Journal of Structural Biology 174 (2011) 203–212

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Self-aligning amelogenin nanoribbons in oil–water system

Xiaodong He [a], Shenping Wu [b], Olga Martinez-Avila [a], Yifan Cheng [b], Stefan Habelitz [a,*]

[a] Department of Preventative and Restorative Dental Sciences, University of California, San Francisco, CA 94143, USA
[b] Department of Biochemistry & Biophysics, University of California, San Francisco, CA 94158, USA

ARTICLE INFO

Article history:
Received 22 September 2010
Received in revised form 29 November 2010
Accepted 30 November 2010
Available online 4 December 2010

Keywords:
Amelogenin
Dental enamel
Self-assembly
Atomic force microscopy
Electron microscopy
Emulsion

ABSTRACT

The highly organized microstructure of dental enamel is a result of protein-guided anisotropic growth of apatite nanofibers. It is established that amelogenin proteins, the main constituent of the developing enamel matrix, form nanospheres in vitro, but the amphiphilic nature of the full-length protein conveys the possibility of generating more complex structures as observed with other surfactant-like molecules. This study tested if the use of metastable oil–water emulsions can induce supramolecular assemblies of amelogenin. Recombinant full-length amelogenin, rH174, was mixed into octanol/ethyl acetate preparations of different ratios to form emulsions at pH 4.5 and 7.4. Atomic force and electron microscopy showed the formation of 16.7 ± 1.0 nm wide nanoribbons which grew to several micrometer length over a period of days. Nanoribbons formed from reverse micelles by enabling hydrophobic tails of the molecules to interact while preventing the formation of amelogenin nanospheres. Ribbon formation required the presence of calcium and phosphate ions and may be localized at a dark central line along the amelogenin ribbons. The ribbons have a strong tendency to align in parallel maintaining 5–20 nm space between each other. The growth rates and number of ribbons were significantly higher at pH 4.5 and related to the metastability of the emulsion. A model for ribbon extension proposes the addition of short segments or amelogenin dimers to the ends of the ribbon. The formation of self-aligning and uniaxially elongating amelogenin structures triggered by the presence of calcium and phosphate may represent a suitable new model for protein controlled mineralization in enamel.

1. Introduction

Self-assembly guides biological organization, is ubiquitous throughout life chemistry and occurs at many length-scales in nature. The formation of a tissue or organ is a hierarchical process with several tiers of self-assembling steps (Monnard and Deamer, 2002). In the case of the enamel matrix, its main component, amelogenin proteins are known to assemble into nanospheres (Fincham et al., 1995; Robinson et al., 1981). The full-length amelogenin protein is a bipolar molecule that is hydrophobic over most of its length, but contains hydrophilic amino acids at the C-terminus. Bipolarity is also a prerequisite for the self-assembly of synthetic materials that can generate a large variety of nanostructures, including fibers, tubes, sheets or membranes and rods (Claussen et al., 2003; Colfen and Mann, 2003; Elie-Caille et al., 2005; Hartgerink et al., 2001; Lindoy and Atkinson, 2000). Some artificial systems also succeeded in generating larger structures through hierarchical self-assembly of the primary nanoconstruct (Choi et al., 1999; Yan et al., 2004).

Self-assembly of amelogenin into nanospheres appears to be dominated by its hydrophobic residues that tend to associate at the non-polar regions and thus shield themselves from the surrounding water (Fincham et al., 1995; Moradian-Oldak, 2001). Amelogenin self-assembly has been studied to a large extend *in vitro*, but due to the absence of this protein in the developed and erupted tooth, investigations were typically performed with recombinant proteins. The size of nanospheres from full-length amelogenin is polydisperse and varies with temperature, concentration, pH, ionic environment and time (Margolis et al., 2006; Moradian-Oldak et al., 2000). In addition, solubility of amelogenin in aqueous solutions is limited. The solubility is strongly dependent on the pH (Tan et al., 1998). Studies on the molecular self-assembly of amelogenin have been performed to a large extend by dynamic light scattering (DLS), atomic force and electron microscopy. At this point it is widely accepted that the full-length protein forms nanospheres of about 15–40 nm in diameter under physiological conditions *in vitro* and at concentrations below the solubility limit (Moradian-Oldak, 2001). A core–shell model for the nanospheres has been proposed, where hydrophilic and negatively charged side chains prevent the agglomeration of hydrophobic cores of the nanospheres (Aichmayer et al., 2005). Recent studies have shown increasing evidence that the full-length protein has the capacity to form chain-like structures consisting of 5–10 nanospheres connected to each other like beads on a string (Aichmayer et al., 2005; Beniash et al., 2005; Wiedemann-Bidlack

