Advances in biomimetic mineralization of tooth enamel based on cell-free strategies

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Abstract. Tooth enamel is a highly-mineralized hard tissue covering the outermost layer of the dental crown, and amelogenesis is inseparable from the participation of necessary components such as ameloblasts, organic matrix proteins, and mineral ions, such as Ca2+ and PO43-. However, mature enamel is an acellular tissue and it is difficult to self-repair once damaged. The current treatment methods for enamel damage are filling or repairing with alloys, ceramics, or composite resins. However, the mechanical properties of these materials are quite different from the natural enamel and they can't ensure a completely closed interface with the remaining enamel surface, which usually causes a series of post-repair problems. At present, the biomimetic mineralization of tooth enamel is a research hotspot in the field of prosthodontics, and has great clinical application needs and prospects, especially the researches on cell-free strategies have made significant accomplishment. Here, based on the cell-free strategies, we review the recent knowledge from ex situ and in situ two dimensions in the remineralization of tooth ename.

Keywords: tooth enamel; enamel-like materials; in situ enamel remineralization; review

1. Introduction

As the hardest tissue in the human body, tooth enamel consists of ~96wt% mineral phase, ~2-3wt% organic phase and ~1wt% water. Based on enamel rods, the highly oriented apatite crystals and the matrix filling in between form a unique and complex hierarchical structure[1], which endows the enamel with near-perfect mechanical properties. Therefore, enamel acts as a natural barrier to protect dentin and pulp tissues from stimuli of external physical and chemical factors.

Enamel is the only tissue in the body which is secreted and then mineralized by epithelial cells. Amelogenesis is dominated by ameloblasts, whose activities can be mainly divided into two stages: secretory stage and maturation stage. In the early stage of secretion, the polarity of ameloblasts changes and the number of organelles increases in preparation for the secretion of organic matrix. Then ameloblasts secrete different types of enamel matrix proteins (EMPs), composed of 80-90% amelogenin, 10% non-amelogenin and proteases in order to create and maintain a unique extracellular matrix environment for the deposition of minerals, following the nucleation and growth of apatite crystals. In the maturation stage, ameloblasts regulate further mineralization of tooth enamel[2]. The initial mineralization product formed by enzymatic reaction is amorphous calcium carbonate (ACC), which is then transformed to more thermodynamically stable amorphous calcium phosphate (ACP), followed by hydroxyapatite (HAp) crystals[3, 4]. Generally, OH^- are easily replaced by CO_3^{2-}, so carbonate hydroxyapatite crystals are the most commonplace forms existing in the enamel, which is more easily to be dissolved by acid and demineralized[4]. Of note, the ameloblasts will gradually transform to a layer of reduced dental epithelium covering the surface of the tooth when the enamel matures, and then turn to junctional epitheliums around the dental neck with the eruption of the tooth[2]. That means mature tooth enamel is acellular tissue and cannot repair itself once it was damaged.

Under physiological conditions, enamel has a series of physicochemical changes in the normal oral cavity even without cells, including demineralization and remineralization, which are an equilibrium system, especially dental caries, the most common oral diseases, which begin to invade from enamel first. The following acid produced by oral cariogenic bacteria break the balance, and the enamel continues to demineralize and even form caries[5]. The existing filling and restoration materials are mainly alloys, composite resins, ceramics etc., whose mechanical properties are different from those of tooth enamel, and an ideal interface cannot be formed between them, which may result in the stress concentration, leading to a series of problems such as secondary caries. Therefore, a new method of tooth enamel restoration is urged.

The biomimetic mineralization of tooth enamel is a hot spot of the current researches, which can be mainly divided into two categories at present. One is based on ex-situ remineralization, imitating the basic structure of

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enamel to artificially prepare enamel-like materials whose properties are as close to those of natural enamel as possible. The other is in-situ remineralization on the surface of the deficient enamel directly, using EMPs as templates and mimicking the microenvironment of amelogenesis, or building a biomineralization frontier to guide the regrowth of HAp crystals. Taking cell-free strategies as the main line, we review the recent advances in biomimetic mineralization of tooth enamel in the past five years ex-situ and in-situ remineralization of enamel in this article.

