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(54) Title: REAGENTS AND METHODS FOR TREATING DENTAL DISEASE

(57) Abstract: The present invention provides reagents and methods for treating dental disease.



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## Reagents and Methods for Treating Dental Disease

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### Cross Reference

This application claims priority to U.S. Provisional Application Serial Nos. 61/490,757 filed May 27, 2011; and 61/509,986 filed July 20, 2011, each of which is incorporated herein by reference in its entirety.

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### Background

Demineralization (loss of mineral content on the surface of tooth) is a major cause of most dental diseases. Loss of mineral can be caused by acidic diet, dry mouth, tooth whitening, abrasion and bacterial plaque formation. If left untreated, demineralization may lead to various problems from pain and sensitivity to systemic infections (e.g. heart disease) and loss of tooth. Depending on the extent of the mineral loss, several treatment options exist to arrest and/or reverse the process and to restore the teeth to proper function with aesthetic materials. In the conventional restorative treatments, the carious part is physically removed and the lost hard tissue is replaced with metal or resin based materials using inorganic adhesives. These materials, however, are usually short lived and may cause toxic products or secondary infections.

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Remineralization of the early lesions is an alternative to currently employed restorative therapies. Fluoride ions have been shown to promote the remineralization of previously demineralized enamel *in vitro* and *in situ* if adequate levels of calcium and phosphate ions are available where the fluoride is applied. It has been shown; however, that prolonged exposure to fluoridated water may cause severe dental fluorosis, skeletal fluorosis, and weakened bones, and that improper implementation of fluoride can also cause acute fluoride poisoning.

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### Summary of the Invention

In a first aspect, the present invention provides isolated amelogenin-derived polypeptides (ADPs) comprising or consisting of

(a) (WP(A/S)TDKTKREEVD)<sub>1-10</sub> (SEQ ID NO: 1)(ADP3);

(b) (PGYIN(L/F)SY(E/A)(K/N/A)SHSQAIN(T/V)(D/A)(R/A)TA)<sub>1-10</sub> (SEQ ID NO: 2) (ADP5);

(c) (LPPLFSMPLSPILPELPLEAWPAT)<sub>1-10</sub> (SEQ ID NO: 3) (ADP6); and

(d) 12-43 contiguous amino acids of (HPP(S/T)HTLQPHHH(L/I)PVVPAQ QPV(A/I)PQQPMMPVPG(H/Q)HSMTP(T/I)QH)<sub>1-10</sub> (SEQ ID NO: 4) (ADP7);

or functional equivalents thereof.

The inventors have discovered that amelogenin-derived polypeptides of this aspect of the invention can be used, for example, to direct dental mineralization and to treat dental disease, as discussed herein.

In a second aspect, the present invention provides recombinant fusion polypeptide, comprising

- (a) the ADP of any embodiment or combination of embodiments of the first aspect of the invention; and
- (b) a heterologous polypeptide.

In a third aspect, the present invention provides compositions, comprising

- (a) the ADP of any embodiment or combination of embodiments of the first aspect of the invention; and
- (b) a non-peptide moiety linked to the amelogenin-derived polypeptide .

In a fourth aspect, the present invention provides pharmaceutical compositions, comprising one or more polypeptides, recombinant fusion proteins, or compositions of the invention and a pharmaceutically acceptable carrier.

In a fifth aspect, the present invention provides oral care products, comprising any embodiment or combination of embodiments of the polypeptides, recombinant fusion proteins, and/or compositions of the invention.

In a sixth aspect, the present invention provides isolated nucleic acids encoding an ADP or fusion polypeptide of the present invention. In a seventh aspect, the present invention provides recombinant expression vectors comprising the isolated nucleic acid of any aspect of the invention operatively linked to a suitable control sequence. In an eighth

aspect, the present invention provides host cells that have been transfected with the recombinant expression vectors disclosed herein, wherein the host cells can be either prokaryotic or eukaryotic.

In a ninth aspect, the present invention provides methods for treating dental disease, comprising administering to a subject in need thereof an amount effective to treat the dental disease of the polypeptide, recombinant fusion protein, composition, pharmaceutical composition or oral care product of any embodiment or combination of embodiments of the invention.

## Description of the Figures

**Figure 1. Identification of amelogenin derived peptides (ADPs).** Panel a, Flowchart showing the design steps for identifying the ADPs. Panel b, High and low similarity amino acid regions among the rM180 and two experimentally selected HABP sets. Each bar represents one amino acid and the amino acid domains above the baseline represent the high similarity while those below represent low similarity regions. The overlapped plot shows the potential calcium ion binding domains. Note that the majority of the highest potential domains coincide with the high similarity regions (arrow heads). Panel c, Computationally determined molecular structure for rM180 amelogenin showing position of (folded) ADP7 within rM180. Panel d, Positions of the ion-binding domains on rM180. Panel e, The locations of the ADPs along rM180.

**Figure 2. Binding, mineralization, and structural characterization of mineral products of the ADPs.** Panel a, Binding constants,  $K_D$ , of the ADPs determined by QCM. Panel b, Calcium consumption rates of mineralization in the presence of ADP5,7 and rM180. Panel c, Calcium phosphate minerals formed in solution in the presence of ADPs. Panel d, The mineral product of ADP7 resembles those formed in the presence of rM180 and these acicular crystallites are consistent with HAp morphology. Panel e, X-ray diffraction (XRD) spectra of the minerals formed by ADPs. Material formed by ADP7 and amelogenin display the characteristic peaks belonging to the HAp crystal structure, while all minerals formed by other ADPs display weak diffraction peaks, consistent with amorphous, or only loosely crystalline outcomes shown in panel c.

**Figure 3. Procedure for *in vitro*-, cell-free synthesis of cementomimetic layer by ADP5 on human root surfaces.** Extracted human teeth are cleaned of any contaminating material

and cylindrical pieces are cut right below the cemento-enamel junction. An aqueous solution of ADP5, the mineralization directing peptide, is applied on the demineralized root surface. The specimen is immersed into a mineralization solution containing calcium and phosphate. Cell adhesion and proliferation is investigated on the re-mineralized root surfaces.

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**Figure 4. Structural and functional characteristics of the cementomimetic layer formed on the root of human tooth by ADP5.** Panels a and b, SEM images of the demineralized (a) and (b) ADP5 formed cementomimetic layer revealing uniform nanocrystals with a Ca/P ratio of 1.67 obtained from EDX (Energy dispersive X-ray) spectra (insets). Panel c, TEM images and the electron diffraction pattern of the newly formed cementomimetic mineral layer in cross-section showing HAp crystallites. Panel d, SEM image of mechanically separated cementomimetic mineral layer displaying uniform thickness of HAp crystallites; Panel e, Attachment of hPDL cells on control and cementomimetic mineral layer. Panel f, Proliferation of the hPDL cells on control, uncoated, root stock compared to ADP induced cementomimetic mineral layer. Panels g and h, Fluorescent microscopy image showing F-actin. Cell attachment without formation of organized actin network on control surface (g) is compared to those on ADP-induced cementomimetic mineral layer that reveals a well-organized actin cytoskeleton and lamellapodia (h).

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**Figure 5.** Procedure for designing amelogenin derived peptides

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**Figure 6.**  $\text{Ca}^{+2}$  consumption rates of the ADPs for the initial 90 minutes

**Figure 7.** Fluorescence microscopy images of the root surfaces coated with no peptide (a) and f-ADP5 (b)

**Figure 8.** Schematic of the sample which nanoindentation characterization was performed.

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20 indentations were made on each region: mineralized dentin, demineralized dentin (~20  $\mu\text{m}$ ), and cementomimetic layer (~10  $\mu\text{m}$ ). Indentations in dentin were made strictly on intertubular dentin.

**Figure 9.** SEM micrographs and the corresponding EDXS spectra of the cementomimetic layer after qualitative mechanical analysis. a) Ultrasonication and b) brushing. (insets: experimental setups)

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**Figure 10.**  $\text{Ca}^{+2}$  consumption rates of the ADP5 mutants for the initial 3.5 hours.

### Detailed Description of the Invention

All references cited are herein incorporated by reference in their entirety. Within this application, unless otherwise stated, the techniques utilized may be found in any of several

well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutscher, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2<sup>nd</sup> Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX).

As used herein, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. "And" as used herein is interchangeably used with "or" unless expressly stated otherwise.

All embodiments of any aspect of the invention can be used in combination, unless the context clearly dictates otherwise.

In a first aspect, the present invention provides isolated amelogenin-derived polypeptides (ADPs) comprising or consisting of

- (a) (WP(A/S)TDKTKREEVD)<sub>1-10</sub> (SEQ ID NO: 1) (ADP3);
  - (b) (PGYIN(L/F)SY(E/A)(K/N/A)SHSQAIN(T/V)(D/A)(R/A)TA)<sub>1-10</sub> (SEQ ID NO: 2) (ADP5);
  - (c) (LPPLFSMPLSPILPELPLEAWPAT)<sub>1-10</sub> (SEQ ID NO: 3) (ADP6); and
  - (d) 12-43 contiguous amino acids of (HPP(S/T)HTLQPHHH(L/I)PVVPAQ QPV(A/I)PQQPMMPVPG(H/Q)HSMTP(T/I)QH)<sub>1-10</sub> (SEQ ID NO: 4) (ADP7);
- or functional equivalents thereof.

The inventors have discovered that amelogenin-derived polypeptides of this aspect of the invention can be used, for example, to direct dental mineralization and to treat dental disease, as discussed herein.

As used herein, a "functional equivalent" of a polypeptide is one that retains the biological activity of the polypeptide in treating dental disease, and included one or more amino acid substitutions, deletions, additions, or insertions. In various embodiments, the functional equivalent is at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, or more identical to the recited polypeptide.

As used throughout the present application, the term "polypeptide" is used in its broadest sense to refer to a sequence of subunit amino acids, whether naturally occurring or of synthetic origin. The polypeptides of the invention may comprise L-amino acids, D-amino

acids (which are resistant to L-amino acid-specific proteases in vivo), or a combination of D- and L-amino acids. The polypeptides described herein may be chemically synthesized or recombinantly expressed. The polypeptides may be linked to other compounds to promote an increased half-life in vivo, such as by PEGylation, HESylation, PASylation, or glycosylation.

5 Such linkage can be covalent or non-covalent as is understood by those of skill in the art.

In various embodiments, the recited polypeptides may be present in 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 copies, preferably in one copy. When present in multiple copies, the copies are contiguous to each other. For example, 2 copies of ADP5 would be:

PGYIN(L/F)SYE(K/N)SHSQAIN(T/V)DRTAPGYIN(L/F)SYE(K/N)SHSQAIN(T/V)DRT

10 A (SEQ ID NO: 5). Further examples will be readily apparent to those of skill in the art based on the teachings herein.

In one embodiment, the isolated ADPs comprise or consist of the amino acid sequence (PGYIN(L/F)SY(E/A)(K/N/A)SHSQAIN(T/V)(D/A)(R/A)TA)<sub>1-10</sub> (SEQ ID NO: 2)

(ADP5), or a functional equivalent thereof. In a further embodiment, the ADP comprises or

15 consists of (PGYIN(L/F)SYE(K/N)SHSQAIN(T/V)DRTA)<sub>1-10</sub> (SEQ ID NO: 24) (ADP5), or a functional equivalent thereof. In preferred embodiments, ADP5 comprises or consists of

(PGYINFSYENSHSQAINVDRTA)<sub>1-10</sub> (SEQ ID NO: 6) (ADP5H),

(PGYINLSYEKSHSQAINTDRTA)<sub>1-10</sub> (SEQ ID NO: 7)(ADP5M), or a functional

equivalent thereof, wherein "ADP5H" refers to ADP5 derived from human amelogenin and

20 "ADP5M" refers to ADP5 derived from mouse amelogenin. In further embodiments, the

polypeptide comprises or consists of an ADP5 mutant, selected from the group consisting of

ADP5(V1):(PGYIN(L/F)SYA(K/N)SHSQAIN(T/V)ARTA)<sub>1-10</sub>(SEQ ID NO: 25);

ADP5(V2):(PGYIN(L/F)SYE(A/N)SHSQAIN(T/V)DATA)<sub>1-10</sub>(SEQ ID NO: 26);

ADP5(V3):(PGYIN(L/F)SYA(A/N)SHSQAIN(T/V)AATA)<sub>1-10</sub>(SEQ ID NO: 27);

25 or functional equivalents thereof, where "V" represents "variant." In further embodiments, the polypeptide comprises or consists of:

(PGYINLSYA(K/N)SHSQAINTARTA)<sub>1-10</sub> (SEQ ID NO: 32);

(PGYINLSYE(A/N)SHSQAINTDATA)<sub>1-10</sub> (SEQ ID NO: 33);

(PGYINLSYA(A/N)SHSQAINTAATA)<sub>1-10</sub> (SEQ ID NO: 34);

30 (PGYINFSYA(K/N)SHSQAINVARTA)<sub>1-10</sub> (SEQ ID NO: 35);

(PGYINFSYE(A/N)SHSQAINVDATA)<sub>1-10</sub> (SEQ ID NO: 36);

(PGYINFSYA(A/N)SHSQAINVAATA)<sub>1-10</sub> (SEQ ID NO: 37);

(PGYINLSYAKSHSQAINTARTA)<sub>1-10</sub> (SEQ ID NO: 38);

(PGYINLSYEASHSQAINTDATA)<sub>1-10</sub> (SEQ ID NO: 39);

(PGYINLSYAASHSQAINTAATA)<sub>1-10</sub> (SEQ ID NO: 40);  
 (PGYINFSYANSHSQAINVARTA)<sub>1-10</sub> (SEQ ID NO: 41);  
 (PGYINFSYEASHSQAINVDATA)<sub>1-10</sub> (SEQ ID NO: 42); and  
 (PGYINFSYAASHSQAINVAATA)<sub>1-10</sub> (SEQ ID NO: 43).

