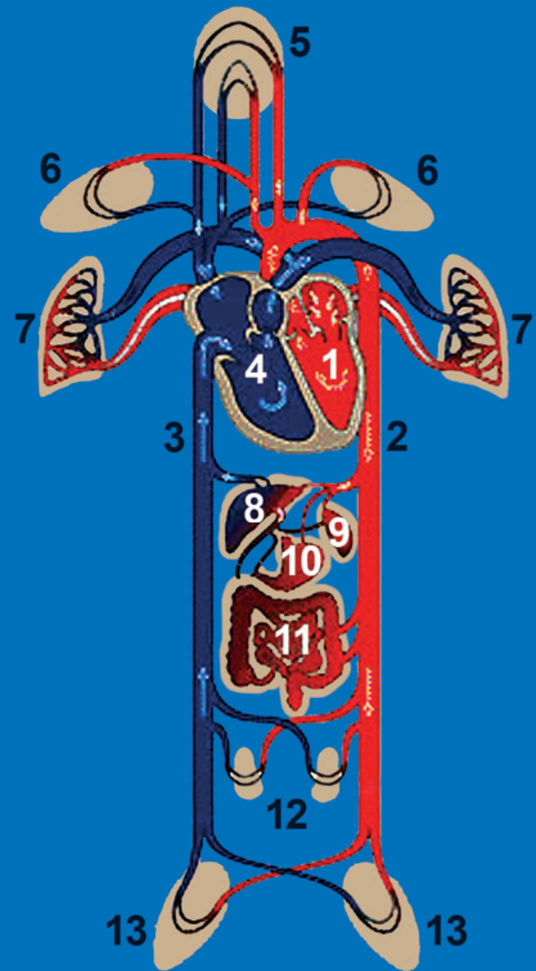
Bio interface

Biointerface

Faculty of Medicine



Clean Sweep: Particle Clearing In The Body

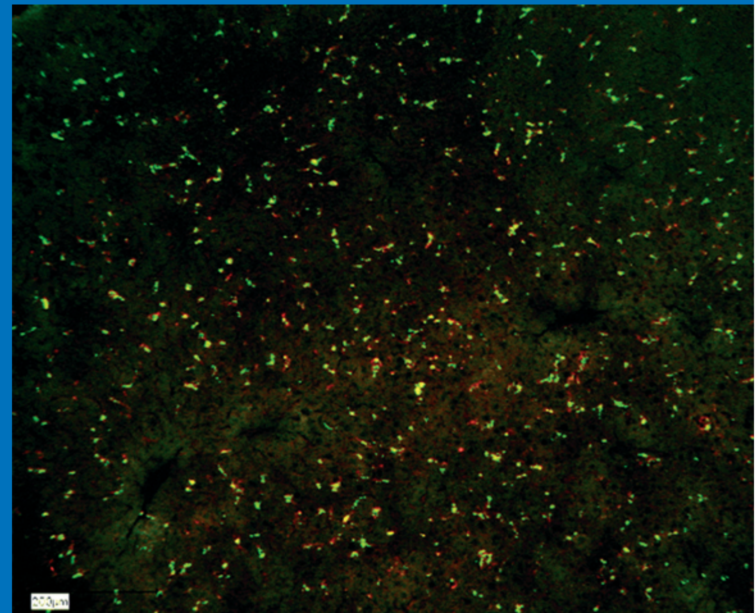
Director

Univ.-Prof. Dr. rer. nat.
Wilhelm Jahnen-Dechent

RWTH Aachen University Hospital
Pauwelsstrasse 30, D-52074 Aachen

Helmholtz-Institute for Biomedical Engineering
Pauwelsstrasse 20

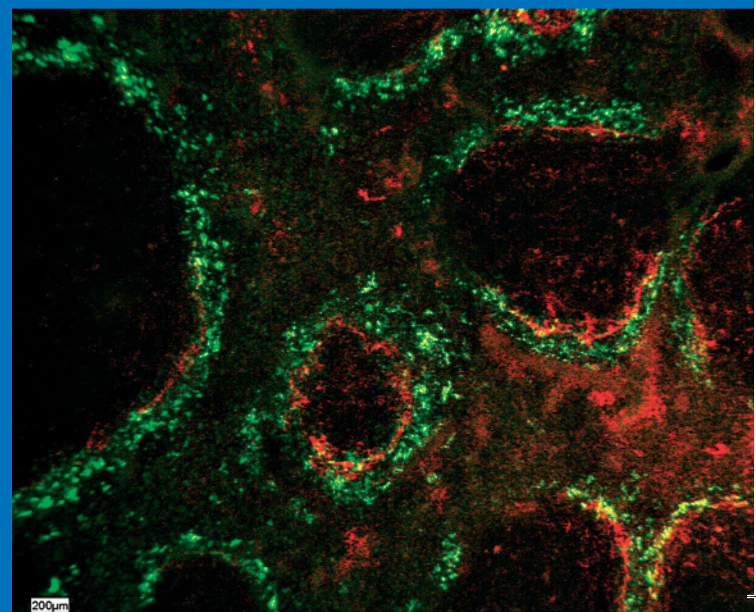
Phone: +49 (0)241 80-80163 (Office)
+49 (0)241 80-80157 (Secretary)
Fax: +49 (0)241 80-82573
Email: rsous@ukaachen.de
Web: http://www.biointerface.rwth-aachen.de