2. Ex-situ remineralization of tooth enamel

Bionics is the development trend of oral restorative materials. At present, various technologies and methods have been used for the preparation of enamel-like materials in vitro, which mimic natural enamel in terms of biosafety, biocompatibility, function, morphology, internal structure, etc.

2.1 Layer-by-layer (LBL) deposition techinique

The LBL deposition technique can modify the surface or prepare nanocomposites with specific structures and functions by using polyanionic electrolytes and polycationic electrolytes to alternately form matrix films. These allow the incorporation of a large number of materials (such as polymers, polysaccharides, etc.) into multilayer films and is now widely used in fields such as optics, energy, and biomedicine[6]. Yeom et al.[7] successfully prepared enamel-like columnar nanocomposites (ZnO/LBL)n in vitro by hydrothermally generating ZnO nanowires on silicon substrates, and then depositing polyallylamine (PAAm) and polyacrylic acid (PAA) around them layer by layer, where n represents the number of cycles of nanowire growth combined with layer-by-layer deposition of the PAAm/PAA matrix. Although the preparation process is in the reverse order of occurrence of enamel, it reproduces the main structural features of tooth enamel more accurately. And the relatively high organic phase content not only does no harm to the hardness, but endowed it with viscoelastic figures of merit (VFOM) similar to or higher than that of natural tooth enamel, these will reduce the low-impact damage and increase the durability. In addition, graphene oxide (GO) can also be introduced into the LBL matrix membrane, which gives it unique antibacterial adhesion properties without impairing its mechanical properties as well, especially the adhesion of Streptococcus mutans[8].

2.2 Laser sintering

Anastasiou et al.[9] are the first to demonstrate that calcium phosphate minerals can be directly laser sintered and attached to enamel without any significant thermal damage to the underlying tissue, due to the use of femto-second laser pulses and the doping of light-absorbing materials in calcium phosphate minerals. A layer of Fe3+-containing fluoroapatite powder is coated on the surface of acid-etched bovine tooth enamel and irradiated with a femto-second pulsed laser, among which Fe3+ absorbs light and transmits heat to adjacent mineral phases, triggering selective sintering and densification of surrounding calcium phosphate crystals, thus forming a dense sintered layer of about 20 μm thickness and bonding with the surface of natural tooth enamel. It was shown that the mechanical properties of the layer are superior to existing restorative materials. However, we need more researches to optimize the properties of the sintered layer, such as hardness, stiffness, and aesthetics, for clinical translation.

2.3 Rotary evaporation

Using silk fibroin (SF) as a template, Wang et al.[10] successfully regenerated enamel-like HAp crystals on different substrates such as enamel and titanium by rotary evaporation. This material is easy to operate and cost-effective because the thickness of the regenerated crystal increases by 50 μm/day without affecting the structure of crystals, 25-50 times faster than that without rotary evaporation. Meanwhile, the growth thickness can be controlled artificially. The crystal size, morphology, arrangement and mechanical properties of the new HAp layers are highly similar to those of natural tooth enamel, but its mechanical properties still remain unsatisfactory.

2.4 New biomimetic ceramic-polymer composites

In recent decades, organic-inorganic composite materials have been widely applied in oral prosthodontics. New biomimetic ceramic-polymer composite materials consist of a ceramic skeleton and an infiltrating resin, which are closer to the natural tooth enamel, now including two main preparation processes.

2.4.1 Polymer Infiltrated Ceramic Network (PICN) Composites

Traditional dental CAD&CAM materials include metal, ceramic or resin-based materials. Because of their different mechanical properties from enamel, various problems often occur after restoration due to stress concentration. The ideal restorative material needs to mimic the mechanical properties of human tooth enamel, including hardness and elastic modulus, so the concept of PICN has been proposed[11-13]. This structure is usually prepared by infiltrating resin monomers into the continuous pores of pre-sintered ceramics, followed by polymerization, which can be prepared down to the nanometer scale at present. The SiO2-PMMA [poly (methyl methacrylate)] composite prepared by Hiroshi Ikeda et al.[14] has hardness compatibility with human enamel, but its elastic modulus is closer to that of dentin. Kawajiri[15] et al. changed the PICN composites by mechanical properties especially elastic modulus, closer to those of enamel, and had good bonding properties with resins. Despite the above advantages, PICN materials still experience mechanical failure during use[16]. The reason behind may be that the microstructure of almost all

CAD&CAM materials is isotropic, which contradicts the anisotropic microstructure of natural tooth tissue[17].

