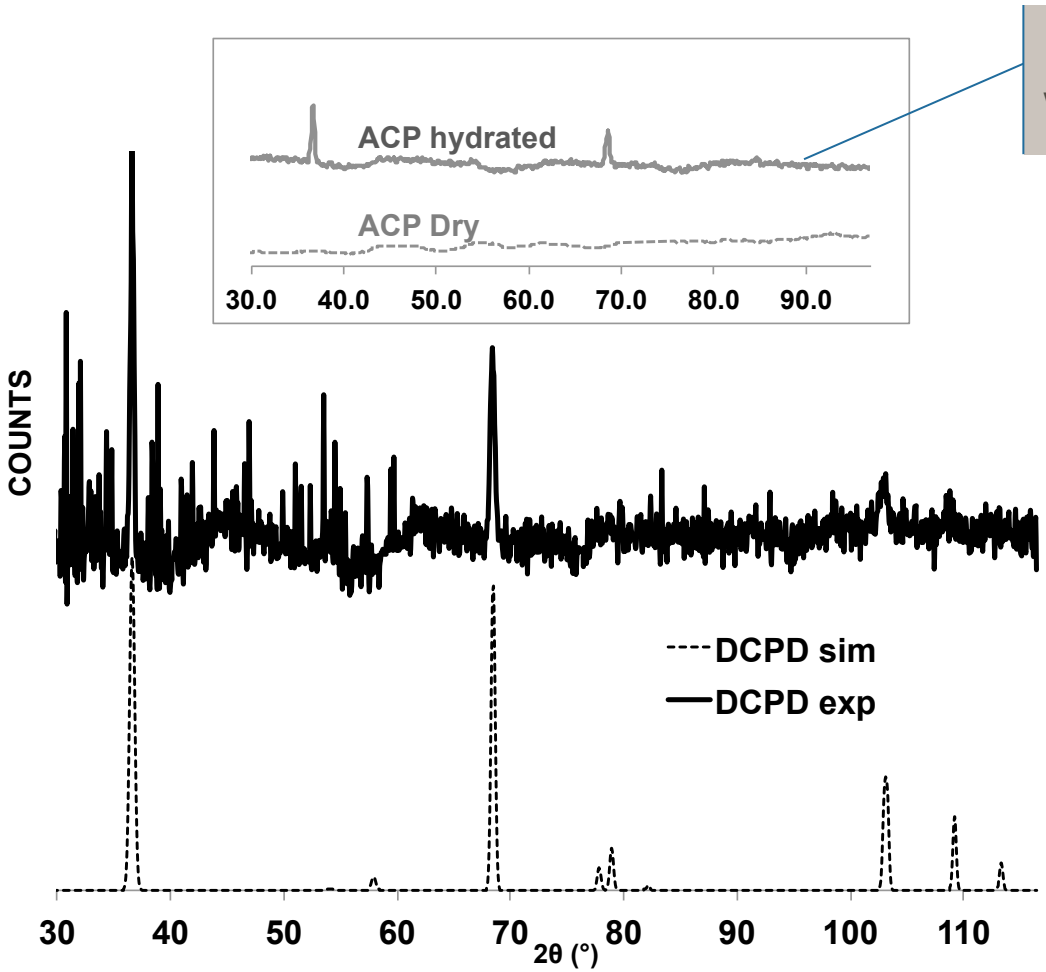
Neutron diffraction pattern of dicalcium phosphate dihydrate (DCPD) and amorphous calcium phosphate (ACP)



