An agarose hydrogel biomimetic mineralisation model for the regeneration of enamel prism-like tissue

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ABSTRACT: Laboratory studies have demonstrated that enamel-like mineralised tissue can be regenerated and used to repair enamel loss. This has implications for the management of non-carious tooth loss due to dental erosion, attrition and abrasion. In this study, we designed a hydrogel biomimetic mineralisation model for the regeneration of enamel-like mineralised tissue with a prismatic structure. The mineralised tissue, which was generated by the model on an etched enamel surface in the presence of 500 ppm fluoride, was analysed with a scanning electron microscope, X-ray diffraction, Fourier transform infrared spectroscopy and the nano-indentation hardness test. The generated tissue had enamel prism-like layers containing well-defined hexagonal hydroxyapatite crystals. The modulus of elasticity and nano-hardness of the regenerated enamel prism-like tissue were similar to those of natural enamel. Thus, the regeneration of enamel using this hydrogel biomimetic mineralisation model is a promising approach for the management of enamel loss.

1. INTRODUCTION

Dental enamel is a highly mineralised tissue made up of approximately 95% substituted hydroxyapatite, 4% water and 1% organic macromolecules.(1) It consists of nanorod-like hydroxyapatite (HA) crystals arranged into well-organised micro-architectural units called enamel prisms.(2) Enamel prisms are an important factor in the remarkable mechanical properties of dental enamel, which protects teeth from fractures and acid attacks.(3) During enamel formation, ameloblasts secrete amelogenin. Amelogenin is approximately 90% organic matrix material. It spontaneously self-assembles into nano-spheres that promote the formation and growth of crystallites, which form a well-organised prism pattern.(4) The hydroxyapatite crystals grow rapidly as the enamel maturates and the amelogenin degrades into small fractions, resulting in the formation of a highly mineralised tissue with a well-organised micro-architecture. The ameloblasts undergo apoptosis after the enamel is formed. Enamel is a non-living mineralised tissue that is susceptible to demineralisation by bacterial and chemical acids in unfavourable environments. It is also subject to mechanical damage through attrition or abrasion.(5)

Previous studies have proposed various methods for regenerating the enamel microstructure and repairing enamel defects based on cell-free strategies, including a hydrothermal method using the controlled release of calcium from Ca-EDTA,(3) hydrothermal transformation of octacalcium phosphate rod to HA nano-rods in the presence of gelatine,(6) surfactant supported HA self-assembly,(7) hydrogen peroxide containing calcium phosphate paste(8) and an electrolytic deposition system at 85°C.(9) However, All of these methods are performed under conditions of high temperature, high pressure or extremely low acidity. Recently, amelogenin was used to control enamel remineralisation to form enamel-like HA nanorods under physiological conditions.(10,11) However, amelogenin has difficulty in the expression and purification, and also very expensive, which limits its clinical application.

The initial formation of enamel apatite in nature occurs when a unique gel-like organic matrix interacts with metabolic and intricate cell activities. The enamel at the secretory or matrix formation stage has a gel-like consistency.(12) In that gel-like micro-environment, the mode of crystal growth is different than in aqueous solutions. Busch and his co-workers developed a method to mimic the gel-like micro-environment.(13) They used Ca^{2+} and HPO_4^{2-} diffusion in gelatine to induce the formation of enamel-like fluorapatite on human enamel. The physio-chemical nature of this gel-like micro-environment more realistically mimics the unique mineralised tissue matrix environment than aqueous solutions.(14) However, gelatine is sol at physiological temperature which limits its application. Glycerine was added to increase the melting temperature of gelatine to 40°C, but this could be a concern on biosafety when applied in oral environment for a long time. Agarose is a natural polysaccharide with good biocompatibility. It has been widely used in biomedicine. The temperature of sol-gel translation of agarose is at about 60°C which can overcome the limitation of gelatine. In this study, we demonstrated that an enamel prisms-like structure can be regenerated by an agarose hydrogel biomimetic mineralisation model under physiological conditions using agarose, without using cell and/or enamel protein.

2. MATERIALS AND METHODS

2.1. Tooth slices preparation. This study was approved by The University of Hong Kong/Hospital Authority Hong Kong

West Cluster Institutional Review Board (IRB UW10-210). Extracted sound human third molars were collected with patients' consent. The teeth were disinfected with 3% sodium hypochlorite and rinsed with phosphate-buffered saline. Tooth slices of 2 mm thickness were prepared perpendicular to the longitudinal axis of each tooth using a low speed diamond saw (IsoMet Low Speed Saw, Buehler, Lake Bluff, Illinois, USA). The slices were polished with silicon carbide papers and then ultrasonically cleaned with deionised water. They were stored in a polyethylene tube at 4ºC.

2.2. Hydrogels and phosphate solution preparation. Calcium chloride ($CaCl_2$) hydrogel was prepared by mixing 0.5g agarose powder (BIOWEST Regular Agarose G-10, Gene Company, Origin, Spain), 1.9g $CaCl_2 \cdot 2H_2O$ and 100ml deionised water. An ion-free hydrogel was prepared by dissolving 0.5g agarose powder in 100ml deionised water. Their pH values were adjusted to 6.5 using 0.1 M NaOH and 0.1 M HCl. The mixtures were allowed to swell at 25ºC for 30 min before being heated to 100ºC. The two hydrogels were kept at 60ºC after complete dissolution before use. The 0.26M phosphate solution (pH adjusted to 6.5) was prepared by dissolving Na_2HPO_4 in deionised water. Sodium fluoride was added to the phosphate solution to obtain a final concentration of fluoride 500 ppm.

2.3. Enamel regeneration in hydrogel biomimetic mineralisation model. Tooth slices were etched with 37% phosphoric acid for 1 min, rinsed with deionised water and put into polyethylene tubes. The slices were first covered with a 2-mm-thick layer of $CaCl_2$ hydrogel and then covered with a 2-mm-thick layer of ion-free hydrogel. After gelification, the polyethylene tubes were filled with 10 mL of phosphate solution. A 4-layer (enamel slice, $CaCl_2$ hydrogel, ion-free hydrogel and phosphate solution) hydrogel biomimetic mineralisation model was then constructed (Figure 1). The polyethylene tubes were sealed and incubated at 37ºC. The phosphate solution was replaced every 24 hours and the hydrogels were replaced every 48 hours. Before replacing the hydrogels, the tooth slices were cleaned ultrasonically with deionised water for 20 sec. They were taken out for examination after incubation for 2, 4 and 6 days.

2.4. Assessment of regenerated enamel and hydrogels. The morphology of the precipitates were characterised by field emission scanning electron microscope (SEM) and atomic force microscope (AFM). For SEM, the slices were sputter-coated with gold for observation (S4800, Hitachi High Technologies America, Inc., Dallas, USA). AFM analysed using a Tapping Model Etched Silicon Probe (Dimension Edge, Bruker, California, USA). The phase composition, structure and orientation of the regenerated tissue were confirmed by X-Ray Diffraction (XRD) (X'Pert PRO, Philips, Almelo, Netherlands). Fourier transform infrared (FTIR) spectra of the regenerated tissue were collected by means of a Multiscope FTIR spectrometer (Nicolet 8700, Thermo Scientific Instrument Co. Friars Drive Hudson, NH, USA). The replaced agarose hydrogels were dehydrated with ethanol and then dried in a critical evaporator for SEM and transmission electron microscopy (TEM) evaluation (Tecnai G2 20, FEI Co., Eindhoven, Netherlands). Selected area electron diffraction (SAED) was used to identify the minerals structures in the hydrogel.

2.5. Evaluation of mechanical properties. The surface of the tooth slices was divided into three sections. The first section was covered with acid-resistant and hydrophobic nail vanish (Revlon, New York, USA) (untreated enamel). The nail vanish was used to protect the enamel surface from treatment such as acid etching and subsequent remineralisation, so that they can be used as control for comparison. The second section was covered with nail vanish after being etched with 37% phosphoric acid for 1 min (etched enamel). The third section was etched with 37% phosphoric acid for 1 min (this was the area where regenerated mineralised tissue formed after biomimetic mineralisation). Three tooth slices were incubated in the hydrogel biomimetic mineralisation model for 6 days. They were then immersed in acetone to remove the nail vanish to expose the three areas of untreated enamel, acid-etched enamel, and regeneration enamel). Subsequently, they were cleaned ultrasonically with deionised water for 20 sec and stored in deionised water at 23ºC. The mechanical properties, namely the elastic modulus and nano-hardness, of the regenerated mineralised tissue on the third section were compared to the properties of the untreated enamel and etched enamel on the same tooth slices surface. A nano-indentation test using the Berkovich tip was used to analyse the elastic modulus and nano-hardness of each section (G200, Agilent Technologies, CA, USA). The tip was calibrated with a fused-silica sample prior to evaluation.

The nano-indentation test consisted of three segments: the loading segment, the peak load holding segment and the unloading segment. The times for both loading and unloading were 15 sec. The holding time was 10 sec. Sixteen indentations were made on each section of three tooth slices. Thus a total of 144 indentations were perform on 9 sections from 3 tooth slices The maximum force applied during loading and unloading was 10 gf (0.098 N). The applied load forces and the depth of penetration into the samples during the indentation were continuously monitored by computer. The data were recorded and processed by Testworks 4 software (MTS Systems Corporation, Eden Prairie, MN, USA), which calculated the elastic modulus and nano-hardness and presented them as force-displacement curves. The differences in the elastic modulus and nano-hardness among the three sections of tooth slices were assessed with a two-way ANOVA. A 5% significance cut-off level was used for the statistical analysis. An optical microscope and SEM were used to examine the residual indent impressions.

3. RESULTS

3. 1. Assessment of regenerated enamel and hydrogels. Rod crystals were found on the enamel surfaces after 2 days of incubation in the hydrogel biomimetic mineralisation model (Figure 2a). The rod crystals extended out the enamel prism surface after 2 day incubation. The *c*-axial orientation of these crystals was perpendicular to the enamel prism (Figure 2b). The newly precipitated rod crystals on the surface were not densely packed and were relatively separated. The spaces between the rod crystals were filled with hydrogel matrix (Figure 2c). The examination of the slices in cross-section found that the rod crystals precipitated from the enamel surface (Figure 2d).

Notably, the rod crystals were not haphazardly distributed and their orientation was almost perpendicular to the underlying enamel. These rod crystals fused with those in the underlying enamel. The lengths along *c*-axis of the crystals were shorter than that of the following growth crystals at 6 days.

Well-defined and orderly distributed rod crystals with a typical apatite hexagonal structure were found after 4 days of incubation in the model (Figure 3a). The hexagonal rod crystals were approximately 150 nm in diameter and 2 μm in length. They were densely packed along the *c*-axis, pushing the rod crystals parallel to each other. Certain rod crystals self-assemble together to form the rudiment of enamel prism-like bundles (Figure 3b Oval). There was a negligible amount of hydrogel matrix left in the spaces between them (Figure 3b Arrow). The crystallographic *c*-axis of these rod crystals shared the same orientation and they were perpendicular to the underlying enamel surface (Figure 3c, d).