2.4.2 Biomimetic nacre-like ceramic-polymer composites

The nacre in the inner surface of seashells is composed of 95vol% aragonite calcium carbonate plates and 5vol% protein sheets, in which the plates are 0.5 μm thick, similar to bricks, and the sheets are 20-30 nm thick, similar to mortar. It is the "brick and mortar" structure that creates a strong and tough material that provides inspiration for enamel biomimetic restorations. Tan et al.[18] used ceramic suspension as raw material to form interconnected "mineral bricks" through bidirectional freezing casting and uniaxial pressing. After surface treatment, resin monomers infiltrated into gaps between bricks and solidified to obtain a new type of bionic ceramic-polymer material, whose mechanical properties are proved closer to enamel than any of ceramic or resin materials, reducing the wear of antagonistic teeth. In addition, the nacre-like ceramic-polymer composites show not only extraordinary fracture toughness, but capacity of energy dissipation under cyclic loading and good machinability. On this basis, Algharaibeh et al.[17] arranged the "mineral bricks" into an anisotropic microstructure, which enhanced both crack growth resistance, flexural strength, elastic modulus and hardness. It is also shown that the 70% ceramic volume fraction is the best combination of strength, elasticity, hardness and toughness. The nacre-like material presents a brilliant future in CAD/CAM repair.

2.5 Multiscale engineered artifical tooth enamel

In order to mimic the hierarchical structure more precisely, Zhao et al.[19] put forward an ingenious design of the microstructure of the enamel-like material. Amorphous intergranular phase (AIP)-coated hydroxyapatite nanowires (HAp@A-ZrO2) were intertwined with polyvinyl alcohol (PVA) to construct a novel enamel-like material from nanoscale to macroscale. The existence of AIP, the strong chemical interfacial bonding force between AIP and HAp nanowires through the coordination of Zr4+ and OH-, PO43- and the confinement of PVA are the reasons for the combination of perfect but seemingly contradictory mechanical properties, such as the high stiffness, strength, hardness, viscoelasticity, toughness, etc., superior to previously reported enamel-like composites and even better than the enamel itself. Besides, the material also overcomes the tricky problem of insufficient thickness of remineralization layer, and can be mass-produced. Therefore, it is most likely the multiscale engineered material become the next-generation tooth enamel restoration material.

3. In-situ remineralization of tooth enamel

The ex-situ mineralization of tooth enamel is realized by the above-mentioned enamel biomimetic materials, although the mechanical properties of tooth enamel can be well simulated, and the ideal thickness can be achieved by design. However, like traditional filling and repairing materials, its function must rely on a good surface active system and bonding system. Therefore, there are a lot of studies by simulating the formation process of enamel,and then directly regenerate enamel-like HAp crystals on the surface of damaged enamel.

3.1 Inorganic strategies

Fluoride[20] is a gold standard for inhibiting demineralization of enamel, and the application of fluoride as a remineralization strategy has been widely used in clinical practice and achieved certain results. However, the restoration of enamel by F- is limited in children under 6 years old, due to the concern that they may swallow the paste by mistake.

3.1.1 Biomimetic hydroxyapatite

The initiation of dental caries is often related to the dissolution and destruction of enamel HAp crystals, the most ideal way is to directly supplement hydroxyapatite. Therefore, a strategy based on fluorine-free replenishment of hydroxyapatite was proposed. Using toothpaste as a carrier and using exogenous zinc carbonate hydroxyapatite nanoparticle formulation[21, 22] regularly can trigger the remineralization reaction and successfully form the deposition of hydroxyapatite nanocrystal coating on the enamel surface. It is not based on physical and chemical changes, but on the coating effect of nano-hydroxyapatite particles on damaged enamel, so as to realize the epitaxial growth of residual enamel rods and smooth the rough enamel surface. Although the newly formed hydroxyapatite continues with the natural enamel, the binding force is far from enough.

