ESRF	**Experiment title:** In-situ study of the inhibition of calcification by added proteins using time-resolved stopped-flow SAXS/WAXS	**Experiment number**: Sc-1652
Beamline:	**Date of experiment**: from: 16.04.2005 to: 19.04.2005	**Date of report**: 08.02.2006
Shifts:	**Local contact(s)**: Dr. T. Narayanan, Dr. M. Sztucki	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

Dr. N. Dingenouts, Polymer-Institut, University of Karlsruhe, Germany*

Dr. S. Rosenfeldt, Physikalische Chemie I, University of Bayreuth, Germany*

B. Haupt, Physikalische Chemie I, University of Bayreuth, Germany*

Dr. A. Heiss, University Hospital Aachen, University of Bayreuth, Germany*

Prof. Dr.W. Jahnen-Dechent, Univerity Clinics, Aachen, Germany

Prof. Dr. M. Ballauff, Physikalische Chemie I, University of Bayreuth, Germany

Dr. T. Narayanan, ESRF Grenoble, France

Report:

Pathological mineralization, i.e. ectopic calcification, is known in humans and in most cases associated with primary diseases such as atherosclerosis. Results obtained in the group of Prof. Jahnen-Dechent with mutant mice suggests that mineralization of calcium and phosphate must be actively prevented by an inhibition mechanism. One important protein inhibitor is α_2-HS glycoprotein/fetuin-A (Ahsg). The lack of this protein in mice results in severe systemic calcification in mice on a genetic DBA/2 background (fig.1).

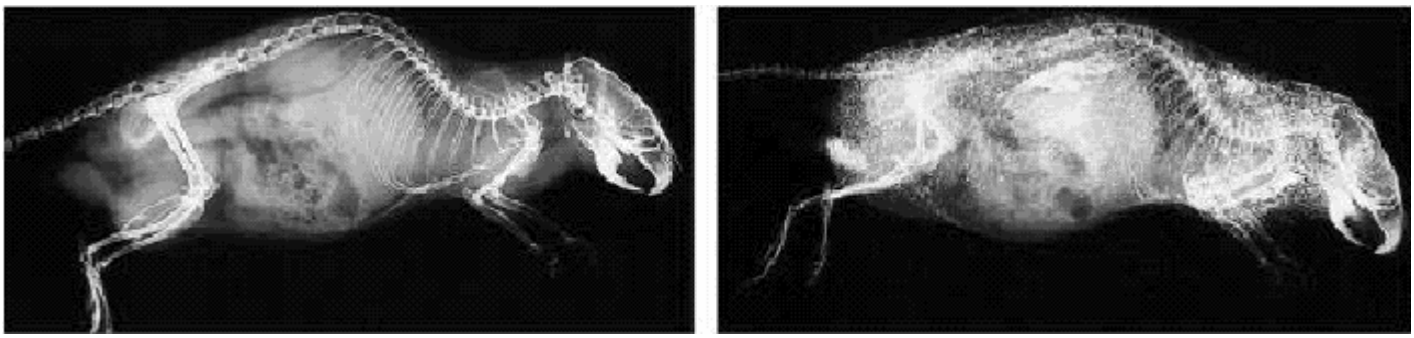

Fig. 1:

Pathological mineralization in mutant mice: Radiological analysis of 9-month-old male $AHSg^{+/+}$ (left) and $Ahsg^{-/-}$ (Ahsg/Fetuin deficient, right) mice. Note the calcification of thorax, kidneys and testes in the $Ahsg^{-/-}$ mouse.[1]

The investigation of the mechanism of inhibition by electron microscopy demonstrated the formation of calciprotein particles, i.e. spherical particles containing protein, calcium and phosphate [1].

We investigated the formation of calcium phosphate with and without added Ahsg in situ by means of time-resolved SAXS. A stopped flow cell was used for rapidly mixing equimolar aqueous solutions of 10mM·$CaCl_2$ $2H_20$ and 6mM Na_3PO_4. In cases where inhibition of the protein were studied the protein was added in the sodium solution (0.1µM, 10 µM or 20µM fetuin) before the mixing process. In contrast to preliminary experiments the pH of the solutions were adjusted to human conditions 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 1ms before mixing process was initiated. The data were acquired in increments of 100-200 ms with an exposure time of about 100 ms.