The regenerated crystals on the enamel surface formed a homogenous and dense layer of mineralised tissue after 6 days of incubation (Figure 4a). The strong attraction between adjacent rods caused them to fuse together to form an extensive layer of well-aligned crystals on the enamel surface (Figure 4b). These regenerated crystals had a tendency to spontaneously aggregate, side by side, to form bundles of enamel prism-like structures along the *c*-axis of the rods (Figure 4c, 4d). The bundled crystals had a width of approximately 1-2 μm and were very similar in appearance to the natural enamel surface. These bundled crystals consisted of agglomerative nano-crystals (Figure 4c). They assembled in a well-organised manner and were densely packed to form a homogeneous layer of mineralised tissue on the enamel surface (Figure 4d). Generally, the long axes of the crystals were radially perpendicular to the underlying enamel surface. The thickness of the enamel prism-like surface was approximately 3.5 μm after 6 days (Figure 4e). The interface between the regenerated tissue and the underlying enamel showed a tight agglomeration and fusion (Figure 4f). There was hardly any hydrogel matrix between the crystal bundles.

Figure 5 showed the surface variation in the spatial direction of crystals in 3D AFM images of acid-etched enamel and the regenerated enamel. The enamel prisms with parallel bundles of hydroxyapatite crystals were observed after the enamel slices acid-etched for 1 min (Figure 5a). The newly precipitated crystals stuck out from the enamel surface. They were relatively separated and not densely packed on the enamel surfaces after 2 days of incubation in the hydrogel biomimetic mineralisation model (Figure 5b). This made the enamel surface rough. The paralleled crystals densely packed along the *c*-axis and form the rudiment of enamel prism-like bundles after 4 days (Figure 5c). The bundles with the enamel prism-like structure were formed after 6 days (Figure 5d) which was similar to sound enamel (Figure 5a). AFM findings corresponded well to the SEM results.

In this hydrogel biomimetic mineralisation model, numerous polymer (agarose fibre)-mineral complex globules were found in the replaced $CaCl_2$ hydrogel adjacent to the enamel surface after 2 days (Figure 6a). These globules were formed by the coalescence of small nano-spheres (Figure 6b and 6c). The blurred ring patterns that formed instead of the sharp and clear arc-shaped patterns in the SAED of the globules suggested an amorphous structure or a very low crystallization (Figure 6d). Very few globules were found in the replaced ion-free hydrogel near the phosphate solution (Figure 6e and 6f).

The XRD patterns of the regenerated crystals after 2, 4 and 6 days of incubation are shown in Figure 7a. The diffraction peaks (002) at 2θ=25.8, (211) at 2θ=31.8, (112) at 2θ=32.2 and (300) at 2θ=32.8 corresponded well to the peaks for fluoridated hydroxyapatite (HA) (JCPDS No. 09-0432),(10) suggesting that the crystals were fluoridated HA.(15) The sharp and intense 002 peak indicated that the crystals were well crystallised and oriented along their *c*-axis; these results were consistent with the observations in the SEM images (Figure 5). After 6 days of incubation, the XRD pattern showed that the diffraction peaks around 2θ of 32° were split and clear, which implied good crystallinity of fluoridated HA. Moreover, the (300) diffraction peak was broad, indicating a lattice strain (microstrain) caused by the shift of the nano-crystals from their normal positions. In addition, the peak around 2θ of 44.6° was very sharp, perhaps due to the microstrain caused by the agglomerative individual nano-crystals.(16) These results were consistent with the SEM observation (Figure 4). The FTIR analysis also confirmed the formation of a typical apatite structure over time. The FTIR spectra shown in Figure 7b demonstrated the presence of phosphate groups on the etched enamel surfaces. The splitting PO_4 υ4 band was present in the region of 660 and 520 cm^{-1}. A well-defined and sharp band was observed in the HA. The split PO_4 υ3 band at about 1037 and 1117 cm^{-1} came from HA crystals.(17) There was no -OH group specific peaks at 1,571 cm^{-1}, indicating that -OH group might be replaced by F group to form fluoridated HA.(15) It should be noted that some information might be lost due to the reflection mode of FTIR, which is difficult to distinguish between FA and HA. It should be analysed together with other methods, such as XRD and morphological change of the crystals.

3. 2. Evaluation of mechanical properties. Figure 8 illustrates the typical loading-unloading curves (8a) and the calculated elastic modulus and nano-hardness (8b) of the untreated, regenerated and etched enamel. The mean (±SD) elastic modulus and mean nano-hardness of the enamel after acid etching were significantly reduced from 90.31±7.63 GPa and 4.28±0.53 GPa, respectively (untreated enamel) to 58.05±9.93 GPa and 1.03±0.31 GPa (etched enamel), respectively. After 6-day incubation, the mean elastic modulus and mean nano-hardness of the enamel were significantly increased to 89.46±11.82 GPa and 3.04±0.75 GPa, respectively (regenerated enamel). There were no significant differences in elastic modulus or nano-hardness between the regenerated and untreated enamel.

Figure 9a shows an optical microscope image of a regenerated enamel surface with 16 nano-indentations. The nano-indentation impressions form a triangular pyramid. No cracks were found around the indentations. The impression area on the surface of the regenerated enamel (Figure 9c) was similar to that on the untreated enamel (Figure 9b) and both were smaller than on the etched enamel (Figure 9d).

4. DISCUSSION

4. 1. An agarose hydrogel biomimetic mineralisation model. The transportation of ions through the organic matrix and the interactions between the ions and the organic matrix are crucial in the regulation of the enamel mineralisation process.(18) The process is mediated by enamel matrix proteins (mainly amelogenin and enamelin) and can be divided into several phases.(18) The proposed molecular mechanisms for the functions of enamel matrix proteins in enamel mineralisation include i) the prevention of the crystal fusion of pre-mature crystal,(19) ii) the control of crystal morphology and subsequent elongation(20) and iii) the control of the nucleation and growth of the crystals.(21)

At the secretion stage, the enamel organic matrix has a gel-like consistency, which results in enamel apatite formation taking place under a unique gel-like organic matrix environment rather than in aqueous solutions. The mode of crystal growth in a gel-like micro-environment is different than in aqueous solutions. During enamel formation, Ca^{2+} and PO_4^{3-} ions are transported from the layer of ameloblasts into the enamel matrix where the mineralisation takes place. In the process of enamel matrix secretion and calcification, ameloblasts withdraw from the mineralising area. There is a unidirectional supply of Ca^{2+} and PO_4^{3-} and this unidirectional ion supply is thought to play a key role in enamel crystal growth and orientation.(22)

In the present study, agarose hydrogel was used to mimic the gel-like organic matrix environment to induce enamel-like tissue regeneration. A unidirectional supply of Ca^{2+} and PO_4^{3-} were achieved in this model. We found no precipitates of calcium phosphate in the ion-free hydrogel (Figure 6e and 6f). This suggested that there was little diffusion of calcium ions from the $CaCl_2$ hydrogel to the ion-free hydrogel. On the contrary, the calcium ions accumulated in the $CaCl_2$ hydrogel layer. Meanwhile, the phosphate ions diffused from the PO_4^{3-} solution through the ion-free hydrogel into the $CaCl_2$ hydrogel and enamel surface. Furthermore, the mineralizing precursor of agarose fibre-mineral complex was formed. The organic matrix can prevent pre-mature crystal-crystal fusion, control the subsequent phase transformations, and control the nucleation and growth of the crystals. In our model, the hydrogel stabilised the mineral precursors and prevented them from transforming into crystal (Figure 6). The mineral precursors in the hydrogel were very small and less readily crystallised. This may have contributed to the strong attractive interaction between the agarose polymer and the inorganic surface, which can in turn arrest nucleation and change the shape and size of the primary clusters. The mechanism of the crystal growth will be discussed below.

Agarose is a natural polysaccharide and consists of a linear polymer with repeating units of D-galactose and 3, 6-anhydro L-galactose. It is low-cost and biocompatible, and has been widely used in biomedicine. Comparing to the glycerine-rich gelatine hydrogel model(13), the temperature of sol-gel transition of agarose is higher than the physiological temperature at which gelatine melts. The mechanical strength of the agarose is higher than gelatine, safer than the glycerine-rich gelatine hydrogel, and with no ethical concerns. The strength and consistency of the hydrogel can be adjusted by varying the concentration and molecular weight of the agarose. These make it easy to transfer to clinical use. On the other hand, the mechanism of regeneration of enamel-like tissue is also different between the gelatine model and our agarose model. In the gelatine model, the amino groups of gelatinous polypeptide can form salt-like bonds to phosphate groups on the apatite surface, and thus to induce the regeneration of enamel-like minerals.(13) The gelatine hydrogel entrapped phosphate groups in the hydrogel, which result in their method as enamel surface-phosphate ions hydrogel. However, in our model, calcium ions were entrapped in the hydrogel, which result in the method as enamel surface-calcium ions hydrogel. If we assembled the hydrogel as enamel surface-phosphate ions hydrogel, no enamel prism-like tissue could be regenerated in our study. Fan et al developed an agarose- amelogenin hydrogel model to regenerate the enamel-like tissue.(23) In their model, agarose was used as a releasing agent to investigate the function of amelogenin in the remineralization of early enamel caries. Although the agarose hydrogel containing calcium and phosphate without amelogenin was used as the control group, the different concentration of the inorganic ions and the different diffusion mode gave different results between our study and theirs. Moreover, in the absence of amelogenin, they did not show the regeneration of enamel-like structure. The aim and function of agarose use, and the results are different between our study and theirs. Ruan et al developed an amelogenin-containing chitosan hydrogel for enamel reconstruction through amelogenin supermolecular assembly, stabilizing Ca-P clusters and guiding their arrangement into linear chains.(11) These amelogenin Ca-P composite chains further fused with enamel crystals and eventually evolved into enamel-like crystals, anchored to the natural enamel substrate through a cluster growth process. Both Fan et al's and Ruan et al's model used amelogenin, but we used an agarose model with no amelogenin to regenerate enamel-like tissue. Our model is simple, low cost, biocompatible, and can be transferred straightforwardly to clinic use. The mechanism need further study. It may contribute to its agarose molecules, concentration of hydrogel, and concentration of calcium, phosphate, and fluoride.

