2010

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Recommended Citation
Uskoković V. Prospects and pits on the path of biomimetics: The case of tooth enamel. *J Biomim Biomater Tissue Eng*. 2010;8:45-78. doi:10.4028/www.scientific.net/JBBTE.8.45.

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Published in final edited form as:

J Biomim Biomater Tissue Eng. 2010 November ; 8: 45–78. doi:10.4028/www.scientific.net/JBBTE.8.45.

Prospects and Pits on the Path of Biomimetics: The case of tooth enamel

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Abstract

This review presents a discourse on challenges in understanding and imitating the process of amelogenesis *in vitro* on the molecular scale. In light of the analysis of imitation of the growth of dental enamel, it also impends on the prospects and potential drawbacks of the biomimetic approach in general. As the formation of enamel proceeds with the protein matrix guiding the crystal growth, while at the same time conducting its own degradation and removal, it is argued that three aspects of amelogenesis need to be induced in parallel: a) crystal growth; b) protein assembly; c) proteolytic degradation. A particular emphasis is therefore placed on ensuring conditions for proteolysis-coupled protein-guided crystallization to occur. Discussed are structural and functional properties of the protein species involved in amelogenesis, mainly amelogenin and enamelysin, the main protein and the protease of the developing enamel matrix, respectively. A model of enamel growth based on controlled delivery of constituent ions or crystalline or amorphous building blocks by means of amelogenin is proposed. The importance of high viscosity of the enamel matrix and a more intricate role that water may play in such a gelatinous medium are also touched upon. The tendency of amelogenin to self-assemble into fibrous and rod-shaped morphologies is considered as potentially important in explaining the formation of elongated apatite crystals. The idea that a preassembling protein matrix serves as a template for the uniaxial growth of apatite crystals in enamel is finally challenged with the one based on co-assembly of the protein and the mineral phases.

Keywords

Amelogenesis; Biomimetics; Biomineralization; Hydroxyapatite; Tooth Enamel

Introduction: The Road of Biomimetics

The ideals of biomimicry are considered as some of the most prosperous ones among materials scientists of the modern day, who increasingly follow the biomimetic approach to design and fabrication of advanced materials [1–6]. Many are material properties that are synthesized by animals and plant and yet find no equivalents in artificially produced substances. Whether it is the iridescence of butterfly wings, the smoothness of dolphin's skin, the stickiness of gecko's foot, the water-repellent properties of duck's feathers, the toughness of abalone shells and the spider's silk, it is claimed that learning from the design of materials that Nature utilizes will lead to new generations of materials with superior properties and performance. Exploring photosynthesis in plants has, for example, led to

creation of transparent photocells that absorb solar rays as they pass through a window, whereas a new trains have reduced energy consumption by modifying the shape of its nose from the tip of a bullet to that of a kingfisher's beak [7]. It may be no surprise that the interest in biomimicry is also growing in parallel with the contemporary cultural shift away from the scientific and technological design that inaugurates man as superior over the rest of his ecosystem and towards the one permeated with ecological awareness, sustainability and the ideals of eco-friendliness. With the latter ideals emphasizing the value of "acting locally, while thinking globally", this critical review paper has adopted a similar stance. On one hand, it focuses on the mimicry of the biogenesis of the tooth enamel, which has been the author's subject of research for the past three years. On the other hand, this paper impends on the prospects and potential drawbacks of the biomimetic approach in light of the analysis of imitation of the growth of dental enamel. In that sense, aside from being a detailed discourse on a particular scientific topic of interest, this paper also presents it enwrapped in a wider context. In this broader sense, this critical review questions the overall prosperity of the trend of exploring the subtle features of Natural design with the purpose of enhancing the properties of artificially produced materials.

The structure of enamel

Dental enamel is the hardest substance in the human body and the only known epitheliumderived mineralized tissue. Unlike dentin and bone, it does not contain collagen and has a markedly higher mineral content compared to collagenous hard tissues: 96 – 98 wt %, with water, lipids and various peptides accounting for the remaining 2 – 4 wt%. Despite being almost pure mineral, dental enamel is unlike ordinary ceramics typified by an exceptional toughness and only moderate brittleness. It displays a high resistance to propagation of cracks, and the reason is its extraordinary microstructure. Namely, enamel is composed of apatite fibres, 40 – 60 nm wide and up to several hundred microns long, assembled in bundles, i.e., rod-shaped aggregates 4 – 8 μm in width (Figure 1). Having length-to-width aspect ratios of up to $3 : 10^4$, apatite crystals in enamel are 1000 times longer than those found in bone (50 × 20 × 3 nm on average) [8]. This is possible since this tissue does not depend on intrinsic cell proliferation, the direct proportion of which is known to exist in relation to apatite crystal size in bone [9]. Approximately 1000 apatite fibres are bundled within each enamel rod, 5 – 12 million of which are found on a single tooth crown lined up in rows. The long axis of the enamel rod within each row is generally perpendicular to the underlying dentin, with the only exception that enamel rods near the cementoenamel junction (CEJ) in permanent teeth tilt slightly toward the root of the tooth.

The process of amelogenesis

The biological formation of enamel tissue is known as amelogenesis and besides specific cells, ameloblasts, it involves macromolecular species that can be divided into families of proteins, proteases and protease inhibitors. The chronology of amelogenesis is typically divided into three stages: the secretory, the processing and the maturation stage. However, in view of the significant overlap of these stages in time, the correctness of this categorization may be questioned. For example, the key components involved in the protein assembly are secreted during the processing stage too, and no precise boundary has been established

between the end of processing and the beginning of maturation. Therefore, the following three events, taking place in parallel during amelogenesis, may be said to more precisely describe this process:

1. Protein expression, secretion and assembly

As a part of this physiological event, ameloblasts express and secrete proteins that make up the enamel matrix, 90% of which will be composed of a single protein, amelogenin. The remaining 10% is comprised of other proteins: ameloblastin, enamelin, serum albumin (which is not expressed by ameloblasts but has been associated with contamination from the adjacent soft tissue), amelotin, and proteolytic enzymes. Together, they assemble into a scaffold that serves as a template for the uniaxial growth of apatite crystals.

2. Nucleation and crystal growth

Proteins involved in amelogenesis are typically divided into hydrophilic and hydrophobic groups. Some proteins, including enamelin and ameloblastin, are said to be hydrophilic in nature, and as such are supposed to act as nucleation sites for crystallization of apatite [11]. This hypothesis is supported by the fact that enamelin is expressed only in the secretory stage (its expression is halted during the maturation stage). Furthermore, whereas genetic knockout targeting ameloblastin and enamelin resulted in the absence of enamel, targeting amelogenin or the major proteases involved in the process produced thin enamel with either disordered apatite rods (typically in the absence of amelogenin) or no rods at all (typically in the absence of the proteases), the condition of which is known as amelogenesis imperfecta [12]. Initial enamel crystals also nucleate along the dentin-enamel junction (DEJ) at an early time point when amelogenin is hardly present in the protein matrix, and the latter is mainly composed of enamelin and ameloblastin. On the other hand, amelogenin is comparatively hydrophobic and has been assumed to inhibit the apatite growth [1]. The reigning model of the crystal growth is built on the assumption that enamel formation thus presupposes that amelogenin proteins self-assemble into polydisperse nanospheres ~ 20 nm in size (Figure 2a) (comprising about 40 – 60 molecules per spherical aggregate of this size), which then align into beaded strings and adhere to the (hk0) faces of the apatite crystals, promoting their growth in the direction of the crystallographic [001] axis. As such, they would prevent the growth and fusion of crystals perpendicular thereto, while aligning them approximately parallel to each other. Such strings were detected to form by merging monodisperse amelogenin nanospheres under specific conditions of aging in aqueous media (Figure 2c).

Attaching the attribute of hydrophobicity to amelogenin as a whole is, however, misleading. Just like every other protein, it contains alternately changing hydrophilic and hydrophobic sequences along its primary structure [14]. As shown in Figure 3, although amelogenin is still more hydrophobic than most proteins, it is, for example, more hydrophilic than human haemoglobin alpha chain. In view of that, recent reports on the ability of amelogenin to promote nucleation of apatite, at least at relatively low concentrations [15], should not be surprising. Tarasevich et al. have shown that the nucleation promoting / inhibiting effect of amelogenin largely depends on its concentration [16], confirming a well-known fact that additives may often exhibit different behaviour depending on their concentration [17]. It has also been demonstrated that amelogenin decreases the nucleation lag time in metastable

calcium phosphate solutions and produced conditions under which the substrate-specific growth of apatite is conditioned by adsorption of amelogenin on the growing crystal surface [18]. By showing that adsorption of amelogenin is the first step in inducing the controlled crystal growth, it confirms the fact that evidence of adsorption does not necessarily imply protein-mineral interactions that hinder the crystal growth on the binding sites. For example, osteocalcin, one of the proteins involved in mineralization of bone, despite aligning and binding on some of the growing crystal planes does not constrain the crystal growth along these directions [19]. Protein assemblies adsorbed on crystal surfaces may act as channels or bridges that transfer the ions from the solution and promote its anchoring onto the growing faces.

These two classes of proteins also differ in amino acid composition [20]. It was only in 1994 that amelogenin was for the first time expressed *in vitro* as a recombinant protein [21]. The gene encoding for amelogenin is in humans present on both the X and Y chromosomes, and both gene copies are expressed during amelogenesis. Although a comparison of the sequences of these two isoforms yields 22 amino acid substitutions, and one deletion/addition (Figure 4), the X chromosome variant accounts for 90% of the overall full-length amelogenin in the enamel matrix. A variety of amelogenin proteins are produced by alternative splicing of pre-mRNA with whole exons or some of their parts deleted during this process. As a low-molecular weight protein, the full-length isoform contains between 160 and 200 amino acids, depending on the species. Thence, the full-length porcine amelogenin (173 amino acid residues) is shorter than the human (175), the human is shorter than the mouse and the rat (179), the mouse is shorter than the one of leopard frog (181), and the leopard frog is shorter than the bovine (197). Amelogenin contains a short, 12-carboxyl-terminal residue sequence of hydrophilic amino acids at the C-terminal, which makes its molecular structure partly amphiphilic. The full-length amelogenin molecules are evidenced to form tightly connected, elongated, high-aspect ratio assemblies comprised of smaller spheres, while the amelogenin cleavage products appear as loosely associated spherical particles (Figure 2c), suggesting that the hydrophilic C-terminus plays an essential role in higher-order assembly of amelogenin [22]. It also contains only one phosphorylated site (^{16}Ser), which makes it different from highly phosphorylated matrix macromolecules that control biomineralization in bone and dentin or the acidic glycoproteins of mollusk shells. Unlike these polyanionic proteins which are readily soluble in water and contain large quantities of charged amino acid residues (for example, dentin phosphophoryn contains close to 70% of charged residues), including aspartic and glutamic acid and phosphorylated serine residues, proline accounts for 25 – 30% of all the amino acids in the peptide chain of amelogenin. Amelogenin also contains relatively high levels of histidine, glutamine and leucine, which explains its considerable hydrophobicity and the corresponding tendency to form aggregates in contact with a polar solvent, even at concentrations lower than 0.05 mg/ml [23].

3. Proteolysis

The action of proteases, such as matrix metalloproteinase-20 (MMP-20, also known as enamelysin), enamel matrix serine protease 1 (EMSP1, also known as kallikrein-4), and cathepsin B, in hydrolysis of amelogenins and other proteins presents a crucial segment of

amelogenesis. Due to its high selectivity of the cleaved peptide bonds, MMP-20 is usually considered as the major protease in this process. It is supposed to act as a regulator that controls the functionality of amelogenins. The latter proteins ought to be removed once the structure of enamel is sufficiently formed, so that the freed space would be filled with additionally crystallized apatite. Thin fibrous crystals are thus allowed to grow in the lateral direction and come in close contact with each other. This is the only way for the formation of 95% mineralized tissue to be achieved. In fact, most of the mineral deposition occurs after the degradation and removal of the enamel matrix [24]. The concentration of the mineral phase in the developing enamel is estimated to increase in this stage from 15 – 20% to its final percentage [25]. Two-thirds of the time spent in the process of amelogenesis thus belongs to the maturation stage. Amelogenins and ameloblastins are eventually removed, leaving predominantly enamelins and tuftelin in trace amounts in the enamel. Before the eruption of the tooth, but after the maturation stage, ameloblasts are broken down also and consequently enamel, unlike other tissues in the body has no way to regenerate itself “from the inside”. Even dentin is able to partially remineralize itself, as the pulp cells form layers of reparative dentin whenever bacterial degradation of teeth reaches the pulp.

The role of ameloblasts

Although crystallization of apatite in enamel is supposed to proceed without the direct involvement of cells in nucleation and growth, other possible roles that ameloblasts may play aside from releasing enamel matrix proteins and monitoring ionic concentrations will be discussed in this section.

The major role of ameloblasts in the process of amelogenesis is expression and secretion of structural proteins and proteases in a timely and spatially organized manner. Ameloblasts enter their first formative state after the first layer of dentin is formed, secreting the enamel matrix and at the same time retreating away from the DEJ, leaving the matrix to mineralize by itself. The Tomes’ process conducted by ameloblasts is responsible for organizing enamel crystals into bundles known as prisms, composed of rod and inter-rod enamel. It is suggested that crystallites grow in a perpendicular direction relative to the plasma membrane, so that the boundary between secretory and non-secretory regions at the ameloblast surface corresponds to the boundary between rod and inter-rod enamel [26]. There are also indications that the Tomes’ process may assist in orienting and elongating the aggregates of apatite crystals by controlling the orientation of the chains of amelogenin nanospheres.

However, it is assumed that while cellular activity primarily controls the enamel microstructure, proteins are in charge of organizing its structure at the nanoscale. After secretion, the enamel matrix assembles without direct cellular intervention, suggesting that apatite crystal morphology and texture are primarily the result of protein-protein and protein-mineral interactions. This assumption lies at the core of attempts to imitate the enamel growth *in vitro* by means of cooperative self-assembly of the given polypeptide species in an acellular environment. Another assumption behind these approaches is that ameloblast components are secreted all at once, with their activity controlled indirectly by reversible inhibitors, pH or local ionic strength. Another possibility would be that

ameloblasts secrete specific components of the enamel matrix ‘on demand” in a spatially and timely controlled manner.

