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Enamel inspired nano-composite fabrication through amelogenin supramolecular assembly

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Abstract

Fabricating the structures similar to dental enamel through the *in vitro* preparation method is of great interest in the field of dentistry and material science. Developing enamel is composed of calcium phosphate mineral, water, and enamel matrix proteins, mainly amelogenins. To prepare a material mimicking such composition a novel approach of simultaneously assembling amelogenin and calcium phosphate precipitates by electrolytic deposition was established. It was found that recombinant full-length amelogenin (rP172) self-assembled into nanochain structures during electrolytic deposition (following increase in solution pH), and had significant effect on the induction of the parallel bundles of calcium phosphate nanocrystals, grown on semiconductive silicon wafer surface. When a truncated amelogenin (rP148) was used; no nano-chain assembly was observed, neither parallel bundles were formed. The coating obtained in the presence of rP172 had improved elastic modulus and hardness when compared to the coating incorporated with rP148. Our data suggest that the formation of organized bundles in amelogenin-apatite composites is mainly driven by amelogenin nanochain assembly and highlights the potential of such composite for future application as dental restorative materials.

Keywords

Electrolytic deposition; Amelogenin; Self-assembly; Enamel; Nanoindentation

Introduction

Biom mineralization concerns the formation of mineral-based highly organized structures by living organisms [1,2]. Structures formed by biological mineralization are utilized as excellent biomimetic models for the design and fabrication of novel and advanced materials with improved mechanical properties [3]. Extracellular matrix proteins play pivotal roles in controlling the nucleation and growth of minerals in many biom mineralizing systems including bone and teeth [1]. Amelogenin is the major extracellular matrix protein in developing enamel. Shortly after secreted by ameloblast, amelogenins are processed by enamel proteases and degrade into shorter segments [4]. The amelogenin molecules spontaneously self-assemble into spherical structures called nanospheres [5]. The supramolecular self-assembly of amelogenin nanospheres into higher ordered structures *in vitro* such as microribbons have also been documented [6]. It is now well accepted that the self-assembly and stepwise degradation

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of amelogenin promote the formation and growth of mineral crystals, which make the mature enamel the hardest tissue in vertebrate [7,8]. These carbonate-containing fluoridated hydroxyapatite crystals, composing >95% wt. of mature enamel, organized into unique hierarchical architecture to provide enamel with extraordinary mechanical and anti-abrasion properties [9,10,11]. *In vitro* fabrication of dental enamel mimicking structures and composites is of great interest [12]. Those advanced biomaterials could be utilized as a future alternative for the current dental restorative materials such as amalgam and resin. Recent attempts to prepare enamel-like microstructures were made by either the hydrothermal method under high temperature and high pressure [13], extreme pH [12], or application of surfactants for the organized assembly of synthesized calcium phosphate crystals [14].

Here, we implement the biomimetic approach in which the enamel protein amelogenin is used to control calcium phosphate crystallization under near physiological conditions (i.e. pH, temperature, and ionic strength), and based on previous studies on amelogenin mineralization, such as the growth of crystals in reconstituted amelogenin gel-like matrices [15,16], on bioactive surfaces [17,18], and in supersaturated solution [19]. Amelogenins were also shown to affect octacalcium phosphate and apatite crystal morphology in such a way as to form crystals with high aspect ratios [19,20]. Recent studies demonstrated the formation of c-axially aligned apatite crystals bundles in the presence of amelogenin in supersaturated solution [19] and a co-operative mineralization of spherical silica from hydrolysis of tetraethoxysilane with amelogenin [21].

