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Biological synthesis of tooth enamel instructed by an artificial matrix

Zhan Huang [a,1], Christina J. Newcomb [b,1], Pablo Bringas Jr. [a], Samuel I. Stupp [b,c,d], Malcolm L. Snead [a,*]

[a] The Center for Craniofacial Molecular Biology, CSA 142, Health Sciences Campus, Herman Ostrow School of Dentistry, University of Southern California, 2250 Alcazar St., Los Angeles, CA 90033, USA
[b] Department of Materials Science and Engineering and Institute for BioNanotechnology in Medicine, Northwestern University, Chicago, IL, USA
[c] Department of Chemistry, Northwestern University, Evanston, IL, USA
[d] Department of Medicine, Northwestern University, Chicago, IL, USA

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ABSTRACT

The regenerative capability of enamel, the hardest tissue in the vertebrate body, is fundamentally limited due to cell apoptosis following maturation of the tissue. Synthetic strategies to promote enamel formation have the potential to repair damage, increase the longevity of teeth and improve the understanding of the events leading to tissue formation. Using a self-assembling bioactive matrix, we demonstrate the ability to induce ectopic formation of enamel at chosen sites adjacent to a mouse incisor cultured *in vivo* under the kidney capsule. The resulting material reveals the highly organized, hierarchical structure of hydroxyapatite crystallites similar to native enamel. This artificially triggered formation of organized mineral demonstrates a pathway for developing cell fabricated materials for treatment of dental caries, the most ubiquitous disease in man. Additionally, the artificial matrix provides a unique tool to probe cellular mechanisms involved in tissue formation further enabling the development of tooth organ replacements.

1. Introduction

The leading cause of enamel loss is dental caries [1], the most prevalent and infectious disease of mankind; in addition, the loss of enamel can also occur with congenital malformation, trauma and mastication. Carious destruction occurs due to a maternally derived biofilm, which produces an acidic environment leading to preferential dissolution of the mineral [2,3]. Replacing the lost enamel currently relies on synthetic restorative materials such as polymers, metals or ceramics, which often fail due to poor adhesion or cracking. As a result, alternative materials and approaches for treating dental caries are currently under investigation [4–10].

Teeth are well known for their durability, a property attributable to the organ's unique structure and hierarchical organization [11,12]. The outermost covering of teeth is enamel, the hardest and most highly mineralized tissue in the vertebrate body. Enamel differs from other mineralized tissues by its origin in the ectoderm rather than the mesenchyme germ layer, its lack of collagen, and the use of a transient protein precursor to produce the highly organized mineral [13]. Mature enamel contains multiple levels of hierarchy to optimize the overall mechanical properties of the tissue [13–19]. It is comprised of nanoscale carbonated hydroxyapatite (HA) crystallites arranged anisotropically in tightly packed bundles called rods (or prisms). The rod is the fundamental structural unit of enamel and each rod contains tens of thousands of mineral crystallites with their *c*-axis aligned along the long axis of the rod. They are organized in distinct patterns to optimize wear resistance [15,20]. Only trace amounts of protein are present in the mature tissue due to proteolytic processing during later stages of development, increasing overall hardness of the tissue.

Highly orchestrated extracellular processes involving proteins, supersaturated mineral ions and proteases direct the arrangement, nucleation and growth of HA crystals. The initial mineral crystals in enamel were first observed to be associated with mineralized collagen from dentin [21], however this theory was later refuted and mineral was instead found to be nucleated independently from the underlying dentin-associated collagen [22]. The enamel crystals originated in the microenvironment provided by supramolecular assemblies of enamel matrix proteins [16,23,24]. This matrix-mediated mineralization occurs in a delineated, extracellular space adjacent to ameloblast cells. Each enamel-forming cell is responsible for the synthesis of a single enamel rod [11,25] and organization of these rods is achieved through highly controlled cell–cell

* Corresponding author. Tel.: +1 3234423178; fax: +1 3234422981.
E-mail address: mlsnead@usc.edu (M.L. Snead).
[1] These authors made equal contribution to the project and are listed alphabetically.

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interactions [15,20]. Ameloblasts secrete a cylinder of matrix proteins principally comprised of amelogenin. Amelogenin self-assembles into nanospheres [23] to regulate the mineral phase by promoting growth of the hydroxyapatite *c*-axis while retarding growth along the *a*- and *b*- faces [26]. Ameloblastin, the second most abundant enamel matrix protein is associated with demarcating rod boundaries and promoting cell to matrix interactions during formation [27–29]. With proteolytic processing of proteins and removal of water, the crystallites replace the organic species and grow to approximately 60 nm in width and up to millimeters in length [17,30].

Enamel formation originates from interactions between ectoderm-derived oral epithelium and neural crest-derived ectomesenchyme [31–33]. During the early stages of enamel formation, the dental epithelium is in contact with the basement membrane, a proteinaceous matrix responsible for providing critical signals to direct cellular proliferation and differentiation mediated in part through cell-based integrin receptors [34,35]. Fibronectin is one of several extracellular matrix (ECM) proteins present in the basement membrane that contains an integrin-based Arg-Gly-Asp-Ser (RGDS, also referred to as RGD) domain [36–38]. Exclusively produced by cells of mesenchyme origin [39], fibronectin has also been shown to induce differentiation of ameloblasts, *in vitro* [40]. The recent identification of a fibronectin-binding domain in a major enamel matrix protein, ameloblastin [41], further supports the central role of integrins in development of enamel tissue. As tissue maturation continues, fibronectin and integrin expression are reduced and enamel organ epithelial (EOE) cells differentiate into secretory ameloblasts that synthesize, secrete and interact with the enamel matrix proteins that control the mineral habit. Defects in enamel matrix protein production or in cell–matrix interactions disturb enamel formation and function. For example, loss of basement membrane proteins or their receptors in the odontogenic epithelia results in deleterious impacts on enamel formation and suggests the requirement for continuous cell – matrix interactions [36,42–47].

