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Experiment Report Form

	Experiment title: T-resolved SAXS/WAXS study on formation of calciprotein particles	**Experiment number**: SC 2029
Beamline: ID 2	**Date of experiment**: from: 10th Nov. 2006 to: 13th Nov. 2006	**Date of report**: 21.02.2008
Shifts: 9	**Local contact(s)**: Dr. T. Narayanan, Dr. M. Sztucki, Dr. S. Finet	*Received at ESRF:*

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Report:

The concentration of calcium and phosphate ions in mammalian blood is much higher than predicted by the solubility product for basic calcium phosphate. [1] However, no precipitation is observed. Recent work has identified the serum protein α_2-HS glycoprotein/Fetuin (Ahsg or Fetuin-A) as an important inhibitor that prevents pathological mineralization in soft tissues and in the extracellular fluid. [1] Once the solubility product is exceeded, colloidal caliprotein particles (CPP) are formed having a diameter of the order of 30-150 nm. [2] SANS experiments reveals a transition from the CCP formed initally to spherical particles consiting of a core of octacalcium phosphate covered by a shell of Fetuin. [2]. In contrast to this experiment we aimed at an understanding of the formation of the initial state in wich the CCP are formed, e.g. time scales below 5 min. Time-resolved small-angle x-ray scattering (TR-SAXS) is a highly suitable tool to pursue this question in detail. The solutions of the reacting species can be mixed rapidly by means of a stopped-flow apparatus and particle density *N/V* as well as the radius *R(t)* can be determined as the function of the time

The present experiment is a continuation of the experiment SC 1652 (In-situ study of the inhibition of calcification by added proteins using time-resolved stopped-flow SAXS/WAXS) and should clear the following questions: Does Fetuin-A act as a nucleating agent or will this protein only stabilize the nuclei of the nanoparticles formed during the precipitation? In the former case Fetuin-A should increase the number *N/V* of the

particles in the volume *V*. If, on the other hand, Fetuin-A only acts as a surface-active component that stabilizes the particles against further aggregation, N/V should not be changed by the presence of Fetuin-A.

A stopped flow cell was used for rapidly mixing equimolar aqueous solutions of 20mM $CaCl_2 2H_20$ and 12mM $Na_3PO_4.12H_2O$. In cases where inhibition effect of the protein were studied, the protein was added in both calcium and phosphate solutions (0 µM ,1 µM, 5 µM or 15 µM Fetuin-A) before the mixing process. The pH of the solutions were adjusted to pH = 7.4 by use of a Tris-buffer. The SAXS images were recorded by an image-intensified CCD camera detector. Data acquisition and counting of the time was hardware-triggered 1 ms before mixing process was initiated. The data were acquired in increments of 100-500 ms with an exposure time of about 100 ms.

In order to study the effect of the glycoprotein on the formation of calcium phosphate complexes, we have first done measurements in the absence of Fetuin-A. We do not observe any change in the scattering intensity after ca. 0.27 s due to the very fast kinetic of the formation of calcium phosphate primary particles. Indeed, scattering patterns recorded for a longer time, (up to 145 s after the mixing process) are completely identical to the ones at 0.27 s. The measurements were repeated under the same experimental conditions, with Fetuin-A added to both reactant solutions before the mixing process. It is important to note that a concentration of 15 µM of Fetuin-A corresponds to the physiological concentration. We observe for samples containing the glycoprotein a slower formation of calcium phosphate. The data sets have been fitted using a model of polydisperse spheres. Fig. 1 gives an example of the experimental data measured after 6.43 s and the corresponding fits.