In order to study the effect of Ahsg on the formation of $Ca_3(PO_4)_2$, we have carried out measurements without the protein.

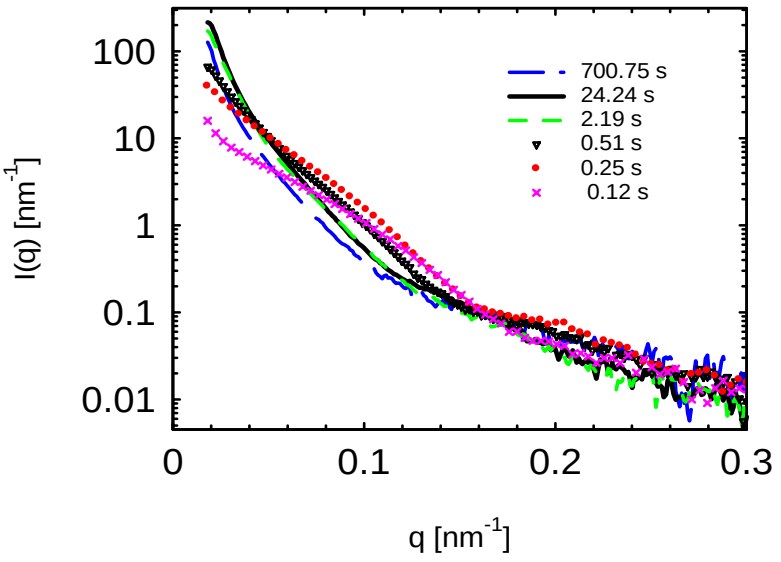

Fig. 2:

Scattering intensities of the formation of $Ca_3(PO_4)_2$ after mixing with a flow rate of 6.7µl/s.

Fig. 2 shows the evolution of the scattering signal after the mixing. Four different regime can be detected:

1. Scattering intensity observed between 0-0.2s. The scattering intensities could be fitted in the q-range between 0.05-0.3nm^{-1} assuming homogenous spheres with a Schulz-Zimm distribution of a polydispersity of ca. 25%, N/V was kept constant). The particle radius was found to be increasing from 16nm for 0.07s to about 26nm for 0.3s.

2. Scattering intensity observed between 0.2-2s. At scattering vectors q smaller than ca. 0.08 a gradual increase of the scattering intensity could be observed which is explained by an aggregation process of primary particles. Porod plots of the experimental data in this time range (not shown) demonstrated that the decay of the SAXS intensities at higher *q* is proportional to q^{-4}. The primary particles may thus be identified as three-dimensional objects with sharp interfaces. From the position of the minima in the Porod plots the radius of the primary particle was estimated to increase further to about 50nm.

3. Scattering intensities from ca. 2s to about 550s. The measured scattering intensities are more or less identical for q>0.4nm^{-1}. A further increase of the primary particle radius can not be excluded. Up to now it is unknown if the derivations for smaller q can be explained by aggregation of primary particles.

4. Scattering intensities over 550s. Here a significant decrease of the scattering intensity and a change in the form of the scattering curve is detected. In this time range we found by visual inspection macroscopic flocs and sedimentation.

The formation of calcium phosphate in the presence of different amount of Fetuin Ahsg were conducted under similar experimental condition. For small time scales (ca. <0.5s) the time dependence of the scattering intensities in addition of 0.1µM Ahsg is similar to that in absence of protein. After that time the overall trends (cf. discussion fig. 1, point 2-4) are also similar, but the corresponding times are delayed. A gradual increase of the scattering intensity at $q<0.03nm^{-1}$ as detected in pure calcium phosphate solution only starts about 0.5s. Until this time also the side maximum at $q\approx0.12nm^{-1}$ remains. Like in the reference case we determined a primary particle radius up to about 45nm from the position of the minima in Porod plots. Between a measuring time ranging from 15 to 280s the form and absolute scale of the scattering curves kept mostly constant for $q>0.4nm^{-1}$. Only for lower q values an upturn of the intensities due to aggregation is seen. Furthermore a small further increase of the primary particle radius in this time range is assumed. After about 280s the total scattering intensity decrease presumable to the formation of fractal like macroscopic flocs. Fig. 3 shows the long-time evolution of the formation of $Ca_3(PO_4)_2$ in the presence of fetuin.

