

Preparation, microstructure, and mechanical properties of enamel-like nanocomposites for dental repair application

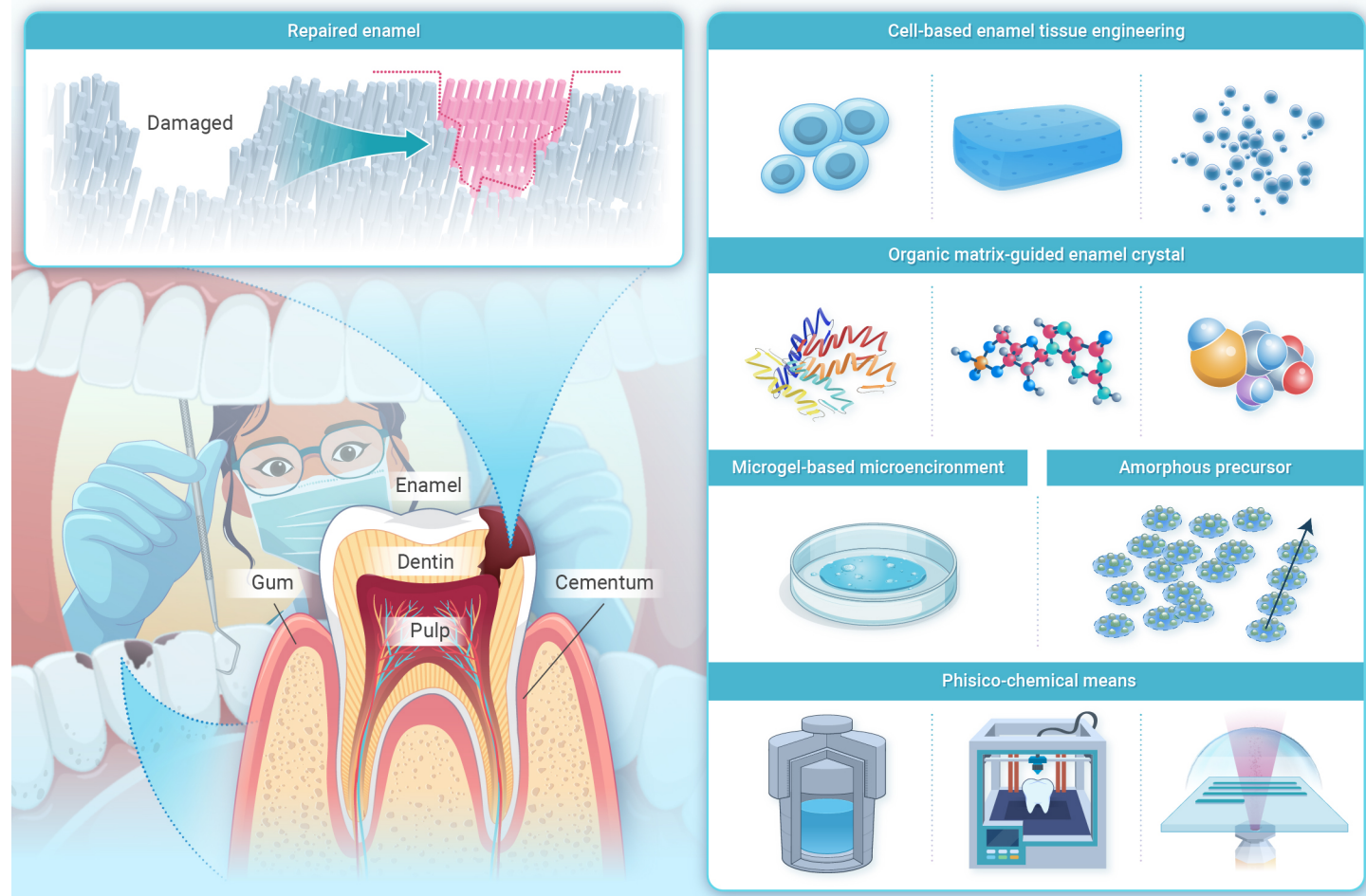
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- The structure-mechanical property-function relationship of natural human tooth enamel was discussed.
- The synthesis, microstructure and mechanical property of biomimetic tooth enamel nanocomposites were reviewed.
- The biosafety and application of nano-enamel composite in dental prosthesis were discussed.

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Tooth enamel, as the hardest and the most resilient bioceramic material (~95.5 wt% apatite minerals) in human body, forms complex, highly ordered, hierarchical hetero-phase array structure over millions of years of evolution. This multiscale complex structure endows tooth enamel with excellent mechanical stability (especially the resistance to fracture, wear, and impact), high chop efficiency, and superb durability. However, in the complex oral environment, several factors such as oral bacteria, acidic foods, and mechanical collisions, can cause the dissolution of apatite crystals and even the damage of the enamel, resulting in a series of lesions such as dental caries that severely affects human health and life quality. Therefore, the urgent need for restoring to the normal function of natural teeth by repairing enamel has motivated researchers to develop advanced synthetic strategies for constructing artificial enamels. In this review, based on the understanding of the hierarchical heterogeneous structure-mechanical property-function relationship of natural human tooth enamel, we firstly introduced several synthetic strategies of biomimetic enamel nanocomposites such as cell-based tissue engineering, organic matrix-guided crystal growth, microgel-based microenvironment mineralization, amorphous precursor mineralization, and physicochemical methods, as well as presenting their microstructures and mechanical properties published in recent years. Finally, we discussed the biological safety of these artificial enamel nanocomposites and their dental repair applications.

INTRODUCTION

Tooth enamel, located at the outermost stratum of natural tooth, is considered to be one of the most fascinating innovations in vertebrates (e.g., human being) over millions of years of evolution. Mammalian mature enamel becomes the hardest tissue owing to its high mineralization (95.0–96.0 wt% minerals, 1.0–2.0 wt% organic matrix, 2.0–4.0 wt% water), which makes it the first shield to protect the whole tooth from daily grinding and chewing.^{1–7} However, in complex oral environments, owing to the bacterial fermentation from free sugars (monosaccharides and disaccharides added to beverages and foods by the manufacturer, cook, or consumer, and sugars naturally presented in honey, syrups, fruit juices, and fruit juice concentrates) to induce acidic by-products, tooth enamel inevitably suffers from demineralization and damage, easily leading to dental caries, which is the local damage of dental hard tissues.^{8,9} Actually, dental caries, affecting more than 3.5 billion people, is the most common disease around the world especially in many low-income and middle-income countries. For instance, tooth decay not only has an impact on young children, but also accompanies and bothers people throughout their entire adolescence and adulthood. The World Health Organization (WHO) usually uses dental caries index (also defined as the Decayed, Missing and Filled teeth (DMFT) index = $n_D + n_M + n_F$, where n_D represents the number of decayed tooth, n_M indicates the number of missing teeth caused by caries, and n_F is the number of filled teeth caused by caries, respectively) to capture an individual's accumulated experience of dental caries. For example, survey data from WHO found that the DMFT index of 12-year-olds in 207 countries and territories reached 1.9,¹⁰ and undoubtedly higher at older ages. While the average value of DMFT reaches 2.7 for 12-year-old children in Americas and Europe, which is about 42% higher than the average value in 207 countries and territories. Chronic dental caries often has serious personal

consequences, as simple as persistent pain and sepsis, or as serious as miss a class, reduced work productivity, and disruption to family life. For early enamel caries, although saliva can be capable of partial remineralization, full mineralization is rarely achieved, especially when the balance of the self-healing phase of demineralization/remineralization is disrupted.^{11–13} Actually, the development and mineralization of enamel are complex processes regulated by a kind of enamel cell called ameloblast. Ameloblasts can secrete enamel matrix proteins. In enamel formation, ameloblasts play a key role in coordinating crystal growth, which includes cellular activities such as transport of minerals ions, pH regulation within the microenvironment, and hydrolysis of proteins. However, after the enamel mineralization completion, ameloblasts undergo programmable death during the time of maturity, resulting in the mature enamel becomes cell-free and cannot be regenerated endogenously after the damage.^{5,14,15} Therefore, there is an urgent need to develop *in situ* enamel repair techniques to improve the longevity of people's second set of teeth.

Natural tooth enamel, as a mechanically robust nanocomposite model, is naturally attracting the attention of researchers, especially with regard to its complex hierarchical structure. Its hierarchical structure can be usually divided into six levels, ranging from molecular scale with the dimension of a few nanometers to dentition on the macro scale.^{16–18} Particularly, the microstructure of apatite crystals in the ordered arrangement is on the basis of the formation of its complex microstructures.¹⁹ Such hierarchical microstructure determines its outstanding mechanical properties, which has been widely explored and recognized in recent years.^{20–23} For instance, the crack resistance can be attributed to the highly-ordered hierarchical arrangement of their organic and inorganic constituents.²⁴ The wear resistance can be related to its highly mineralized structure.²⁵

In recent years, there have been developed advanced characterizations and computational-simulation methodologies. Significant insights into the morphologies, chemical compositions, structures, and mechanical properties of human enamel can be obtained by scanning electron microscopy (SEM),²⁶ high-resolution scanning transmission electron microscopy (STEM),²⁷ atomic force microscopy (AFM),²⁸ micro (nano)-computed tomography (Micro (Nano)-CT),²⁹ X-ray diffraction (XRD),³⁰ Raman spectroscopy,³¹ Fourier transform infrared spectroscopy (FTIR),³² thermogravimetry (TGA),³³ nanoindentation,²⁴ impact wear,³⁴ and contact fatigue,³⁵ etc. Notably, these characterization techniques can further deepen our knowledge of the composition-structure-property relationship of human enamel, especially when combined with finite element (FE) simulations and molecular dynamics (MD).^{36,37} In other words, this collective information enables us to achieve the enamel composition, surface properties, and complex hierarchical structure more precisely. Besides, these advanced technologies can also accelerate these discoveries of fine microstructure and mysterious mineralization mechanisms, contributing to developing advanced techniques of artificial enamel regeneration.

Traditional dental treatments use un-structural non-apatite materials such as alloys, ceramics and polymers to fill in some damaged enamels.^{38–40} However, owing to the weak bonding interactions between the unstructured material and natural enamel, it is easy to attract bacteria at the interface, leading to secondary cavities.^{41–43} In the past decades, some advanced techniques (e.g., biomimetic mineralization, tissue engineering, and layer-by-layer assembly) have been successfully developed to synthesize enamel-like

structural materials which can be potentially applied to repairing human enamel.⁴⁴⁻⁴⁶ For example, through imitating the mineralization process of natural hydroxyapatite (HAP, $\text{Ca}_5(\text{PO}_4)_3\text{OH}$) nanorods' directional growth mediated by amelogenin, researchers used amyloid-like proteins to induce the orientated growth of HAP crystals, constructing the enamel-like HAP array structural nanocomposite.⁴⁴ Additionally, by using enamel tissue cells, such as enamel organ epithelial cells, dental pulp cells, and ameloblast lineage cells, this allows for the generation of enamel *in vivo*.⁴⁵ Besides, abiotic tooth enamel could be also synthesized by a layer-by-layer method depositing nanorods in polyelectrolyte matrix.⁴⁶

In this review, we firstly outlined the composition, structure and mechanical properties of human tooth enamel, and explored the relationship between them. Secondly, synthetic strategies for enamel-like nanocomposites were presented in the last decade, referring to (i) cell-based enamel tissue engineering; (ii) organic matrix-guided enamel crystal growth; (iii) microgel-based microenvironment mineralization; (iv) amorphous phase precursors induced mineralization; and (v) physicochemical methods. Thirdly, mechanical properties of enamel-like nanocomposites are further summarized and compared, including hardness, elastic (Young's) modulus, strength, toughness and viscoelasticity. And the biosafeties of some artificial repairing enamels are evaluated by the implantation *in vivo*. Finally, we provided our views on future research directions, emphasizing the need to construct artificial enamel-like materials in a safe, environmentally friendly, and controllable manner to address the challenge of loss of dental of function for all humanity.

Hierarchical structure and composition of human tooth enamel

Human tooth enamel is a peculiar hierarchical structure ranging from the molecular scale of inorganic crystals/organic matrixes to the macroscale of the dentition. It primarily consists of apatite, water and organic matrix, forming a natural nanocomposite with the thickness between 1~2 mm. Its excellent mechanical properties can be primarily attributed to the hierarchical structure formed by the arrangement and orientation of the nano-apatite crystals.^{36,54} Depending on the terminology used to describe the details of the enamel structure, it can be divided into six levels of hierarchy (Figure 1A), denoted Level 0 to Level V: (0) individual HAP crystals and organic molecules, (I) crystallite orientation, (II) modules, (III) enamel types, (IV) schmelzmuster, and (V) dentition.¹⁶⁻¹⁸ Lower level structures can be orderly arranged and assembled into increasingly complex higher level structures. In the following, the microstructural features are discussed at different levels.

Level 0 — The smallest building block of the enamel is HAP crystallite (Figure 1B), a hexagonal calcium apatite.¹⁸ HAP crystallites are needle- or lath-type crystallites aligned with their crystallographic *c*-axis direction. Typical crystals have polygonal long-axis sections with long and short sides measuring 70~170 nm and 20~50 nm, respectively.⁵³ A defect called central dark line (CDL) can be observed in the central region of the crystal, and initial dissolution of caries appears to be more likely to occur in the CDL.²⁷

Level I — The directional arrangement of the HAP crystallites varies. The main difference in the orientation of the crystal arrangement is that the crystal plates in the central three-dimensional region of the rod are often parallel to the longitudinal axis of the rod while those near the edge of the rod usually have an angle.^{55,56} The crystal plates are indeed all parallel in spatial geometry, but they are not co-oriented. Actually, the angle distribution of their *c*-axes within a rod is usually 30°–90°.³⁶

Level II — Module is a repeatable 3D structure in the enamel layer. The enamel rod acts as a regular enamel module with bundled crystals as its main part, with a locking 'fish scale' or 'keyhole' shaped in cross section and a 4–8 μm diameter, extending regularly from the enamel-dentine junction (EDJ) to the outer enamel surface (OES) at an angle (In other words, the enamel rod is characterized by continuous enamel thickness).^{16,18,57,58} The crystal bundle of the enamel rod is restricted to its outer surface by the glazing rod sheaths, which is a distinct microcrystalline interrupted surface. Enamel rods are encapsulated in an organic matrix (consisting primarily of proteins) called the enamel rod sheath, which enables adjacent enameled rods to be bonded together. The organic matrix of the rod sheath is susceptible to corrosion during acid etching of the enameled sheet, giving it a cracked appearance.⁵⁹ In fact, the organic matrix is present throughout the whole enamel layer (Figure 1C), but it is more concentrated in the inner enamel layer

where mineral abundance is slightly lower. In addition to the rod sheaths, larger organic structure (called enamel tufts) is present at the EDJ together with the inner enamel region. A very small organic fraction made of small peptides, amino acids and insoluble protein substances, also exist in the rod sheath and EDJ regions of mature enamel.^{48,60} In general, the arrangement of the rods as modules makes up the enamel layer and simultaneously determines the next level of the unit enamel type.

Level III — Enamel types. Four basic enamel types are identified in prismatic enamel: radial enamel, tangential enamel, Hunter-Schreger bands (HSBs) and irregular decussation.¹⁷ The types of human tooth enamel have radial enamel and HSBs. The radial pattern is presented by a rad band on one third of the thickness of the enamel layer with typical Retzius lines.¹⁶ The HSBs as a common enamel type (exhibiting rod decussation) in enamel sections is the dominating enamel type in humans.¹⁵ The HSBs is mainly composed of one or several rows of light and dark bands, and the enamel rod between adjacent light and dark bands intersects at a certain angle, exhibiting a repetitive alternating light and dark band on the tooth section.^{59,61} HSBs begin at the EDJ and generally curve in a cuspal direction toward the outer enamel, fading out at approximately two-thirds of the enamel thickness.⁶² Regional differences in the packing densities (number of HSBs per unit size) of HSBs were found in different human teeth. HSBs packing densities were the highest in areas with the greatest functional and occlusal loads, which seemed to correlate with the function of each tooth.⁶³ The path of the enamel rods in the enamel type, such as any decussations or bending, correlates with the movement of the ameloblast. In the process of enamel formation, individual ameloblast is responsible for the creation of an enamel rod, and the highly coordinated movement of the ameloblasts leads to the formation of the final decussating pattern.^{63,64}

Level IV — Schmelzmuster and Level V — Dentition. The schmelzmuster refers to the 3D spatial structure composed of the enamel type of a single tooth, while dentition expresses the Schmelzmuster of a person's different teeth. Typically, the schmelzmuster can be divided into two parts, inner/outer enamel, where the inner enamel near the EDJ is the HSBs enamel which accounts for about two-thirds of the enamel thickness, and the outer enamel near the enamel surface is the radial enamel.^{18,63,65} On next hierarchical Level V, obviously different Schmelzmusters appear on different teeth. For example, there have areas of enamel without HSBs at the cervical quarter of the maxillary canine and at the incisal edge of the incisor, many HSBs in the mandibular molar does not reach the outer surface of the enamel, and the mandibular central incisor by Striae of Retzius appears to interrupt areas of HSBs.⁶³ In general, the above discrepancies can be due to differences in the functional role of the individual teeth.

