Published in final edited form as:

J Struct Biol. 2008 December ; 164(3): 314–321. doi:10.1016/j.jsb.2008.09.005.

The cooperative self-assembly of 25 and 23 kDa amelogenins

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Abstract

Self-assembly of the extracellular matrix protein amelogenin is believed to play an essential role in regulating the growth and organization of enamel crystals during enamel formation. The full-length amelogenin uniquely regulates the growth, shape, and arrangement of enamel crystals. Protein hydrolysis will ultimately facilitate a tissue with high mineral content. Protein processing is however highly specific suggesting a functional role of the cleaved amelogenins in enamel maturation. Here we hypothesize that the cooperative self-assembly of the recombinant full-length amelogenin 25 kDa and the 23 kDa proteolytic cleavage product is a function of pH, mixing ratio and incubation time and is associated with the isoelectric point of the protein. Self-assembly of amelogenin into nanospheres which increased in size with increasing pH was observed by atomic force microscopy. Elongated structures of about 100nm length and 25 nm width formed over several days for amelogenin 25 and 23 kDa predominantly at pH-values of 6.5 and 7.5, respectively. When both proteins 25 and 23 kDa were mixed, self-assembled nanostrings of 200–300 nm length consisting of fused nanospheres were obtained at pH around 7.0 within 24 h. The protein nanostrings formed links over time and a continuous mesh was obtained after 7 days. Electrical conductivity data also showed gradual changes when both amelogenins were mixed in solutions supporting the idea that elongated structures form over extended periods of time. We propose that due to the difference in the isoelectric point, self-assembled nanospheres composed of 23 or 25 kDa amelogenin have opposite ionic charges at pH-values around 7.0 and thus experience ionic attraction that enables cooperative self-assembly.

Keywords

Enamel; Amelogenin; Cooperative self-assembly; Nanospheres

1. Introduction

The incorporation of nanosized mineral crystals into proteinaceous tissue has been a successful strategy to obtain biological structures of improved mechanical strength and hardness. Bone and dentin are two examples where mineralization is used to reinforce an organic matrix. While the matrix remains almost unchanged, mineral is added to the organic structure which in return significantly improves the load bearing characteristics of the tissue (Balooch et al., 2008; Weiner et al., 2000). The formation of enamel, however, differs from the way these collagen-based tissues mineralize. Enamel not only mineralizes through a protein-controlled process, but also generates its unique structure and mechanical characteristics through enzymatic

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hydrolysis which removes almost the entire organic matrix and leaves behind a tissue composed of about 98 wt% mineral (Robinson et al., 1995).

Amelogenin proteins, which are secreted by cells of the inner enamel epithelium (ameloblasts), constitute up to 90% of the extracellular protein matrix in developing enamel. Amelogenins mainly contain hydrophobic domains, with the exception of the hydrophilic C-terminal (Fincham et al., 1995). The full-length 25 kDa amelogenin is localized exclusively in the region of newly formed mineral, thus suggesting a unique role of the full-length molecule in the control of crystal shape and organization during enamel formation (Uchida et al., 1991; Wiedemann-Bidlack et al., 2007). Full-length amelogenin is expressed during the secretory stage of amelogenesis and rapidly cleaved by metallo-matrix protease-20 into several proteolytic products (Bartlett and Simmer, 1999). Extracts isolated from developing porcine enamel showed the presence of at least four MMP-20 cleavage products, including the 5.3, 20 and 23 kDa amelogenins, as well as the 6.5, 18, 25, and 27 kDa amelogenin splice variants (Yamakoshi et al., 1994). While the main component of the matrix at the very early stage of the secretory enamel was found to be the full-length 25 kDa amelogenin, the matrix composition of the developing enamel is highly dynamic and changes continuously over time and with distance from the cell layer.

