64. D'Haese PC, Spasovski GB, Sikole A *et al.* A multicenter study on the effects of lanthanum carbonate (Fosrenol) and calcium carbonate on renal bone disease in dialysis patients. *Kidney Int Suppl* 2003; 85: S73–S78
65. Teng M, Wolf M, Lowrie E *et al.* Survival of patients undergoing hemodialysis with paricalcitol or calcitriol therapy. *N Engl J Med* 2003; 349: 446–456
66. Aladren Regidor MJ. Cinacalcet reduces vascular and soft tissue calcification in secondary hyperparathyroidism (SHPT) in hemodialysis patients. *Clin Nephrol* 2009; 71: 207–213
67. Floege J, Raggi P, Block GA *et al.* Study design and subject baseline characteristics in the ADVANCE Study: effects of cinacalcet on vascular calcification in haemodialysis patients. *Nephrol Dial Transplant* 2010; 25: 1916–1923
68. Raggi P, Chertow G, Block G *et al. A Randomized Controlled Trial To Evaluate the Effects of Cinacalcet Plus Low-Dose Vitamin D On Vascular Calcification in Hemodialysis Patients* 2010. National Kidney Foundation; 2010. Spring Clinical Meetings Abstracts
69. Chonchol M, Locatelli F, Abboud HE *et al.* A randomized, double-blind, placebo-controlled study to assess the efficacy and safety of cinacalcet HCl in participants with CKD not receiving dialysis. *Am J Kidney Dis* 2009; 53: 197–207
70. Cannata-Andia JB, Fernandez-Martin JL. Mineral metabolism: Should cinacalcet be used in patients who are not on dialysis? *Nat Rev Nephrol* 2009; 5: 307–308
71. Helas S, Goettsch C, Schoppet M *et al.* Inhibition of receptor activator of NF-kappaB ligand by denosumab attenuates vascular calcium deposition in mice. *Am J Pathol* 2009; 175: 473–478

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A red herring in vascular calcification: 'nanobacteria' are protein–mineral complexes involved in biomineralization

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Abstract
Biomineralization at pathological extraosseous sites (i.e. vasculature and soft tissues) is associated with increased morbidity and mortality. So-called 'nanobacteria' have been described as pathogenic agents causing many diseases including calcification. Initially, their appearance, and having a content consisting of nucleic acids plus proteins and properties of growing structures, suggested that they were living organisms. However, it could be demonstrated that the so-called nanobacteria were in fact mineralizing nanoparticles that contain mineral and non-mineral compounds, that these particles bind to charged molecules and that supersaturation enables *in vitro* growth of these nanoparticles. Recent data indicate that nanoparticles consisting of protein–mineral complexes can be seen both *in vitro* and *in vivo* as precursors of matrix calcification.

Keywords: biomineralization; nanobacteria; nanoparticles; vascular calcification

Introduction

In living vertebrates, biomineralization is a highly regulated cell-autonomous process, usually restricted to the skeleton and teeth. However, biomineralization may also occur as pathological extraosseous calcification in the vasculature or soft tissues, leading to an increased morbidity and/or mortality. Until approximately one decade ago, extraosseous calcification was mainly studied with regard to the chemical process of precipitation due to supersaturation of calcium and phosphate ions. Since it was discovered that tissues involved in pathological calcification also expressed genes initially discovered in bone metabolism, these putative osteogenic processes (active calcification) contrasted with the chemical precipitation of calcium salts (passive calcification). It is most likely that both processes contribute to extraosseous calcification [1, 2]. It is well-known that calcium, phosphate and mineralizable matrix-like collagen fibres are sufficient to induce tissue calcification in the absence of osteoblasts [3]. Dead cells and necrotic tissues form an excellent mineralizable matrix, and in this case, the process is called 'dystrophic calcification'. One and a half decades ago, so-called 'nanobacteria' were described as pathogenic agents causing calcification. However, recent results demonstrate that this approach was merely a 'red herring', which put us on the wrong track.

