Biomineralization

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"Hidden" Hierarchy of Microfibrils within 3D-Periodic Fluorapatite–Gelatine Nanocomposites: Development of Complexity and Form in a Biomimetic System**

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The living world creates organic–inorganic composites in the form of biominerals that act as functional materials, and which frequently also show fascinating forms and patterns on various length scales.[1,2] Mimicking the processes of biomineralization to gain deeper insight into these complex events constitutes a great challenge, not only for basic research but also for materials science and applications.[3] Since 1996 our specific interest in biomimetic processes and hybrid materials has been focused on (fluor-)apatite–gelatine nanocomposites[4] bearing a strong resemblance to the biosystem hydroxyapatite–collagen, which plays a decisive role in the human body as a functional material in the form of bone[5] and teeth.[6]

The biomimetic system apatite–gelatine is investigated by a double-diffusion arrangement in which the ions migrate into a gelatine gel from opposite reservoirs containing aqueous solutions of calcium and phosphate/fluoride, respectively.[7,8] This system is perfectly suited to obtaining information on processes of self-organization, and may help in gaining insight into the essentials of the formation of organic–inorganic nanocomposites of biological relevance.[9]

Herein, we report a recent observation concerning the cooperative interplay between gelatine microfibrils and the nano-apatite–gelatine composite, which creates a new quality at an increased level of complexity that may be considered as a key scenario in the development of biological/biomimetic patterns of organic–inorganic nanocomposites. In particular, we would like to contribute to the general question of dumbbell formation during the fractal morphogenesis[3,4,7,8] of the fluorapatite–gelatine nanocomposite.

The fractal morphogenesis of this nanocomposite (containing about 2.3 wt% gelatine)[8] starts with an elongated hexagonal prism, which develops through outgrowth areas at both ends to form the first dumbbell state. This development is clearly seen in Figure 1, in which scanning electron microscopy (SEM) images of different growth states are superposed and differently colored: the central "young" seed grows into a "mature" one (violet areas in Figure 1) and subsequently splits up to form the first dumbbell state (yellow-green area in Figure 1).

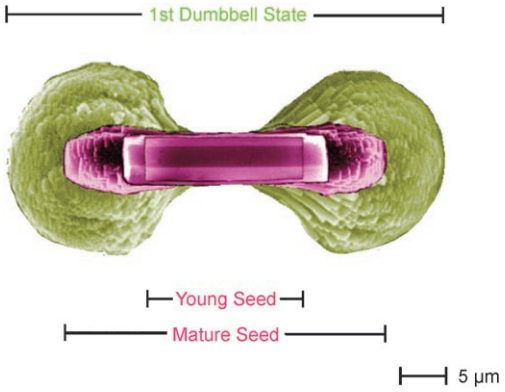

Figure 1. Superposition of SEM images of the initial growth stages of (fractal) fluorapatite–gelatine nanocomposite aggregates.

It has already been shown by X-ray diffraction (synchrotron radiation source) that the young seed exhibits scattering properties representative of a single crystal.[8] The same is true for the mature seed (see Figure 4), although contour steps on the prism faces near the basal planes of the individual nanocrystals are clearly indicated. The X-ray pattern of the first dumbbell state is then characterized by sickle-shaped diffraction maxima with tendencies to form diffraction rings, which indicate the change in particle orientation.[8]

Until now, the fractal growth mechanism was predominantly described (and simulated)[4,7–9] as a splitting procedure that develops directly from the basal planes of the (young) seed. This model was certainly oversimplified, as can immediately be seen from Figure 1, and it was speculated[10] that the splitting scenario is more or less an outgrowth rather than an upgrowth phenomenon. This, however, would imply that even the young seed bears the intrinsic conception for its future shape development. No experimental evidence for such a kind of "equipment/talent" has been obtained so far, apart from the fact that the young seed builds up an intrinsic electrical dipole field,[11] which, however, is not a sufficient condition for the shape development alone. An additional condition should be expected to explain the observation of controlled outgrowth from the inner seed volume in more detail.