Staff

Sous, Renate, Administrative Assistant

Dietzel, Eileen, cand.-Biol.
Dreymüller, Daniela, Dipl.-Biol.
Floehr, Julia, cand.-Biol.
Gräber, Steffen, CTA
Herrmann, Marietta, Dipl.-Biol.
Kinkeldey, Anne, Dipl.-Biol.
Pan, Yu, M.Sc.
Schäfer, Cora, Dr. rer. nat.



RWTH AACHEN UNIVERSITY

UNIVERSITÄTSKLINIKUM AACHEN

Introduction

The Biointerface Laboratory continues work on the structure and function of secreted (type 3) cystatin protein family members, the fetuins (fetuin-A and fetuin-B) and histidine-rich glycoprotein (HRG). These proteins occur in all vertebrates. They are next neighbours in the human and mouse genomes. Fetuins and HRG are plasma proteins predominantly made in the liver. We study their biological function by gene knockout in mice. We have generated gene knockouts for all three proteins and we maintain the genetically modified mice on various genetic backgrounds and in combination with related gene knockouts. Thus we have published first results obtained with a double knockout of fetuin-A and apolipoprotein E.

Calciprotein particles and mineral chaperones

In all vertebrates, the extracellular fluids are supersaturated with respect to the mineral ions calcium and phosphate. Local supersaturation can even be much higher than the ion concentrations in the blood, for example in bone remodeling in the bone marrow or during transport and concentration of primary urine in the renal epithelium. Therefore, potent inhibitors of spontaneous mineral precipitation must exist to counterbalance this thermodynamic driving force. Otherwise ectopic calcification in blood or in the soft tissues may occur. As our previous studies have shown, the serum protein fetuin-A is a particularly potent systemic inhibitor of soft tissue calcification. Fetuin-A is highly effective in the formation and stabilization of protein-mineral colloids, so-called calciprotein particles, CPPs. Recently we showed, that CPPs mature in a two-stage process by a morphological transformation from spherical particles to larger ellipsoids. Employing a combination of light scattering spectroscopy and electron microscopy we could demonstrate that the secondary particles emerged by strongly anisotropic growth of the primary particles.

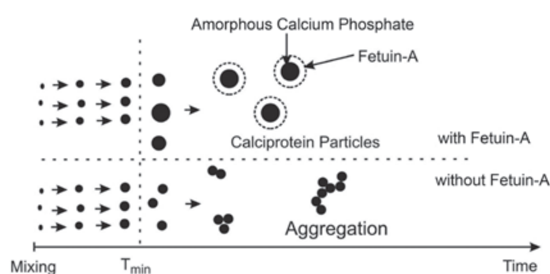


Figure 1: The role of fetuin-A in the early stages of mineralization: calcium phosphate particles spontaneously form when the ion concentrations exceed the solubility product. In this early phase of mineralization, fetuin-A has virtually no influence. The primary particles are stabilized, first by fetuin-A only, then by further acidic serum proteins. Without protein inhibitors or mineral chaperones, mineral nuclei grow into large aggregates, which ultimately precipitate.

We investigated the earliest stages of formation of CPPs with time resolved small angle X-ray scattering methods (TR-SAXS) and stopped-flow analysis. It was determined that fetuin-A has no influence on the formation of mineral nuclei. However fetuin-A effectively prevented within milliseconds the aggregation of mineral nuclei and thus mineral precipitation. The shield formed by fetuin-A leads to stable CPPs in the first phase of mineralization. Fetuin-A is therefore indispensable during the earliest stage of formation of protein-mineral complexes in order to prevent uncontrolled mineralization (figure 1).

The electron microscopic analysis of ascites fluid of a patient with sclerosing calcifying peritonitis revealed for the first time secondary CPPs in humans. Unlike in vitro generated CPPs, ascites CPPs contained relatively low amounts of fetuin-A, but large quantities of serum albumin (figure 2). This prompted us to investigate the combined role of fetuin-A and other serum proteins in the formation and stabilization of CPPs. Fetuin-A was necessary and sufficient for primary CPP stabilization.

