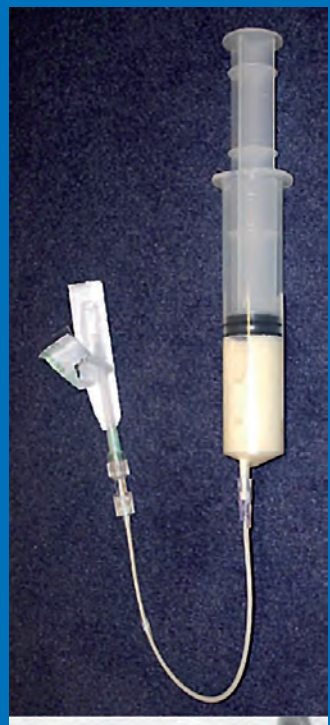
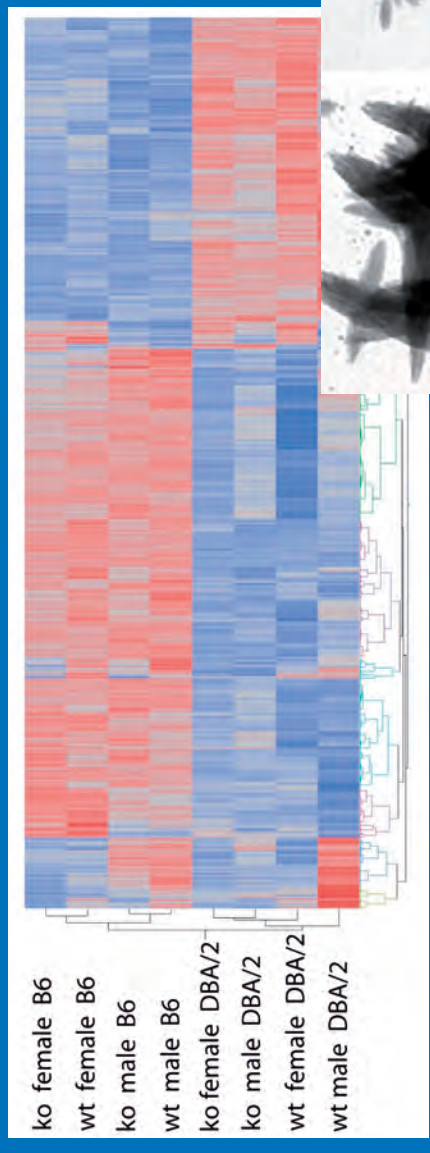
Biointerface

Faculty of Medicine

Return Of The Opsonin: Common Features of Mineralization Biology and Nanoparticle Clearing





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Calcifying mice and men, stiff arteries and bone turnover

We start our report of 2007 by quoting the editorial of the March, 26 issue of the Journal Nephrology Dialysis and Transplantation:
»The occurrence of extraosseous calcification and its prevention is one of the "hot" topics in nephrology. A number of inhibitors of ectopic calcification have been identified and among them fetuin-A has received the most attention. In this elegant study, using wild-type and fetuin-A-deficient mice, further evidence for the "protective" role of fetuin-A was provided. The authors could demonstrate that fetuin-A deficiency, chronic kidney diseases and a high phosphate diet act synergistically in the pathogenesis of extraosseous calcification.«

In this article we studied the role of fetuin-A in chronic kidney disease (CKD)-associated calcification using a mouse model of graded renal insufficiency generated by nephrectomy and high phosphate diet. We used wildtype and fetuin-A deficient mice on the calcification resistant genetic background C57BL/6 to study the influence on calcification of CKD, dietary phosphate and fetuin deficiency.

To gain further insight into the molecular mechanisms governing pathological mineralization and to reveal putative anti-calcification mechanisms we performed comparative microarray analysis of C57BL/6 (B6) and DBA/2 wildtype (wt) and knockout (ko) mice. The bottom figure on the title page shows the hierarchical clustering of differentially regulated genes in the kidneys of mice. The gene clusters are arranged according to genetic background DBA/2 or B6, fetuin-A genotype and sex. A single glance at these data suggests a more prominent effect of fetuin-A deficiency in the DBA/2 than in the B6 strain, reflecting the calcification phenotype. In addition the clustering of DBA ko mice and the female wt mice confirm a long-held contention that females may be pre-disposed to unwanted calcification.