It is well established that amelogenin proteins self-assemble into nanospheres in vitro (Fincham et al., 1994; Moradian-Oldak, 2007) and there are indications that similar structural elements exist in the developing enamel as well (Fincham et al., 1995; Robinson et al., 1981). The observed MMP-20 cleavage sites and an solid-state NMR-study on the leucine rich amelogenin peptide (LRAP) led to the conclusion that the charged C-terminus is exposed on the surface of the nanosphere. The self-assembly is driven by inter-molecular hydrophobic interactions that result in the formation of a nanosphere which keeps the hydrophobic part away from water, while the hydrophilic end is exposed to the surface. The size of the assembled structures depends on pH, temperature, and protein concentration (Beniash et al., 2005; Habelitz et al., 2006; Moradian-Oldak, 2001). Using small angle X-ray scattering (SAXS) and dynamic light scattering (DLS) analyses, Aichmayer et al. (2005) have provided additional support to show that amelogenin nanospheres consist of a hydrophobic and electron dense core that is surrounded by a less electron dense and hydrophilic corona comprised of the charged C-terminus.

Recent studies have also shown that the full-length amelogenin protein further aggregates into chains of connected nanospheres (Moradian-Oldak et al., 2006; Wiedemann-Bidlack et al., 2007) revealing a potential for hierarchical self-assembly into elongated structures that could provide guidance to the highly anisotropic growth of apatite crystals in enamel. Collinear arrays of amelogenin nanospheres were observed during protein assembly experiments using solutions with high protein concentrations in a hanging drop over extended periods of time suggesting that the full-length molecule could provide a scaffold during enamel maturation into which apatite crystals grow with high-aspect ratio and parallel alignment (Moradian-Oldak et al., 2006).

The timely cleavage and the removal of proteolytic products are essential to enamel development. The charged C-terminal of amelogenin has significant apatite binding affinity and the potential to control morphology and organization of the crystals (Beniash et al., 2005; Moradian-Oldak et al., 2002). Several studies have indicated that removal of the C-terminal hydrophilic residue of amelogenin does not affect its ability to inhibit crystal growth of calcium phosphates (octacalcium phosphate and hydroxyapatite) in directions perpendicular to the c-axis. (Beniash et al., 2005; Iijima and Moradian-Oldak, 2005). Furthermore it was shown that crystals that formed in the presence of the hydrophilic C-terminus were organized into parallel arrays, whereas crystals forming in the absence of the hydrophilic C-terminus

under the same conditions were randomly arranged. (Beniash et al., 2005). It was therefore proposed that the hydrophobic portion of amelogenin inhibits crystal nucleation and growth, while the C-terminal domain plays a role in the parallel alignment of crystals within an enamel rod.

The expression and secretion of hydrophobic proteins into the extracellular space is commonly seen as a cellular activity to provide space for mineral formation. In contrast hydrophilic proteins are regarded as molecules that promote crystal nucleation and growth and thus can precisely define the location and size of mineral crystallites (Addadi and Weiner, 1992). Ravindranath et al. showed that murine amelogenin specifically binds to recombinant ameloblastin via its tyrosine-rich domain (amino acids 33–45), suggesting the possible formation of a heteromolecular assembly (Ravindranath et al., 2004). Moradian-Oldak et al. examined the effects of amelogenin and the 32 kDa enamelin on the induction of synthetic apatite crystals precipitated in 10% gelatin gel and proposed that the 32 kDa enamelin bound to amelogenin serves as a potential nucleator for apatite crystals (Bouropoulos and Moradian-Oldak, 2004). While these findings introduce the idea of a cooperative effort of enamel proteins to control mineral nucleation, other studies introduced the aspect of cooperative mineralization, suggesting that functional self-assembly of the organic matrix molecules can only occur in situ and synergistically with enamel crystal growth and alignment (Margolis et al., 2006). All in all it can be hypothesized that the protein components of the developing enamel matrix are highly interactive and may function in a cooperative fashion to ultimately serve as a suitable template for biomineralization and the fabrication of the highly ordered enamel tissue.