5 In various embodiments, ADP5, ADP5M, ADP5H, or any of the variations thereof is present in 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 copies. In a preferred embodiment, ADP5, ADP5M, ADP5H, or any of the variations there is present in 1 copy. ADP5 is shown in the examples that follow to mimic the mineralization promotion effect of the wild type amelogenin protein on a given solid surface, including dentin on human tooth, rapidly, and thus can be used in  
 10 any of the methods disclosed herein. In a most preferred embodiment, the ADP5 comprises or consists of ADP5H, or any of the variations thereof, preferably in one copy.

In another embodiment, the isolated ADPs comprise or consist of the amino acid sequence (HPP(S/T)HTLQPHHH(L/I)PVVPAQQPV(A/I)PQ QPMMPVPG(H/Q)HSMTP(T/I)QH)<sub>1-10</sub> (SEQ ID NO: 4)(ADP7), or a functional equivalent  
 15 thereof. In a preferred embodiment, ADP7 comprises or consists of (HPPSHTLQPHHHLPVVPAQQPVAPQQPMMPVPGHHSMTPTQH)<sub>1-10</sub> (SEQ ID NO: 8)(ADP7M); (HPPHTLQPHHHIPVVPAQQPVIPQQPMMPVPGQHSMTPIQH)<sub>1-10</sub> (SEQ ID NO: 9)(ADP7H), or a functional equivalent thereof, wherein “ADP7H” refers to ADP7 derived from human amelogenin and “ADP7M” refers to ADP7 derived from mouse amelogenin. In various embodiments, ADP 7, ADP7M, or ADP7H is present in 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 copies. In a preferred embodiment, ADP7, ADP7M, or ADP7H is present in 1 copy. ADP7 is shown in the examples that follow to control hydroxyapatite (HA) crystallography, and morphology, in a solution phase, and thus can be used in any of the methods disclosed herein.  
 20 ADP7 can also be used, for example, to target and immobilize bioactive molecules of interest (therapeutics, labeling molecules, other polypeptides of interest, etc.) to HA surfaces on the tooth. In a most preferred embodiment, the ADP7 comprises or consists of ADP7H, preferably in one copy.

In one embodiment, the isolated ADPs comprise or consist of the amino acid sequence  
 30 WP(A/S)TDKTKREEVD)<sub>1-10</sub> (SEQ ID NO: 1)(ADP3), or a functional equivalent thereof. In a preferred embodiment, ADP5 comprises or consists of ((WPATDKTKREEVD)<sub>1-10</sub> (SEQ ID NO: 10)(ADP3M) or (WPSTDKTKREEVD)<sub>1-10</sub> (SEQ ID NO: 11)(ADP3H), or a functional equivalent thereof, wherein “ADP3H” refers to ADP3 derived from human amelogenin and “ADP3M” refers to ADP3 derived from mouse amelogenin. In various



embodiments, ADP3, ADP3M, or ADP3H is present in 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 copies. In a preferred embodiment, ADP3, ADP3M, or ADP3H is present in 1 copy. ADP3 is a low similarity HA binder, and thus may be used in any of the methods of the invention. ADP3 can also be used, for example, to target and immobilize bioactive molecules of interest (therapeutics, labeling molecules, other polypeptides of interest, etc.) to HA surfaces on the tooth. In a most preferred embodiment, the ADP3 comprises or consists of ADP3H, preferably in one copy.

In various other preferred embodiments, the isolated polypeptide comprises or consists of a polypeptide selected from the group consisting of:

- (i) (HTLQPHHH(L/I)PVV)<sub>1-10</sub> (SEQ ID NO: 12) (ADP1);
  - (ii) (VPG(H/Q)HSMTP(T/I)QH)<sub>1-10</sub> (SEQ ID NO: 13) (ADP2)
  - (iii) (HPP(S/T)HTLQPHHH(L/I)PVV)<sub>1-10</sub> (SEQ ID NO: 14) (ADP4);
  - (iv) (PAQQPV(A-I)PQQPMMP)<sub>1-10</sub> (SEQ ID NO: 15) (ADP8);
  - (v) (HTLQPHHHLPVV)<sub>1-10</sub> (SEQ ID NO: 16) (ADP1M);
  - (vi) (HTLQPHHHIPVV)<sub>1-10</sub> (SEQ ID NO: 17) (ADP1H);
  - (vii) (VPGHHSMTPTQH)<sub>1-10</sub> (SEQ ID NO: 18) (ADP2M)
  - (vii) (VPGQHSMPTIQH)<sub>1-10</sub> (SEQ ID NO: 19) (ADP2H)
  - (ix) (HPPSHTLQPHHHLPVV)<sub>1-10</sub> (SEQ ID NO: 20) (ADP4M);
  - (x) (HPPTHTLQPHHHIPVV)<sub>1-10</sub> (SEQ ID NO: 21) (ADP4H);
  - (xi) (PAQQPVAPQQPMMP)<sub>1-10</sub> (SEQ ID NO: 22) (ADP8M); and
  - (xii) (PAQQPVIPQQPMMP)<sub>1-10</sub> (SEQ ID NO: 23) (ADP8H);
- or functional equivalents thereof.

In various embodiments, the recited polypeptides may be present in 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 copies, preferably in one copy. Each of these polypeptides bind to HA, and thus, even apart from their mineralization effects, they can be used to target and immobilize bioactive molecules of interest (therapeutics, labeling molecules, other polypeptides of interest, etc.) to HA surfaces on the tooth. Furthermore, ADP7 is a combination of ADP4 + ADP8 + ADP2, while ADP1 is completely contained within ADP4. Thus, each of these polypeptides can also be used, for example, in synthetically preparing ADP7.

All of these embodiments can be combined with any other embodiment, unless the context clearly dictates otherwise.

In a second aspect, the present invention provides recombinant fusion polypeptide, comprising

(a) the ADP of any embodiment or combination of embodiments of the first aspect of the invention; and

(b) a heterologous polypeptide.

As used herein, a “heterologous polypeptide” is any polypeptide that either (a) is not derived from amelogenin; or (b) is a different ADP than the ADP recited in component (a) of the recombinant fusion polypeptide. The recombinant fusion polypeptide does not include wild type amelogenin. Thus, for example, the recombinant fusion polypeptide may comprise or consist of a fusion of ADP5 and ADP7, so long as the fusion polypeptide is not amelogenin.

The recombinant fusion polypeptides of the invention can be used, for example, to treat dental disease. Thus, providing the ADPs as fusion polypeptides is a useful strategy for providing increased functionality when carrying out such treatments, or for any other suitable use.

In one embodiment, the heterologous polypeptide is a detectable polypeptide, to allow for detection of the recombinant fusion, for example, during administration to treat dental disease. In another embodiment, the heterologous polypeptide is an affinity tag of any type (including but not limited to FLAG tags, hexa-histidine tags, and myc tags) that, for example, can be used to assist in isolation of the polypeptide.

In various other embodiments, the heterologous polypeptide provides added functionality, for example, when the fusion polypeptides are used to treat dental disease. Exemplary such heterologous polypeptide include, but are not limited to anti-microbial polypeptides (inhibiting bacterial infection), biomineralization-promoting polypeptides (i.e.: any polypeptides that are useful for controlling or promoting biomineralization), inorganic material-binding polypeptides, three-dimensional scaffold-forming polypeptides, collagen, chitosan, amphiphilic peptides, protein-binding polypeptides, enamel-derived polypeptides, tuftelin-derived peptides, statherin-derived polypeptides, dentin-derived polypeptides, bone sialoprotein-derived polypeptides, osteocalcin-derived polypeptides, osteopontin-derived polypeptides, proteins with caries inhibitory activity, casein, and bone morphogenetic-derived polypeptides. As will be understood by those of skill in the art, the recited heterologous polypeptide may comprise or consist of the full length protein, or functional polypeptides derived therefrom. Such heterologous polypeptides are known to those of skill in the art. In various non-limiting embodiments, such heterologous polypeptides include, but are not limited to dentin or polypeptides derived therefrom; cementum, or polypeptides derived therefrom; or other amelogenin-derived peptides including but not limited to:

MPLPPHPGSPGYINLSYEVLTPWKYQSMIRQPPLSPILPELPLEAWPATDKTK  
REEVD (SEQ ID NO: 28)

MPLPPHPGSPGYINLSYEVLTPWKYQSMIRQPYPSYGYEPMGGW (SEQ ID  
NO: 29);

5 LPPHPGSPGYINLSYEVLTPWKYQSMIRQPYPSYGYE (SEQ ID NO: 30); and  
SPILPELPLEAWPATDK (SEQ ID NO: 31).

In a third aspect, the present invention provides compositions, comprising

(a) the ADP of any embodiment or combination of embodiments of the first  
10 aspect of the invention; and

(b) a non-peptide moiety linked to the amelogenin-derived polypeptide .

As used herein, a “non-peptide moiety” is any non-peptide moiety, whether naturally  
occurring or synthetic, that provides added functionality to the ADPs of the invention. For  
example, the non-peptide moiety may be any organic or inorganic nanostructure, or hybrids  
15 thereof. The compositions can be used, for example, to treat dental disease. Thus, providing  
the ADPs as the recited compositions is a useful strategy for providing increased functionality  
when carrying out such treatments, or for any other suitable use.

In one embodiment, the non-peptide moiety may be a non-peptide detectable moiety,  
including but not limited to radioactive isotopes, non-peptide affinity tags, isotope-coded  
20 affinity tags, and fluorophores, to allow for detection of the composition, for example, during  
administration to treat dental disease.

In other embodiments, the non-peptide moiety may comprise organic nanostructures  
(ex: micelles, liposomes, and dendrimers) or inorganic nanostructures (ex: metallic,  
semiconducting and dielectric), including but not limited to nanotubes, nanowires,  
25 nanoparticles, atomic clusters, quantum dots, single layer or multilayer atomic materials,  
inorganic ions or ion clusters, and organic-inorganic hybrid nanostructures, and non-peptide  
polymers. These embodiments can be used, for example, to provide structural features of  
interest for a given application of the compositions.

In other embodiments, the non-peptide moiety may comprise non-peptide entities that  
30 provide for additional therapeutic benefit to a subject being treated, including but not limited  
to fish flour, amino acids (including but not limited to glycine), and non-peptide polymers.

In each of the second and third embodiments, the heterologous polypeptide or non-  
peptide moiety can be linked to the ADP through covalent bonding, including, but not limited  
to, disulfide bonding, hydrogen bonding, electrostatic bonding, recombinant fusion and

conformational bonding. Alternatively, the heterologous polypeptide or non-peptide moiety can be linked to the ADP by means of one or more linking compounds. Techniques for conjugating moieties to polypeptides are well known to the skilled artisan.

Any suitable detectable polypeptide or detectable non-peptide moiety can be linked to the ADP in the second and third aspects of the invention, including but not limited to enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals, and nonradioactive paramagnetic metal ions. The tag used will depend on the specific detection techniques and/or methods used. Enzymes typically conjugated to polypeptides to permit their visualization are well known and include, but are not limited to, acetylcholinesterase, alkaline phosphatase, beta-galactosidase, glucose oxidase, horseradish peroxidase, and urease. Typical substrates for production and deposition of visually detectable products are also well known to the skilled person in the art. The ADPs can be labeled using colloidal gold or they can be labeled with radioisotopes, such as  $^{33}\text{P}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^3\text{H}$ , and  $^{125}\text{I}$ . ADPs of the invention can be attached to radionuclides directly or indirectly via a chelating agent by methods well known in the art.

In a fourth aspect, the present invention provides pharmaceutical compositions, comprising one or more polypeptides, recombinant fusion proteins, or compositions of the invention and a pharmaceutically acceptable carrier. The pharmaceutical compositions of the invention can be used, for example, in the methods of the invention described below. The pharmaceutical composition may comprise in addition to the polypeptide of the invention (a) a lyoprotectant; (b) a surfactant; (c) a bulking agent; (d) a tonicity adjusting agent; (e) a stabilizer; (f) a preservative and/or (g) a buffer.

In some embodiments, the buffer in the pharmaceutical composition is a Tris buffer, a histidine buffer, a phosphate buffer, a citrate buffer or an acetate buffer. The pharmaceutical composition may also include a lyoprotectant, e.g. sucrose, sorbitol or trehalose. In certain embodiments, the pharmaceutical composition includes a preservative e.g. benzalkonium chloride, benzethonium, chlorohexidine, phenol, m-cresol, benzyl alcohol, methylparaben, propylparaben, chlorobutanol, o-cresol, p-cresol, chlorocresol, phenylmercuric nitrate, thimerosal, benzoic acid, and various mixtures thereof. In other embodiments, the pharmaceutical composition includes a bulking agent, like glycine. In yet other embodiments, the pharmaceutical composition includes a surfactant e.g., polysorbate-20, polysorbate-40, polysorbate-60, polysorbate-65, polysorbate-80 polysorbate-85, poloxamer-188, sorbitan monolaurate, sorbitan monopalmitate, sorbitan monostearate, sorbitan monooleate, sorbitan trilaurate, sorbitan tristearate, sorbitan trioleate, or a combination thereof. The

pharmaceutical composition may also include a tonicity adjusting agent, e.g., a compound that renders the formulation substantially isotonic or isoosmotic with human blood. Exemplary tonicity adjusting agents include sucrose, sorbitol, glycine, methionine, mannitol, dextrose, inositol, sodium chloride, arginine and arginine hydrochloride. In other  
5       embodiments, the pharmaceutical composition additionally includes a stabilizer, e.g., a molecule which, when combined with a protein of interest substantially prevents or reduces chemical and/or physical instability of the protein of interest in lyophilized or liquid form. Exemplary stabilizers include sucrose, sorbitol, glycine, inositol, sodium chloride, methionine, arginine, and arginine hydrochloride.

