Biominerals

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The Nucleation Mechanism of Fluorapatite–Collagen Composites: Ion Association and Motif Control by Collagen Proteins**

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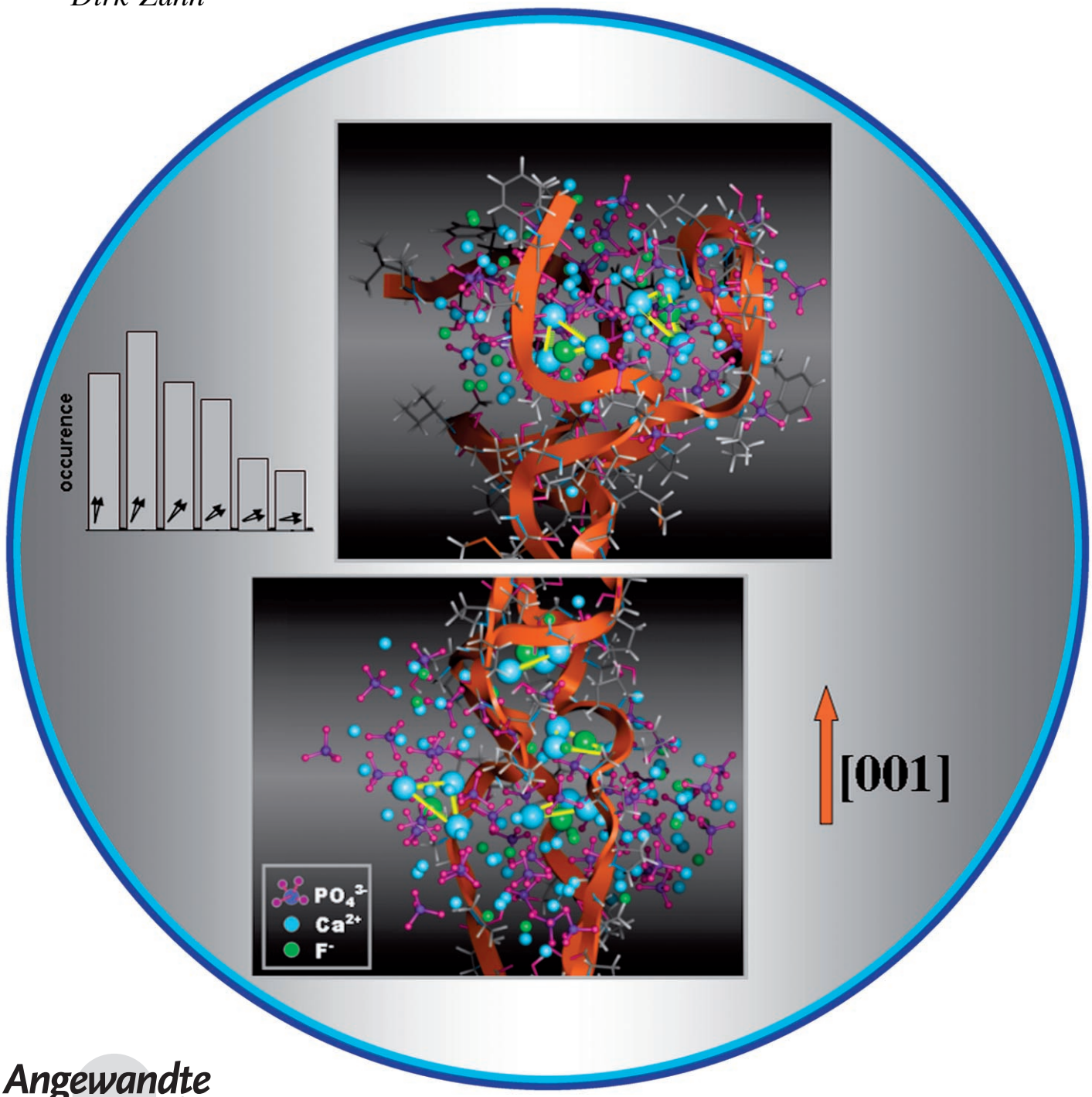