Maturation of ACP give crystalline structure

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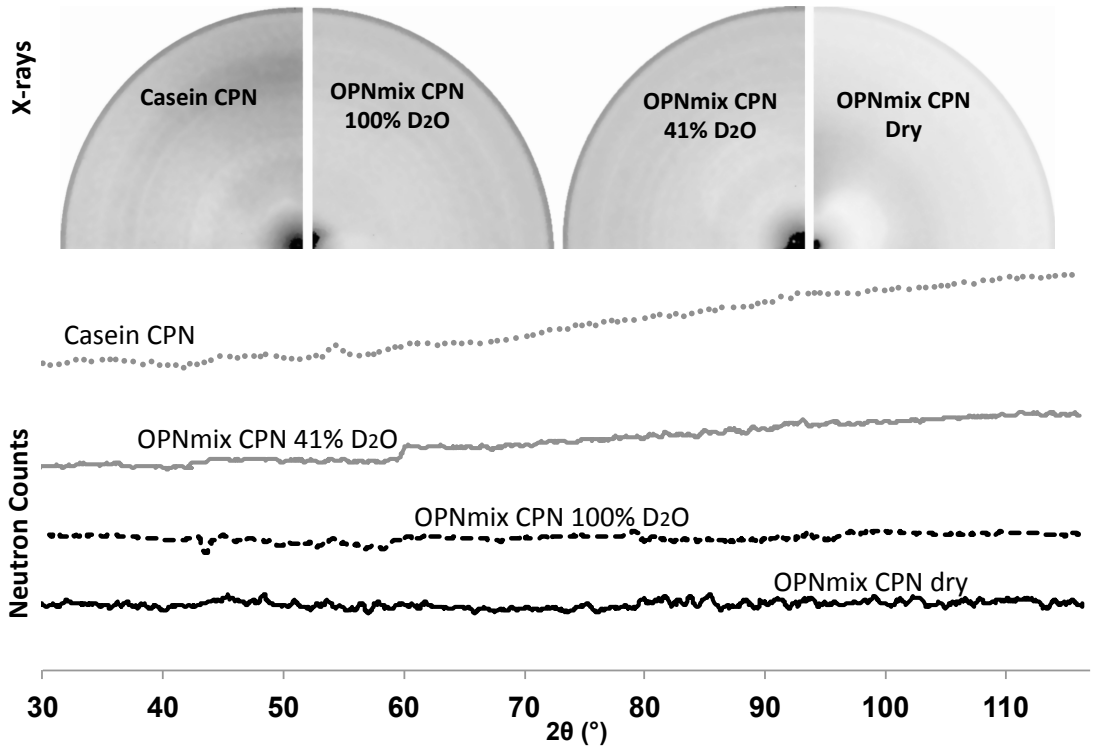
X-ray diffraction patterns for the CPN samples in the range of 2.07-20Å and neutron diffraction patterns of the OPNmix and pronase digested CPN samples.



No crystalline structure with sequestering peptides/proteins

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Conclusion

The lack of higher-order structure in the CaP core

- may be required for sequestration by the peptide
- favor calcium bioavailability
  - through higher rate of solution since amorphous phases lack the lattice energy of calcium phosphate crystals

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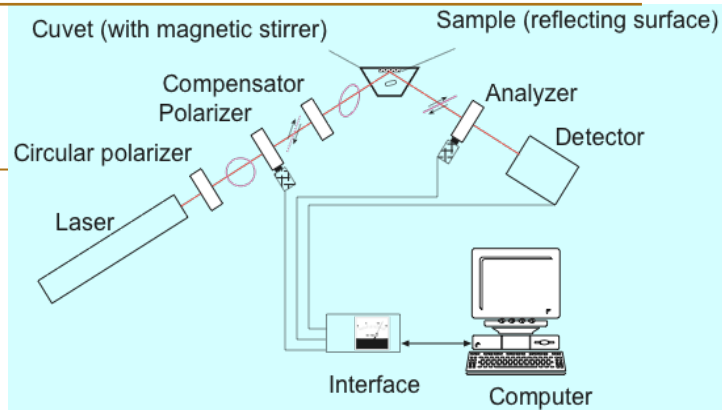
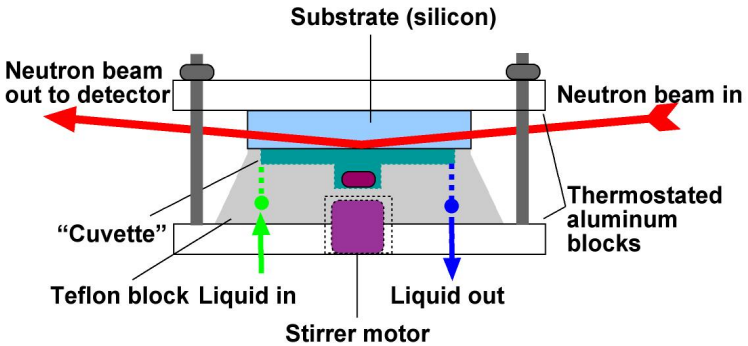
β-Casein –CPN at interfaces