Protein self-assembly is clearly affected by changes in pH, which can induce ionization of the amine and carboxyl groups as well as the functional side-chains. It has been shown by others (Aggeli et al., 2003; Zhao et al., 2003) that intermolecular interactions of macromolecules are often enhanced at their isoelectric pH, since electrostatic repulsion is minimal and molecules can approach each other closely. The calculated isoelectric pH (IP) for monomeric amelogenin 25 kDa is 6.62 and 6.85 for the 23 kDa amelogenin (http://ca.expasy.org/tools/pi_tool.html). The true IP of these molecules may vary significantly from these theoretical values depending on the secondary structure of the protein. Surface charges of self-assembled structures become relevant when cooperative assembly between two different proteins is aspired. Electrostatic attraction between different proteins may occur when either the dissolved macromolecules or the initially self-assembled nanostructures carry opposite charges, providing an opportunity for close protein to protein interaction that could stimulate a cooperative self-assembling behavior.

In this study, we aimed to advance our understanding of the fundamental mechanisms of molecular interactions involved in the formation of higher ordered structures with a focus on amelogenin and its first MMP-20 cleavage product. The effects of pH, mixing ratio and incubation time on cooperative self-assembly of the human full-length 25 kDa amelogenin and its 23 kDa proteolytic cleavage product were investigated with respect to changes in the nanostructure and electric conductivity as evaluated by AFM and conductivity measurements.

2. Materials and methods

2.1. Protein preparation

The human full-length amelogenin protein rH174 (25 kDa) and the proteolytic cleavage product rH163 (23 kDa) were obtained through a recombination process and expressed by *Escherichia coli*, as described previously (Li et al., 2003). The protein was purified using C4 beads and freeze-dried. MALDI-ToF analysis showed high purity (99%) for both proteins.

Amelogenins rH174 and rH163 were dissolved at 0.5 mg/ml in 25–50 mM Tris/HCl or Bis-tris/HCl added, which increased the pH to values around 9. From these stock solutions, protein solutions that contained either single type rH174 or rH163 amelogenin or mixtures of the two were prepared at different ratios with a total protein concentration of 0.1 mg/ml by adding KCl (final concentration: 150 mM) and HCl to obtain pHs in the range between 5.5 and 8.1 with steps of approximately 0.5 pH points. The pH was measured at each time point at 37 °C using a calibrated micro glass-electrode (Orion 8220 BNWP, Thermo Electron Co.). Proteins were stored in solution at 37 °C for up to 7 days. The pH values varied within ± 0.1 points over the seven day period. At 1 h and then every 24 h, 20 μ l of protein-solution were pipetted onto microscope glass slides and kept for 1 h in a wet cell to immobilize proteins onto glass slide avoiding significant evaporation. Subsequently, solutions were washed off with a few drops of deionized water, immediately dried with dust-free canned air and placed onto AFM scanner head for imaging. A total of three samples for each condition were studied.

2.2. Atomic force microscopy

Microstructure of the immobilized proteins was observed using atomic force microscopy (AFM) (Nanoscope III; Digital Instruments, Santa Barbara, CA, USA). Images were obtained in dry conditions using tapping mode with a Si-tip ($r < 10$ nm, $l \sim 125$ μ m) (Tap 300, Budgetsensors) at a resonance frequency of ~ 300 kHz. Si-tips with tip radius below 5 nm (Nanoworld, SSS-NCH, Switzerland) were used for high resolution images with scan sizes of 1 μ m or smaller. The average nanospheres sizes were measured from the AFM graphs by using Nanoscope software and determining the average width of at least 50 nanospheres. Values were corrected for tip broadening assuming a tip radius of 5 nm. The nanostring was defined as chain-like nanoparticles structure with about 100 nm length (i. e. at least three linked nanoparticles). The amount of nanostrings present on the substrates was determined from at least three AFM graphs of scan sizes 2×2 μ m². The percentage of nanostrings coverage was defined as the ratio of area covered by nanostrings to the total area of protein coverage. Larger random and multilayered agglomerates of proteins were not considered as nanostrings while spheres that evidently lined up into strings but still showed a clear demarcation as shown in Fig. 1h were included in the definition of nanostrings. The average aspect (L/W) ratio of nanostrings were calculated from the mean length of nanostrings and the average diameter of the comprised, closely associated nanospheres.

