Amelogenin Nanospheres Supra-Molecular Assembly and the Preferential Orientation of Apatite Crystals

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ABSTRACT

Dental enamel is a bio-ceramic formed through a cascade of intra and extra-cellular events among which protein self-assembly is a critical step. Amelogenin self-assembly into nanospheres has been proposed as a key factor in controlling the oriented and elongated growth of enamel crystals. This manuscript is a short review of our recent in vitro study on the process of amelogenin self-assembly into higher order structures. We report characterization of birefringent micro-ribbons formed by the supramolecular assembly of amelogenin nanospheres (10-25nm) through nano-chains of >100nm in length. Mineralization of the micro-ribbons resulted in the formation of remarkably ordered apatite crystals oriented with their c-axes parallel to the long ribbon axes. The strong tendency of amelogenin to form nanospheres which self-assemble into linear structures provides a new insight into the function of amelogenin as a template for the oriented growth of crystals during enamel biomineraization.

***Keywords*:** tooth enamel, biomineralization, amelogenin nanospheres, self-assembly

1. INTRODUCTION

Tooth formation is an ideal model for the study of biomineralization processes [1]. Enamel is the outermost layer covering the tooth and it possess a unique combination of mechanical functions including wear and fracture resistant [2]. While collagen serves as the template for the growth of bone and dentin crystals, enamel crystals are formed within an "amelogenin-based" matrix [3]. The mineralized tissue is formed through a series of controlled intra and extra- cellular events including: 1. protein secretion, 2. protein self-assembly , 3. crystal nucleation, 4. oriented crystal growth, 5. protein processing and degradation. The initial secretory stage enamel matrix is composed of about 50-60% water, 20-30% protein, and 15-20% mineral by volume [4]. The bulk of the extracellular "organic matrix framework" is processed in a stepwise and controlled manner and eventually removed from the extracellular space. Notably, massive protein degradation events occur simultaneously with the completion of minerallization, at late maturation stage, creating a highly organized structure, which is almost completely inorganic (>99%) possessing sophisticated mechanical properties.

Amelogenin proteins secreted by ameloblasts constitute the primary structural entity (>90%) of the developing dental enamel extracellular matrix. The "core" of the amelogenin sequence is enriched with the hydrophobic amino acids proline and leucine while the majority of the charged residues of the sequence are concentrated at the C-terminal region providing the molecule with a hydrophobic-hydrophilic polarity. Amelogenin sequences at both carboxy- and amino terminal regions across species are highly homologous suggesting that these regions play specific functional roles during the matrix mediated enamel biomineralization.

Amelogenin "nanospheres" were first described in 1994 when the self-assembly properties of a recombinant mouse amelogenin (rM179) were studied employing dynamic light scattering (DLS), size exclusion chromatography, atomic force and transmission electron microscopy [5,6]. It was then reported that full-length amelogenin molecules undergo a self-assembly process to generate spherical structures (Fig.1). Following the description of amelogenin "nanospheres" these structures were identified in histological sections of developing mouse molars as electron lucent "beaded rows" (15-20 nm in diameter) surrounding the tiny enamel crystallites at the very early stage of enamel formation [7]. It was then proposed that amelogenin molecules self assemble to form nanospheres constituting the basic structural entities of enamel extracellular marix and providing the organized microstructure for the initiation and oriented growth of enamel crystals.

Numerous in vitro studies have collectively supported the notion that amelogenin nanospheres interact with calcium phosphate crystals in a selective manner affecting morphology, habit and orientation of the crystals [8,9].