10       The polypeptides of the invention may be the sole active agent in the pharmaceutical composition, or the composition may further comprise one or more other active agents suitable for an intended use, including but not limited to anti-microbial polypeptides (inhibiting bacterial infection), biomineralization-promoting polypeptides (i.e.: any polypeptides that are useful for controlling or promoting biomineralization), inorganic  
15       material-binding polypeptides, three-dimensional scaffold-forming polypeptides, collagen, chitosan, amphiphilic peptides, protein-binding polypeptides, enamel-derived polypeptides, tuftelin-derived peptides, statherin-derived polypeptides, dentin-derived polypeptides, bone sialoprotein-derived polypeptides, osteocalcin-derived polypeptides, osteopontin-derived polypeptides, proteins with caries inhibitory activity, casein, and bone morphogenetic-derived  
20       polypeptides.

      The pharmaceutical compositions described herein generally comprise a combination of a compound described herein and a pharmaceutically acceptable carrier, diluent, or excipient. Such compositions are substantially free of non-pharmaceutically acceptable components, *i.e.*, contain amounts of non-pharmaceutically acceptable components lower  
25       than permitted by US regulatory requirements at the time of filing this application. In some embodiments of this aspect, if the compound is dissolved or suspended in water, the composition further optionally comprises an additional pharmaceutically acceptable carrier, diluent, or excipient. In other embodiments, the pharmaceutical compositions described herein are solid pharmaceutical compositions (*e.g.*, tablet, capsules, *etc.*).

30       These compositions can be prepared in a manner well known in the pharmaceutical art, and can be administered by any suitable route. In a preferred embodiment, the pharmaceutical compositions and formulations are designed for topical administration, and may include ointments, lotions, creams, pastes, gels, drops, sprays, liquids and powders.

Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

The pharmaceutical compositions can be in any suitable form, including but not limited to tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments containing, for  
5 example, up to 10% by weight of the active compound, soft and hard gelatin capsules, sterile injectable solutions, and sterile packaged powders.

In a preferred embodiment the pharmaceutical compositions are in the form of a dental care product, including but not limited to toothpaste, toothpowders, mouthwash, dental  
10 floss, liquid dentifrices, dental tablets, topical gels, troches, chewing gums, dental pastes, gingival massage creams, gargle tablets, lozenges, and food products. Thus, in a fifth aspect, the present invention provides oral care products, comprising any embodiment or combination of embodiments of the polypeptides, recombinant fusion proteins, and/or compositions of the invention. Such oral care products can be used, for example, in treating  
15 dental disease.

In a sixth aspect, the present invention provides isolated nucleic acids encoding an ADP or fusion polypeptide of the present invention. The isolated nucleic acid sequence may comprise RNA or DNA. As used herein, "isolated nucleic acids" are those that have been removed from their normal surrounding nucleic acid sequences in the genome or in cDNA  
20 sequences. Such isolated nucleic acid sequences may comprise additional sequences useful for promoting expression and/or purification of the encoded protein, including but not limited to polyA sequences, modified Kozak sequences, and sequences encoding epitope tags, export signals, and secretory signals, nuclear localization signals, and plasma membrane localization signals. It will be apparent to those of skill in the art, based on the teachings herein, what  
25 nucleic acid sequences will encode the polypeptides of the invention.

In a seventh aspect, the present invention provides recombinant expression vectors comprising the isolated nucleic acid of any aspect of the invention operatively linked to a suitable control sequence. "Recombinant expression vector" includes vectors that operatively link a nucleic acid coding region or gene to any control sequences capable of effecting  
30 expression of the gene product. "Control sequences" operably linked to the nucleic acid sequences of the invention are nucleic acid sequences capable of effecting the expression of the nucleic acid molecules. The control sequences need not be contiguous with the nucleic acid sequences, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter

sequence and the nucleic acid sequences and the promoter sequence can still be considered "operably linked" to the coding sequence. Other such control sequences include, but are not limited to, polyadenylation signals, termination signals, and ribosome binding sites. Such expression vectors can be of any type known in the art, including but not limited to plasmid and viral-based expression vectors. The control sequence used to drive expression of the disclosed nucleic acid sequences in a mammalian system may be constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of inducible promoters including, but not limited to, tetracycline, ecdysone, steroid-responsive). The construction of expression vectors for use in transfecting prokaryotic cells is also well known in the art, and thus can be accomplished via standard techniques. (See, for example, Sambrook, Fritsch, and Maniatis, in: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989; *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX). The expression vector must be replicable in the host organisms either as an episome or by integration into host chromosomal DNA. In a preferred embodiment, the expression vector comprises a plasmid. However, the invention is intended to include other expression vectors that serve equivalent functions, such as viral vectors.

In an eighth aspect, the present invention provides host cells that have been transfected with the recombinant expression vectors disclosed herein, wherein the host cells can be either prokaryotic or eukaryotic. The cells can be transiently or stably transfected. Such transfection of expression vectors into prokaryotic and eukaryotic cells can be accomplished via any technique known in the art, including but not limited to standard bacterial transformations, calcium phosphate co-precipitation, electroporation, or liposome mediated-, DEAE dextran mediated-, polycationic mediated-, or viral mediated transfection. (See, for example, *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press; *Culture of Animal Cells: A Manual of Basic Technique*, 2<sup>nd</sup> Ed. (R.I. Freshney. 1987. Liss, Inc. New York, NY). A method of producing a polypeptide according to the invention is an additional part of the invention. The method comprises the steps of (a) culturing a host according to this aspect of the invention under conditions conducive to the expression of the polypeptide, and (b) optionally, recovering the expressed polypeptide. The expressed polypeptide can be recovered from the cell free extract, but preferably they are recovered from the culture medium. Methods to recover polypeptide from cell free extracts or culture medium are well known to the man skilled in the art.

In a ninth aspect, the present invention provides methods for treating dental disease, comprising administering to a subject in need thereof an amount effective to treat the dental disease of the polypeptide, recombinant fusion protein, composition, pharmaceutical composition or oral care product of any embodiment or combination of embodiments of the invention.

As shown herein, the inventors have discovered that the polypeptides of the invention can be used to treat a variety of dental diseases. While not being bound by a specific mechanism of action, the inventors believe that the polypeptides of the invention direct mineralization of dental lesions to form a “dento-mimetic” mineral layer, which also serves to reduce dental hypersensitivity and bacterial infiltration. It is further believed that some of the polypeptides exert their activity via binding to hydroxyapatite (HA) surfaces on the tooth to kinetically promote remineralization, occluding of dentin tubules to prevent/limit stimulants from reaching the tubules, and/or rebuilding lost mineral to create a physical barrier against bacteria. Thus, the methods of the invention provide a great improvement over prior art methods for treating dental disease.

The subject may be any subject that can contract dental disease, including but not limited to mammals. In various embodiments, the mammal is a human, dog, cat, horse, cow, sheep, goat, pig, or other pet or food/dairy animal. In a preferred embodiment, the subject is a human.

As used herein, dental disease is any disease or condition affecting the teeth, gums, or other tissues of the mouth that involves (directly or indirectly) demineralization (i.e.: loss of mineral content on the surface of the tooth. In a healthy person, tooth is in a cycle of demineralization and remineralization through the exchange of ions between the surface of the tooth and the surrounding saliva. When the rate of demineralization exceeds the rate of remineralization, it leads to dental disease. Demineralization is a major cause of most dental diseases. Loss of mineral can be caused by any means, including but not limited to acidic diet, dry mouth, tooth whitening, abrasion and bacterial plaque formation.

Such dental diseases may include, but are not limited to, periodontitis, tooth erosion, hypersensitivity, dental caries (also known as tooth decay/cavities), dental fluorosis, tooth resorption, craniomaxillofacial bone disease, and gingival recession.

As used herein, “periodontitis” is a set of inflammatory diseases affecting the periodontium, i.e., the tissues that surround and support the teeth. Periodontitis involves progressive loss of the alveolar bone around the teeth, and if left untreated, can lead to the loosening and subsequent loss of teeth. Periodontitis is caused by microorganisms that adhere



to and grow on the tooth's surfaces, along with an overly aggressive immune response against these microorganisms.

As used herein, "tooth demineralization" or "tooth erosion" is erosion of tooth enamel. This erosion may be caused by any number of factors, including bacterial infection, teeth grinding, abrasion, erosion, and abfraction.

As used herein, "hypersensitivity" is an increased exposure to the environment of nerves inside the dentin of teeth, causing an increased nerve response, which can be mild to severe. The sensitivity may be caused by any factor, including but not limited to wear, tooth decay or exposed tooth roots.

As used herein, "dental caries" is an infectious disease usually bacterial in origin that causes demineralization of the hard tissues (enamel, dentin and cementum) and destruction of the organic matter of the tooth. The bacteria most commonly responsible for dental cavities are *Streptococcus mutans* and *Lactobacillus*. If left untreated, dental caries can lead to pain, tooth loss and systemic infection.

As used herein, "dental fluorosis" is a developmental disturbance of dental enamel caused by excessive exposure to high concentrations of fluoride during tooth development. The risk of fluoride overexposure is greatest between the ages of 3 months and 8 years. In its mild forms, fluorosis often appears as unnoticeable, tiny white streaks or specks in the enamel of the tooth. In its most severe form, tooth appearance is marred by discoloration or brown markings. The enamel may be pitted, rough and hard to clean. The spots and stains left by fluorosis are permanent and may darken over time.

As used herein, "tooth resorption" is a process by which all or part of a tooth structure is lost. In "external resorption" the root surface is lost; this can be caused, for example, by chronic inflammation, cysts, tumors, trauma, reimplantation of a tooth, or by unknown causes. "Internal resorption" involved resorption of dentin and pulpal walls centrally within the root canal; the cause can sometimes be attributed to tooth trauma, but often there is no known cause.

As used herein, "gingival recession" is root (cementum) that is exposed to the environment as a result of retraction of the gumline from the crown of the teeth. Cementum is a calcified tissue that supports attachment of the tooth to mandibular or maxillary bone through periodontal ligaments. Exposed cementum can result in resorption of cementum and exposure of the underlying dentin, therefore, hypersensitivity and pain.

In one embodiment, "craniomaxillofacial bone disease" refers to disease of dental bone structures.

As used herein, “treating dental disease” means accomplishing one or more of the following: (a) reducing the severity of the dental disease; (b) limiting or preventing development of symptoms characteristic of the dental disease(s) being treated; (c) inhibiting worsening of symptoms characteristic of the dental disease(s) being treated; (d) limiting or preventing recurrence of the dental disease(s) in patients that have previously had the disorder(s); (e) limiting or preventing recurrence of symptoms in patients that were previously symptomatic for the dental disease(s); and (f) limiting development of the dental disease in a subject at risk of developing the dental disease, or not yet showing the clinical effects of the dental disease.

As used herein, an “amount effective” refers to an amount of the polypeptide that is effective for treating and/or limiting dental disease. The polypeptides are typically formulated as a pharmaceutical composition, such as those disclosed above, and can be administered via any suitable route, including orally, parentally, by inhalation spray, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. In a preferred embodiment, the pharmaceutical compositions and formulations are topically administration, such as in the form of ointments, lotions, creams, pastes, gels, drops, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Dosage regimens can be adjusted to provide the optimum desired response (e.g., a therapeutic or prophylactic response). A suitable dosage range may, for instance, be 0.1 ug/kg-100 mg/kg body weight; alternatively, it may be 0.5 ug/kg to 50 mg/kg; 1 ug/kg to 25 mg/kg, or 5 ug/kg to 10 mg/kg body weight. The polypeptides can be delivered in a single bolus, or may be administered more than once (e.g., 2, 3, 4, 5, or more times) as determined by an attending physician.

In each of these embodiments, or combinations of embodiments, the polypeptide may be any embodiment or combination of embodiments of the first, second, or third aspects of the invention. Thus, in one embodiment, ADPs comprise or consist of

- (a) (WP(A/S)TDKTKREEVD)<sub>1-10</sub> (SEQ ID NO: 1) (ADP3);
- (b) (PGYIN(L/F)SY(E/A)(K/N/A)SHSQAIN(T/V)(D/A)(R/A)TA)<sub>1-10</sub> (SEQ ID NO: 2) (ADP5);
- (c) (LPPLFSMPLSPILPELPLEAWPAT)<sub>1-10</sub> (SEQ ID NO: 3) (ADP6); and
- (d) 12-43 contiguous amino acids of (HPP(S/T)HTLQPHHH(L/I)PVVPAQ QPV(A/I)PQQPMMPVPG(H/Q)HSMTP(T/I)QH)<sub>1-10</sub> (SEQ ID NO: 4) (ADP7);

or functional equivalents thereof. In various embodiments, the recited polypeptides may be present in 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 copies, preferably in one copy.