Macrophages are potential effector cells controlling pathologic mineralization

Interestingly we detected osteopontin (OPN), a bone matrix protein, at all calcified lesions. OPN expression is regarded by many as a marker of osteogenic differentiation of cells actively driving calcification. We had previously noted strong osteopontin expression also at calcified lesions of our spontaneously calcifiying fetuin-A deficient DBA/2 mouse (Figure 2). Osteopontin was clearly associated with macrophages not osteoblastic cells. This led us to revisit the potential role of macrophages in pathological calcification. We suggest that calcification - like atherosclerosis - may at least in part be a chronic inflammatory

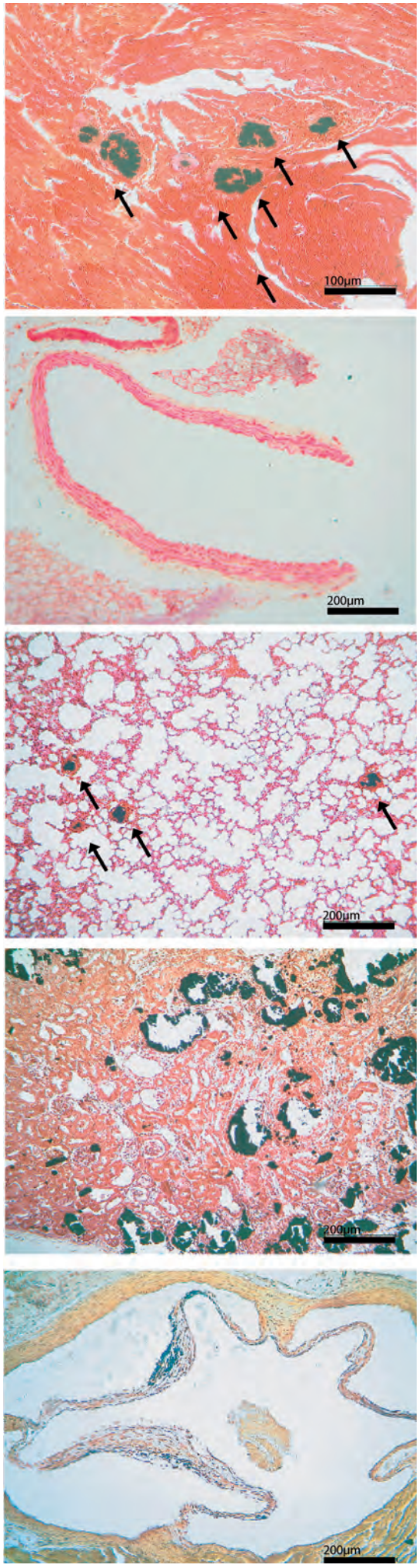

Figure 1: Extent and localization of soft tissue calcification. photomicrographs of von Kossa stained sections from Ahsg-deficient mice suffering CKD. Shown are (from top to bottom) myocardium, aorta, lung, kidney and heart valves. Calcified lesions (black stain) were found in all tissues except the aorta.