The discovery of nanobacteria and evidence for their existence

Around 15 years ago, nanoscopic life forms called nanobacteria entered the stage [4, 5] and eventually were described as

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the causative agent of many diseases including calcification. Initially, Folk [4] had studied rock specimens by electron microscopy and observed tiny structures with a cell-like appearance i.e. resembling cell walls and filamentous surface projections. These particles were only 10–200 nm in size and thus were much smaller than any other known bacteria. Therefore, Folk [4] called these tiny particles nanobacteria. A few years later, McKay *et al.* [5] observed similar tiny structures within a Martian meteorite, which caused considerable excitement, as these and other structures pointed to the possibility of extraterrestrial life. In 1998, Finnish researchers described similar structures (50–500 nm) with nucleic acids and proteins as potential pathogens in their cell culture (Figure 1) [6]. Interestingly, these small particles could change from small spherical bodies to films and clumps of mineralized material containing hydroxyapatite, the main mineral of bone. Further research revealed that similar structures existed in body fluids including blood and urine. These entities were deemed 'infectious' because 'inoculating' mineral-containing fluids with these entities caused a slow 'reproduction' of these entities. Ultimately, nanobacteria were considered the causative pathogens for many diseases from kidney stones to cancer [7, 8]. Polycystic kidney disease was assumed to be caused by these agents [9], and nanoparticles were associated with calcified blood vessels [10] as well. These particles seemed to contain DNA, proteins and synthesized RNA. These findings caused a major boom for nanobacteria, to the extent of receiving tabloid press coverage followed by the founding of highly promising start-up companies.

Nanobacteria do not exist and simply represent nanoparticles

However, in the year 2000, Cisar *et al.* [11] explored the so-called nanobacteria and determined that phospholipids could bind and, thereby, facilitate the formation of calcium–phosphate crystals which resembled these nano-sized structures. Secondly, it was observed that the crystalline structures were shown to grow and replicate *in vitro* as if they were alive. Thirdly, it was demonstrated that the nucleic acid sequences thought to be a diagnostic marker of nanobacteria were in fact common sequences of nucleic acid frequently contaminating laboratories [11].

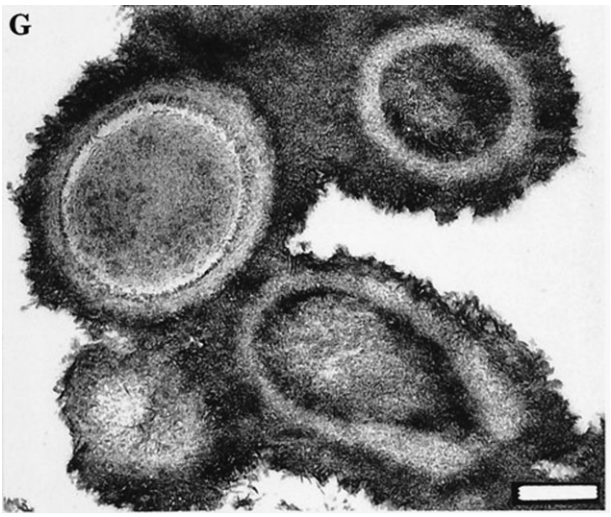

Fig. 1. Transmission electron micrograph of so-called 'nanobacteria' after a 3-month culture period (bar = 200 nm). Photograph taken with permission from [6] (copyright 1998 National Academy of Sciences, USA).