In one preferred embodiment, the methods comprise administering a polypeptide comprising or consisting of the amino acid sequence

5 (PGYIN(L/F)SY(E/A)(K/N/A)SHSQAIN(T/V)(D/A)(R/A)TA)<sub>1-10</sub> (SEQ ID NO: 2) (ADP5), or a functional equivalent thereof. In a further embodiment, the ADP administered comprises or consists of (PGYIN(L/F)SYE(K/N)SHSQAIN(T/V)DRTA)<sub>1-10</sub> (SEQ ID NO: 24) (ADP5), or a functional equivalent thereof. In preferred embodiments, the administered ADP5 comprises or consists of (PGYINFSYENSHSQAINVDRTA)<sub>1-10</sub> (SEQ ID NO: 6)(ADP5H), (PGYINLSYEKSHSQAINTDRTA)<sub>1-10</sub> (SEQ ID NO: 7) (ADP5M), or a functional equivalent thereof. In further embodiments, the polypeptide comprises or consists of an ADP5 mutant, selected from the group consisting of

ADP5(V1):(PGYIN(L/F)SYA(K/N)SHSQAIN(T/V)ARTA)<sub>1-10</sub> (SEQ ID NO: 25);

ADP5(V2):(PGYIN(L/F)SYE(A/N)SHSQAIN(T/V)DATA)<sub>1-10</sub> (SEQ ID NO: 26);

15 ADP5(V3):(PGYIN(L/F)SYA(A/N)SHSQAIN(T/V)AATA)<sub>1-10</sub> (SEQ ID NO: 27);

or functional equivalents thereof, where “V” represents “variant.” In further embodiments, the polypeptide comprises or consists of

(PGYINLSYA(K/N)SHSQAINRTA)<sub>1-10</sub> (SEQ ID NO: 32);

(PGYINLSYE(A/N)SHSQAINRDATA)<sub>1-10</sub> (SEQ ID NO: 33);

20 (PGYINLSYA(A/N)SHSQAINRTAATA)<sub>1-10</sub> (SEQ ID NO: 34);

(PGYINFSYA(K/N)SHSQAINRVARTA)<sub>1-10</sub> (SEQ ID NO: 35);

(PGYINFSYE(A/N)SHSQAINRVDATA)<sub>1-10</sub> (SEQ ID NO: 36);

(PGYINFSYA(A/N)SHSQAINRVAATA)<sub>1-10</sub> (SEQ ID NO: 37);

(PGYINLSYAKSHSQAINRTA)<sub>1-10</sub> (SEQ ID NO: 38);

25 (PGYINLSYEASHSQAINRDATA)<sub>1-10</sub> (SEQ ID NO: 39);

(PGYINLSYAASHSQAINRTAATA)<sub>1-10</sub> (SEQ ID NO: 40);

(PGYINFSYANSHSQAINRVARTA)<sub>1-10</sub> (SEQ ID NO: 41);

(PGYINFSYEASHSQAINRVDATA)<sub>1-10</sub> (SEQ ID NO: 42); and

(PGYINFSYAASHSQAINRVAATA)<sub>1-10</sub> (SEQ ID NO: 43).

30 In various embodiments, ADP5, ADP5M, ADP5H, or any of the variations thereof is present in 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 copies. In a preferred embodiment, ADP5, ADP5M, ADP5H, or any of the variations there is present in 1 copy. ADP5 is shown in the examples that follow to mimic the mineralization promotion effect of the wild type amelogenin protein on a given solid surface, including dentin on human tooth, rapidly, and thus can be used in

any of the methods disclosed herein. In a most preferred embodiment, the ADP5 comprises or consists of ADP5H, or any of the variations thereof, preferably in one copy.

In another preferred embodiment, the methods comprise administering a polypeptide comprising or consisting of the amino acid sequence

5 (HPP(S/T)HTLQPHHH(L/I)PVVPAQQPV(A/I)PQ  
QPMMPVPG(H/Q)HSMTP(T/I)QH)<sub>1-10</sub> (SEQ ID NO: 4) (ADP7), or a functional  
equivalent thereof. In a preferred embodiment, the ADP7 comprises or consists of  
(HPPSHTLQPHHHLPVVPAQQPVAPQQPMMPVPGHHSMTP(T/I)QH)<sub>1-10</sub> (SEQ ID NO: 8)  
(ADP7M);

10 (HPPHTLQPHHHIPVVPAQQPVIPQQPMMPVPGQHSMTP(I)QH)<sub>1-10</sub> (SEQ ID NO: 9)  
(ADP7H), or a functional equivalent thereof. In various embodiments, ADP 7, ADP7M, or  
ADP7H is present in 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 copies. In a preferred embodiment, ADP7,  
ADP7M, or ADP7H is present in 1 copy. In a most preferred embodiment, the ADP7  
comprises or consists of ADP7H, preferably in one copy.

15 In a further embodiment, the polypeptide for use in the methods comprises or consists  
of the amino acid sequence WP(A/S)TDKTKREEVD)<sub>1-10</sub> (SEQ ID NO: 1) (ADP3), or a  
functional equivalent thereof. In a preferred embodiment, ADP3 comprises or consists of  
((WPATDKTKREEVD)<sub>1-10</sub> (SEQ ID NO: 10) (ADP3M) or (WPSTDKTKREEVD)<sub>1-10</sub> (SEQ  
ID NO: 11) (ADP3H), or a functional equivalent thereof. In various embodiments, ADP3,  
20 ADP3M, or ADP3H is present in 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 copies. In a preferred  
embodiment, ADP3, ADP3M, or ADP3H is present in 1 copy. In a most preferred  
embodiment, the ADP3 comprises or consists of ADP3H, preferably in one copy.

In various further embodiments, the polypeptide for use in the methods comprises or  
consists of a polypeptide selected from the group consisting of selected from the group

25 consisting of

- (i) (HTLQPHHH(L/I)PVV)<sub>1-10</sub> (SEQ ID NO: 12) (ADP1);
- (ii) (VPG(H/Q)HSMTP(T/I)QH)<sub>1-10</sub> (SEQ ID NO: 13) (ADP2)
- (iii) (HPP(S/T)HTLQPHHH(L/I)PVV)<sub>1-10</sub> (SEQ ID NO: 14) (ADP4);
- (iv) (PAQQPV(A-I)PQQPMMP)<sub>1-10</sub> (SEQ ID NO: 15) (ADP8);
- 30 (v) (HTLQPHHHLPVV)<sub>1-10</sub> (SEQ ID NO: 16) (ADP1M);
- (vi) (HTLQPHHHIPVV)<sub>1-10</sub> (SEQ ID NO: 17) (ADP1H);
- (vii) (VPGHHSMTP(T/I)QH)<sub>1-10</sub> (SEQ ID NO: 18) (ADP2M)
- (viii) (VPGQHSMTP(I)QH)<sub>1-10</sub> (SEQ ID NO: 19) (ADP2H)
- (ix) (HPPSHTLQPHHHLPVV)<sub>1-10</sub> (SEQ ID NO: 20) (ADP4M);

- (x) (HPPTHTLQPHHHIPVV)<sub>1-10</sub> (SEQ ID NO: 21) (ADP4H);
  - (xi) (PAQQPVAPQQPMMP)<sub>1-10</sub> (SEQ ID NO: 22) (ADP8M); and
  - (xii) (PAQQPVIPQQPMMP)<sub>1-10</sub> (SEQ ID NO: 23) (ADP8H);
- or functional equivalents thereof.

5 In various embodiments, the recited polypeptides may be present in 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 copies, preferably 1 copy.

All of these aspects/embodiments disclosed herein can be combined with any other aspect/embodiment, unless the context clearly dictates otherwise.

10

## Example 1

### Abstract

Cementum is the outer-, mineralized-tissue on the tooth root. It is part of the system of periodontal tissue that anchors the tooth to the bone. Periodontal disease results from the destructive behavior of the host elicited by an infectious biofilm adhering to the tooth root and left untreated, may lead to tooth loss. Tissue specific extracellular-matrix proteins for repairing damaged mineralized tissues of the teeth have been limited to date in therapeutic applications. Here, we describe a novel protocol for identifying peptide sequences from native proteins associated with controlling hydroxyapatite biomineralization. Using amelogenin as a case study and a bioinformatics scoring matrix, we identified similarity regions, for amelogenin with a set of hydroxyapatite-binding peptides, previously selected by phage display. Among these regions, referred to as amelogenin derived peptides, a 22-amino acids long peptide (ADP5) was shown to facilitate cell-free formation of a cementum-like hydroxyapatite mineral layer on demineralized human root dentin that, in turn, supported attachment of periodontal ligament cells *in vitro*. Our findings have several implications in peptide-assisted mineral formation that mimics biomineralization events. By further elaborating the mechanism for protein control over the biominerals formed in these systems we afford new insights into the evolution of protein-mineral interactions. By exploiting small peptide domains of native proteins, our understanding of structure-function relationships of biomineralizing proteins can be extended and these peptides can be utilized to engineer the mineral formation. Finally, the ADPs have clinical application to treat dental disease, such as through repair of diseased root surfaces caused by caries and periodontal disease, so as to promote the regeneration of periodontal tissues and thereby reduce the morbidity associated with tooth loss.

## Introduction

The distinguishing characteristics of dental bioceramic hard tissues, e.g., enamel, cementum, and dentin, are achieved due to the amount of the hydroxyapatite (HAp) crystallites formed, their dimensional structural characteristics and their overall architecture. These characteristics originate from the tissue-specific extracellular matrix (ECM) proteins found within each tissue during its development (1-5) and which act as regulators of nucleation and growth of biological apatite (1, 2, 6). Periodontal disease is caused by the host's inflammatory reaction to the bacterial biofilms that adhere to the tooth surface (7) and results in periodontal tissue destruction. It is one of the most prevalent infections of mankind, and left unchecked, will result in the loss of the teeth and surrounding tissue, including bone (7, 8). The use of dental ECM proteins as therapeutic agents to repair damage caused by such diseases has been limited so far due to technical and financial limitations in the identification, extraction and purification of these proteins. We previously used the enamel protein, amelogenin, to identify similarity regions, i.e., similar amino acid sequences, with a set of HAp-binding peptides (HABP) that we previously experimentally selected (23). Amelogenin is expressed during tooth enamel formation and contributes to the hardest and most highly mineralized tissue in the human body by exerting control over the dimension and directionality of HAp crystals formed within the assembled protein matrix (17-19). Although certain regions of amelogenin amino acid sequence are proposed to interact with calcium phosphate minerals, an extensive mapping of putative crystal-interaction or mineral nucleation domains is still lacking (20, 21). Here, we show that functional peptides can be derived from amelogenin using phage display-selected peptides as a knowledge base and identifying next generations of peptides by bioinformatics. We then show that these peptides can function to direct the cell-free re-mineralization of the tooth root resulting in a cementomimetic (e.g., cementum-like) material and thus can contribute to cell-based regeneration of periodontal tissue.

## Materials and Methods

### Combinatorial Selection of Peptides

Selection of the peptides by phage display and the HAp binding and mineralization characterization were carried out as previously described (22) (see Supplemental Information).

### Similarity Analysis

The amino acid sequences obtained from the combinatorially selected peptides have been used as the data source for our knowledge-based design. Using the methods previously described by Oren and colleagues (23), two scoring matrices, HAp12I and HApC7CI, were derived for the peptides selected from the 12- and c7c-phage libraries (New England BioLabs Inc., USA), respectively. The Point Accepted Mutation 250 (PAM 250) was chosen as the seed matrix to optimize the new matrices specific to the selected peptides. The PAM matrix gives substitution probabilities for sequences that experience a certain number of point mutations in every hundred amino acids. Therefore, PAM 250 reflects the probabilities for 250 point mutations for every 100 amino acids. PAM 250 was modified to compensate for the relative abundance of amino acids within the libraries used to select the HAp-binding peptides (HABP) and for the codon usage of the host organism (*E. coli* K12 ER2738) used to amplify the phages. To identify the sequence similarities between the selected peptides with the recombinant mouse 180 amino acid long amelogenin (rM180), we divided the rM180 amino acid sequence into segments with the same length as those from the phage libraries (i.e., 7- or 12-amino acids). Each iteration generated a segment starting from the next amino acid, therefore, creating all possible 7 or 12 amino acid segment lengths for the whole M180 sequence. Then, each segment was compared with each HABP and was given a similarity score. Once every combination of the HABPs and amelogenin segments were compared, regions that demonstrated high similarity scores against both libraries were overlapped. These coinciding high similarity regions were picked as the putative strong binding regions (see Supporting Information). In the same way, coinciding low similarity regions were picked as the putative weak binding regions. These regions were then refined by protein structure prediction,  $\text{Ca}^{+2}$  ion binding domain predictions and meta-functional signature analyses (see Supporting Information). A schematic description of the design process is shown in Figure 1a.

### Peptide Synthesis

rM180 amelogenin was created as described previously by Moradian-Oldak and colleagues (24). The ADPs were synthesized by standard solid phase peptide synthesis technique on Wang resin using F-moc chemistry and HBTU activation. CSBio 336s (CSBio, USA) automated peptide synthesizer was used for the synthesis. The resulting resin-bound peptides were cleaved and side-chain-deprotected using Reagent K (trifluoroacetic acid /thioanisole/ $\text{H}_2\text{O}$ /phenol/ethanedithiol (87.5:5:5:2.5)) and, precipitated by cold ether. The crude peptides obtained were purified by reverse phase high performance liquid chromatography up to a >98% purity (Gemini 10 $\mu$  C18 110A column). The masses of the

purified peptides were checked by mass spectroscopy using a MALDI-TOF mass spectrometer (Bruker Daltonics, USA).

#### **Binding Analysis via QCM**

Calcium phosphate coated QCM electrodes were purchased commercially. (Q-Sense, Sweden) The diameter of the crystals and electrodes were 8.8 mm and 5.0 mm, respectively. The oscillation electronic circuit was a typical Collpits oscillator, which had a buffer amplifier. A 12 V DC current was applied to the oscillator circuit to drive the crystal, and the frequency was measured with a Hewlett-Packard 53131A frequency counter sampling at 225 Hz (Universal Counter, Agilent Technologies). After the crystal was mounted in the cell, they were cleaned and dried with high-purity nitrogen gas and used immediately. To establish a stable baseline, a sufficient amount of buffer solution was introduced into the cell before adding the peptide. The frequency change of the crystal in pure buffer solution was recorded for 30 to 60 minutes. After this, the desired amount of ADPs was introduced into the cell and the frequency change was recorded continuously.