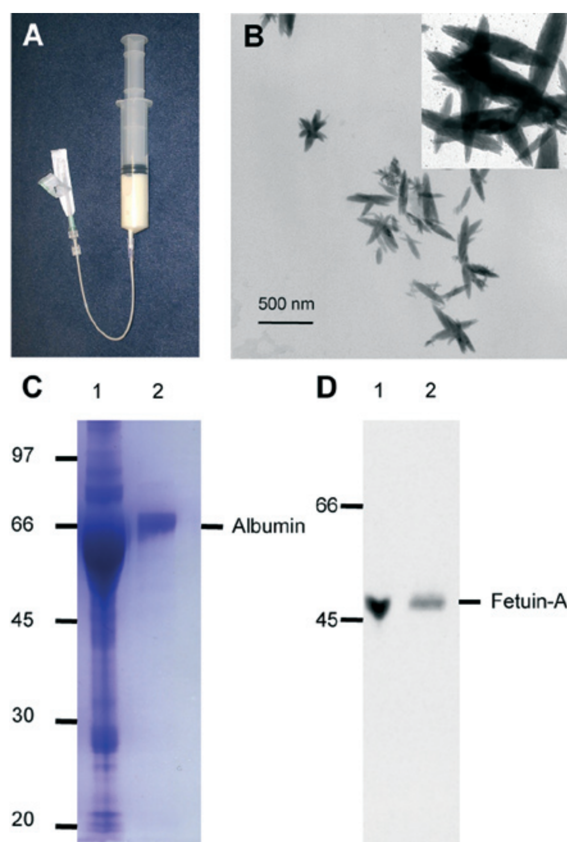
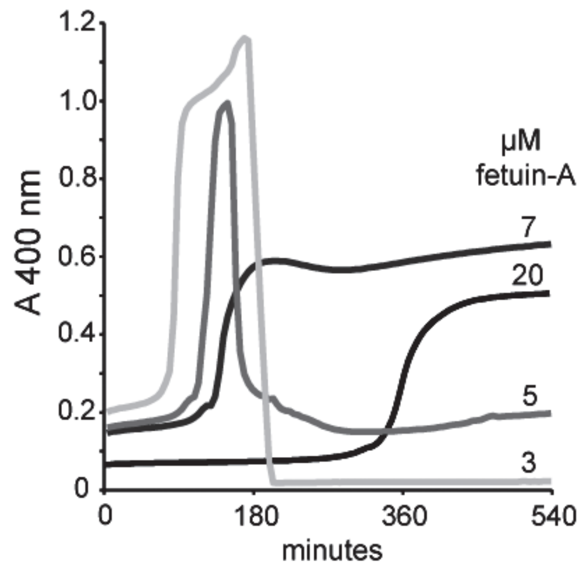


Figure 2: Morphology and composition of calciprotein particles isolated from human ascites. A) Strongly mineralized ascites fluid of a patient with sclerosing calcifying peritonitis contained large quantities of CPPs. B) Morphologically these CPPs were identical with the laboratory-produced fetuin-A-mineral complexes (inset). C), D) Unlike laboratory derived CPPs the patient CPPs contained large amounts of serum albumin, which could replace a large proportion of fetuin-A in long-term stabilization of CPPs.

Serum albumin and other acidic proteins facilitated the fetuin-A-mediated formation of secondary CPPs. Albumin was able to replace considerable quantities of fetuin-A without loss of inhibition of calcium-phosphate precipitation (figure 3). This

Biointerface

observation may help to explain why pathological deposition of minerals in the body is unlikely, even if low fetuin-A serum levels prevail, as long as sufficient albumin is available. Thus fetuin-A can be substituted with acidic plasma proteins mediating the stabilization, transport and removal of insoluble calcium phosphate as colloidal complexes. Therefore we call these transport proteins mineral chaperones.



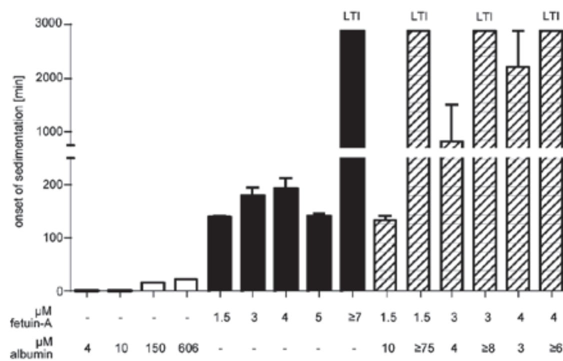


Figure 3: Calciprotein particles, CPPs are colloidal complexes of fetuin-A, calcium and phosphate that form spontaneously from supersaturated solutions given sufficient amounts of fetuin-A are also present. In the absence of fetuin-A precipitation occurs immediately. The formation of stable primary CPPs requires relatively low fetuin-A concentrations. Stable secondary CPPs form when fetuin-A concentrations exceed 7 µM (top panel). In the presence of high concentrations of serum albumin much lower fetuin-A levels suffice to form long-term stable CPPs. Shown here is an example of a mixture of 1.5 µM fetuin-A and > 75 µM albumin effecting long term inhibition (LTI, bottom panel).