4.2. The mechanism of growth of the enamel prism-like crystals. Classical crystallization pathway is thermodynamic process where ion-mediated crystallization proceeds via a one-step route to the final mineral phase.(24) Non-classical crystallization pathway is a kinetic process where crystallization proceeds by a sequential process involving structural and compositional modifications of amorphous precursors and crystalline intermediates. Crystallization often involves an initial amorphous phase (such as ACP) that may be nonstoichiometric, hydrated, and susceptible to rapid phase transformation.(25) In biology, a biomineralisation process is an organic matrix particle-mediated non-classical crystallisation pathway.(26) The organic matrix controls the mineral crystallites through the molecular interaction between the polymer and minerals with a sequestering mechanism.(27) The amorphous primary particles that are formed by ion or cluster binding at the organic surface can undergo coupled

matrix-mediated mesophase transformations resulting in oriented crystallisation with iso-oriented mosaic textures.(24)

Fluoride has an effect on crystal growth during enamel mineralisation.(10) In the pilot experiment of this study, well-defined rod-like crystals were formed when 500ppm fluoride was added into the phosphate solution. Ribbon-like crystals were observed when the fluoride concentration was reduced to 100ppm and only plate-like crystals were found on the enamel surface in the absence of fluoride. (Figure S1, S2 in the Supporting Information).

The agarose hydrogel in our model functioned as an organic matrix to control the agarose fibre-nano ACP complex precursors (Figure 6). There was strong attractive interaction between abundant hydroxyl OH groups of agarose molecules and Ca^{2+}. This interaction could arrest nucleation and also change the shape and size of the primary mineral clusters when phosphate ions diffusing into the $CaCl_2$ agarose hydrogel layer. The ACP nanoparticles coalesced and aggregated consisting of inorganic cores surrounded by an organic component (Figure 6b). In this way, stabilised nano-ACP or low crystallisation HA with anchored organic ligands was produced in the hydrogel matrix (Figure 6). This corroborated with the observation that there were little mineral complex formed in the layer of ion-free hydrogel (Figure 6e, 6f). Furthermore, the agarose hydrogel acted as a reservoir to replenish mineral precursors and as a dynamic interface to transport the mineral precursors to the enamel surface for the mineral mesocrystal transformation (Figure 10a). With the consistent diffusion of PO_4^{3-} and F^- into the hydrogel, the metastable ACP nanoparticles kinetically nucleated on the lattice of the enamel HA crystals. The matrix-mediated crystal growth processed by mesophase transformation and aggregation of preformed crystalline building blocks (a mesoscale assembly process, Figure 10b, 10c) resulting in oriented fluoridated HA crystallisation, and creating single crystals with iso-oriented mosaic textures (Figure 4d, 10d).

In this study, the regeneration of enamel prism-like tissue was monitored over 6 days of incubation. The mineral precursor nucleated on the enamel surface by heterogeneous nucleation after 2 days. It grew along its *c*-axis extension. The resultant crystals were perpendicular to the enamel prism surface. Because of the different orientations of the enamel prisms and the nuclei sites, the fluoridated HA oriented disorderly. However, the fluoridated HA crystals were perpendicular to the enamel prism surface where they nucleated. The fluoridated HA crystals were unevenly distributed on the enamel surface. The size of the initial grown crystals in *c*-axis was shorter than the following grown crystals after 4 and 6 days of incubation (Figure 3, 4). Moreover, the nuclei sites were relatively apart, and the hydrogel matrix involved in the nuclei sites. This could provide more space for fluoridated HA crystal growth. As a result, the diameter of the crystals after 2 days (Figure 2c) was larger than the crystals after 4 and 6 days (Figure 3, 4). This finding also corroborated that the involvement of agarose hydrogel in controlling the crystal growth. As more nuclei sites were formed with the progress of the crystal growth, and the amount of agarose hydrogel was gradually reduced. The agarose hydrogel 'draw back' from the mineralizing areas (Figure 3, 4). This phenomenon was similar to the natural enamel formation where enamel proteins degraded resulting in enamel crystal maturing to form prismatic structure. Therefore, the well-defined hexagonal crystals were evenly distributed on the enamel surface after 4 days (Figure 3). The crystals grew along the *c*-axis and densely packed. This mode of crystal growth forced the rod-like crystals to progressively align parallel to each other. The long, large hexagonal crystal probably acted as a primary crystal (seed crystal) enabling the short nanorods to fuse and align parallel to the primary crystal surface and formed crystal bundles. However, the nuclei sites were still relatively apart at 4 days, and most of the crystals were still immature intermediates during the crystal self-assembly or phase transformation. Thus, the morphology of these crystals was different from natural enamel crystal. However, certain rod crystals self-assemble together to form the rudiment of enamel prism-like bundles (Figure 3b Oval). With the crystal growth and assembly, enamel prism-like structure was observed after 6 days (Fig.4). The cross-sections of the enamel slices revealed formation of a layer of highly mineralised tissue, which comprised of parallel-oriented and densely packed homogeneous crystals. The mechanism of enamel prism-like tissue assembly is shown in Figure 10 which was based on the SEM observations in our study and referring to mechanism of the non-classical crystallization pathway.(24)

4.3. Mechanical properties of the regeneration tissue. The nano-indentation test was used to compare the mechanical properties of the regenerated enamel surface with the untreated and etched enamel surfaces. The nano-indentation test is a useful tool for studying mechanical properties at a nano-scale.(28) This depth-sensing technique allows the indentation of minute areas of a few square micrometres, so that the elastic modulus and hardness of small volumes of enamel can be measured.(29) The elastic modulus and nano-hardness obtained from this test should be reasonably acceptable, as no cracks were found around the indentations. It is important to note that the calculation of elastic modulus is based on the unloading curve. The nano-indentation method is therefore applicable to the study of linear, isotropic materials. Enamel consists of mainly apatite crystals and is an orthotropic material. Therefore, errors could arise if there is a "pile-up" or "sink-in" of the enamel surface on the edges of the indent during the indentation process.

The elastic modulus (90 GPa) and nano-hardness of the untreated enamel (4.3 GPa) were comparable to the results reported by Habelitz and his co-workers(30) (elastic modulus: 88 GPa, nano-hardness 4.3 GPa). To minimise the variations of elastic modulus and hardness caused by the different orientations of the enamel prism, the mechanical tests were performed on the untreated, regenerated and etched enamel from the same slices. The elastic modulus (89.5 ± 11.82 GPa) and nano-hardness (3.04 ± 0.75 GPa) of the regenerated tissue on the enamel surface after 6 days of incubation was comparable to those of the untreated enamel surface. The mechanical examination demonstrated that the enamel prism-like layer was characterised by similar mechanical properties as the untreated enamel, probably due to their similar microstructures. Similar results were also reported by Busch et al.(13)

Wei used fluorapatite cement pastes to fill enamel defects. However, the setting time is very long and at the initial setting stage it is easily washed away by the saliva. Furthermore the fluorapatite crystals in the cement are haphazardly arranged and different from the enamel structure.(15) Many researchers prefer using biomimetic mineralisation to regenerate prsim-like enamel tissue. This study demonstrated that enamel prism-like tissue can be regenerated in a hydrogel biomimetic mineralisation model. Compared to non-hydrogel solution studies, the physio-chemical nature of this enamel regeneration process vividly mimics the unique mineralised tissue matrix environment.(14) It is noteworthy that this model only mimics the gel-like environment in which the initial formation of enamel apatite occurs. Although it is a simplified model, it provides a basis for future research on enamel regeneration. Further studies should add an organic matrix, such as enamel proteins, to the hydrogel biomimetic mineralisation model to mimic the enamel matrix. On the other hand, this biomimetic mineralisation model can be transferred for future clinical applications such as treatment of erosive wear caused by acidic food and inappropriate brushing habit. The $CaCl_2$ agarose hydrogel, ion-free agarose hydrogel, and phosphate agarose hydrogel can be made commercially. They can be overlapped layer-by-layer to form a sandwich structure inside a tray which the patient will wear overnight. Furthermore, the saliva containing calcium and phosphate ions, and also F^--containing mouthwash can be used as the supplement of mineral ions for the mineralisation.

5. CONCLUSIONS

A hydrogel biomimetic mineralisation model to regenerate enamel prism-like tissue was designed. The hydrogel regulated the habit, size and mineral phase of the growing crystals through cooperative interactions with calcium, phosphate and fluoride ions. The regenerated apatite crystals were found to be highly oriented along the *c*-axis, with good crystallinity. The present study provides an important basis for future attempts to develop enamel prism-like material. Hopefully, such material could be used as an alternative treatment in clinical dentistry and other biomedical or industrial applications.

FIGURES

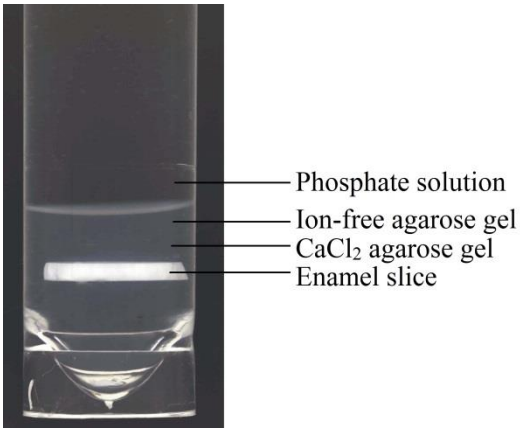

Figure 1. The four-layer hydrogel mineralisation model.