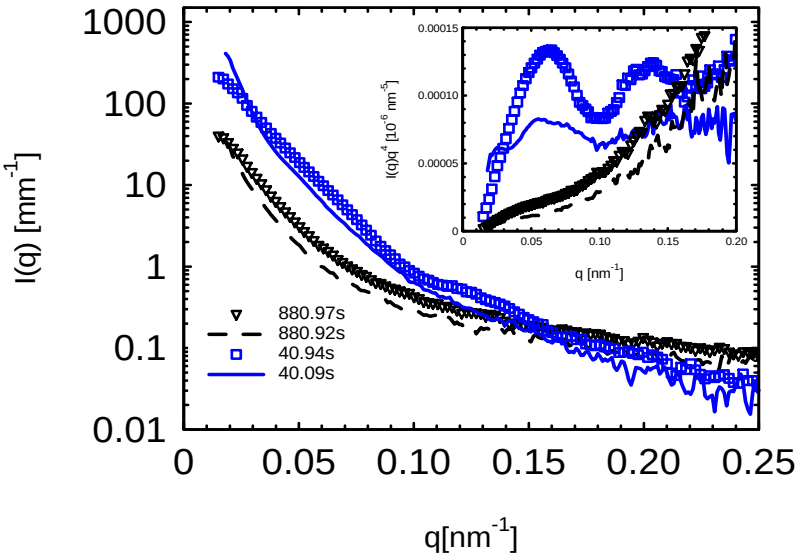

Fig. 3:

Long-time evolution of the scattering intensities of the formation $Ca_3(PO_4)_2$ of with (symbols) and without (lines) 0.1µM Ahsg.

Inset: Porod plot of the experimental data.

Fig. 4 represents the time evolution of the scattering intensities of the formation $Ca_3(PO_4)_2$ with (symbols) and without (lines) 10µM Ahsg. The inset shows a Porod plot of the experimental data about 880s. After addition of a higher amount of fetuin the formation of primary particles seems to be further delayed. The formation of primary particles starts only after about 0.5s and went on to ca. 1s. During this time range the experimental data could be fitted after $q\approx0.04nm^{-1}$ assuming homogenous spheres with a Schulz-Zimm distribution (ca. 25% polydispersity, N/V varied). The particle radius was found to be increasing to about 20nm for 1s. Over times of 1s the maximum seen in the q-rage between 0.05-0.2 decrease, therefore the intensity at lower q values increase to a maximum at about 80s. For even higher times the overall shape of the scattering intensity stays constant, but the absolute height decrease slowly. A possible explanation of this fact is

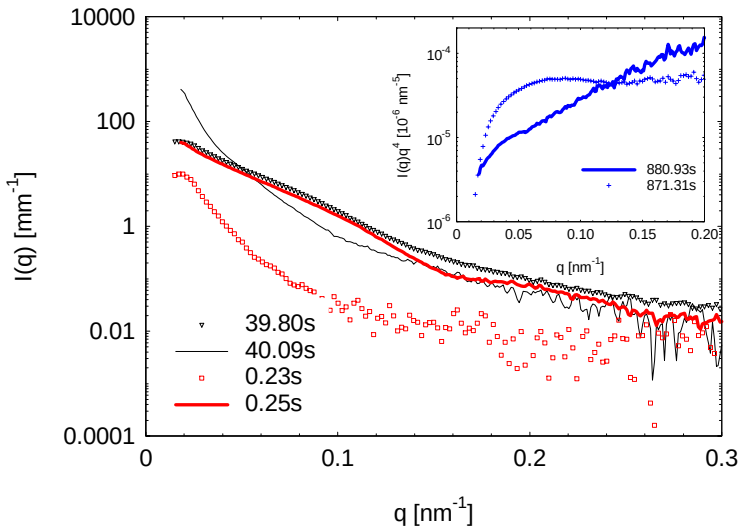

Fig. 4:

Time evolution of the scattering intensities of the formation $Ca_3(PO_4)_2$ with (symbols) and without (lines) 10µM Ahsg for low and intermediate times.