#### ***In vitro* Solution Biomineralization**

An alkaline phosphatase (AP) based mineralization model was used as described before to investigate the mineralization behaviors of the peptides (22) (see Supporting Information for details).

#### ***Ex vivo* Re-mineralization of Tooth Root**

The cementum-root stock blocks were prepared from single rooted, extracted adult teeth collected at the University of Washington Dental School clinics. No individual identifiers were used and the use of such material complied with the Institutional Review Board guidelines. The teeth were kept in 70% ethanol solution at 4°C until the specimens were prepared. Cylindrical blocks of 4 mm diameter were cut from the acellular cementum, close to the cementoenamel junction using a trephine bur. The teeth were kept immersed in 70% ethanol-phosphate buffered saline (PBS) during the cutting to prevent heat damage due to friction. The cut cylindrical blocks were cleaned of contaminating material using PBS and demineralized with 35% phosphoric acid gel for 10 seconds. The specimens were stored in 70% ethanol-PBS solution at 4°C until used.

Prior to coating the specimens with the peptide, the previously cut cylindrical blocks were rehydrated and equilibrated for 2 hours in 24mM Tris-HCl buffer, pH 7.4. The lyophilized ADP was dissolved in 24mM Tris-HCl buffer, pH 7.4 to a final concentration of 0.4 mM. A 50 µl drop of the ADP solution was dropped on top of the specimens and left on the specimen



for 10 minutes at room temperature in a water saturated chamber to prevent evaporation. Control specimens were identically prepared only by dropping 50  $\mu$ l of buffer alone. At the end of 10 minutes, the samples were rinsed twice with deionized water. Fluorescent microscopy analysis was performed with fluorescein isothiocyanate-labeled ADP (fADP).

5 Solutions of 9.6mM  $\text{CaCl}_2$  and 5.6mM  $(\text{PO}_4)^{-3}$ , a mixture of  $\text{NaH}_2\text{PO}_4\text{-H}_2\text{O}$  and  $\text{Na}_2\text{HPO}_4\text{-7H}_2\text{O}$ , were prepared in 24mM Tris-HCl buffer, pH 7.4. The peptide coated and non-coated control specimens were placed in 300 $\mu$ l of  $\text{Ca}^{+2}$  solution and an equal volume of  $(\text{PO}_4)^{-3}$  solution was added to achieve a final concentration of 4.8mM of  $\text{Ca}^{+2}$  and 2.8mM of  $(\text{PO}_4)^{-3}$ . The specimens were incubated in the mineralization solution for 2 hours at 37°C in a  
10 water saturated atmosphere, removed from the mineralization solution and rinsed with 24mM Tris-HCl buffer, pH 7.4. The specimens were kept in 70% ethanol solution at 4°C until the time of characterization. A schematic explanation of the *ex vivo* re-mineralization is shown in Figure 3.

#### Mechanical Properties of the Cementomimetic Layer

15 Mechanical properties of the cementomimetic layer were assessed by nanoindentation and qualitative mechanical abrasion tests. The samples were prepared by first infiltrating from the top of the cementomimetic layer with room-temperature-cure epoxy (Allied High Tech, Inc., U.S.A.) to provide a continuous volume for indentation characterization. After the epoxy was cured, the sample was ground to expose the interior in the cross-section of the  
20 specimen, then ultra-microtomed to achieve a smooth surface for nanoindentation. Twenty indentations each were taken on mineralized dentin region (indentations were made on intertubular dentin), demineralized dentin regions (adjacent to the cementomimetic layer) and the cementomimetic layer (see Supporting Information, Figure 8).

Qualitative mechanical analysis was done by ultrasonication and mechanical abrasion.  
25 For ultrasonication, specimens were mounted on scanning electron microscopy (SEM) sample mounts and placed in 70% ethanol in a glass vial. The probe was set to 3cm above the specimens and ultrasound energy was applied for 15 seconds yielding a total of 10 J. Mechanical abrasion was applied to the same specimens using an electric toothbrush intended for home use (Oral B, series 4000). The specimens and the toothbrush were fixed in position  
30 allowing the bristles to be brought into contact with the specimens for 1 minute, after which the samples were rinsed with 70% ethanol and analyzed with SEM.

#### Cell Adhesion and Growth Assays

Cell adhesion experiments were done using cultured human periodontal ligament (hPDL) fibroblasts. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (FBS) supplemented with 100 units/ml penicillin, 100µg/ml streptomycin and 2mM glutamine (PSG). The cells were used  
5 between the seventh and ninth passage. Before starting the cell adhesion assays, the mineralized specimens were taken out of the 70% ethanol solution and equilibrated in serum-free DMEM for 2 hours. The confluent hPDL cells were suspended with 0.05% Trypsin-EDTA and counted using a hemocytometer. The equilibrated tooth specimens were placed in 24-well plates, with 4 specimens per well performed in triplicates. The suspended hPDL cells  
10 were prepared in serum-free DMEM and  $3 \times 10^4$  cells per well were seeded on top of the specimens. The specimens were incubated with the cells for 2 hours at 37°C and 5% CO<sub>2</sub> atmosphere. After two hours the specimens were rinsed with the media and cells remaining on the surface were recovered from the surface of the specimen with 0.05% trypsin-EDTA. The cells so obtained were counted using CyQUANT cell proliferation assay kit (Invitrogen,  
15 USA).

After fixing with 2% glutaraldehyde in PBS for 10 minutes the cells were permeabilized with 0.1% Triton X in PBS for 2 minutes and blocked with 1% bovine serum albumin (BSA) for 30 minutes and stained with Phalloidin-Alexa fluor 488 (Invitrogen, USA) for fluorescence microscopy observations. The cells were observed and recorded using  
20 a TE 300 L microscope at an appropriate wavelength and gated filters (Nikon, Japan).

For proliferation assays, hPDL cells were prepared the same way as described above for the cell adhesion assays. The specimens were equilibrated in media and placed in 24 well plates, 4 specimens per well and performed in triplicate wells. The suspended hPDL cells were prepared in serum-free DMEM and  $3 \times 10^4$  cells per well were seeded on top of the  
25 specimens. After 24 hours, the specimens were rinsed and transferred into new plates to prevent possible contamination of the cells that grow on the bottom of the plate. The cells were maintained in a water-jacketed incubator at 37°C in 5% CO<sub>2</sub> saturated to H<sub>2</sub>O and the media was exchanged every 48 hrs 2 with DMEM supplemented with 2% FBS. Triplicate samples were terminated after 2-, 6-, 10- and 15-days duration. At the end of each time point,  
30 the specimens were taken out of the wells, rinsed with serum-free DMEM, then with PBS. The cells were recovered from the specimen surface with 0.05% trypsin-EDTA and counted using CyQUANT cell proliferation assay kit (Invitrogen, U.S.A.).

## Results

One component in the newly developed cementomimetic mineral construction is HAp-forming peptides that operate as mineral synthesizers and control deposition of a confluent nanostructured HAp layer. The procedure for designing these peptides is schematically described in **Figure 1**. Using a phage display approach, we combinatorially selected more than 100 HABP from a 7-amino acid and a 12-amino acid phage peptide library and characterized their binding affinity to the HAp mineral under near-physiological conditions (22). Since not all of the peptides selected have the same affinity to HAp, we next categorized them into three classes as strong-, moderate-, and weak-binding peptides. Using bioinformatics classification protocols (23), we derived similarity-scoring matrices for both sets of selected septa- and dodeca-peptides. These matrices were used to systematically compare and identify similarity regions, i.e., domains of similar amino acid sequences between the experimentally selected HABPs and rM180. The comparisons yielded high and low similarity regions along the amelogenin (Fig. 1b). By overlapping the high similarity regions from both libraries, putative crystal binding sequences were identified, referred to as amelogenin-derived peptides, ADPs (Fig. 1b,e). The similarity analysis were refined and supported by other computational tools, i.e.; structure prediction, meta-functional signature and ion binding domains analyses (Fig. 1c,d) (see Supplemental Information). Many short amino acid sequences can be generated by this procedure and each has the potential to be used for specific applications requiring control over HAp formation and growth. The putative HAp interacting regions of amelogenin having the highest similarity, i.e., ADP1, ADP2, and ADP4, were synthesized chemically (see Supplemental Information). For comparison, we also synthesized amelogenin-derived peptides (ADP3, ADP6) corresponding to the previously proposed putative mineral-binding regions near the C-terminus (25-28), non-mineral interactive (e.g., low similarity score) regions (ADP5, ADP8) and finally ADP7, a peptide that included ADP1, ADP2 and ADP8 (Fig. 1e and Table 1). The HAp-binding affinities of these peptides were examined *via* quartz crystal microbalance (QCM) and their propensity to control formation of calcium phosphates was carried out via *in vitro* solution mineralization assays and *ex vivo* tooth remineralization experiments.

**Table 1** Amino acid sequences, physico-chemical properties and the dissociation constant ( $K_d$ ) of the ADPs used in this study.

|      | M.W.    | pI   | Charge      | G.R.A.V.Y. | K <sub>D</sub> (μM) |
|------|---------|------|-------------|------------|---------------------|
| ADP1 | 1414.60 | 7.10 | 0           | -0.360     | 1,395               |
| ADP2 | 1328.40 | 7.00 | 0           | -1.067     | 1,611               |
| ADP3 | 1574.70 | 4.94 | (-4, +3) -1 | -1.862     | 6,397               |
| ADP4 | 1833.00 | 7.16 | 0           | -0.713     | 1,431               |
| ADP5 | 2465.60 | 7.16 | (-2,+2) 0   | -0.959     | 50                  |
| ADP6 | 2630.10 | 3.79 | +2          | 0.592      | 6,247               |
| ADP7 | 4645.30 | 7.28 | 0           | -0.824     | 1,148               |
| ADP8 | 1519.80 | 5.96 | 0           | -0.743     | 3,14                |

### Binding Analysis via QCM

The dissociation constants (K<sub>D</sub>) of the peptides were determined. The K<sub>D</sub> is an equilibrium constant that represents the concentration necessary to achieve 50% surface coverage. Experimental binding assays demonstrated that ADP1, ADP2, ADP4 and ADP7 exhibit strong affinity to HAp with K<sub>D</sub> values on the order of 1μM, as was predicted by the similarity analysis (Fig. 2a, Table 1). Likewise, the binding affinities of the ADP3, ADP6, ADP5 and ADP8 were lower as predicted, with ADP5 having a significantly lower K<sub>D</sub> (50mM). (Figure 2a, Table 1)

### *In vitro* Solution Biomineralization

We noted that there were three distinct trends of mineralization among the tested ADPs. The majority of the peptides (ADP1, ADP2, ADP3, ADP4, ADP6 and ADP8) exhibited similar kinetics to the negative control, where no peptide was present. The phage display selected HABP1 and the ADP7 identified in this study exhibited a slow mineralization trend (Fig. 2b). Interestingly, full-length rM180 and the ADP5 exhibited a fast mineralization trend, where more than half of the available free Ca<sup>2+</sup> was consumed at the end of the 90 minutes (Fig. 2b) (See Supplementary Information figure 6 for the mineralization trends of all ADPs).

The microstructural and crystallographic analysis of the synthesized minerals via SEM, transmission electron microscopy (TEM) and x-ray diffraction (XRD) revealed another interesting consequence regarding the relationship between the mineral binding and mineralization activity. Similar to the mineralization kinetics, three distinct trends of mineral morphologies were observed. The majority of the peptides (ADP1, ADP2, ADP3, ADP4, ADP6, ADP8 and HABP1) produced spherulitic particles consistent with the formation of spherical amorphous calcium phosphate (ACP) and transformation into crystalline phases

(29-31). The amount of radiating crystalline blade-like particles emanating from the spherulites was slightly higher for ADP1, ADP2, ADP4 and HABP1 (strong binders) compared to ADP3, ADP6, ADP8 (weak binders) and to the no peptide control, an order indicating that the amorphous to crystalline transformation rates were slightly different (Fig. 2c). In the case of rM180 and ADP7, however, a completely different morphology was observed. Needle like nano-crystals were organized into bundle-like assemblies were observed for rM180 and ADP7 (Fig. 2d). The bundle-like assemblies appeared to be better organized in the case of rM180, which is consistent with the self-assembly properties of amelogenin and the *in vitro* mineralization behavior of recombinant amelogenin (24, 32-34).

The particles formed in the presence of ADP5 were much smaller spherulites with a less electron-dense core and smaller radiating crystals. (Figure 2c) The smaller particles sizes in the presence of ADP5 may be due to its nucleation dominate regime, as suggested from the data describing the mineralization kinetics.

The crystallographic analysis via XRD spectroscopy also confirmed that the observed morphological differences were due to different crystal structures. In all cases except rM180 and ADP7, the minerals yielded a broad peak around  $2\theta$  25-30° indicating a poorly crystalline phase (Fig 2e). In the presence of rM180 and ADP7, however, the XRD patterns consisted of numerous sharp peaks indicative of crystalline HAp. The major peaks were observed at  $2\theta$  = 31.8° ( $d = 2.81\text{\AA}$ ) and 32.1° ( $d = 2.78\text{\AA}$ ) corresponding to (211) and (300) planes of HAp, respectively.