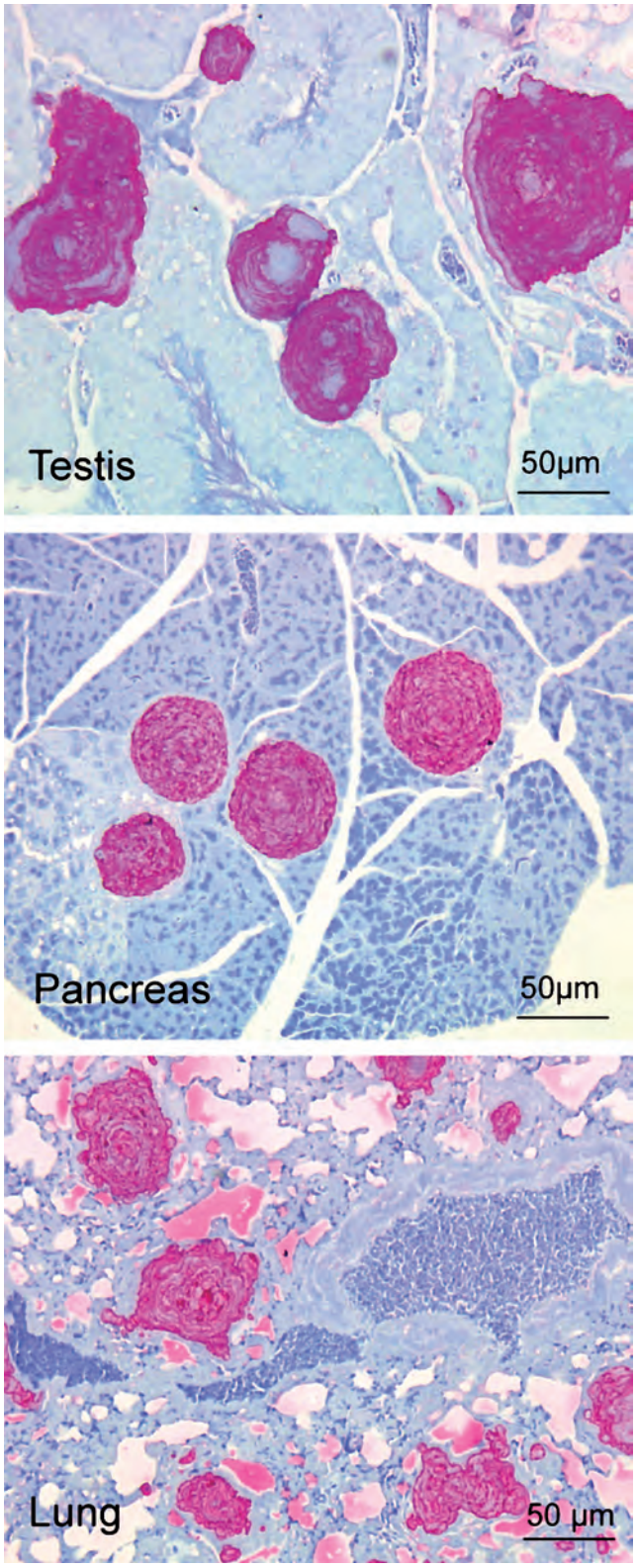

Figure 2. Immunohistochemistry of fetuin-A-deficient mouse for osteopontin (OPN) (purple) using paraffin sections of decalcified testis, pancreas, and lung shows intense immunostaining associated with the mineral deposits, often presenting as repeating concentric rings of OPN likely reflecting alternating periods of mineral growth and inhibition by macrophage-derived OPN secreted at the calcification sites.

disease associated with defective clearing by macrophages, a »scavenging disease«.

Immunohistochemical analysis using macrophage-specific antibody F4/80 showed that the calcified lesions are indeed surrounded by a dense layer of macrophages (Figure 3).

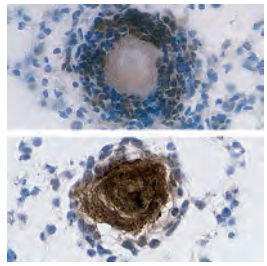

Figure 3: Light microscopic view of calcified lesion in the lung of a fetuin-A-deficient mouse showing a dense layer of F4/80 positive macrophages (top panel) surrounding the von OPN-positive mineral debris (bottom panel).

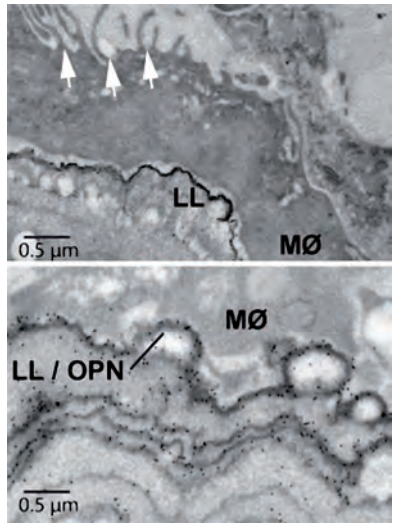

Figure 4: Transmission electron microscopy of macrophage interactions with ectopic calcification, and immunogold localization of osteopontin (OPN). Mineral deposits in lungs of fetuin-A-deficient mice show engulfment by macrophages (MØ). These cells project lamellipodial extensions along the surface of spherulitically shaped mineral deposits coated by a thin layer of organic material (LL, lamina limitans) rich in OPN that is labeled with immunogold.

Electron micrographs depicted in Figure 4 confirmed that macrophages are present at extracellular calcified lesions in fetuin-A-deficient mice. The resident macrophages secrete large amounts of osteopontin (OPN), 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 also regulates the production of inflammatory cytokines and nitric oxide in macrophages. A substantial percentage of the secreted OPN from macrophages adsorbs directly onto mineral at pathologic 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. OPN is thus promoting opsonization. At present, it is still unknown if these macrophages remove mineralized remnants in a non-inflammatory fashion like osteoclasts do in bone, or whether they are pro-inflammatory like atherosclerosis-associated foam cells, which worsen the disease. Alternatively, they could become “frustrated” macrophages, which enter anergy like macrophage-derived foreign body giant cells arising from attempts to clear intentionally implanted or intrusive materials.