As far as the ionic species are considered, even though the entry point of calcium and phosphate ions are blood vessels, ameloblasts control the ionic milieu in the enamel fluid by either directly “pumping” ions or delivering peptide species that either catalyze ionic reactions (such as the formation of bicarbonate or hydrogen phosphate ions by secreting carbonic anhydrase or alkaline phosphatase, respectively) or capture ionic species at the precise rate and amount, and thus maintaining the supersaturation at low levels to prevent spontaneous crystallization of the mineral phase. Studies have indicated that the apatite crystallites are oriented perpendicular to the ameloblast surface with some of them even touching the wall of the ameloblasts, thus suggesting their possible role in super-structurally organizing the primary acicular crystallites [27]. Ameloblasts also appear to be able to monitor the pH of the enamel fluid, and their rhythmical change from smooth to ruffled ended cells is accompanied by a local pH-change in the surrounding enamel fluid from nearly physiological (7.2 – 7.4) to slightly acidic (6.1 – 6.8) [28]. As it has been verified that amelogenin assemblies in aqueous suspension change size by almost two orders of magnitude in the region close to neutral pH (i.e., they possess about 20 – 30 nm in size at pH 2 – 5.5 and pH 7 – 12, and have almost a micron in size in the mid-region) [29], the purpose of these variations may be to modulate the aggregation character of amelogenin monomers and thereby finely tune their function in the crystal formation. It is highly doubtful whether these fine variations in pH, the precise monitoring and delivery of ions and a highly coordinated retreat of this biological ionic delivery system away from the crystallization front could be imitated *in vitro* without involving the presence of the cellular ingredients.

Considering that biomineralization *in vivo* proceeds as a sort of a non-equilibrium process in which cells continuously supply the extracellular space with matrix proteins and monitor concentration of inorganic ions through the activity of ion pumps, it seems to be unrealistic to expect that precipitation from the solution as an equilibrium process could yield the same functional structure as the one obtained in the biological process. The use of experimental devices able to replicate the dynamic environment similar to the one existing *in vivo* - like those based on controlled supply of ions so as to maintain the low degree of saturation - may thus be essential [13]. If this ion-delivery-at-a-controlled-rate function of ameloblasts could be imitated by a convenient experimental apparatus, then the last major role that ameloblasts play would be the one of expressing and secreting the right secondary proteins and proteases at the right time. If these could be identified, reproduced and introduced in the right sequence so as to perform the exact self-assembly process as the one taking place *in vivo*, they would not be needed anymore for the production of enamel structures.

On the other hand, after the decades of crowning nucleic acids as crucial factors in determining the structure and function of biological systems, biology is nowadays increasingly turning its interests onto the role that proteins are playing in determining cellular behaviour. After all, as DNA encodes proteins, whereby some of these proteins are responsible for replicating and maintaining the correct structure and pathways of DNA expression, it becomes obvious that cellular behaviour can be explained only in terms of feedback cycles described by the concept of autopoiesis [30]. In this sense, it was proposed

that both intact, full-length amelogenin and some of its portions may act instructively to specific cells, signalling them to differentiate into ameloblasts, osteoblasts or cementoblasts lineages [31]. This feedback interaction is supported by the effect of the disrupted protein structures on ameloblasts [12]. In fact, unlike MMP-20, which is a tooth-specific metalloproteinase secreted by only ameloblasts and odontoblasts, amelogenin is not restricted to enamel and hard tissues since it has been found in soft tissues including the brain and cells of the hematopoietic system [32]. Amelogenin is known to be expressed in dental pulp, and amelogenin knock-out mice have been shown to exhibit periodontal malformations, implying an important role that this protein may play in the development of periodontal tissues. As a matter of fact, the only current drug on the market that contains amelogenin is primarily used in regenerating periodontal ligaments. Amelogenin can also act as a cell adhesion protein [33], which can be visually observed in terms of high surface tension of its aqueous suspension. However, amelogenins found in tissues other than oral tissues are claimed not to be the full-length variant. They are splice products formed by the expression of a lesser number of exons compared with the full-length variant which has so far been only found in the developing enamel.

The structure and role of amelogenin

Amelogenin is normally thought to constrict rather than foster the crystal growth. It is assumed to affect the mineral habit by guiding the crystal growth along preferential directions and possibly organizing the acicular crystals into hierarchical forms. This idea is consistent with the observed poorly organized mineral layer formed in the amelogenin knock-out mouse [34]. On the other hand, it is known that enamel formed in the absence of amelogenin is pathologically thin, indicating that amelogenin might be involved if not in crystal nucleation then in the catalysis of the process of extension of the primary crystals. Elongated apatite crystals have been obtained by other methods, including hydrothermal processing and precipitation in the presence of various additives, not necessarily involving slow crystallization events [35], implying that the formation of thin apatite crystals is not unique to amelogenesis. Alternatively, compared to octacalcium phosphate for which the crystal growth is a more energetically favorable than nucleation, the free energy of these two processes approximately the same for apatite, and that for a wide window of saturation conditions, implying that the formation of elongated monocrystals of apatite is not a strictly favourable process. This also explains why the mechanism of formation of needle-shaped apatite crystals based on the aggregation of smaller subunits is often proposed [36, 37].

The typically observed morphology of amelogenin aggregates is the one of nanospheres with the size at the order of tens of nanometers [38]. Recent combined small-angle X-ray scattering (SAXS) and dynamic light scattering (DLS) experiments have indicated that a certain ellipticity (with the aspect ratio in the range of 0.45℃0.5) may be attributed to amelogenin assemblies [39]. Limited proteolysis experiments and those based on polyelectrolyte multilayers have indicated that regions at both C- and N- termini are exposed on the surface of the nanospheres [40, 41]. Accordingly, the structure of amelogenin nanospheres is usually depicted as the one with both C- and N- terminal regions present at the nanosphere surface (Figure 5), with the hydrophilic C-terminal supposedly in contact with apatite [8]. Experiments in which C-terminal was cleaved prior to the interaction with

apatite have demonstrated a reduced ability of amelogenin cleavage products to interact with apatite [42, 43]. With both C- and N- terminals exposed on the nanosphere surface, it is expected that C-terminal would be involved in the attachment onto the mineral surface, while N- terminal and the hydrophobic core of the protein would be involved in protein-protein interactions.

The knowledge on secondary and tertiary structures of amelogenin is, however, still poor. Diffraction studies have been impeded by the pronounced hydrophobicity of the protein, which tends to clump the molecules together and prevent the monomers from adopting a crystalline arrangement. More than 10^5 crystallization attempts are thus informally said to have failed. Only the amino acid sequence of amelogenin is currently known, although there is a prospect that both evolutionary structural alignment simulations [45] and *ab initio* modeling will provide an insight into the other structural levels of this protein. Despite the fact that the sequence of amelogenin is 90% evolutionary conserved, its primary structure is quite unique in the animal world, whereas there is only 24% similarity to the next closest protein in the human body. Of course, although there are examples of exceptionally high structural similarity between proteins who share only 20% of sequence similarity (haemoglobins, e.g.), substitution of one or a few out of hundreds of residues in a protein sequence often results in drastic changes in its secondary and tertiary structures [46]. The main challenge for these studies, however, comes from a high proportion of proline residues: 49/175 residues in the complete X chromosome sequence of human amelogenin – which includes the exon 4 otherwise missing in the full-length amelogenin secreted in the enamel matrix - and 42/175 in the complete Y chromosome sequence. The pervasive appearance of proline residues along the primary structure of amelogenin presents a considerable limitation due to their structurebreaking role and deviations from the regular secondary structure elements that they induce. The Raman Amide I band of recombinant full-length human amelogenin detected at 1620 cm^{-1} indicated intermolecular extended chains [18], and is in agreement with the results of circular dichroism (CD) studies, which have suggested the existence of polyproline type II structure in porcine amelogenin [47, 48]. The secondary structure of amelogenin is also highly affected by changes in pH, temperature and the presence of multivalent ions. As the aggregation behaviour of amelogenin is affected by the same factors, it is estimated that the secondary structure is important in regulating protein-protein interactions that lead to aggregation, self-assembly and, therefore, the crystal growth. Also, as it is known that folding mechanism is not solely determined by the amino acid sequence (as proposed by the now classical Anfinsen's model), but by numerous environmental factors that proteins are inherently sensitive to, and that changes in the tertiary structure can produce a drastic effect on the secondary structure of the individual peptide chains, including the occasional transformations of α-helices to β-sheets [49], it is natural to expect modifications of the structure of monomers depending on the size of their aggregates.

High content of proline residues, however, does not predispose the protein for adopting poly-L-proline helix type II in aqueous solution, similar to the one adopted by native collagen or many globular proteins (10% of individual amino acid residues in proteins exist in form of the polyproline conformation, and each protein on average contains one poly-

proline helix, although most of them are short, ranging from 4 – 6 residues in length) [50]. Whereas the sequence of collagen is composed of the repeating sequence of Gly-Pro-Y (with Pro residues preventing collagen from adopting α-helix and instead imposing a left-handed helix with ~ 3 residues per turn), proline residues in amelogenin are not positioned in such a periodic manner. Despite that, there are certain structural insights that can be derived from the high content of proline residues. First of all, the side chains of residues in the poly-proline helix protrude outward from the axis of the helix and are considerably separated by the extended nature of the helix, thus precluding hydrogen bonding interactions between adjacent side chains. As a result, both hydrophilic and hydrophobic side chains become exposed on the surface, providing favourable conditions for protein-protein interactions. The majority of side chains and backbone carbonyl and amide groups are thus also solvent-exposed, which is readily visible as kinks or bulges produced by a proline residue in the middle of an α-helix or β–sheet, respectively [51]. Unlike secondary structures with extensive intra-molecular hydrogen bonding, such as α-helix, the backbone carbonyl oxygen atoms are free to participate in hydrogen bonds across protein surfaces. Poly-proline secondary structures also exhibit a significant conformational stability, which additionally contributes to their exploitation as binding sites. Proline-rich sequences are, in fact, common recognition sites for protein-protein interaction modules [52]. An intrinsic predisposition of amelogenin for intermolecular interactions and for the formation of functional assemblies naturally follows.

The Amelogenin sequence also has a relatively high content of glutamine: 26/174 residues. The only exception among side chains that preclude the formation of intra-molecular interactions between side chains of a poly-proline protein is glutamine, as it can participate in hydrogen bonding with the backbone carbonyl oxygen of the preceding residue [52]. On the other hand, just as proline residues tend to participate in the formation of isolated extended strands that are conformationally distinct from poly-proline helices, glutamines have also been implicated in the formation of aggregates through the extended strand formation. Polyglutamines are also some of the peptides that readily adopt the poly-proline helical structure. Most proteins in human parotid and submandibular saliva, in fact, belong to the family of proline-rich proteins. On average, proline, glycine and glutamine account for 70 – 80% of all the amino acids within these proteins that are, however, not unique to salivary glands in the oral cavity, but are found in the respiratory tract and pancreas [53]. These proline-rich proteins are known for their ability to bind calcium and thus presumably assist in buffering the concentration of ionic calcium in saliva. They have also been shown to adhere strongly to apatite, exhibiting a lubricating effect and contributing to the formation of dental pellicle. However, owing to a high content of the three amino acids, their sequence is, unlike the one of amelogenin, highly repetitive [53].

Macromolecules involved in biomineralization tend to have high contents of carboxylate groups, which promote interaction with the precipitating cations and attachment to the crystal surfaces. The amount of charge is also an important factor. For example, elongated particles of apatite are precipitated in the presence of poly(L-lysine), whereas in the presence of more charged poly(L-glutamic acid) nanocrystals of apatite are formed under otherwise identical conditions of precipitation [54]. It was hypothesized that the higher the

charge of the heterogeneous nucleation surface, the more cations would be attracted thereto and more nuclei will be formed, resulting in smaller particles.

The main mammalian lineages show highly conserved residues in the hydrophilic N- and C-terminal regions, while the central region of amelogenin molecules seems to be more variable [56]. Hence, it is natural to expect that C- and N-terminals play the major role in the protein-guided crystal growth. As expected, the similarity in the structure of the molecular end groups is reflected in the similarity in the nanostructure of enamel among different mammalian species. The fact that the microstructural organization is subject to more variation among individual species can be correlated with a difference in molecular interactions that become activated at the later stages of amelogenesis, when controlled proteolysis may be a dominant factor in the protein assembly. A difference in the monitoring, secreting and migratory action of ameloblasts can also be considered as causing these structural differences in spite of the highly conserved amino acid sequence of amelogenin in mammalian species (Figure 6). For example, human enamel is similar to the one in pigs, bovine and dogs, but quite different from the enamel in rodents. Many other peculiarities exist in the animal kingdom, including the enamel layers entwined with dentin in horses' teeth (apparently producing a strengthening effect on the resulting hard tissue) and incisor teeth in mice that grow continuously during their lifetime. The fact that the structure of amelogenin is albeit well preserved during the evolution points at other factors being involved in shaping enamel other than the self-assembly of sole amelogenin. Whereas the similarity in the sequence could be moderate, as in the case of 66 and 45% homology between frog and *Xenopus*, and frog and mouse, respectively, the hydrophobicity plots of amelogenin retain distributions of relatively high similarity, as shown in Figure 7.

Evidencing specific morphological forms *in vitro* does not automatically imply the existence of the same morphologies *in vivo*. Even the general model for the very nucleation of apatite in bone, assuming that the negatively charged surface of osteocalcin, the most abundant non-collagenous protein in bone, coordinates five calcium ions in a spatial arrangement that corresponds to hydroxyapatite lattice, has never been confirmed with an *in vitro* experiment. Hence, whether amelogenin aggregates are in form of globules or beads or fibres during amelogenesis is still not known. What is known is that investigating the structure of a peptide (and consequently the function) or any other biological entity in one environmental context could not be directly conversed to another context. For example, transmembrane surface receptors do not exhibit almost any structure in an isolated state, but when returned to their *in vivo* surrounding they immediately display well-defined helices through the transmembrane domain. Finding the exact interrelating molecules and the exact chemical environment that would sufficiently resemble the native biological environment and trigger the natural, biological behavior of amelogenin presents a challenging task. Studies of the interaction of amelogenin with other peptide molecules present in the developing enamel matrix, altogether with the empirical study of the effects of various other chemical variables of the applied microenvironment, may thus present a potentially fruitful approach.

A precise empirical foundation is needed in the study of enamel formation, although the degree of complexity of the investigated process and differences in context in which *in vitro* results are obtained and the corresponding models proposed to take place *in vivo* make this

task more difficult. As shown in Figure 8, there are three chemical aspects that simultaneously take place and influence each other: crystal growth, protein self-assembly and protease activity. Yet, each of these aspects essentially depends on all the others, which furthermore increases the overall complexity.

The role of the proteases

One of the most interesting features of amelogenesis comes from the fact that not only does its' final product, the tooth enamel, present the hardest tissue in the vertebrate body, but its' high mineral content coupled with an ultrafine architecture implies that in this process the extracellular matrix directs not only the crystal growth processes, but its' own constructive degradation too. In that sense, the enamel protein matrix is unique in the realm of biomineralization as it fulfills the old truism of biology: "Intercellular matrix exists to be destroyed". Its role can also be neatly described by ancient Biblical verses: "Verily, verily, I say unto you, except a corn of wheat fall into the ground and die, it abideth alone: but if it die, it bringeth forth much fruit" [58]. This also makes amelogenesis a significantly more intricate mineralization process compared to dentinogenesis during which the collagenous protein matrix essentially remains kept in the same place. Studies of proteolysis of the enamel matrix are thus of particular importance in attempts to understand amelogenesis.