We applied a novel technique of electrolytic deposition (ELD) to fabricate an enamel-mimicking composite coating, at near physiological pH and ionic strength, from a solution containing calcium, phosphate ions, and soluble recombinant amelogenin proteins. In the process of ELD a coating is deposited from the solution onto the cathode by controlled increase of the pH through electrolysis. ELD has been demonstrated to be an effective method in preparing a collagen-mineral composite coating [22]. The advantage of this technique is that local pH near the cathode is increased gradually by continuous electrochemical reactions and results in supersaturation with respect to the calcium phosphate mineral phases of interest and their nucleation on the surface of the cathode [23]. This increase of pH also induces simultaneous amelogenin self-assembly [24]. The pH change in ELD is relevant to the previous investigations of cyclic regular pH pattern from pH 5 to 7 in normal developing teeth [25]. *In vitro* studies have reported that amelogenin solubility, its assembly properties and its effect on apatite crystal growth were all dependent on pH [26,27,28]. In these studies purified recombinant amelogenin in expressed in *E. Coli* was used and proved to be an appropriate model for such structural and functional studies. Here, we applied two engineered pig recombinant amelogenins to prepare enamel mimicking composite coating and to study their effect on the growing crystals in the nano-composite coating. One is the full-length recombinant rP172, analogue to the full-length native porcine amelogenin P173 [29] and the other one rP148, lacking the hydrophilic C-terminal 25 amino acids, is the homologue to the major component of native enamel proteolytic product P148 [30,6]. Comparison between the assembly behavior of rP172 and that of rP148 during ELD reaction as well as their effect on the surface topography provided insight on the function of amelogenin self-assembly in controlling the surface topography of nano-composite coating.

Experimental

Amelogenin expression and purification

Purified recombinant porcine amelogenin rP172 and truncated rP148 were prepared as described previously [29,30]. The rP172 protein has 172 residues (amino acids 2-173) and is an analogue to the full-length native porcine P173, and rP148 has 148 residues (amino acids 2-149) from P173, both lacking the N-terminal methionine and a phosphate group on Ser16

[31]. Generally, the protein was expressed in *Escherichia Coli* strain BL21-codon plus (DE3-RP, Strategene), purified by ammonium sulfate precipitation, and reverse-phase high performance liquid chromatography (HPLC, C4-214TP510 column, Vydac, Hesperia, CA).

Electrolytic deposition (ELD)

Silicon wafers (100), boron-doped 500 μm thick, 7 mm $\times$ 20 mm slide, resistivity 0.006–0.2 Ohm.cm (Silicon Quest International, Santa Clara, CA) were used as ELD coating substrate. Electrolyte was prepared as 3.6 mM $[\text{PO}_4]$ and 6.0 mM $[\text{Ca}^{2+}]$ from $\text{NH}_4\text{H}_2\text{PO}_4$ (purity >99.5%, J.T. Baker, Phillipsburg NJ) and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (purity >99.8%, J.T. Baker, Phillipsburg NJ). All proteins were dissolved in the buffer solution at the desired concentrations: rP172, 0.5 mg/mL, and rP148, 0.4 mg/mL. The pH of the cathode electrolyte, monitored by Metrohm 718 STAT pH meter, was adjusted to 4.8 by 0.1M NaOH, and then the electrolyte was filtered through a 0.45 μm PVDF filter (Millex-HV, Millipore, MA) before ELD. A potentiostat (Gamry PCI4/300, Gamry Instruments, Warminster, PA) with a three electrode system was used to control the electrolytic deposition. A Si wafer was used as a cathode, a platinum foil as the counter anode and Ag/AgCl, (Sat. NaCl) electrode as the reference electrode. A 2% agar gel salt bridge containing 1M KCl was used to connect cathode and anode chambers. Constant potential $\varphi = -1.6$ V was applied on the cathode at 20°C for 10–60 min for ELD. The sample with coating was rinsed with deionized water thoroughly for 20 seconds, followed by air drying. The electrolyte pH in the cathode chamber was tested by Litmus paper at about 1 mm distance to cathode.

Characterization of composite coatings

The dried samples of ELD coating were sputtered with 5 nm Carbon or Iridium for conductivity and secondary electron images were acquired by scanning electron microscope (SEM, 5–10 kV, Leo 1550 VP). High performance liquid chromatograph (HPLC, Varian Prostar 210) with a Vydac analytical C4-215TP54 column was used to detect the protein in the coating. 100 μL electrolyte or 150 μL 0.1% tetrafluoride acetate dissolved from one piece of coating was used as one injection amount of sample.