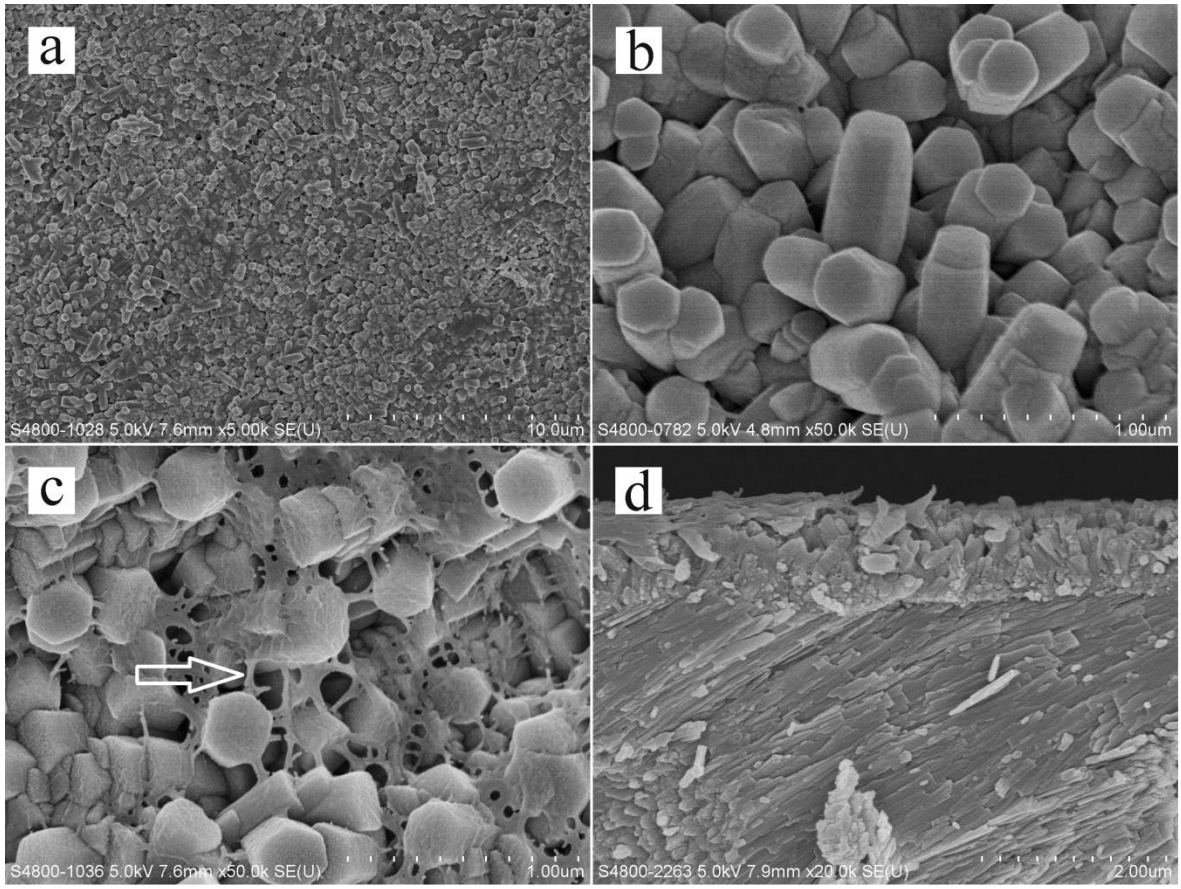

Figure 2. SEM micrographs of the regenerated mineralised tissue after 2 days. (a) Rod-like crystals regenerated on the enamel surface after 2 days. (b) Magnified micrograph of (a) to show that rod-like crystals grew along the *c*-axis. (c) Magnified micrograph of (a) to show the crystals and the hydrogel matrix (Arrow). (d) Cross-sectional view of the regenerated mineralised tissue to show the crystal orientation and prototype of the enamel prism-like structure.

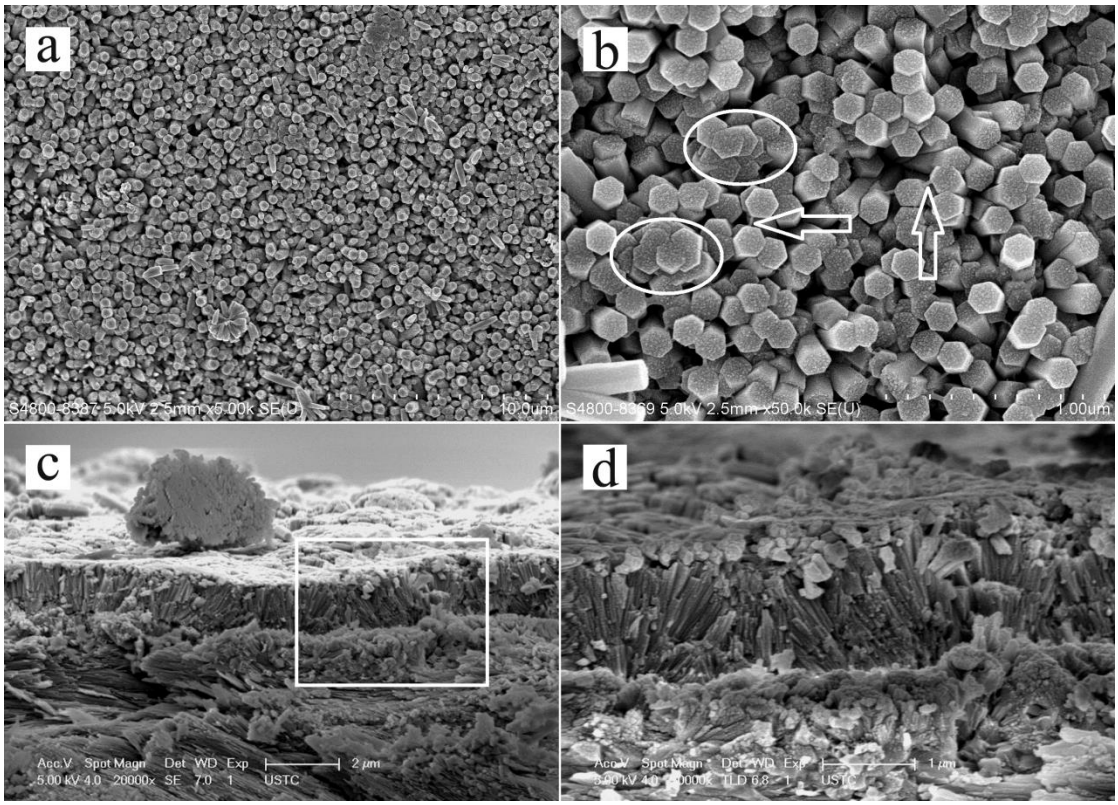

Figure 3. SEM micrographs of the regenerated mineralised tissue after 4 days. (a) Rod-like crystals with a typical apatite hexagonal structure regenerated on the enamel surface. (b) Magnified micrograph of (a) to show the paralleled crystals, the rudiment of the enamel prism-like bundles (Oval) and the hydrogel matrix (Arrow). (c) Cross-sectional view of (a). (d) Magnified micrograph of the rectangular area of (c).

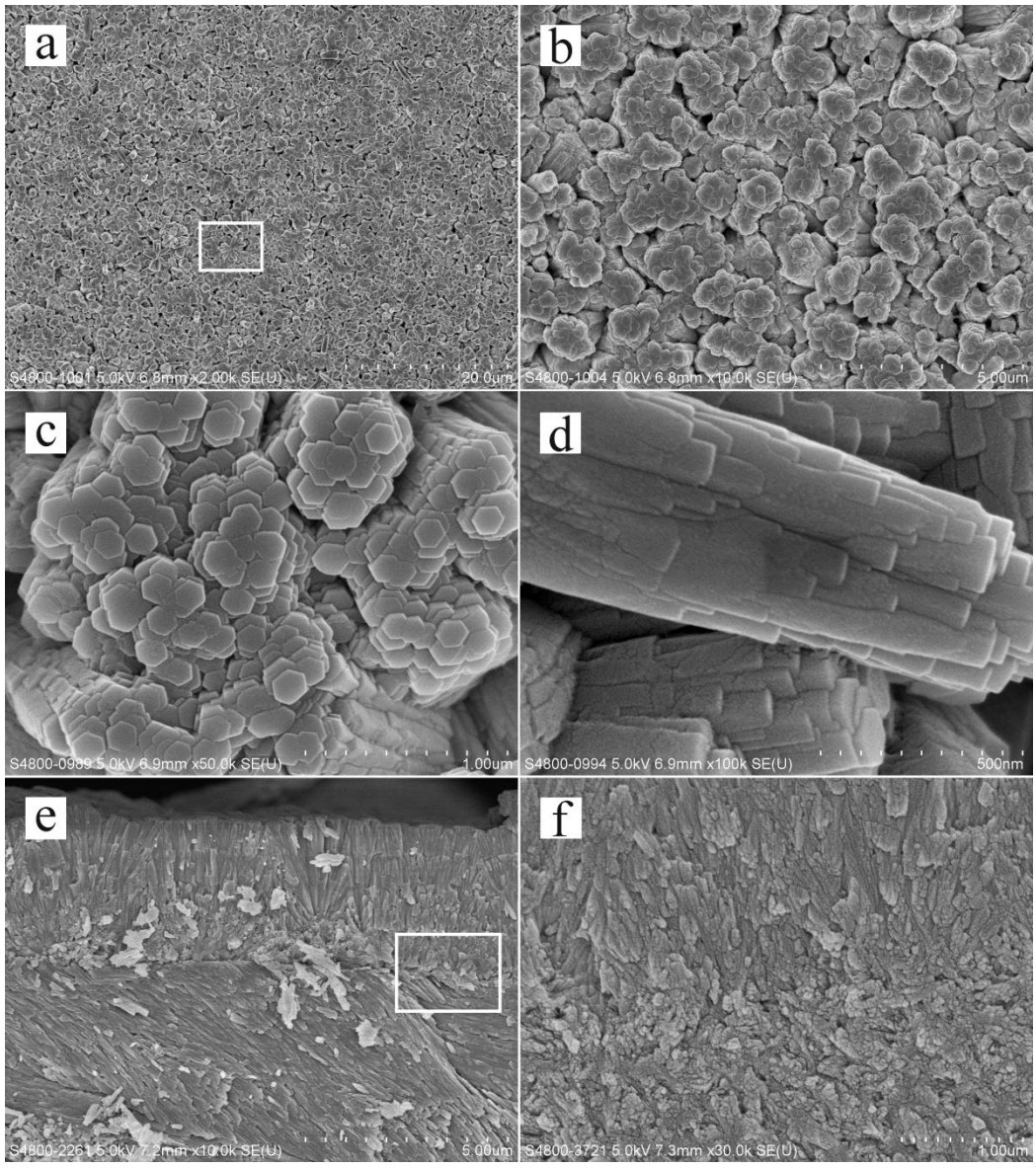

Figure 4. SEM micrographs of the regenerated mineralised tissue after 6 days. (a) Mineralised tissue with enamel prism-like crystals regenerated on the enamel. (b) Magnified micrograph of (a) to show the paralleled bundles. (c) Magnified micrograph of (a) to show the agglomerative hexagonal crystals. (d) Magnified micrograph of the rectangular area of (a) to show the side view of the crystal bundle. (e) Cross-sectional view of (a) to show that the regeneration layer was perpendicular to the underlying enamel. (f) Magnified micrograph of the rectangular area of (e) to show the interface between the regeneration tissue and the underlying enamel.