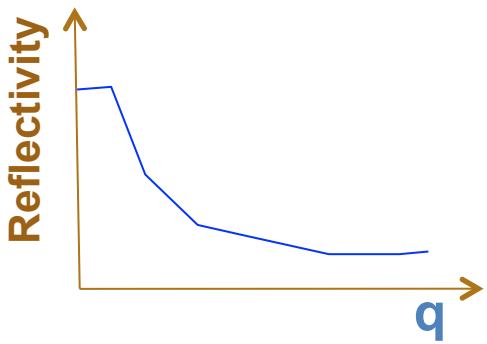
Some techniques used

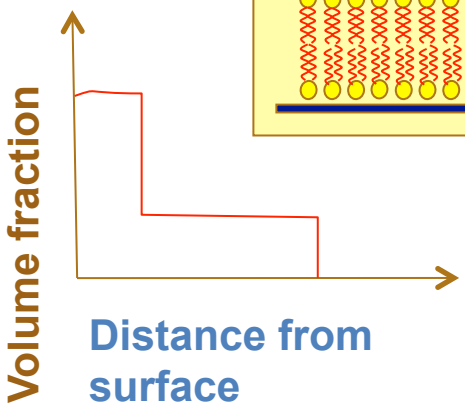
- Ellipsometry gives “optical” thickness and “dry” mass versus time

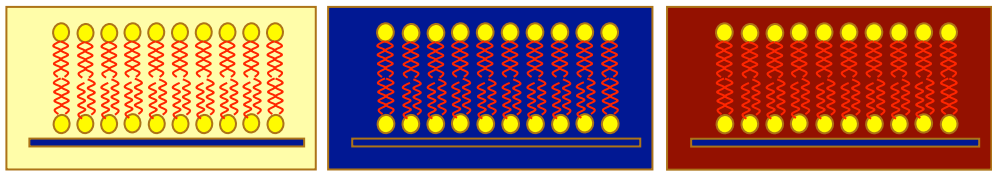


- Neutron Reflectometry gives density profile of the interfacial layer and selective deuteration + contrast matching gives composition









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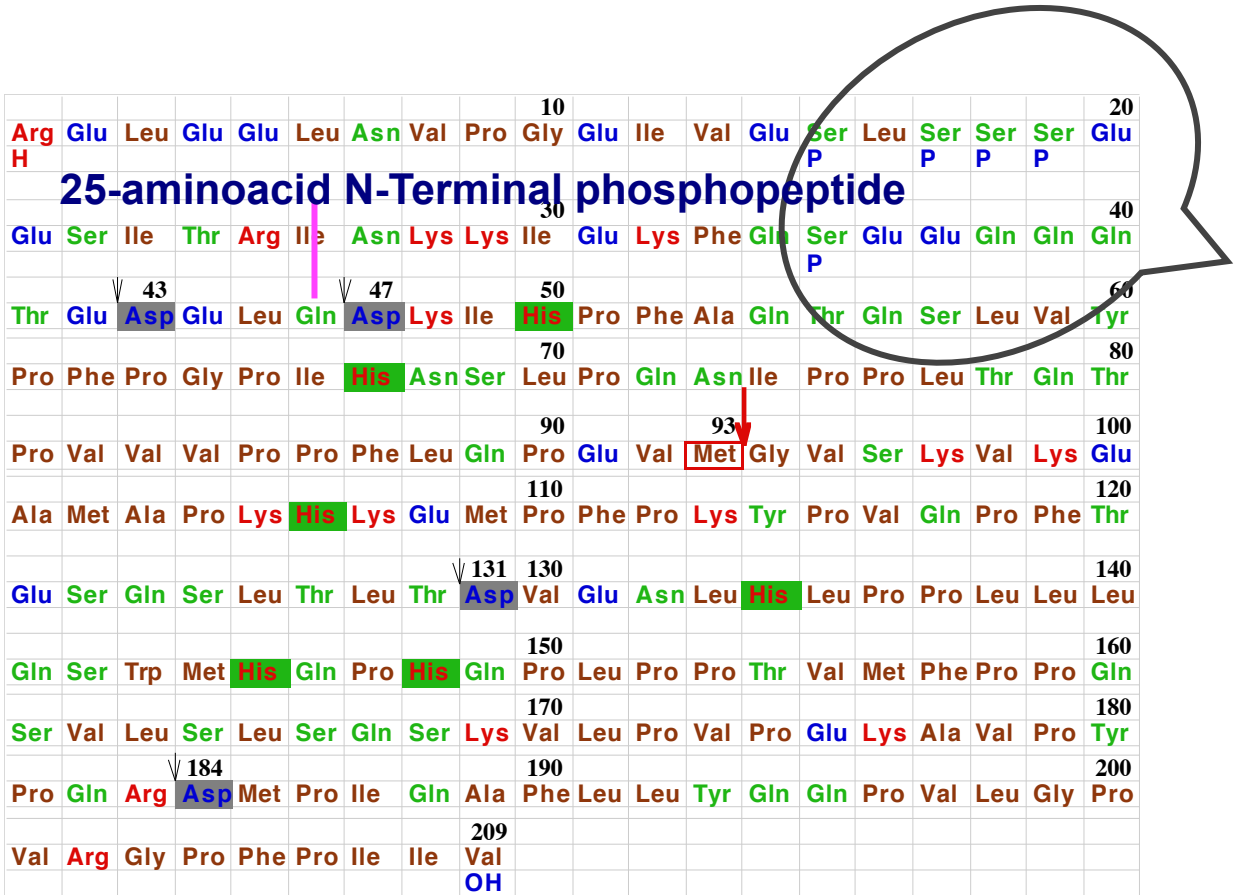
Endoproteinase Asp-N can cleave β-casein at four different sites, where two are located in the hydrophilic region (residues 43 and 47)