2.3. Conductivity measurements

The conductivity measurements were carried out using an Accumet conductivity meter model AB 30 in the conductivity range from 0.001 μ S to 500 mS (milli-Siemens) with accuracy about $\pm 0.5\%$. The MI-900 series Conductivity Microelectrode (Microelectrodes, Inc.) was used and its cell constant (K) at 25 °C was calibrated using the 0.01 N KCl solution method as determined by Jones and Bradshaw (Jones and Bradshaw, 1933). The cell constants were obtained from seven samples and were in agreement with a deviation of $\pm 0.2\%$. The protein solutions 1 ml were contained in glass vessels at 37 °C for up to 7 days of incubation time and conductivity was measured every 24 h.

3. Results

3.1. Effect of pH on the self-assembly of amelogenin 25 and 23 kDa

Tris/HCl or Bis-tris/HCl buffered samples of rH174 and rH163 amelogenin were immobilized on glass slides and the development of self-assembled structures studied by AFM, as shown in Fig. 1. Amelogenin self-assembled into predominantly spherical particles at all pHs. But at pH 6.5 and 7.5 (Fig. 1b and g), amelogenin rH174 and rH163, respectively formed elongated structures. These elongated structures were comprised of closely associated nanospheres (25

± 3.1 nm in width), had a mean length of 100 ± 16.2 nm and a mean aspect (L/W) ratio of 4.0 ± 1.8 .

Particle dimensions were obtained by direct measurement of AFM micrographic images and are summarized in Table 1. The average diameter of both kinds of amelogenins increased with pH. The full-length amelogenin rH174 was found to have a larger size than its rH163 counterpart. The removal of the 11-amino-acid hydrophilic C-terminus from the parent molecules generally resulted in a decrease in particle size that is either due to a decrease of intermolecular electrostatic repulsion or an altered arrangement of molecules with the amelogenin nanosphere.

Self-assembly is a kinetic process and will require time to obtain an equilibrium state. Hence, incubation time will affect self-assembly of amelogenins. During the initial stages of assembly (examined at 1 day), both the full-length amelogenin and its cleavage product were found to predominantly form spherical particles along with some elongated assemblies that consisted of tightly connected small spheres (Fig. 1b and g). However, after 3 days of incubation, tightly connected small spheres grew into chain-like structures (Fig. 1d and h).

3.2. Effect of pH on the cooperative self-assembly of amelogenin 25 and 23 kDa mixtures

During the secretory stage of enamel formation, the full-length amelogenin is continuously cleaved by enamel proteases into various proteolytic products. The potential interactions between amelogenin rH174 and rH163 were evaluated and the results are shown in Fig. 2. Comparing with single-type protein (Fig. 1), elongated assemblies were more readily obtained when the rH163 and rH174 amelogenins were combined at a ratio of 1:1. In particular at a pH of 6.5 and 7.0 the morphology of the protein assemblies changed into chain-like structures (Fig. 2b and c) which had a length of $200\text{--}300 \pm 21.2$ nm and a mean aspect (L/W) ratio of 11.4 ± 4.8 . At other pH-values, nanospheres were predominant as shown for pH 5.5 and 7.5 in Fig. 2a and d, respectively. However, also at these pHs alignment of spheres like pearls on a string could be seen. Larger agglomerates were also observed on these substrates and appeared as elevated white areas in the AFM micrographs. Such aggregates formed multilayers which made it impossible to resolve their micro/nanostructure their structure in regards to self-assembly by AFM.