Apatite–collagen composites belong to the most abundant biominerals in both humans and animal life forms. The importance of this material as the predominant component of bone and teeth motivated a large number of biomimetic experimental and theoretical studies.[1–3] Despite these efforts, we are still at the beginning of understanding biomineral formation. This motivated the development of biomimetic setups which reduce the complexity inherent to metabolic processes, but nevertheless describing the key aspects of composite nucleation.[2] Based on electron microscopy and computer simulation studies, recent investigations of biomimetic apatite–gelatine composites could reveal a series of insights into the growth mechanisms at the mesoscopic scale.[4] On the μm scale, the morphogenesis of the composite is controlled by the arrangement of the biomolecules, the polar nature of which gives rise to an intrinsic dipole field.[5] A prismatic seed is initially formed; with increasing size the composite experiences increasing electrostatic interactions which induce branching patterns, and finally lead to the development of spherical morphologies.[6]

The fundamental principles of this mesoscale morphogenesis are closely connected to phenomena occurring on much smaller length scales. A central issue is the alignment of the collagen fibers, which was shown to correlate with the c-axis of the apatite crystal structure.[2,5] This can only be rationalized from exploring the interplay of the apatite-forming ions and the collagen proteins at the atomistic scale.

Electron microscopy proved to be a powerful tool for identifying nanoscale patterns of apatite–collagen composites.[2,4,6] However, for interpretation purposes, it is useful to apply atomistic simulation techniques.[3] Along this line, we recently developed a specialized simulation method for the investigation of nanocrystal aggregation from solution.[7] Apart from simple ion solutions, our approach may also address more complex systems having growth-controlling molecules in solution.[8] Along this line, model studies mimicking the association of single ions to collagen fibers in aqueous solution provided an atomistic understanding of collagen stiffening or bending by calcium or phosphate preimpregnation, respectively, which accounts for changes in the growth mechanisms governing the composites form development at the mesoscale.[3,9]

Herein, the mechanisms of aggregate formation and growth-control by collagen fibers at the atomistic scale are investigated. The focus is on the embryonic stage of the nucleation process, both in water and in aqueous solutions containing collagen molecules. The selforganization is then explored in different environments to elaborate insights into the atomistic interplay governing the formation of apatite–collagen composites. Our studies address ion aggregation promoted by the tails of a collagen fiber and calciumphosphate nucleation at the triple-helical backbone of collagen. Both aspects are explored in separate simulation setups and are contrasted with a third model system corresponding to calcium, phosphate, and fluoride ion aggregation from aqueous solution in the absence of collagen molecules.

Modeling of the ion–ion and ion–water interactions was done by empirical force fields.[10] The telopeptide model was adopted[11] and the triplehelical backbone is mimicked by a $(Gly\text{-}Pro\text{-}Hyp)_{12}$ model. The choice of the latter model was motivated from the studies of Radmer and Klein[12] and Periskov et al.,[13] which established $(Gly\text{-}Pro\text{-}Hyp)_n$ as the most stable sequence occurring in the collagen helical domain that captures all the significant features of its unique characteristics. Each biomolecule/aggregate system is considered in aqueous solution. Our model studies are based on a recently developed iterative simulation procedure for investigating ion-by-ion aggregate growth.[7] It is designed to tackle nucleation from very dilute solutions by making specific use of the characteristics related to crystallization processes of compounds of low solubility.[8] Along this line, ion diffusion to the aggregate is mimicked by a docking procedure, rather than treating ion solutions counting millions of water molecules explicitly. Explicit solvent is only considered for studying the relaxation of the aggregate, for which a solvent skin of 2.5 nm extension (typically around 10000 water molecules) was found to be sufficient.