The real structure of the young seeds has already been investigated by high-resolution TEM (HRTEM) meth-

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ods.[12,13] These results revealed that the inner architecture of the young seeds consists of a parallel rod-stacking of elongated hexagonal-prismatic nanocomposite subunits oriented with their long axes parallel [001] to the seed. Each nanocomposite subunit grows around a central protein triple helix[14] and is characterized by a significant structural mismatch of the nano-apatite areas.

The nanostructured collective is shown schematically in Figure 2 and represents a highly mosaic-controlled nanocomposite superstructure.[12,13] This kind of solid matter is also called a "mesocrystalline" state,[15] which is characterized by

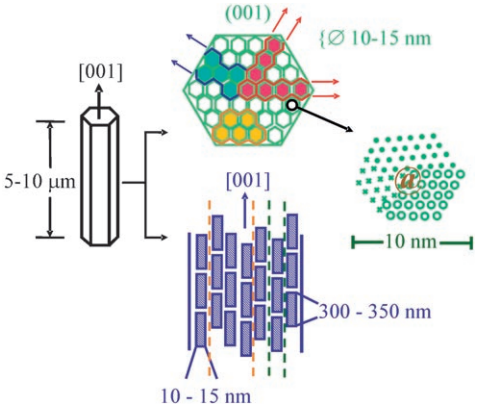

Figure 2. Diagram of the nanocomposite inner architecture of a young fluorapatite–gelatine seed derived from HRTEM investigations.[12,13] Left: elongated hexagonal-prismatic habit of the seed. Middle bottom: parallel-rod stacking of (self-similar) elongated nanosubunits along [001] as well as "accidental" variations in rod clustering by mechanical treatment (-----). Middle top: nanostructure in (001) with grain boundaries representative of a hexagonal material, as well as variations in accidental clustering by breaking (different colors) and preferred fracture directions (red and blue arrows). Right: Nanomosaic structure about 10 nm in diameter nucleated by (around) a central gelatine macromolecule (@).

an arrangement of individual nanocrystals aligned with a common crystallographic orientation, thereby giving rise to scattering properties similar to those of single crystals. As can be seen from Figure 2, the protein macromolecules within the nanocomposite are in a parallel orientation with the long axis of the young seed, which is the crystallographic *c* axis. The polar triple helices exhibit opposite charges at their ends, and by adding up all these microscopic dipoles a macroscopic electric dipole of the nanocomposite is formed.[11]

Thin cuts (II [001]) of young seeds with a perfect hexagonal-prismatic habit, and without any signs of contour steps on their prism faces, were prepared by careful treatment with a focused ion beam (FIB) technique and investigated by TEM. The TEM image (Figure 3) clearly shows that the 3D nanocomposite arrangement (as presented schematically in Figure 2) is distinctively overlaid by a pattern consisting of gelatine microfibrils with diameters scaling around 10 nm. The variations in orientation (and concentration) of the microfibrils lead to a spatial subdivision of the young seed consisting of three distinct areas (marked 1–3 in the TEM image, Figure 3):

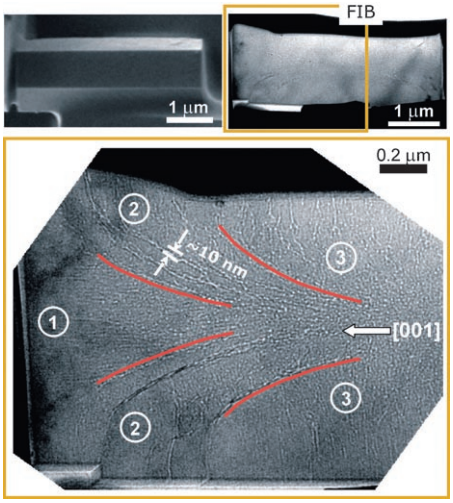

Figure 3. Top left: scanning ion image of a young composite seed before FIB thinning, which shows the perfect elongated hexagonal-prismatic habit. Top right: TEM overview image of a longitudinal FIB thin cut of the young seed. Imperfections (steps, cracks) at both ends of the thin slice of the young seed are caused by the mode of FIB preparation (W mask/Si substrate). If amorphous regions are formed during the FIB treatment they may contribute to an enhanced noise level within the electron micrograph, but do not produce an artificial pattern. The gold-colored frame represents the zoomed area. Bottom: enlarged section (gold frame) as visualized by TEM, which reveals a microfibril pattern dramatically superposing the nanocomposite matrix (see Figure 2). Red lines indicate the borders between the distinct areas 1–3.