Two major enamel proteases have been identified so far: metalloproteinase enamelysin (MMP-20) and serine protease kallikrein 4 (ESMP1 or KLK4). They are secreted into the extracellular space by ameloblasts with the role of catalyzing hydrolysis of specific peptide bonds in amelogenin molecules. An increasing amount of evidence suggests that the cleavage products carry out different secondary self-assembly-related functions in the developing enamel matrix [59]. Hence, it was shown that mixtures of the full-length human amelogenin (rH174) and the first proteolytic cleavage product formed in the reaction with MMP-20, rH163, possess a markedly higher propensity for the formation of more complex, fibrous protein assemblies from the initial nanospheres compared with the pure rH174 [60]. The structure of amelogenin is thus thought to be modular, in a sense that it may contain several functional domains that become activated for different purposes and at different stages of amelogenesis [44]. This idea is supported by the findings that point out that enamel matrix proteases are expressed early during development. MMP-20 is expressed primarily during the secretory stage, whereas during the maturation stage its levels significantly drop, similar to those of amelogenin proteins [61]. Furthermore, an unchanging pattern of enamel matrix protein bands in the advanced secretory stage of amelogenesis is a sign of a steady, well-balanced state in which the ratio between enamel matrix components is kept constant during relatively long periods of time [26]. Since proteolysis takes place at this stage too, this implies that the rate of generation and secretion of a given peptide corresponds to the rate of its cleavage, which is different for each peptide in the matrix. In view of this, enamel proteases might carry out not only the function of degrading the enamel proteins and providing the space for the additional mineralization, but also act as essential regulators of the activity of the enamel matrix proteins.

This viewpoint is supported by studies that have shown that the mutations not only in amelogenin genes, but in those that encode MMP-20 cause *amelogenesis imperfecta*, a

pathological state typified by abnormal and significantly weakened enamel [62,63]. Experiments on MMP-20 knockout mice evidenced the formation of hypoplastic enamel composed of thin, shorter crystallites with undefined prisms [26]. The reason may be that smaller peptides formed and activated by the proteolytic action of MMP-20 are required to activate the extension of crystals after the primary apatite fibres are formed although they have yet to grow laterally. Inhibition of the activity of MMP-2, MMP-9 and MMP-20 by marimastat similarly led to an impairment of the mineralization of dental tissues in mice [64]. That KLK4, another major protease in amelogenesis, plays an equally crucial role has been evidenced by demonstrating that the mutation g.2142G>A on the gene coding for this protease causes an abnormal enzymatic activity, resulting in the enamel crystals of normal length but of an insufficient thickness [65].

At least 7 different cleavage sites on the full-length molecule in its interaction with MMP-20 were identified, but it is still not clear what factors are involved in the selectivity of MMP-20 in cleaving specific regions in the nascent molecule. What is known is that recombinant MMP-20 cleaves pig amelogenin *in vitro* at the identical sites as *in vivo*, that is, after residues 45, 63, 105, 107, 136, 148, and 162 [66]. On the other hand, there are indications that cleavage sites may vary depending on the conformation of amelogenin molecules, which is expected to be different for suspended amelogenin and amelogenin bound to apatite surface. However, although the digestion products are well defined, MMP-20 is not selective in terms of the cleavage sequence (with the exception of the 12 residues long sequence at the C-terminal, which is known to be the first one to be cleaved off), which is known to be subject to change depending on the experimental conditions applied. While the action of MMP-20 pertains to producing smaller functional polypeptide units, the role of the other major protease in this process, KLK4, is thought to be the one of complete digestion of amelogenin [67]. The latter argument is supported by *in vitro* studies in which an aggressive degradation of amelogenin by the action of KLK4 was observed [26]. On the other hand, KLK4 can be said to be relatively selective too as it was found to have a higher cleavage specificity for peptide bonds involving lysine. In general, different proteases are expressed during different stages of the process, presumably serving different functions. The peak in mRNA expression of KLK4 comes after the one of MMP-20, during enamel maturation. MMP-20, in contrast, is maximally expressed during the secretory and early transition stages of the process.

The initially secreted nascent proteins are present in the enamel matrix in a transient form and are quickly processed to generate a wide spectrum of smaller peptides. The nascent amelogenin is thus broken down to several fragments that serve specific roles in the assembly of protein and the mineral growth. As the mineral grows into an increasingly intricate and refined superstructure, the requirements for an progressively finer interaction between the peptides and the crystals arise. This might explain the cleavage of the large nascent molecule and the formation of smaller peptide chains that should be more flexible, intruding and precise in the finer self-assembly arrangement. In fact, the full-length amelogenin molecule is shown to be able to regulate crystal growth in the surface layer of secretory-stage enamel but not in deeper layers as well [23]. The nascent, intact amelogenin and its C-terminal cleavage products are found exclusively in the region of newly formed

enamel [68], that is, in the region of outer enamel, within 40 μm from the enamel surface [26]. Enamelin is similarly present only at the mineralization front within 1 μm away from the enamel surface. On the other hand, amelogenin and enamelin cleavage products are located in the rod and inter-rod enamel (i.e., between individual apatite fibres). Yet, by lacking the C-terminal, the amelogenin cleavage products have been shown to possess a rather low affinity for enamel crystals.

Hence, whereas enamel proteins are only present at the surface, in the outer enamel layer, cleavage products are exclusively found in the deeper, inner enamel layers where they also tend to organize into specific compartments. The C-terminal-containing cleavage products also tend to position at the enamel surface and are rarely found in the deeper layers [26], suggesting that the full-length molecules might be involved in the crystal growth only in the first stage during which the formation of elongated particles is initiated and is followed by self-assembly of these fibrous crystals into rods through a finer peptide-mineral interaction mediated by the C-terminal-lacking peptides that are small enough to protrude and line up in the inner enamel regions.

Deposition of the mineral in the deeper secretory and maturing enamel is thus considered as controlled by either amelogenin cleavage products or non-amelogenin proteins. Amelogenin extracts isolated from developing porcine enamel contained 7% of the full-length, P172 protein, and 11% of P161 and 40% of P144 proteolytic degradation products, with the rest being smaller peptides [67]. The relatively high content of small peptides suggests their important role in conducting the crystal growth. The use of smaller polypeptides that are either splice variants or cleavage products of the nascent amelogenin could correspondingly present a logical step in investigation of the mechanism of amelogenesis. At least 13 amelogenin isoforms, based on the combination of 9 exons, are currently known. One of them is leucine-rich amelogenin peptide (LRAP), a polar molecule often said to be a suitable candidate for self-assembly and supramolecular organization of apatite crystals. Its calcium binding affinity was measured to be more than 6 times higher compared to that of the full-length amelogenin [69]. However, despite its ability to form nanospheres, it has not been shown to accelerate the apatite crystal growth comparable to the levels provided by the full-length amelogenin [70]. The reason may lie in the fact that LRAP is composed of combined amino acid sequences of C- and N- terminals of the fulllength amelogenin, and as such lacks the central, hydrophobic part of the molecule which endows stability to it (e.g., by slowing the hydrolysis in reaction with MMP-20). Needless to add, an optimized balance between bioactivity and stability is the property of all functional biomolecules. The other candidate may be tyrosine-rich amelogenin peptide (TRAP) obtained by cleaving a short sequence of amino acids (44) at the N-terminal of the nascent molecule.

In relation to this, it is worth recollecting that short peptide chains that correspond to the active sequences in given proteins may be designed to possess the same functionality as the full-length proteins, and theoretical methods for determining these active sequences have been proposed [71,72]. On the other hand, the fact that nascent amelogenins expressed by ameloblasts are phosphorylated at only one residue (^{16}Ser), whereas the recombinant proteins expressed by *E. coli* and used in most studies do not comprise any post-translational modifications, often causing concerns about the functional discrepancies that may occur by

this slight structural difference, particularly in view of the fact that it is well-known that phosphorylated groups are especially important in the formation of calcium phosphate minerals (including dentin, the formation of which is directed by the phosphophoryn protein family that contains numerous repeats of the sequences Asp-Ser(P)-Ser(P)- and Ser(P)-Asp) [73]. Not only have studies on transgenic mice shown that the lack of C-terminal or deletion of 42 amino acids from the N-terminal induce severe defects in the produced enamel [74–76], but one study came to the conclusion that a single point mutation in the amelogenin-coding gene, resulting in a single amino acid substitution (proline to threonine) causes a type of *amelogenesis imperfecta*, related to severe dental enamel malformation [77]. A single Pro-41 to Thr mutation in recombinant full-length human amelogenin has been shown to result in significantly lower rates of apatite growth compared with the wild-type [78]. In view of the fact that the nearest proteolytic cleavage site lies between the residues 45 (Trp) and 46 (Leu), and that this mutation significantly reduces the enzymatic hydrolysis of amelogenin in the reaction with MMP-20, it has been suggested that proline residues might play a major role in aligning the cleavage-site residues along the active site of the enzyme [79]. In fact, the concentration of proline residues along the amelogenin sequence typically increases in the vicinity of the sites that are subject to proteolytic cleavage. These results have implied that the diminished enzymatic interaction between amelogenin and MMP-20 may be the cause of *amelogenesis imperfecta* [79]. Aside from innumerable cases wherein single-point mutations disrupt the functionality of proteins (e.g., substitution of valine with glutamic acid in the β-chain of haemoglobin resulting in sickle cell anemia), single-point mutations have also been shown as able to modify the peptide self-assembly [80]. Yield in the synthesis of calcium molybdate by precipitation induced by phage peptides was reduced by one-third after a single serine residue was replaced by aniline [81]. As amelogenesis is a complex biological process that involves a network of feedback interactions between cells, multiple polypeptide species and crystals, it is inherently predisposed to exhibit a significant sensitivity towards slightest changes in the boundary conditions.

The effects of the slow rate of crystal growth

Crystal growth within enamel occurs at an exceedingly slow pace. Growing at the appositional rate of ~ 2 – 4 μm per day, it takes 4 years for enamel to get completely formed and mineralize the entire crown, although the rate of the process varies with the tooth type and the species in question [28]. The appositional growth rate for enamel in rat incisors is thus higher than in humans: 13 μm/day vs. 4 μm/day. In fact, amelogenesis takes more time to complete than is needed for the embryo to be formed *in utero*, which speaks well in favour of its extraordinary complexity. The mechanism of crystallization of enamel is still an enigma. For example, whether the crystal growth proceeds by adsorption of ions or by aggregation of nanosized sub-units or as a back-and-forth stream of crystallization/dissolution/crystallization events, in which “mistakes” are made but recognized and subsequently corrected, as is otherwise typical for biological syntheses [82,83], is not known.

The low metastable levels of supersaturation appear to be crucial for providing the right conditions for protein-guided crystal growth. Low rates of nucleation and crystal growth naturally favour the formation of elongated crystals. For example, when controlled

degradation of urea is used to slowly increase alkalinity of the solution and provide conditions for precipitation, apatite crystals formed are either plate-shaped or needle-shaped [84]. Single-crystal apatite fibres of 20 – 60 μm in length and 100 – 300 nm in diameter were thus obtained by precipitation using decomposition of urea [85]. Attempts to initiate nucleation and crystal growth at a higher rate by increasing the supersaturation ratio (S) would deprive amelogenins from their ability to direct the crystallization events [86]. The concentrations of calcium and phosphate ions in the fluid of developing enamel are 0.5 mM on average, and 2 – 5 mM, respectively, resulting in a degree of saturation below 12. It is interesting that approximately the same ratio between the concentration of calcium and phosphate ions (markedly different from the one within hydroxyapatite crystals, i.e., Ca/P = 1.667) is present in saliva, suggesting its favourableness for both the growth of enamel during amelogenesis and the natural remineralization of enamel. Much of the calcium ions are furthermore bound to peptides, which contributes to an even lower supersaturation with respect to apatite in the developing enamel. Finding the optimal supersaturation levels is, however, challenging, especially since it is not certain whether amelogenins assemble the crystal phase using individual ions or individual nanosized (crystalline or amorphous) particles of calcium phosphate as building blocks. In the former case, nucleation (for which calcium phosphates have a rather high tendency, implying that larger particles typically form through aggregation of separately nucleated clusters) would be thoroughly avoided, whereas in the latter case it would present the initial stage of crystallization.

As it was shown that precipitation conditions under which supersaturation ratio increases gradually from $S = 0$ to $S > 1$ leads to favourable conditions for substrate-specific growth of apatite in the presence of amelogenin [18], the growth of enamel may be said to resemble the classical model of growth of silicon nanowires, as proposed by Wagner and Ellis [87]. According to this model (Figure 9), nanodroplets of gold deposited on top of silicon wafers attract silicon atoms from the vapour and after becoming supersaturated with respect to silicon begin to precipitate it, building well-aligned nanowires oriented perpendicular to the underlying surface [88]. A gradual increase in saturation levels in this case allows for the highly substrate-specific and oriented growth of the elongated crystals, and it is hypothesized hereby that similar conditions need to be provided for the proper bio-imitational growth of the enamel-like crystals of apatite. It has been shown that adsorption of amelogenin onto apatite substrates is the first step prior to their controlled growth [18,38,86], with the first nuclei forming within the amelogenin deposits, as in agreement with the aforementioned model. Consequently, the role of amelogenin in channelling and controllably delivering constituent ions or crystalline or amorphous building blocks can be reasonably assumed. One such model based on hypothesized β-spirals formed by a series of β-turns in the secondary structure of folded amelogenin and their channelling of Ca^{2+} ions to the mineralization front was previously proposed [89,90]. Amelogenin may be thus said to act not as an inhibitor of crystal growth, as the currently reigning model proposes, but as a bridge between the ionic solutes or complexes and the crystalline surface that they are anchored to, as shown in Figure 10. The presence of hydrophobic domains within amelogenin structure may be important in ensuring the proper “gating” of the units of growth, as already known to exist in the case of ionic channels on cell membranes [91].

The elongated morphology and highly crystalline nature of apatite in enamel compared to smaller, nanosized and much less crystalline apatite particles formed in bone and dentin implies that different models of growth should be applicable for these two cases of biomineralization. Indeed, the model involving template-based catalysed nucleation and limited growth by means of hydrophilic proteins, such as osteocalcin, valid for bone and dentin can thus be claimed not to be applicable for the case of enamel. Instead, a model based on: (a) slow crystal growth; (b) gradual increase of supersaturation levels; and (c) the role of amelogenin in channelling the growth units onto the growing apatite surface, is given here as an alternative to the standard models of biomineralization that depict nucleation events as taking place on foreign organic surfaces, governed by their hydrophilic character and precisely matching lattice spacing, and crystal growth as proceeding while being inhibited by the adsorbing bioorganic particles.