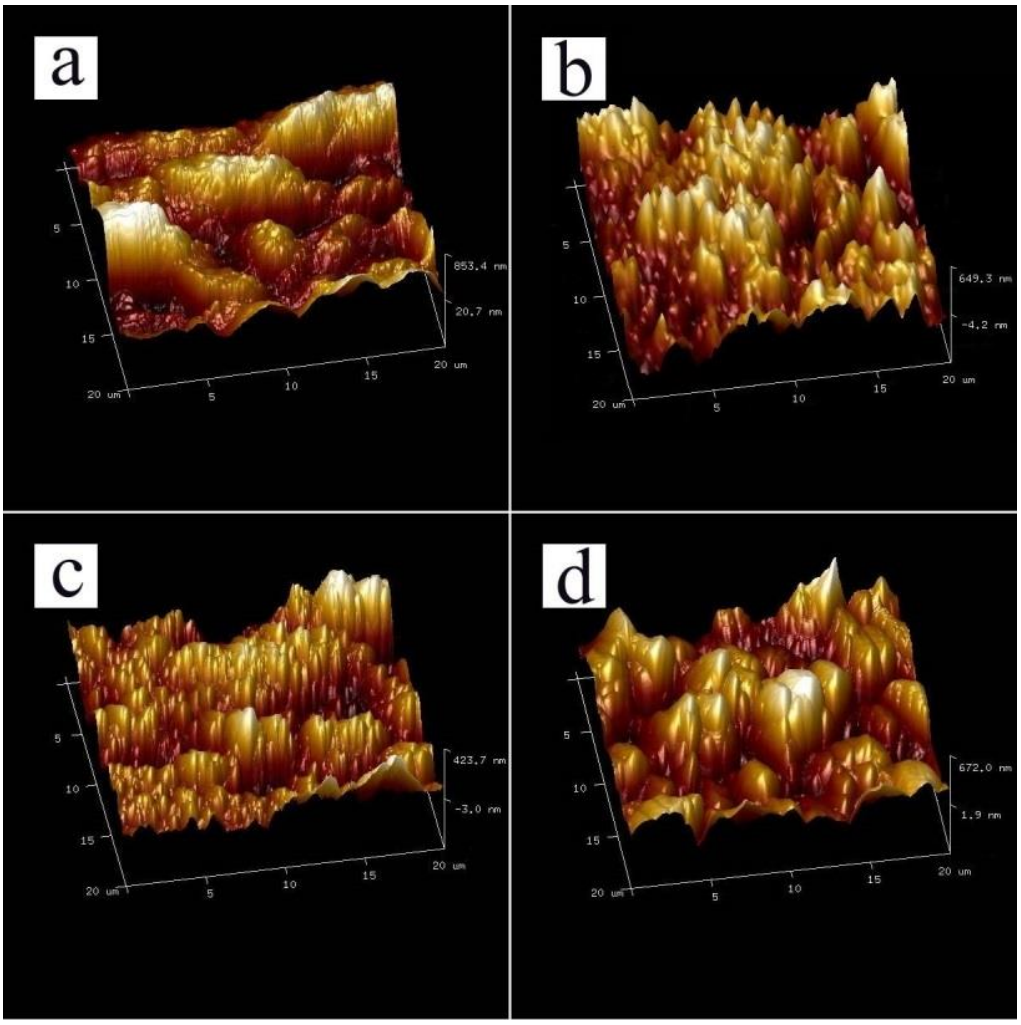

Figure 5. 3D AFM tapping-mode images of the etched enamel and regenerated mineralised tissue. (a) Etched enamel. (b) Regenerated mineralised tissue after 2 days. (c) Regenerated mineralised tissue after 4 days. (d) Regenerated mineralised tissue after 6 days.

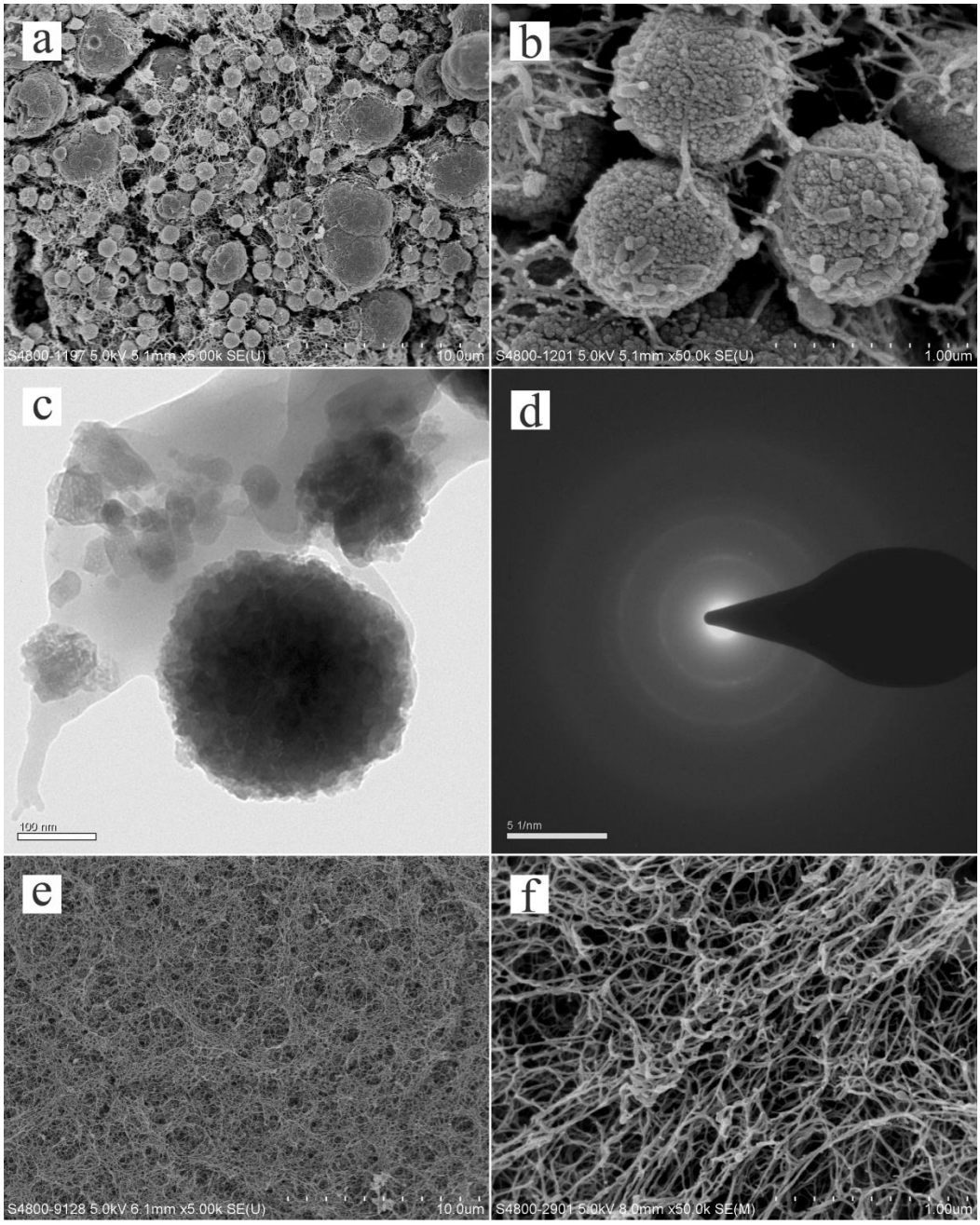

Figure 6. SEM and TEM micrographs of the replaced hydrogel after 2 days. (a) Polymer (agarose fiber)-mineral complex globules in the replaced $CaCl_2$ hydrogel. (b) Magnified micrograph of (a) to show the coalescence of nano-spheres. (c) TEM micrograph of the polymer (agarose fiber)-mineral complex globules. (d) SAED pattern of the mineral globule showing no evidence of reflective arcs. (e) A few globules in the replaced ion-free hydrogel. (f) Magnified micrograph of (e).

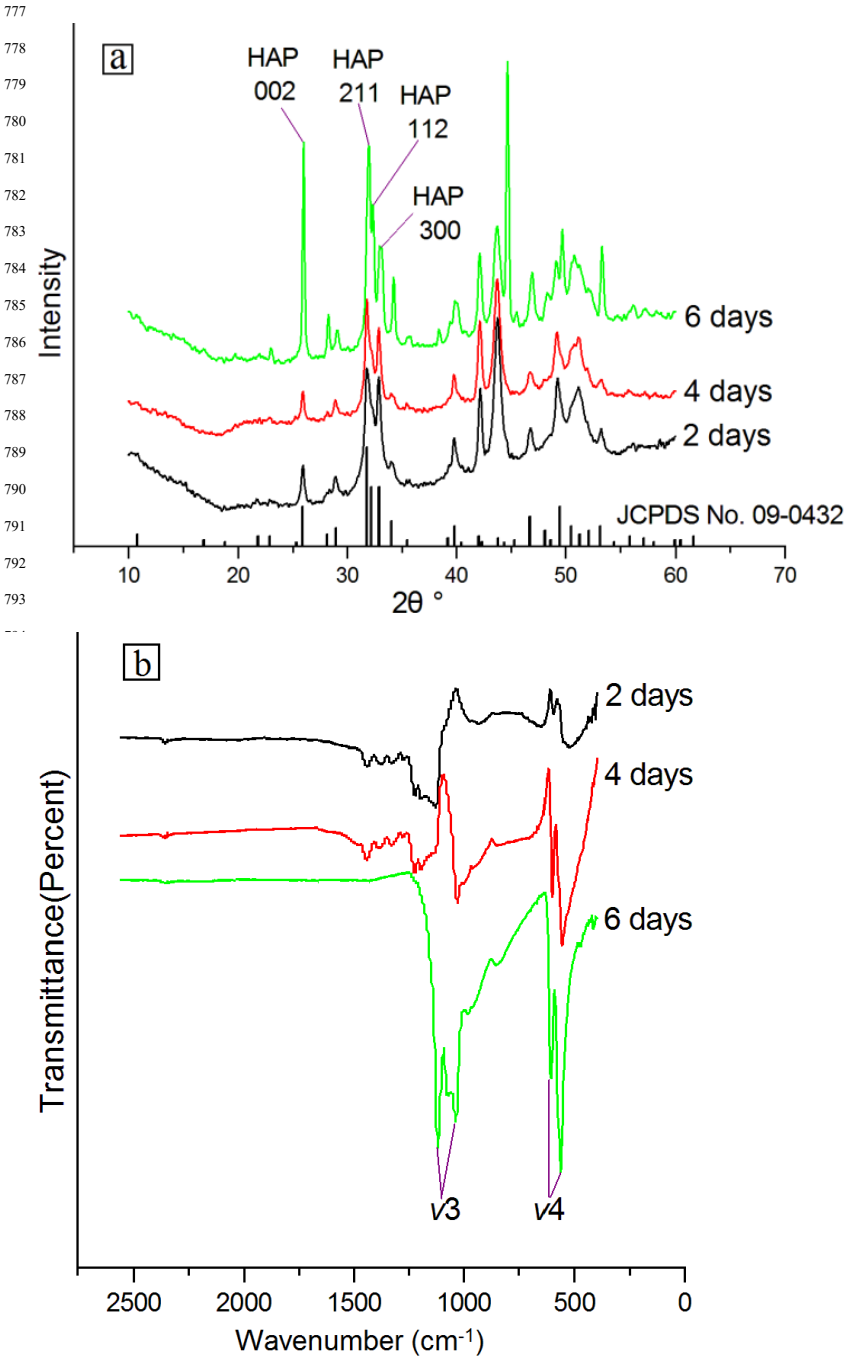

Figure 7. XRD (a) and FTIR (b) spectra of the regeneration layer on the enamel surface after 2, 4 and 6 days.