FT-IR (Fourier Transformed Infrared) spectrum of the coating was collected on an Attenuated Total Reflection (ATR) DuraScope diamond accessory (SensIR Technologies, Danbury, CT) connected to a Nicolet Flourier Transformed Infrared spectrometer (Thermo Electro Co. Waltham, MA). For each spectrum an integral of 1024 scans at the resolution of 4 cm^{-1} was recorded. FT-Raman spectrum of the coating was collected by a Renishaw Raman System RM1000 (Renishaw plc, Wotton-under-Edge, UK) connected to an optical microscope Leica DMLM (Leica Microsystems, Wetzlar, Germany). The Ar laser (514.5nm) was applied at 50 mW.

Transmission Electron Microscope (TEM) (JEM-1200EX, JEOL, Japan) was used at 80 kV. A carbon-coated TEM copper grid was directly used as the ELD cathode to collect the precipitation in 10–15 min. The protein was dissolved in pH=4.6~4.8 25 mM sodium phosphate solution at 0.5 mg/mL. After electrolytic deposition, the grids were rinsed with de-ionized water twice, then treated with Karnovsky' fixation for 30 min, and then rinsed again by de-ionized water then air dried.

Dynamic light scattering (DLS)

DLS measurement was performed by a DynaPro-99EMS/X instrument (Wyatt Technology, Santa Barbara, CA, USA) equipped with a solid-state laser operating at 655 nm with temperature control at 20°C as previously described [27]. For each measurement, at least 15 valid data points was collected and repeated 3 times for each sample. The distribution of hydrodynamic radius was calculated as isotropic spheres model and the optimized time cut-

off was selected to obtain the best regularization fit. The rP172 was dissolved in 50 mM sodium phosphate solution or calcium phosphate solution (3.6 mM [PO₄] and 6.0mM [Ca]) at pH=4.6–4.8 and then filtered by a 0.45 micron filter. Different pH was adjusted by 2M HCl or 2M NaOH carefully before the DLS measurement.

Nanomechanical Testing

A nano indenter XP (MTS, Oak Ridge, TN, USA) was used with a Berkovich tip under continuous stiffness measurement for thin film. rP172/CaP, rP148/CaP composite coating, and polished human enamel were measured. Human third molars, extracted following the standard procedures for extraction at the University of Southern California School of Dentistry and handled with permit by Institutional Review Board, was embedded in Buehler EPO-Thin resin and polished with 0.1 μm diamond paste at the occlusal surface. On each sample at least 50 indents controlled as 1000 or 1500 nm depth limit were made and the data was recorded and processed by software Testworks 4 to calculate the elastic modulus and hardness. The modulus of coating on Si was calculated when the harmonic modulus vs. displacement curve has a step at 50–200 nm depth and there was a complete indent correspondingly under optical microscope. Critical fracture toughness of enamel was calculated by the below equation by measuring the length of indenter produced cracks on SEM images.

$$K_{Ic} = B \left(\frac{E}{H} \right)^{1/2} \left(\frac{W}{c^{3/2}} \right)$$

B is an empirical constant, 0.016 for Berkovich indenter. *E* is the elastic modulus (GPa). *H* is hardness (GPa). *W* is the applied force (N). *c* is the crack length after indentation (m) [32,33]. Since few equations were established for calculating thin coating, the fracture toughness of ELD coating was estimated by this equation.