Regression of pathological Mineralization

Ectopic soft tissue mineralization resembles physiological bone mineralization in many ways. Markers of mineralizing bone also are present during soft tissue mineralization. We postulate that it may be possible to reverse soft tissue mineralization by applying selected principles of bone catabolism, namely mineral dissolution and phagocytosis. We consider putative strategies for therapeutic intervention to maximize the clearing of calcified debris particles. In particular, we study the roles of the plasma protein fetuin-A/alpha2HS-glycoprotein and the mineral-binding protein osteopontin (OPN) in the prevention and possible regression of mineralization in disease.

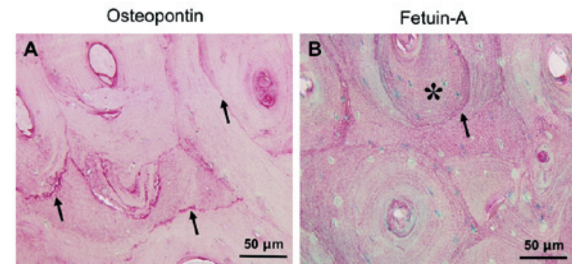


Figure 4: The mineral chaperones osteopontin and fetuin-A are present in high amounts in naturally mineralized bone matrix (shown here are osteons of mouse tibia cortical bone). Macrophages may be used to treat pathological calcification.

We focus on novel strategies of direct and active reversal of pathological calcification. Anecdotal clinical evidence demonstrates that removal of even excessive metastatic/tumorous calcification may be possible without medication using existing, intrinsic physiological mechanisms. While clearly not understood at this time, a better elucidation of "mineral clearing" would potentially enable the harnessing of these processes for future anti-calcification therapies. Physiological "demineralization" that occurs during bone turnover involves osteoclast activities that lower extracellular pH to levels that dissolve apatitic mineral. Thus, it would seem reasonable that similar processes might be activated intracellularly (and possibly also extracellularly) at pathological calcification sites by osteoclasts or their lineage-related macrophages. Electron micrographs depicted in figure 5 show that macrophages are present at extracellular calcified lesions in fetuin-A-deficient mice. In all these cases, the resident macrophages secrete large amounts of osteopontin, an inhibitor of vascular calcification with an RGD cell adhesion sequence, and an autostimulatory cytokine promoting recruitment and retention of macrophages and T-cells to sites of inflammation. OPN, in turn, regulates the production of inflammatory cytokines and nitric oxide in macrophages.

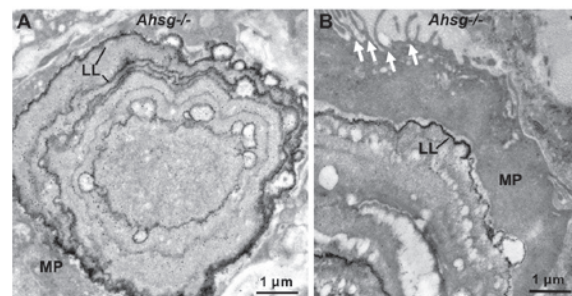


Figure 5: Transmission electron microscopy of macrophage interactions with ectopic calcification. A), B) Black dots in the lamina limitans (LL) show OPN localization with immunogold. Mineral deposits in the lungs of fetuin-A-deficient (Ahsg-/-) mice are surrounded by macrophages (MP). B) The macrophages have typical lamellipodiae cell processes (white arrows).

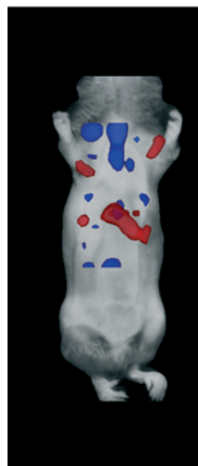
A substantial percentage of the secreted OPN from macrophages adsorbs directly onto mineral at pathological calcification sites in fetuin-A-deficient mice, presumably there acting as a calcification inhibitor and/or opsonin. This immobilization of OPN onto the mineral phase simultaneously provides an anchor for the protein to then present its cell adhesion sequences (such as RGD) to macrophages and other cells. In terms of the opsonization abilities of OPN, mineralized bone and tooth debris particles at a surgical site are coated with macrophage-derived OPN, and then internalized into macrophages by phagocytosis.