In addition, Martel and Young [12] performed a series of experiments on the origin of putative nanobacteria. Indeed, non-mineral compounds such as proteins interfered with the crystallization process. Surprisingly, calcium phosphate, together with non-mineral compounds, grew into nanoparticles that resembled the putative nanobacteria in structure and shape. Moreover, these nanoparticles showed a high-binding capacity to charged molecules such as ions, carbohydrates, lipids and nucleic acids. Depending on the ratio of mineral to non-mineral compounds, either crystallization to hydroxyapatite or to more complex forms took place [13, 14]. Raoult *et al.* [15] found that the main protein of the so-called nanobacteria was fetuin-A. Besides fetuin-A, other proteins such as albumin or apolipoproteins could also be identified [16]. Moreover, Young *et al.* [16] determined that polyclonal antisera raised against nanobacteria strongly cross-reacted with fetuin-A and albumin. Consequently, the term 'nanobacteria' was discarded and replaced with the term 'nanoparticles' [17].

Taken together, the putative living nanobacteria have been shown to be non-living nanoparticles containing both mineral and non-mineral compounds (Table 1) such as the calciprotein particles (CPPs) shown in Figure 2, which represent a possible multitude of calcifying nanoparticles in a very idealized form regarding both shape and composition. Nevertheless, the interaction of minerals with calcium-binding proteins suggests that these nanoparticles are part of the body's defence mechanisms against unwanted calcification. Thus, mineral–protein complexes seem to be part of the normal mineral homeostasis. If the mineral supersaturation, and thus the balance of mineral versus mineral-binding proteins (i.e. calcification inhibitor proteins), is tilted towards the mineral component, crystallization can take place. In stages of disease i.e. kidney stones or vascular calcification, this imbalance, and consequentially calcification, can take place [21].

Nanoparticles and their role in extraosseous calcification

Crystal formation starts with nucleation and subsequently proceeds to growth (Figure 3). Nucleation starts with small

Table 1. Nanoparticles as putative living organisms turned out to be a biochemical phenomenon

Nanoparticles as putative living organisms		Nanoparticles as biochemical phenomenon
Form and shape similar but much smaller than bacteria	→	Nanoparticles of mineral and non-mineral compounds
Content: nucleic acid and proteins	→	*In vitro* high binding to charged molecules
Growing structures	→	Saturated solution enables *in vitro* growth
Antibodies against nanobacteria	→	Antibodies against albumin and fetuin-A

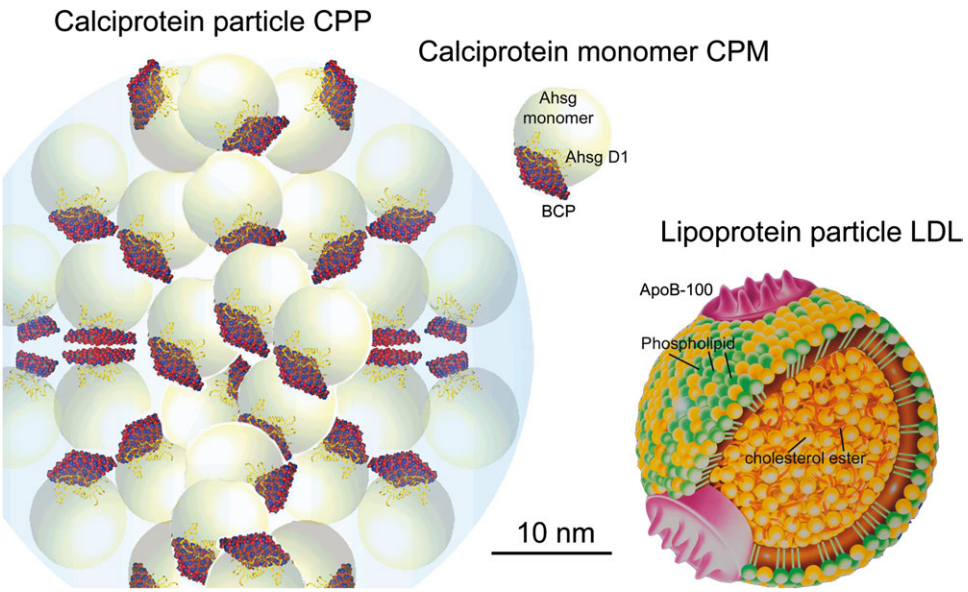

Fig. 2. Illustration of a CPP, calciprotein monomer (modified after [18, 19]) and a low-density lipoprotein (LDL) particle. The LDL particle is ~22 nm in diameter and contains many esterified cholesterol molecules in the hydrophobic core, cholesterol, phospholipids and a few apolipoprotein B-100 molecules in the hydrophilic coat (modified after [20]).