The role of water and minor components

In this section, the discussion will question the role of some of the often times neglected components of the enamel organ: fluoride and carbonate ions, water, and the underlying dentin.

Irrespective of whether it is found in enamel, dentin, cementum or bone, biogenic apatite is always impure and non-stoichiometric. The major impurity is carbonate (3 – 8 wt%), and minor impurities include sodium (0.5 – 1 wt%), magnesium (0.4 – 1.2 wt%), potassium (0.03 – 0.08 wt%), chloride (0.01 – 0.3 wt%), and fluoride (0.01 – 0.06 wt%). Most of these impurities, except fluoride, increase the solubility of apatite [92].

As enamel naturally presents a carbonate-containing apatite [93], a perfect imitation of amelogenesis would account for the presence of carbonate ions. Precipitation under atmospheric conditions leads to a natural transfer of dissolved carbonate ions to the solid state. Carbonate ions substitute phosphate ones in the stoichiometric formula of hydroxyapatite, but in order to maintain the electroneutrality of the compound, either a decrease in the stoichiometric amount of calcium or a co-substitution with another ion present in the solution, such as chlorine or potassium, takes place.

Although adding fluoride to biomimetic experiments aimed at replicating amelogenesis would be a natural approach in view of its presence in natural enamel apatite, exceeding amounts thereof are known to result in increased porosity and weakening of the enamel structure [94]. It was also shown that increased levels of fluoride in developing enamel decreased the activity of MMP-20 [95], resulting in the condition known as fluorosis. The role of fluoride ions in promoting elongation of apatite crystals has, however, been well documented. In one set of experiments, only the combination of amelogenin and fluoride led to formation of rod-like apatite crystals, while merely octacalcium phosphate precipitated in the absence of fluoride [96,97]. On the other hand, it was demonstrated that fluoride ions do not directly interact with amelogenins, but limit their effect on the process of amelogenesis to their incorporation into the apatite crystal lattice [98]. Another set of experiments came to the conclusion that the epitaxial growth of hydroxyapatite on flouroapatite substrates results in the formation of needle-shaped crystals [99]. The role of other ions, including magnesium

and sodium, known to be present in the natural enamel, should not be underestimated, especially in view of their inhibiting effect on apatite growth rates.

Enamel can be seen as developing through a complex interaction between three components: the organic matrix, the mineral, and the aqueous fluid. Early secretory enamel consists of 50 – 60 vol% of water, 20– 30 vol% of protein, and about 15 – 20 vol% of mineral. High concentrations of amelogenin (~ 200 – 300 mg/ml) in the developing enamel matrix imply that the latter resembles a gel more than an aqueous solution [100]. Growing apatite in gelatinous media rather than in ordinary aqueous solutions thus presents a natural biomimetic choice [101,102]. Crystallization of apatite from such dense media may favour the slow and controlled growth. Precipitation of fluoroapatite in gelatin, without amelogenin present, thus resulted in spherical composites consisting of needle-shaped crystals and around 2% of organic matter [103,104]. However, due to the limited supply, typical studies have dealt with concentrations of less than 0.4 mg/ml, which means that self-assembly behaviour of amelogenin at significantly higher concentrations has not been carefully evaluated.

Density of the aqueous medium is higher compared to ordinary aqueous solutions not only in the enamel matrix, but in biological environments. Under such circumstances, water exhibits modified structure and properties. Cytoplasm typically contains about 400 g/dm^3 of macromolecules, which occupy 5 – 40% of the total cell volume with an average separation between them of 1 – 2 nm. Within such nano-confined conditions, water possesses an altered hydrogen bonding structure in comparison with the bulk water. In addition, by playing various structural roles, water presents an essential component of a fully functional protein. Although it has been shown that structure and functionality of some enzymes can be preserved in non-polar media or even in vacuum (albeit the preservation of bound water even under such circumstances), it is suggested that water “lubricates” the peptide chains and provides conditions for favourable molecular recognition effects. Consequently, the concepts of diffusion and solubility limits should be redefined with the transition to complex and dense media such as those from which enamel crystal grow.

The effects of minor additives and environmental effects in experiments that tend to replicate amelogenesis should not be underestimated. Different salts can have different effects via either changing the dielectric constant of the solution or by screening interactions vital for stabilizing the protein structure. Some salts, such as *guanidium* chloride, may destabilize the proteins and even act as denaturants, whereas others, so-called osmolytes, may act as stabilizers against thermal unfolding. Although insertion of individual charged species into the non-polar environment of the protein globule interior is highly unfavourable, this effect could be done by non-polar solutes, such as alcohols that reduce the stability of proteins in water. A recent computer model has shown that ions with low charge density become adsorbed preferentially at the surface of protein or any other hydrophobic particle, leading to micelle-like clusters of particles that are as such saved from further aggregation and precipitation, whereas the ions with high charge density tend to be depleted from the particle surface and also lead to the formation of similar clusters of dispersed hydrophobic particles [105]. Only in the intermediate case, the conditions for dispersion of individual particles, avoiding the formation of clusters, exist. This example demonstrates that hydration

effects could not be reduced to a simple rule of a thumb, and that in synergy with other species present in the reaction, salt effects are difficult to predict.

Precipitation of apatite increases acidity of the surrounding solution, according to the following equation.

$$10Ca^{2+}+6HPO_4{}^{2-}+2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2+8H^+ \quad \text{(Eq. 1)}$$

Namely, for each unit cell of hydroxyapatite formed, 8 protons are released into the solution, although this number may be lower depending on the amount of carbonate (or other ions present in the solution) incorporated in the lattice and also whether hydrogen or dihydrogen phosphates are consumed in the process as precursor phosphate ions. Buffers are typically used to maintain a constant pH during precipitation, although their side effects on the protein assembly are not known. A variety of effects by different buffers on enzymatic activities have been previously detected in far simpler conditions [106]. Also, as already mentioned, pH during amelogenesis varies within the range by more than one unit (i.e., from 6.0 to 7.2) [107]. Dramatic changes in pH are, in fact, known to occur with the onset of biomineralization, which is why pH is often considered as one of the most important parameters in controlling it [108]. However, the mechanism by which the enamel matrix buffers the formation of HAP is still not well understood. All three of the most important buffers in biological systems – phosphate, carbonate and protein – may play their role. Another buffering mechanism proposed relates to a constant flushing of the formed protons by a dynamic flow of ions through the enamel matrix.

The mineralized dentin substrate may play a role in orienting the amelogenin nanospheres by protruding into the enamel matrix at the dentin-enamel boundary [109]. Although enamel crystals are formed outside of the area of contact with the underlying dentin [110], this does not speak against the possibility of an initial epitaxial influence that dentin may exert on the self-assembly of enamel crystals or rods, the first of which are nucleated along the DEJ. Although there are claims that despite the fact that epitaxial effects can be responsible for controlling the nucleation events, and thereby determining the crystal location, orientation and phase, the final morphology and size of the growing crystals will be determined by events taking place in the areas unaffected by the nature of the substrate [17]. Quite contrary, once the initial order is established, even if it occurs within a small range along the axis of growth, an ordered attachment of the subsequently formed crystals becomes favoured. Any mechanism that explains how non-equilibrium biomineral morphologies defying the symmetry of the underlying lattices form has to rely on organic-inorganic interactions that take place in a highly coordinated fashion in the 3D environment surrounding the crystal growth.

Epitaxial effects been proven many times as essential in self-assembly procedures [111], and many biomineralization mechanisms (e.g., crystallization of thin flakes of nacre in mollusc shells) depend on the interfacial structural matching between an organic substrate and an inorganic phase. Despite the fact that the hardness of enamel is a result of its nanoscale superstructural organization, the strength of enamel is also highly dependent on the supporting dentin. This supporting interaction between the dentin substrate and

superstructurally organized enamel crystals may be also critical for any replication of this intricate assembly of fibrous apatite crystals *in vitro*. It is also known that signals originating from the dental papilla are required to activate the expression of amelogenin [94], which points to an even wider scope of amelogenesis, in view of which the prospects of replicating the process by focusing only on a selected number of species and control parameters can be subjected to reasonable scrutiny.

Was it chicken or egg that came first? Who is older in the process of amelogenesis: the mineral or the protein assemblies?

Each self-assembly phenomenon can be seen as a bidirectional process in which the assembling system and its environment serve as templates for the other's structural evolution [112,113]. Hence, it has been suggested that not only assembled amelogenin spheres and ribbons guide the growth of apatite crystals, but that calcium ions and mineralization may be required for the proper assembly of the protein. The formation of amelogenin fibres by aggregation of nanospheres could be shown as greatly facilitated in the presence of calcium and phosphate ions, invoking hypotheses according to which calcium ions may act as bridges that link and align individual amelogenin nanospheres. Although amelogenin nanospheres have a tendency to align and merge into fibres even without the presence of calcium or phosphate ions [114], the formation of nanochains of amelogenin is enhanced in the presence of calcium [115]. Self-assembly of other proteins and organic molecules has been shown to be dependent on the supply of calcium ions [116]. In fact, many proteins become readily cross-linked, aggregated and precipitated in the presence of calcium ions, which explains why through a constant pumping of these ions across the cellular membrane and into the extracellular medium cells maintain the cytoplasmic concentration of calcium at around 0.1 μM, which is by four orders of magnitude less compared to the extracellular fluid [1]. It has been, furthermore, argued that the self-assembly of amelogenin needs to be coordinated with the precise control of pH and calcium and phosphate concentrations. Supersaturation levels and the growth rate of the mineral would thus be coordinated with the assembly of amelogenin nanospheres. The notion of "co-assembly" is thus increasingly finding usage in explaining the mutual fibrilization of the protein and mineral phases during amelogenesis [117].

A model in which gel-like properties of the enamel matrix were correlated with the protein/mineral co-assembly was proposed by Eastoe in 1963 [118]. He stressed that the enamel matrix may exhibit thixotropic behaviour, which has up to this date remained an unconfirmed hypothesis [28]. In this state, local increases in pressure caused by the growing mineral would cause a protein flow, resulting in a mutual form of self-assembly [100]. Another similar hypothesis was proposed by Fearnhead in 1960 [119]. He suggested that streaming of the organic molecules as they are secreted by ameloblasts causes the specific orientation of the apatite crystals. Both hypotheses highlight the importance of the dynamic nature of the physicochemical medium in which enamel forms. Cölfen and Mann have recently proposed a similar model according to which a simultaneous assembly of the protein and mineral phases occurs under the influence of stress. The stress field is conceived as originating internally through progressive changes in densification, or externally through

the action of adjacent cells. Thus, initial spherical apatite particles would correspond to spherical protein aggregates, whereas a mutual elongation of the two phases occurs afterwards. The idea of the formation of elongated crystalline structures by aggregation of smaller subunits presents another aspect of this model, according to which the "formation of macroscopic architectures with embedded structures involves a continuous supply of hybrid nanoparticle precursors to the growth sites of crystals formed initially in association with mesoscopic aggregates" [36]. This model is still not confirmed and remains as a point of disagreement between different research groups over whether the individual calcium carbonate plates of nacre are monocrystalline or composed of smaller crystalline subunits.

Two major scenarios for the interaction between the protein and the mineral phase are usually outlined. According to one scenario, proposed by Margolis, there is a cooperative and simultaneous assembly of the two phases into elongated, ribbon-like morphologies. The second scenario, most notably propagated by Moradian-Oldak, pre-assembled amelogenin nanospheres align so as to form strings and ribbons that subsequently play the role of a template for an oriented growth of apatite crystals [19].

In view of interrelated amelogenin assembly, proteolytic hydrolysis and the crystal growth, we have proposed a triadic nature of amelogenesis as the basis for its biomimicry, as shown in Figure 8. The proper assembly of amelogenin will thus depend on the proteolytic hydrolysis thereof, whereas the assembly of amelogenin spheres of fibrils will affect the rate and selectivity of proteolysis by exposing/concealing specific active groups. Proteins in native globular conformations are known to be more stable to the proteolytic action compared to unfolded ones, and amelogenin assemblies may be subject to similar proteolytic sensitivity. Also, crystallites precipitated in the presence of monomeric rM179 and rM166 comprised acicular morphologies, whereas the pre-assembled full-length rM179 had no influence on the crystal morphology [115], indicating that conditions for a co-assembly in which both phases would structurally change need to be established (rather than attempting to achieve a mere "template" effect wherein the growing phase adopts the shape of an unchanging matrix) to promote a successful protein-crystal interaction.

Assembly of amelogenin nanospheres and factors affecting the transition to nanofibres

The tendency of amelogenin to form nanosized spherical assemblies in water and other polar solvents is explained by its hydrophobic nature [120], which is furthermore confirmed by the stability of amelogenin monomers in non-polar acetonitrile [121]. Also, amelogenin nanospheres with the hydrophilic C-terminal cleaved off have been observed to possess a higher tendency towards aggregation [122]. As the nascent molecule is processed at a relatively high rate by the action of proteases in the biological conditions, it is expected that these nanospheres cannot last for a long time, let alone be a crucial factor in the formation of enamel. As shown in Fig.2c, the introduction of proteases induces broadening of the size distribution of the protein globules, which is a natural consequence of the existence of multiple peptide species following the proteolytic digestion.

The question whether uniform amelogenin nanospheres have any biological meaning can thus be naturally posed. It is realistic to expect that the prompt hydrolysis of amelogenins *in vivo* would lead to morphologies of a more complex symmetry. On the other hand, although aggregates of identical polypeptide species are in most cases indications of pathological states (and that particularly when these aggregates are fibrous in morphology [123]), there are a number of proteins functional only within aggregates of the same species, including most notably the components of cytoskeleton: actin, myosin, tubulin, etc. In fact, proteins comprising a left-handed helical polyproline conformation (including abductin, titin and elastin) are known to be playing a pivotal role in the genesis of elasticity owing to extensive protein-protein interactions that this structure promotes. The common characteristic of all of these proteins is the propensity for the formation of fibrils, structural flexibility (due to extended helices and the absence of intra-chain hydrogen bonds), and involvement in molecular recognition and multi-molecular self-assembly processes [124].