3.1.2 Polyphosphate particles

Supplementation of mineral raw materials such as phosphate and calcium can inhibit demineralization and promote remineralization. The donor of phosphate in vivo is polyphosphate (polyP) mostly produced by platelet degradation, which consists of 40-100 phosphate units connected by high-energy phosphoric anhydride bonds. Although polyP is not a constituent of HAp crystals, the studies have shown that polyP released from platelets contributes to the growth of HAp crystals, and adding amorphous calcium polyphosphate microparticles to toothpaste become a potential remineralization method[23, 24]. A protective layer of Ca-polyP deposits will be firstly formed on the surface of damaged tooth enamel, and then polyP will be hydrolyzed by alkaline phosphatase (ALP) rich in saliva, producing a large amount of orthophosphate and energy, also lowing the local ambient pH at the same time. Under the effect of

activated carbonic anhydrase, the deposition of calcium phosphorus accelerates, and the phase transition of ACP to HAp crystals expedites[3]. Studies have shown that the Ca/P ratio in enamel deposits is significantly higher than that in toothpaste, indicating that the particles can fuse with enamel and promote the remineralization of demineralized enamel surfaces. Although the transformation of ACP to HAp takes a longer time, compared with direct supplementation of HAp, the regenerated layer is more tightly bound to the enamel surface, able to resistant to short ultrasound by a high-power (320W) ultrasound machine[4]. Even more attractive, the layer can seal dentinal tubules, repair dentin and enamel at the same time, which reveal a brilliant future for polyphosphate particles as an in-situ remineralization material.

3.1.3 Amorphous calcium phosphate(ACP)

Amorphous calcium phosphate may be a precursor of hydroxyapatite crystals, which is quite unstable when it exists alone and it is easy to aggregate and nucleate causing remineralization failure. Studies have shown that casein phosphopeptides (CPP)[25, 26] and some non-collagenous protein analogues (NCPs), such as polyacrylic acid (PAA)[27] and poly(amidoamine) (PAMAM)[28], can stabilize ACP nanoparticles. They use liquids, pastes, gels, etc. as carriers to form an immediate protective layer on the surface of damaged tooth enamel, and then gradually transform into a layer of hydroxyapatite crystals through the principle of dissolution-precipitation or solid-solid phase transformation. However, the remineralized layer is not the same as the natural tooth enamel, and the reason may be that ACP particles are adsorbed on the surface of HAp, causing a discontinuous particle-crystal interface, leading to the polycrystalline growth of HAp[29]. Shao et al.[30] proposed an improved method to construct a biomineralization frontier on the surface of damaged enamel through triethylamine (TEA)-stabilized calcium phosphate ion clusters (CPICs)—residual hydroxyapatite crystals are completely wrapped by amorphous precursors ACP to form a structurally continuous HAp-ACP interface and realize the perfect epitaxial growth. The hierarchical structure of tooth enamel was successfully replicated in 48 hours, and the thickness was up to 2.7μm. It is surprisingly completely consistent with the natural tooth enamel, and integrated with the remaining enamel without any gap. These realize the complete regeneration of enamel, which can be regarded as a permanent restoration. The use of TEA, a volatile organic compound, can stabilize ACP before use, and be completely volatilized without residue at room temperature, which ensures the safety of use and the mechanical strength of the mineral phase. Regarding the thickness of the repair layer, it has shown that the ideal thickness can be achieved by cyclic processing.

3.2 Strategies based on functions of organic matrix

Amelogenesis is a biomineralization process mediated by enamel matrix proteins (EMPs), which are composed of 90%-95% amelogenin and 5%-10% non-ammelogenin, synergistically controlling the initiation, organization, and orientation of enamel crystal mineralization, and are gradually degraded and cleared by proteases during enamel maturation.