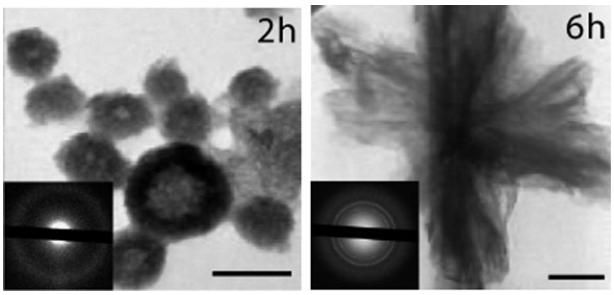

Fig. 3. Electron microscopic picture of synthetic CCPs [22]. The CCPs initially have a diameter of 30–150 nm (2 h, 37°C) and are amorphous as shown by diffraction analysis. Nextly, the CPPs are transformed, dependent on temperature, mineral ion supersaturation and fetuin-A concentration, into larger and crystalline mineral particles [23]. These particles are still soluble until ~24 h at 37°C. Similar particles have been detected in ascites of patients with sclerosing calcifying peritonitis [24]. Scale bars represents 100 nm. This research was originally published in [22] © the American Society for Biochemistry and Molecular Biology.

mineral ion clusters forming in supersaturated solutions of constitutive mineral ions. Coordination of calcium ions by proteins and phospholipids may regulate these events [25]. A critical size is required for stable nuclei, otherwise redissolution may occur. In the presence of preformed nuclei, crystal growth proceeds at a fast pace even in the absence of supersaturation. Fetuin-A has been shown *in vitro* to inhibit mineralization on the level of crystal growth by the transient formation of soluble protein–mineral complexes containing fetuin-A, calcium and phosphate [22, 26]. These CCPs start out as nanoscopic (50–150 nm diameter) colloidal spheres. Initially, they are amorphous and soluble but become progressively more crystalline and insoluble in a time- and temperature-dependent fashion [22]. Amorphous mineral phases admixed with protein are now widely recognized as the earliest manifestations of biomineralization, both in the mollusc shell and in the vertebrate skeleton [27, 28]. The mineral part itself has also been shown to contain spherical mineral particles with nanocrystalline needles of ~10 × 100 nm [29]. Self-assembly of nano-sized apatite particles seems to constitute a mechanism for the generation of larger biological mineral crystals [30]. Ultra high-resolution electron microscopy revealed that microcalcifications of 20–500 nm contained nanocrystals 2–10 nm in size [31]. Similar nanocrystals 20–25 nm in size were demonstrated in vascular calcifications [29, 32]. In addition, phosphate may induce calcification by enhancing nanocrystal formation [33]. These findings collectively suggest that early mineralization products are nanocrystalline and contain protein in the form of protein–mineral complexes. Furthermore protein–mineral complexes play an early and essential role in both physiological and pathological calcification. Not surprisingly, protein–mineral complexes have been described in experimental animal models of calcification [34] and, most recently, also in dialysis patients who are known to be at high risk of calcification [35]. Protein–mineral complexes/CPPs/calcifying nanoparticles should, however, not be confused with larger cell-derived and membrane-delineated vesicles including matrix vesicles or calcifying apoptotic vesicles that originate from actively mineralizing cells like osteoblasts or calcifying chondrocytes or from calcifying apoptotic or necrotic cells. Calcifying vesicles may nevertheless harbour protein–mineral complexes like the fetuin-A-rich vesicles associated with calcifying smooth muscle cells [36].