### ***Ex vivo* Remineralization of the Tooth Roots**

Human cementum discs were prepared as described in "Materials and Methods" and as illustrated in figure 3. Fluorescent microscopy analysis with fluorescein-labeled ADP5 (fADP5) showed that the ADP5 readily adsorbs on the demineralized surface of human root stock cementum and remains on the surface after extensive washing. Since the microscope was calibrated to the emission of the control samples, the background emission was eliminated in both samples. The human samples after cleaning and etching reveal the expected dentinal tubules characteristic of the dentinoenamel junction, with diameters about 1  $\mu\text{m}$  are clearly visible on the f-ADP5 coated samples (See Supporting Information, Figure 7)

ADP5 resulted in a substantial difference in the re-mineralization profiles of the human root stock cementum surface as observed via SEM and energy-dispersive x-ray

spectroscopy (EDXS) analysis. At the end of 2 hours, no substantial re-mineralization occurred on the control samples consisting of no peptide coating (Fig. 4a). On the other hand, a continuous layer of mineral covering the whole surface of the specimen was observed on the ADP5 coated specimens (Fig. 4b). The morphology of the newly formed mineral was plate-like crystals growing out of the surface of the underlying dentine. Elemental composition analysis by EDS showed no observable Ca or P peaks from the control sample containing no peptide, where, in contrast, substantial Ca and P peaks were observed from the ADP5 coated samples (Fig. 4a,b inserts, respectively). Both SEM and TEM cross-sectional analysis showed that ADP5 yielded a 10-15 $\mu$ m thick mineral layer that appears to be well integrated with the underlying dentin (Fig. 4c,d). Moreover, the thickness of the cell-free biomimetic cementum layer compares well to the thickness of native human acellular cementum.

### Mechanical Properties of the Cementomimetic Layer

As shown in Table 2, the cementomimetic mineral layer exhibited comparable elastic modulus and hardness to the native human cementum (35). The large standard deviation observed for the cementomimetic layer was noted and was likely due to the less homogeneous distribution of mineral than that observed for native mineralized dentin or cementum.

**Table 2.** Mechanical properties of cementomimetic layer as measured by nanoindentation, n = 20 indentations.

|                             | $E_r$ (GPa) | H (GPa)       |
|-----------------------------|-------------|---------------|
| <b>Cementomimetic Layer</b> | $19 \pm 7$  | $0.7 \pm 0.5$ |
| <b>Native Cementum</b>      | $15 \pm 4$  | $0.8 \pm 0.3$ |
| <b>Mineralized Dentin</b>   | $26 \pm 3$  | $1.0 \pm 0.2$ |
| <b>Demineralized Dentin</b> | $5 \pm 3$   | $0.2 \pm 0.2$ |

We also tested whether the newly formed mineral layer can survive mechanical stresses produced by ultrasonication and mechanical abrasion (brushing). SEM observations showed that the plate-like morphology of the minerals was somewhat distorted after ultrasonication and brushing. However, as confirmed with EDXS, the mineral was still attached to the underlying dentin. (See supplementary information, Fig. 9)

### Cell Adhesion and Growth Assays

Cell attachment assays showed that the hPDL cells attach to the mineralized surfaces more effectively compared to their non-mineralized control surfaces. Quantification of cells recovered after 2 hours adhesion period showed that 23.2% of the cells were adherent on the non-mineralized control surface whereas 60.6% of the cells adhered to the biomimetic mineralized surface after 2 hours of incubation. (Fig. 4e)

Proliferation assays showed that the hPDL cells grow more efficiently on the biomimetic mineralized surfaces compared to non-mineralized control surfaces. At the end of 15 days, hPDL cells have reached more than twice the number cells on the biomimetic mineralized surfaces compared to the control surface (Fig. 4f). This data suggests that the cementomimetic mineral layer provides both a favorable environment for the hPDL cells to attach and to proliferate. Fluorescence imaging showed that, after initial adhesion, the cells organize their cytoskeleton on the mineral layer better (Fig. 4h) than the same cells growing on control surfaces (Fig. 4g).

### Discussion

The quantitative diffraction and microscopy analyses of the minerals produced by the ADPs revealed that, among the designed peptides, only ADP7, the longest of the similarity regions, and native rM180 produced needle-like-, crystalline-HAp particles, while the others formed crystalline spherulite particles (Figure 2c-e). Interestingly, although the N-terminal ADP5, which revealed no binding affinity to HAp, exerted a kinetic control over calcium phosphate nucleation at a level similar to full length rM180 amelogenin protein (Fig. 2b). The fact that the ADP5 did not demonstrate a high affinity to HAp indicates that control over nucleation is governed by interactions with precursor ions.

Taken together, these data indicate that the strong binding affinity by a peptide to its mineral substrate, does not necessarily always translate into mineralization directing activity. The fact that the ADP5 showed no significant binding affinity towards HAp but resulted in faster kinetics implies that the ADP5 might be interacting with the soluble precursor ions rather than the mineral surface. The two adjacent pairs of oppositely charged residues at positions 9-10 and 19-20 within ADP5 may be responsible for attracting soluble  $\text{Ca}^{2+}$  and  $\text{PO}_4^{-3}$  and creating an increased local super-saturation and thereby, decreasing the nucleation barrier. In contrast, the strong interaction between ADP7 with the mineral surface indicates that a change in the mineral-solution interfacial energy is responsible for the observed

mineralization behavior. It has been reported that biomolecules, such as citrate, can alter the interfacial energy of kinetically favorable meta-stable calcium phosphate phases and trigger the phase transformation towards thermodynamically favorable HAp (41, 42). A similar effect may be responsible, due to the relatively high affinity of ADP7 to the mineral surface.

5 Binding of a peptide on a crystal surface is determined by both the conformation (side chain availability) of the peptide and the atomic configuration of the crystal at the binding interface (43-45). We speculate that the formation of HAp instead of other metastable CaP phases, such as ACP, OCP or TCP, in the presence of ADP7 is due to a specific conformation of the peptide and the arrangement of Ca and P sites on the crystal surface that maximizes the  
10 binding energy and, in turn, reduces the interfacial energy barrier required to overcome phase transformation.

In summary, the binding and biomineralization behaviors of ADP5, ADP7 and the rM180 imply an interesting mechanism for amelogenin mediated mineralization during the formation of enamel. The fact that two different amelogenin derived peptides emulate  
15 different aspects of the mother protein brings up the question whether these peptides might be different functional regions within amelogenin. Namely, ADP5 may be the region responsible for increased mineralization kinetics through increasing local super-saturation and ADP7 may be the region responsible for favoring the phase transformation towards HAp through altering the interfacial energy. However, the answers to these questions are the focus of another  
20 ongoing study.

The range of characteristics exhibited by these individual ADPs represents potential molecular agents to consider for engineering and biomedical applications involving hard tissue repair and replacement where specific control features for the mineral phase are desired. Peptide-assisted re-mineralization of dental tissue defects offers a significant  
25 potential for clinical use. Toward this end, we asked whether or not the biomimetic cementum-like mineral layer has a favorable effect on cell behavior, *in vitro*. Cultured human periodontal ligament cells (hPDL) were selected for investigation since these are the predominant cell types that root surfaces are exposed to *in vivo*, and further, these cells, when appropriately triggered, can carry on important functions in the repair and regeneration of  
30 periodontal tissues (46-50). Cell adhesion and cell proliferation assays, performed on the surface of ADP5 mediated cementum-like tooth material in the absence of serum-derived factors, showed that the nanostructured mineral layer favors both the adhesion and proliferation of cells derived from the periodontium (Fig. 4e, f).



In conclusion, the work described here has several significant implications in peptide-assisted biomineralization. First, we describe a new protocol for identifying peptide sequences from among native proteins associated with HAp containing tissues that are critical to their control over biomineralization. A similar analysis of extracellular matrix proteins associated with biominerals formed by unicellular organisms (e.g., magnetotactic bacteria), invertebrate (sponges, mollusks) and vertebrate animals can reveal other unique amino acid domains that regulate the mineral formation and growth for each of their variety inorganic compounds (magnetite, silica and calcium carbonate) (51). Biomineralization is fundamental to many living organisms and the range of precipitated minerals reflects the elements of the periodic table. Elaborating the mechanism for protein control over the biominerals formed in these systems will afford new insights into the evolution of protein to mineral interactions (52). In the case of enamel, amelogenin is a member of the class of intrinsically disorganized proteins (53). By restricting a structural analysis to but a small domain of a larger protein, such as that defined by ADP5, it is possible to expand our understanding of structure-function relationships, portending the capacity to decipher a relationship between a peptide and a chemical precipitate, and then to use such information for their practical utility to engineer material formation technologies (e.g., bio-manufacturing). Secondly, as demonstrated in this study, strong binding affinity of a peptide to an inorganic solid is not necessarily an indication of its mineral formation capability. Thirdly, the cementomimetic layer formed by ADP5 has a clinical application potential to repair diseased root surfaces, both those caused by caries and periodontal disease, and to promote regeneration of periodontal tissue, reducing the morbidity associated with tooth loss.

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## Supporting Material for Example 1

### Combinatorial selection of peptides

Commercially available Ph.D. Phage Display Peptide Library Kits (c7c and 12) were  
 15 used as the biocombinatorial library (New England BioLabs Inc., USA) and synthetic HA powder was used as the target substrate (a generous gift from Ivan Transevica Institute, Department of Materials Science, Ukraine). Prior to exposing to the peptide - phage library, the HA powder was cleaned by ultrasonication in a 1/1 methanol/acetone mixture followed by ultrasonication in isopropanol. Cleaned HA powder was incubated with the phage-peptide  
 20 library overnight in a potassium phosphate/sodium carbonate (PC) buffer (pH 7.4), containing 0.1% detergent (Tween 20 and Tween 80, Merck, USA) at room temperature with constant rotation. The detergent prevents non-specific interactions between phage particles and enables individual phage particle to powder surface interaction to occur. After incubating the HAp powder substrate with the phage library, the powder was washed several times with  
 25 PC buffer containing 0.1% detergent to remove any non-specifically or weakly bound phage particles from the HA surface. The phages remaining on the HA surface were then eluted by 0.2 M Glycine HCl (pH 2.2) solution, followed by ultrasonication. Eluted phage were transferred to an early-log phase *E.coli* ER2738 culture and amplified for 4 hours. The amplified phage are isolated by polyethylene glycol (PEG) precipitation. After the third  
 30 round of panning, the phage obtained from each round of selection are plated on LB-Agar media containing 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (Xgal) and isopropyl- $\beta$ -D-thiogalactopyranosid (IPTG) in serial dilutions to obtain single phage plaques. Since ER2738 strain lacks the *lacZ $\alpha$*  gene, only the cells infected by M13mp 19 bacteriophage,

carrying the *lacZα* gene can produce βgalactosidase and provide a chromogenic change in color when exposed to Xgal. Therefore, infected cells can hydrolyze X-Gal and form blue phage plaques. After growing the phages on LB-Agar plates, single blue plaques containing clonal expanded phage particles were picked, amplified and the amino acid sequence of the  
5 corresponding polypeptide segment was identified by DNA sequencing.

### Semi-quantitative analysis of HA binding

The binding affinity of each clone was assessed by immunofluorescence fluorescence microscopy and enzyme-linked immunosorbent assay (ELISA). For the fluorescence  
10 microscopy, cleaned HAp powder was incubated overnight with selected monoclonal phages at  $10^{11}$  PFU/mL concentration in PC buffer containing 0.1% detergent at room temperature and with constant rotation. Wild type M13mp19 bacteriophage with no random peptides displayed on the pIII protein was used as negative control. After incubation, the powder was washed three times with the same buffer. After the washing, the phage remaining bound to  
15 the surface are labeled with mouse anti-M13 IgG (Amersham Biosciences, USA) and Alexa Fluor 488-anti-mouse IgG2a conjugate (Invitrogen Corporation, USA). After labeling, the powder suspension was transferred to a microscope slide and observed using a TE 300L microscope (Nikon, Japan). The approximate surface coverage of the phage particles was calculated using MetaMorph Imaging System Ver. 6.2 (Photometrics UK Ltd., UK, formerly  
20 Universal Imaging Co., USA) by comparing the approximate surface area of the powder in the bright field image to the approximate phage coverage in the fluorescence image.

For ELISA analysis, cleaned HAp powder was incubated overnight with selected monoclonal phages at  $10^{11}$  PFU/ml concentration in PBS buffer containing 2% BSA at room temperature with constant rotation. Wild type M13mp 19 bacteriophage, displaying no  
25 peptides on the p(III) protein, was used as negative control. The powder was washed 3 times with PBS buffer containing 0.05% detergent. After the washing, the bound phages on the surface were labeled with horseradish peroxidase (HRP)-anti-M13 IgG conjugates (Amersham Biosciences, USA) in PBS buffer containing 2% BSA for 30 minutes with constant rotation and washed 4 times with PBS buffer. The reaction ready HRP substrate 3,  
30 3', 5', 5' tetramethylbenzidine (TMB)-ELISA (Pierce, USA) was added into the solution. The oxidation of the TMB by HRP yields a diimine form of TMB. The reaction was stopped after 2 minutes by adding 1 M H<sub>2</sub>SO<sub>4</sub>, which results in a yellow color with maximum

absorbance at 450nm. The optical density (OD) of the reaction solution was measured at 450 nm wave length using a microplate reader (Tecan Trading AG, Switzerland).