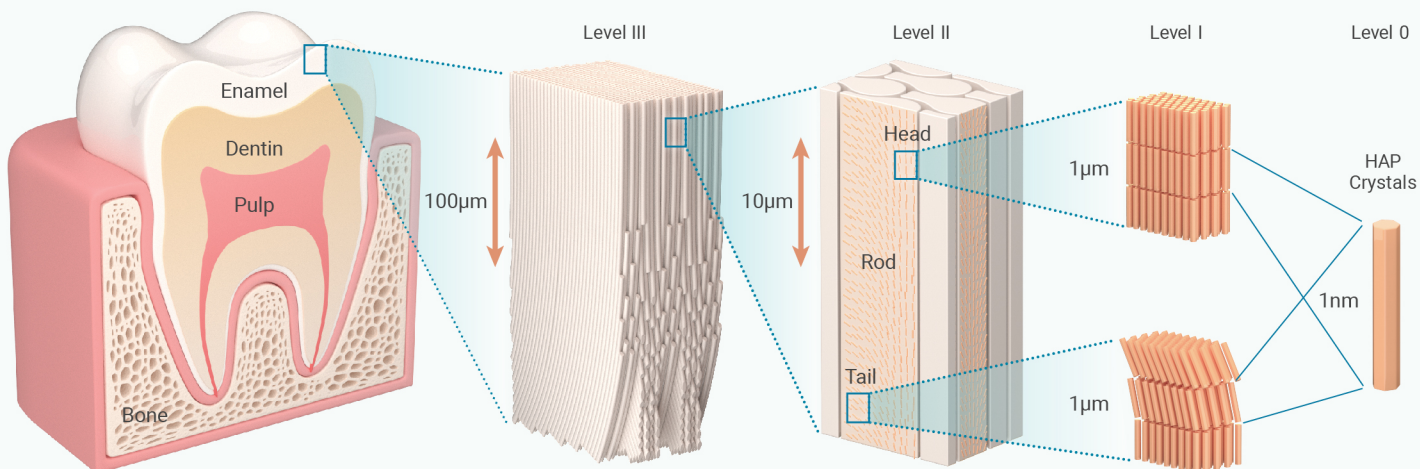
Mechanical and functional properties of human tooth enamel

The excellent mechanical properties of natural tooth enamel enable it to perform the unique functions such as tearing, cutting, and grinding food during chewing. Therefore, the insight into their mechanical properties (hardness (*H*), stiffness (Young's modulus (*E*)), fracture strength (α_f) and fracture toughness (K_{IC})) and functional properties will help to develop reference standard for optimizing and enhancing the performance of enamel-like materials.

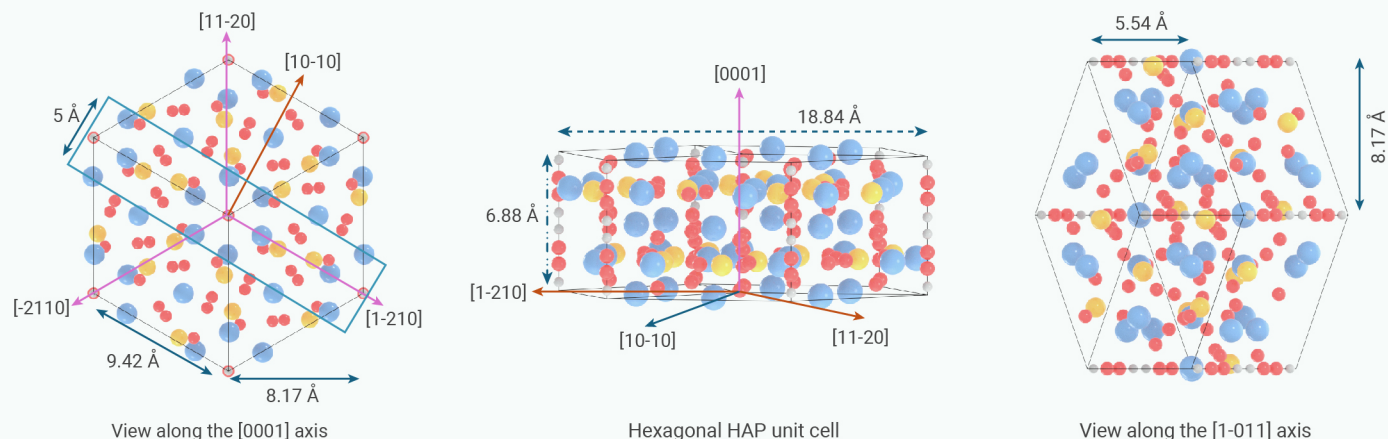
H and *E* are usually used to describe the deformation resistance properties of materials. In the early days, the values of microhardness and *E* of human enamel were obtained by microhardness tester and compression tester, respectively,⁶⁹⁻⁷¹ but now can be measured in a more accurate method by nanoindentation technology, with the experimental results indicating the *H* of 3~6 GPa and *E* of 70~120 GPa for human tooth enamel.^{20-22,72-73} It can be found that the quantitative results obtained from different studies are different, which is attributed to different experimental techniques and sample being used.^{72,74} However, the qualitative change tendency obtained from different studies are consistent. The *H* and *E* both reduce gradually from the OES to DEJ, as shown in Figures 2A-C.^{66,67,74-76} Researchers⁵⁶ found that there was an exponential relationship between the *H* and *E* of enamel and its normalized thickness (*x*) as ($E(x)=111.64x^{0.18}$ ($R^2=0.94$) and $H(x)=4.41x^{0.16}$ ($R^2=0.87$) GPa), respectively.

α_f and K_{IC} can evaluate a material's tolerance to damage. α_f relates to the stress at which a material fractures, while K_{IC} is a measure of the intensity of stress field around the crack tip.^{18,77} The results of microcantilever tests on

A The hierarchical structure of tooth enamel



B The principal inorganic component of enamel



C The principal organic component of enamel

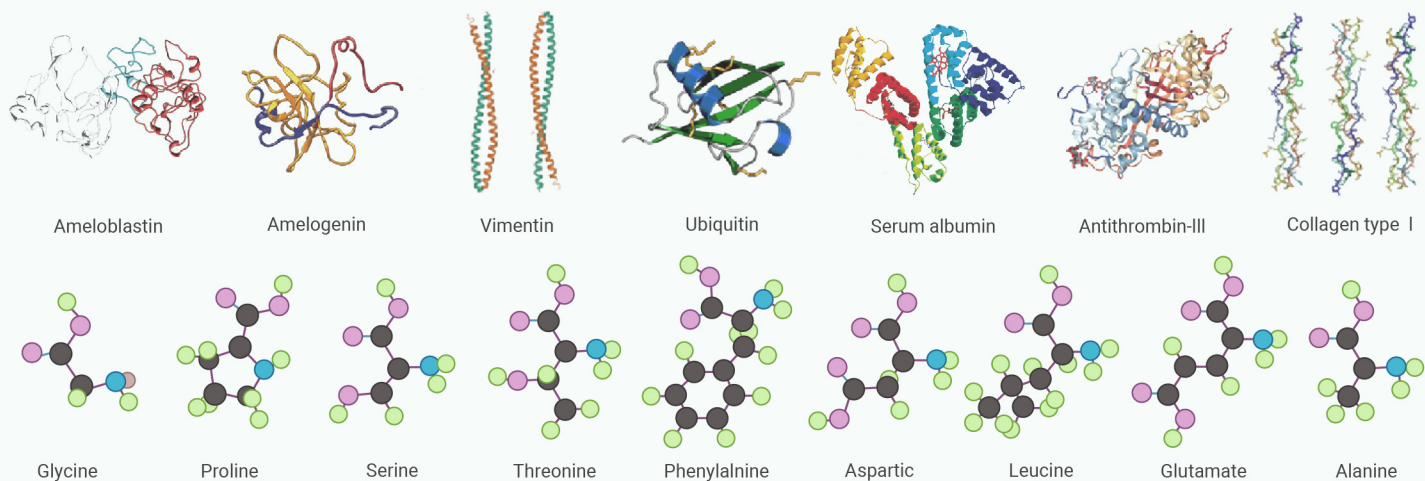


Figure 1. The hierarchical structure and composition of human tooth enamel (A) Schematic of the cross-section and hierarchical structure of human teeth. Its structure features include the enamel, dentin and pulp chamber. The figure displays a schematic of the structure of tooth enamel with different magnification from level 0 to level III. In addition to the levels shown in the figure, two more levels exist: Level IV and Level V. Adapted with permission from ref.^{18,47} Copyright 2020/2018, Elsevier. (B) Three-dimensional schematic of a hexagonal HAP unit cell. Ca, P, O, and H atoms are in blue, yellow, red, and gray, respectively. Adapted with permission from ref.²⁷ Copyright 2021, MDPI. (C) The primary proteins in the erupted human enamel and their relevant amino acids.^{48–51} Reproduced with permission from ref.^{44,52,53} Copyright 2020/2008, Wiley-VCH.

human primary enamel show a flexural strength of 640~1420 MPa when loaded parallel to the rods direction.⁷⁸ The synergistic effect of various toughening mechanisms in the microstructure has a significant impact on the frac-

ture behavior. K_{IC} of the enamel ranges from 1.79 to 2.37 MPa m^{0.5}, quantified by using controlled crack extension experiments (compact tension, CT). The internal enamel possesses strong fracture resistance, and the increase in

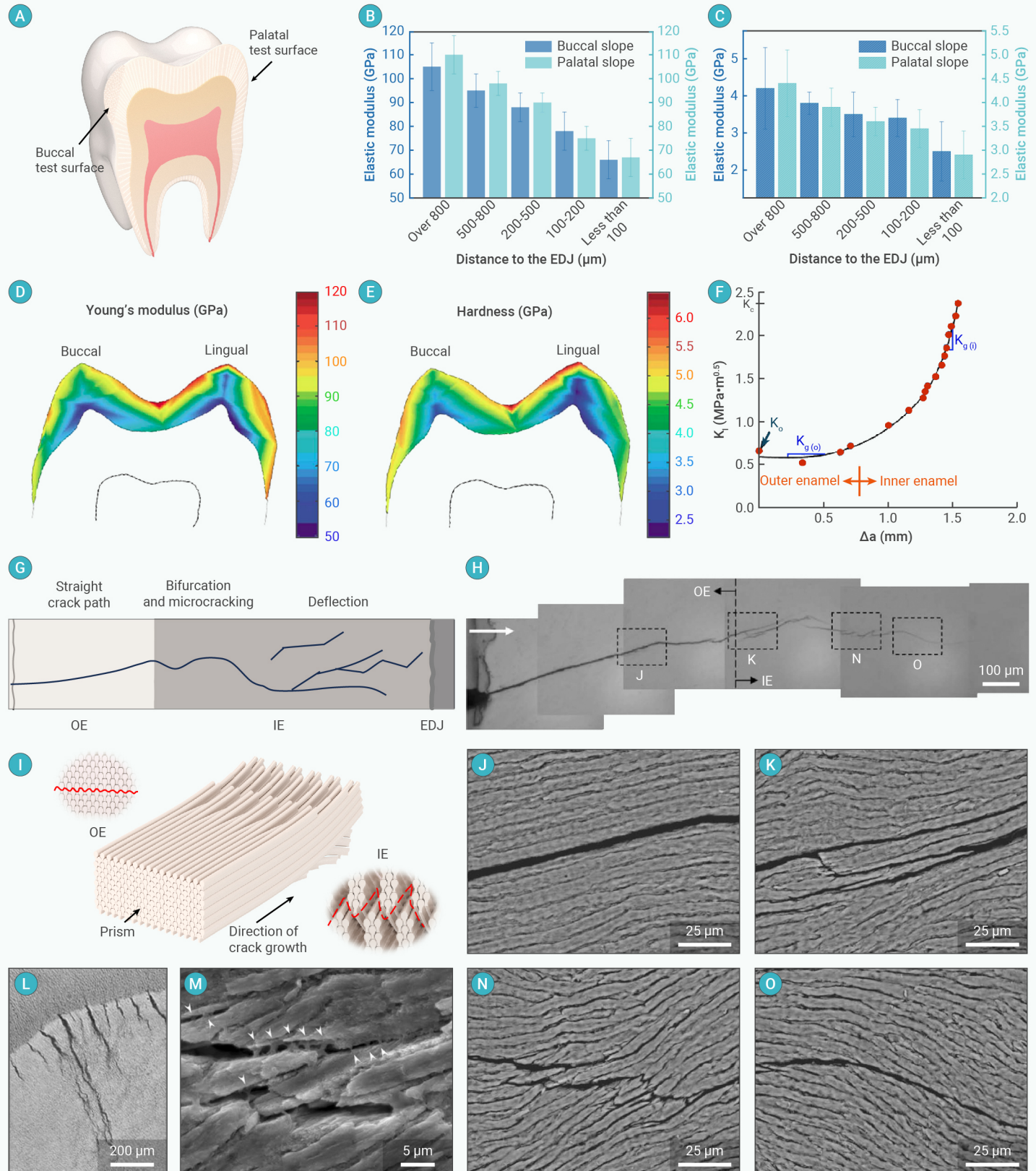


Figure 2. Mechanical properties and strengthening and toughening mechanisms of human tooth enamel (A) Schematic of test surface sampled on the buccal slope and palatal slope of the buccal cusp of the premolar. (B, C) Elastic modulus (B) and hardness (C) of the enamel at different thicknesses of enamel. Adapted with permission from ref.⁶⁶ Copyright 2013, Elsevier. (D, E) Digitized mapping showing the hardness (D) and elastic modulus (E) distribution in a human molar tooth section. Reproduced with permission from ref.⁶⁷ Copyright 2002, Elsevier. (F) Typical crack-resistant growth curves for human enamel samples. Adapted with permission from ref.⁶⁸ Copyright 2009, Elsevier. (G) Schematic of typical crack paths on enamel from OE to EDJ. Reproduced with permission from ref.¹⁸ Copyright 2020, Elsevier. (I) Schematic of the enamel prism orientation in the outer and inner sites. Reproduced with permission from ref.⁶⁸ Copyright 2009, Elsevier. (L) A segment of transverse section view of human molars. Cracks were found to initiate from tufts. Reproduced with permission from ref.⁸² Copyright 2009, SAGE. (M) Ligament bridges formed by crack growth (yellow arrows). (H), Optical micrographs of crack extension (from left to right, shown by white arrows) in enamel. (J) The relatively straight prism results in a straighter crack path introduced from the outer enamel. (K) Crack bifurcation. (N) Ligament bridges formed by crack extension. (O) Crack extension near the DEJ occurs with deflections and stalls. Reproduced with permission from ref.²⁴ Copyright 2009, Elsevier.

crack resistance from the outside to the inside can be attributed to the increased crack propagation resistance (i.e. rising *R*-curve, Figure 2F).⁶⁸

The average masticatory load of human is about 220 N, with actual contact load of approximately 15–30 min per day.⁷⁹ The wear of enamel can be chiefly attributed to the depletion of surface material caused by mechanical action. When saliva is used as a lubricant, the wear rate of the enamel is only 0.55 mg/1000 cycles at a load of 16.2 kg.⁸⁰ In a study, using a reciprocating sliding wear test machine to investigate the wear of titanium alloys relative to human tooth enamel, experimental results demonstrated that after 1,000 cycles, the depth of abrasions was approximately a few microns and there was no significant deformation of the enamel rods.⁸¹

Structure-property relationship of human tooth enamel

The excellent mechanical properties of human tooth enamel are determined by its hierarchical heterogeneous structure at different length scales. In the following, the relationship between the mechanical properties of the enamel and its hierarchical structure are briefly discussed:

At the crystal level (level 0), inorganic mineral crystals endow the essential hardness and modulus, which are related to the degree of mineralization (e.g., CaO/P₂O₅ content).⁶⁷ Individual HAP crystal usually has an obvious anisotropy, with the elongated axis in the enamel HAP being stiffer and harder (axial direction: 125.9 ± 1.6 GPa for *E* and 8.8 ± 0.4 GPa for *H*; basal direction: 135.1 ± 1.3 GPa for *E* and 9.7 ± 0.1 GPa for *H*).⁸³ Owing to the nanoscale size of HAP crystals, its fracture strength and fracture toughness are difficult to precisely evaluated by the nanoindentation technique. At the nanoscale, the tribological behavior of enamel is very complex, and the wear properties of enamel crystals depend on the hardness of the mineral and the fracture of the microcrystals. As far as the fracture behavior of microcrystals is concerned, when enamel responds to masticatory loads, individual hydroxyapatite fibers will respond in three ways in sequence with the gradual increase of load, including (1) plucking, (2) plastic deformation, and (3) fragmentation, which is conducive to the consumption of loads on the microscopic scale and thus creates excellent wear-resistant properties.⁸⁴

At the level I, the origin of the anisotropy in mechanical properties of enamel is mostly a result of the anisotropic alignment of apatite crystals.^{21,85} Because of the variation in crystal orientation in the tail region of the enamel rods, the *E* and *H* in this region are lower than those in the head of the rods where the crystals show parallel alignment.²¹ Among the crystals arranged in parallel, the wear resistance is the highest because the contact area is the smallest when the crystals are spatially perpendicular to the wear test surface.¹⁸

At the level II, it is demonstrated that the direction parallel to the enamel rod is greater *H* and *E* than the direction perpendicular to it. This point can be also attributed to the anisotropy of the enamel crystal.²¹ It is found that both directions of crack growth exhibit an ascending *R*-curve behavior. Although the fracture resistance is independent of the orientation of enamel rods, the degree of toughening per unit crack extension (*K_{IC}*) and the apparent (*K_{IC}*) are both highest in the longitudinal direction of the enamel rods.⁸⁶ The crack is very difficult to cut through the enamel rod, since the low density of rods sheath has the accumulation of water and residual protein, which play a transfer of shear stress and promote energy dissipation. Therefore, the crack usually forms a crack bridge (Figure 2M) along the weak path of propagation, i.e. the rod boundary.^{34,55,86–88} The contact wear rate (the true wear rate) on the occlusal surface of the enamel rod is all very low, and such a dependence of the wear resistance orientation can be due to the variation of the hardness at the level of the single rod.⁸⁹

At the level of III, the *H* and *E* of radial enamel are higher than those at HSBs.⁷⁵ The decussation structure has major contribution to the fracture toughness of enamel (Figure 2I). Cracks occurs usually on the outer surface of the enamel and are initiated and propagated from contact damage. Cracks mainly expand along the boundary of adjacent rods within radial enamel. However, the extension path becomes more tortuous when the cracks encounter the decussated rod region (Figure 2H). At the microscopic level, toughening mechanisms refer to crack bridging constituted by rod bundles, crack deflection, and crack bifurcation imposed by the rod curvature (Figures 2J–O).^{68,88,90,91} Meanwhile, cracks may also emerge from the tufts of the EDJ, as shown in Figure 2L, where there have many edge cracks from the EDJ to

the OES of the enamel.⁸² As for wear resistance, radial enamel owns better wear resistance than decussated enamel types because decussated structure the presence of crystals that are geometrically parallel to the wear surface, resulting in faster wear.