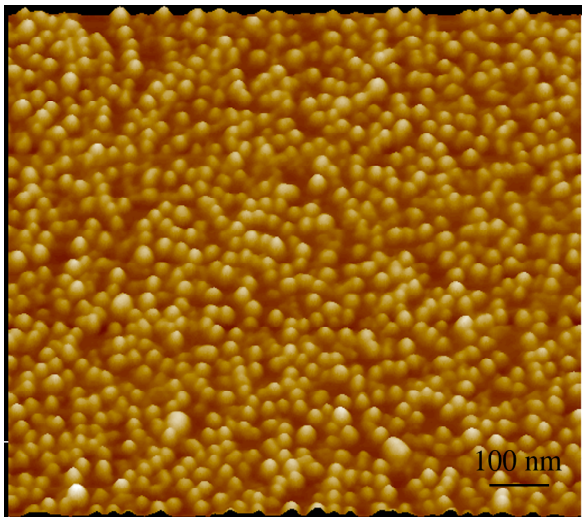

Fig. 1. Amelogenin nanospheres imaged by atomic force microscopy (in tapping mode). A recombinant mouse amelogenin (rM179) at concentration of 0.3mg/ml was dissolved in Tris-Hcl buffer, adsorbed on mica and fixed as described previously [10].

This paper is a summary of our most recent finding on the process of amelogenin self-assembly in vitro, and exploration of higher levels of ordered structures from the nanospheres [11]. We report the spontaneous assembly and hierarchical organization of birefringent "micro-ribbons" of a full-length recombinant porcine amelogenin (rP172). The formation and c-axial oriented organization of hydroxyapatite on the self-assembled amelogenin micro-ribbons during an in vitro mineralization study was also observed. We propose that the significant alignment of amelogenin nanospheres into long chains is relevant to the mechanism of their function as a template in facilitating the elongated and oriented growth of apatite crystals during enamel biomineralization

2. EXPERIMENTAL STRTATEGIES

Amelogenin micro-ribbons were formed by the classic techniques of hanging drop for protein crystallization, under a wide variety of solvent conditions as described by Du et al, [11]. A recombinant porcine amelogenin (rP172) which was expressed in e-coli and purified by means of reversed-phase HPLC was used. The fibre diffraction patterns for amelogenin ribbons were collected at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The self-assembly process was analysed by dynamic light scattering (DLS) measurements using the DynaPro-99E-MS/X instrument (Proterion Corporation, Piscataway, NJ). Atomic force microscopy (AFM) was performed in tapping mode in air with a NanoScope® IIIa Scanning Probe Microscope System (Digital Instruments, Inc., Santa Barbara, CA) with a tapping mode etched silicon probe (model OTESPA). For TEM experiments, the protein solution samples (5 μl) were pipetted onto carbon-coated electron microscopy grid, either fixed or not with Karnovsky fixative, and stained with 2% uranyl acetate. The samples were observed in a JEOL JEM-1200EX microscope operating at 80 kV. For in vitro mineralization of amelogenin micro-ribbons, the samples were washed thoroughly with de-ionized water, and then immersed into the mineralizing solution (150 mM NaCl, 10 mM HEPES, 2.5 mM $CaCl_2$, 1.5 mM K_2HPO4) at pH 7.4 and room temperature for 7 days. Some samples were pretreated with 20 μg/ml phosvitin for one day, in an attempt to promote nucleation, and transferred to mineralizing solution for 7 days. The in situ mineralized ribbons on the glass plates were sputter-coated with Au and observed under SEM (Cambridge 360, 15 kV). For TEM observation, the samples were mechanically dissected and dispersed in 70% ethanol and transferred to carbon-coated grid.

3. RESULTS AND DISCUSSION

The recombinant porcine amelogenin formed micro-ribbons under a wide variety of solution conditions (i.e. pH and ionic strength) (Fig. 2). The highly birefringent micro-ribbons had a x-ray diffraction pattern that indicated a preferential orientation of periodic units along their long axis (Table 1) [11].

meridional	equatorial	near equatorial
0.26 (*strong*)	0.39 (*strong*)	0.43 (*weak*)
0.34 (*weak)*	0.53 (*strong*)	
0.52 (*medium*)		

Table 1. X-ray diffraction spacing (nm) from an amelogenin micro-ribbon. The ribbon was oriented edge on or face on with respect to the x-ray beam direction. The meridional direction is along the long axis of the ribbon. Following the proposed β-spiral model with helical parameters of h=0.251 nm and n=3.95 [12], and assuming a periodicity of approximately 1 nm, we predict that the meridional reflections at 0.52, 0.34 and 0.26 nm correspond to the second, third, and fourth order respectively. Note that the presence of diffractions along the equatorial axis at 0.39 and 0.53 nm suggests an ordered lateral assembly among the crystalline units.