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Objective:

- To reveal the effect of calcium phosphate nanocluster (CPN) on the interfacial behaviour of β-casein
- Implication for biomineralisation?

- METHOD
- Neutron reflectivity measurements, which allows measurement of the layer density profile!
- SLD in D_2O = $2.78x10^{-6}Å^{-2}$
- Concentration 0.1mg/ml BCN, 20 mM Imidazole pH 7, 17 mM $CaCl_2$

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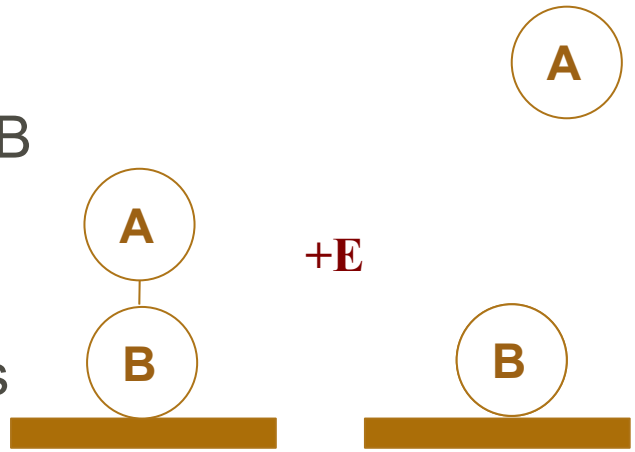
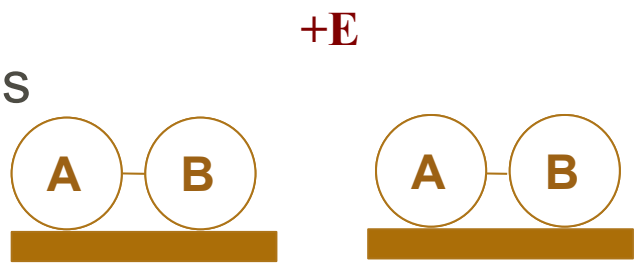
Chemical contrast variation

- Consider
  - A protein , which consist of two parts, A-B (for β-casein A is hydrophilic part and B hydrophobic part)
  - A highly specific enzyme, E, that cleaves A-B bond (endoproteinase Asp-N)



- A-B perpendicular to surface: enzyme access and change in thickness:
- A-B parallel to surface: no enzyme access and no change in thickness:



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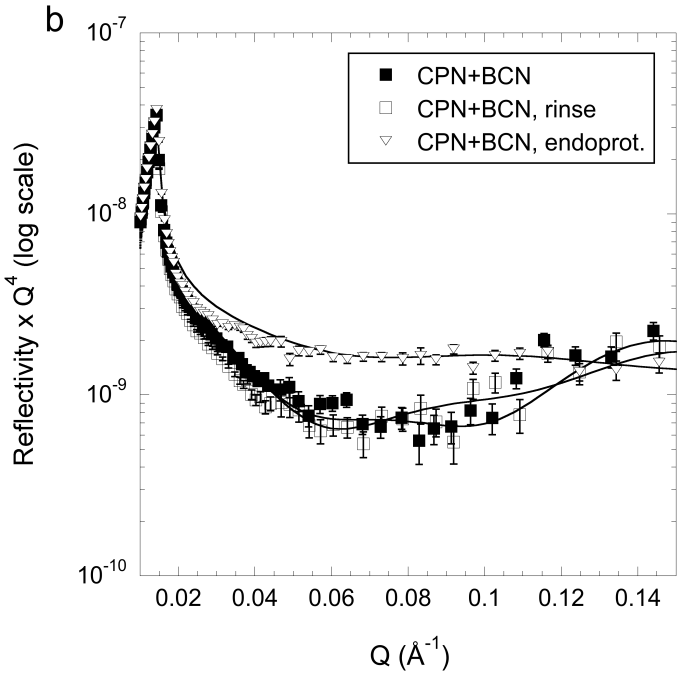
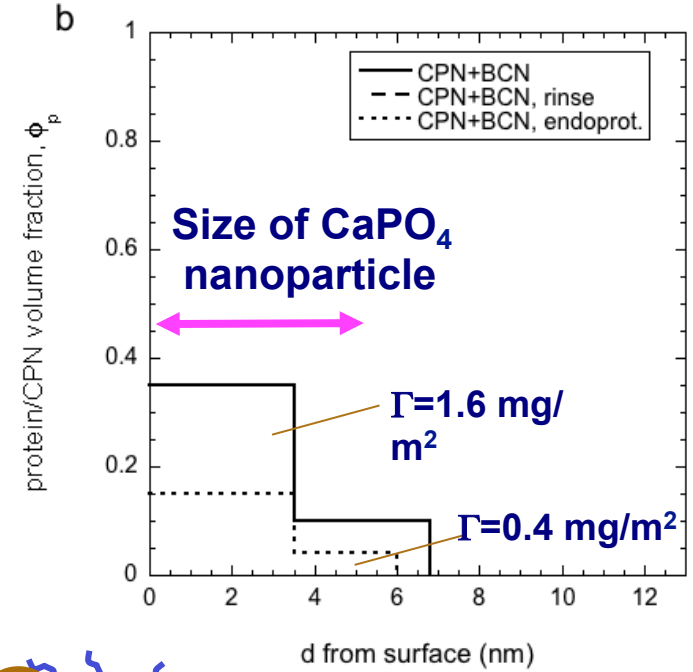
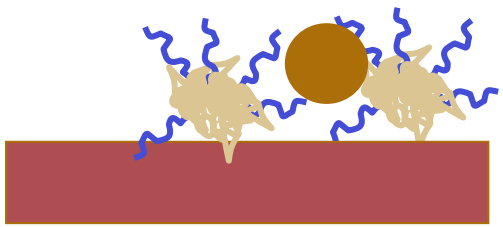
Neutron reflectivity and Volume fraction of β-casein as a function of distance from the silica surface obtained from two layer model fit

0.1 mg/ml β-Casein and 0.1 mg/ml CPN on hydrophilic silica surface in 0.02 M imidazole (pH 7) and 17 mM $CaCl_2$ pH 7







Note: It takes several hours to reach steady state=>
Slow adsorption kinetics – Bulk interactions