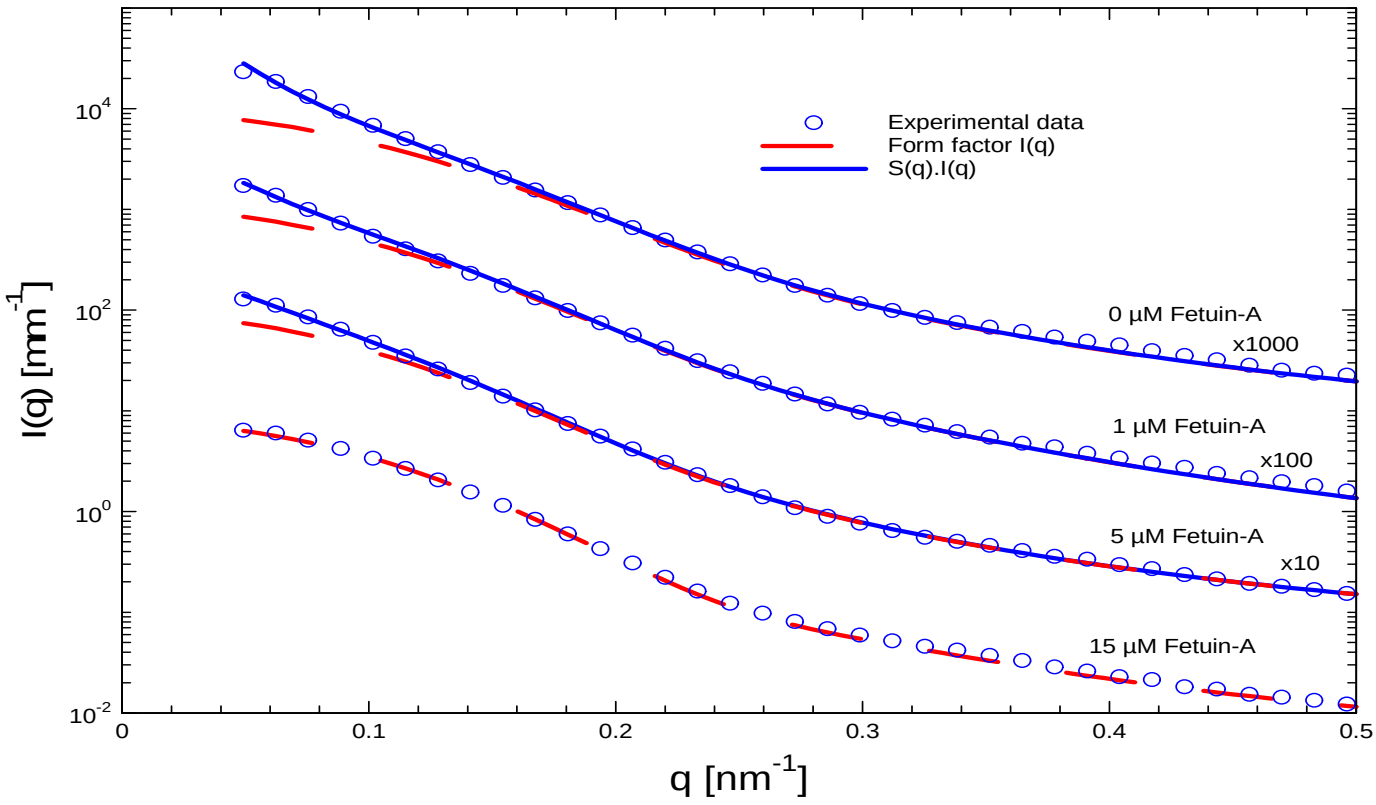

Figure 1: Experimental data (circles), form factor (dashed lines) and corresponding theoretical intensity by taking into account the structure factor (full line). Age: 0.891 sec after the mixing process.

From the fits the number density *N/V* and the radius of the primary particles R_{psp} can be determined as function of time. Fig. 2 displays the main result.