**Table 3.** Number of the characterized peptides from the 7 amino acids cyclic (c7c) and 12 amino acids linear phage-peptide libraries.

|                | <b>Strong</b> | <b>Moderate</b> | <b>Weak</b> | <b>Total</b> |
|----------------|---------------|-----------------|-------------|--------------|
| <b>PhD c7c</b> | 12            | 27              | 18          | 57           |
| <b>PhD 12</b>  | 16            | 20              | 13          | 49           |

### Protein Structure Prediction

Contemporary protein structure prediction methods use known structures as variable length templates for construction assembly. We have developed an initial set of incomplete structure alignments in the form of meta-predictions using 3D-Jury (web site /bioinfo.pl/meta) (1). and incorporated these as templates for the development of many full length models using the Protinfo protein structure prediction protocol (web site protinfo.compbio.washington.edu/protinfo\_abcmfr) (2) (RAMP: A suite of programs to aid in the modeling of protein structure and function (web site compbio.- washington.edu/ramp/) (3). In addition we have retrieved the full-length models from the I-TASSER server (web site zhang.bioinformatics.ku.edu/I-TASSER) (4). Next, we have used an all-atom conditional probability discriminatory function, RAPDF (5), to select the best models. These models were iteratively refined using template-, geometry- and physics-based methods included within the RAMP suite (3).

We used RAPDF to select the best five models among all of the decoys created for the full-length M180 amelogenin. We then have picked the best combination of regions from the top 5 models, using a graph-theoretic clique finding approach, in the creation of an optimized conformation model (6).

### Ca<sup>2+</sup> Ion Binding Domain Predictions

We have performed an exhaustive grid-based search placing ions into all solvent exposed sites in the 3D structure. The sites were scored with a grid based exhaustive sampling algorithm, clustered using a variant of an unsupervised hierarchy clustering algorithm, and refined with Monte Carlo minimization. The scoring function was developed



from geometrical parameters observed for inorganic single atom ions coordinating small molecule organics found in many crystallography structures publicly available through the PDB (7). This scoring function was verified by accurately ranking the binding affinities between proteins and ions, predicting the naturally occurring ion binding sites of proteins and recovering the native conformation of protein-substrate binding (web site  
5 protinfo.compbio.washington.edu/soak) (8)

### Metafunctional Signatures

The Meta-Functional Signature (MFS) method comprises five sub-scores. The  
10 compilation of these scores was trained on two databases of functionally important residues (9) using a simple logit model (web site protinfo.compbio.washington.edu/mfs). (10-12) The MFS method can also be applied without structural analysis, using only scores based on sequence information, hidden Markov model (HMM), State-to-Step Ratio (SSR), and amino acid type (AA\_type), to produce highly accurate identification of amino acids known to be  
15 important to function. (10)

*SSR* - The evolutionary context for a region is modeled by creating a phylogenetic tree for the sequence surrounding each amino acid in a protein, using multiple sequence alignments of PSI-BLAST results (13). The root of the tree represents the theoretical ancestral sequence, while each leaf of the tree represents a PSI-BLAST result sequence. We  
20 quantify the evolutionary divergence of each amino acid from its evolutionary context through the phylogenetic tree. SSR describes the ratio of the number of different amino acids presenting at a position across the multiple sequence alignments, to the total number of changes between the input protein and the base sequence within the phylogenetic tree. (10)

*HMM* - We have compiled a HMM from the multiple sequence alignments described above,  
25 using the HMMER package (14). We then have compared emission frequency estimates from the HMM model with the amino acid background frequency given by karlin.c of the BLAST program package (13), to produce the HMM relative entropy score for each amino acid position (15).

*AA\_type* - We have assigned a simple score to each amino acid type, developed from the  
30 frequency of each amino acid type appearing within active sites in two large databases.

*Structural Stability scores – virtual mutations* - We have evaluated the importance of each residue to the structural stability of the protein by performing a series of virtual mutations to the 19 alternate amino acids, modeling induced changes in tertiary structure, and comparing simulated energy function scores (RAPDF; (5)) for the mutation set for each residue.

RAPDF\_spread, the variation of RAPDF scores across all possible mutants for the residue, indicates the structural volatility of the position, and therefore the contribution of the position to stability. The RAPDF\_dif structural stability score represents the sum of the simple differences between the RAPDF score for the input structure and all 19 alternatives, which indicates the effect of the naturally occurring amino acid identity on stabilization of the structure (11).

The structural stability score was implemented both within MFS and used separately for identifying fold bearing regions not likely to contribute to function but may create specific conformations important to function for the regions they span.

### ***In vitro* Solution Biomining**

To investigate the effect of peptides on in vitro calcium phosphate nucleation, an alkaline phosphatase (AP) based mineralization model was used. (16) This model mimics the biological matrix vesicle mediated mineralization, where inorganic phosphate is cleaved from an organic phosphate compound by AP and reacts with calcium inside the matrix vesicle. Prior to the mineralization experiments the activity of AP was measured in the presence of the peptides, to assess the effects of either peptide on the enzyme activity.  $\beta$ -Glycerophosphate ( $\beta$ -GP) solutions at 14.4 mM containing no peptide and 0.4 mM peptides were prepared in 25 mM Tris-HCl buffer (pH 7.4). The reaction was started by adding  $1.4 \times 10^{-6}$  g/mL bacterial AP (Invitrogen, U.S.A.) to the solution. The reaction was carried at 37°C, and samples were collected at times 0.25-, 1-, 3-, 16- and 24-hr. The phosphate released in each solution was measured using PiPer Phosphate Assay Kit (Invitrogen Co., U.S.A.) and plotted against time.

For the mineralization experiments, mineralization solutions containing 24 mM  $\text{Ca}^{2+}$  and 14.4mM  $\beta$ -GP and 0.4 mM peptide was prepared in 25 mM Tris-HCl buffer (pH 7.4). A mineralization solution containing no peptide was prepared as the negative control. Mineralization reactions were commenced by adding bacterial AP to the solution at a final concentration of  $1.4 \times 10^{-6}$  g/mL. Subsequent mineral formation kinetics was monitored by continuous measuring of absorbance at 820 nm wave length using a Tecan Safire microplate reader (Tecan Trading AG, Switzerland) as well as by periodic assays of calcium ions throughout the reaction. For periodic assays of calcium and phosphate, 10  $\mu$ L of the reaction solution was collected at 0.25-, 0.5-, 1- and 1.5-hr. The calcium concentration in the solutions was determined using QuantiChrome Calcium Assay Kit (Bioassays, U.S.A.). All mineralization reactions were carried out at 37°C. Samples for electron microscopy analysis

were prepared by placing a 10  $\mu$ L aliquot from the reaction solution onto a carbon coated TEM grid. After 1 min, liquid on the TEM grid was carefully wicked away and the grids were vacuum-dried for 5 min. The dried grids were stored in a desiccated container until analyzed. SEM imaging was performed at 10 kV using a JSM 7000F (JEOL, Japan) SEM in secondary electron imaging mode. TEM imaging and electron diffraction analysis were performed using an EMS 420T TEM (FEI, Inc., USA; formerly Philips, The Netherlands) operated at 120 kV.

We noted that there were three distinct trends of mineralization among the tested ADPs. (Figure 2) The majority of the peptides (ADP1, ADP2, ADP3, ADP4, ADP6 and ADP8) exhibited similar kinetics to the negative control, where no peptide was present. The phage display selected HABP1 and the ADP7 exhibited a slow mineralization trend. Interestingly, full-length rM180 and the ADP5 exhibited a fast mineralization trend where more than half of the available free  $\text{Ca}^{2+}$  was consumed at the end of the 90 minutes (Figure 6). The microstructural and crystallographic analysis of the synthesized minerals via SEM, TEM and XRD revealed another interesting consequence regarding the relationship between the mineral binding and mineralization activity. Similar to the mineralization kinetics, three distinct trends of mineral morphologies were observed. The majority of the peptides (ADP1, ADP2, ADP3, ADP4, ADP6, ADP8 and HABP1) produced spherulitic particles consistent with the formation of spherical amorphous calcium phosphate (ACP) and transformation into crystalline phases. (17-19) The amount of the radiating crystalline blade like particles from the spherulites were slightly higher for ADP1, ADP2, ADP4 and HABP1 (strong binders) compared to ADP3, ADP6, ADP8 (weak binders) and no peptide, an order indicating that the amorphous to crystalline transformation rates were slightly different. In the case of rM180 and ADP7, however, a completely different morphology was observed. Needle like nanocrystals were organized into bundle-like assemblies observed for rM180 and ADP7. The bundle-like assemblies appeared to be better organized in the case of rM180, which is consistent with the self-assembly properties of amelogenin and the *in vitro* mineralization behavior of recombinant amelogenin (20-22). The particles formed in the presence of ADP5 were much smaller spherulites with a less electron-dense core and smaller radiating crystals. The smaller particles sizes in the presence of ADP5 may be due to its nucleation dominated regime, as observed from in data describing the mineralization kinetics. The crystallographic analysis via XRD spectroscopy also confirmed the morphological differences were due to different crystal structure. In all cases except for rM180 and ADP7, the minerals yielded a broad peak around  $2\theta$  25-30° indicating a poorly crystalline phase. In the presence of rM180

and ADP7, however, the XRD patterns were consisted of numerous sharp peaks indicative of crystalline HAp. The major peaks were observed at  $2\theta = 31.8^\circ$  ( $d = 2.81\text{\AA}$ ) and  $32.1^\circ$  ( $d = 2.78\text{\AA}$ ) corresponding to (211) and (300) planes of HAp, respectively. (Figure 2d)

## 5 References for example 1 Supporting Material

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## Example 2

Inherent antibacterial properties of the calcium phosphate mineral (Tin-Ooet al., Archives of Orofacial Sciences, 2007. **2**: p. 41-44; Ingram, et al., Plastic and Reconstructive Surgery, 1996. **98**(6): p. 1119-1119; Owadally, Endodontics & Dental Traumatology, 1994. **10**(5): p. 228-232) may also create a less viable environment for bacterial colonization and result in a decrease in bacterial colonization. To test this, preliminary experiments were carried out on the peptide-formed mineral layer with *S. epidermidis*, a potent biofilm forming bacteria. Bacterial viability assays clearly show that the mineral layer exhibits a bactericidal effect (data not shown). In the live/dead assay performed, the live bacteria were stained green, while the dead bacteria were stained red since the red dye can only penetrate compromised cell walls. The percent of the dead bacteria on the mineral layer was much higher than that of on exposed dentin. The total bacterial coverage on the bare dentin and the peptide-formed mineral was 41% and 58% respectively. Although the total bacterial coverage was higher on the mineral layer, 40% of the bacteria were dead, while on bare dentin, only ~6% of the bacteria were dead. The observed effect has significant implications in preventing further infiltration of exposed dentin tubules and arresting tooth resorption. Despite the antibacterial effect, the peptide-formed mineral layer exhibits an opposite effect on eukaryotic cells such as PDL fibroblasts and cementoblasts, as shown above

## Methods for Example 2

### Bacterial viability assays

*S. epidermidis* culture was adjusted to an optical density of 1.0 (approximately  $1 \times 10^8$  cells/ml) and the cell suspension was placed in all wells with the previously prepared mineralized specimens and non-mineralized control specimens. The bacteria were incubated for 2.5 h at 37°C. Loosely adherent bacteria were removed by gently rinsing the wells three times with PBS. Adherent bacteria were stained using Live/Dead BacLight™ Bacterial Viability stain kit (Invitrogen, USA) according to the manufacturer's protocol. Excess liquid was aspirated from surfaces and cells viewed with an epifluorescent microscope (Zeiss Axioskop 2, Jena, Germany) equipped with an Optronics MagnaFire™ Digital Camera System (Optronics, Goleta, CA), a 100x oil immersion objective, and appropriate filters. Five random fields were selected per well and imaged using the MagnaFire™ software program. Bacteria coverage was quantified with the aid of ImageJ™ software.

## Example 3

**ADP5 Mutations:**

The two adjacent pairs of oppositely charged residues (E9-K10 and D19-R20) in ADP5 may be responsible for attracting soluble  $\text{Ca}^{+2}$  and  $\text{PO}_4^{-3}$  and creating an increased local super-saturation in the proximity of the polypeptide. To further test this assumption and assess the functionality of the polypeptides, we have tested the effect of eliminating the charged residues on mineralization using three mutations where only positively charged, only negatively charged and all charged residues were replaced with alanine (A). The amino acid sequences of the mutant peptides generated are listed in Table 4.

**Table 4:** Amino acid sequences and the calculated physicochemical properties of the ADP5 mutants

|                      | Amino Acid sequence    | M.W.    | pI   | G.R.A.V.Y. |                 |
|----------------------|------------------------|---------|------|------------|-----------------|
| <b>ADP5</b>          | PGYINLSYEKSHSQAINTDRTA | 2465.60 | 7.16 | -0.959     | (SEQ ID NO: 7)  |
| <b>ADP5 (+ A)</b>    | PGYINLSYAKSHSQAINRTA   | 2363.60 | 9.72 | -0.477     | (SEQ ID NO: 38) |
| <b>ADP5 (- A)</b>    | PGYINLSYEASHSQAINDDATA | 2323.40 | 4.35 | -0.414     | (SEQ ID NO: 39) |
| <b>ADP5 (+, - A)</b> | PGYINLSYAASHSQAINTAATA | 2221.40 | 7.16 | 0.068      | (SEQ ID NO: 40) |

Adapting the standardized mineralization assays described herein using the mutant ADP5 peptides showed that the oppositely charged adjacent residues indeed play a role in the promotion of nucleation during the biomineralization process. The deletion of both the positively and the negatively charged residues resulted in complete loss of function as observed via calcium depletion assays. Deletion of only positively or negatively charged residues, on the other hand, resulted in a partial loss of function. The promotion of the nucleation was inhibited to a lesser extent when the positively charged residues were deleted and the negatively charged residues were retained (Figure 10).