At levels IV and V, note in Figures 2D–E that there has a significant decrease in *H* and *E* can exceed 50% as the probe moves from the enamel surface (*H* > 6 GPa and *E* > 115 GPa) to the EDJ (*H* < 3 GPa and *E* < 70 GPa).⁶⁷ The variation of *H* and *E* on different surfaces (buccal and lingual sides) of the teeth is also obvious. In general, the values in both *H* and *E* are clearly higher along the surface of the lingual cusp than those along the buccal cusp, while the opposite is true in its interior.⁶⁷ Changes in *E* and *H* can be related to the complex structure within the enamel, the degree of mineralization, and composition gradient. The schmelzmuster of individual teeth is constitutive of a combination of enamel types that can prevent catastrophic failure (A typical crack path in enamel thickness is displayed in Figure 2G). At both levels, the wear resistance of the enamel is related to its whole layer thickness.

Sound human enamel has high fracture strength, high stiffness, exceptional toughness, and excellent wear-resistance. The above systematic analysis of the composition-structure-property relationship of natural human enamel has laid the foundation for the development of enamel-like structured materials which are expected to be applied in the field of dental repair.

PREPARATION METHODS OF ENAMEL-LIKE NANOCOMPOSITES

Nowadays, a formidable challenge to construct enamel-like nanocomposites with comparable mechanical properties, excellent biocompatibilities and aesthetics on damaged enamels or biocompatible substrates exists and bothers people. Although there are some published reviews on enamel restoration,^{15,92,93} we firstly described in detail and summarized such systematic strategies of enamel-like nanocomposites. Given the developments in this field over the past decades, the preparation methods of enamel-like nanocomposites can be divided into the following five categories: (i) cell-based enamel tissue engineering, referring to induced proliferation and differentiation of stem cells to obtain bioengineered whole teeth with intact tooth characteristics; (ii) organic matrix-guided enamel crystal growth, mimicking the process where the enamel matrix mediates the formation of natural enamel; (iii) microgel-based microenvironment mineralization, simulating a unique microenvironment where enamel mineral crystals are formed; (iv) mineralization process induced by amorphous phase precursors; (v) physico-chemical methods under extreme conditions (such as high/low temperature, high pressure), as well as mild conditions. Above all, whatever the methods are used to synthesize the enamel-like nanocomposite, the ultimate goal is for clinical application in dental repair. The following are details about the five preparation methods of enamel-like nanocomposites.

Cell-based enamel tissue engineering

Cell-based tissue engineering usually involves stem cells, scaffold materials and associated growth factors which are most likely to restore the physiological structure and the enamel function.^{94,95} In order to better utilize the cell-based tissue engineering to regenerate tooth enamel, it is necessary to understand the different stages during the formation of the enamel from a perspective of tissue development. As shown in Figure 3A, tooth morphogenesis is a gradual process controlled by sequential and reciprocal interactions between the epithelial and mesenchymal tissues: ① the epithelial cells of the dental lamina with odontogenic potential initiate tooth development by sending signal molecules, which are responded by mesenchymal cells; ② epithelial thickening and condensation of the surrounding mesenchyme generate a tooth bud; ③ the enamel knot is the signal center of coordinated morphogenesis appearing in the cap stage; ④ odontoblasts and ameloblasts appear at the end of the bell-shaped stage to differentiate and secrete their respective matrices at the interface of epithelial cells and mesenchymal cells, and then the mineralization happens; ⑤ after the tooth root is formed, the tooth begins to erupt into the mouth.^{96,97} Because of the important potential of stem cells located in the oral epithelium in the process of odontogenesis,⁹⁸ researchers are committed to inducing the differentiation and culture of various stem cells, and fabricating biodegradable scaffolds to culture stem cells and achieve cell-based tooth tissue regeneration.

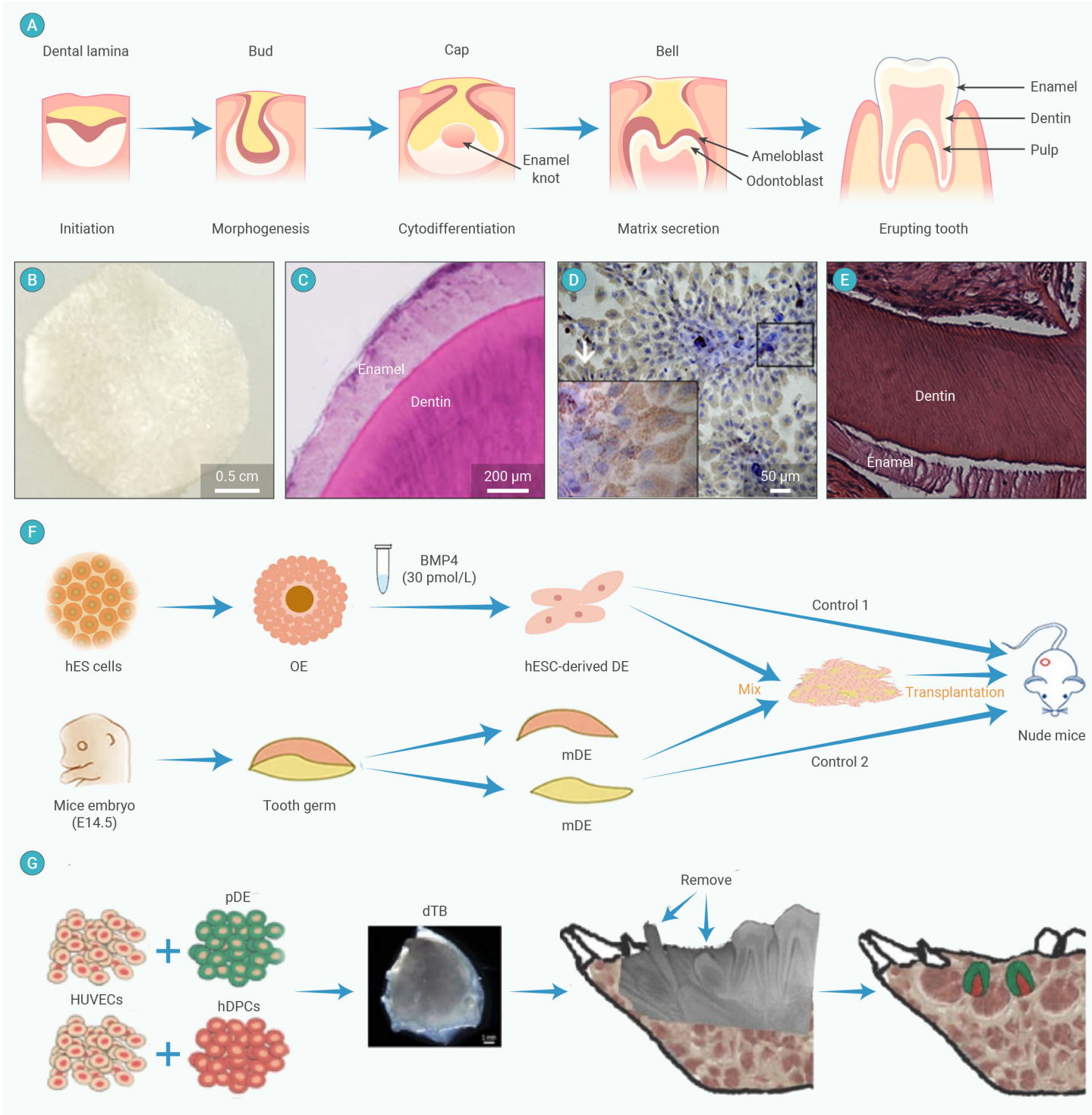


Figure 3. Tooth formation and regeneration process by tissue engineering (A) Tooth formation process. Adapted with permission from ref.⁹⁶ Copyright 2011, Future Medicine. (B) Enamel product after the compaction and partial removal of organic phases. Reproduced with permission from ref.⁹⁹ Copyright 2021, Wiley-VCH. (C) Histological analysis of the crown area in the bioengineered tooth. Reproduced with permission from ref.¹⁰⁰ Copyright 2017, Springer Nature. (D) Acquisition of the characteristics of ameloblasts for the skin epithelial cells under the induction of DPMCs *in vitro*, with some cells displaying a prismatic appearance. Reproduced with permission from ref.¹⁰¹ Copyright 2013, Wiley-VCH. (E) The implant tissues exhibit multiple calcified recombinant tissues including tooth crowns. Reproduced with permission from ref.¹⁰² Copyright 2014, Wiley-VCH. (F) The *in vivo* experimental schematic diagram showing the formation of tooth-like structures introducing hDE-like cells mixed with mDM *in vivo* oral ectoderm (OE). Adapted with permission from ref.¹⁰³ Copyright 2019, Springer Nature. (G) Schematic diagram of dTB (decellularized tooth bud) implantation process. (hDPC, human dental pulp cell); (HUVEC, human umbilical vein endothelial cell); (pDE, pig dental epithelial cell). Adapted with permission from ref.¹⁰⁴ Copyright 2017, SAGE.

Autologous stem cells usually have no problems on host immune responses.⁹⁴ Cultivation or induction of autologous cells related to dental development is important for enamel regeneration. Bazina, F. et al.⁹⁹ used serotonin to induce the formation of enamel-like materials from reconstituted mouse oral epithelial cells *in vitro*. The newly formed crystals were densified under pressure and vacuum to remove partial organic phases, as

shown in Figure 3B. The size of the material is comparable to those of tooth enamel. Autologous transplantation of canine bioengineered tooth germs reconstructed by using germ cells from permanent teeth can regenerate tooth with correct tooth structure (Figure 3C) and function after birth in canines. However, more verification and evidence are required to demonstrate its application in other mammals.¹⁰⁰ In addition, cells of nondental

epithelial-derived were used for the construction of bioengineered teeth. It was shown that dental papillary mesenchymal cells efficiently induced skin epithelial cells in newborn rats and were transformed into functional ameloblasts (Figure 3D).¹⁰¹ The odontogenic potential extracted and cultured dental cell (from postnatal porcine teeth and human third molar wisdom tooth dental pulp) was investigated, and the results indicated that the reconstituted 3D dental constructions formed calcified tissues including enamel (Figure 3E).¹⁰² In vitro expansion of gingival epithelial cells (derived from adult gingival tissue) was used to co-culture with mouse embryonic dental mesenchymal cells, capable of forming bioengineered teeth containing enamel.¹⁰⁵

Growth factors usually released from cells are directly interacted with adjacent extracellular matrix to cell surface receptors to activate various mechanisms and pathways involved in tissue engineering.⁹⁵ That is because platelet-rich fibrin (PRF) is abundant in growth factors, the porcine tooth bud cell-fibrin glue-PRF composite was autologously returned to the original alveolar sockets, and enamel-containing tooth regeneration was achieved after 36 weeks.¹⁰⁶ Sonic hedgehog and Fibroblast growth factor 8 treated human keratinocyte stem cells increased the rate of the tooth-like structures formation after reconstructing human-mouse chimeric tooth germs with mouse dental mesenchyme. Although the regenerated tooth enamel was softer than that of human children, the intact prisms of the neonatal enamel operated in a similar pattern to that of human enamel.¹⁰⁷ Zhang, Y. et al.¹⁰⁸ induced mouse embryonic stem (mES) cells to differentiate into dental epithelium by regulating the function of bone morphogenetic protein 4 (BMP4) by time control. Subsequently, the epithelial cells were combined with mouse tooth mesenchyme and implanted into the mouse kidney capsule, and eventually a complex structure, including teeth, was formed. After that, this study was further broadened that BMP4 was used to induce differentiation of human embryonic stem (hES) cells that become dental epithelium (DE), which was mixed with embryonic mesenchymal teeth (mDM) from mice born at 14.5 days and transplanted into the kidney capsule of mice (Figure 3F), forming teeth with enamel and dentin 30 days after *in vivo* transplantation. However, if only using (hESC-derived DE) or (mDM), the result was not repeatable.¹⁰³

Three-dimensional scaffolds with biocompatibility, degradability and excellent mechanical properties can provide ideal environmental conditions for stem cell proliferation, differentiation and migration to promote the formation of new tissues.^{109,110} Duailibi, S. E. et al.¹¹¹ seeded cultured rat dental bud cells on a biodegradable polymer scaffold (PGA/PLLA) and (PLGA), and then implanted in a jaw of an adult rat hosts to form a small, organized bioengineered tooth crown containing tooth enamel. Shinmura, Y. et al.¹¹² demonstrated that sub-cultured epithelial cell rests of Malassez bound to primary pulp cells transplanted onto collagen sponge scaffold formed the enamel-like tissue after 8 weeks of transplantation. Furthermore, sub-cultured odontogenic epithelial cells combined with fresh dental mesenchymal cells or sub-cultured enamel organ epithelial cells combined with primary dental pulp cells were implanted onto collagen sponge scaffolds, respectively, and both culture systems produced ameloblast-like cells.^{45,113} Compared to polymer and collagen sponge scaffolds, the use of decellularized natural tissue scaffolds not only retains many characteristics of natural tissues, but also promotes the differentiation of reseeded cells.¹¹⁴ As shown in Figure 3G, dTBs scaffolds made from un-emerged porcine tooth buds (TBs) were used to guide the differentiation of reimplanted cells, forming a completed bioengineered tooth. This demonstrated the possibility of whole tooth regeneration by using pig of natural dTBs scaffolds.¹⁰⁴

In a word, the shape and size of bioengineered teeth obtained in mouse, rat, pig, and canine models hardly reach the level of natural formation in the organism, letting alone their mechanical properties. But this brings a new concept to restorative dentistry. The introduction of stem cell-based treatments in the clinic promises to revolutionize regenerative dentistry. However, stem cell-based tissue engineering for regenerative dentistry still faces many problems, such as how to obtain sufficient numbers of human stem cells, how to maintain the long-term survival and differentiation ability of the implanted cells, and how to shorten the formation time of bioengineered teeth.