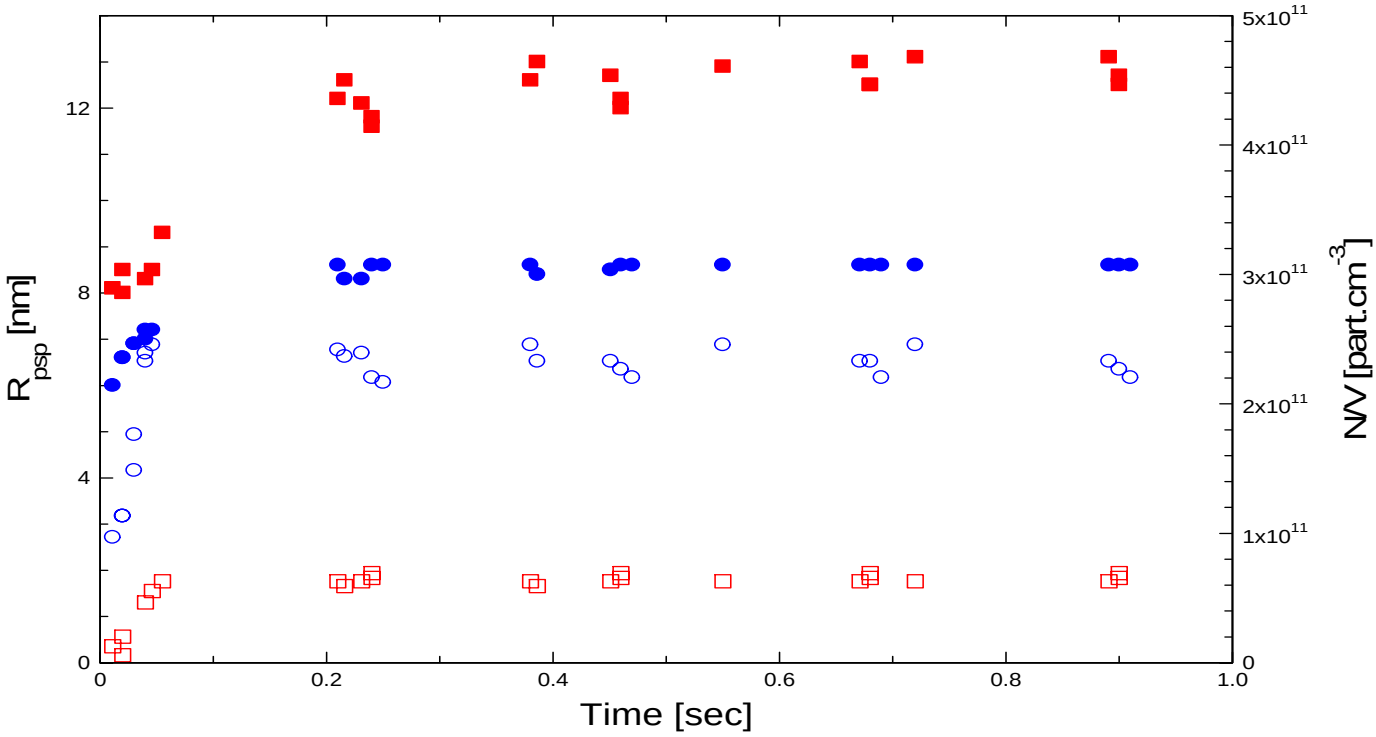

Figure 2: Time-evolution of the radius of primary spherical particles ($R_{psp}(t)$; filled symbols) and of the number of particles per volume (N/V; empty symbols). Red squares represent sample with 15 µM of Fetuin-A, blue circles the one without protein.

We note a growth of the radius of the primary spherical particles and a decrease of the number of particles per volume with the increase of glycoprotein. Hence, the results obtained here in presence of Fetuin-A do not permit to highlight the inhibitor effect [1] of the protein since we observe a growth of the primary particles in size with the addition of Fetuin-A. Additionally, the average number of primary particles per aggregate has been obtained and is plotted in fig. 3.