* Corresponding author. Fax: +1 415 476 0858.
E-mail address: stefan.habelitz@ucsf.edu (S. Habelitz).

1047-8477/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jsb.2010.11.027

et al., 2007). The string-like nanosphere aggregates were also observed to align themselves parallel to the *c*-axis of apatite crystals (Habelitz et al., 2004). The aggregation processes were dependent on the pH and appeared to produce organized tertiary structures when approaching the isoelectric point of the protein (Wiedemann-Bidlack et al., 2007). A recent small angle X-ray scattering study showed that nanospheres are actually ellipsoidal and thus may not be isotropic, a possible prerequisite to forming chain-like structures (Aichmayer et al., 2010). Mesh-like networks of full-length amelogenin were also formed during the electrolytic deposition of apatite suggesting that the presence of calcium was a key-factor in the nanochain formation of the protein (Fan et al., 2007). In TEM studies in combination with turbidity measurements a transition from individual nanospheres to string-like arrangements were observed within 150 min of sample preparation (Wiedemann-Bidlack et al., 2007), but only if the protein contained both the hydrophilic and the hydrophobic portions. MMP-20 cleavage products of amelogenin lack the hydrophilic portion and have a lower tendency to form chain-like aggregates (Kwak et al., 2009).

The observation of a transition from a nanosphere to string-like arrangements requires kinetic energy for movement and reorganization of the spheres and chain length increases with incubation time. AFM studies have shown that numerous strings of amelogenin can form over a period of days or weeks and lead to the formation of featureless fibers that lack the pattern of the initial nanospheres, also indicating that the nanosphere may not be the thermodynamically most stable structure amelogenin prefers to maintain (He et al., 2008). The kinetics for such fibrillogenesis was significantly improved when a mixed population of amelogenins containing the full-length protein (rH174) and the MMP-20 cleavage product (rH163) were used and supported the hypothesis that surface charges are involved in controlling the hierarchical assembly of amelogenin (He et al., 2008). Zeta-potential measurements showed values of 6.8 as the isoelectric point of the full-length molecule which coincides well with the increased tendency for aggregation around this pH (Uskokovic et al., 2010). Surface charges are critical in self-assembling systems and have been fundamental for the design of self-assembling peptide amphiphiles. Several studies in the Stupp laboratory have shown the relevance of the pH to induce supramolecular self-assembly and gelation of peptide amphiphiles. Gelation occurred when the attractive Van der Waals forces overcame the electrostatic repulsion between the counterion double-layer that surrounded a particle or molecule (Stendahl et al., 2006).

Oil–water mixtures are ubiquitous in nature and are particularly important in biology and industry (Leunissen et al., 2007) When oil and water are mixed, micro-emulsions develop either by the formation of micrometer sized oil-droplets or micelles dispersed in a supersaturated ionic aqueous solution or in the inverse system based on reverse micelles where water droplets form and are stabilized by the use of surfactants e.g. AOT [sodium bis-(2-ethylhexylsulfo succinate)] surfactants or DDAB [didodecyldimethyl-ammonium bromide]. Such micro-emulsions have been used to create unique crystal morphologies at the nanometer scale (Colfen and Mann, 2003; Qi et al., 2001). Several studies were successful in controlling the growth of inorganic materials, including calcium phosphates, by forcing the crystals to adapt the micelle or the intermicellar structure (Chen et al., 2007). Walsh and Mann were able to produce interconnected fibrous apatite crystals using DDAB in an oil–water system at temperatures below the melting point of the oil phase (Walsh and Mann, 1995). Margolis et al. reported that a calcium phosphate material with structural features resembling tooth enamel (bundles of co-aligned filaments) was synthesized from a highly viscous reaction solution containing reverse micelles and micro-emulsions, stabilized by AOT (Margolis et al., 2006). That study also emphasized the analogies between the action of the surfactant and the amphiphilic protein amelogenin.