To study this important aspect of calcification we have begun to take a closer look at macrophage biology.

Pro- and anti-inflammatory macrophages

Mice and men offer a wide variety of professional phagocytes that vary in their activation pattern and immunological activity. Pro-inflammatory macrophages are primed to stimulate both the innate and adaptive immune system to fend off pathogenic invaders to kill bacteria and virus. In contrast, alternatively activated macrophages are predominantly anti-inflammatory. These cells clear debris and apoptotic cells without overt inflammation. The clearing of dead cells and debris is mostly performed by sessile macrophages of the reticulo-endothelial system, RES. We have derived two different populations of macrophages from bone marrow (BMMØ) and from mouse embryonic stem cells (ESMØ) to study this phenomenon. BMMØ were differentiated from primary bone marrow stem cells. Upon activation BMMØ express pro-inflammatory cytokines and chemokines. In the embryo inflammation is absent and macrophages remain »silent« while dutifully clearing apoptotic cells that are left behind during tissue remodeling. Besides, embryonic wounds heal scar-free, again suggesting that pro-fibrotic stimuli of the inflammatory type that are largely derived from macrophages are absent.

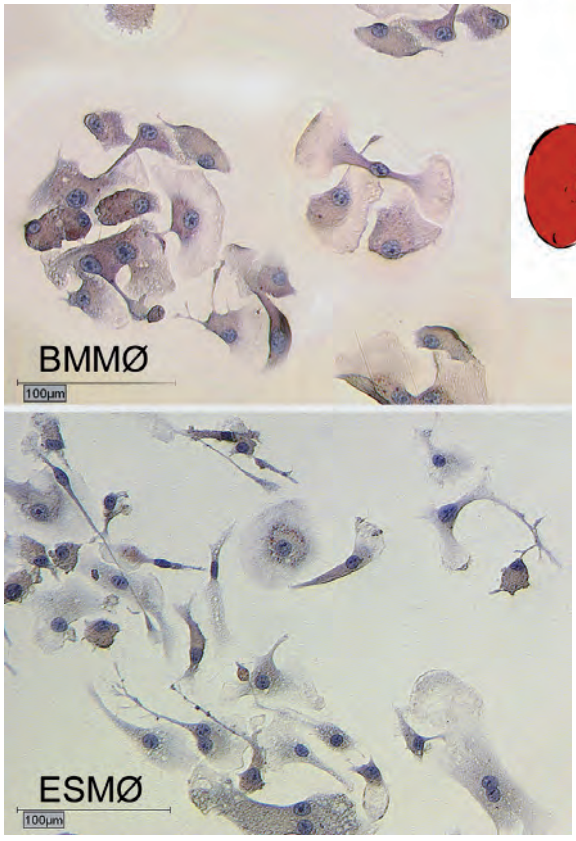

Figure 5: Macrophages derived from bone marrow (BMMØ) and from mouse embryonic stem cells (ESMØ). The cells differ in morphology, proliferation and gene expression. Note the elongated morphology of ESMØ.

The ESMØ shown in Figure 5 were derived from embryonic stem cells and thus should resemble these early macrophage subtypes.

Clearing of calcified debris is a part of physiological bone remodeling

Previously we suggested a clearing pathway for calciprotein particles, CPPs, a mineral-protein complex consisting of the serum protein fetuin-A, calcium and phosphate. The structure, function and biology of CPPs show intriguing similarities with lipoprotein particle metabolism. In 2007 we have detected CPPs for the first time in a human patient, a peritoneal dialysis patient suffering from calcifying peritonitis. The picture on the cover page shows a syringe containing peritoneal aspirate. The milky appearance is caused by rice-grain shaped protein-mineral particles identical in morphology and composition to CPPs. We can now refine this pathway to include bone mineral turnover.