Clearing of CPPs

To investigate the clearing process of CPPs in vivo we generated fluorescence-labeled CPPs in vitro and injected them into mice to study the clearance rate and the target tissues. For comparison of the clearing process and to identify potential target cells, fluorescent polystyrene beads of about the same size as CPPs (diameter: 170 nm and 50-150nm, respectively) were also injected and the colocalization with cell type specific markers was analyzed by fluorescence microscopy (see cover figures). The major organs of the reticuloendothelial system involved in clearing are the liver (No. 8 in the top image) and the spleen (No. 9). The nanoparticles (green fluorescence) were found mainly in Kupffer cells (center panel) and in the marginal zone of the spleen (bottom panel). Macrophages in the red pulp are stained red.

In vivo imaging

The exact localization of the initiation points of the microcalcifications in fetuin-A-deficient mice and the processes involved in further progression of the mineralization are under investigation. In cooperation with the group of Professor Kiessling we investigate the distribution of mineral precipitates or



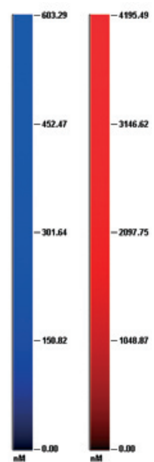


Figure 6: Fluorescence mediated tomography of a fetuin-A-deficient mouse showing calcification in blue color and protease activity and thus inflammation in red color.

Some live more dangerous than others: Whole genome expression studies may reveal novel calcification inhibitors.

microcalcifications in fetuin-A-deficient mice using fluorescence mediated tomography (FMT) and highly sensitive molecular probes for hydroxyapatite and inflammation-associated protease activity (figure 6). This method allows the investigation of the progression of soft tissue calcification and associated inflammatory processes in the same animal for long periods of time.

We have previously shown that fetuin-A deficiency in combination with the calcification sensitive strain DBA/2 (D2), but not in combination with the C57BL/6 (B6) genetic background results in widespread soft tissue calcification (figure 7). Thus B6 mice are better protected against pathological calcification even in the absence of fetuin-A.

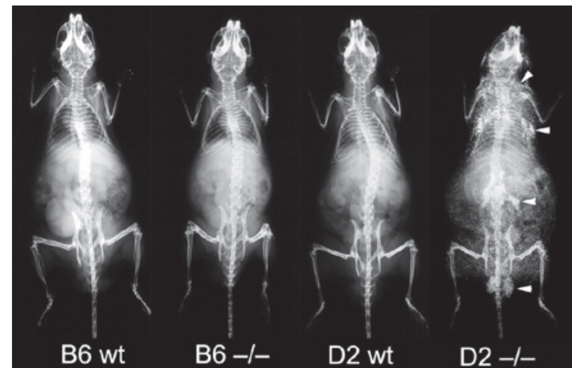


Figure 7: B6 mice are resistant to soft tissue calcification in the absence of fetuin-A: X-ray examination reveals widespread and systemic ectopic calcification in fetuin-A-deficient mice on D2 but not on B6 genetic background.

To gain further insight into the molecular mechanisms governing pathological mineralization and to reveal additional anti-calcification mechanisms we performed comparative microarray analysis of B6 and D2 wildtype and KO mice. Soft tissue calcifiation in D2 fetuin-A-deficient mice occured in several organs including the kidney. The kidney plays an outstanding role in mineral homeostasis therefore

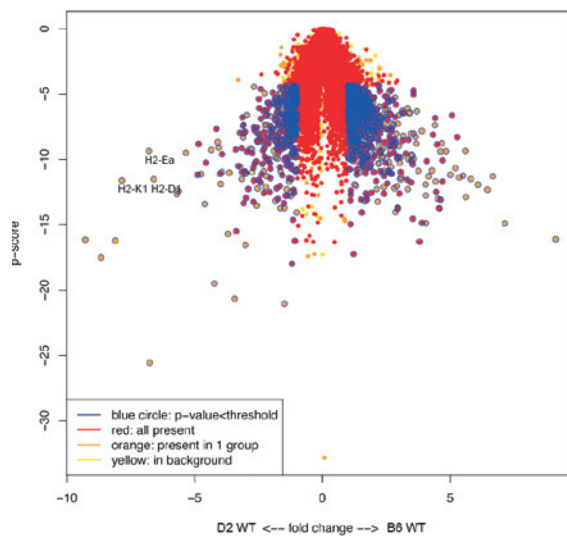
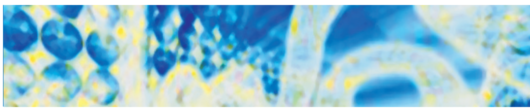


Figure 8: Comparative gene expression analysis of kidney tissue from D2 and B6 wildtype mice. The volcano plot reveals strain specific differences in the gene expression profile, e.g some histocompatibility antigens as marked out.



kidney tissue was chosen to detect candidates for calcification inhibition in B6 mice respectively modulators of the calcification phenotype in D2 mice. Genes with strain specific differences in expression values were excluded by a comparison between B6 and D2 wildtype mice (figure 8). A heatmap representation of the candidate genes is shown in figure 9, genes with low expression values are drawn blue, red color encodes "hot" candidates with high expression values. Putative inhibitors of calcification should differ in their expression levels between B6 fetuin-A-deficient (B6 KO) and all other mouse groups as is indicated by a different color distribution. A large group of potential modulators of the calcification phenotype in D2 mice is shown by an aberrant expression profile in D2 fetuin-A-deficient (D2 KO) mice.