Results and discussion

Analysis of surface topography and composition

Typically, a gel-like layer could be seen around the cathode after 20 min ELD in the presence of rP172/CaP and a composite coating of 300 nm was obtained in 30 min. The thicker coating (300–600 nm) in the presence of amelogenin were prepared when compared to the 100 nm thickness of the calcium phosphate control without amelogenin, indicating the amelogenin promoted mineral deposition. High performance liquid chromatography (HPLC) analysis of the protein in the dissolved coating and the protein in the electrolyte solution demonstrated the composition of full-length rP172 amelogenin in the coating without any degradation (Fig. 1). FT-IR and Raman spectroscopy also confirmed the presence of amelogenin in the composite coatings (Fig. 2). IR spectrum of the rP172/CaP coating showed the composition of phosphate mineral (1057 cm⁻¹ ν₃ PO₄ stretch and 550–610 cm⁻¹ ν₄ PO₄ bend) and proteins (1653 cm⁻¹ Amide I, 1553 cm⁻¹ Amide II). The mineral phase in rP172/CaP composite was close to octacalcium phosphate, OCP (601 cm⁻¹ and 558 cm⁻¹). The broader peak at 558 cm⁻¹ indicated the presence of the amorphous phase. The spectrum of rP172/CaP coating gave the typical fluorescence broad background and overlaid were significant bands of Amide I (1667 cm⁻¹), Amide III (1245 cm⁻¹), alkyl vibration and stretch (2930 and 1447 cm⁻¹), and proline backbone (1336 cm⁻¹) attributed to amelogenin, and 953 cm⁻¹ (ν₁ PO₄ stretch), 1010 cm⁻¹ (ν₁ HPO₄ stretch), and 1043 cm⁻¹ (ν₃ PO₄ stretch) bands attributed to OCP [34]. Although the nature of the mineral phase was revealed to be mostly OCP, the presence of amorphous calcium phosphate (ACP) in the coating cannot be ruled out.

Significant effects of rP172 have been observed under scanning electron microscopy (SEM) on the surface topography (Fig. 3c) and crystal morphology (Fig. 3d) of the coating when

compared to those of the control in the absence of protein (Fig. 3a, 3b). The control surface appeared more porous with numerous cracks, and flaky crystals could be seen on the surface. However, the surface of rP172/CaP appeared to be much denser with fewer cracks.

Rod-like crystalline bundles with 30 nm in diameter and 100–200 nm in length were observed on the surface of the rP172/CaP under SEM observation (Fig. 3c, Inset). The rods transversing the crack aligned to be parallel to the substrate surface (arrows in Fig. 3d). In a short range (500×500nm), the rods had organized oriented structures and some were parallel to each other (Fig. 3c, Inset). The formation of similar organized apatite crystal bundles nucleated on the surface of titanium and bioglass in the presence of full-length amelogenin has also been previously described [17,18]. Transmission electron microscope (TEM) imaging of scraped coating revealed bundles of dense crystals; and the selected area (4 mm) electron diffraction pattern of these bundles indicated the d-spacing of 0.344 and 0.278 nm (Fig. 4), attributing to apatite or octacalcium phosphate (OCP) [35]. Based on these observations, we consider the rodlike crystal bundles of about 100–200 nm long in the rP172/CaP composite coating (Fig. 3c, Inset) to be apatite or octacalcium phosphate crystals formed following the collinear alignment of amelogenin nanospheres [27]. We therefore propose that the potential of amelogenin to control crystal morphology and surface topography is attributed to its strong tendency to self-assemble into nano-chain structures [27].

The coating of calcium phosphate with amelogenin lacking the hydrophilic C-terminus (rP148) showed globular structures, which is different from that of rP172 regarding the surface topography and crystal morphology (Fig. 3e, 3f). Calcium phosphate nanocrystalites of about 18 nm aggregated into globules as large as 200 nm also in diameter; and the globules attached each other irregularly to form a porous structure (Fig. 3f). The difference of the crystal morphology between rP172/CaP and rP148/CaP is postulated to be the result of different self-assembly behaviors of amelogenins, which was induced by the increase of solution pH around the cathode.