3.3. Effect of mixing ratio on the cooperative self-assembly of amelogenin 25 and 23 kDa mixture in Tris/HCl buffered samples

Due to hydrolysis of the full-length amelogenin in the secretory and maturation stage of amelogenesis the amount of proteolytic products gradually increases and the molar ratio of full-length amelogenin to proteolytic products progressively decreases. Hence, it is expected that molar ratio between amelogenin rH174 and rH163 will affect the cooperative assembly. AFM was used to study self-assembled structures at pH 7.0 at various protein ratios, while maintaining the total protein concentration constant at 0.1 mg/ml. Prolonged nanostrings composed of fused nanospheres were found in all samples with different molar ratio (Fig. 3). However, comparing with samples at molar ratio of 1:1 (Fig. 2c), it was noticed that the amount and length of the strings were reduced when the ratio between amelogenin rH174 and rH163 was disproportionate.

3.4. Effect of incubation time on the cooperative assembly of amelogenin 25 and 23 kDa mixture in Tris/HCl buffered samples

The cooperative assembly of amelogenin rH174 and rH163 depends not only on pH but also on incubation time. The effect of incubation time on the cooperative assembly of amelogenin rH174 and rH163 (molar ratio 1:1) at pH 7.0 was evaluated by AFM as shown in Fig. 4. After 3 days of storing the protein suspensions at 37 °C (Fig. 4b), the mixtures contained

predominantly chain-like structures that were distinguishable in morphologies from those observed after 1 day of incubation. Isolated nanospheres were rarely observed and the number of elongated chain terminals observed after 5 days (Fig. 4c) was dramatically reduced indicating that the chain length was increasing with incubation time. These fibrous structures comprised of closely associated smaller nanospheres (18 ± 3.1 nm in width) as comparing with Fig. 1b and g, had a mean length of 350 ± 26.2 nm and a mean aspect (L/W) ratio of 19.4 ± 4.8 . However, after 7 days of incubation at 37 °C, significant differences were observed. As shown in Fig. 4d, terminals of elongated chains were scarcely observed; instead a fibrous mesh of self-assembled continuous nanostrings had formed.

AFM scans at high resolution revealed that the nanostrings formed during protein incubation were initially composed by individual nanospheres that gradually fused together to form a continuous fiber. As shown in Fig. 5, between day 1 and 3 individual nanospheres are still apparent with the nanostrings. Nanospheres align in short segments of three to ten spheres (Fig. 5b). The strings are not continuous and have dead ends. At this point the shape of the nanospheres is often not perfectly spherical; moreover the spheres appear deformed and often flattened at the areas where they connect with each other (Fig. 5c). The diameter of the nanospheres decreased by about 2–3 nm during string formation, indicating that there is mass transport and rearrangement of molecules occurring. At day 7 the outlines of nanospheres are no longer apparent. Instead the strings are continuous and have formed a fibrous meshwork. The fiber width is about 15 nm and has decreased from the width of the nanostrings at day 5, indicating further mass transport and rearrangement of amelogenin molecules within the self-assembled structures.

3.5. Effect of incubation time on the conductivity of amelogenin 25 and 23 kDa mixture

Conductivity curves from suspensions containing 0.1 mg/ml of rH174 and rH163 amelogenin and mixtures thereof were obtained and compared to protein-free Tris/HCl solution at certain pH at different incubation time points (Fig. 6). There was an increase in conductivity when protein was added to the solutions, indicating that the protein itself may be able to carry charges. Single-type protein suspensions showed little to no change in conductivity overtime. However, protein mixtures showed a decrease of conductivity after 5 days of storage. The change in conductivity was about 1 mS for suspensions prepared at pH 7.0, while it only dropped by about 0.2 mS at pH 6.6.