Although the idea of representing protein particles as charged spheres may be useful as the starting point in conducting amelogenin-guided growth and assembly of rod-shaped apatite crystals, representing protein molecules that are abundant with sites that enable sophisticated molecular recognition effects in terms of inert charged spheres is oversimplified. It is well established that one of the ways by which organic surfaces facilitate the nucleation of complementary inorganic phases is precise matching of the distribution of charges across the organic surface with the distribution between cations and anions in the nucleated mineral lattice (although this may present an oversimplified depiction of biomineralization processes; namely, the biomineralization process is too dynamic to support the static conditions required for epitaxial growth, whereas organic surfaces are rarely flat and well-ordered, hardly fitting the approximation of constant ligand spacing). However, this form of surface charge matching occurs at a significantly finer scale compared to interactions described by merely invoking electrostatic potentials of the interacting surfaces. Whereas merging of the pre-assembled amelogenin entities could be possibly explained by invoking surface charge effects, the subsequent re-organization of the new assemblies could be described only by referring to molecular recognition effects that take place at significantly finer scales. That biomineralizing interactions between the respective organic and inorganic phases necessarily involve fine molecular recognition is demonstrated in Figure 11.

As mentioned earlier, identifying a specific self-assembled morphology of amelogenin molecules *in vitro* does not necessarily imply their presence in biological conditions in which their full functionality becomes exhibited. In other words, any attractive morphology of amelogenin assemblies detected does not necessarily imply their relevance for the enamel development. Due to the natural tendency towards superior molecular recognition effects, amelogenin spheres could be probably assembled into a wide array of architectures under different chemical conditions, but none of the formed structures would guarantee its biological significance for the process of amelogenesis. For example, observing cytosine molecules assemble into nanochains on gold surfaces [125] does not mean that these assemblies appear in any biological situation. Similarly, DNA molecules have been evidenced to assemble into a variety of forms and morphologies, including cubes, triangles, pentagons, hexagons and octahedrons [126], but none of which has probably had any role in

the biological function of DNA, even in evolutionary terms. The aforementioned macrophage peptidepromoted precipitation of inorganic salts at atypically low temperatures does not mean that these inorganic/organic interactions possess any biological meaning. A high potential for molecular recognition actually predisposes organic molecules and particularly polypeptides to assemble into an endless variety of morphologies depending on the environmental conditions that they are naturally structurally sensitive to [127]. It is certain that combination of peptides formed by random sequestration of the full-length amelogenin sequence would under some conditions assemble into a variety of attractive morphologies, none of which would be an analogue to the biological processes in which amelogenin acts. Hence, what is observed in a simplified biomimetic set of conditions may present only an indication that similar structures and mechanisms may occur *in vivo* as well.

Spherical amelogenin nanoparticles were identified in histological sections of developing mouse molars in form of “beaded rows” that surround the enamel crystals at the early stages of its formation, which is in accordance with previous observations of the spherical nanoparticles in the vicinity of developing enamel crystals [67]. Despite this, it has never been proven that these were indeed amelogenin spheres, let alone that they actually attach to the crystal phases and direct the crystal growth. The composition of these spheres remained unidentified, and it was even suggested that these spheres, reminiscent of irregularities and steps on the crystal surface [67], might be particles of amorphous mineral precursors that subsequently fuse into larger crystals [100]. Surface irregularities in terms of steps and terraces exist on all biominerals, reflecting the natural inclination of biosystems towards imperfection. The topography of enamel crystals similarly exhibit surface irregularities, the size of a single hydroxyapatite unit cell, which hypothetically corresponds to the tendencies to increase protein binding in the process of biomineralization [128]. In fact, there is still a debate among the researchers involved in the study of amelogenesis over the question whether the apatite ribbons grow by diffusion of ions or form by aggregation of smaller particles. It is known that long and defect-free filaments with the aspect ratios of up to 10,000 can be obtained by the aggregation mechanism [129], overthrowing the idea that so grown apatite fibres would be prone to fracture due to many defects. In fact, in the context of a highly anisotropic organization of apatite crystals in enamel, such a slightly imperfect crystalline nature could be even proven as favourable.

Therefore, nanospheres may be the simplest self-assembled structures of amelogenin, typically formed in simple laboratory settings. Fibres may present a step forward [130, 114]; however, it is not known whether even the elongated amelogenin assemblies serve any function in directing the crystal growth. True significance from the biomimetic point of view might lie in far more complex (and maybe not even that attractive in their symmetry) supramolecular architectures.

When thermally induced, denaturation of amelogenin does not proceed as a sharp transition. Instead, there is a continual change in the tertiary structure as the temperature is raised, as detected in both DSC and UV-CD studies [131]. Also, amelogenin heated up to 80°C has been detected with a different structure compared to the same molecule subjected to chemical denaturation, suggesting that the thermally degraded sample at 80°C may still possess a specific secondary structure. In fact, fibrous proteins are known for their generally

higher resistance to denaturation when compared to globular ones. Furthermore, this thermal transition is shown to be fully reversible. Deconvolution of the broad DSC peak (different from sharp DSC peaks of both globular proteins such as enzymes, and fibrous ones, such as collagen) that corresponds to this transition yielded three peaks and the double transition (Figure 12), signifying that in contrast with most proteins, amelogenin is prone to exhibit flexible changes in the tertiary structure, and not only as a function of temperature. Such changes may expose different key groups at the surface, which could be crucial for the interaction with the mineral at the different phases of its formation. It is known that the distribution of charges within a single amelogenin nanosphere is asymmetric [109], which altogether with a significantly less ordered positioning of proline residues within the amelogenin sequence compared to that of collagen, may indicate a higher conformational entropy and flexibility of the tertiary structures than amelogenin molecules adopt.

It has been known that denaturation of some types of proteins cannot fit the standard model where only two states are invoked: native/folded and denatured/unfolded. Instead, there are numerous intermediate and metastable states through which the protein structure can pass on its way towards either denaturation or folding. In general, native states of proteins correspond to folded, typically globular conformations, whereas denatured states correspond to unfolded, typically fibrous morphologies. It may be the case that amelogenin similarly undergoes a seeming denaturation on its transition to fibrous morphology, but eventually reaches a functional metastable state. Such is the case of so-called molten globules and other hardly defined protein conformations when instead of a sharp transition, one detects only a variation in a continuum of conformationally heterogeneous states, especially under conditions in which the native structure is mildly stable. A typical example of this phenomenon occurs at relatively low pH values when protonation of the carboxyl group occurs, resulting in the lack of stabilizing ligand interactions and destabilization of the whole molecule. Also, there are indications, supported by CD studies, that unfolded proteins may adopt a type of polyproline structure instead of being considered as random-coil conformations [52]. The α–helix of native human lysozome has been, for example, observed to melt to a polyproline conformation in a partially denatured protein. Be that as it may, unfolded and denatured protein states are best described not as completely unstructured coils randomly sampling backbone conformations, but as composed of segments structured to various extents, possibly depending on the unfolding/denaturation mechanism.

The amphiphilic nature of amelogenin molecules has given rise to speculations that their nanospheres may present reverse micelles [132]. In a polar medium, such as water, the hydrophilic C-terminals would be positioned at the surface of the spheres, whereas the hydrophobic parts would tend to be oriented towards the core. In case of other fibrous proteins, there is evidence that hydrophobic alkyl tails could be packed on the inside of the fibre, with the acidic moieties exposed on the surface [73]. The possibility that amelogenin may adopt a micellar structure could be strengthened by the pronounced structural homology with β–casein, the milk protein which also contains relatively high amounts of proline and glutamine residues and is known to form micelles as calcium-protein complexes [133,134]. Like amelogenin itself, this protein has not been crystallized yet due to its hydrophobic nature. However, representing globular proteins as polymeric micelles of thoroughly hydrophobic interiors and hydrophilic surfaces supplies a simplistic picture.

Proteins are neither amphiphilic oil drops nor crystalline particles. It has been calculated that on average 83% of non-polar side chains are buried in the interior of the folded protein, but so are 63% of polar side chains and 54% of the charged side chains [135]. The energy landscape of a protein is ridged, implying that not necessarily the most favourable local tendencies (such as hydrophobic side chains being buried in the interior of the folded protein) are satisfied within a folded peptide structure. Secondary structural elements of any given protein are normally not stable alone, i.e., without them being incorporated in the protein as a whole, which indicates a significant involvement of non-local effects in determining the protein structure. Also, the role of water in stabilizing proteins has been well documented, which explains why the interior of a globular protein could not be imagined as “dry”, as in the case of ordinary micelles [136]. In fact, even with a perfect close packing, ~ 25% of the interior of a protein molecule would be empty space.

Thinking of amelogenin molecule as a peptide amphiphile prone to assemble into micelles may lead us to useful insights in the quest for the ways to reach control over nanosphere-to-nanofibre transition, which many claim to be a crucial step in imitating the process of amelogenesis *in vitro*. For example, if considered as micelles, protein nanospheres could be forced to transform from spherical to elliptical or rod-shaped by manipulating the micelle curvature parameter. In this context, the example of cetyltrimethylammonium bromide (CTAB) and the transition of its micelles and reverse micelles from spherical to elliptical ones could be instructive. This transition can be achieved by the addition of a proper co-surfactant, such as benzene or a long-chain alcohol. Penetrating into the micellar interface, co-surfactant molecules affect the mean distance between the polar head-groups of surfactant molecules and reduce the electrostatic repulsion between them, promoting the spherical-to-cyllindrical micelle transition[137–139]. This results from the fact that the curvature of a micellar aggregate is dependent on the interfacial tension between micelle and the surrounding phase [140], and is strongly influenced by the ratio of the effective head-group crosssectional area to the effective cross-sectional area of the aliphatic chain. With decreasing this ratio, the surfactant aggregate shapes should follow the following trend: spheroidal micelles → worm-like (rod-shaped) micelles → bilayer structures → reverse structures [137]. A decrease in the headgroup repulsion with the subsequent sphere-to-rod transition can also be brought about by counterions which insert between the charged groups or by other solutes located in the head-group region [138]. In addition, the further the point in the phase diagram is from the critical micelle concentration (CMC), the higher the possibility of forming cylindrical micelles is. Therefore, increasing the concentration of the amphiphile might result in this transition, which implies that high-density, gel-like amelogenin may have a higher propensity to elongate its assemblies. This transition can sometimes be catalyzed by minor effects, such as single-ion substitutions [138].

Linear surfactants, such as CTAB, in general tend to form ellipsoid micelles more often than branched surfactants. For example, linear cetyltrimethylammonium 4-vinylbenzoate in water forms cylindrical micelles of 4 nm in diameter and thousands of nanometers long [141], whereby branched sodium bis(2-ethyl hexyl) sulfosuccinate (AOT) forms exclusively spherical micelles. Linearization of the amelogenin peptide chain by means of the right experimental conditions, additives or proteolysis may thus favour the formation of ribbon-

like structures [132]. In correlation with the mechanism by which co-surfactants and salts [99, 142] influence elongation of micelles, it might be expected that calcium or phosphate ions could screen the electrostatic interaction between the polar C-terminals of the peptide spheres and thus promote their elongation. On the other hand, the formation of ribbons was observed even without the presence of calcium and phosphate ions, the reasons for which may lie in the effect of another screening substance. The use of many components in the preparation procedures, typically including organic buffers to stabilize the pH, zinc ions to activate the metalloproteinase, sodium azide to sterilize the solution and potassium chloride to simulate the physiological ionic strength makes it exceedingly difficult to eliminate the probability of an undesired side effect of any of these substances on this or any other crucial transition in these biomimetic experiments.

CTAB reverse micelles have been used to obtain elongated hydroxyapatite particles. Hydrothermal processing of a reverse micelle containing precipitate thus resulted in organized bundles of hydroxyapatite fibres with aspect ratios of up to one thousand [129]. Mesoscopic assemblies of other surfactants, including AOT, were similarly employed with the idea that, due to the amphiphilic nature of amelogenin, the interaction of apatite with other surfactants might bring insight into the mechanism of the enamel formation [143]. Precipitations in the presence of various polysaccharides [144] as well as in simulated body fluid [145] also resulted in needle-shaped crystals of apatite. It is known that anions can have a crucial effect on the morphology of precipitated powders [146] (versus cations which are usually used for coagulation purposes), which provides another biomimetic task: qualitatively and not only quantitatively (through the physiological ionic strength) replicating the ionic milieu in which the enamel grows.

It is worth recalling in this context that the actual biological site at which amelogenin nanospheres assemble is still not resolved; whether they assemble in ameloblasts or the extracellular, enamel matrix medium is not known. What is known is that primary amelogenin molecules in the intracellular medium of ameloblasts exist encapsulated in form of vesicles and are in this form inserted into the enamel matrix. Adding that the formation of some hard tissues, such as cartilage, begins within phospholipid vesicles, an approach where amelogenin/apatite interactions would be studied in the presence of micelles or vesicles seems promising. Namely, negatively charged phosphatidyl serine that forms the vesicle surface initially builds complexes with calcium ions, which in turn attract hydrogen phosphate ions, thus leading to the nucleation of apatite crystals along the vesicle membrane [1]. It has been hypothesized that nanospheres composed of up to six monomers, measuring ~ 5 nm in size, are formed intracellularly and are as such released to the enamel matrix [147]. Only then are they predicted to assemble into larger, ~ 20 – 60 nm sized spheres. The nanospheres detected in aqueous media may thus be composed of smaller subunits, somewhat similar to those shown in Figure 2c. In fact, amelogenin nanofibres most commonly adopt a proto-fibrous form wherein aligned and interconnected nanospheres may be visible at high resolution [130]. The phenomenon of aggregation of smaller subunits in the formation of colloidal particles has been well known for both inorganic [148,149] and organic particles[150], and apatite particles with high symmetries have been, for example, often shown to be sub-structured. On the other hand, the exceptional structural flexibility and the molecular recognition propensities of peptides preclude the possibility that

amelogenin nanospheres permanently exhibit a sub-aggregate structure. It is more probable that these sub-aggregates would become merged in any aggregates thereof. An illustration of the structural flexibility of protein assemblies comes from the phenomenon of quasi-equivalence, that is an event where protein spheres in viral protein coatings interact with identical side chains and yet in one case form a hexagonal and in another case a pentagonal symmetry [151]. In that sense, it is protein assemblies, and not aggregates, that one should refer to whenever a supra-organization of the primary amelogenin nanospheres or molecules is observed [152].

Return to the beginnings: Final questions to be shed upon the biomimetic path

Returning to the purpose of this paper, as outlined in the introduction, we may once again be reminded of the entwined basic and practical aspects of the research into enamel formation.

Namely, studies of amelogenesis possess both fundamental and practical significances. First, studying interactions between the mineral and macromolecular components of this biosynthetic process may provide us with insights relevant for understanding biomineralization phenomena in general, in addition to those from which the reparative and preventive dentistry may benefit [67]. Second, there is a prospect that the basis for fabrication of artificial structures with superior performance by controlled imitation of these natural processes could be established. By learning how to control the interaction between scaffold proteins and the growing crystals, a path for the synthesis of novel materials using protein self-assembly mechanisms could be opened. If we define the practical aim of the biomimicry of amelogenesis to produce the exact replicas of the natural enamel, and the fundamental aim to gain insight into the nature of the interaction between the protein species and the mineral in this process, we would quickly realize that pursuing one side without the other would have no meaning at all. The fundamental and practical aspects are thus interrelated and a parallel study of the both is, as always, crucial in ensuring a productive research.