The influence of the collagen fibers on aggregate formation is particularly strong for the uptake of calcium ions. The latter species is offered a variety of favorable association sites in which the Ca^{2+} ions are coordinated by carbonyl oxygen atoms (partial charges of −0.51 to −0.57) and oxygen atoms from hydroxy groups of the HYP residues (partial charge of −0.61). Typically, four to six Ca···O electrostatic bonds are formed, as illustrated by yellow lines in Figure 1. At the very beginning of aggregate formation, there are only few ion–ion contacts and protein–ion interactions are clearly dominating. Unlike the phosphate and fluoride ions, the calcium ions may be incorporated between the strands of the triple helix. This incorporation involves the opening of one to two hydrogen bonds connecting the triple helix, a situation which, however, does not lead to unfolding. Instead, the incorporated calcium ions replace the connecting hydrogen bonds by electrostatic O···Ca···O interactions while keeping the overall triplehelical structure.[9] The strong calcium–collagen interactions cause a preferential uptake of Ca^{2+} at the very early stage of aggregate growth, though in each aggregate growth step, the ionic species is chosen randomly according to the apatite composition.

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

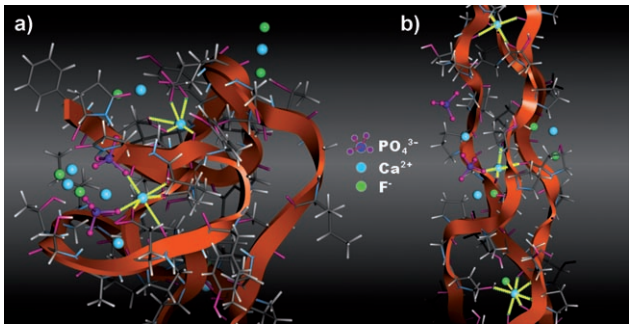

Figure 1. Snapshots taken from an early stage of ion association to a) one of the tails of a collagen fiber (N-telopeptide, with $9Ca^{+}$, $2PO_4^{3-}$, and $6F^{-}$), and b) to a tropocollagen model (with $6Ca^{+}$, $2PO_4^{3-}$, and $4F^{-}$). The backbone of the triple helix is illustrated by red ribbons. The yellow lines indicate favorable electrostatic interactions inducing Ca^{2+} coordination by oxygen atoms (purple) of the peptides and the phosphate ions. The corresponding Ca···O distances range from 2.2 to 2.4 Å. The solvent (ca. 10000 water molecules) and ions which are dissolved are omitted for clarity.

In the course of further ion uptake, association to the protein is complemented by an increasing number of ion–ion interactions and at first aggregates are formed. From Figures 2 and 3 it can be clearly seen that the ionic clusters are

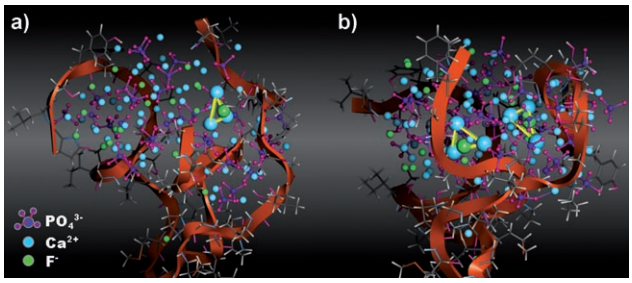

Figure 2. Snapshots taken from a) intermediate ($[Ca_{63}(PO_4)_{33}F_{19}]^{8+}$) and b) late ($[Ca_{85}(PO_4)_{47}F_{24}]^{5+}$) stages of ion aggregation at the tails of a collagen triple helix. Yellow lines are used to illustrate Ca_3F motifs (highlighted as larger spheres). The Ca···F and Ca···Ca distances range from 2.0 to 2.5 Å and 3.6 to 4.7 (2.29 Å and 4.04 Å in the apatite crystal structure[1]), respectively, and the angles of the triangles vary by up to 20° from the ideal value of 120°. The average displacement of the fluoride ions from the triangle center is 0.3 Å.

not arbitrarily attached to the collagen proteins, but display correlation, that is, intergrowth of both components of the developing composite material. The telopeptide model reflects the more flexible ends of the collagen strands, and therefore undergoes strong structural changes in the course of ion association (Figure 2). This flexibility is widely believed to account for preferential apatite nucleation at the fiber tails.[1,2] Indeed, the triple-helical model of the fiber backbone undergoes only minor rearrangements during aggregate formation (Figure 3). Nevertheless, the discrimination between ion accumulation and ionic ordering brings up a more complex picture as described below.