4. Discussion

Amelogenins uniquely regulate the growth, shape, and arrangement of enamel crystals. While 90% of the matrix secreted by ameloblasts is comprised of full-length amelogenin, enzymatic processing quickly produces cleavage products, thus reducing the amount of 25 kDa amelogenin while fragments like the 23 and 20 kDa and the tyrosine-rich amelogenin peptide (TRAP) are prone to dominate the matrix over time. Although a detailed analysis of the constituents of the developing enamel is still lacking, it is reasonable to assume that the interaction between these amelogenin fragments and the full-length molecule may play a significant role in enamel maturation and mineralization. The fibrous apatite nanocrystals of enamel are a product of protein-guided growth suggesting that the protein itself may provide a dynamic scaffold that forms elongated structures directing the growth of the mineral. The possibility of generating elongated structures and fibrillar organic matrices through cooperative self-assembly of amelogenins in vitro can be a valuable model to understand the remarkable anisotropic growth that apatite crystals undergo in vivo during enamel maturation.

The presented data confirms that amelogenins self-assembled initially into nanospheres in vitro at all pHs (Moradian-Oldak, 2007). However, single-type protein suspensions containing full-length rH174 or cleaved rH163 amelogenins showed the development of self-assembled

elongated structures consisting of apparently connected small spheres (Fig. 1b and g). These structures form within a narrow range of pH around 6.5 and 7.5 for the 25 and rH163 protein, respectively. Changes in pH affect protein charges through ionization of carboxyl and amino groups as well as functional side chains. Such changes will affect both intra- and intermolecular protein interactions and are reflected as the ζ -potential of particles in a suspension. It has been shown in a number of artificial as well as biological systems that self-assembly is enhanced when the electrostatic repulsion is reduced or the ζ -potential is close to zero (Yu et al, 2006).

We observed hierarchical self-assembly of amelogenin from the nanosphere into nanostrings by alignment and fusion of spheres. This hierarchical assembly was pH and time dependent. Since the calculated isoelectric point for the full-length amelogenin is at pH 6.62 and at pH 6.85 for rH163 molecule, nanostrings formation was rarely observed in the pH range below 6.5. For the individual protein suspensions, elongated structures were observed around pH 6.5 for the rH174 amelogenin and around pH 7.5 for the rH163 amelogenin. Electrostatic repulsion appears to be reduced at these pHs indicating that the ζ -potential is small enough to facilitate protein to protein interactions. Since intermolecular repulsion of proteins is minimal at their isoelectric pH, we estimate that the IP of the rH174 and rH163 amelogenin nanosphere to be around pH 6.5 and pH 7.5, respectively.

Fig. 7 shows a schematic that illustrates the effect of the pH on surface charges and the possibility of intermolecular interactions. Comparing with single-type protein the mixed proteins more frequently assembled into chain-like structures at pH around 7.0. After one day of incubation, chain-like structures of amelogenin rH174 were observed at pH 6.5 on about 20% of the area studied (Fig. 1b), about 15% for amelogenin rH163 at pH 7.5 (Fig. 1g), but 55% for the proteins mixture at pH 7.0 (Fig. 2c) (see Table 2). While for the single-type amelogenin suspension hierarchical self-assembly was a slow process and it took several days to generate elongated structures, this process was accelerated when both the rH174 and the rH163 protein were combined in the suspension at pH-values around 7.0. This behavior is attributed to certain surface charges on the protein nanospheres. In contrast to the single type protein suspension where the ζ -potential was minimized to allow protein to protein interactions, in the case of protein mixtures we hypothesize that at pH 7.0 nanospheres will have significant surface charges, but of opposite sign. According to the model shown in Fig. 7, at pHs close to 7.0, nanospheres comprised of the rH174 protein will be negatively charged while nanospheres from the rH163 protein will be positively charged. Hence the two particles will experience electrostatic attraction and are likely to surround themselves with particles of opposite charges. The strongest tendency to form nanostrings was observed for a weight ratio of 1:1, while the amount and length of strings were limited when the concentration of either amelogenin rH174 or rH163 dominated over the other. This observation is in good agreement with the model of Fig. 7. The even ratio between the two proteins is the most likely to ensure that 25 and rH163 amelogenins form alternating chains nanospheres, if the spheres are composed of approximately equal numbers of monomers. Due to the additive electrostatic interactions, prolonged strings of nanoparticles were obtained within 24 h of incubation. Subsequently these nanospheres fused together to first form short strings of up to 350 nm length and then over a period of 7 days a tight meshwork of fibers that lacked dead-ended terminals developed (Figs. 4d and 5d). Such prolonged timeline for fibrillar assembly is not unusual among biomolecules. Amyloid proteins are suitable examples for fibrillogenesis that requires periods of weeks and months (Kelly, 1997). This type of self-assembled structure was not observed in the single-type protein suspensions, neither were such structures present in mixed protein suspensions when the pH was 6.0 and lower or above 7.5. At these pH-values protein nanospheres will have the same charge signs even when composed of different monomers and repulse each other, thus preventing enhanced cooperative assembly.