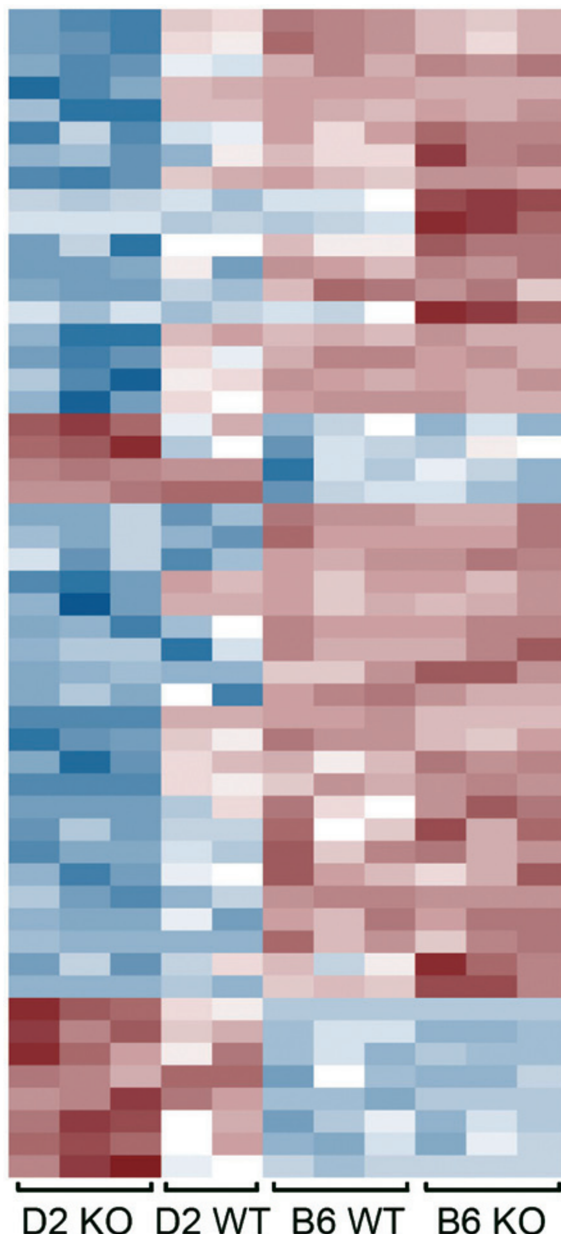


Figure 9: Heatmap representation of selected candidate genes: Differences in the expression profile indicated by the color distribution reveal putative compensatory upregulated genes in B6 fetuin-A-deficient (B6 KO) or modulators of the calcification phenotype in D2 fetuin-A-deficient (D2 KO) mice.

The Lost Treasure – Gold Nanoparticles too small to see

Gold endocytosis can also be quantified by measuring the decreasing gold content in the supernatant using atomic absorption spectroscopy. This technique demonstrates substantial binding and endocytosis of the particles within half an hour and subsequent release, probably by necrotic cells

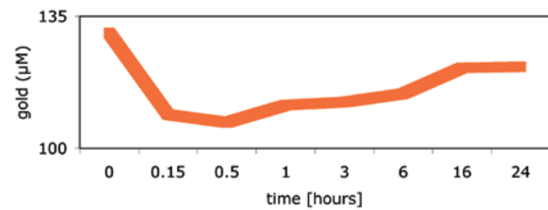


Figure 10: Indirect quantification of gold nanoparticle uptake by HeLa cells by measuring the remaining gold content in the supernatant.

from 3 hours onward (figure 10).
We study the cytotoxicity of gold nanoparticles (AuNP). Endocytosis is a prerequisite of cellular toxicity, but is not easily studied in ultrasmall AuNP, because they defy most routine analytical methods. We have previously shown that Au1.4MS (gold nanoparticles of 1.4 nm diameter) are cytotoxic. Here we studied cellular uptake and trajectories of these particles and of larger particles (15 nm) by transmission electron microscope (TEM), silver enhancement, cell fractionation combined with neutron activation analysis, and atomic absorption spectrometry (AAS) measurement of gold atom concentration in cell culture supernatant.