Organic matrix-guided enamel crystal growth

Many biologically generated organic matrices play a key role in regulating

the formation processes of inorganic materials, especially during enamel formation.¹¹⁵ The organic matrix, secreted by ameloblasts, is essential for controlling the formation of enamel apatite crystals and consists of amelogenin, enamelin, and ameloblastin.¹¹⁶ Amelogenin, consisting of 90% of the total enamel proteins, are comprised of a charged C-terminal telopeptide (C-Ame) that mediates parallel alignment of crystals and an N-terminal amelogenin peptide with a central domain (N-Ame) that is essential for the amelogenin self-assembly into amyloid-like microribbons to template the growth of HAP crystals.^{44,117-120} The nanoribbon template is assembled by amelogenin nanospheres formed by hydrolytic cleavage of amelogenin in C-Ame, enabling the formation of fibrous apatite nanocrystals with high *in vitro* aspect ratios.¹²¹⁻¹²⁴ In the view of the extraordinary contribution of amelogenin in enamel crystal formation, it or its fragments and its cleavage products, as well as analogues that mimic the function of amelogenin presented on the enamel surface, are very important for achieving biomimetic mineralization. Typical biomimetic mineralization takes place in simulated saliva, which not only has buffering capacity but also plays an active role in enamel remineralization as a carrier of alkaline ions such as fluoride, calcium and phosphate, where organic matrices are adsorbed onto etched enamel surfaces to achieve ion enrichment and direct themineral crystal growth.¹²⁵ The following are specific strategies to achieve biomimetic mineralization through proteins, peptides, amino acids, dendritic macromolecules, etc.

It is both uneconomical and difficult to extract and apply amelogenin because it is largely degraded by proteases during the development of enamel maturity.¹²⁹ Thus, the *in vitro* self-assembly and mineralization behavior and characteristic of the artificial amelogenin were investigated, demonstrating the formation of rod-shaped HAP crystals after 3 days of induction.¹³⁰ It was shown that modified amelogenin (insert the mineralized AMTN-derived motif SSEEL in its internal and c-terminal ends) could also promote hydroxyapatite growth.¹³¹ Amyloid-like amelogenin was used to mimic the N-Ame and C-Ame within natural enamel to form a (PTL/C-AMG) matrix on the enamel surface (Figure 4A), inducing the *in vivo* and *in vitro* synthesis of HAP crystals (Figure 4B).⁴⁴ In addition, this study found that some water-soluble matrices (WSM) containing proteins and organic macromolecules could promote biomineralization. Li et al.¹³² used the extracted nacre WSM as a template to successfully promote the good arrangement of HAP nanorods and accelerate the growth of HAP crystals. It was inferred that the two carboxyl oxygen atoms of WSM protein like claws could cooperate with calcium ions and play an adhesive role. The experimental results indicated that WSM could isolate from abalone water-soluble substrate (AWSM) to efficiently induce remineralization of HAP. Moreover, the effect of remineralization increased with increasing AWSM concentration.¹³³ Besides mediating hydroxyapatite nucleation, the protein also mediates fluorapatite nucleation. Fluoride is a well-known common preventive agent to reduce and prevent enamel demineralization. This is due to fluoride entering the apatite structure by replacing the hydroxide ions in HAP crystals to form fluorapatite (FHAP), which has lower solubility and more resistance to low pH than HAP.¹³⁴⁻¹³⁷ In a study on fluorapatite, the solution of molecules of elastin-like recombinase (ELR) could form a membrane on the substrate that was incubated in the mineralized solution under physiological conditions (Figures 4C-D). The ELR matrix not only facilitated the formation of fluorapatite phases with atomic ratios similar to those of apatite crystals and hard dental tissues, but provided a template to the growth of unique hierarchical mineralized structures as well (Figures 4E-H).¹²⁶

Because peptides have high biocompatibility, structural stability, and sequence diversity, they have important applications in the biomineralization process.¹³⁸ Peptides P26 and P32 are synthetic peptides that retain important functional domains of natural amelogenin. As shown in Figure 4I, a solution of the peptide is coated on the demineralized enamel window, left to dry and then immersed in the artificial saliva for mineralization. The amelogenin-inspired peptides controls and guides nucleation, while controlling the HAP crystals being formed (size and orientation), ultimately enabling cyclic remineralization of the controlled thickness (Figure 4J).¹²⁷ ADP5 is a peptide consisting of 22 amino acids that reflects the unique characteristics of the natural protein amelogenin. A further shortened derivative peptide consisting of 15 amino acids (ShADP5) was used as the active ingredient to form the mineralized layer, producing a dense and fairly smooth HAP mineralized

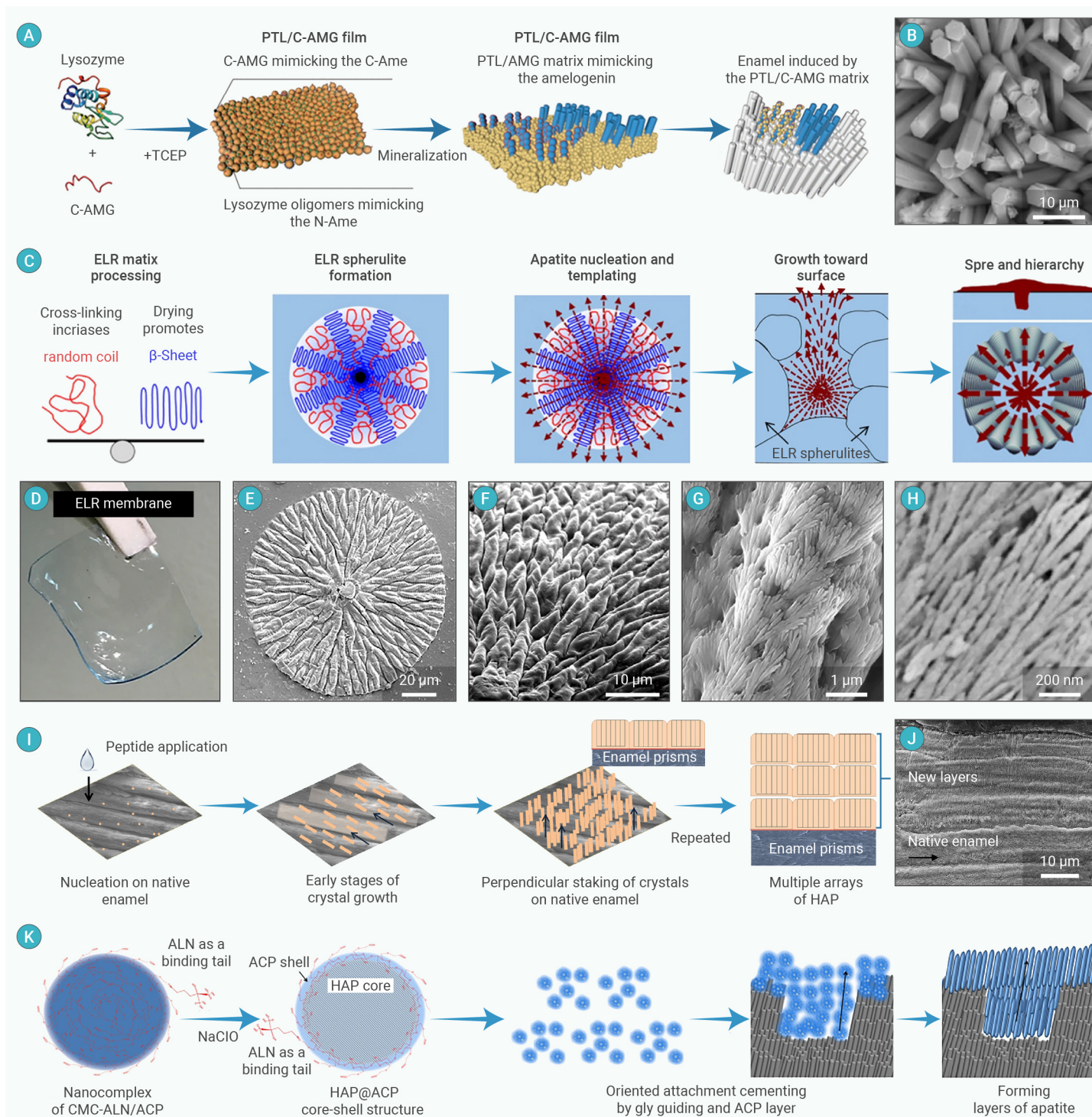


Figure 4. Schematic diagram and SEM images of regenerated enamel of biomimetic mineralization guided by protein matrix (A) Schematic exhibition of the phase-transited lysozyme/synthetic peptide (PTL/C-AMG) matrix to mediate on enamel for in situ remineralization. (B) SEM image of the newly formed HAP layer. Adapted with permission from ref.⁴⁴ Copyright 2020, Wiley-VCH. (C) A schematic diagram demonstrating the mechanism of ELR matrix assembly and calcium phosphate nucleation & growth. (D) Digital photograph of a transparent, flexible, and robust statherin-ELR membrane before mineralization. (E–H) SEM images of the top surface of a hierarchically ordered Arg-Gly-Asp-Ser-ELR membrane with different magnification after mineralization. Reproduced with permission from ref.¹²⁶ Copyright 2018, Springer Nature. (I) Schematic illustration depicting the regrowth of enamel-like HAP layers mediated by peptides. (J) Enamel-like growth in situ under the action of amelogenin-inspired peptides. Adapted with permission from ref.¹²⁷ Copyright 2018, American Chemical Society. (K) Schematic representation of the role of Gly in the linear alignment of HAP@ACP nanoparticles and formation of apatite crystals. Adapted with permission from ref.¹²⁸ Copyright 2017, Springer Nature.

crystal layer with 10 μm -thickness.¹³⁹ The etched enamel surface was treated with acid E125 polypeptide. After mineralization, the resin was penetrated into the regenerated enamel layer, enabling the inorganic-organic composite with excellent mechanical properties.⁴² Combining natural antimicrobial materials (such as tannic acid (TA) and carboxymethyl chitosan (CMC)) with peptides for bionic mineralization is also a promising strategy. A modified peptide

inspired by saliva-acquired pellicle (SAP) was constructed by replacing phosphoserine with aspartic acid residues and can be specifically adsorbed to HAP. The modified peptide is linked to natural cysteine known as SAP-TA, which has specific adsorption properties, antibacterial properties and promotes mineralization. Experiments have shown that if iron ions are added to TA cross-link to form SAP-TA/Fe(III), a better biomimetalization effect will

be achieved.¹⁴⁰ CMC with antimicrobial properties is a suitable stabilizer for amorphous calcium phosphate (ACP) nanoparticles.¹⁴¹ The chimeric synthesized peptide shows that it is effective to remineralize the demineralized enamel. It consists of two peptide chains with functional motifs and a linking part, one of which can direct the degradation of CMC/ACP particles by NaClO to form orientated ordered crystals, while the other one can bind to the enamel surface.¹⁴² What comparable to the above design is the direct modification of the antimicrobial peptide so that the active molecule phosphoserine-histone 5 (SP-H5) with remineralization ability can be designed and synthesized. This is because H5 is an antimicrobial peptide naturally produced in saliva by the human parotid and submandibular glands, while SP is a key part determining the binding to hydroxyapatite. Therefore, the active molecule can be safely applied to prevent dental caries and promote mineralization effectively.¹⁴³ Notably, researchers constructed a self-standing apatite film made up of *c*-axis oriented nanorods at the gas-liquid interface by means of method (two-step crystal growth) controlled by artificial peptides and D-aspartic acid.¹⁴⁴ The simpler peptides such as 8DSS (DSS, Aspartate-serine-serine).¹⁴⁵ Peptide-7 (Asp-Asp-Asp-Glu-Glu-Lys-Cy) have been reported to attach to the enamel surface and induce mineralization.¹⁴⁶

As a basic component of proteins, amino acids have a small molecular weight compared to proteins and peptides, and their role in mediating crystal orientation in biomimetic mineralization has been widely studied.^{41,128} Apatite particles (20 nm) pre-adsorbed on the enamel surface were assembled by glutamate (Glu), which accounted for 15~20% of the amino acid composition of amelogenin, to achieve enamel crystal regeneration. This is attributed to the strong calcium ion complexation ability of Glu, whose carboxyl groups at both ends provide active sites to bridge two calcium ions. Glu molecules can be selectively adsorbed on the surface of apatite particles (001) and induce their directional aggregation using the terminal carboxyl groups, leading to their ordered aggregation or crystallization along the *c*-axis.⁴¹ In addition, there have been reports on the glycine-guided directed crystallization of nanoparticles, as shown in Figure 4K, starting with the preparation of CMC/ACP incorporating alendronate (ALN), followed by degrading the CMC/ALN matrix with hypochlorite to obtain core-shell nanoparticles (HAP@ACP) consisting of a HAP nucleus and an amorphous outer layer, culminating in ordered rod-like apatite crystals in the presence of glycine.¹²⁸ It is noteworthy that the interaction between the core-shell nanoparticles and the enamel surface is achieved by replacing two phosphate groups on the enamel surface by two phosphate groups of the ALN molecule.¹⁴⁷

Dendrimers are polymeric nanomaterials known as artificial proteins, which are nanoscale monodisperse macromolecules with controlled three-dimensional structures, unique physical and chemical properties.^{147,148} Since polyamide (PA) dendrimers have the potential to work as organic templates for controlling the crystal growth during the crystallization of hydroxyapatite, dendrimers with various surface groups have been developed successively.^{149,150} The carboxylated PA dendrimers have a notable effect on the morphology of hydroxyapatite nanostructures. However, although PA-COOH can be used as a template for remineralization, it is not strong enough to bind to the enamel surface.¹⁵⁰ However, the carboxyl group has a slightly weaker affinity for calcium ions in HAP compared to the phosphate group, which can form ligand bonds with calcium ions. Each PA-PO₃H₂ molecule has an average of 26 phosphate groups, and its strong adsorption capacity controls the subsequent accumulation of calcium and phosphate, thereby generating new crystal layers.¹⁴⁸ The aminated PA also achieves effective remineralization on the demineralized enamel surface, and the smoother surface of the regenerated enamel reduces the adhesion of *Streptococcus mutans*.¹⁵¹ To immobilize (PA-COOH) on the surface of the enamel sheets, ALN-linked dendritic, i.e., ALN-PA-COOH were applied and mineralized in artificial saliva environment for 28 days to be able to induce HAP mineralization on acid-etch-treated enamel substrates. The newly formed HAP bundle crystals with parallel nanorod-type are almost identical in morphology and are oriented essentially parallel to the orientation of the substrate enamel crystals.¹⁴⁷

In general, the crucial thing to initiate the biomimetic mineralization process is not only enriching the calcium ions and guiding the nucleation and growth direction of the crystals through protein matrices and analogues, but more importantly for them to adsorb on the damaged enamel surface to

occupy the most favorable position. Although it is possible to reconstruct hydroxyapatite mineral layers *in vivo* and *in vitro* using organic matrices, it still differs somewhat from the case of crystal growth under real natural conditions, and it is even more difficult to replicate the complex hierarchical structure of enamel, mainly due to the fact that tooth enamel growth in living organisms is an extremely complex procedure including gene expression, protein secretion-folding-assembly, mineral growth, protein degradation, and slow physiological process.⁹²

Microgel-based microenvironmental mineralization

Since enamel apatite initially forms in a gel-like environment rich in organic matrices, the gel system simulates the unique gel-like microenvironment where biomineralization occurs more vividly than mineralization in simulated saliva or body fluids. Cao and Li et al. devised a hydrogel biomimetic mineralization model (Figure 5A) that achieved an unidirectional supply of ions by placing enamel slices on the lower part of a bilayer agarose hydrogel several millimeters thick containing calcium ions and injecting a solution containing fluorine and phosphate above the hydrogel.^{152,158} After about a week of mineralization, it is interesting to note that adjacent crystal rods spontaneously fuse to form a well-aligned crystal layer closely resembling the surface of natural enamel (Figures 5B-C). It is noteworthy that due to the formation of mineral precursors of the agarose fiber-mineral complexes, the hydrogel acts as a stabilizer of mineral precursors and prevents their transformation into crystals. Later, they developed an electrophoresis-assisted agarose hydrogel system (Figure 5D) that rapidly regenerated the microstructure of dental tissue in only 12 hours because of electromigration movement of ions in the hydrogel due to the presence of an electrical potential difference.¹⁵³ This protocol first restores the dentin with the assistance of electrophoresis and subsequently regenerates needle-like hydroxyapatite crystals similar to enamel on its surface, which promotes an integrated restoration of dentin and enamel (Figure 5E). As shown in Figure 5F, an anodic aluminum oxide (AAO)-assisted gel system was developed, which can attract calcium ions due to the formation of polydopamine (PDA) after coating dopamine on a titanium substrate. While the double-layer gel in the system directs the unidirectional migration of ions to the substrate, the porous AAO membrane strictly limits and directs the inflow of ions, which ultimately leads to the formation of highly oriented HAP crystals.¹⁵⁴ Fletcher, J. et al.¹⁵⁹ prepared a film formed by a viscous suspension containing ACP particles, ethanol and poly(vinylpyrrolidone) (PVP) onto dental slices impregnated with artificial saliva, immediately forming a hydrogel mat, which can then be mineralized in a humid environment to produce crystals of fluorinated hydroxyapatite. The chitosan/calcium phosphate microgel model developed by Marin et al.¹⁶⁰ promotes calcium phosphate nucleation and the growth on demineralized enamel surfaces, which can effectively enhance remineralization of early caries sites. In addition, reports of bionic synthesis of fluorapatite layers on human enamel using gelatin mineralization models under *in vivo* conditions have also shown that surface defects in enamel can be effectively treated.¹⁶¹ In order to keep the hydrogel in good contact with the teeth and free from other substances in the mouth, agarose hydrogel was loaded into customized trays and applied to the rabbits, resulting in oriented densely parallel aligned HAP layers.¹⁶²