In contrast to a regular micelle, the reverse micelle is defined by the hydrophilic portion of the surfactant being at the interior of the micelle which contains water, while the hydrophobic portion is exposed to the surrounding non-polar matrix or oil phase. Reverse micelles, thus, provide a restrictive aqueous environment for controlled chemical or biological reactions and have been depicted as passive nano-reactors (Uskokovic and Drofenik, 2007). Reverse micelles may have been the precursors to the formation of membranes of a biological cell. They are also critical in current approaches for the immobilization and encapsulation of macromolecules for medical and pharmaceutical purposes (Trivedi and Kompella, 2010) and have been used for protein extraction through the affinity partitioning process (Kelley et al., 1993).

This study used an oil–water system in which full-length amelogenin molecules play the role of the surfactant. Different compositions of the oil phase were used and the pH of the ionic solutions was adjusted until metastable emulsions formed. When analyzed by TEM and AFM elongated, ribbons of 16.7 nm width composed of recombinant full-length amelogenin molecules formed in such emulsion. A model for the process of self-assembly is described.

2. Materials and methods

The full-length human amelogenin protein (rH174) was obtained via recombination and expression in BL21(DE3) plysS *E. Coli*, as described previously (Li et al., 2003). The protein was purified by incubating the extract with C4 hydrophobic beads in 30% acetonitrile with 0.1% trifluoacetate (TFA) for 30 min at room temperature. After washing, the protein was eluted by 80% acetonitrile in 0.1% TFA and lyophilized to obtain aliquots of about 1 mg rH174 per test-tube. Amelogenins rH174 were dissolved in double deionized water to prepare different concentration of stock solutions according to experimental demand. Stock solutions of calcium chloride dehydrate and potassium dihydrogen phosphate were prepared from reagent grade chemicals. The molar ratios of the solution components were varied in a series of experiments. Octanol (99% reagent grade, Fisher Scientific Inc) and ethyl acetate (99.7% reagent grade, Fisher Scientific Inc.) were used as received. Sodium azide was added to all reaction solutions at 0.02 vol.%.

2.1. Preparation of amelogenin stabilized emulsion

For preparing amelogenin stabilized water-in-oil emulsion, the choice of the oil phase is the first step and is based on the principle of like dissolves like. Octanol was chosen as primary oil phase composition due to its low Hildebrand solubility parameter γ of 21.1 $(J/cm^3)^{0.5}$ (Sepassi and Yalkowsky, 2006). Ethyl acetate was used to modify the interfacial tension at the oil–water interface during amelogenin self-assembly (vanBuuren et al., 1996). The addition of ethyl acetate, which most likely acts as a co-surfactants, was required for emulsification as indicated by a creamy white or turbid protein/water/oil mixtures that formed after vortexing. Ethyl acetate (EA) concentrations were adjusted between ratios of 9;1 and 6:4 for $Vol_{octanol}$:Vol_{EA} to achieve stability of the emulsion. Emulsion remained turbid for extended time at a volume ratio of octanol and ethyl acetate equal to 7/3, oil–water ratio equal to 4/1 and acidic pHs. With increasing the pH to 7.4 the emulsion stabilized significantly and we did not observe a significant phase separation after 48 h. After seven days only a small rim of separated water was visible. The final concentration of 3.7 mg/ml of amelogenin rH174 was used in all experiments. The protein concentration is based on the assumption that the protein is distributed in the water phase only and not in the oil phase. Raman spectroscopy confirmed the absence of protein in the oil phase.