1) A conelike area with a low concentration of microfibrils running perpendicular to the basal plane and thinning out in that direction.
2) An area with bent microfibrils opening like a flower and extending in the direction of the edges between basal and prism faces.
3) An area with the largest parts of the prism faces forming its outer boundaries. This area will be called the "waist" area in the case of the mature seed. Microfibrils arrange themselves to finally find an orientation perpendicular to the prism faces.

This intrinsic pattern of gelatine microfibrils embedded within the periodic matrix of the fluorapatite–gelatine nanocomposite represents an increased level of complexity, which provides the concept for future form developments and is already present within a young composite seed with its perfect hexagonal-prismatic habit. This observation is far from trivial, as it sheds new light on the essential question of up- versus outgrowth of succeeding composite generations during morphogenesis. The clear observation of a previously unknown ("hidden") microfibril pattern within young composite seeds was only made possible by using the FIB technique (see Experimental Section) for the careful preparation of oriented thin slices. Now it also becomes evident that the microfibril pattern within mature composite seeds, first reported a year ago,[3,11] is nothing else but a continuous pattern development passed forward by the intrinsic concept of the young seeds.

The morphological details of a mature seed are shown in Figure 4 (top right) with the characteristic face developments extending at both ends of the individual. These specimens are still at the morphogenetic state of a seed, with an X-ray

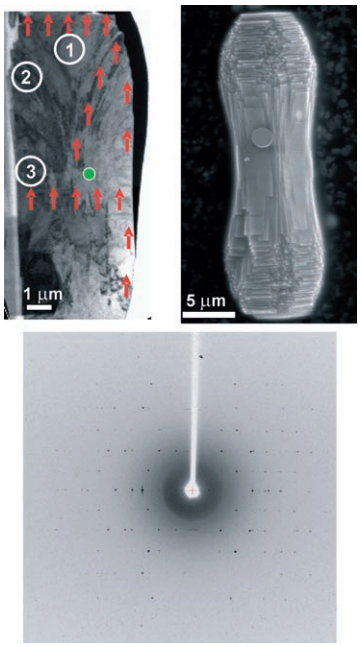

Figure 4. Top right: SEM image of a mature seed. Top left: TEM image of a FIB thin cut II [001]. Areas 1–3 correspond to Figure 3. Red arrows indicate crystallographic orientations determined by TEM at the given positions (see text). The green circle indicates the area investigated by TEM (Figure 6). Bottom: X-ray diffraction pattern of a mature seed obtained by 30° oscillation around the *c* axis. Hexagonal unit cell with $a = 9.20$ and $c = 7.07$ Å.

pattern that is consistent with an overall 3D-periodic structural arrangement of the inorganic component of the composite (Figure 4, bottom). The red arrows in the TEM overview image of a mature seed (Figure 4, top left) indicate the crystallographic orientation (*c* axis directions) of small areas as determined by HRTEM. Thus, it is evident that even the crystallographic orientation of small regions of the mature seed is consistent with periodicity of the nanostructured collective, and is independent of the embedded pattern of microfibrils as well as of the development of the face-steps that dominate the outer shape (habit) of the mature individual.

Also indicated in Figure 4 (top left) are distinct areas of microfibril orientation (1–3), which are significantly enlarged compared with the situation for the young seed (see Figure 3). Details of the arrangement of microfibrils within a mature seed are presented in Figure 5. The most interesting microfibril pattern is developed in the waist area (enlarged image of square 2 in Figure 5), with a long and bent fibril strand clearly separating area 2 from area 3 (see Figure 3 and text: bunch of bent microfibrils opening like a flower; microfibrils arrange themselves to finally find an orientation perpendicular to the prism face).