Taken together, nanoparticles consisting of protein–mineral complexes can be seen both *in vitro* and *in vivo* as precursors of matrix calcification. The interaction of mineral with mineral-binding proteins and low-molecular weight inhibitors thus constitute important facets of mineral transport and homeostasis. Fetuin-A, as well as other proteins, contained in soluble protein–mineral particles

may be viewed as mineral chaperones [37] fulfilling a role in the stabilization, transport and recycling of water-insoluble mineral, similar to the role of lipoproteins in the metabolism of lipoprotein particles that contain water-insoluble lipids [20].

Conflict of interest statement. None declared.

References

1. Schinke T, McKee MD, Karsenty G. Extracellular matrix calcification: where is the action? *Nat Genet* 1999; 21: 150–151
2. Jahnen-Dechent W, Heiss A, Schafer C *et al.* Fetuin-a regulation of calcified matrix metabolism. *Circ Res* 2011; 108: 1494–1509
3. Murshed M, Harmey D, Millan JL *et al.* Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone. *Genes Dev* 2005; 19: 1093–1104
4. Folk RL. SEM imaging of bacteria and nannobacteria in carbonate sediments and rocks. *J Sediment Petrol* 1993; 63: 990–999
5. McKay DS, Gibson EK Jr, Thomas-Keprta KL *et al.* Search for past life on Mars: possible relic biogenic activity in martian meteorite ALH84001. *Science* 1996; 273: 924–930
6. Kajander EO, Ciftcioglu N. Nanobacteria: an alternative mechanism for pathogenic intra- and extracellular calcification and stone formation. *Proc Natl Acad Sci U S A.* 1998; 95: 8274–8279
7. Ciftcioglu N, Bjorklund M, Kuorikoski K *et al.* Nanobacteria: an infectious cause for kidney stone formation. *Kidney Int* 1999; 56: 1893–1898
8. Sedivy R, Battistutti WB. Nanobacteria promote crystallization of psammoma bodies in ovarian cancer. *APMIS* 2003; 111: 951–954
9. Hjelle JT, Miller-Hjelle MA, Poxton IR *et al.* Endotoxin and nanobacteria in polycystic kidney disease. *Kidney Int* 2000; 57: 2360–2374
10. Miller VM, Rodgers G, Charlesworth JA *et al.* Evidence of nanobacterial-like structures in calcified human arteries and cardiac valves. *Am J Physiol Heart Circ Physiol* 2004; 287: H1115–H1124
11. Cisar JO, Xu DQ, Thompson J *et al.* An alternative interpretation of nanobacteria-induced biomineralization. *Proc Natl Acad Sci U S A.* 2000; 97: 11511–11515
12. Martel J, Young JD. Purported nanobacteria in human blood as calcium carbonate nanoparticles. *Proc Natl Acad Sci U S A.* 2008; 105: 5549–5554
13. Young JD, Martel J, Young D *et al.* Characterization of granulations of calcium and apatite in serum as pleomorphic mineralo-protein complexes and as precursors of putative nanobacteria. *PLoS One* 2009; 4: e5421
14. Wu CY, Martel J, Young D *et al.* Fetuin-A/albumin-mineral complexes resembling serum calcium granules and putative nanobacteria: demonstration of a dual inhibition-seeding concept. *PLoS One* 2009; 4: e8058
15. Raoult D, Drancourt M, Azza S *et al.* Nanobacteria are mineralo fetuin complexes. *PLoS Pathog* 2008; 4: e41
16. Young JD, Martel J, Young L *et al.* Putative nanobacteria represent physiological remnants and culture by-products of normal calcium homeostasis. *PLoS One* 2009; 4: e4417
17. Ciftcioglu N, McKay DS. Pathological calcification and replicating calcifying-nanoparticles: general approach and correlation. *Pediatr Res* 2010; 67: 490–499
18. Schlieper G, Westenfeld R, Brandenburg V *et al.* Inhibitors of calcification in blood and urine. *Semin Dial* 2007; 20: 113–121