Regular TEM employing contrast agents is well suited to determine the uptake and intracellular localization of 15 nm AuNPs, but not of 1.4 nm gold nanoparticles (figure 11). To detect intracellular

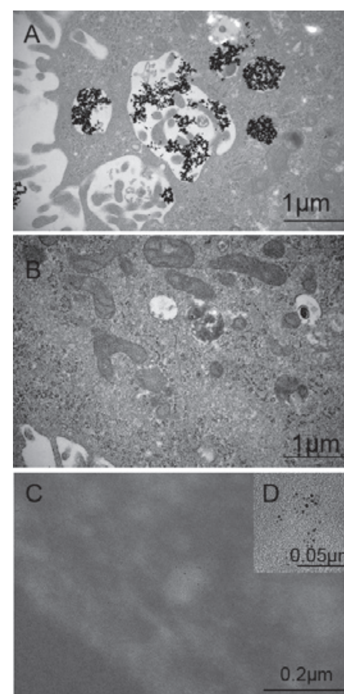


Figure 11. A) Endocytosis of Au15MS by HeLa cells is readily detected by routine TEM using conventional contrasting technique. B) Ultrasmall Nanoparticles of 1.4 nm diameter (Au1.4MS) remain invisible using this routine. To visualize these small particles inside cells non-contrasted sections (C) have to be viewed at high magnification (D) using a high-contrast camera.

Au14MS we had to employ TEM without contrast enhancement, rendering cellular organelles barely visible. Silver enhancement may be used to monitor the uptake of 15 nm

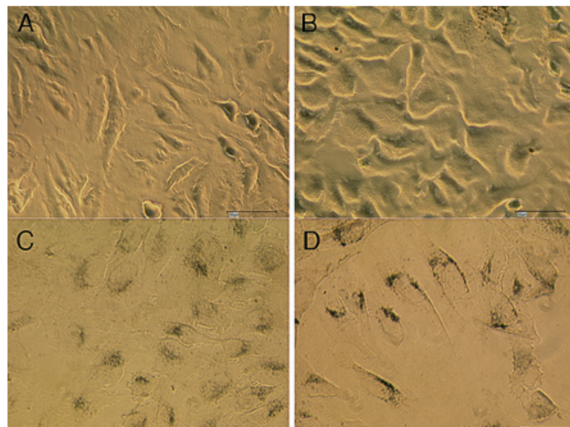


Figure 12: Endocytosis of Au15MS by HeLa cells. A) untreated HeLa; B-D) HeLa cells incubated with Au15MS for 1, 6, 12 hours respectively. Gold nanoparticles that are normally invisible in light microscopy can be visualized using silver enhancement as black precipitate inside the endocytic compartment.

gold nanoparticles using light microscopy (figure 12).
Thus a combination of chemical analysis, special electron and light microscopy and cell biology enable the study of nanoparticles even though they may not be visualized using conventional techniques.

Publications

[1] Westenfeld R, Schäfer C, Krüger T, Haarmann C, Schurgers L, Reutelingsperger C, Ivanovski O, Drueke T, Massy ZA, Ketteler M, Floege J, Jahnen-Dechent W.: Fetuin-A protects against atherosclerotic calcifications in mice with renal failure. J Am Soc Nephrol 2009, in press.

[2] Sabine N, Schneider RKM, Tietze L, Knüchel R, Jahnen-Dechent W.: Secretion of fibrinolytic enzymes facilitates human mesenchymal stem cell invasion into fibrin clots. Cells Tissue Organs, 2009, in press.

[3] Rochette CN, Rosenfeldt S, Heiss A, Narayanan T, Ballauff M, Jahnen-Dechent W.: A Shielding Topology Stabilizes the Early Stage Protein-Mineral Complexes of Fetuin-A and Calcium Phosphate: A Time Resolved Small Angle X-Ray Study. ChemBioChem. 2009; 10 (4):735-740.

[4] Bozkurt A, Deumens R, Beckmann C, Olde Damink L, Schügner F, Heschel I, Sellhaus B, Weis J, Jahnen-Dechent W, Brook GA, Pallua N.: In vitro cell alignment obtained with a Schwann cell enriched microstructured nerve guide with longitudinal guidance channels. Biomaterials. 2009; 30 (2): 169-179.

[5] Lee C, Bongcam-Rudloff E, Söllner C, Jahnen-Dechent W, Claesson-Welsh L.: Type 3 cystatins; fetuins, kininogen and histidine-rich glycoprotein. Frontiers in Bioscience. 2009; 14: 2911-2922.

[6] Jahnen-Dechent W, Simon U.: Function follows form: shape complementarity and nanoparticle toxicity. Nanomedicine, 2008; 3 (5): 601-3.

[7] Rydengård V, Shannon O, Lundqvist K, Kacprzyk L, Chalupka A, Olsson AK, Mörgelin M, Jahnen-Dechent W, Malmsten M, Schmidtchen A.: Histidine-rich glycoprotein protects from systemic Candida infection. PLoS Pathogen, 2008; 4 (8): e1000116.