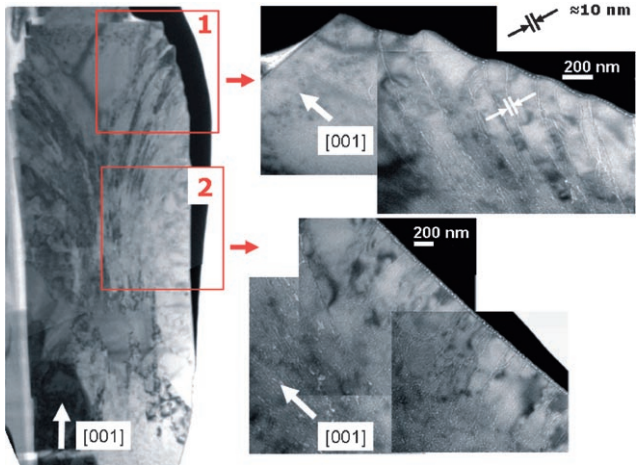

Figure 5. Left: TEM overview image of a FIB thin cut II [001] of a mature seed. Right: enlarged sections (from red frames 1 and 2 of the overview image) as visualized by TEM, which reveal the microfibril patterns. For further details see text.

Figure 6 gives an impression of how microfibrils attach to the nanocomposite structure. The chosen position is marked with a green circle in Figure 4 (top left) and belongs to the expected inner volume of the former young seed, slightly outside and "above" its center but well within area 2 (see

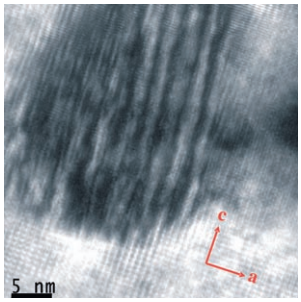

Figure 6. HRTEM image of the FIB thin cut shown in Figure 4 (top left) at the position of the green circle. Formation and attachment of a microfibril embedded in the nanocomposite matrix and slightly tilted against [001]. To image the fibrils at optimum contrast a specific defocus was chosen, thus avoiding Scherzer focus which is suitable for higher-resolution imaging. The nanocomposite matrix appears blurred whereas the microfibril appears highlighted.

Figure 3 and text). The TEM image (Figure 6) shows a more or less parallel bundle of at least five fiber-protein macromolecules (diameter of a triple-helical molecule ≈ 1.5 nm),[5] which form (part of) a microfibril that is slightly tilted against the *c* axis direction of the nanocomposite. The composite structural pattern remains unaffected and, in principle, the orientation of the microfibril is consistent with the picture of developing a bent strand that is characteristic for area 2 (see Figure 3 and text) of the young seed.

A summary of the experimental observations concerning the intrinsic pattern development of gelatine microfibrils within a growing nanocomposite seed (from young to mature)

is given in Figure 7. The central (orthogonal) part of Figure 7a (marked in black) represents the young seed which—in accordance with areas 1–3 in Figure 3—is subdivided into an orange region (1), a region with bent microfibrils (2; red curves), and a waist region (3). By taking into account the experimental evidence for the presence of an electrical potential around a growing young seed,[11] the orientation of the microfibrils (red curves in Figure 7a) follows the directions of electrical field lines generated inside the permanent dipole matrix given by the apatite–gelatine nanocomposite. During the morphogenesis of the hierarchically structured system, microfibrils are formed by the intrinsic electrical field. It is clearly this field that determines the orientation and bundling of polar triple-helical macromolecules from the surrounding gelatine gel.

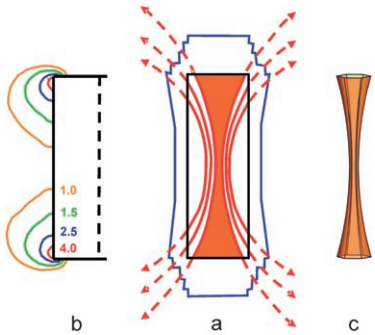

Figure 7. a) Bunch of bent microfibrils (red/dashed lines) within a young seed (black outline) and extending to the mature seed (blue outline). b) Energy isolines (kJ mol^{-1}) around a young seed, which represent the areas of largest divergence for fibril attachment from the [001] direction.[11] c) 3D representation (elongated hexagonal saddle prism) of the central (orange) area given in (a).