The width and height of nanospheres in chains observed at 5 days for the full-length protein was significantly smaller than that observed at 1 day. The continuous reduction in the width of the self-assembled nanostructures indicates that a reorganization of amelogenin nanoparticles occurs during the formation of the elongated structures. Interestingly nanospheres connect on opposite sides to form strings suggesting that either the distribution of amelogenin molecules within the nanospheres is not homogenous and isotropic or the connecting event generates redistribution of amelogenin molecules and certain anisotropy within a nanosphere. Most likely such reorganization is associated with a change in the secondary structure of the amelogenin proteins and may be the origin of changes observed in Raman spectra from full-length amelogenin during micro-ribbon formation (Moradian-Oldak et al., 2006).

The current studies were performed at relatively low protein concentrations (0.1 mg/ml), but future studies at concentrations above the solubility limit of the rH174 protein are warranted, which will more closely mimic in vivo conditions of the enamel matrix. The 25 kDa is found in vivo primarily around the Tomes' process and most likely is assembled as nanospheres. Hydrolysis by MMP-20 will thus occur on amelogenin molecules within the nanosphere. It is conceivable that entire nanospheres could be transformed from 25 kDa to shorter fragment nanospheres without losing their nanosphere character (Fukae et al., 2007). According to our model of Fig. 7 these processed nanosphere would then interact with non-processed 25 kDa nanospheres and generate hierarchical structures. It is on the other hand also plausible that partially processed nanospheres containing various cleavage products could interact with each other due to electrostatic attraction based on charge differences between different amelogenin fragments. The presence of multiple amelogenin species within one nanosphere may further increase the possibility of generating a sphere containing an anisotropic distribution of molecules which in return may enhance the formation of amelogenin strings through alignment of spheres.

The electrical conductivity data confirms the occurrence of structural changes due to cooperative assembly of amelogenins over time. Comparing with single-type molecular chain or nanoparticle, the mobility of the elongated structures renders more difficult due to increased translational entropy. With the formation of chain-like structures the property to transmit electric current, namely conductivity is expected to decrease. The samples of single-type amelogenin rH174 and rH163 at pH 7.0 were not forming chain-like structures readily because of the strong electrostatic repulsion. Hence the conductivity only showed slight changes during incubation. However, conductivity decreases for the mixed proteins because of the gradual formation of chain-like structures that may also evolve into a continuous network that barely moves in an electric current. Hence we propose that the decrease in conductivity of mixed protein suspensions over time is associated with a cooperative self-assembly process and conductivity measurements could be a suitable method to determine the physical-chemical conditions for the formation of superstructures through hierarchical self-assembly.