[8] Heiss A, Eckert T, Aretz A, Richtering W, van Dorp W, Schäfer C, Jahnen-Dechent W.: Hierarchical Role of Fetuin-A and Acidic Serum Proteins in the Formation and Stabilization of Calcium Phosphate Particles.
J Biol Chem. 2008; 23; 283(21): 14815-25.

[9] von Walter M, Steffens GCM, Hermanns B, Jahnen-Dechent W, Rüger M, Erli HJ.: Biomimetic modification of the TiO2/glass composite Ecopore with heparinized collagen and the osteoinductive factor BMP-2. Acta Biomater. 2008 Jul;4 (4): 997-1004.

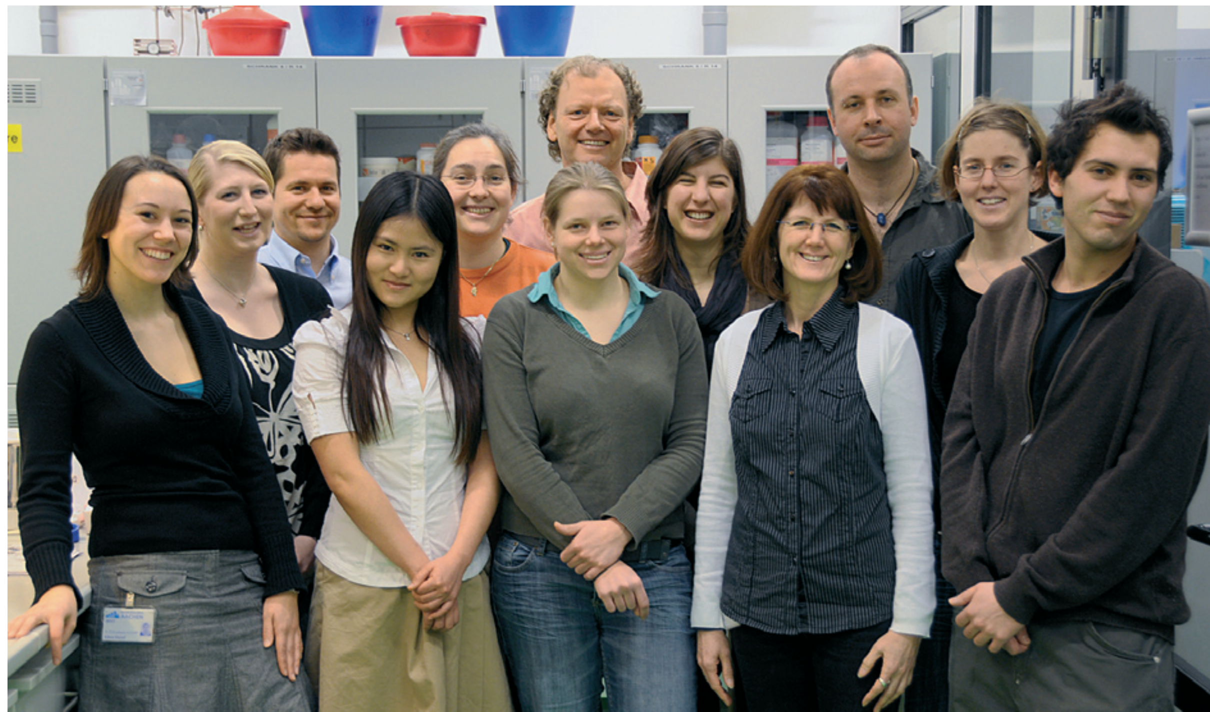
[10] Jahnen-Dechent W, Schäfer C, Ketteler M, McKee M.: Mineral chaperones: A role for fetuin-A in the inhibition and regression of pathologic calcification. J Mol Med. 2008; 86 (4): 379-89.

[11] Neuss S, Apel C, Buttler P, Denecke B, Dhanasingh A, Ding X, Grafahrend D, Gröger A, Hemmrich K, Herr A, Jahnen-Dechent W, Mastitskaya, S, Perez-Bouza A, Rosewick S, Salber J, Wöltje M, Zenke M.: Assessment of stem cell/biomaterial combinations for stem cell-based tissue engineering. Biomaterials. 2008; 29: 302-313.

Book chapter

[12] Denecke, B., Wöltje, M., Neuss, S. & Jahnen-Dechent, W. : Tissue Engineering – Combining Cells and Biomaterials into Functional Tissues. In: Bioengineering in Cell and Tissue Research (Artmann, G. M. & Chien, S., eds.), Springer Berlin, Heidelberg, 2008; Vol. III, 19.

Team



The Biointerface Lab members in March 2009