19. Genge BR, Wu LN, Wuthier RE. In vitro modeling of matrix vesicle nucleation: synergistic stimulation of mineral formation by annexin A5 and phosphatidylserine. *J Biol Chem* 2007; 282: 26035–26045
20. Price PA, Nguyen TM, Williamson MK. Biochemical characterization of the serum fetuin-mineral complex. *J Biol Chem* 2003; 278: 22153–22160
21. Heiss A, DuChesne A, Denecke B *et al.* Structural basis of calcification inhibition by alpha 2-HS glycoprotein/fetuin-A. Formation of colloidal calciprotein particles. *J Biol Chem* 2003; 278: 13333–13341
22. Aizenberg J, Weiner S, Addadi L. Coexistence of amorphous and crystalline calcium carbonate in skeletal tissues. *Connect Tissue Res* 2003; 44 (Suppl 1): 20–25
23. Mahamid J, Aichmayer B, Shimoni E *et al.* Mapping amorphous calcium phosphate transformation into crystalline mineral from the cell to the bone in zebrafish fin rays. *Proc Natl Acad Sci U S A.* 2010; 107: 6316–6321
24. Sandin K, Kloo L, Nevsten P *et al.* Formation of carbonated apatite particles from a supersaturated inorganic blood serum model. *J Mater Sci Mater Med* 2009; 20: 1677–1687
25. Robinson C. Self-oriented assembly of nano-apatite particles: a subunit mechanism for building biological mineral crystals. *J Dent Res* 2007; 86: 677–679
26. Schlieper G, Aretz A, Verberckmoes SC *et al.* Ultrastructural analysis of vascular calcifications in uremia. *J Am Soc Nephrol* 2010; 21: 689–696
27. Becker A, Epple M, Muller KM *et al.* A comparative study of clinically well-characterized human atherosclerotic plaques with histological, chemical, and ultrastructural methods. *J Inorg Biochem* 2004; 98: 2032–2038
28. Neven E, De Schutter TM, De Broe ME *et al.* Cell biological and physicochemical aspects of arterial calcification. *Kidney Int* 2011; 79: 1166–1177
29. Price PA, Williamson MK, Nguyen TM *et al.* Serum levels of the fetuin-mineral complex correlate with artery calcification in the rat. *J Biol Chem* 2004; 279: 1594–1600
30. Hamano T, Matsui I, Mikami S *et al.* Fetuin-mineral complex reflects extraosseous calcification stress in CKD. *J Am Soc Nephrol* 2010; 21: 1998–2007
31. Reynolds JL, Skepper JN, McNair R *et al.* Multifunctional roles for serum protein fetuin-a in inhibition of human vascular smooth muscle cell calcification. *J Am Soc Nephrol* 2005; 16: 2920–2930
32. Jahnen-Dechent W, Schafer C, Ketteler M *et al.* Mineral chaperones: a role for fetuin-A and osteopontin in the inhibition and regression of pathologic calcification. *J Mol Med* 2008; 86: 379–389
33. Libby P. Inflammation in atherosclerosis. *Nature* 2002; 420: 868–874
34. Jahnen-Dechent W. Lotś wifeś problem revisted: how we prevent pathological calcification. In: Baeuerlein E, (ed). *Biomineralization Progress in Biology, Molecular Biology and Application*. Weinheim: Wiley-VCH; 2004:245–267
35. Heiss A, Pipich V, Jahnen-Dechent W *et al.* Fetuin-A is a mineral carrier protein: small angle neutron scattering provides new insight on fetuin-a controlled calcification inhibition. *Biophys J* 2010; 99: 3986–3995
36. Wald J, Wiese S, Eckert T *et al.* Structure and stability of calcium phosphate - fetuin-A colloids probed by time-resolved dynamic light scattering. *Soft Matter* 2011; 7: 2869–2874
37. Olde Loohuis KM, Jahnen-Dechent W, van DW. The case: milky ascites is not always chylous. *Kidney Int* 2010; 77: 77–78

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