3.3 Peptide strategies based on secondary structure of amelogenin

Amelogenin is the main component of organic matrix in the process of enamel biomineralization. When amelogenin is secreted from ameloblasts, it can self-assemble into nanospheres with a diameter of 10–20nm after proteolytic cleavage, playing a key role in the mineralization and organization of tooth enamel due to highly conserved N-terminal domains and C-terminal domains of amelogenin (N-Ame and C-Ame). N-Ame folds through β-sheet stacking and promotes the self-assembly of amelogenin into functional amyloid aggregates[31], serving as a template to guide the growth of HAp crystals, while C-Ame mediates the oriented growth and parallel alignment of HAp crystals. With the aggregation of ACP nanoparticles and the further phase transition of ACP to HAp, a sophisticated hierarchical structure is formed. Synthesis of full-length amelogenin can guide the orderly growth of enamel-like HAp crystals in bundles, but it demands a lot. and the protein is prone to contamination, denaturation, and difficulty in storage. Therefore, it is crucial to completely mimic N-Ame and C-Ame in a flexible and low-cost manner without using the full sequence of amelogenin for the successful application of amelogenin-inspired biomimetic strategies.

A leucine-rich amelogenin peptide (LRAP), composed of 56 non-phosphorylated amino acids, retaining only N-Ame and C-Ame splicing products, is shown to have a regulatory effect similar to full-length amelogenin. Kwak et al.[32] combined non-phosphorylated LRAP and CaP formation inhibitor PPi for biomimetic mineralization of enamel by exposing acid-etched enamel to a PPi-stabilized supersaturated CaP solution containing 0.04 mg/ml LRAP, seamless epitaxial growth of tooth enamel was successfully achieved. When PPi came into contact with HAp crystals, its original inhibitory effect on mineralization was reversed, and enamel regeneration was initiated preferentially on the acid-etched enamel surface. While LRAP preferentially interacted with the enamel crystal plane parallel to the c-axis and prevented the crystal from forming on the ab plane growth to promote directional elongation of the crystal. To simulate the physical and chemical environment of enamel formation, Fang et al.[33] took the interaction of non-amelogenin and amelogenin into the consideration of the construction of the EMPs model. First, an N-terminal modified LRAP (mLRAP) and a non-amelogenin analog (NAA) were artificially synthesized, then their interaction was realized through specific-designed domains. Due to the strong Ca2+ binding ability of NAA, when NAA and mLRAP assemble together, the

aspect ratio of CaP crystals increases significantly, the co-assembled structure increases the stability of transient ACP, and the glycosylation and phosphorylation sites present in NAA can interact with HAp, which promote crystal nucleation. Although the hierarchical structure of the regenerated HAp crystals differs from that of natural tooth enamel, it is also meaningful to try to make different models for biomimetic mineralization.

An amelogenin-mimicking product PTL/C-AMG matrix film based on mimicking N-Ame and C-Ame with specific domains, with good biocompatibility, also successfully achieved epitaxial growth of HAp crystals[34]. The phasetransited lysozyme (PTL), a biomimetic of N-Ame, which is formed by lysozyme folding into amyloid aggregates in vitro based on protein phase transitions. As an interfacial anchor, it can form a layer of strong adhesive on various substrate surfaces, free from the limitation of residual HA crystals required by traditional strategies. The synthetic peptide C-AMG with the C-terminal functional domain of amelogenin added to this coating promoted the directional alignment of ACP and transformed it into ordered enamel-like HAp crystals. The interface between the regenerated HAp crystals and the remaining enamel has good bonding stability, and the hardness reaches or even exceeds that of natural tooth enamel. The only disadvantage is that its regeneration efficiency is low, and the thickness can only be limited to the micron level.

3.3.1 Hydrogel systems based on natural polysaccharide polymers

The hydrogel system can be prepared by physical or chemical cross-linking. Compared with the solution system, the physical and chemical properties of the gel-like environment are closer to the enamel matrix in the secretory stage and maturation stage, and it is easier to operate clinically and stay on the tooth surface for a long time with effective concentration. Natural polysaccharide polymers with good biosafety, compatibility and degradability are often used in the biomimetic mineralization of tooth enamel in the form of hydrogels. It can both fix the nanoscale building units of HAp crystals well, and serve as non-collagen biomaterials templates to guide the growth of HAp crystals.