The protein–ion interplay induces structural motifs, which at this early stage of the nucleation process do not occur in ion

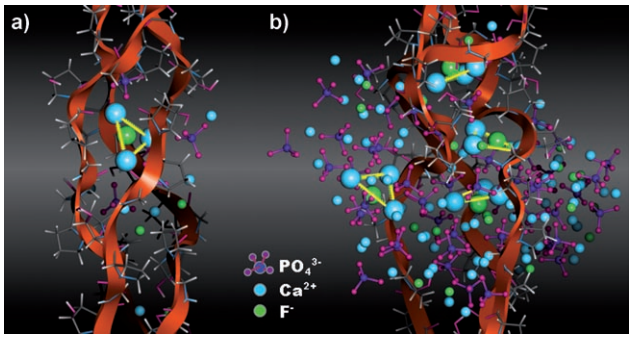

Figure 3. Snapshots taken from a) intermediate ($[Ca_{11}(PO_4)_4F_5]^{5+}$) and b) late ($Ca_{87}(PO_4)_{49}F_{26}]^{+}$) stages of ion aggregation on/in a collagen triple helix. Yellow lines are used to illustrate Ca_3F motifs (highlighted as larger spheres), which emerge at a much faster rate as the ion association to the telopeptide tails (see also Figures 2 and 4). Although the apatite-like motifs are initially incorporated into the triple helix, later stages of aggregate growth also exhibit Ca_3F motif formation lateral to the biomolecule. The range of ion–ion distances corresponds to that of the Ca_3F motifs observed in the telopeptide simulations (Figure 2). Strikingly, incorporation in the collagen triple helix induces the orientation of the triangle motifs (see also Figure 5).

clusters grown during aggregation from aqueous solutions without collagen molecules. This is best described by means of a peculiar feature of the apatite crystal structure which is represented by the coordination of the fluoride ions. The latter are located in the center of triangles formed by calcium ions. The number of such Ca_3F motifs as a function of the aggregate size is given in Figure 4. To avoid artifacts arising from incomplete motifs at the aggregate surface, only ions which do not exhibit water molecules in their nearest neighborhood are considered. Strikingly, in our ion association runs performed in aqueous solution without including a collagen model, the tendency to form Ca_3F motifs is much smaller compared to aggregate growth on a collagen fiber. While no Ca_3F motifs were observed in aggregates of up to 160 ions grown from aqueous solution containing no collagen, the design of such motifs corresponding to the apatite crystal structure is observed for both the collagen tails and the fiber backbone. This tendency is particularly pronounced in the

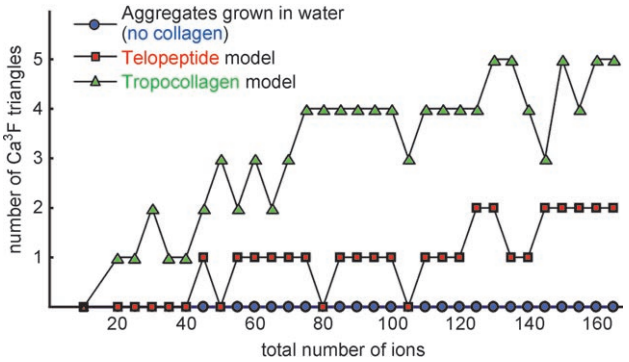

Figure 4. Number of Ca_3F motifs in the aggregate core as a function of the total number of ions accumulated. The motifs emerge at fastest rate during aggregation in/at the collagen triple helix. In aqueous solution containing no collagen molecules, the rate of motif formation is too small to be captured.

triple-helical body of the biomolecule. Moreover, the backbone of the protein fiber induces orientation correlation of the apatite motifs and the triple helix. This phenomenon may be seen in Figure 3b (see also the Supporting Information), and more quantitatively, by plotting the occurrence profile of the angle defined by the normal vector of the calcium triangles and the long axis of the fiber protein (Figure 5).