As indicated in Figure 7b, the calculated energy isolines (kJ mol^{-1}) around a young seed, which represent the areas of largest divergence of fibril attachment from the [001] direction, are strongest close to the corners where the prism faces meet the basal planes. From these calculations[11] it becomes clear that the morphogenetic step from the young to the mature seed constitutes the continuation of the preformed pattern of the microfibrils. At the same time, the 3D apatite pattern of the nanocomposite is also continued but affected in such a way that the growth rates in the direction of the developing fibrils (regions of largest electrical field and high concentration of bent microfibrils) are slightly increased, thereby forming the characteristic growth-steps at both ends of a mature seed (Figure 7a, blue outline).

In consequence, it can also be expected that the growing face-steps at the ends of a mature seed will significantly influence the pattern and the local strength of the electrical potential around the growing individual. The corresponding local changes are assumed to be responsible for the development of the following (first) fractal generation growing out of the mature seed (see Figure 1, yellow-green area). This morphogenetic step will be the next challenge to be investigated to clarify the mechanism of orientational changes of the hexagonal nanocomposite subindividuals. For the mature seed shown in Figures 4 and 5, a very small structural misfit of 2–3° with respect to the overall composite–crystal orientation was observed at the very ends of the most outgrowing face-steps only.

Finally, two further details concerning the microfibril pattern inside a (young/mature) composite seed remain to be mentioned. These observations build the bridge to general aspects of shape in nature[16] and to the mathematics of biostructures[17] dealing with 3D spatial systems and their shape development. In this connection the following points should be emphasized. 1) In 3D space, the conelike area with a low concentration of microfibrils running perpendicular to the basal plane and thinning out in that direction (for example, area 1 in Figure 3) actually extends over the volume of an elongated hexagonal saddle prism (Figure 7c). This macroscopic polyhedron is consistent in symmetry with the overall symmetry of the seed morphology (point group 6/*m*). Clearly, the elongated hexagonal saddle prism represents the volume in which the electrical field lines are oriented parallel (or close to parallel) along [001] within a seed (see Figure 7b). 2) In 3D space the volume of the waist area is given by (parts of) the prism faces and the outer surface of a compressed hexagonal saddle prism surrounding the elongated saddle prism. Within this volume the microfibrils arrange themselves to finally find an orientation perpendicular to the prism faces. The driving force for this particular microfibril development remains unclear at present and is the subject of an ongoing theoretical investigation.[18]

The biomimetic system apatite–gelatine is perfectly suited to obtaining new information on processes of self-organization and the formation of hierarchically organized structures closely related to biological composites. It turns out that the system under investigation is able to create an increased level of complexity, which clearly exceeds the periodicity of nanocomposite arrangements and gives rise to the development of new qualities by introducing cooperative phenomena. The situation can be summarized as follows. Polar (triple-helical) biomacromolecules with opposite charges at their ends provide nucleation sites for the oriented formation of nano-apatite and support the development of a highly mosaic-controlled 3D nanocomposite arrangement. By adding up all the microscopic dipoles a macroscopic electric dipole is formed, thus producing an intrinsic electrical field, which affects neighboring polar macromolecules to form microfibrils that line up in the direction of the developing electrical field.

Hence, a scenario is generated that is perfectly suited to the transfer of information into a macroscopic volume by development of a predefined pattern of microfibrils. The combination of the chemical components under consideration has been optimized during processes of evolution, and plays a decisive role in the living world. Although on a reduced level of complexity (compared with a living system based on cell activities), the dramatic events of self-organization observed in the biomimetic system are assumed to be similarly representative of (single) steps in the biomineralization of bone and teeth.