Biomimetic mineralization induced by the involvement of organic matrix in the gel microenvironment is a well-suited strategy that more closely resembles the enamel synthesis process of the organism itself than the above constructed microgel environments that do not contain organic matrix to guide mineralization. Cao et al.¹⁶³ used enamel matrix derivatives (EMD) extracted from porcine fetal teeth in an agarose hydrogel model to induce the formation of highly crystalline hydroxyapatite. As shown in Figure 5G, a hydrogel system containing myelinogenin (rP172) was applied to treat artificial enamel caries by remineralizing them.¹⁵⁵ Ruan et al.¹⁵⁶ developed chitosan (CS) hydrogel containing amelogenin (AMEL) protein for enamel reconstruction (Figure 5H). The continuous growth of crystals in the CS-AMEL (rP172) hydrogel environment formed a tight bond between the new growth layer and the enamel, which prevented secondary caries and provided effective protection of the tooth. It shows that full-length amelogenin impedes the formation of HAP, leading to nanoscale defects in HAP crystals, reduced crystallinity, and softer texture than normal enamel. The addition of

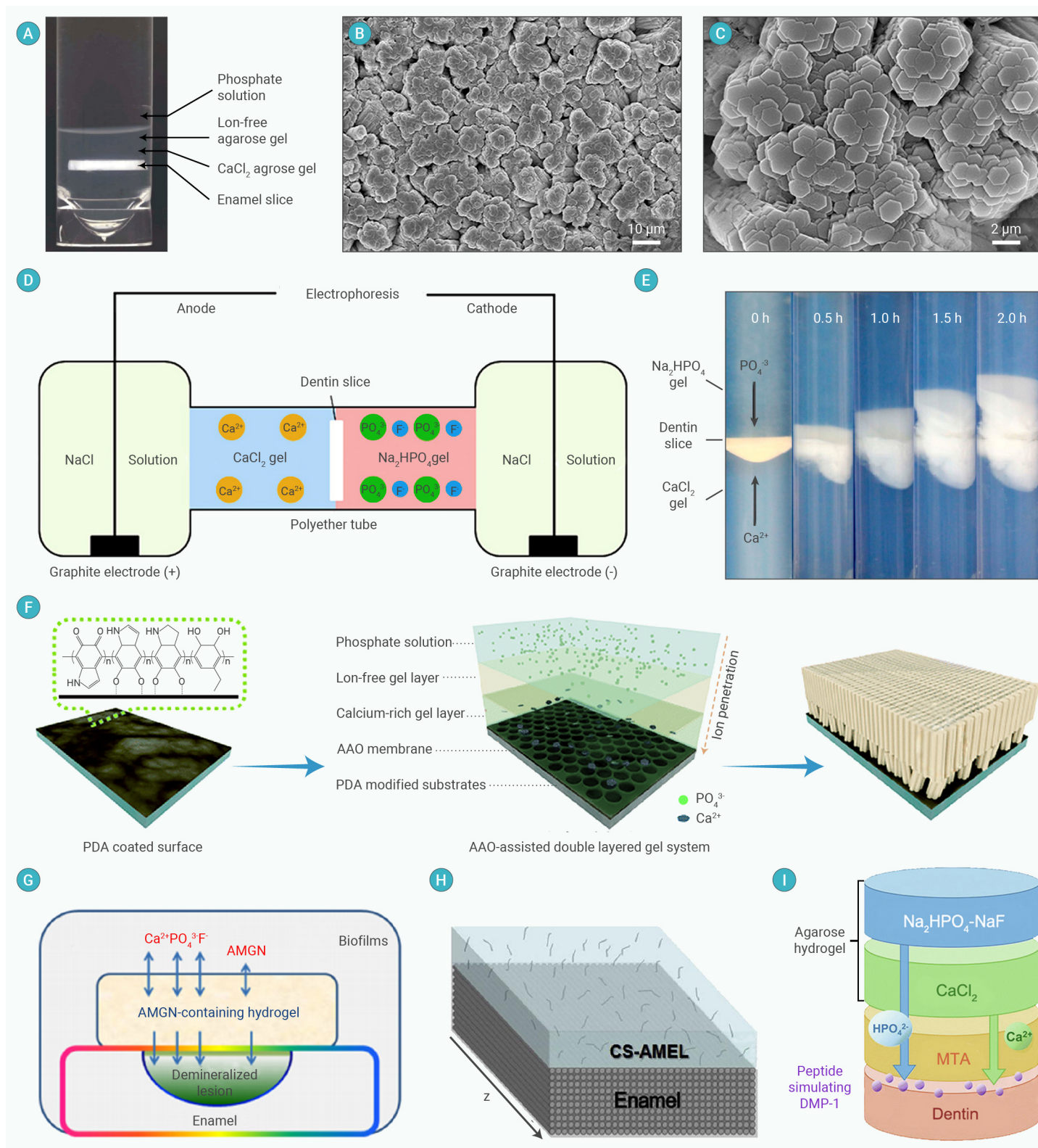


Figure 5. Schematic and electron microscope images of enamel regeneration by a gel microenvironment mimicking a biomineralization process (A) Agarose hydrogel biomimetic mineralization model, (B, C) SEM images with different magnification of the regenerated mineralized tissue after culture for 6 days. Reproduced with permission from ref.¹⁵² Copyright 2013, American Chemical Society. (D) Schematic representation of a hydrogel mineralization system assisted by electrophoresis, (E) Thickness of the mineral layer formed on the dentin substrate with the increasing of time. Adapted with permission from ref.¹⁵³ Copyright 2014, American Chemical Society. (F) Schematic diagram of the AAO-assisted double-layered gel system (ADGS). Adapted with permission from ref.¹⁵⁴ Copyright 2019, Royal Society of Chemistry. (G) Schematic representation of tooth restoration by hydrogel containing amelogenin (rP172). Reproduced with permission from ref.¹⁵⁵ Copyright 2012, SAGE. (H) Schematic diagram demonstrating of the CS-AMEL hydrogel for enamel repair. Reproduced with permission from ref.¹⁵⁶ Copyright 2013, Elsevier. (I) Schematic diagrams of the DMP-1@MTA@AHBMM biomimetic mineralization model. Adapted with permission from ref.¹⁵⁷ Copyright 2019, Royal Society of Chemistry.

MMP-20 to CS-AMEL hydrogels prevented unwanted protein blockage in apatite crystals.^{129,164} As shown in Figure 5I, a more complex model enables

the tissue regeneration of enamel-like fluorinated hydroxyapatite crystals in the (DMP-1@MTA@AHBMM) biomimetic mineralization model: ① mineral

trioxide aggregate (MTA) releases calcium ions in a stable and continuous manner to induce the ACP formation, ② oligopeptide stimulating dentine matrix protein 1 (DMP-1) is used to stabilize ACP, and ③ agarose hydrogel bionic mineralization template (AHBMM) contributes to the nucleation and growth of HAP crystals.¹⁵⁷ Fang, Z et al.¹⁶⁵ used a mimetic enamel matrix protein consisting of a modified leucine-rich enamel protein peptide (mLRAP) and a non-enamel protein analogue (NAA) to regenerate enamel. Hydrogels can also be formed upon addition of calcium chloride to self-assembled oligopeptide amphiphiles (OPA) solutions.¹⁶⁶ OPA is more stable than natural proteins and induces the formation of organized hydroxyapatite crystal bundles on enamel, and when it is repeatedly exposed to solutions containing calcium and phosphate ions, amorphous calcium phosphate can nucleate and grow along the self-assembled OPA nanofibers.

Amorphous phase-induced mineralization

Amorphous phase is thought to be a temporary precursor of biomineralization in numerous tissues, with a unique structure and many active sites. In tooth enamel, it is the transient ACP phase that comprises the initial mineral which transforms into apatite crystalline minerals over time.^{9,167} It has been verified that the growth units of hydroxyapatite crystals are clusters rather than simple ions. The nano clusters are real in SBF solutions and randomly aggregate into spherical objects with a diameter of around 30~100 nm so that the typical ACP morphology can be formed, acting as building blocks during the formation of ACP and the clusters.¹⁶⁸⁻¹⁷¹ However, the conversion mechanism of ACP to HAP is controversial, possibly including dissolution recrystallization,¹⁷² reorganization of Posner's clusters,¹⁶⁸ formation of crystalline phases directly within the ACP phases,¹⁷³ and surface-mediated transformation.¹⁷⁴ In addition, the exact location of nucleation has also been debated, and according to previous reports,¹⁷⁵⁻¹⁷⁷ nucleation may occur on the surface of ACP spheres, at the ACP-solution interface or in the interior of ACP. Although the ACP instability poses a problem for its production, these ACP preparation methods (including wet chemical methods, mechanical and thermal techniques) have been successfully developed in recent years, which will facilitate studies on the biomimetic mineralization of tooth enamel.^{178,179}

Artificial enamel can be induced by using pressed amorphous calcium phosphate nanoparticle substrates in a near physiological solution containing fluorine.¹⁸³ In the initial stage of mineralization, ACP nanoparticles are slightly dissolved on the substrate surface and transformed to 10~20 nm HAP nanoparticles directly with low crystallization. Therefore, the HAP nanorods with highly oriented structure can be formed on the HAP interlayer with poor crystallization via geometric selection. Notably, the orientation and crystallinity of HAP nanoparticles were observed to be enhanced with the gradual increase of fluoride concentration during the growth, facilitating the growth of HAP nanorods along their *c*-axis. In further studies on amorphous substrates, it was found that the low doses of fluorine have noticeable influence on the growth behavior of octacalcium phosphate (OCP) and hydroxyapatite (HAP), as shown in Figure 6A. The transformation of OCP to HAP is due to the preference for a thermodynamically more stable state. The concentration range of the fluoride affecting the growth of both crystals was accurately determined. The OCP crystals with plate type were completely substituted by closely arranged HAP nanorods at the threshold concentration.¹⁸⁰

The biomineralized crystalline-amorphous boundary can be cleverly mimicked during natural enamel development. As shown in Figure 6B, Shao and Liu et al.¹⁸¹ prepared the materials consisting of calcium phosphate ion clusters (CPICS) to fabricate precursor layers so that the epitaxial growth of enamel apatite crystals can be induced. Specifically, a gel-like drop of CPICS is placed on an etched enamel plate and the entire enamel surface is flattened. With the volatilization of triethylamine and ethanol at room temperature, CPICS is converted into ACP, thus achieving the conversion of the continuous ACP layer formed on the natural HAP into an artificial HAP layer (Figures 6C-E). The HAP layer regenerated by epitaxial growth can be bonded to the natural tooth enamel to form a permanent restoration, and the method can be recycled to easily control the thickness of the restoration layer.

By adding simple inorganic ion, polymer, and molecules, ACP stability can be improved, facilitating the manufacture of apatite crystals for the restoration of tooth enamel. Magnesium ions (Mg^{2+}) can stabilize ACP and are widely used in biological fluids to regulate the crystallization of HAP.^{184,185} The

Mg-rich amorphous layer growing on a polymer substrate is crucial in the following epitaxial growth of Mg^{2+} (FPN-M) arrays, and acts as an interlayer phase between the PVP/PAA matrix and the minerals to strengthen the interfacial bonding after mineralization.¹⁸⁶ As shown in Figure 6F, the in vitro remineralization of the enamel with white spot lesions was achieved by a transportation system (PAA-ACP@aMSN) of amine-functionalized expanded pore silica (aMSN) which is loaded with amorphous calcium phosphate (PAA-ACP).¹⁸² ACP can also be stabilized by the formation of composite materials or nanocomposites. Carboxymethyl chitosan and lysozyme-coated amorphous calcium phosphate nanogels (CMC/LYZ-ACP) enable controllable delivery of ACP to form a prismatic enamel layer. The ACP transformed into hydroxyapatite by being separated from the negatively charged CMC/LYZ-ACP nanogel with a spherical structure in high NaCl concentration.¹⁸⁷ Casein phosphopeptide (CPP) as a biomolecule can stabilize ACP because CPP-amorphous calcium fluoride phosphate (CPP-ACFP) can stabilize the release of fluoride, phosphate, and calcium ions on the outer-surface of tooth and facilitate remineralization.¹⁸⁸

Physicochemical methods

Although the research on the biomimetic mineralization of enamel has shown unique advantages, the preparation of large quantities of apatite in a short time still relies on physicochemical methods. The reaction time and temperature of hydrothermal path can control the crystal growth and the aspect ratio with good repeatability, wide reaction temperature range and simple operation procedure. The hydrothermal method that does not use a template but only introduces sodium bicarbonate as a pH buffer can control the nucleation and growth during crystallization to obtain hydroxyapatite nanorods with uniformity and high purity.¹⁸⁹ Manafi, S et al.¹⁹⁰ synthesized one-dimensional hydroxyapatite nanorods with hexagonal heads and rods with a diameter of about 25~50 nm and a length of around 120 ± 30 nm by hydrothermal method. Jin et al.¹⁹¹ used sodium citrate as an additive to regulate the morphology of HAP, prepared hydrophilic HAP at 150°C, and investigated the effect of sodium citrate/calcium ion molar ratio (CT/CM) on the structure, size and morphology of HAP nanorods. Cao et al.¹⁹² successfully synthesized HAP nanorods by a biomolecule-assisted hydrothermal method using adenosine triphosphate (ATP)-derived $ATPNa_2$ as a reactant, offering the possibility of its application in biomedical fields. In addition, HAP nanorods can also be fabricated by a hydrothermal method using a template, which is faster and easier to obtain uniform crystal sizes than direct synthesis. Wang et al.¹⁹³ synthesized HAP nanorod arrays with oriented structures using hillebrandite $[Ca_2(SiO_3)(OH)_2]$ with nanobelts-type as solid templates, which transformed into the shape of nanorods by internal splitting along the stripe direction during hydrothermal process. The newly formed nanorods have the diameter of around 10~20 nm and length of 100~200 nm. Zhang et al.¹⁹⁴ mediated the conversion of montmorillonite to hydroxyapatite by organophosphate surfactants and gelatin under hydrothermal conditions. The organophosphate groups therein destabilized the montmorillonite phase and tended to split it into a more stable rod-like hydroxyapatite phase, while the gelatin made the HAP nanorods more compact and better aligned. However, the aforementioned hydrothermal synthesis methods make it difficult to build enamel layers directly on the enamel surface or on biocompatible substrate. Li et al.¹⁹⁵ prepared fluorapatite array on porous zirconia ceramic disk after surface pretreatment by hydrothermal method, and then infiltrated it with composite resin to achieve a tight connection between highly ordered FHAP array and zirconia substrate.