Chitosan (CS) is a natural cationic polysaccharide that can form stable hydrogel networks through chemical cross-linking, whose chemical structure is similar to the main component of extracellular matrix, with antibacterial activity and strong adhesion to both soft and hard surfaces. Ruan et al.[35] established the CS-AMEL-CaP hydrogel system and introduced amelogenin into the chitosan hydrogel system. The new HA layer grows directly on the original enamel and fully adheres to the acid-etched enamel, which can reduce secondary caries caused by poor interfaces. And the hardness and elastic modulus were increased by 9 times and 4 times, respectively compared with acid-etched enamel. Under natural conditions, amelogenin cannot function without a protease, matrix metalloproteinase-20 (MMP-20), which plays a role in hydrolyzing full-length amelogenin. On the one hand, it can ensure the good growth law of HAp crystals, and on the other hand, it can prevent amelogenin from being trapped in the growing HAp crystals and prohibit protein occlusions in the enamel crystals. Ruan et al.[36] added MMP-20 to the above hydrogel system, which improved the morphology and structure of the regenerated HAp crystals, and the elastic modulus and hardness were significantly increased by 1.8 times and 2.4 times, respectively.

Agarose (A), a family member of natural polysaccharides extracted from red algae, can promote the growth of HAp crystals by forming a thermal reversible gel. A hydrogel system of chitosan (CS) and agarose (A) simultaneously working to achieve biomimetic mineralization of dental enamel was first reported recently. The CS-A hydrogel was remineralized in artificial saliva for 7 days, resulting in a calcium-to-phosphorus ratio (1.64) similar to that of natural tooth enamel, and the microhardness recovery of the enamel-like layer was 77.4%[37].

3.4 Other strategies

Mineralization repair of tooth enamel can also be achieved by rationally-designed small peptides. The self-assembled peptide P11-4 monomers and its β-sheet aggregates can diffuse below the early enamel carious lesions, triggering self-assembly through specific environments, and forming a three-dimensional network with surface features of the enamel matrix. These trigger the nucleation of HAp crystals and growth, and remineralize the surface of early carious lesions. CT showed that the remineralized specimen in the presence of P11-4 increased the mineralization rate by 68% within two weeks, which was statistically significant compared to the placebo control group[38].

Chang et al.[39] achieved efficient biomimetic mineralization for the first time using low-complexity protein segments (LCPSs). LCPSs containing phosphate or phosphonate groups serve as a novel biocompatible peptide scaffold with high water solubility, structural flexibility, and ability to form network morphologies due to weak multivalent interactions. Integrating biomineralization-related components such as matrix proteins, regulatory additives and osteogenic stimulators, LCPCs synergistically promoted the epitaxial growth of HAp crystals on the surface of acid-etched enamel, Besides the remineralized layer can reduce the adhesion of Streptococcus mutans and prevent secondary caries.

4. Summary

Natural tooth enamel has a complex and well-organized structure, so successfully regenerating tooth enamel is an extremely challenging task. For the ex-situ remineralization of enamel, namely the preparation of enamel-like materials, more attention should be paid to the design of the microstructure to achieve a finer bionic from the nanometer to the macroscopic scale. At the same time, the bonding technology should be developed accordingly to form a well-sealed interface between the restoration material and the remaining enamel surface.

For the in-situ remineralization of tooth enamel, it is necessary to fully explore the self-assembly principle of biological macromolecules such as proteins, polypeptides, DNA[40], so as to provide a more accurate template for the nucleation and growth of HAp crystals. And it is still necessary to continue to study the process and mechanism of enamel occurrence in order to realize the precise construction of the enamel mineralization microenvironment in vitro.

In terms of clinical translation, enamel-like materials are faced with the difficulty of mass production while insufficient thickness for in-situ remineralization of enamel. The diseases that lead to enamel defects, including caries, trauma, abrasion, etc., often invade enamel and dentin at the same time, but the mechanical properties of dentin and enamel differs. Thus, how to achieve the simultaneous repair of both in one method requires further research. Materials specialists and physicians working side by side in a complementary manner will certainly shed further light on the ideal remineralization materials and on their translation from the bench to the bedside.

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