Experimental Section

Details of the preparation of fluorapatite–gelatine nanocomposites have already been reported.[3,4,7] The composite aggregates were isolated from the gelatine-gel matrix by treatment with water and final drying at 40 °C. Seeds were deposited from ethanol suspensions onto thin silicon wafer bars to perform FIB preparation and TEM experiments.[11] FIB cuts of young seeds were performed with a dual-beam Quanta 3D microscope at the FEI Company (Eindhoven, Netherlands). The TEM experiments were carried out at the Special Laboratory Triebenberg for Electron Holography and High-Resolution Microscopy of the TU Dresden. A field-emission microscope CM 200 FEG/ST-Lorentz (FEI, Eindhoven) equipped with a Gatan 1×1 k camera was used. The analyses of the TEM images were realized by Digital Micrograph software (Gatan, USA).

X-ray diffraction investigations on mature seeds were carried out on a Rigaku R-axis Spider diffractometer with a rotating anode microsource and mirror optic at a wavelength of 0.5608 Å ($Ag_{K\alpha}$ radiation).

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[1] *The Diatoms* (Eds.: F. E. Round, R. M. Crawford, D. G. Mann), Cambridge University Press, Cambridge, **1996**.
[2] *Wie Schnecken sich in Schale werfen* (Ed.: H. Meinhard), Springer, Heidelberg, **1997**.
[3] *Biomineralization I, II* (Ed.: K. Naka), Springer, Heidelberg, **2007** (*Top. Curr. Chem.* **2007**, *270–271*); "Fluorapatite–Gelatine Nanocomposites": R. Kniep, P. Simon in *Biomineralization I*, **2007**, pp. 73–125.
[4] R. Kniep, S. Busch, *Angew. Chem.* **1996**, *108*, 2788 – 2791; *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 2624 – 2626.
[5] *Bone Formation* (Eds.: F. Bronner, M. Farach-Carson), Springer, Heidelberg, **2003**.
[6] *Development, Function and Evolution of Teeth* (Eds.: M. F. Teaford, M. M. Smith, M. W. J. Ferguson), Cambridge University Press, Cambridge, **2000**.
[7] S. Busch, H. Dolhaine, A. DuChesne, S. Heinz, O. Hochrein, F. Laeri, O. Podebrand, U. Vietze, T. Weiland, R. Kniep, *Eur. J. Inorg. Chem.* **1999**, 1643 – 1653.
[8] S. Busch, U. Schwarz, R. Kniep, *Adv. Funct. Mater.* **2003**, *13*, 189 – 198.
[9] S. Busch, U. Schwarz, R. Kniep, *Chem. Mater.* **2001**, *13*, 3260 – 3271.
[10] C. Göbel, P. Simon, J. Buder, H. Tlatlik, R. Kniep, *J. Mater. Chem.* **2004**, *14*, 2225 – 2230.
[11] P. Simon, D. Zahn, H. Lichte, R. Kniep, *Angew. Chem.* **2006**, *118*, 1945 – 1949; *Angew. Chem. Int. Ed.* **2006**, *45*, 1911 – 1915.
[12] P. Simon, W. Carrillo-Cabrera, P. Formanek, C. Göbel, D. Geiger, R. Ramlau, H. Tlatlik, J. Buder, R. Kniep, *J. Mater. Chem.* **2004**, *14*, 2218 – 2224.
[13] P. Simon, U. Schwarz, R. Kniep, *J. Mater. Chem.* **2005**, *15*, 4992 – 4996.
[14] H. Tlatlik, P. Simon, A. Kawska, D. Zahn, R. Kniep, *Angew. Chem.* **2006**, *118*, 1939 – 1944; *Angew. Chem. Int. Ed.* **2006**, *45*, 1905 – 1910.
[15] H. Cölfen, M. Antonietti, *Angew. Chem.* **2005**, *117*, 5714 – 5730; *Angew. Chem. Int. Ed.* **2005**, *44*, 5576 – 5591.
[16] *Structure in Nature is a Strategy for Design* (Ed.: P. Pearce), MIT Press, Cambridge, **1978**.
[17] *Mathematics of Biostructures and Biodynamics* (Eds.: S. Andersson, K. Larsson, M. Larsson, M. Jacob), Elsevier, Amsterdam, **1999**.
[18] J. Brickmann, ongoing DFG project, BR 306/34-1. *Computer Simulations on the Development of Patterns in Nanostructured Apatite–Gelatine Systems.*