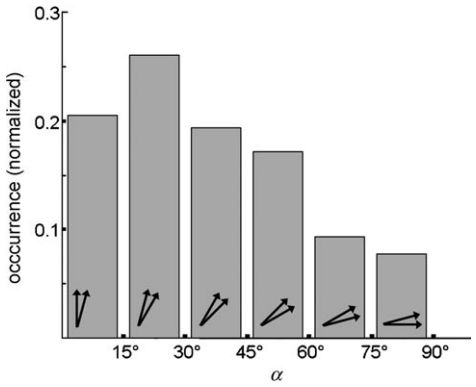

Figure 5. Occurrence profile of the orientation of Ca_3F motifs formed during aggregation in/at the collagen triple helix. The distribution of the angle α of the triangle normal and the fiber axis is normalized by a factor of $\frac{1}{\sin\alpha}$. For the angles of randomly oriented vector pairs in three-dimensional space, this normalization implies a constant occurrence profile.

In the apatite crystal structure, the Ca_3F motifs are staggered along the *c* axis, and the normal vectors of the calcium triangles are oriented (anti)parallel to this direction. Whereas apatite–collagen composites exhibit a mosaic structure on the nanometer scale, over lengths of several micrometers, the collagen triple helices are directed along the *c* axis of the composite.[2,4,6] This phenomenon, known from TEM investigations, can be explained on the basis of our aggregate growth simulations which demonstrate the incorporation of Ca_3F motifs into the collagen triple helix at their preferential orientation. This growth control mechanism originates from the embryonic stage of the formation of the nanocomposite. It is commonly assumed that the flexibility of the fiber ends assists the catching of ions from the solution. However, from our simulations, the ordering in favor of the apatite crystal structure is shown to be predominantly induced by the triple helix of tropocollagen. Indeed, we suggest the design of Ca_3F motifs to account for the peculiar nature of apatite–collagen composites, that is, the intergrowth of both components at correlated orientation.

This motif control by collagen is a consequence of the specific collagen–ion interactions. We also explored the evolution of other motifs, such as the coordination polyhedra around the phosphate ions (which are each coordinated by nine calcium ions in the apatite crystal structure). However, at this early stage of aggregate growth, the coordination of the phosphate ions is still subject to fluctuations. Moreover, the diversity of calcium phosphate structures with similar coordination polyhedra complicates the analysis of characteristic motifs. On the other hand, F^- coordination by three calcium ions reflects a much more peculiar feature of the apatite crystal structure. Nature's choice of the Ca_3F triangles for motif design and orientation control by collagen molecules hence appears quite reasonable.

Whereas the computational efforts for our studies of aggregate growth up to 160 ions amount to several CPU years, the observation of full ordering in terms of apatite nanocrystals would require much more extensive simulations. Nevertheless, it is reasonable to assume that the effect of the design of Ca_3F motifs by collagen is carried throughout the aggregate during further growth, inducing ionic ordering in favor of the apatite crystal structure. This concept is supported by the observation of both, incorporated and lateral Ca_3F motifs in larger aggregates as illustrated in Figure 3b. A thorough investigation of full ionic ordering during further aggregate growth surely requires the accumulation of thousands to millions of ions. Nevertheless, the design of Ca_3F motifs induced by incorporation into the triple helix during the embryonic stage of ion association represent nucleation seeds for the formation of the apatite crystal structure oriented in accordance to the alignment of the collagen fibers. Accordingly, ion association to collagen followed by motif design and orientation is suggested as an atomistic mechanism of growth control governing the nucleation of apatite–collagen composites.

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