We have proposed that the lattice spacing along this direction results from a unique conformation and secondary structure of folded amelogenins. The

structure that can fit such periodicity is the β-spiral (a repetitive β-turn structure) which was previously predicted by Renugopalakrishnan et al. [12] on the basis of a molecular mechanics-dynamics study of a bovine amelogenin. Computational analysis of the rP172 sequence has predicted a high probability for the formation of concatenated β-turns in the proline-rich regions that provide a high probability for a β-spiral structure [11]. It is noteworthy that the proline-rich sequence has been highly conserved among amelogenins from different species [13]. Overall, the diffraction pattern suggests that the molecules, or certain domains of the molecules, can evolve into a relatively ordered array during the formation and maturation of the micro-ribbons.

Systematic analysis of particle size distribution by DLS revealed that typical nanospheres with hydrodynamic radii (R_H) of 13.2-27.5 nm were the most stable assembling form (over 75% of the mass) in either acetate buffer (pH 4.5) or distilled water (pH ~6.5). Monomers and discrete oligomers such as dimers, trimers and hexamers were detected in the diluted protein solutions using 60% aqueous acetonitrile as a solvent. Larger aggregates (>100 nm in size) existed in small amounts. Direct visualization by TEM, SEM, and AFM has revealed these larger aggregates to be the result of further association of nanospheres in a linear arrangement (Fig. 2 B, inset).

Mineralization of the micro-ribbons resulted in the formation of ordered and oriented crystals, aligned approximately along the long axis of the ribbons. TEM and the corresponding electron diffraction patterns of crystals obtained from the tips of the mineralized micro-ribbons confirmed the formation of oriented apatite crystals along their c-axial direction (Fig. 3 A). SEM micrographs of micro-ribbons that have been pre-treated with phosvitin (a highly phosphorylated protein which was used in our experiments as a model for a potent nucleator) followed by mineralization revealed the remarkable orientation of the crystals grown on the surface of these micro-ribbons (Fig. 3B). We suggest that the linear alignment of nanospheres in vitro creates a pattern of functional groups and therefore an ideal framework that facilitates the oriented growth of apatite crystals regardless of the presence of an acidic protein such as phosvitin. The oriented nucleation of such crystals could also be promoted through the interactions of the structured amelogenin framework with the highly phosphorylated protein, phosvitin.

The finding that amelogenin protein has a strong tendency to self-assemble into nanospheres which then align themselves in a linear arrays has notable biological significance and it provides insight into underlying mechanisms of control over orientated crystal growth during enamel biomineralization.

The control over crystal nucleation and growth orientation by ordered arrays of acidic functional groups has been widely implicated in the biomineralization world [14]. In the case of enamel mineralization the ordered hydrophobic/hydrophilic partitioning of amelogenin molecules could create an oriented array of acidic peptides on a hydrophobic substrate. This control can also be achieved by the interaction of amelogenin and the non-amelogenin proteins such as the acidic enamelin or ameloblastin.