To avoid extreme reaction conditions like high temperature, high pressure and uncommon pH, some milder methods have been developed in recent years. Yin et al.¹⁹⁹ established an aqueous solution method for constructing the prismatic structure of fluoridated hydroxyapatite directly on the surface of natural tooth enamel. Specifically, the etched enamel was placed in a suspension containing HEDTA-Ca, KH_2PO_4 and KF, and bathed in water for 8 days at 37°C. The pretreated Ti plates were immersed in ion-doped pH-controlled 7.4 phosphate buffer solution at 60°C. FHAP crystals with a diameter of 10~20 nm, good organization, and tight alignment were grown after two weeks of immersion.²⁰⁰ Zhuang et al.²⁰¹ synthesized highly orientated hexagonal HAP crystals along the *b*-axis with an average diameter of 10 μm at the gas-liquid interface through an enzyme reaction between urea and

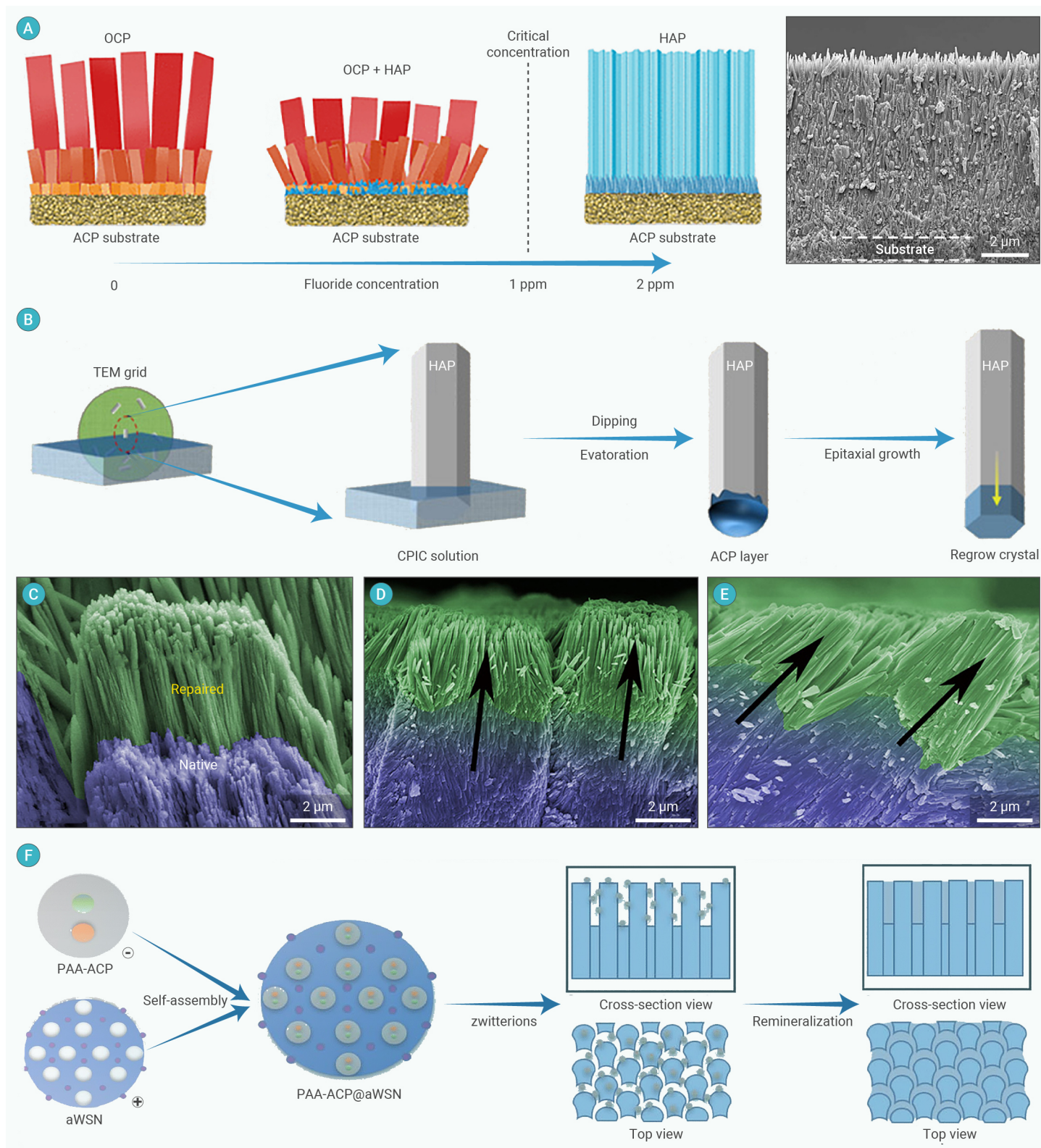


Figure 6. Repair of enamel due to amorphous-crystalline phase transition (A) Materials evolution growing on an ACP substrate with different fluoride concentration. Adapted with permission from ref.¹⁸⁰ Copyright 2018, American Chemical Society. (b) Schematic diagram of the epitaxial growth of crystalline HAP from the construction of the amorphous frontier on its surface. Reproduced with permission from ref.¹⁸¹ (C–E) SEM images of the native and repaired enamel, (C) and enamel rods with different orientations (D, E). Reproduced with permission from ref.¹⁸¹ Copyright 2019, AAAS. (F) Schematic diagram for the synthetic route of PAA-ACP@aWSN and its remineralization mechanism. Reproduced with permission from ref.¹⁸² Copyright 2019, Springer Nature.

urease after the starting solution was continuously heated at 50°C for 96 h. Xu et al.²⁰² prepared enamel-like composite films of hydroxyapatite and chondroitin sulfate by a solution-air interfacial method at 80°C. Wang et al.¹⁹⁶ used rotary evaporation (70°C) for the rapid regeneration of enamel-like crys-

tals, which is due to the enamel sheet repeatedly entering and exiting the mineralized solution during the rotation. When the enamel sheet is rotated out of the mineralizing solution, evaporation causes a sharp increase in the local concentration of the mineralizing solution adsorbed on the surface of

the enamel sheet to promote heterogeneous nucleation, which eventually leads to a uniform and orderly growth of HAP crystals. If silk fibroin (SF) as a heterogeneous nucleation template is added to the mineralization solution, the size of the regenerated crystal is smaller than that without addition, but the method of rotary evaporation is extremely advantageous in terms of the mineralization effect.

Layer-by-layer (LBL) method is a flexible and ingenious technique that combines seemingly contradictory properties in enamel-like nanocomposites and gives them the potential to outperform natural properties. Therefore, LBL method can be used to assemble bionic enamels with excellent performance.^{203,204} As shown in Figure 7D, firstly zinc oxide nanorod blanket was grown on silicon substrate by hydrothermal method, and then the polymer matrix was deposited around it for many times to achieve cross-linking to obtain a monolayers composite film (ZnO/LBL)_n. The polyelectrolyte produces a hydrophilic layer on the top of the ZnO blanket to smooth the surface, which supports the deposition of new ZnO nanoparticles so that the ZnO nanorods can be repeated in the same growth stage. This realizes the *in vitro* replication of enamel columnar nanocomposites benefiting from the interface interaction between the organic-inorganic layers.⁴⁶ Similar to the above method, Wei et al.²⁰⁵ assembled rutile titanium dioxide nanorods, chitosan, and graphene oxide (GO) layer by layer to form artificial enamel materials with organic-inorganic structures and controllable thickness. It is important to note that GO is rich in hydroxyl and carboxyl groups, dangling bonds and defects to offer numerous bonding spots for the polymetric matrix and TiO₂ nanorods so that a strong interlayer bonding can be achieved. Unlike the above two examples, the stacked fluorapatite nanorod blanket prepared by Wong et al.²⁰⁶ is a biomimetic antibacterial adhesion enamel material that has similar composition to natural enamel. And the polyelectrolyte matrix interlayer was deposited by immersion method instead of spin coating. It is predictable that LBL technology will break the limitation of small area and small size of bionic enamel material, thereby opening up a broader application space.

Lasers have a thermal, photochemical and bio-stimulatory effect on biological tissues and have been used in a variety of surgical applications. The rapid mineralization of enamel was achieved by using the synergistic effect of laser and chelating agent. The laser not only accelerates the mineralization of the crystals, but also allows precise control of the growth of fluorapatite crystals. The 15 µm thick fluorapatite (FHAP) crystals are densely grown on the enamel substrate within twenty minutes, which offered the possibility of bionic restoration of tooth enamel.¹⁹⁷ However, this method has obvious disadvantages in practice, such as the tendency to cause the overheating of the pulpal neuron cells of the laser light source and the permanent black embedded graphite layer that makes the mineralized teeth lose their aesthetic appearance and appear light black. Chen et al.²⁰⁷ used the ablation phenomenon of laser at atmospheric pressure and room temperature to irradiate α-tricalcium phosphate (α-TCP) into particles and insert them into the interstices of enamel rods to restore hardness. The deposited α-TCP layer was hydrolyzed by artificial saliva to form HAP films for the purpose of enamel restoration.

Electrodeposition is an effective method for the preparation of apatite coatings, which is expected to repair enamel lesions. Wei et al.²⁰⁸ used electrochemical methods to control the morphology of HAP precipitated on the cathodic titanium surface by controlling the concentration of ions in the electrolyte, and obtained a uniform coating after 2 h of electrochemical treatment. Liao et al.²⁰⁹ successfully prepared a coating of needle-tipped hydroxyapatite with a thickness ranging from 1 µm to 10 µm by placing the etched enamel near the working electrode at 55°C. Zhang et al.²¹⁰ prepared the HAP coating by electrodeposition on a flexible mesoporous graphene/single-walled carbon nanotubes hybrid film. By pretreating the carbon film, the size and composition of the HAP can be regulated. The carbon film and electrodeposition processes are combined to prepare independent, flexible HAP composite films that can be used as patches and strips for enamel repair.

In recent years, 3D printing (additive manufacturing) technology has been gradually developed and applied, which has the prospect of being widely used in medicine to create the main supporting parts of missing or damaged organs and tissues. And of course, 3D printed teeth are also being studied accordingly. Zirconia ceramic dental implants prepared by 3D inkjet printing

are prone to brittle fracture due to the high hardness of the sample.²¹¹ The 3D printed resin teeth exhibit loaded vertical fracture of the cusps without chipped deformation, which provides sufficient fracture resistance to artificial teeth.²¹² Zhao et al.¹⁹⁸ used HAP nanorods mixed with resin-based composites as inks to obtain the crowns with high precision and multi-scale highly ordered HAP structures through extrusion-based 3D printing technology (Figure 7E), which can provide customized components for clinical dental restorations. The 3D printing technology has completely changed the traditional concept of preparing or restoring dental tissues in a bionic simulation. However, achieving high speed printing with a wider range of materials to build highly reliable prostheses remains a huge challenge associated with production practices.²¹³

Plasma spraying is an effective technology to modify the surface of a material, which enhances the resistance to surface wearing, corrosion, and oxidation under high temperature. Zheng et al.²¹⁴ deposited hydroxyapatite coatings on stainless steel substrates by suspended plasma spraying, controlled the temperature of the plasma flame by varying the magnitude of the arc current, and investigated its effect on the crystallinity of the coatings. Under high arc current conditions, the HAP coating showed high surface density and crystallinity, and the HAP nanorods with lengths of 300~400 nm were formed. In addition, cold plasma treatment can be used to improve the retention of fluoride in enamel. The increased uptake of fluoride on the surface of teeth treated with cold plasma leads to a decrease in acid solubility on the enamel surface, which has the effect of strengthening enamel and preventing dental caries.^{215,216} El-Wassefy et al.²¹⁷ studied the combined impact of cold plasma and bioglass-phosphate paste on the remineralization of demineralized enamel, demonstrating that cold plasma treatment improved remineralization of bioglass on the demineralized enamel.

Our group and cooperators have designed an artificial tooth enamel (ATE, HAP@A-ZrO₂@PVA) that retains the structural complexity of the biological prototype. The artificial tooth enamel has a hierarchical structure of an amorphous intergranular phase (AIP) of zirconium dioxide structure encapsulating HAP nanowires and interlaced with PVA, which is assembled into a large machinable mass firstly by the ice stencil method, and is prepared into a tooth by sanding and polishing (Figure 7A).²¹⁸ Once such a material has been applied on a large scale, the problem of cavities in human beings in the future will be solved. In fact, as early as 2020, our group has developed a crystalline-amorphous interface construction technique and created an in-situ growth repair strategy of the hard tissue to homogeneously grow an amorphous zirconia ceramic repair layer on the surface of the destructed tooth enamel (Figure 7B).⁹ This strategy not only allows amorphous zirconia to penetrate between the damaged enamel rods to form a physically anchored connection and enhance the interfacial force, but also promotes to forming chemical interactions (e.g., hydrogen bonding, covalent bonding, etc.) on the enamel surface to further enhance the adhesion with the substrate and enhance stability. Recently, we have developed a large-area, three-dimensionally interlaced array of HAP nanosheets reinforced ceramic composite separators using in situ bio-mineralization and annealing, which have superb mechanical properties and good resistance to thermal shrinkage, as shown in Figure 7C.²¹⁹ Using this mineralization method, restoration of enamel may be possible by simply replacing the flexible substrate with damaged enamel.

The growth rate of enamel is concerned with the efficiency of restoring enamel, as shown in Figure 8A, which demonstrates the growth rate (the number of nanometers produced per hour) of various strategies for growing enamel. It can be seen that the growth rate under the organic matrix mediated by proteins is generally slow, but is witnessed with a slight increasing growth rate in the constructed gel environment and a faster rate when the amorphous phase is mediated. Since the nucleation process of apatite crystals is influenced by multiple factors, the nucleation kinetics is still required to be investigated in depth to develop a fast way to obtain enameled materials. Although the above apatite crystals prepared by using different strategies differ from natural enamel in orientation, size, and arrangement, it is these explorations that have brought light to the restoration of enamel. Table S1 summarized the advantages and disadvantages of each preparation strategy in the hope that it will inspire researchers to figure out a more complete strategy for restoring enamel.

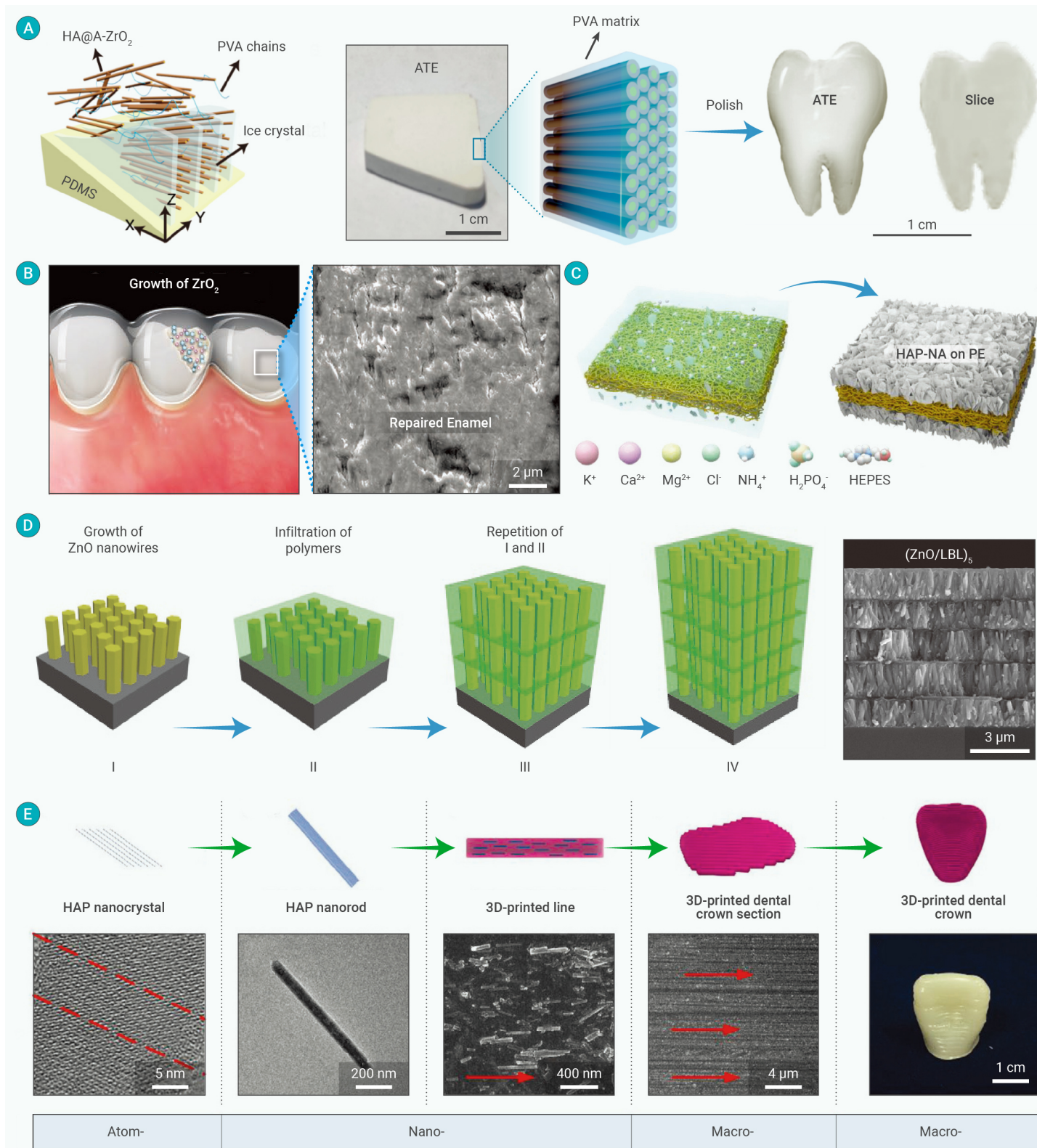


Figure 7. Schematic diagram and electron microscope images of enamel regeneration by physicochemical methods (A) Schematic diagram of the artificial tooth enamel by assembling HAP@A-ZrO₂ nanowires with PVA via the ice stencil method, and digital photographs of the teeth prepared after grinding. Reproduced with permission from ref.²¹⁸ Copyright 2022, AAAS. (B) Schematic illustration of the structure of the tooth enamel after bionic repair by the *in situ* growth of an amorphous ZrO₂ layer as well as SEM images of repaired tooth enamel from the top view. Reproduced with permission from ref.⁹ Copyright 2020, Wiley-VCH. (C) Schematic diagram of the formation mechanism of hybrid microstructures of HAP that are interlocked and mutually supported with the substrate. Reproduced with permission from ref.²¹⁹ Copyright 2023, Wiley-VCH. OCP-NA: octacalcium phosphate-nanosheet array. HAP-NA: hydroxyapatite nanosheet array. (D) Schematic diagram showing the preparation methods and structural evolution of the bio-inspired columnar composites constructed by sequential LBL infiltration of ZnO nanowires with polymers (left). SEM image of cross-section of (ZnO/LBL)₅ (right). Reproduced with permission from ref.⁴⁶ Copyright 2017, Springer Nature. (E) Schematic diagram of the 3D printing synthetic route on the basis of the highly ordered HAP-monodispersed resin-based composites inks (up). From microscopic TEM images of HAP nanocrystals to macroscopic digital photographs of 3D printed dental crowns (down). Reproduced with permission from ref.¹⁹⁸ Copyright 2021, Wiley-VCH.