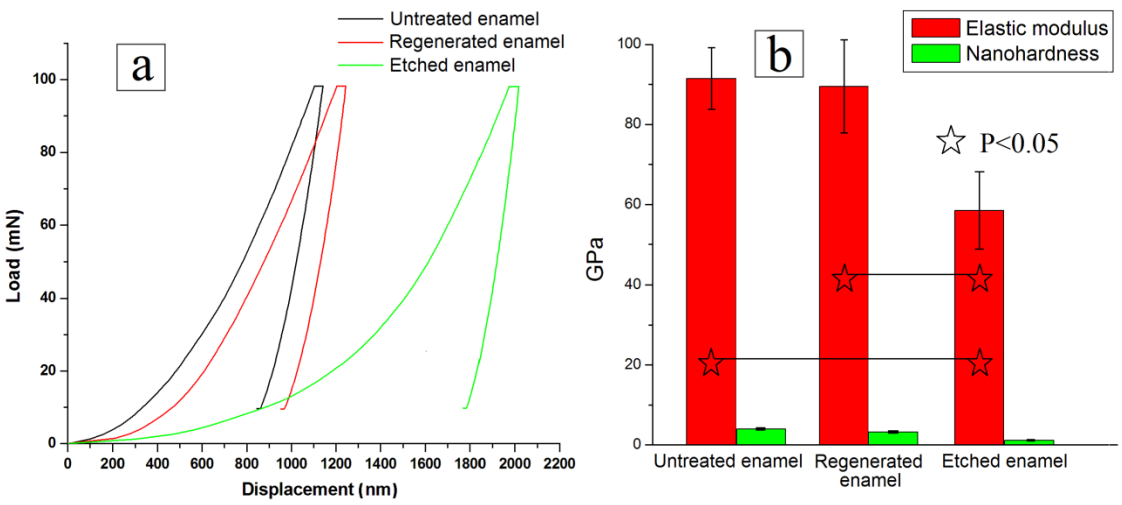

Figure 8. Typical Loading-unloading curves (a) and elastic modulus and nano-hardness (b) on the untreated, regenerated and acid-etched enamel after 6 days.

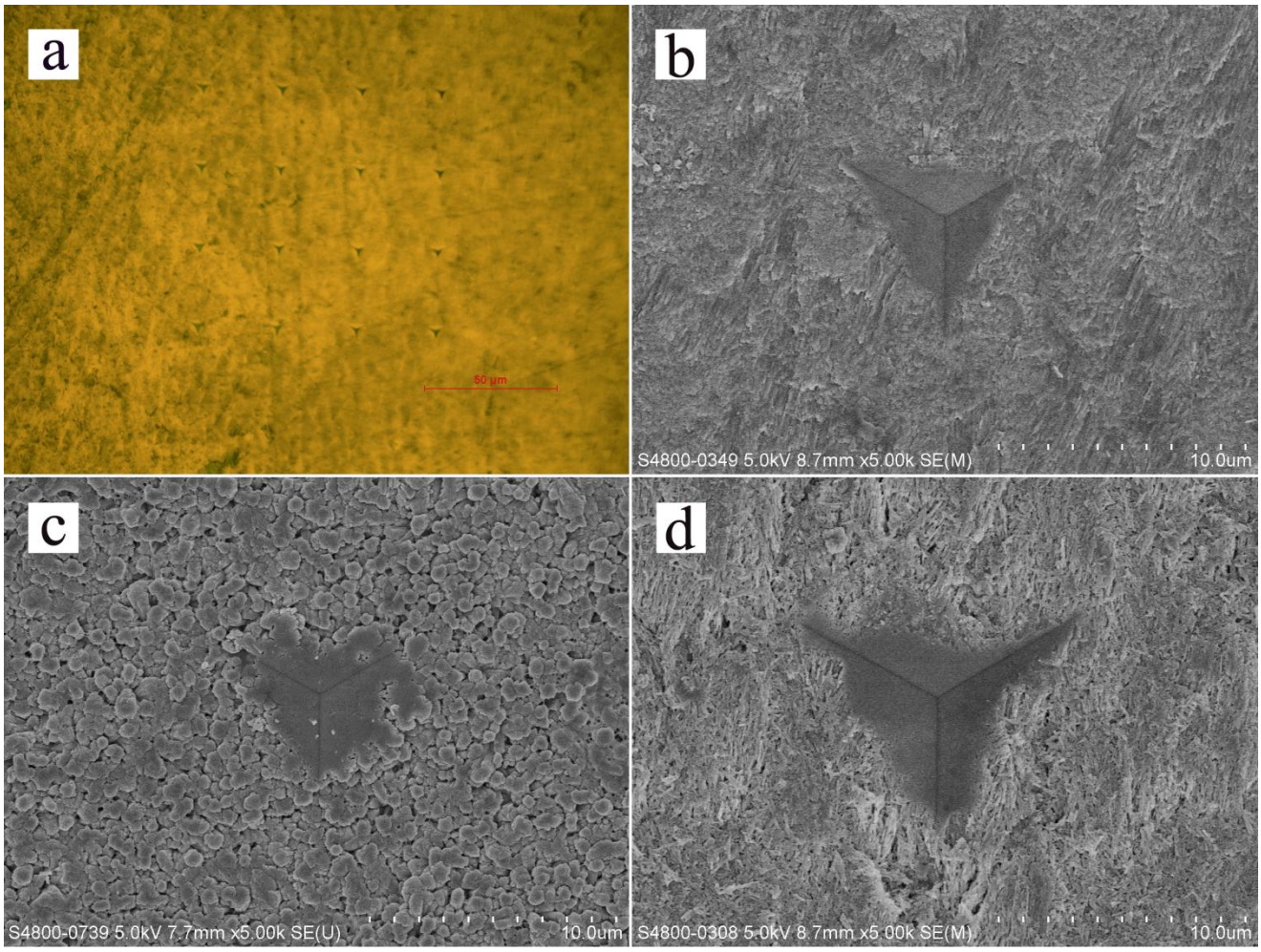

Figure 9. Optical microscope image of the regenerated enamel surface with nano-indentations (a). SEM images of the indentation impression on the surface of untreated enamel (b), regenerated enamel (c) and etched enamel (d).

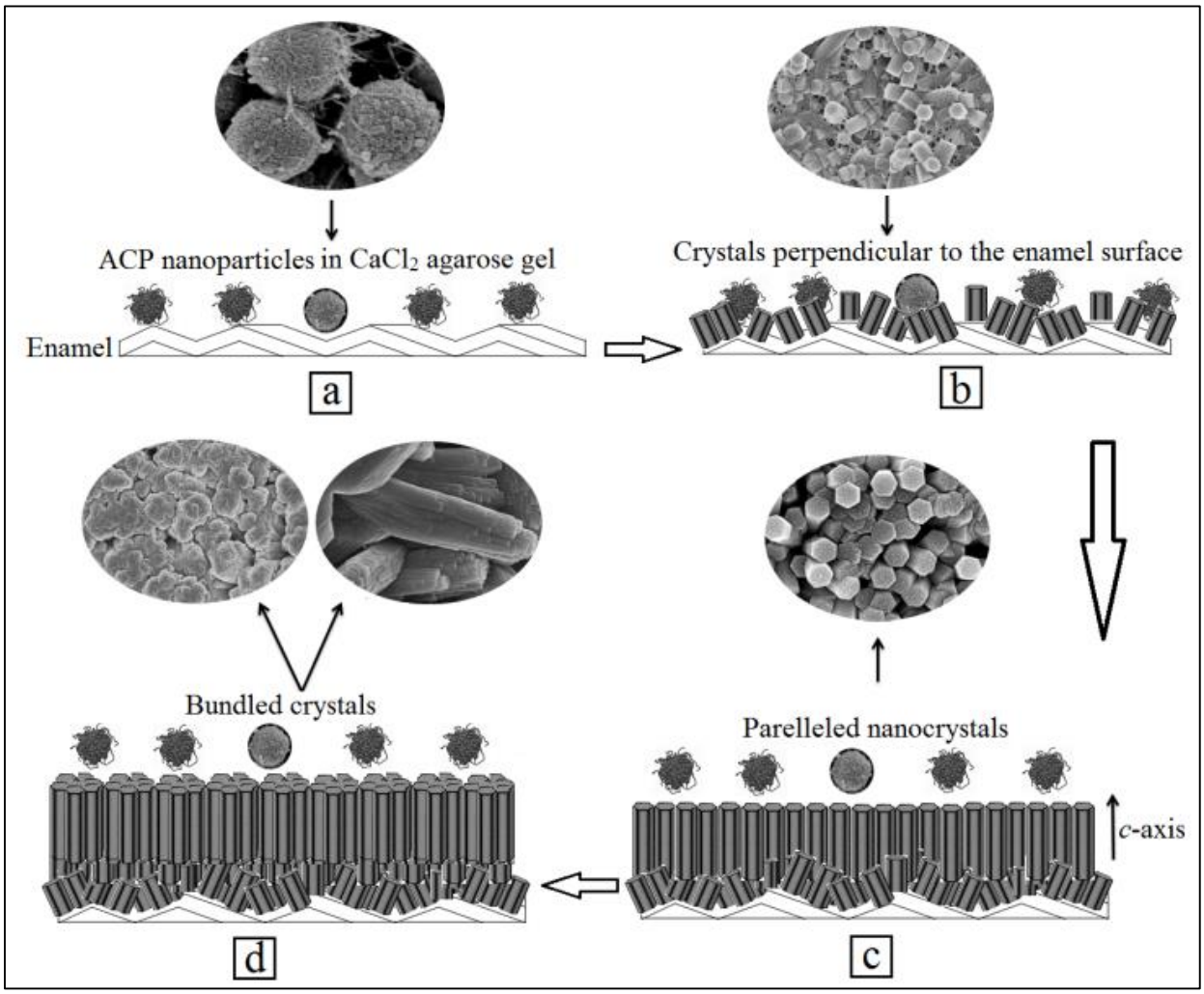

Figure 10. Schematic diagrams demonstrating the non-classical crystallisation pathway. (a) ACP nanoparticles nucleated on the lattice of the enamel HAP crystals. (b) The initial precipitated crystals grew along its *c*-axis which is perpendicular to the enamel prism surface. (c) Well-defined hexagonal crystals were evenly distributed and densely packed on the enamel surface. The mode of crystal growth forced the rod crystals to align parallel to each other. (d) A long, large hexagonal crystal acted as a primary crystal in the center of the bundles, and short nanorods fused and aligned parallel to the surface of the primary crystal to form enamel prism-like structure.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION

The crystals formed in the agarose hydrogel model under different fluoride concentrations are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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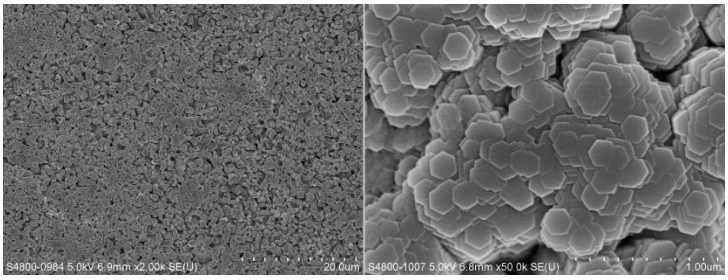
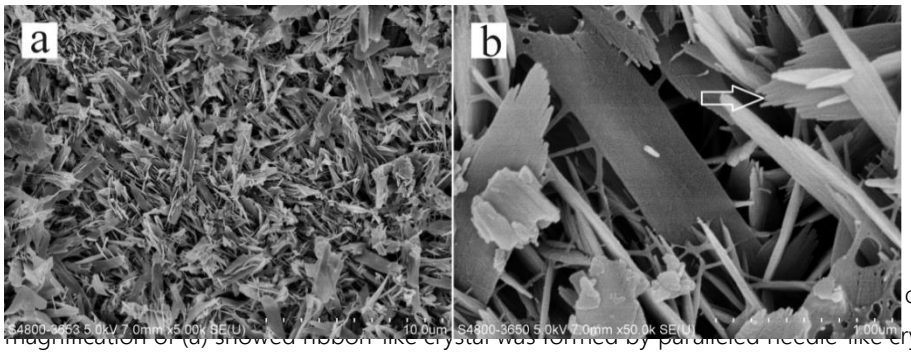


Supporting information

Iijima et al(1) found the fluoride was crucial for the organized rod-like apatite crystal formation. In our experiment, we found that fluoride affected the morphology of the calcium phosphate crystals in the biomimetic mineralization of enamel.