Wherever we look around, there are reasons to wonder at the immaculate precision and elegance which living creatures are pervaded with. Many are biological materials and processes that seem superior in their characteristics and performance over the man-made ones. This is why biomimetics presents an ideal growing among chemists of the modern day. Humans since the beginning have been turning to everyday natural processes in search for a metaphoric inspiration in their problemsolving approaches, but the trend of systematically learning from natural synthetic pathways on how to synthesize superior functional materials in the lab is relatively new. However, despite the fact that undoubtedly we, as humans, can learn a lot from biosynthetic pathways and utilize them for the purpose of production of new materials and devices, it is this authors' opinion that researchers should look for the balance between imitatively looking backwards and innovatively looking forward. In view of that, the notions of "bioimitation" and "biomimicry" should, literally speaking, cede their places to the one of "bioinspiration", which would correspond to neither our blind following of the synthetic pathways that Nature relies on nor a complete neglect thereof. Besides, as the number of atomic elements available to humans in the design of

advanced materials and structures far exceeds that employed by the living organisms, it is natural to expect that not merely imitating and copying, but being inspired by, learning from and going beyond the natural design is the way forward for materials science and engineering of the future [153]. Such a point of view is in agreement with the concept of co-creation, which has been earlier proposed by the author [154–156]. The basic idea behind this concept is that every result of our perception and the entire experience of ours become shaped at the intersection between: (a) the sphere of our own creative powers defined by our knowledge, values, presumptions, intentions, anticipations and biological predispositions, and (b) objective qualities of the physical reality to which we belong. The latter we can never detect in the way it really is, that is, without co-defining its qualities by means of exhibiting our own intrinsic creativity. Consequently, whatever our endeavours are, we should rely on our own creative potentials and the voice from the inside on one side and the incentives and potentials that Nature abounds with, including its metaphoric messages that inspire our creativity, on the other.

The question is why considering novel artificially produced materials as products of, literally, non-natural design. Anything that humans produce inevitably belongs to the productive repertoire of biological creatures. Therefore, any type of artificial productivity inherently retains the attribute of natural. Insisting on the aesthetics of uniform particles in material systems thus actually presents a step forward in the direction of diversification of the repertoire of naturally existing material structures, and not attempts to go against the principles of the natural design, which, as you may remember, is based on an inherent tolerance of imperfections in approach and, partially, even the results [82]. The structures of bone and hard tissues with their mild imperfections are a natural consequence of the formational limitations imposed by biological environments and may present one example.

Experiments on using phage peptide libraries [81,157] and engineering novel specific protein sequences [73] based on adsorption studies present some of such attempts to go beyond Nature. Trial-and-error use of polypeptides for the purpose of formation of materials previously obtainable only under much more complex processing conditions is an instance of a similar approach [158–160]. Another example may be the freeze-drying synthesis of stacked plate-shaped calcium carbonate structures that resemble nacre [161], demonstrating a solid matching with the imitated biosystem, although using a non-biomimetic approach to synthesis. In fact, as all biomimetic scientists are by default limited to the use of significantly simpler experimental conditions than those existing *in vivo*, the question is how far they should strive to attain the ideals of absolutely faithful biomimicry (which is ultimately an unattainable destination, strictly speaking) and to what extent they should integrate the non-biomimicking ideas in their approach. To elaborate this idea, we return to the complexity of amelogenesis, the perfect replication of which *in vitro* seems to be more than challenging, if not thoroughly impossible. For example, reports on the role of enamel matrix proteins other than amelogenin in the enamel formation are relatively scarce [162]. Nonetheless, it is known that enamelin also has a high affinity for apatite crystals, and that it also gets cleaved soon after its secretion [67]. Also, a study showing how mutations on the enamelincoding gene result in severe phenotypic *amelogenesis imperfecta* also points to an essential role it plays in amelogenesis [100]. This highly acidic glycoprotein is observed as

forming a sheath around the growing apatite crystals (so that their c-axes are perpendicular to the β–sheets of the surrounding enamelin), which complies with the reigning model that describs the way extracellular mineralization proceeds. According to it, hydrophobic proteins, such as amelogenin, collagen or cellulose, are involved in the build-up of the insoluble macromolecular matrix of the developing hard tissue, whereas hydrophilic proteins are involved in attracting the precursor ions and providing the nucleation surfaces. Whereas the low concentration of enamelin in the enamel matrix can be thought of as a sign of its low importance, that is not necessarily so. There are many examples of macromolecular or amphiphilic additives that exhibit a cooperative effect on the assembly of the precipitated phase at low concentrations only [1]. Morphological specificity, such as preferential adsorption of the additive molecules along specific planes of the crystalline phase, would in such cases diminish at higher concentrations.

Ameloblastin is also presumed to carry a significant function, not only because of its localization at the secretory end of ameloblasts where the crystal growth is initiated, but because of knowing that both an exaggerated and inhibited expression of ameloblastin results in *amelogenesis imperfecta* [100]. The roles of even less abundant components of the enamel matrix, such as KLK4, keratin K14, DLX3 or biglycan protein, the mutant expressions of which are also known to produce the conditions of *amelogenesis imperfecta* [163], have not been investigated thoroughly, and it is doubtful that Nature would ever allow for the presence of functionless ingredients in Her biosynthetic pathways. This can be linked to an important function that cross-linked, noncollagenous protein molecules have in mineralization of dentin. The absence of intrafibrillar mineral in the early stages of dentinogenesis is known to produce conditions of *dentinogenesis imperfecta*, whereas unsuccessful attempts to remineralize dentin *in vitro*, despite the preserved collagenous matrix, were blamed on the loss of these phosphorylated and hydrophilic peptide species. Then, one portion of amelogenin proteins comes from recombination of exons, whereas the other portion originates as cleavage products, which introduces intricate relationships between the processes of proteolytic digestion and transcription to the whole process. Despite the claims of insignificance of the single post-transcriptional modification in amelogenin, this single-residue phosphorylation has been evidenced as crucial in enabling the proper development of enamel. Following on, there is the presence of various inhibiting factors that limit the activity of the actual proteases, including the supply of zinc ions and the expression of inhibiting macromolecules. In the end, epigenetic expression pathways comprise a circular loop in which DNA encodes RNA and proteins that in turn maintain and replicate the DNA sequence, and it would be no surprise that a similar self-regulating feedback loop involving the species of proteins, proteases, protease inhibitors and the mineral, even with the presumed minimal monitoring action of ameloblasts, exists in the process of amelogenesis as well.

Conclusion

As a conclusion, an important question emerges. At which point should the pursuance of fundamental insights be substituted with that of simpler but more practical ideas? It seems that as much as the fundamental and practical aspects of this research are inherently connected, they are also complementary in large extent, and require a smart balance that

would maximize insights and achievements at the both levels. Nevertheless, it is for sure that only the combined efforts between the fundamental insights and the practical, experimental knowledge that could bring future prospects and attainment of the aims drawn.

In summary, the current state-of-the-art mechanisms in the study of enamel formation and the attempts to imitate them *in vitro* appears like a tip of an iceberg. Slow progress is evident, and if this trend continues, enamel might be grown in laboratories in future. However, it is doubtful whether elimination of one out of a hundred components of this process would bring about satisfying results. The true replication of amelogenesis requires knowledge and the ability to control interactions between a multitude of polypeptide species. In the end, it is logical to expect that the major aspects of amelogenesis – the assembly of amelogenin and other framework and acidic proteins, the proteolytic activity and crystallization – need to be in precise synergy with each other in order to produce the desired outcome.

Today, it is hard to estimate which aspect of the process is more difficult to penetrate: the fundamental or the practical. In any case, conceiving original experimental approaches to mimic amelogenesis presents the key, although two eyes need to be used to analyse the outcomes. The proteomic eye to follow the protein-related aspects of the process, and the materials science eye to follow the crystal formation facets. Needless to add, these two eyes need to look in the same direction and in synergy from the top of the aforementioned pyramid (Figure 8) in order for the path of biomimicry of tooth enamel to be walked upon successfully. In this way, there is a chance that the future development of this field will transcend the broad speculations that dominate the contemporary literature reports on amelogenesis-related studies, and yet not become blind to the crucial effects of some of the minor components of this process. For, if the science of the enamel growth teaches us something profound, it is that “small is beautiful” and that a tiny detail of this Universe, such as the enamel, hides many mysterious patterns, diligent in-depth research of which may open the doors to understanding the greater secrets of the physical reality in which we abide.

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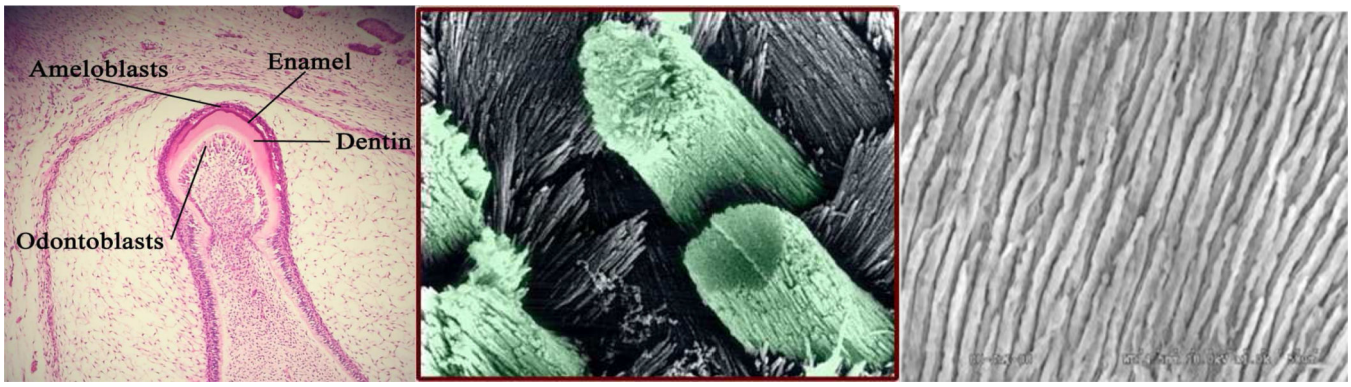

Figure 1.

(left) Histological section of the developing tooth in the maturation stage Note that the growing dentin and enamel are in contact with each other, whereas odontoblasts responsible for directing the growth of dentin and ameloblasts responsible for directing the growth of enamel are thoroughly separated at this stage. Although coming from different tissues (ameloblasts from the epithelial, and odontoblasts from mesenchymal tissue), these two types of cells are engaged in intensive communication prior to initiation of an almost simultaneous crystallization of dentin and enamel. (center) Parallel arrangement of mouse enamel rods composed of smaller rod-shaped crystals with a similar parallel organization in space, and interspersed within the crystals of inter-rod enamel. (right) A micrograph of human enamel showing much lesser presence of the inter-rod enamel compared to that of mouse. Reprinted with permission from the web page of Brodie Laboratory of Craniofacial Genetics at the University of Illinois, Chicago, IL, and Ref.[10].

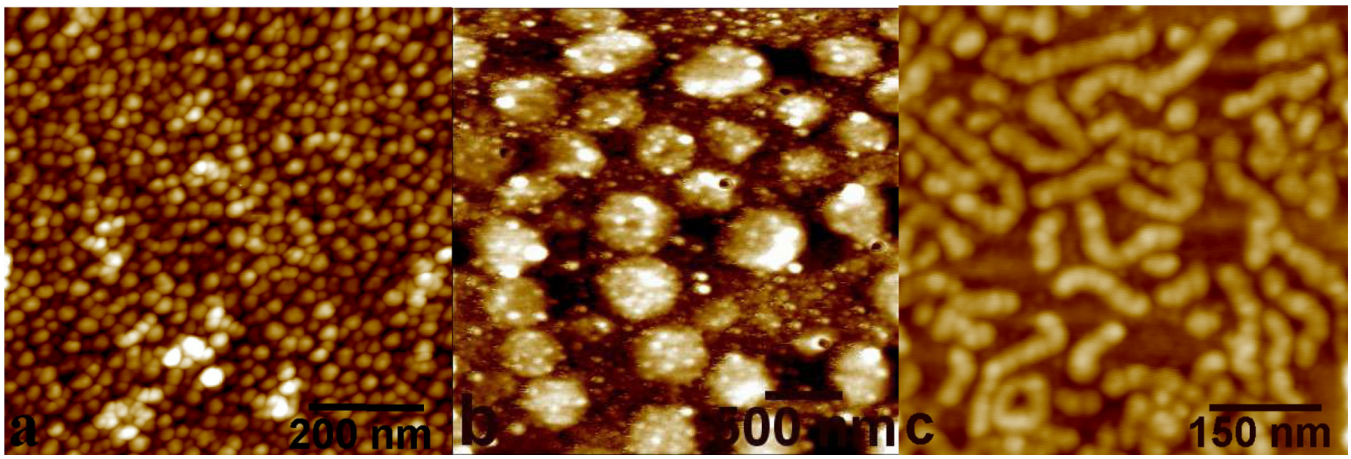

Figure 2.

(a) Recombinant full-length human amelogenin nanospheres, size 20 – 40 nm. (b) Amelogenin aggregates formed following the cleavage of the full-length human amelogenin by the action of MMP-20. Note how the aggregation of smaller spherical sub-units leads to formation of larger spheres with the consequent broadening of the aggregate size distribution. (c) Nanostrings formed by mixing amelogenin and water Reprinted with permission from Ref. [13].