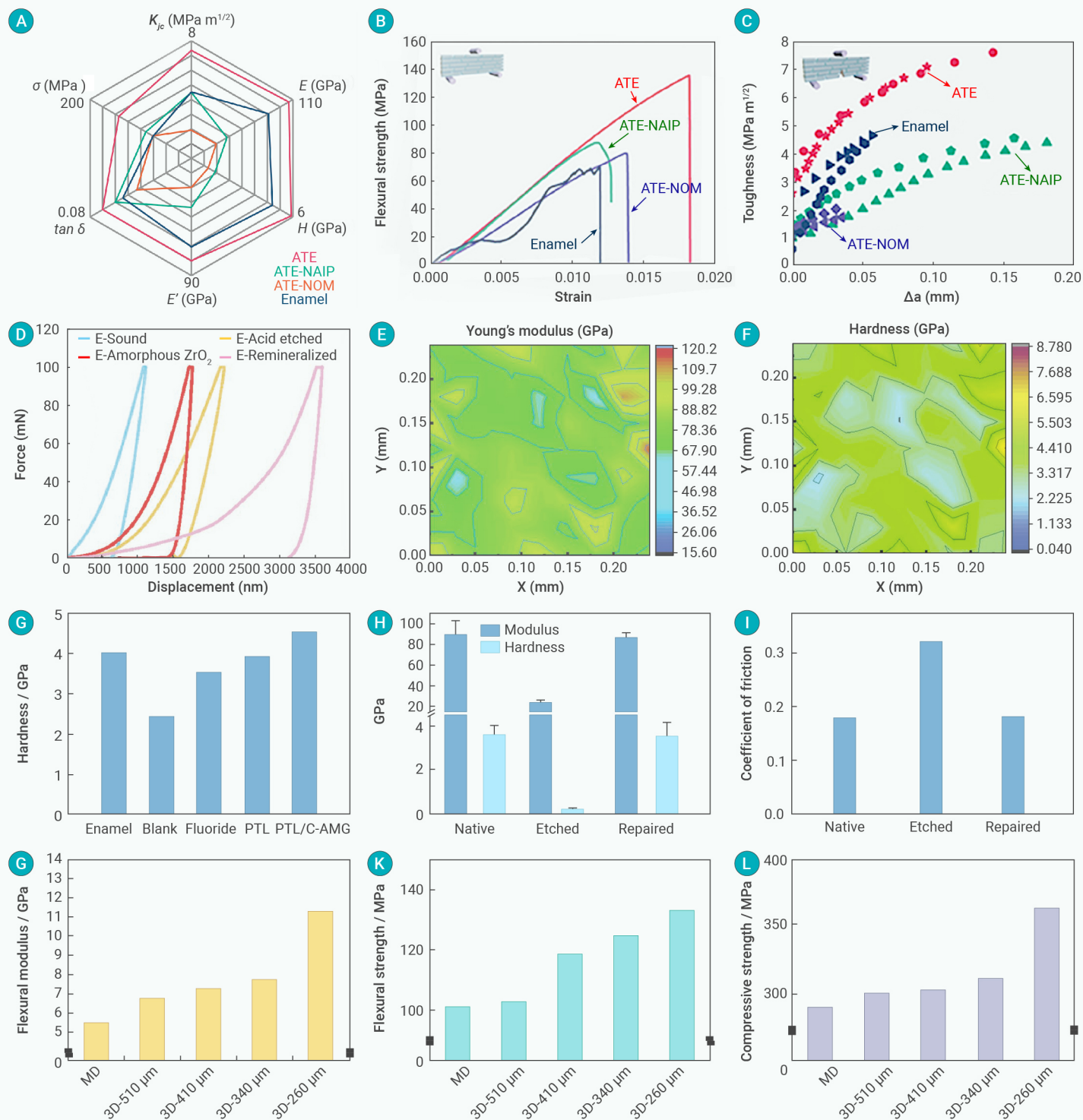


Figure 8. The growth rates as well as relative recovery rates of modulus and hardness in various biomimetic enamel materials (A) Direct comparison of growth rates. Data were estimated from SEM images and arranged from the smallest to largest. PAMAM-NH₂,¹⁵¹ ADGS,¹⁵⁴ Peptide-7,¹⁴⁶ Oligopeptide amphiphile,¹⁶⁶ Glutamine,⁴¹ ALN-PAMAM-COOH,¹⁴⁷ PAMAM-PO₃H₂,¹⁴⁸ HEDTA,¹⁹⁹ Agarose hydrogel,¹⁵² SAP-TA/Fe(III),¹⁴⁰ PTL/C-AMG,⁴⁴ CPICs,¹⁸¹ EDTA+Evaporation,²⁰⁶ Mg²⁺(FPN-M),¹⁸⁶ Bioengineered tooth germ,¹⁰² CS-rP172 hydrogel,¹⁵⁶ Phosphoserine-Histatin5,¹⁴³ P26,¹²⁷ DMP-1@MTA@AHBMM,¹⁵⁷ Serotonin,⁹⁹ ACP nanoparticles,¹⁸³ Rotary evaporation.¹⁵⁶ (B) Relative recovery rates of modulus and hardness.

Mechanical properties of enamel-like nanocomposites

Various strategies for enamel-like nanocomposites have been described above. However, it is of utmost importance for clinical applications to assess whether the mechanical properties of regenerated enamel-like nanocomposites can reach the comparable performance of natural enamel. Nanoindentation, a popular technique that encompasses both static and dynamic mechanical testing for efficient and convenient characterization of the

mechanical properties of materials, is commonly used to test the hardness, elastic modulus and viscoelastic parameters of nanocomposites. In addition, the scratch method is widely used to quantify the bonding force at the interface between regenerated enamel and original enamel. The mechanical properties of regenerated enamel for each strategy are summarized in detail below, which corresponds to the preparation of enamel-like materials described in the previous part of this paper. In terms of cell-based tissue

engineering to regenerate teeth or enamel, since there are almost no relevant publications to test the mechanical properties, our presentation in this part will start directly from the organic matrix-mediated enamel crystal growth, and we also hope that the subsequent researchers will strengthen the mechanical attention.

The amyloid-like amelogenin (PTL/C-AMG) mimic has achieved good results in controlling enamel remineralization. The hardness and elastic modulus of the regenerated enamel were measured using a Berkovich diamond tip. As shown in Figure 9G, the hardness (H , 4.5 ± 0.2 GPa) of the regenerated HAP layer based on the PTL/C-AMG film reached the level of natural tooth enamel.⁴⁴ WSM was used as a template to prepare a dense and well-aligned HAP crystal layer with a surface Vickers hardness (HV) of 242.78 ± 18.29 kg m⁻², so that the hardness reached 76.4% of the original enamel.¹³² After the mineralization formed through the interaction of ELR spherical crystals of supramolecular organic skeleton with apatite crystals, the mechanical properties of the material reach a level superior to the mechanical properties of dentin due to its special hierarchical structure and non-vertical crystal orientation.¹²⁶ Composite enamel material formed by treating regenerated enamel samples with E125 and Glu and then cured by dental resin infiltration showed that composite enamel was comparable to the original enamel as measured by Knoop hardness and nanohardness of the surfaces.⁴² The mechanical properties of restored enamel under the influence of peptide P26 and P32 were measured by the continuous stiffness method, and the Young's modulus and hardness of the regenerated enamel increased by 1.7~1.8 and 1.8~1.9 times, as compared with that of demineralized enamel, respectively.¹²⁷ The average Young's modulus and hardness of the mineralized layer guided by ShADP5 were 58.60 ± 4.70 GPa and 2.23 ± 0.23 GPa respectively by twenty nano-indentation tests.¹³⁹ The mechanical properties of the regenerated enamel treated with by SAP-TA/Fe(III) were tested using the Knoop microhardness method (50 g acting for 10 s) and the scratch method (scratch rate: 3 mm/min, loading rate: 80 N/min). The test results that 80% of the surface microhardness was restored, and the measured adhesion force of 64.85 N indicated that the regenerated enamel was well bonded to the original enamel interface.¹⁴⁰ Nanoindentation tests (constant load: (100 mN), loading time: 15 s) on enamel layers formed using chimeric peptide-guided biomimetic remineralization showed that the modulus of elasticity and hardness of [(CMC/ACP) + peptide A] were 66.7 ± 2.4 GPa and 0.70 ± 0.21 GPa, respectively.¹⁴² Regenerating enamel materials obtained by the addition of 8DSS had an average hardness and Young's modulus of 58.7% and 74.6% of that of native enamel, respectively, under the same testing conditions.¹⁴⁵ Nanoindentation tests of the restoration layer formed by the combined action of Glu and nano-apatite particles pre-adsorbed on the enamel surface showed a high elastic modulus of 96.9 ± 4.0 GPa and a hardness similar to that of natural enamel at a maximum loading force of 2000 μ N.⁴¹ The mechanical properties were measured by nanoindentation instrument at 100 mN loading for 10 seconds. Whether for the Group A that is directly mineralized in the mineralization solution or the Group B, C, and D with different special designs, the new enamel has better Young's modulus and hardness than the etched enamel. It may be due to the stronger anchoring effect of ALN, Group C and D than the Group B. Because the Group D was further added with glycine, the elastic modulus and hardness recovery rate were higher, 68.6% and 49.4%, respectively.¹²⁸ The surface hardness of the enamel formed by dendritic molecules (ALN-PAMAM-COOH)-mediated formation was tested using a microhardness tester with a Knoop diamond indenter, and the results of a 10-second test at a load of 50 g showed that 95.5% of the microhardness of the natural enamel had been restored.¹⁴⁷

The enamel grown in agarose hydrogel mineralization had a Young's modulus of 89.5 ± 11.82 GPa and a nanohardness of 3.04 ± 0.75 GPa, which is almost comparable to natural enamel.¹⁵² The (AAO)-assisted two-layer gel system can prepare well-oriented crystals, with a scratch test showing an adhesion of 21.44 ± 5.18 N (test load and time: increase of load from 0-30 N within 1 minute).¹⁵⁴ The elastic modulus and nanohardness of the enamel slices treated with EMD-CaCl₂ hydrogel were 78.45 ± 14.01 GPa and 2.15 ± 0.61 GPa, respectively, which reached 86.6% and 51.2% of the natural enamel, respectively.¹⁶³ For the enamel restored after the addition of rP172 to chitosan hydrogel, the modulus and hardness reached 45.7% and 25.0% of the natural enamel, respectively.¹⁵⁶ Additionally, the adding of MMP-20 to CS-

AMEL (rP172) hydrogel could regulate the composition and microstructure of the artificial tooth enamel, thereby improving the mechanical properties. For example, the modulus and hardness reached 86.4% and 88.4% of the natural enamel, respectively.¹⁶⁴ Hydrogels formed by adding calcium chloride to the oligopeptide solution were used for biomimetic mineralization, and the Vicker's hardness of the demineralized enamel was significantly improved to 77.3% of that of the native enamel.¹⁶⁶

The simple compression molding of the active amorphous nanoparticle substrate was able to effectively guide the formation of an intermediate layers, which in turn led to the formation of an enamel-like structure, with mechanical property tests showing a hardness and elastic modulus of 2.9 ± 0.4 GPa and 63.4 ± 9.2 GPa, respectively.¹⁸³ The hardness and modulus (constant strain rate of 0.2 nm/s) of the enamels prepared by epitaxy of CPICs were 3.8 ± 0.2 GPa and 87.3 ± 3.7 GPa (Figures 9H-I), respectively, and the coefficient of friction (constant load of 500 mN) was 0.18 ± 0.008 , which is at the level of natural enamels in terms of mechanical properties.¹⁸¹ Nanoindentation measurements showed that the Mg²⁺(FPN-M) array have favorable mechanical properties, with the Young's modulus (81.5 ± 0.6 GPa) and hardness (2.42 ± 0.01 GPa) of the array are comparable to those of the natural array, and it is worth mentioning that due to the presence of Mg²⁺, the compacting of the arrays is induced and a unique magnesium-rich amorphous reinforced structure forms, which results in the improvement of the mechanical.¹⁸⁶ The surface microhardness of the enamel samples mineralized by PAA-ACP@amSN was measured by a Vickers microhardness tester, and the recovery ratio of the surface microhardness (loading force of 50 g for 10 s) was 75.2% as compared to the untreated control sample.¹⁸² The average hardness (3.8 ± 0.3 GPa) and Young's modulus (80.3 ± 5.0 GPa) (measured at 100 mN for 10 seconds) of regenerated enamel after mineralized by CMC/LYZ-ACP nanogel coating were comparable to those of the biological enamel.¹⁸⁷

Yin et al.¹⁹⁹ constructed fluorinated hydroxyapatite directly on the natural enamel substrate using a mild chemical method, eventually restoring more than 90% of the Young's modulus of the original enamel. The nanohardness and elastic modulus of tooth enamel regenerated by rotary evaporation (NOF-SF-HAP group), were 66.1 ± 7.6 GPa, 2.4 ± 0.3 GPa, respectively.¹⁹⁶ The Young's modulus (39.8~44 GPa) and hardness (1.65~1.7 GPa) of the composite bionic enamel material of ZnO nanorod blanket and polyelectrolyte matrix stacked with three or five layers (ZnO/LBL)_n varied with the depth of probe indentation, and moreover, thanks to the well-designed structure, (ZnO/LBL)_n has an excellent viscoelastic figure of merit (VFOM), which can resist vibration damage.⁴⁶ Based on the idea of the preparation of (ZnO/LBL)_n, the enamel-like (TiO₂-CS/GO)₄ and (TiO₂-PVA/GO)₄ nanocomposites were further constructed, achieving comparable mechanical properties of (ZnO/LBL)_n. The Young's modulus and hardness of (TiO₂-CS/GO)₄ were 81.0 ± 2.7 and 1.56 ± 0.05 GPa, respectively.²⁰⁵ The (FHAP-CS/GO)₈ material prepared by EDTA involved in nucleation, using evaporation method, bottom-up self-assembly method and layer-by-layer technique was measured as 83.4 ± 10.4 GPa for E and 3.3 ± 0.2 GPa for H (40 mN hold loading for 10 s).²⁰⁶ Sun et al.¹⁹⁷ measured Young's modulus and hardness of the regenerated enamel layer assisted by laser and chelating agent under the maximum load of 1000 μ N, reaching 65% and 30% of the original enamel, respectively. The hydroxyapatite coating prepared by electrodeposition was tested using a Vickers micro-hardness tester (load of 10 g, time of 30 s), and enamel hardness was restored to about 84.0% of its original value.²⁰⁹ As shown in Figures 9J-L, the flexural modulus (E_f), flexural strength (S_f) and compressive strength (S_c) of the 3D printed HAP-based hybrid resin composite crown were measured using a universal testing machine, and it can be seen that the E_f , S_f and S_c of the fabricated crowns exhibited different properties affected by the nozzle diameter of the 3D printed nozzles and were optimal for the nozzle diameter of 260 μ m, with the E_f , S_f and S_c values of 11.2 ± 1.1 GPa, 134.1 ± 3.9 MPa and 361.6 ± 8.9 MPa, respectively.¹⁹⁸ As shown in Figure 9A, the ATE nanocomposites assembled by the ice stencil method simultaneously exhibited high stiffness (105 GPa), high hardness (5.9 GPa), high viscoelasticity (VFOM, 5.5 GPa), high strength (143 MPa, (Figure 9B), and high toughness (7.4 MPa m^{1/2}, (Figure 9C)), which exceeded the properties of natural tooth enamel and all of the reported HAP matrix composites and ceramic polymer-based composites. Such structure and