In conclusion, the results of the present study strongly support the hypothesis that full-length amelogenin and its cleavage products have cooperative self-assembly capabilities. Mixtures of the 25 and 23 kDa amelogenins generate nanostrings under physiological conditions within 24 h and form continuous fibrous meshes with 7 days by fusion of individual nanospheres. The driving force for this hierarchical self-assembly process is electrostatic interactions that derive from opposite electric charges of the amelogenin nanospheres at pH 7.0 according to their isoelectric points. These observations are of relevance to understanding amelogenesis, since the tightly controlled changes in pH in the secretory enamel matrix may serve to regulate and precisely design self-assembled structures of the full-length amelogenin, its splice variants and cleavage products.

Acknowledgments

Funding was granted by NIH/NIDCR Grants R01-DE017529 and R01-DE015821. Drs. Sally J. Marshall and Grayson W. Marshall (UCSF) for use of AFM.

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Fig. 1. AFM images of amelogenin rH174 (a–d) and rH163 (e–h): at (a) pH 5.5 ($z = 26$ nm), (b) pH 6.5 ($z = 32$ nm), (c) pH 7.5 ($z = 39$ nm), (d) pH 6.5 ($z = 30$ nm), (e) pH 5.5 ($z = 15$ nm), (f) pH 6.5 ($z = 20$ nm), (g) pH 7.5 ($z = 29$ nm), (h) pH 7.5 ($z = 24$ nm) and after 3 days incubation (d and h).

Fig. 2. AFM images of amelogenin rH174 and rH163 mixture (1:1 molar ratio and incubation time 1 day) at (a) pH 6.4 ($z = 31$ nm), (b) pH 6.6 ($z = 34$ nm), (c) pH 7.0 ($z = 30$ nm) and (d) pH 7.5 ($z = 31$ nm).

Fig. 3. AFM images of amelogenin rH 174 and rH 163 mixture (pH 7.0 and incubation time 1 day) at mixture ratio of (a) 3:1 ($z = 28$ nm), (b) 2:1 ($z = 28$ nm), (c) 1:2 ($z = 30$ nm) and (d) 1:3 ($z = 33$ nm).

Fig. 4. AFM images of amelogenin rH 174 and rH 163 mixture (pH 7.0 and molar ratio 1:1) after being incubated for (a) 1 day ($z = 30$ nm), (b) 3 days ($z = 27$ nm), (c) 5 days ($z = 25$ nm) and (d) 7 days ($z = 20$ nm) at 37 °C.

Fig. 5. High-resolution AFM-micrographs of self-assembled amelogenin mixtures of rH174 and rH163 molecules at a ratio of 1:1 after different incubation times: (a) 1 h ($z = 35\text{nm}$), (b) 3 days ($z = 21\text{ nm}$), (c) 5 days ($z = 24\text{nm}$), (d) 7 days ($z = 13\text{ nm}$).

Fig. 6. Conductivity as a function of incubation time curves of amelogenin rH174, rH163 and their mixture at different pH.

Fig. 7.
Schematic illustration of electrostatic interactions between amelogenin 23 and 25 kDa due to different isoelectric points (IP) and varying ionic surface charges (ζ -potential) with pH.

Table 1

The average diameter of amelogenin nanospheres at different pH

		pH5.5	pH6.5	pH7.5
rH174	Nanoparticles (nm)	20.4 ± 2.9	26.1 ±2.5	29.3 ± 2.3
rH163	Nanoparticles (nm)	17.2 ±1.6	21.9±2.1	28.7 ±3.2

Table 2

The percentage of nanostrings of at least 100 nm length for amelogenin rH174, rH163 and their mixtures at different incubation time

		Percentage of nanostrings (%)		
		1 day	3 days	7 days
rH174	pH6.5	20	25	35
	pH7.0	5	5	5
	pH7.5	0	5	5
rH163	pH6.5	0	0	5
	pH7.0	5	5	10
	pH7.5	15	30	40
rH174/rH163(1:1)	pH6.5	35	45	55
	pH7.0	55	75	Fibrous mesh
	pH7.5	30	35	50