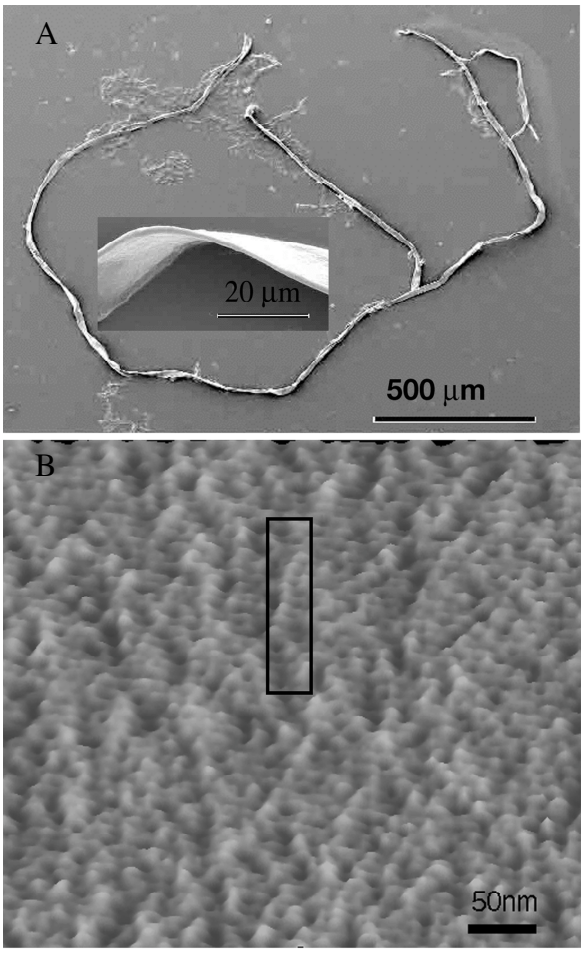

Fig. 2. Amelogenin micro-ribbons imaged by **A:** scanning electron microcopy. Inset in A is a high magnification demonstrating the sharp edges of these ribbons. **B**: Tapping mode atomic force miscroscope image of the surface of a micro-ribbon showing the linear array of amelogenin nanospheres (inset) [11].

Amelogenin nanospheres have been detected in vivo as "beaded rows" along the c-axis of developing enamel crystallites suggesting their close interaction with the crystal surface [7]. We propose that the organized assembly of amelogenin nanospheres into collinear arrays is critical at the initial stage of mineral deposition, and adjacent to the dentino-enamel junction, when oriented nucleation occurs and prior to amelogenin processing and degradation. This will occur most likely as a result of other enamel interacting with the structured amelogenin framework [15].

The outcomes of our finding may have a potential impact on the field of matrix-mediated biomineralization, protein self-assembly, formation of ordered nano-structures, development of biomaterials and bioceramics as well as hard tissue regeneration.

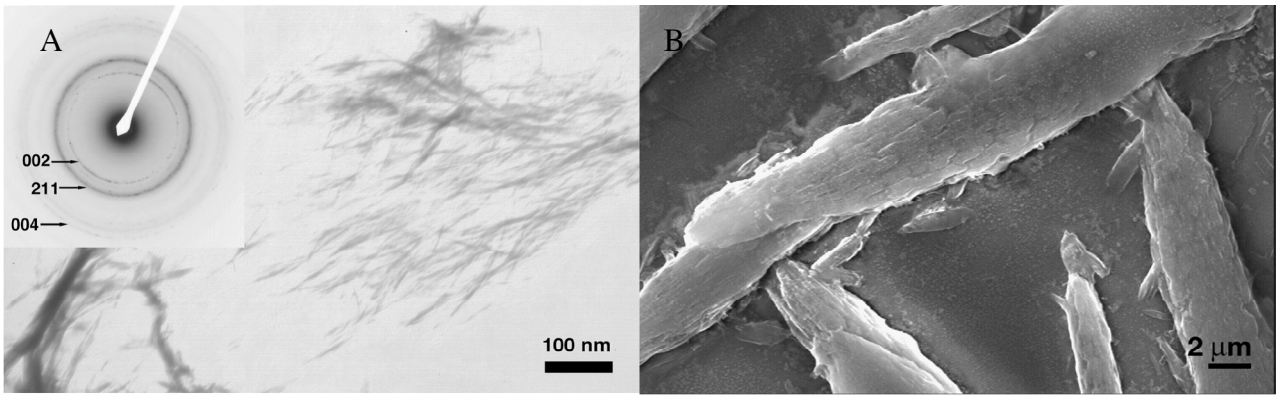

Fig. 3. A. TEM micrographs and corresponding electron diffraction pattern of apatite crystals (inset) isolated from the tips of mineralized amelogenin micro-ribbons demonstrating the preferential orientation of the crystals parallel to their c-axis. **B.** SEM micrograph of apatite crystals grown on amelogenin micro-ribbons pre-treated with phosvitin showing crystal alignment with the long axes of the micro-ribbons [11].

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