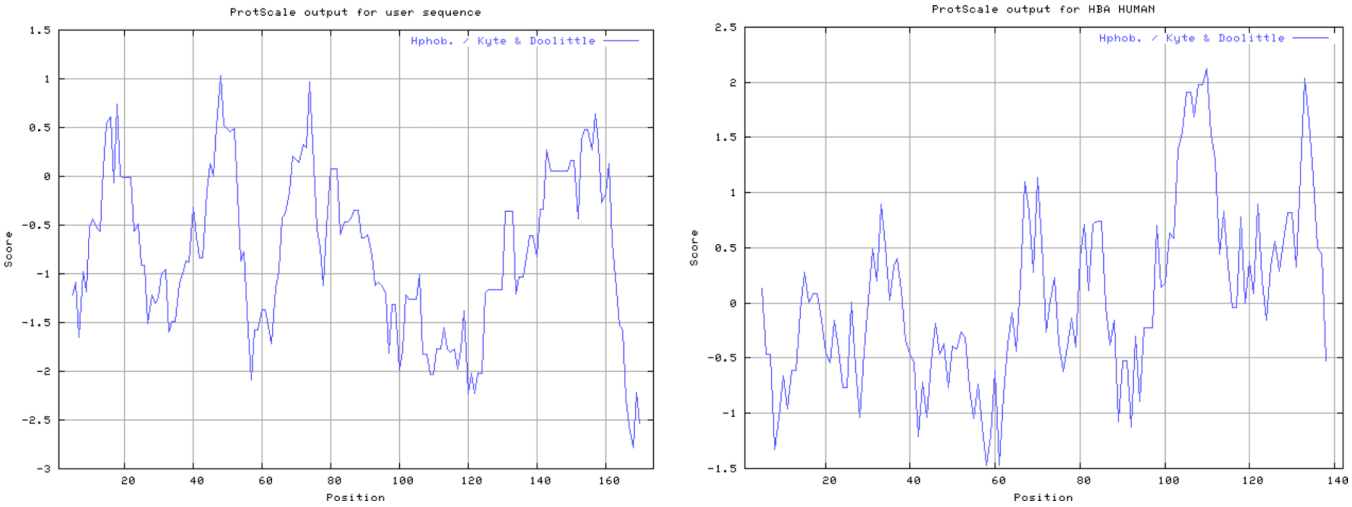

Figure 3.

Hydrophobicity plots obtained using ExPASy ProtScale Kyte & Doolittle model for human amelogenin (left) and human haemoglobin alpha chain (right). The positive score on the diagrams denotes hydrophobic sequences.

MGTWILFACLL(/V)GAAFAMPLPPHPGHPGYINFSYEVLTPLKWYQS(M)IRPPYP(/S)SYGY
EPMGGWLHHQIIPVL(/V)SQQHPP(/L)THTLQP(/S)HHHIPVVPAQQPVIP(/RVR)QQPM(/AL)
MPVPGQH(/Q)SMTPI(/T)QHHQPNLPP(/L)PAQQPY(/F)QPQPVQPQPHQPMQPQPPVH(/Q)P
MQPLP(/L)PQPPLPPMFPMQ(/LR)PLPPM(/I)LPDL(/H)T(/)LEAWPS(/A)TDKTKR(/Q)EEVD

Figure 4.

The primary structure of the X chromosome variant of the full-length human amelogenin, with the denoted variations for the Y chromosome variant. Blue-coloured sequence belongs to the exon 4, which in the recombinant full-length sequence typically substitutes for the preceding 18-residue long sequence at the N-terminal coloured in yellow.

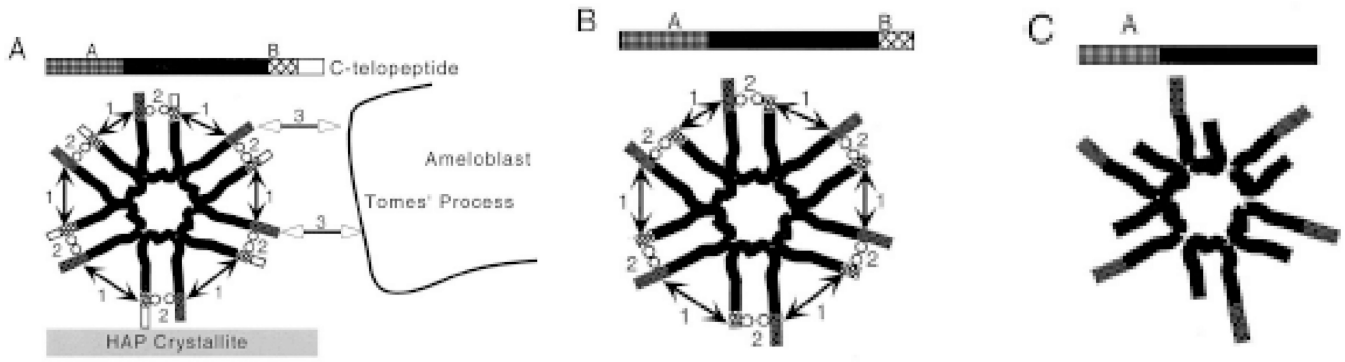

Figure 5.

Hypothesized structure of the amelogenin nanosphere, comprising both the C- and N-terminals exposed at the surface. Note how the successive proteolytic shortening of the amelogenin primary structure disrupts the uniform spherical geometry that amelogenin nanospheres initially display. Reprinted with permission from Ref.[44].

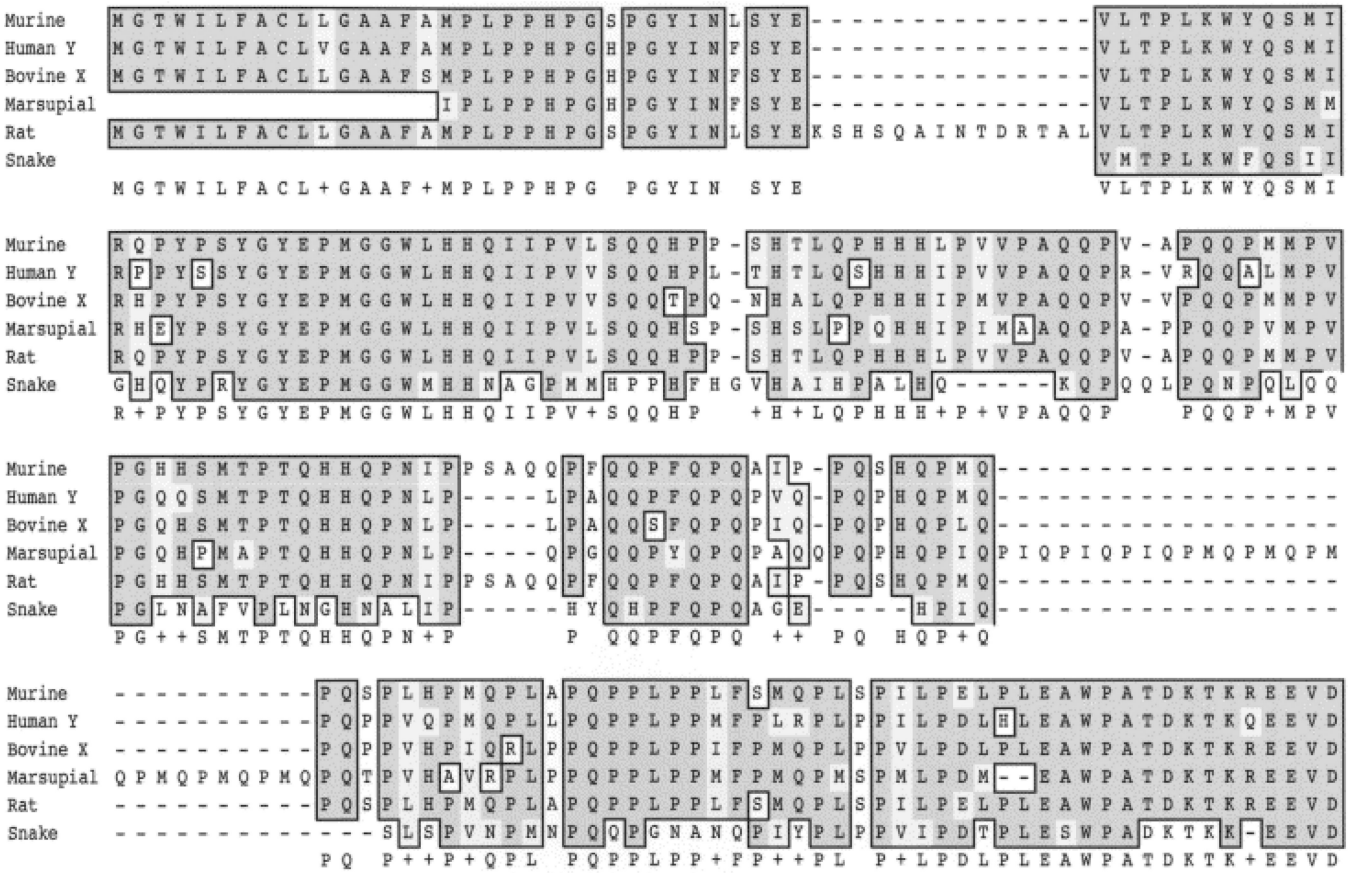

Figure 6.

Aligned amino acid sequences of amelogenins from murine, human, bovine, marsupial, rat and snake species. Reprinted with permission from Ref.[55].

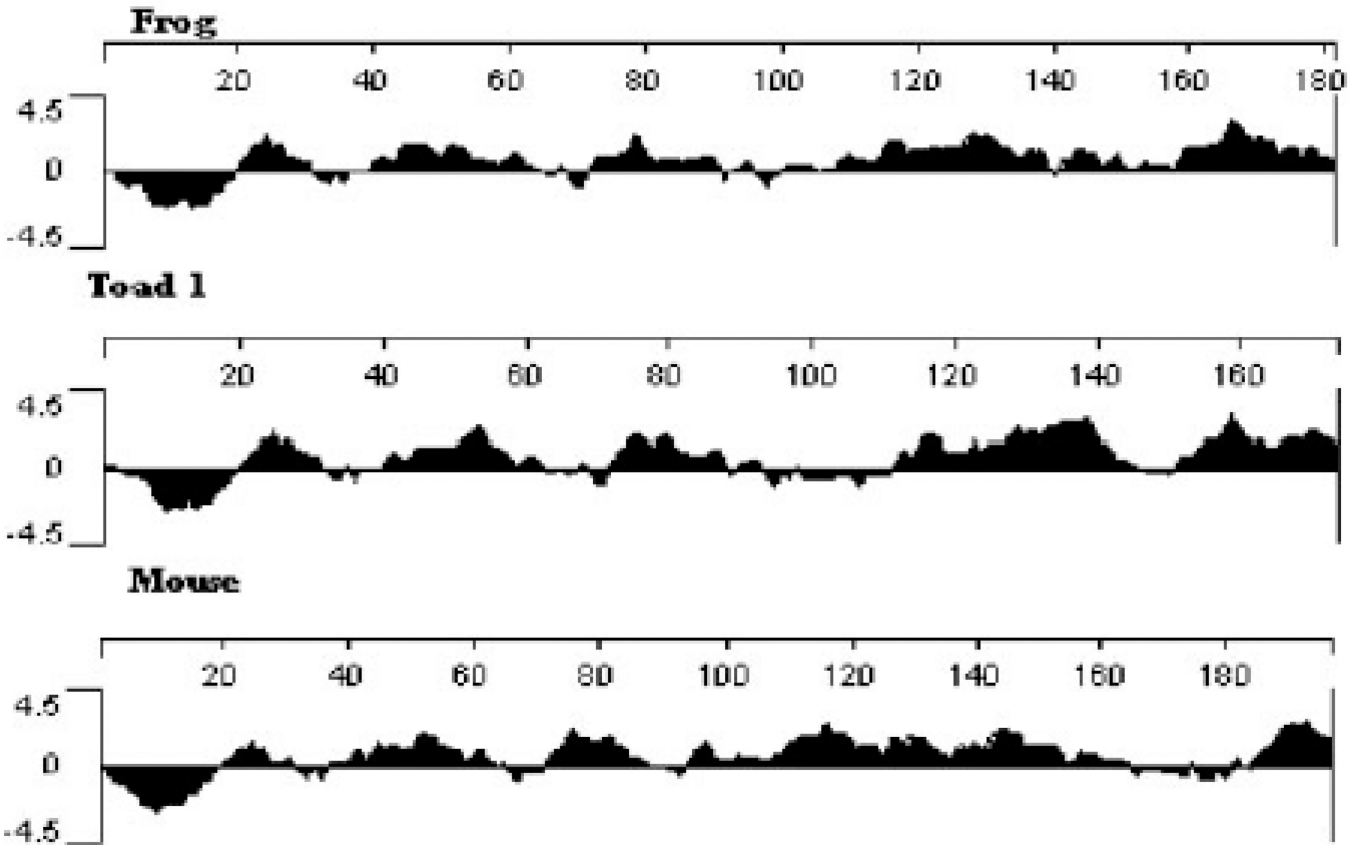

Figure 7.
Hydrophobicity plots of frog, toad and mouse amelogenin. Reprinted with permission from Ref.[57].

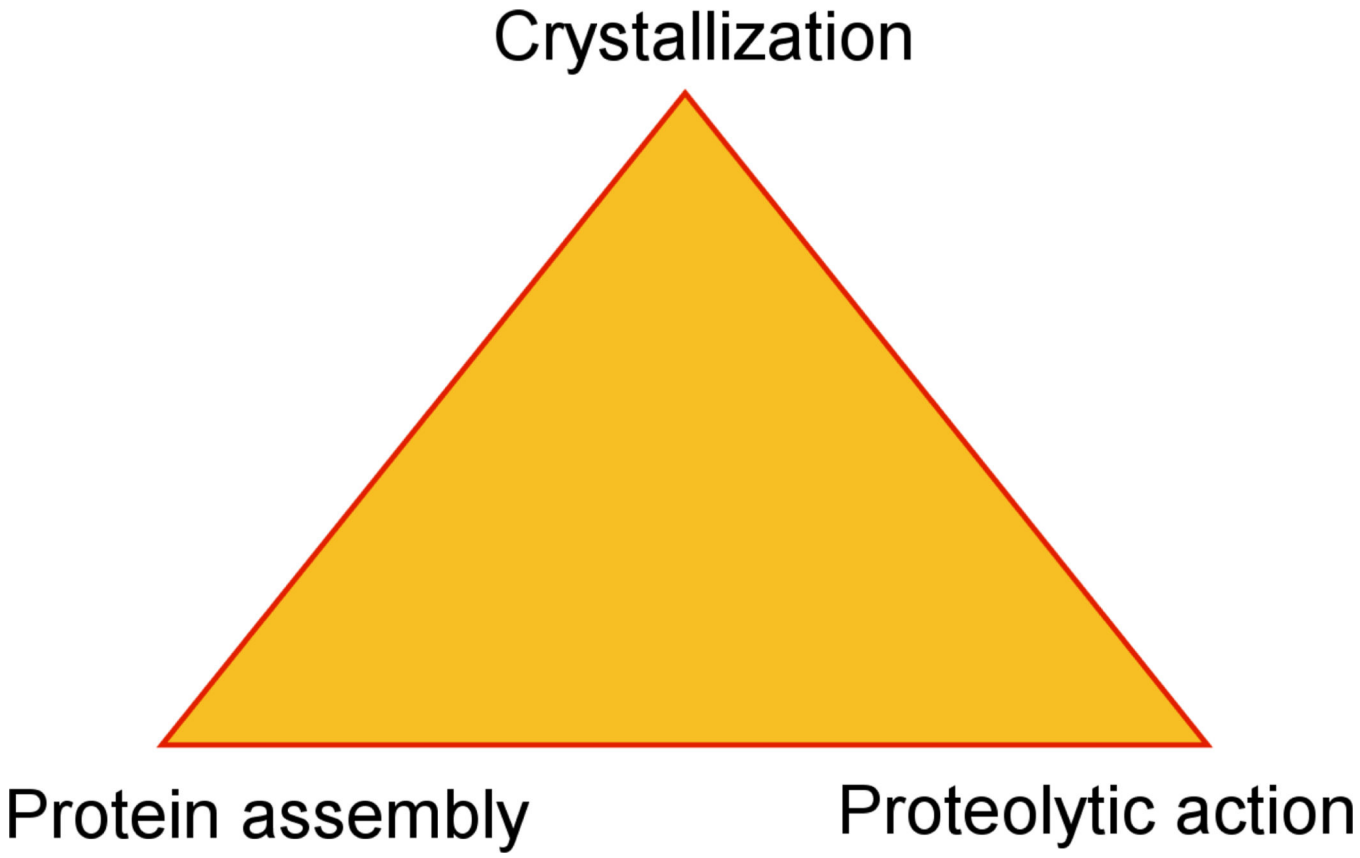

Figure 8.
A triangle depicting the interlacement of the three main aspects of amelogenesis.