DLS Analysis of pH induced self-assembly and mineralization

During the ELD, with the co-precipitation of rP172 and calcium phosphate, the local pH increase near the cathode (1mm distance) was from 4.8 to 8 and that in the solution was 4.8 to 5.7. Therefore, the pH induced self-assembly of rP172 in phosphate solution was analyzed by dynamic light scattering (DLS) under the similar pH change of ELD (Fig. 5a). The plots summarize variation in particle size distribution (average hydrodynamic radii) of amelogenin (rP172), calcium phosphate solution, and the mixture of the two (rP172/CaP) as a function of solution pH. The hydrodynamic radius of rP172 was 3.8 nm (dimers, trimers, or tetramers) at pH 3–5, where the low polydispersity values (error bar in the curve) indicated homogenous size distribution of protein assemblies [24]. Large particles with radii of 200 nm and more were observed at pH 5.5 or higher in rP172 solution and at pH 6.5 in calcium phosphate. In the solution containing both calcium phosphate and rP172, the relative homogeneous particles with radii of about 300 nm at pH 6 and higher were considered as consequence of co-precipitation of larger particles formed by calcium phosphate and rP172.

The unique rP172 nanospheres and chain structures could be observed under TEM after 10 min ELD of amelogenin rP172 onto a copper grid at an initial pH of 4.8 and 25 mM sodium phosphate solution, even without negative staining (Fig. 5b). The contamination of copper containing deposits was ruled out by EDS and selected area electron diffraction (data not shown). The electron density that appears in the nanospheres is due to the compact structure of assembled amelogenin molecules. The nanospheres of rP172 have a uniform diameter of 56 ± 8 nm. These chain structures are similar to supramolecular chains by self-assembly of amelogenin nanospheres promoted in the presence of polyethylene glycol (PEG) [27] or calcium ions [19]. Chain-like structures were also seen when calcium ions were added to the

ELD system. The spherical calcium phosphate containing precipitates however had a larger size of 100–130 nm (Fig. 5b). The notion of protein-mineral aggregate formation during enamel development was originally reports by Robinson *et. al* [36]. The concept of cooperative mechanism of protein self-assembly and apatite mineralization has been also recently proposed [14,19,21]. The selected area (4 mm) electron diffraction pattern of the co-precipitate composite at the stage prior to its deposition on the cathode did not show any crystalline form indicating the presence of amorphous calcium phosphate. The absence and presence of calcium and phosphorus in the Fig. 4 samples were confirmed by electron dispersive spectroscopy (EDS) under SEM (data no shown).

In order to examine the notion that amelogenin supramolecular self-assembly was the basis for the structure of the protein-mineral coating, the rP172/CaP coating on silicon was demineralized in EDTA and the remains were a continuous mesh-like structure of the protein as documented by SEM and EDS analysis (Fig. 6). Spherical structures of about 50 nm in diameter are seen in the SEM image (arrows in Fig. 6). However, removal of the protein by sodium hypochlorite resulted in the complete loss of the coating structure. These observations indicated that the framework for the composite coating of rP172/CaP in Fig. 3 is self-assembled rP172.

No collinear chains of nanospheres were found in ELD precipitation of rP148 in sodium phosphate buffer (Fig. 5d). Instead, large aggregates and irregular spheres from 10 nm to 500 nm in diameter were observed. Recent DLS analysis of rP148 has confirmed the formation of large aggregates at pH=7.4 ~ 8 buffer solution [30,6]. These differences of self-assembly properties in ELD between rP172 and rP148 clearly demonstrate that the supramolecular assembly of amelogenin into nano-chains is the driving force for the formation of organized nano-rod composite structures.

Characterization of nanomechanical properties

Ribbons of carbonate-containing fluoridated hydroxyapatite crystals are the basic building units of natural enamel. Due to the unique hierarchical crystal organization, enamel has excellent properties of elastic modulus, hardness, and fracture toughness when compared to pure apatite mineral and other calcium phosphate composites. Nanoindentation was applied to compare the mechanical properties of the ELD composite coatings (rP172/CaP) with human enamel. For this purpose thicker coatings (about 500 nm) were prepared. SEM observation of the thicker rP172/CaP coating indicated the rod structures were covered by 5–10 nm dense particles (Fig. 7). The top layer particles showed the typical morphology of amorphous calcium phosphate phase, which was considered to form after the depletion of amelogenin concentration in the electrolyte solution in the later stage.