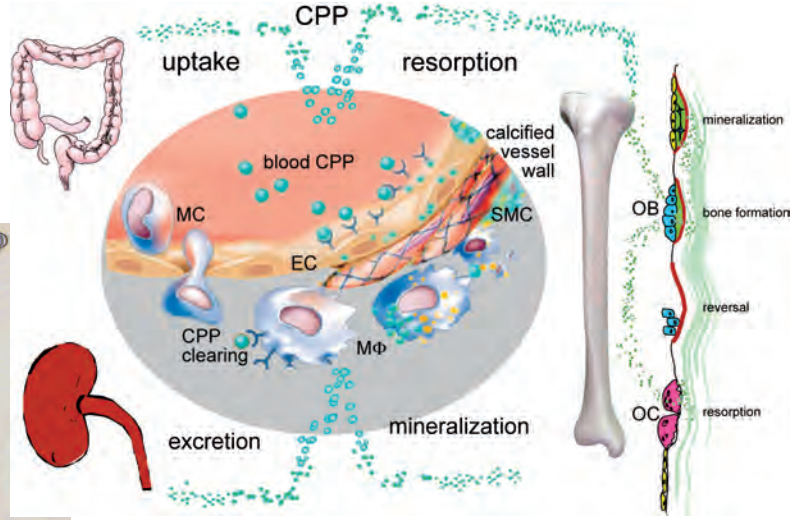

Figure 6: Proposed clearing pathways for calciprotein particles, CPP in health and disease. From Jahnen-Dechent et al., 2007.

The next logical step will be unraveling the clearing of CPPs in vivo. Several reports indicate that innate immune cells like macrophages and neutrophils create an inflammatory environment in response to basic calcium phosphate (BCP) thus mediating progression of calcification. Down-regulation of proinflammatory cells and enhancement of phagocytosis by opsonization by plasma proteins like fetuin-A are believed to enhance clearing of calcified remnants. In a first series of phagocytosis assays using synthetic CPPs and a macrophage cell line we observed highly efficient clearing of CPPs and comparatively less uptake of free monomeric fetuin-A.

Studying clearing in whole animal studies is now possible. We expect that cells of the reticulo-endothelial-system (RES) will be important in removing calcified remnants and CPPs very efficiently. Experiments to this effect are ongoing.

We have shown on many levels of complexity that fetuin-A stabilizes mineral and helps cells to handle excessive

mineral loads. A clear picture is emerging of fetuin-A fulfilling multiple roles in the prevention of calcification. Firstly fetuin-A stabilizes calcium phosphate in solution, in the form of CPPs (see above). Next, fetuin-A allows cells to handle elevated extracellular calcium and phosphate without calcifiying. Last not least fetuin-A opsonizes mineral debris facilitating its removal by the RES.

Size dependent cytotoxicity of gold nanoparticles

In 2007 we started a new collaborative research project funded by the Deutsche Forschungsgemeinschaft. Together with colleagues from the Institute for Inorganic Chemistry, RWTH Aachen (Prof. Simon) and University of Essen (Proff. Schmid and Brandau) we study the cellular trajectories and the cytotoxicity of noble metal nanoparticles. Initally we concentrated on gold nanoparticles (AuNP), because they can be synthesized in a wide size range essentially using the same stabilizing ligand chemistry.
Gold nanoparticles are widely used in biomedical imaging and diagnostic tests. Based on their established use in the

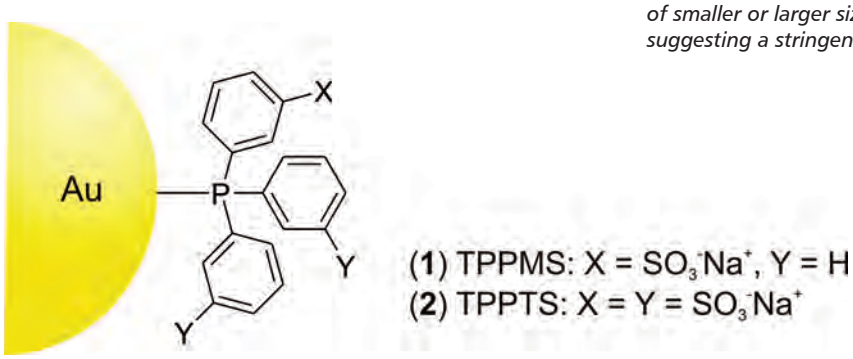

Figure 7: A gold nanoparticle with ligand shell

laboratory and the chemical stability of Au0, gold nanoparticles were expected to be safe. The recent literature however, contained conflicting data regarding the cytotoxicity of gold nanoparticles. Against this background we undertook a systematic study of water-soluble gold nanoparticles stabilized by triphenylphosphine monosulfonate (TTPMS) derivatives ranging in size from 0.8 nm to 15 nm.