Inset: Porod plot of the experimental data at a time about 880s.

a aggregation process of primary particles to larger objects. Nevertheless for all measured times (0-2400s) the overall shape of the scattering intensities leveled off in a Porod plot at higher scattering angles. This shows that the particle is composed of well defined three-dimensional particles. Note that this is not the case for pure $Ca_3(PO_4)_2$ solution.

A possible reorganisation of already formed particles can be analyzed further by the integral scattering of an ensemble of particles, e.g. the invariant *Q*. The invariant is fully independent of the specific spatial arrangement of the particle within the sample and dominated by the structure near the particle surface at high enough *q* values. Fig. 5 shows the variation of *Q* as a function of time for the formation of $Ca_3(PO_4)_2$ with and without Ahsg.

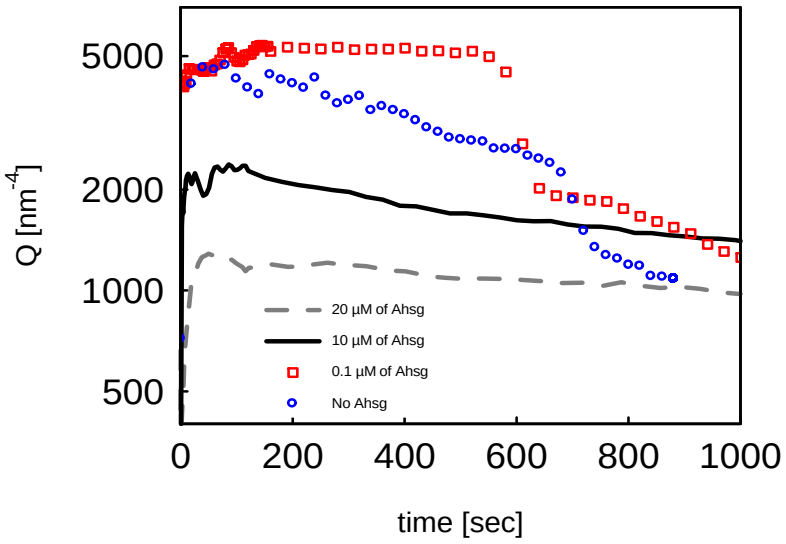

Fig. 5:

Variation of Q as a function of time for the formation of $Ca_3(PO_4)_2$ with and without Ahsg.

The addition of Fetuin is influencing the time evolution of the invariant. The highest values for the invariant *Q* is found after about 80s and adding 0.1 µM Ahsg. This value is even higher than for the pure reference solution. Another feature in need of further explanation is the following: In the time range between 600 and 800s there is a sudden drop of the *Q* value, but only for the reference solution and for the formation of particles in presence of 0.1µM Ahsg. In the presence of 10µM Ahsg and 20µM Ahsg only a constant slow decrease after about 80s is observed. To investigate and understand the influence of the concentration of Fetuin on the time dependence of the invariant *Q* more measurements are needed, especially in the concentration range around 0.1 µM Ahsg.

Conclusion

We have studied the formation of calciprotein particles [1] by time-resolved SAXS. Up to know we deduct from that the formation of the calciprotein particles starts by formation of small primary spherical particles, which grows to a size of about 100nm in diameter. During this growing process they already begin to aggregate. We plan to analyze the possible reorganisation process of the already formed particles in further detail. In particular, more measurements are needed using amounts of Ahsg between 0.1-10 µM.

Literature

[1] C. Schäfer, A. Heiss, A. Schwarz, R. Westenfeld, M. Ketteler, J. Floege, W. Müller-Esterl, T. Schinke, W. Jahnen-Dechent, *J. Clin. Invest. 112* (2003) 357.