The differences in nanomechanical properties of the composite coatings was compared with other standard substrates as seen in the elastic modulus - displacement curves in Fig. 8a and the calculated elastic modulus and hardness in Table 1. The rP172/CaP and rP148/CaP coatings were measured as about 400–600 nm in thickness. When the nanoindenter tip penetrated through the coating thickness and indented into Si substrate, the modulus increased and showed a short plateau or a step at the displacement of 200–400 nm, and then increased to the modulus of Si. The moduli of the coatings were calculated at that plateau or step of the curve.

SEM images of the indents on different samples in Fig. 8 (b–d), demonstrate the different indent geometry after interaction with the indenter probe. As a control, human enamel at the occlusal surface was tested. The enamel elastic modulus ($E=87$ GPa) and hardness ($H=4.6$ GPa) obtained in the present test matched the data previously reported by Habelitz *et al.* [37], $E=88$ GPa, $H=4.3$ GPa. The fracture toughness of enamel was calculated as $0.33 \text{ MPa} \cdot \text{m}^{1/2}$, which is lower than the previous reported $0.6\text{--}0.9 \text{ MPa} \cdot \text{m}^{1/2}$ on enamel [38]. From the SEM image

of the indent on rP148/CaP in Fig. 8c, the coating appeared weak, brittle, and was delaminated from the substrate following indentation. Therefore reliable measurements of modulus and fracture toughness were not obtained. As compared from the curves in Fig. 8a, rP172/CaP coating demonstrated higher elastic modulus than rP148/CaP coating. When the indenter indented through this coating, the cracks formed from the indenter corner and propagated to a limited distance as shown in Fig. 8b. The coating was deformed and piled up a 350 nm height hump at each edge of the triangle indent which indicating sufficient elasticity of the coating. The wrinkle like feature on the hump as pointed by arrow in Fig. 7b also indicated that the coating was made up of nano-sized crystal grains, which were cohered to each other by self-assembled amelogenin. This hierarchical structure made the coating much tougher [39]. The estimated fracture toughness on rP172/CaP ($0.47 \pm 0.09 \text{ MPa} \cdot \text{m}^{1/2}$) is comparable to that achieved on the enamel surface ($0.33 \pm 0.03 \text{ MPa} \cdot \text{m}^{1/2}$) in the same testing condition. A separate testing using cubic corner tip, expecting to provide more accurate fracture toughness data (not shown here) showed that 0.2g load was sufficient to produce cracks on mature human enamel surface, while higher load (1.5g) on rP172/CaP composite coating was necessary to cause cracks. Because the synthesized rP172/CaP coating contains more protein than mature enamel, its fracture toughness is expected to be higher than that of mature enamel. When comparing the elastic modulus and hardness in Table 1, the rP172/CaP coating is much stronger and harder than rP148/CaP coating, which is attributed to the interlocking effects of rod-like structures in the rP172/CaP coating. Due to the loose structure of rP148/CaP coating an estimate of its fracture toughness was not possible neither any cracks were formed following indentation.

Conclusions

Based on growing evidence for effectiveness of amelogenin protein in controlling calcium phosphate crystal growth [15,16,19,21,34], we applied ELD as a novel method to synthesize an enamel bio-inspired composite material in the presence of amelogenin. ELD allows preparation of composite coatings by simultaneous co-precipitation of self-assembled amelogenin and calcium phosphate mineral, at physiological conditions. By comparing the effect of recombinant full-length amelogenin (rP172) and a truncated mutant amelogenin (rP148) lacking the hydrophilic C-terminal we present data to demonstrate that the supramolecular self-assembly of amelogenin nanospheres into nanochains is the driving force for the formation of parallel bundles of apatite. Truncated rP148 without the hydrophilic C-terminal did not form ordered elongated structures (nanochains) and therefore such bundles were not obtained. The ordered nano-rod structures of the rP172/CaP composite and its improved nanomechanical properties, gave a good indication that a synthesized composite of amelogenin and calcium phosphate is a promising biomaterial.

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Fig 1.