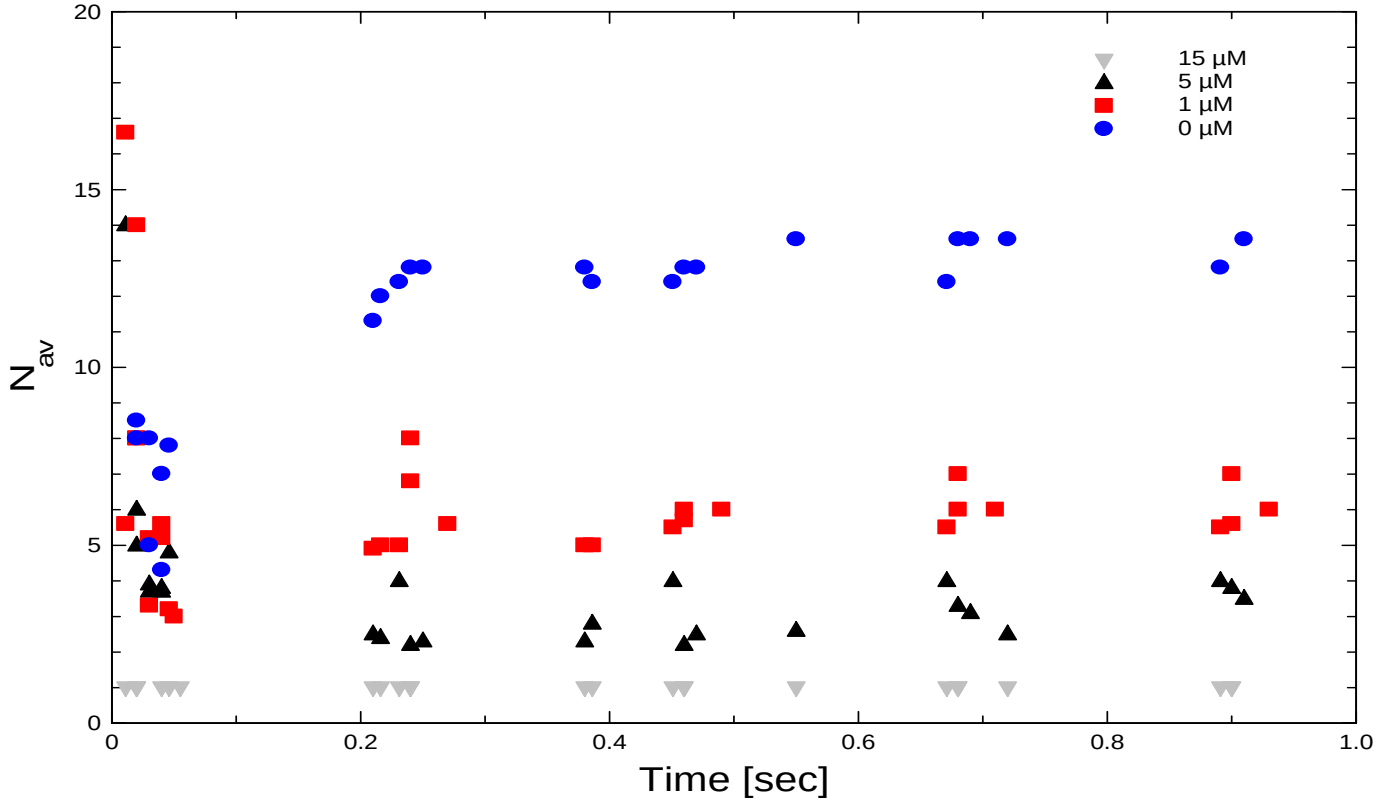

Figure 3: Time-evolution of the average number of primary particles per aggregate.

The size of the aggregates decrease with the addition of Fetuin-A. This could be compared with the work of Bolze and co-workers [3] who found an up-turn at small *q* regime, which is significant of the formation of aggregates with time. The value of the aggregate size for the sample without any additional protein (~24 nm) is in agreement with the recent work of Heiss and co-workers [2] through SANS (26 nm). A publication is in preparation.

Literature

[1] Heiss, A.; Duchesne A.; Denecke, B.; Grötzinger, J.; Yamamoto, K.; Renné, T.; Jahnen-Dechent, W., *J. Biol. Chem*. **2003**, *278*, 13333.
[2] Heiss, A.; Jahnen-Dechent, W.; Endo, H.; Schwahn, D. *Biointerphases*, **2007**, *2*, 16.

[3] Pontoni, D.; Bolze, J. ; Dingenouts, N.; Narayanan, T.; Ballauff, M., *J. Phys. Chem. B*, **2003**, *107*, 5123.