In our agarose hydrogel model with phosphate solution containing 500ppm fluoride, hexagonal rod crystals were formed after 6 days of incubation (Figure 2, 3, 4). Enamel prism-like bundles consisted of aggregated rod crystals were found (Figure 4). When the fluoride concentration was reduced to 100ppm, ribbon-like crystal was formed (Figure S1), and they consisted of paralleled needle-like crystals (Figure S1b Arrow). In the absence of fluoride, plate-like crystals were found on the enamel surface (Figure S2). Enamel has a high packing density of apatite crystals but the precipitated plate-like and ribbon-like apatite layer was a loose aggregate of the crystals with a porous structure. The layer formed with plate-like or ribbon-like crystals thus could not offer any mechanical function of hard tissues.



of 100ppm fluoride after 4 days. (b) High-magnification of (a) showed ribbon-like crystal was formed by paralleled needle-like crystals.

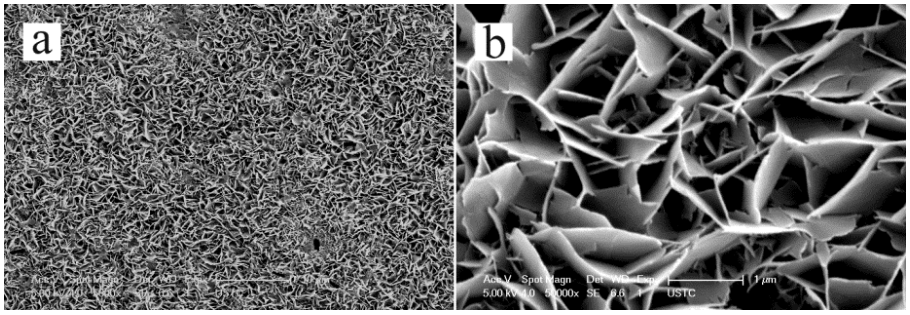


Figure S2. (a) Plate-like crystals regenerated on the enamel surface without fluoride after 4 days. (b) High-magnification of (a) showed ribbon-like crystal was formed by paralleled needle-like crystals. (c)

References:

1. Iijima, M.; Moradian-Oldak, J., Control of apatite crystal growth in a fluoride containing amelogenin-rich matrix. *Biomaterials* **2005,** *26* (13), 1595-603.

Reviewer's comment	Authors' response
Reviewer #1: Recommendation: Do not publish. Comments: There is nothing new in this report and the data are poorly interpreted. The study mostly repeats what has been already done using slightly different experimental setting and different gel. The authors used calcium containing agarose-based gel to remineralize etched enamel surfaces. Electron microscopy, XRD and FTIR were used to characterize the composition of the product. Mechanical properties were accessed by Berkovith nanoindentation tip. General Concerns to be addressed: The strategy used in this report is very similar to what has already been reported by other investigators using gelatin (Busch et al 2004), agarose (Fan et al 2012), and recently chitosan-amelogenin (Ruan et al 2013).	Regeneration of enamel structure with acellular method is an important field of study in biomaterial science and dentistry. Studies so far demonstrated success in partially mimicking the assembly steps of enamel crystals and enamel-like structure formation. However, there is no study (including the articles suggested by the reviewer) can demonstrate regeneration of enamel microstructure. Therefore, it is essential to develop model for enamel regeneration. Our study is unique because: 1) Busch et al. Regeneration of human tooth enamel. Angew. Chem. Int. Ed. 2004:43:1428-31. Busch et al. used gelatine hydrogel for regeneration of enamel-like mineral, but we used agarose. The agarose used in our model is not a simple replacement of materials, the mechanism is also different. The specialty and advantages are discussed in our paper (page 1 lines 68-76, page 4 lines 390-395, 398-411, marked in red). 2) Fan et al. Novel amelogenin-releasing hydrogel for remineralization of enamel artificial caries. J Bioact & Compat Polym 2012;27:585-603. Fan et al used agarose as a releasing agent to study the function of amelogenin in remineralization. In the absence of amelogenin, they did not show the regeneration of enamel-like structure. We constructed a model using agarose without cell and/or protein for enamel remineralization. The aim and function of the use of agarose by Fan et al are different from those by our study and theirs. Therefore, the two studies cannot be compared. This is discussed in page 4 lines 412-423, marked in blue.

	3) Ruan et al. An amelogenin-chitosan matrix promotes assembly of an enamel-like layer with a dense interface. Acta Biomaterialia. 2013;9: 7289-97. Ruan et al developed an amelogenin-containing chitosan hydrogel for enamel reconstruction. Ruan et al used amelogenin, but we used an agarose model with no amelogenin to regenerate enamel-like tissue. This is discussed in page 4 lines 423-432, marked in red.
The development of fluorapatite cement for dental enamel defect has been also reported lately (fluorapatite cement (Wei et al 2011).	Wei et al. Development of fluorapatite cement for dental enamel defects repair. J Mater Sci: Mater Med. 2011; 22:1607-1604. Wei et al used apatite cement pastes to fill enamel defects. We used a biomimetic mineralization model to induce enamel-like tissue formation, and the prospective application of our model will be different from their study. This is discussed in page 6 lines 581-587, marked in red.
This is also too similar to what has been reported in 2006 by Chen et al.	Chen et al. Acellular Synthesis of a Human Enamel-like Microstructure. Adv. Mater.2006; 18:1846-1851. Chen et al used a hydrothermal technique (121ºC) on various substrate plates, such as iron and titanium, to produce enamel-like tissue. The theory, conditions and prospective application of hydrothermal technique are different from our study method. This is discussed page 1 lines 42-50, marked in red.
The experimental procedures lack details and the there are problems with the data and their interpretation.	Further information was added (page 2 lines 98-108, marked in red).
Based on the morphology and size of the crystals, it appears that the authors grew fluorapatite and not hydroxyapatite.	Agree. The regenerated crystals were fluoridated hydroxyapatite. This was amended in page 3 lines 279-284, marked in red.
The mechanical testing was performed only on one sample with nine points and this is not adequate for statistical analysis. At least three different samples for each need to be tested.	We performed the mechanical test using 3 samples with a total of 144 points as suggested by the reviewer. This is added in Method (page 2 lines 161-162, 180-183, 190-193) and in Results (page 3 lines 327-328, marked in blue) and Figure 9.
For specific comments see below: Title: The word “Novel” is overused in the title.	

	The title is amended. The word “Novel” is replaced by “agarose”.
The novelty of using agarose gel in enamel remineralization is questionable. See Fan et al 2012, in Bioactive and Compatible Polymers	The difference between our study and Fan et al 2012 was discussed (page 4 lines 412-423, marked in blue).
It is not clear what makes this strategy “biomimetic”??	We use agarose hydrogel to mimic the gel-like micro-environment during enamel formation. The hydrogel also maintains a unidirectional Ca^{2+} and PO_4^{3-} supply. Moreover, our study was performed at 37°C, but not at high temperature or at high pressure. This is discussed in page 4 lines 363-379, marked in blue.
Is this a “gel” or “hydrogel”?? Both terms are used throughout the text for agarose.	Done. The ‘gel’ is changed to “hydrogel”.
What is the concentration??	The concentration is 0.5%. This is added in page 2 lines 98-103, marked in red.
Introduction: The notion that electro deposition system was done at 85 c degree in the presence of amelogenin is wrong.	Amended. The reference should be Ye et al 2007 (page 1 lines 47-48 and page 16 line 954, marked in red).
What is the advantage of agarose over gelatin and other gels??	The advantage of agarose over gelatin and other gels is added in page 1 lines 68-76, page 4 lines 390-395, marked in red.
Materials and Methods The experimental details for calcium and phosphate concentration as well as agarose concentration are missing.	Done. The details are added in page 2 lines 98-108, marked in red.
Results and Figures: Section 3.1 and Fig 2 can be removed since it does not add any new information and similar findings have already been reported in the literature.	Done. Section 3.1 and Fig 2 are removed.
Figure 3: There is no indication that the crystals are aligned parallel to each other.	Agree. The crystals were perpendicular to the enamel prism (page 2 lines 201-211, marked in blue and Figure 2).
In the presence of 500ppm F those hexagonal prisms cannot be HAP but they are fluorapatite crystals.	Agree. The regenerated crystals were fluoridated hydroxyapatite (page 3 lines 279-284, marked in red).