High performance liquid chromatography (HPLC) spectra of a) electrolyte solution with rP172 before ELD, b) electrolyte solution with rP172 after ELD, c) dissolved ELD composite coating in 0.2% trifluoroacetate acid. The results indicated that the amelogenin is stable before and after electrodeposition. The elution time (45.5 min) of the protein from the dissolved coating and the electrolyte are similar, which showed the coating contained the original rP172 amelogenin molecules. The smaller peak at 43–44 min is the aggregated amelogenin.

Fig 2.

a) ATR-FTIR (Attenuate total reflection- Fourier Transformed Infrared) spectrum from the coating of CaP, rP172/CaP, and standard hydroxyapatite (HAP std, standard reference materials, hydroxyapatite #2910, obtained from NIST). b) Micro FT-Raman spectrum on the composite coating and the control ELD coating without amelogenin.

Fig 3.

Characterization of the ELD coating by scanning electron microscopy. a & b) the calcium phosphate coating without amelogenin. There are numerous cracks and no significant ordered structures are observed. c & d) coating in the presence of full-length amelogenin, rP172/CaP. Inset in (c) is the 2 times higher magnification than (d). Nano-rod structures of about 30 nm width are apparent. Arrows in (c) and (d) indicate the parallel bundles. e & f) coating in the presence of the C-terminus truncated amelogenin, rP148/CaP. The structure is characterized by mainly globular aggregates of crystallites.

Fig 4. Characterization of the composite scraped ELD coating by transmission electron microscopy. a) Bright field image and b) selected area electron diffraction pattern of the rP172/CaP coating shows the dense bundles of calcium phosphates. The dashed ring in b) indicates the size of select area aperture in the electron diffraction, about 4 μm in diameter.

Fig 5. Analysis of amelogenin supramolecular self-assembly in the absence and presence of calcium phosphate. a) Particle size distribution of amelogenin assemblies, calcium phosphate precipitates, and the mixture of the two as the function of pH following the condition of ELD (but not during ELD), as analyzed by dynamic light scattering (DLS). Data presented are the majority of the mass in the solution and polydispersity is shown as an error bar. b–d) TEM images of the precipitation obtained on the cathode 10 minutes after the initiation of ELD: b) the assembled amelogenin rP172 in 25 mM sodium phosphate buffer, c) rP172 in the presence of 6.0 mM calcium, 3.6 mM phosphate, d) rP148 in the 25 mM sodium phosphate buffer.

Fig 6. SEM images of the EDTA demineralized 172-CaP composite coating. No Calcium and Phosphorus was detected by electron dispersive spectroscopy (EDS) after demineralization by 7% EDTA and 2% glutaraldehyde for 30 mins. It can be seen that rP172 in the composite coating is a continuous mesh-like structure and nano-sized spherical aggregates of about 50 nm in diameter as indicated by arrowhead. Note the alignment of three nanospheres indicated by the arrows.

Fig 7.
SEM image of rP172/CaP coating showing a crack formed in the 500 nm thick coating. The parallel nano-rods were observed inside the cracks.

Fig 8.

a) Elastic modulus of amelogenin composite coating compared to enamel, silicon, and silica surfaces during the nanoindentation. The measured values for silicon and silica modulus matched the theoretical numbers. b–d) SEM images of the indent patterns on the surface of b) rP172/CaP coating, c) rP148/CaP coating and, d) human enamel. Arrow in b) indicates a hump wrinkle produced by nanoindentation.

Table 1

Mechanical properties of synthesized composite coating and reference materials by nanoindentation

	Elastic Modulus GPa	Hardness GPa
Human Enamel [*]	86.9 ± 7.3	4.55±0.41
rP172/CaP coating on Si [*]	55.6 ±13	1.8 ±0.6
rP148/CaP coating on Si [*]	16.0 ± 3.7	0.53 ± 0.51
Amorphous CaP on Si [40]	68.5	0.40
Crystalline CaP on Si [40]	127	2.3
Silicon [*]	174.2 ± 3.9	12.9 ± 0.8
Fused silica [*]	70.6 ± 4.6	9.1 ± 0.7

^{*} Data were achieved by the average of 16 indent measurements and expressed as average ± standard deviation.