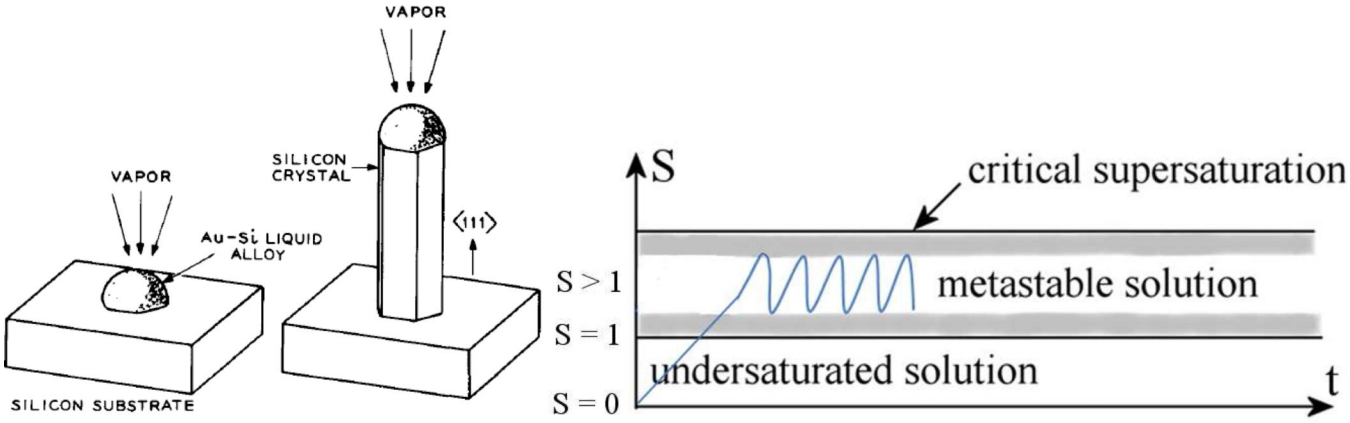

Figure 9.

A schematic drawing that depicts the formation of silicon nanowires (left), according to Ref. [87]., and a slow increase in supersaturation ratio that enables it (right).

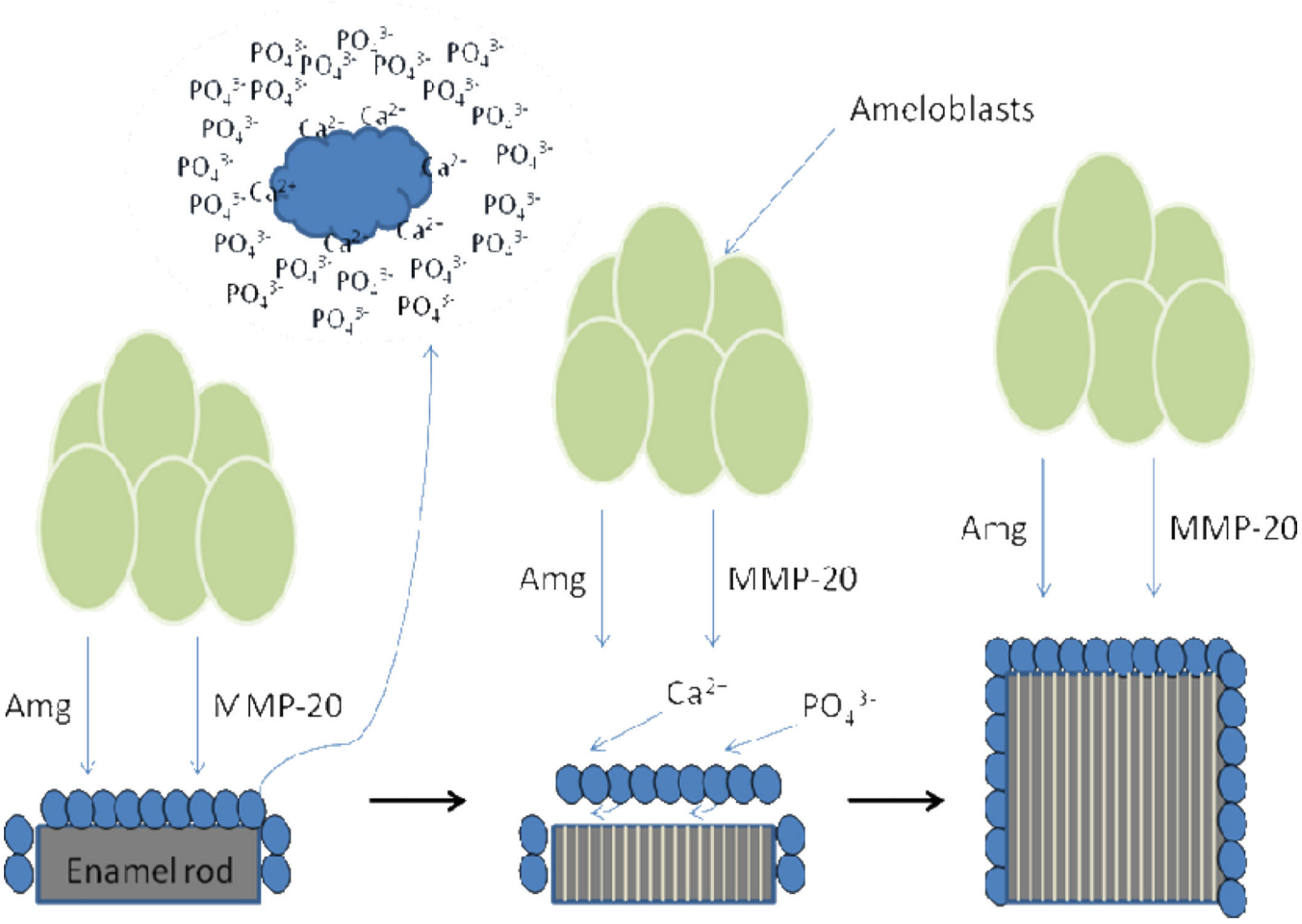

Figure 10.

Hypothesized model for the protein-controlled crystal growth in amelogenesis based on the adsorbed amelogenin assemblies, such as nanospheres or nanofibres, channelling calcium and phosphate ions from the solution or amorphous calcium phosphate entities formed through a precise coordination of Ca^{2+} bound to the protein and phosphate ions diffusing in the hydrodynamic layer of the protein particles onto the growing crystal surface.

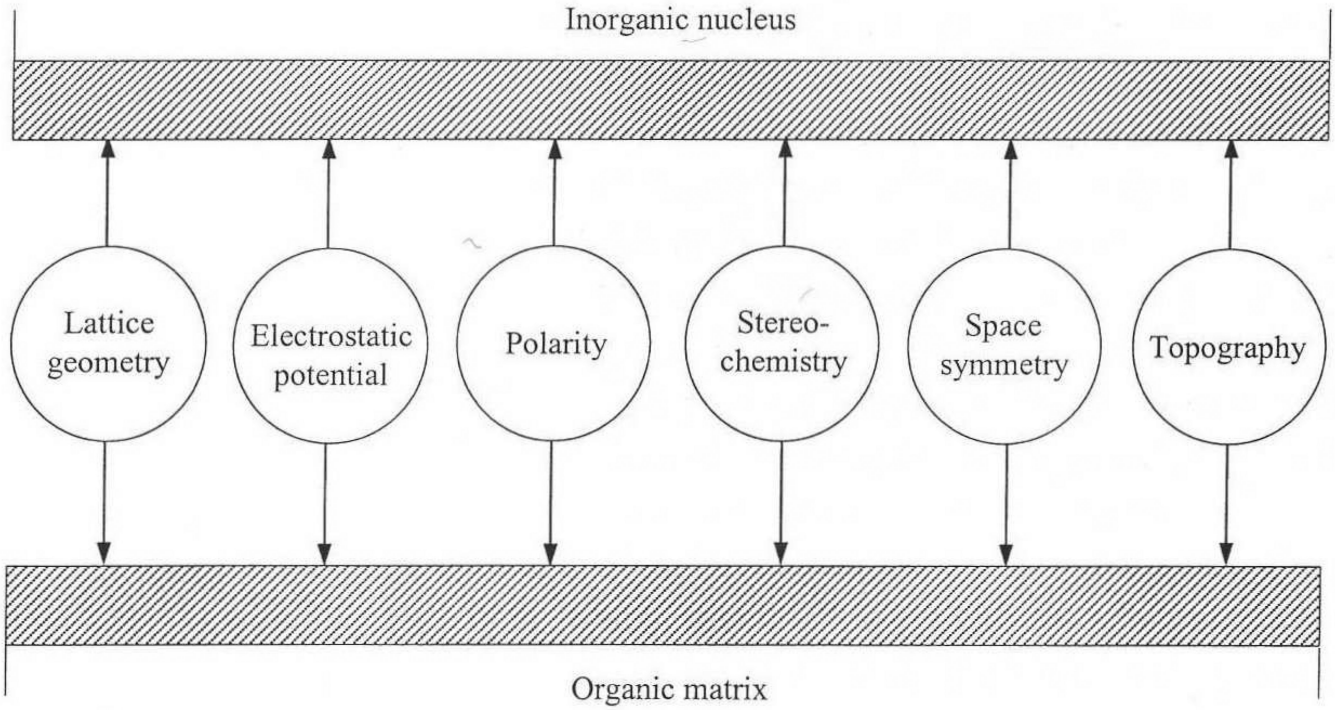

Figure 11.
Various aspects of molecular recognition interactions between organic and inorganic phases in a biomineralization process. Reprinted with permission from Ref.[1].

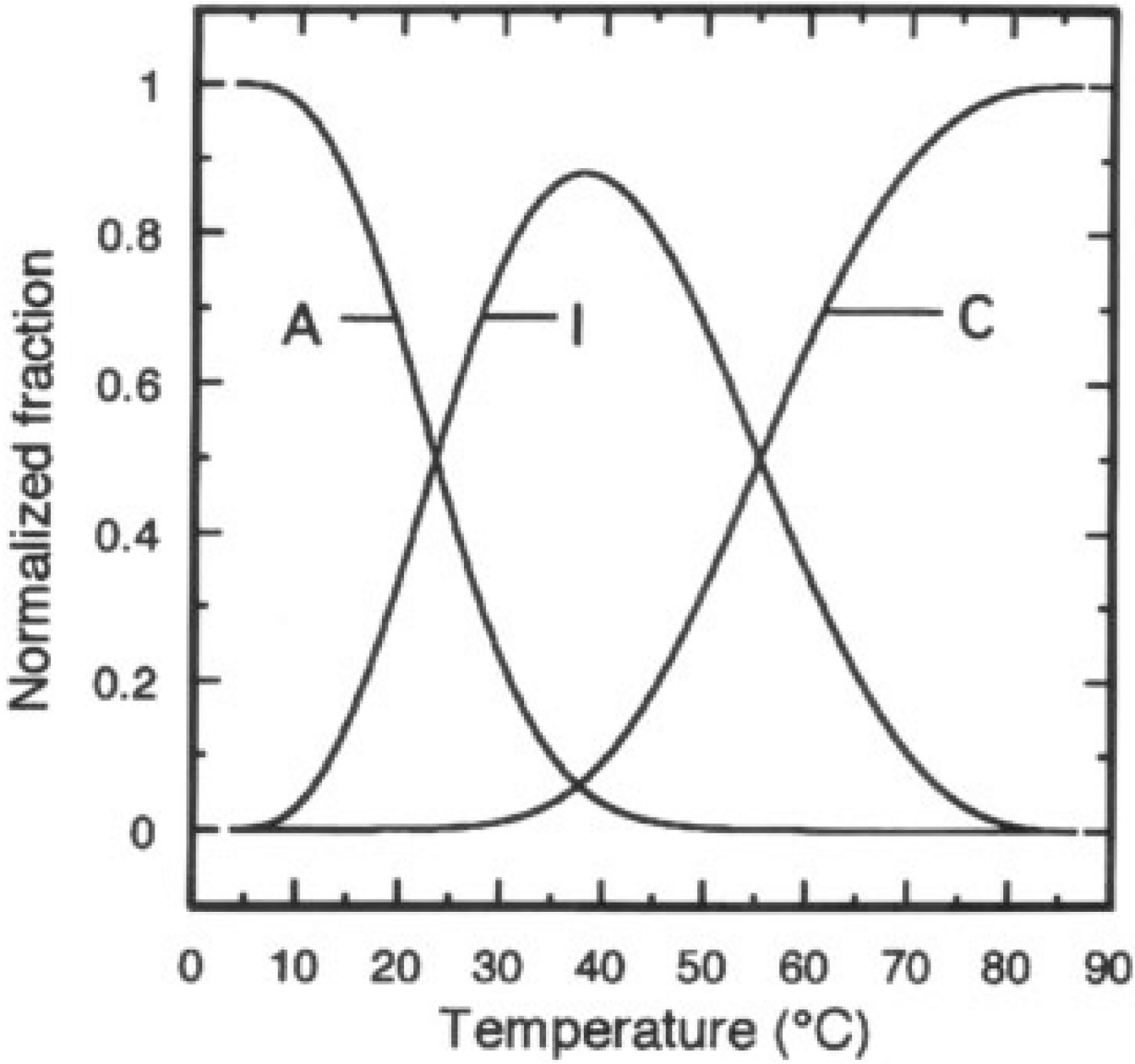

Figure 12.

Three structural modifications of amelogenin as a function of temperature. Notice there is an equilibrium between the low T phase and the physiological phase at 23.4°C, and between the high T phase and the physiological phase at 55.3°C, whereas at exactly the physiological T the low and high T phases are essentially non-existent. Reprinted with permission from Ref. [131].