We tested the cytotoxicity of these particles in four cell lines representing major functional cell types with barrier

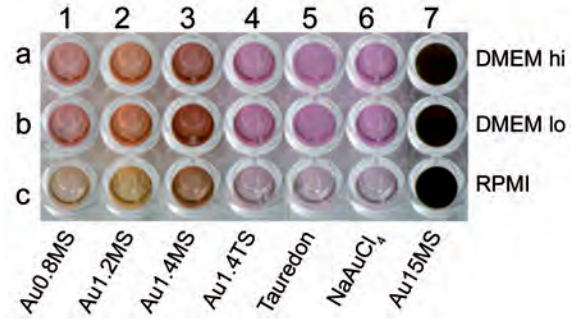

Figure 8: Stability of AuNP in cell culture media. Macroscopic view of Au compounds dissolved in cell culture media RPMI, DMEM/low glucose (lo), high glucose (hi) medium.

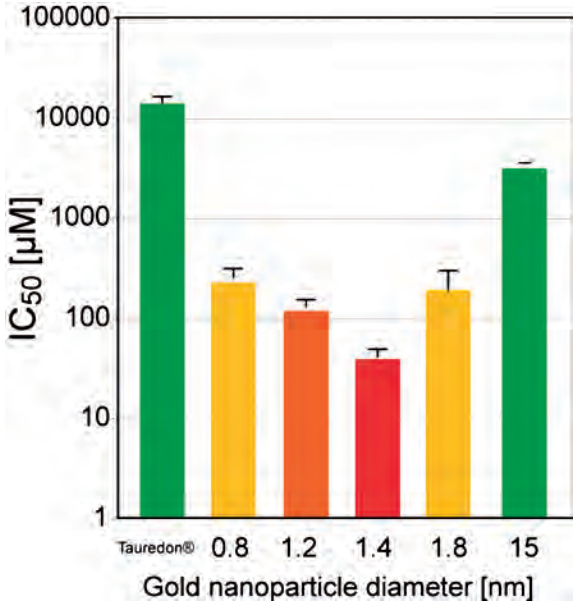

Figure 9. Cytotoxicity of Au compounds during the logarithmic growth phase of HeLa cells. Note that the IC50 values of Au1.4MS were lowest and that Au compounds of smaller or larger size were progressively less cytotoxic suggesting a stringent size dependency of cytotoxicity.

and phagocyte function. Connective tissue fibroblasts, epithelial cells, macrophages and melanoma cells proved most sensitive to gold particles of 1.4 nm size resulting in IC50 values ranging from 30-56 µM depending on the particular Au1.4nm compound-cell line combination. In contrast, gold particles of 15 nm size and Tauredon® (gold thiomalate) were non-toxic up to 60-fold and 100-fold higher concentration, respectively. The cellular response was size-dependent in that 1.4 nm particles caused predominantly rapid cell death by necrosis within 12 hours while closely related particles of 1.2 nm diameter effected predominantly programmed cell death by apoptosis.

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Further reading

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Cooperations

- Simon, Anorganische Chemie RWTH Aachen
- Richtering, Physikalische Chemie RWTH Aachen
- Möller, TexMC RWTH Aachen
- Universität Bayreuth
- Universität Bonn
- Universität Duisburg – Essen
- Forschungszentrum Jülich
- Australian National University
- University College London
- Mc Gill University Montreal
- University Uppsala

Patent applications

- Treatment of Diseases with Nanoparticles Having a Size-Dependent Cytotoxicity, EP 07 006 822.6; EP 07 008 035.3; US patent pending
- Verwendung des Serumproteins Fetuin-B und dessen natürlichen, synthetisch oder gentechnisch hergestellten Surrogaten zum Nachweis von Fertilitätsstörungen sowie zur Steuerung der Fertilität in Mensch und Tier. DE 10 2007 045 691.5

Team

Figure 10: The Biointerface group at the time of writing in April 2008.

In October 2007, Alexander Heiss moved to Forschungszentrum Jülich and Sven Brinckers to a private laboratory in Düsseldorf. In November Jennifer Goldstein finished her PhD »with distinction« and took a job with a large International Pharmaceutical Company. We congratulate our colleagues to their successful career moves and we will stay in touch...

Labout 2007

Figure 11. Labout 2007 of Biointerface and Cell Biology groups, and staff from IZKF BioMAT. central laboratory at »Industriepark Duisburg Youth Hostel«.