We chose to develop an artificial matrix and cell-based strategy for regeneration of enamel by using bioactive nanostructures to trigger biological events involved in enamel formation. The artificial matrix we used is based upon self-assembling molecules known as peptide amphiphiles (PAs). Peptide amphiphiles are small molecules comprised of a hydrophobic alkyl segment covalently conjugated to a hydrophilic peptide head group. Under physiological conditions, salts screen electrostatic repulsion between PA molecules and induce self-assembly, promoting formation of high-aspect-ratio nanofibers nanometers in diameter and up to microns in length [48–50]. The assembled nanoscale fibers mimic the ECM and display biological moieties on their surfaces in three dimensions to instruct surrounding cells to proliferate and/or differentiate [51–54]. Peptide amphiphiles exhibit biocompatibility [55] and have been designed for multiple biological applications including promoting biomineralization [56–59] and controlling the differentiation pathway of neural [52] and vascular precursors [51,60]. Additionally, PAs displaying an integrin-specific RGDS moiety have been shown to promote cell adhesion, proliferation, and differentiation [53,54]. The density of RGDS epitopes presented on the PA surface can be controlled by using branched, linear, and even cyclic architectures [53,61]. In the context of enamel regeneration, we chose to use a branched RGDS-bearing PA to provide a synthetic extracellular environment similar to that at the time of ameloblast differentiation. Additionally, the branched architecture of PAs has demonstrated increased signaling capacity relative to their linear counterparts [53,61,62]. We report here the use of self-assembling PAs to trigger the formation of dental enamel. We used a mouse incisor model that involves injecting the PA among dental epithelial cells followed by transplantation of the incisor under the kidney capsule of a host mouse.

2. Materials and methods

2.1. Peptide amphiphile synthesis and purification

The branched RGDS peptide amphiphiles (bRGDS PA) and its control scrambled (Scr) bRGDS PA shown in Fig. 1A and B, respectively were synthesized using standard 9-fluorenyl methoxy carbonyl (Fmoc) solid phase peptide synthesis [61]. Palmitic acid was attached by first removing a 4-methyltrityl (Mtt) protecting group from the ε-amine of a lysine residue and coupling the palmitic acid to the resulting free amine. The branched architecture was achieved by a similar method where the bioactive peptide sequence was coupled to the ε-amine of a lysine side chain [61]. Fmoc deprotection was performed using 30% piperidine in dimethylformamide (DMF) twice for 10 min each. Amino acid and palmitic acid coupling reactions were performed with a mixture of 4 M equivalents of protected amino acid or palmitic acid, 3.95 equivalents of 2-(1H-benzotriazol-1-yl)-1,1,2,2-tetramethyluronium hexafluorophosphate (HBTU) and 6 equivalents of diisopropylethylamine (DIEA) in a solvent mixture of 50% DMF, 25% dichloromethane (DCM) and 25% N-methyl pyrrolidine (NMP) for a minimum of 2 h. Kaiser tests were performed following amino acid and palmitic acid coupling to confirm a negative result for the presence of free amines. If necessary, the coupling was repeated until the test read a negative result. Molecules were cleaved from the resin and protecting groups removed using a mixture of 92.5% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), 2.5% 1-2 ethanedithiol (EDT) and 2.5% water for 3 h. Excess TFA and scavengers were removed by rotary evaporation and the remaining solution was triturated with cold diethyl ether to form a white precipitate. MBHA Rink amide resin, Fmoc protected amino acids, Boc-Lys (Boc)-OH, Fmoc-Lys(Mtt)-OH and HBTU were purchased from EMD Chemicals, Inc. All other reagents were purchased from VWR.

All molecules were purified using reverse phase high performance liquid chromatography (HPLC) on a Varian Prostar Model 210 preparative scale HPLC system equipped with a Phenomenex Gemini NX column (C_{18} stationary phase, 5 μm, 100 Å, 30 × 150 mm). A mobile gradient of water and acetonitrile with 0.1% TFA was used at a flow rate of 25 mL/min. The elution of the molecules was monitored by UV absorbance at 220 nm and 280 nm. Purified fractions were characterized by electrospray ionization mass spectrometry (ESI-MS) using an Agilent 6510 quadrupole time-of-flight (Q-Tof) instrument. Pure fractions were collected and combined and excess organic solvent was removed using rotary evaporation. The molecules were lyophilized and stored at −20 °C until use.

For visualization, a rhodamine tagged PA was prepared using 5-rhodamine carboxylic acid (Anaspec) activated with neat thionyl chloride (Sigma–Aldrich). The fluorescent dye was coupled to the ε-amine of the terminal lysine following Mtt deprotection in place of the bioactive peptide sequence.

2.2. Cryogenic transmission electron microscopy

Cryogenic transmission electron microscopy (cryoTEM) was performed on a JEOL 1230 microscope with an accelerating voltage of 100 kV. A Vitrobot Mark IV equipped with controlled humidity and temperature was used for plunge freezing samples. A small volume (5–10 μL) of the peptide amphiphile at 1% (w/v) in phosphate buffered saline (Hyclone Inc.) was deposited on a copper TEM grid with holey carbon support film (Electron Microscopy Sciences) and held in place with tweezers mounted to the Vitrobot. The specimen was blotted in an environment with 90–100% humidity and plunged into a liquid ethane reservoir that was cooled by liquid