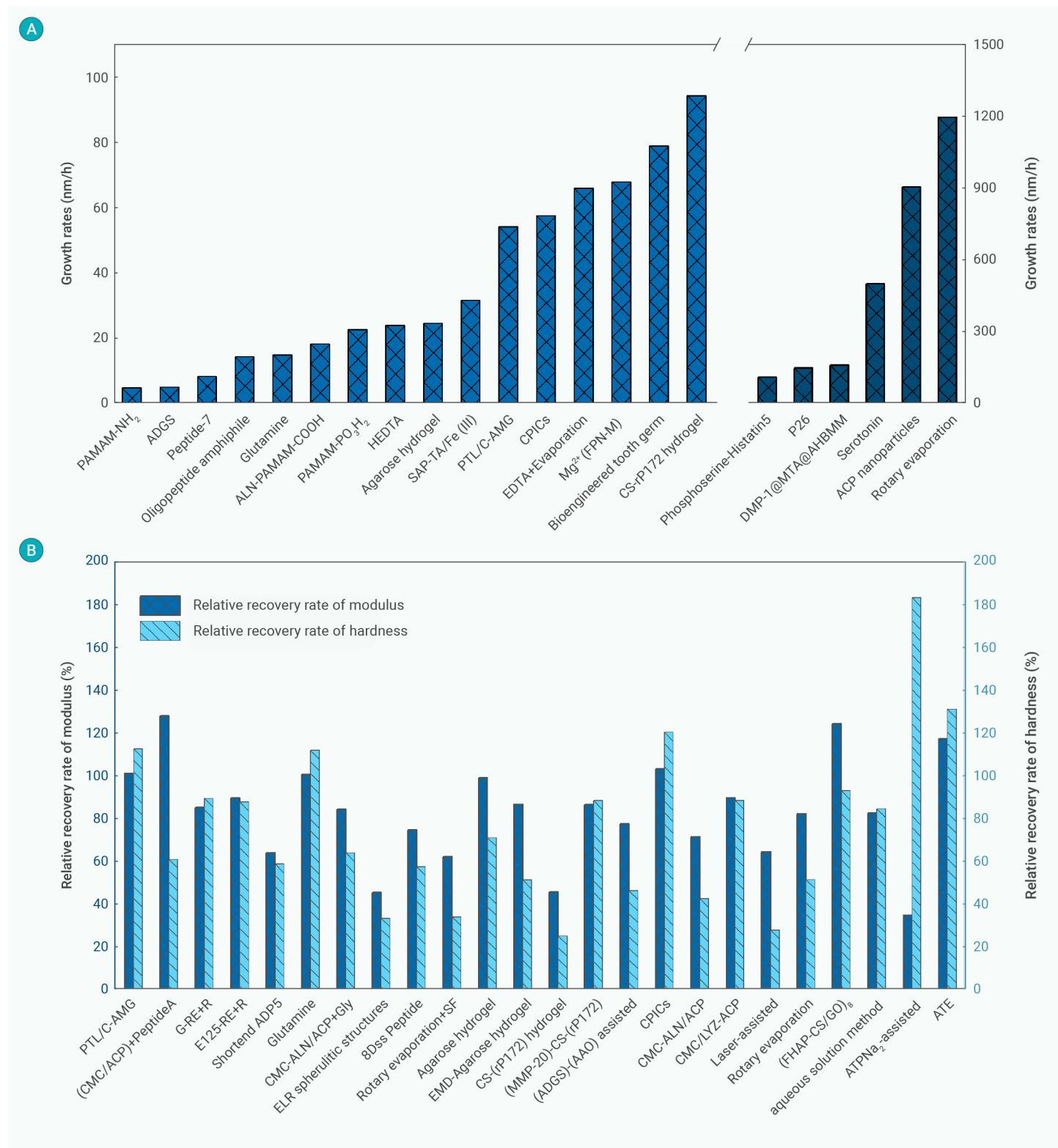


Figure 9. Mechanical performances of the regenerated enamel-like nanocomposites (A-C) Mechanical performance of different enamel-like composites, samples including ATE, ATE-NAIP (without AIP), ATE-NOM (random arrangement), and enamel. Adapted with permission from ref.²¹⁸ Copyright 2022, AAAS. (D-F) Load-displacement curves of different samples during nanoindentation (D), Contour mappings of the (E) Young's modulus and (F) hardness of the E-Amorphous ZrO₂. Reproduced with permission from ref.⁹ Copyright 2020, Wiley-VCH. (G) Surface hardness of the newly formed HAP minerals on PTL/C-AMG films. Natural enamel, demineralized enamel, fluoride treatment, and PTL were only used as controls. Adapted with permission from ref.⁴⁴ Copyright 2020, Wiley-VCH. (H,I) Comparison for mechanical (H) and microtribological (I) properties of enamel samples. Adapted with permission from ref.¹⁸¹ Copyright 2019, AAAS. (J-L) Mechanical properties of samples prepared by 3D printing under different diameter nozzles. Adapted with permission from ref.¹⁹⁸ Copyright 2021, Wiley-VCH.

performance of the composites have the potential to be used as the novel dental repair materials for the next generation.²¹⁸ Thanks to the biomimetic hydroxyapatite crystal-amorphous zirconia interface configuration, the

mechanical properties of amorphous zirconia-restored enamel are restored to the level of healthy enamel (modulus and hardness of 82.5 GPa and 5.2 GPa (Figures 9D-F), respectively, and excellent viscoelasticity (tanδ)). In addi-

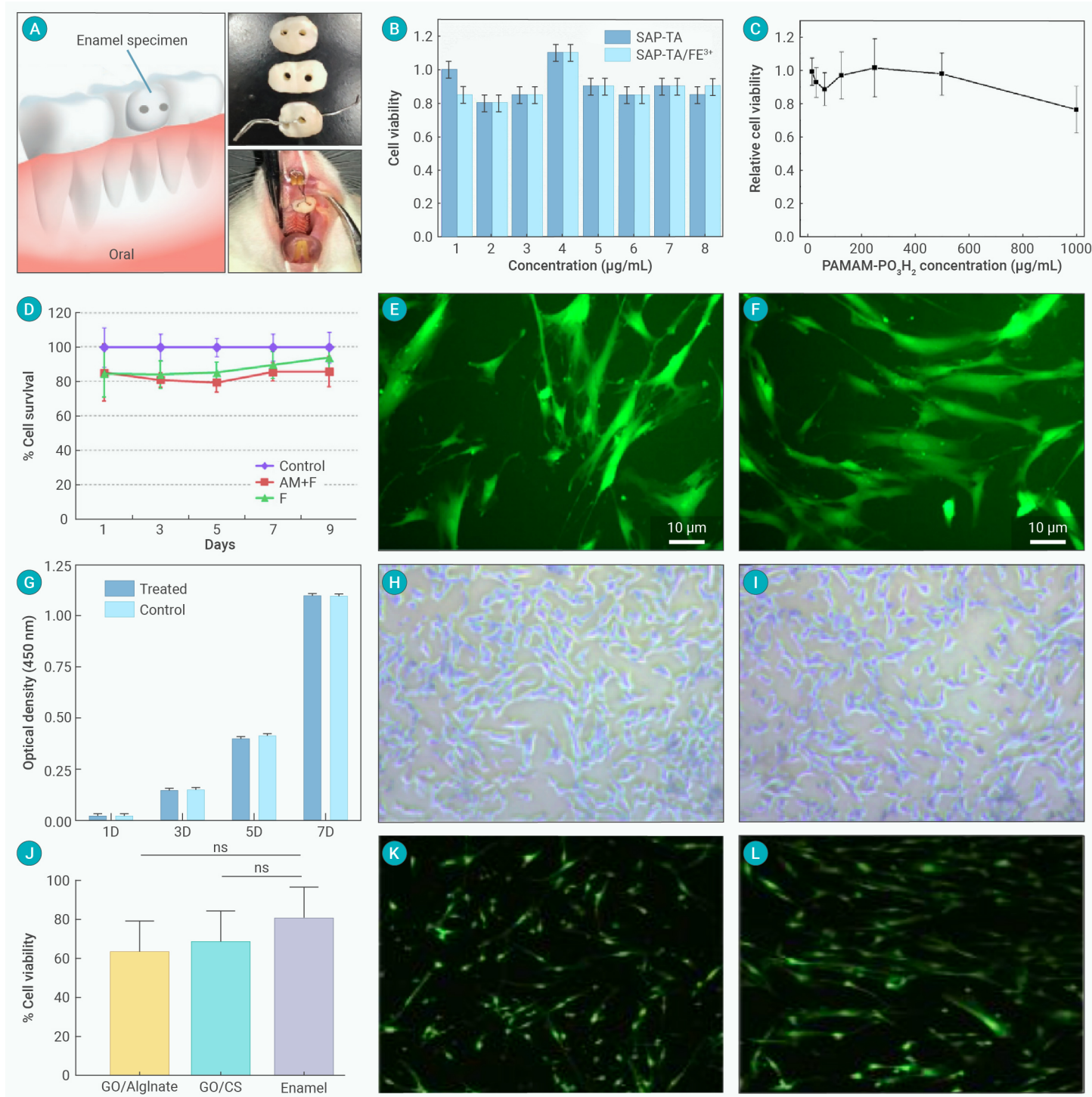


Figure 10. In vivo mineralization test and cytotoxicity test (A) Schematic diagram of experiments with demineralized tooth enamel immobilized in the oral cavity of rats. Adapted with permission from ref.⁴⁴ Copyright 2020, Wiley-VCH. (B) Measurement of SAP-TA and SAP-TA/Fe (III) cytotoxicity in MG63 cells by MTT assay. Reproduced with permission from ref.¹⁴⁰ Copyright 2017, American Chemical Society. (C) Measurement of the effect of different concentrations of PAMAM-PO₃H₂ on L929 cells by MTT assay. Adapted with permission from ref.¹⁴⁸ Copyright 2014, Elsevier. (D–F) The safety of mineralized hydrogels was tested with PDL fibroblasts. (D) Cell survival after 9 days of the experiment. Fluorescence microscopy images of cells, (E) control group, (F) experimental group. Reproduced with permission from ref.¹⁵⁵ Copyright 2012, SAGE. (G–I) Assessment of cytocompatibility of BMSCs in mLRAP/NAA group and control group. (G) CCK-8 assay showing the proliferation of BMSCs. The light microscope micrographs of experimental group (H) and control group (I). Reproduced with permission from ref.¹⁶⁵ Copyright 2021, Elsevier. (J–L) Biosafety of prepared biomaterials tested by HGF-1 cells. (J) Evaluation of cell viability of biomimetic tooth enamel bioactive materials and natural enamel. (K,L) Fluorescence images of cell proliferation on the surfaces of bioactives (K) and enamel (L). Reproduced with permission from ref.²⁰⁶ Copyright 2021, Elsevier.

tion, the restorations are tightly bonded to the enamel, withstand up to 15000 toothbrush abrasion cycles and 600 hot/cold cycles, and long service life with stable performance.⁹

To better visualize the Young's modulus and hardness of the regenerated enamel-like nanocomposites, the relative recovery rates were plotted as histograms (Figure 8B) compared with the mechanical properties of the origi-

nal enamel of each documented human, and their detailed mechanical properties are shown in Table S1.

Security assessment and practical applications

The relevant techniques and materials used in the repair of dental tissues must be ensured to be safe and harmless, which makes the evaluation of the

biosafety of biomaterials of particular importance. To test the effects of mineralization in a realistic biological environment, rats have made an extraordinary contribution (Figure 10A), and a common method is to tie pieces of enamel to rat teeth or to the inside of face clips for mineralization experiments.⁴⁴ In the context of safety testing, the effect of the toxicity assessment is usually expressed in terms of the amount of cell viability. The cytotoxicity of SAP-TA and SAP-TA/Fe (III) was detected using MG63 cells, and the cell viability could be maintained at 80~100% within a certain concentration range, which indicated that it has low cytotoxicity (Figure 10B).¹⁴⁰ The cytotoxicity of PAMAM-PO₃H₂ was assayed using the L929 cell line, and the results showed that this dendrimer has very low cytotoxicity in a wide concentration ranging and be can be applied in biomedicine (Figure 10C).¹⁴⁸ In addition to the viability of the cells, the morphology of the cells indirectly reflects the safety of the sample being measured. Human periodontal ligament (PDL) fibroblasts were used to co-culture with the remineralized hydrogel, which showed that the survival rate of cells directly contacting with the hydrogel was above 80%, with a little change in cell shape and viability (Figures 10D-F).¹⁵⁵ Moreover, bone mesenchymal stem cells (BMSCs) were used to evaluate the cytocompatibility of artificial extracellular matrix (mLRAP/NAA) applied to enamel mineralization, and no significant variation in cell proliferation and cell morphology was observed after 7 days of culture (Figures 10G-I).¹⁶⁵ As shown in Figures 10J-L, HGF-1 cells were used to test the biosafety of preparing enamel-like materials, and the cell viability shown by enamel-like materials synthesized with GO-CS or GO/Alginate as a substrate still differed from that of natural tooth enamel, but there was no significant difference in cell proliferation on the surface of the enamel-like material and that of natural enamel.²⁰⁶

All the developments of methods for the restoration of enamel are naturally aimed at practical applications. Adding restorative active ingredients that can be deposited on the surface of tooth enamel to our daily toothpaste or mouth rinse is a more convenient way to repair enamel. In 1980, the world's first nano hydroxyapatite toothpaste was introduced by the Japanese company Sangi and sold in huge volume.²²⁰ Recently, Bossù, M et al.²²¹ evaluated the remineralization effect of biomimetic hydroxyapatite toothpaste on enamel, showing that the use of toothpaste containing hydroxyapatite is a highly promising method for caries prevention. In addition, Amaechi, B. T. et al.²²² also showed that toothpaste containing nano-HAP was effective to remineralize initial caries and inhibit the demineralization of healthy enamel. Studies have shown that after prolonged use of mouth rinse containing hydroxyapatite, the surface of tooth enamel was completely covered with remineralized HAP and witnessed better whitening effect.²²³ However, the use of toothpaste or mouth rinse as a medium to restore tooth enamel requires long-term consistent use, which does not satisfy patients with severely damaged teeth and urgent restorative needs. To meet the individual needs of the patient, the patient's tooth was used as a model to create a custom tray, which has space only in the defective area of the tooth, then injected a reaction solution into the limited space of the custom tray and subsequently conditioned to complete the restoration.⁹ Depending on the degree of damage to the teeth, we see the following technical routes for future practical applications in dentistry. The first is the repair of minor surface scratches and superficial enamel breakage with specially designed daily used oral products. The second is to repair moderate damage of visible volume by filling with a large number of mineral precursors that are biocompatible and easy to control the shape of the mineral after aggregation. The third is the direct replacement of a tooth that has lost function after rapid *in vitro*-reconstruction by means of advanced medical technology.

CONCLUSIONS AND PERSPECTIVES

Thanks to innovations in characterization and measurement techniques, the structures and properties of enamel composites are becoming more and more clearly revealed. Enamel, as a structurally and morphologically complex material, has hierarchical anisotropy on multiple length scales. It has always been a desire to repair broken natural enamel with artificial enamel in order to have a perfect set of teeth.

In recent years, researches on the fabrication of artificial tooth enamel materials have flourished. We have summarized almost all preparation methods with their advantages and disadvantages in Table S2, hoping to bring

convenience and inspiration to subsequent researchers. Based on preceding parts of the text summary of the latest structural features, fabrication methods and mechanical properties of bionic enamel composites, there are still some challenges to be overcome with the current approach that has the potential to restore tooth enamel, such as achieving a cost-effective and green synthesis of block-grade artificial enamel, attaining perfect integration of artificial enamel with the natural enamel interface, and enabling rapid repair of enamel. In order to make artificial enamel materials into practice and benefit society in the future, ingenious design at the micro and nano scale and optimization of the preparation technology are still required.

To date, research on bionic enamel materials has only focused on how to grow vertically aligned apatite arrays, whether prepared hydroxyapatite, doped and modified fluorapatite, or magnesium-rich apatite, which only accomplished the imitation of the zero and I level of the hierarchical structure of natural tooth enamel. Although the elastic modulus and hardness of the prepared bionic enamel can be comparable to those of human tooth enamel, the gap between the present bionic enamel materials and natural enamel in terms of hierarchical structure will lead to poor fracture resistance. The chemical composition, size, shape and arrangement of enamel crystals are also important factors to affect their properties. Therefore, the regulation of the components and structure of the artificial enamel material at several levels is the key to obtain excellent properties. If the composition-structure-performance relationship is utilized for cross-scale programming and multi-level controllable preparation of enamel bio-nanocomposites, it is expected to achieve the precise spatial and directional arrangement of enamel crystals. However, it is still necessary to discover the effects of each design parameter on the mechanical properties of the material, such as stiffness, strength, and elastic modulus before we explore the design methods of the hierarchical structure-based bionic enamel material. Moreover, the dental repair materials will suffer from corrosion under an acid environment in the oral cavity but there is few published paper investigating the corrosion performance of the material. Therefore, it is in an urgent requirement to study the corrosion behaviors of the dental repair materials.

In spite of the fast-growing development of the research on tooth enamel and the tremendous potential of existing achievements to be landed into clinical applications, only a few large-scale implementations have occurred.²¹⁹ The main obstacles are the long duration of the biomimetic mineralization process because it is complicated and expensive to fabricate artificial enamel materials with the thickness of millimeter scale. Therefore, future research should focus on developing low-cost methods to obtain block-grade artificial enamel materials in a short period of time for large-scale practical applications. Although significant challenges still exist, we believe that based on continuous effort on experimental fabrication and multi-scale modeling, enamel-like synthetic materials will be used in dentistry to improve people's oral health.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

DATA AND MATERIALS AVAILABILITY

Data are available from the corresponding author upon reasonable request.

SUPPLEMENTAL INFORMATION

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