Figure 4’” these structures are not typical to enamel crystals. The authors are encouraged to look at some literature for the morphology of enamel crystals.	The crystals (Figure 3) are intermediates during the crystal self-assembly. Certain rod crystals self-assemble together to form the rudiment of enamel prism-like bundles (Figure 3b highlighted in Oval). This is added in page 3 lines 221-223 and discussed in page 5 lines 528-534, marked in red.
Figure 5: What is the relation between C and d??	In Figure 4, 4c (bundle surface) and 4d (side view of the bundle) are images at high magnifications from 4a. (Page 9 lines 699-703, marked in red).
Figure 6: The TEM in f is not crystalline while the claim for b and e states that they are crystalline. It is not clear what the authors want to convey with showing this figure??	Figure 6b and 6d suggests an amorphous structure. This is added in page 3 lines 267-275, marked in blue.
Figure 7: The XRD pattern is indicative of fluorapatite (FA) (see Jie Wei et al , J Mater Sci 22, 1607, 2011, Chen et al 2006 in the reference list).	Agree. The result is amended (page 3 lines 279-284, marked in red). The two references are added in page 16 lines 940-942, 965-967, marked in red.
The FTIR is not detailed enough to distinguish between FA and HAP.	Agree. This is added in page 3 lines 304-310, marked in red.
Figure 8: The results of Fig 8b is very surprising because the elastic modulus of the regenerated enamel is unusually high and similar to enamel.	The elastic modulus of the regenerated tissue was comparable to those of the untreated enamel surface, probably due to their similar microstructures. Similar results were also reported by *Busch et al (2004).* This is discussed in page 5 lines 571-579, marked in blue.
The use of one sample for these mechanical testing is not enough and at least three samples of each needs to be tested and averaged. Nine points on each sample is also minimal.	Agree. 144 points of indentations from 3 samples were performed. This is added in Method (page 2 lines 161-162, 180-183, 190-193), Results (page 3 lines 327-328, marked in blue) and Figure 9.
It is not clear what figure 10 is based on??	Figure 10 illustrates the process of the enamel prism-like crystals growth in our study. It is based on our findings, especially the SEM observations and referring to the mechanism of non-classical crystallization pathway. This is discussed in Discussion 4.2 (page 4 lines 440-457, page 5 lines 468-493, 539-543, marked in blue).
What is the base of classical crystallization pathway?? As oppose to n	

Additional Questions: Is this paper in the top 20% of manuscripts in the field?: No If this paper is not in the top 20% of manuscripts in the field: It is unlikely to be improved to be in the top 20%. Is it appealing to a broad audience?: No Does the manuscript give a complete description of the procedures that could be reproduced by others in the field?: No Are the literature references appropriate and up to date?: Yes Provides significant insight into or the development of an important application: Poor Work is original and significant: Poor Conclusions adequately supported by data: Poor Clarity of presentation: Fair Potential for impact in materials science and engineering: Fair	Classical crystallization pathway is thermodynamic process where ion-mediated crystallization proceeds via a one-step route to the final mineral phase. Non-classical crystallization pathway is a kinetic process where crystallization proceeds by a sequential process involving structural and compositional modifications of amorphous precursors and crystalline intermediates. (Page 4 lines 440-446, marked in blue).

Reviewer's comment	Authors' response
Reviewer #2: Recommendation: Publish after revisions noted. Comments: The authors present interesting data in regenerating enamel prism-like structure through a novel hydrogel biomimetic model. The study is well-designed and the paper is well written. The reviewer would suggest publication after its revision suggested below.	The authors appreciate the reviewer's encouraging comments.
In details: 1. In the agarose gel model, in addition to the Ca and Phospate sources, a sodium fluoride solution has been added to the system.	Experiment of the effects of concentration of fluoride on crystal regeneration is provided as supporting information (page 5 lines 459-466, page 16 lines 929-933, marked in red).
What is the role of F- in the synthesis of HAP crystals?	The role of fluoride was discussed in the supporting information (page 16 lines 929-933, marked in red).
Any fluorapatite or fluor-hydroxyapatite formation? Please provide experimental data and discuss.	Yes. The crystals formed were fluoridated hydroxyapatite (page 3 lines 279-284, 304-310, marked in red). Experiment of the effects of concentration of fluoride on crystal regeneration is provided as supporting information (page 5 lines 459-466, marked in red) and discussed in the supporting information (page 16 lines 929-933, marked in red).
2. Please show and discuss how this biomimetic model would be transferred for future clinical applications.	Further information was added in Discussion (page 6 lines 598-602, marked in blue).
How the saliva containing oral environment affect the synthesis of HAP crystals?	Further information was added in Discussion (page 6 lines 606-608, marked in blue).
How is this model going to target the common enamel subsurface lesions?	Further information was added in Discussion (page 6 lines 602-605, marked in red).
3. There is a lack of details in the preparation of this agarose gel system, which should be provided for the repetition of the experiment.	Done This is added in page 2 lines 98-108, marked in red).

4. The authors should explain the mechanisms of the breakdown of agarose matrix while the HAP crystals are undergoing maturation process. Additional Questions: Is this paper in the top 20% of manuscripts in the field?: If this paper is not in the top 20% of manuscripts in the field: Is it appealing to a broad audience?: No Does the manuscript give a complete description of the procedures that could be reproduced by others in the field?: No Are the literature references appropriate and up to date?: Yes Provides significant insight into or the development of an important application: Good Work is original and significant: Good Conclusions adequately supported by data: Good Clarity of presentation: Good Potential for impact in materials science and engineering: Fair	The mechanism of the breakdown of agarose matrix was discussed in page 5 lines 508-520, marked in red.

Reviewer's comment	Authors' response
Reviewer #3: Recommendation: Publish after revisions noted. Comments: This manuscript describes a novel hydrogel biomimetic mineralization model for the regeneration of enamel prism-like tissue. SEM, TEM, XRD, FTIR and the nano-indentation hardness test have been used for analyzing the physicochemical properties of the regeneration enamel and the agarose.	
Whereas, for reliable surface characterization, parameters describing surface variation in the spatial direction are additionally needed, so I would consider adding the 3D surface analyze by using AFM. The results showed that this novel hydrogel biomimetic mineralization model is useful for the regeneration of enamel prism-like tissue.	3D surface analysis by using AFM was performed as suggested by the reviewer. This is added in Figure 5 and page 2 lines 131-132, 134-136, page 3 lines 251-265, marked in red.
Overall, the paper is well written, well organized. The topic and results of this study are interesting. I would recommend this manuscript for publication in ACS Applied Materials & Interfaces, provided the authors reasonably address the following points.	The authors appreciate the reviewer's encouraging comments.
The following are concerns that should be deliberated in the paper: 1. The tooth slices were covered with nail vanishes. What is the purpose of using nail vanishes and which kind of nail vanishes was used?	The nail vanish was used to protect the enamel surface from treatment such as etching and subsequent remineralisation, so that they can be used as control for comparison. Further information was added in page 2 lines 152-156, marked in red.
2. Which kind of statistics analysis was used for elastic modulus and nanohardness test (Figure 8b)?	Two-way ANOVA was used (page 2 lines 190-193, marked in blue).
3. "The regenerated crystals on the enamel surface formed a homogenous and dense layer of mineralized tissue after 6 days of incubation.	Amended. We redid the mechanical test using 3 samples after 6 days of incubation. Further information was added in page 2 lines 161-162,

"But in evaluation of mechanical properties, the tooth slices were incubated for 4 days. 4. The magnification of SEM micrographs was not unified. Etched human enamel was used as control but the magnification of SEM micrographs of etched human enamel (Figure 2) was not the same as SEM micrographs of the regenerated mineralized tissue after 2,4 and 6 days (Figure 3,4 and 5). Why it didn't have SEM micrographs of c-axis and cross-sectional view after 4 days (Figure 4)? 5. Did the diameter of rod crystals changed over time? As shown in Figure3c and Figure4b, the diameter of rod crystals was expressed remarkably different with the same magnification. If it changed, what the reason it is? If it not changed, why looked different in same magnification? 6. I would like to see the experiments using this regenerated enamel in vitro study. Additional Questions: Is this paper in the top 20% of manuscripts in the field?: No If this paper is not in the top 20% of manuscripts in the field: It could be improved to be in the top 20% with appropriate revisions. Is it appealing to a broad audience?: Yes Does the manuscript give a complete description of the procedures that could be reproduced by others in the field?: Yes Are the literature references appropriate and up to date?: Yes Provides significant insight into or the development of an important application: Good	180-183, 190-193, page 4 lines 327-328, marked in blue. Figure 9 was amended. SEM micrographs were unified now. SEM micrographs of c-axis and cross-sectional view after 4 days are added (Figure 3). Yes. The diameter of rod crystals changed over time. The reason was discussed in page 5 lines 508-520, marked in red. This model is an in vitro study. We plan to do in vivo study in near future.

Work is original and significant: Good Conclusions adequately supported by data: Good Clarity of presentation: Good Potential for impact in materials science and engineering: Good	

Editor's comment	Authors' response
On the basis of the reviewer comments and my own assessment of the manuscript, I am willing to consider a revised version of this paper for publication in ACS Applied Materials & Interfaces pending a second round of external review.	
In preparing the revision, carefully consider all of the comments made by the reviewers. In particular, you must address the novelty issues of this work raised by Reviewer 1.	Done. A point to point response is made.
We would like to receive your revision as soon as possible, by 03-Dec-2013 at the latest.	We have asked for extension of the due date to 31 Dec 2013 and have been approved.
In addition to addressing the reviewers' comments, please make each of the technical corrections listed below:	
1) For the benefit of reviewers during the second round of external review, you must incorporate/include all your responses to reviewer comment directly into the revised manuscript, and not just in the Response Letter. In your Response Letter, you must also indicate the page and line number in the manuscript where your responses/corrections have been incorporated	Done. A point to point response is made and amendment in response or as suggested by the reviewers are made accordingly in the manuscript. These amendments are highlighted for reference by the reviewers in the revised manuscript submitted as supporting document.
2) NEED new Journal Publishing Agreement. Please go to your Paragon Plus website and click the link "Forms to be completed" and follow the instructions to submit the electronic journal publishing agreement form.	Done.
a) As your JPA was addressed to Chemistry of Materials, we would like to know if this manuscript was previously submitted to Chemistry of Materials and the outcome of previous submission.	This manuscript was submitted to Chemistry of Materials, and the editor of Chemistry of Materials considered it more suitable for journal that specializes in applied materials or biomedical materials.
3) Supporting Information (SI): If needed in the revised manuscript, a Supporting Information paragraph should be included after the acknowledgment paragraph. The paragraph should describe the contents of the SI section, and the last line should read as follows: "This information is	Done.

available free of charge via the Internet at http://pubs.acs.org/.	
4) Reference Formatting: The references are not formatted according to journal standard. See the journal at http://pubs.acs.org/journal/aamick for example of proper format.	Done.
a) Use CASSI abbreviations for all journal names. See http://www.cas.org/sent.html for list of journal name abbreviations.	Done.
b) All Refs missing end page numbers; Ref 2 missing volume and page numbers, not found on CASSI ; Ref 12 incomplete reference; Ref 13,15,23 incorrect journal name abbreviation; Ref 27 journal name not abbreviated	Amended. (Ref 2, 12, 13, 16, 26 and 29 are highlighted in blue in page16, 17)
5) Figures and tables need to be reformatted. a) Axis labels and tick markers in figures/plots are too small. Increase font sized used.	Done.
b) Please provide error limits on data in tables/plots.	Done.
c) Figure 10 missing labels in caption	Done.
6) On resubmission, please provide 2 copies of the final manuscript file:	
a) The final revised manuscript file that does not contain any highlighting or editing marks. This file should be uploaded as the primary manuscript document file.	Done.
b) A marked copy of the revised manuscript that shows changes made on revision clearly highlighted. This file should be uploaded SEPARATELY FROM THE FINAL MANUSCRIPT FILE as Supporting Information for Review.	Done.
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