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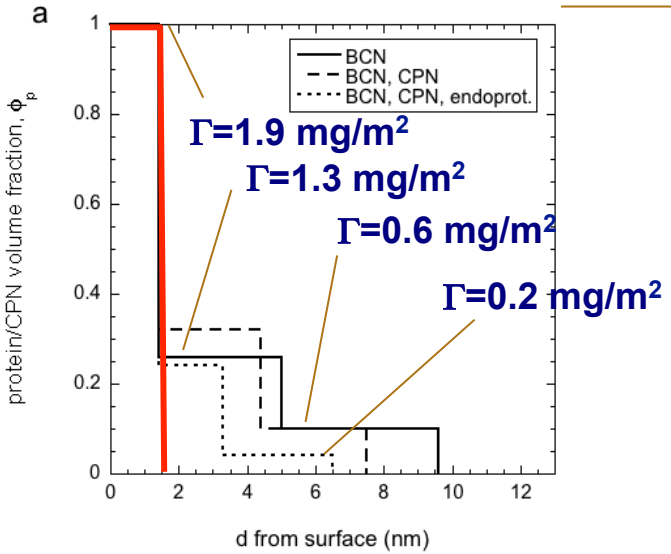
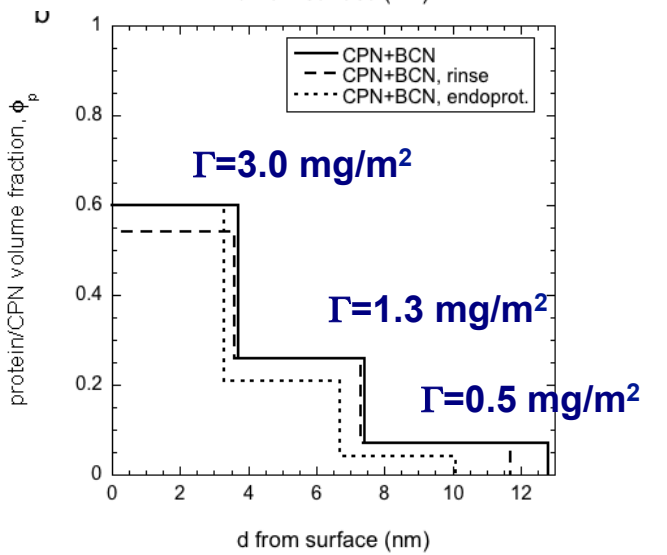
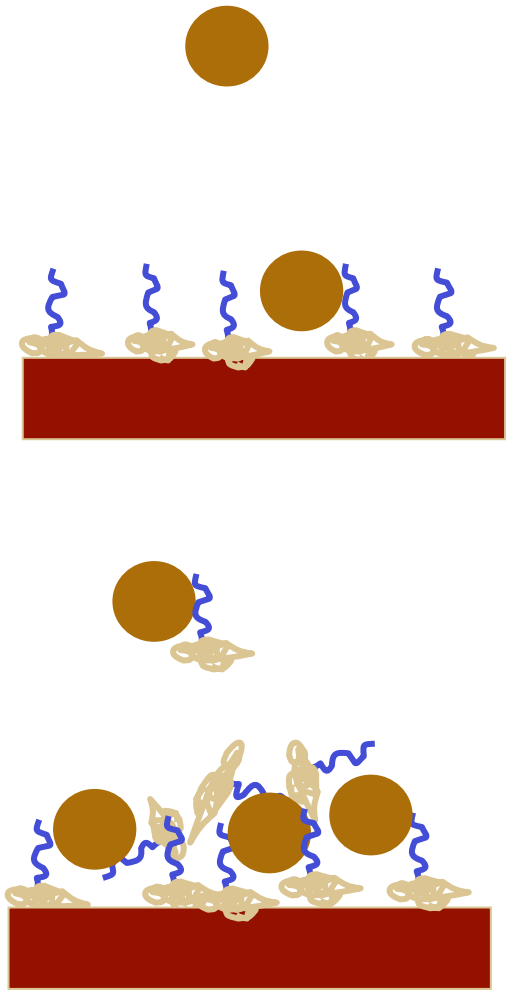
Neutron reflectivity x Q^4 versus momentum transfer (Q) for sequential versus simultaneous addition of β-casein (0.1 mg/ml) and CPN (0.1 mg/ml) on hydrophobic silica surface in 0.02 M imidazole an 17 mM $CaCl_2$ pH 7

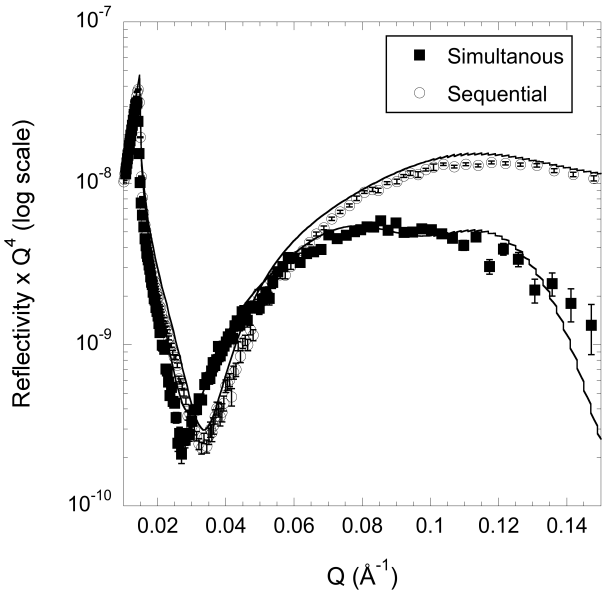
Previous data showed much larger effects of Asp-N without CPN







- The order of adding CPN makes a difference



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