As a result of these interactions, we have been able to control the biomineralization process, in particular the rate of reactions. Here we find that ADP5 stands out as a clinically potential peptide where promotion of nucleation may be necessary to provide a rapid mineralization. Furthermore, each mutant now has a different affect for rate of biomineralization. This may be useful if one wants to control this rate, for example, for shape formation of a biomineralized entity.

**Fragments of ADP7:**

In the case of ADP7, the strong interaction between the polypeptide and the mineral indicate that a change in the mineral-solution interfacial energy is significant in the formation of HA in preference to OCP. The formation of HA is a rather complicated process and can occur via different routes. At pH 9 and above, ACP directly transforms into HA. [1] It is  
5 thought that the ACP formed at elevated pH is structurally less defined but more similar to HA compared to those form at lower pH, thus, energetically it is more favorable to transform into HA.[1,2] Below pH 9, however, the transformation is more complicated, where an ACP to OCP transformation precedes an OCP to HA transformation. The ACP particles forming below pH 9 have more defined structures with randomly positioned ordered clusters  
10 resembling hydrated OCP-like structures.[1] Therefore, ACP to OCP transformation is more favorable below pH 9. Later, an OCP to HA transformation may occur under the right conditions, mainly by the hydrolysis of the OCP. [3]

As stated above, the strong interaction between the polypeptide and the mineral indicate that a change in the mineral-solution interfacial energy is involved in the  
15 mineralization behavior observed with ADP7. However, the fact that the HA precipitation was only observed in ADP7 and none of the other strong binders, *i.e.*: ADP1, ADP2 and ADP4, indicates that binding alone is not responsible for the observed effect. One possible explanation of the observed effect is that the ADP7 may act as a heterogeneous nucleation site for the HA, thus, promoting the precipitation of HA in preference to other phases,  
20 possibly through molecular recognition. ADP7 comprises of the strong binders ADP1 and ADP2 and the weak binding region, with ADP8 in between. Mineralization experiments done to compare the effect of the whole ADP7 vs. the ADP1, ADP2 and ADP8 separately show that HA formation occurs only when the ADP7 is present as a single polypeptide in the solution (Figure 2 a, b). This suggests that structural features, which may be induced upon  
25 surface binding, are essential for the observed function. One interesting feature of the ADP7 is the abundance of basic histidine (H) residues in the mineral binding regions (ADP1 and ADP2) and the absence in the non-binding middle region (ADP8). Histidine is an amino acid commonly found in the calcium-binding proteins [4, 5, and 6] and may be playing a role in ADP7 function as well. Moreover, the molecular structure predictions done using RAMP  
30 software show that ADP7 tends to form a loop-like structure both on the full protein and as a single polypeptide. This conformation brings the histidine residues in close proximity, which further implies the importance of the histidine residues.

Although we know that ADP7 promotes the precipitation of HA in preference to OCP, the binding and biomineralization behaviors of ADP5 and ADP7, however, imply an



interesting mechanism for amelogenin mediated mineralization during the formation of enamel. The fact that two different amelogenin derived polypeptides emulate different aspects of the parent protein brings up the point that these polypeptides are different functional regions within the original amelogenin sequence. Namely, ADP5 may be the region responsible for increased mineralization kinetics through increasing local supersaturation and ADP7 may be the region responsible for favoring the phase transformation towards HA through altering the interfacial energy.

### References for Example 3

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6. Pathak, R.K., R.G.W. Anderson, and S.L. Hofmann, Histidine-rich calcium binding protein, a sarcoplasmic reticulum protein of striated muscle, is also abundant in arteriolar smooth muscle cells. *Journal of Muscle Research and Cell Motility*, 1992. **13**(3): pp. 366-376.

We claim:

1. An isolated amelogenin-derived polypeptide consisting of an amino acid sequence selected from the group consisting of:

(a) (WP(A/S)TDKTKREEVD)<sub>1-10</sub> (SEQ ID NO: 1) (ADP3);

5 (b) (PGYIN(L/F)SY(E/A)(K/N/A)SHSQAIN(T/V)(D/A)(R/A)TA)<sub>1-10</sub> (SEQ ID NO: 2) (ADP5);

(c) (LPPLFSMPLSPILPELPLEAWPAT)<sub>1-10</sub> (ADP6) (SEQ ID NO: 3); and

(d) 12-43 contiguous amino acids of (HPP(S/T)HTLQPHHH(L/I)PVVPAQ QPV(A/I)PQQPMMPVPG(H/Q)HSMTP(T/I)QH)<sub>1-10</sub> (SEQ ID NO: 4) (ADP7);

10 or functional equivalents thereof.

2. The isolated amelogenin-derived polypeptide of claim 1, wherein the polypeptide consists of the amino acid sequence

(PGYIN(L/F)SY(E/A)(K/N/A)SHSQAIN(T/V)(D/A)(R/A)TA)<sub>1-10</sub> (SEQ ID NO: 4) (ADP5), or a functional equivalent thereof.

15 3. The isolated amelogenin-derived polypeptide of claim 1, wherein the polypeptide consists of the amino acid sequence (PGYINFSYENSHSQAINVDRTA)<sub>1-10</sub> (SEQ ID NO: 6) (ADP5H), or a functional equivalent thereof.

4. The isolated amelogenin-derived polypeptide of claim 1, wherein the polypeptide consists of 12-43 contiguous amino acids of

20 (HPP(S/T)HTLQPHHH(L/I)PVVPAQQPV(A/I)PQ QPMMPVPG(H/Q)HSMTP(T/I)QH)<sub>1-10</sub> (SEQ ID NO: 4) (ADP7), or a functional equivalent thereof.

5. The isolated amelogenin-derived polypeptide of claim 4, wherein the polypeptide is selected from the group consisting of

25 (i) (HTLQPHHH(L/I)PVV)<sub>1-10</sub> (SEQ ID NO: 12) (ADP1);

(ii) (VPG(H/Q)HSMTP(T/I)QH)<sub>1-10</sub> (SEQ ID NO: 13) (ADP2)

(iii) (HPP(S/T)HTLQPHHH(L/I)PVV)<sub>1-10</sub> (SEQ ID NO: 14) (ADP4);

(iv) (PAQQPV(A-I)PQQPMMP)<sub>1-10</sub> (SEQ ID NO: 15) (ADP8);

(v) (HPP(S/T)HTLQPHHH(L/I)PVVPAQQPV(A/I)PQQPMMPVPG

30 (H/Q)HSMTP(T/I)QH)<sub>1-10</sub> (SEQ ID NO: 4) (ADP7);

(vi) (HTLQPHHHLPVV)<sub>1-10</sub> (SEQ ID NO: 16) (ADP1M);

(vii) (HTLQPHHHIPVV)<sub>1-10</sub> (SEQ ID NO: 17) (ADP1H);

(viii) (VPGHHSMTPTQH)<sub>1-10</sub> (SEQ ID NO: 18) (ADP2M)

(ix) (VPGQHSMTPIQH)<sub>1-10</sub> (SEQ ID NO: 19) (ADP2H)

- (x) (HPPSHTLQPHHHLPVV)<sub>1-10</sub> (SEQ ID NO: 20) (ADP4M);
- (xi) (HPPTHTLQPHHHIPVV)<sub>1-10</sub> (SEQ ID NO: 21) (ADP4H);
- (xii) (HPPSHTLQPHHHLPVVPAQQPVAPQQPMMPVPGHHSMPTQH)<sub>1-10</sub>  
(SEQ ID NO: 8) (ADP7M);
- 5 (xiii) (HPPTHTLQPHHHIPVVPAQQPVIPQQPMMPVPGQHSMPTIQH)<sub>1-10</sub>  
(SEQ ID NO: 9) (ADP7H);
- (xiv) (PAQQPVAPQQPMMP)<sub>1-10</sub> (SEQ ID NO: 22) (ADP8M); and
- (xv) (PAQQPVIPQQPMMP)<sub>1-10</sub> (SEQ ID NO: 23) (ADP8H);
- or functional equivalents thereof.

10 6. The isolated amelogenin-derived polypeptide of claim 1, wherein the polypeptide is selected from the group consisting of

- (i) (WPATDKTKREEVD)<sub>1-10</sub> (SEQ ID NO: 10) (ADP3M); and
- (ii) (WPSTDKTKREEVD)<sub>1-10</sub> (SEQ ID NO: 11) (ADP3H),
- or functional equivalents thereof.

15 7. A recombinant fusion polypeptide, comprising:

- (a) the amelogenin-derived polypeptide of any one of claims 1-6; and
- (b) a heterologous polypeptide.

8. The recombinant fusion protein of claim 7, wherein the heterologous polypeptide is selected from the group consisting of detectable polypeptides, polypeptide affinity tags,  
20 peptide markers, FLAG-tags, anti-microbial polypeptides, biomineralization-promoting polypeptides, inorganic material-binding polypeptides, three-dimensional scaffold-forming polypeptides, collagen, chitosan, amphiphilic peptides, protein-binding polypeptides, enamel-derived polypeptides, tuftelin-derived peptides, statherin-derived polypeptides, dentin-derived polypeptides, bone sialoprotein-derived polypeptides, osteocalcin-derived  
25 polypeptides, osteopontin-derived polypeptides, proteins with caries inhibitory activity, casein, and bone morphogenetic-derived polypeptides.

9. A composition, comprising:

- (a) the amelogenin-derived polypeptide of any one of claims 1-6; and
- (b) a non-peptide moiety linked to the amelogenin-derived polypeptide.

30 10. The composition of claim 9, wherein the non-peptide moiety is selected from the group consisting of radioactive isotopes, non-peptide affinity tags, isotope-coded affinity tags, fluorophores, organic (micelles, liposomes, dendrimers) or inorganic (metallic, semiconducting and dielectric) nanostructures, including nanotubes, nanowires, nanoparticles, atomic clusters, quantum dots, single layer or multilayer atomic materials,

inorganic ions or ion clusters, organic-inorganic hybrid nanostructures, fish flour, amino acids, and non-peptide polymers.

11. A pharmaceutical composition, comprising
  - (a) the polypeptide, recombinant fusion protein, or composition of any one of claims 1-10; and
  - (b) a pharmaceutically acceptable carrier.
12. An oral care product, comprising the polypeptide, recombinant fusion protein, or composition of any one of claims 1-10.
13. The oral care product of claim 12, wherein the dental care product is selected from the group consisting of toothpaste, toothpowders, mouthwash, dental floss, liquid dentifrices, dental tablets, topical gels, troches, chewing gums, dental pastes, gingival massage creams, gargle tablets, lozenges, and food products.
14. An isolated nucleic acid encoding the polypeptide or recombinant fusion protein of any one of claims 1-8.
15. A recombinant expression vector comprising the isolated nucleic acid of claim 14.
16. A recombinant host cell comprising the expression vector of claim 15.
17. A method for treating dental disease, comprising administering to a subject in need thereof an amount effective to treat the dental disease of the polypeptide, recombinant fusion protein, or composition of any one of claims 1-10, the pharmaceutical composition of claim 11 or 12, or the oral care product of claim 12 or 13.
18. The method of claim 17, wherein the dental disorder is selected from the group consisting of periodontitis, tooth erosion, hypersensitivity, bacterial plaque, dental fluorosis, tooth decay, carries, tooth resorption, and gingival recession.
19. An isolated polypeptide, comprising one or more amino acid sequences selected from the group consisting of:
 

ADP5(V1):(PGYIN(L/F)SYA(K/N)SHSQAIN(T/V)ARTA)<sub>1-10</sub> (SEQ ID NO: 25);

ADP5(V2):(PGYIN(L/F)SYE(A/N)SHSQAIN(T/V)DATA)<sub>1-10</sub> (SEQ ID NO: 26);

and

ADP5(V3):(PGYIN(L/F)SYA(A/N)SHSQAIN(T/V)AATA)<sub>1-10</sub> (SEQ ID NO: 27).
20. The isolated polypeptide of claim 19, comprising one or more amino acid sequences selected from the group consisting of
 

ADP5(V1): PGYIN(L/F)SYA(K/N)SHSQAIN(T/V)ARTA (SEQ ID NO: 25);

ADP5(V2): PGYIN(L/F)SYE(A/N)SHSQAIN(T/V)DATA (SEQ ID NO: 26);

ADP5(V3): PGYIN(L/F)SYA(A/N)SHSQAIN(T/V)AATA (SEQ ID NO: 27);

PGYINLSYA(K/N)SHSQAINVARTATA (SEQ ID NO: 32);

PGYINLSYE(A/N)SHSQAINVDTATA (SEQ ID NO: 33);

PGYINLSYA(A/N)SHSQAINVATAATA (SEQ ID NO: 34);

PGYINFSYA(K/N)SHSQAINVARTATA (SEQ ID NO: 35);

5 PGYINFSYE(A/N)SHSQAINVDTATA (SEQ ID NO: 36);

PGYINFSYA(A/N)SHSQAINVATAATA (SEQ ID NO: 37);

PGYINLSYAKSHSQAINVARTATA (SEQ ID NO: 38);

PGYINLSYEASHSQAINVDTATA (SEQ ID NO: 39);

PGYINLSYAASHSQAINVATAATA (SEQ ID NO: 40);

10 PGYINFSYANSHSQAINVARTATA (SEQ ID NO: 41);

PGYINFSYEASHSQAINVDTATA (SEQ ID NO: 42); and

PGYINFSYAASHSQAINVATAATA (SEQ ID NO: 43).

21. A recombinant fusion polypeptide, comprising:

(a) the isolated polypeptide of any one of claims 19-20; and

15 (b) a heterologous polypeptide.

22. A composition, comprising:

(a) the isolated polypeptide of any one of claims 19-20; and

(b) a non-peptide moiety linked to the polypeptide.

23. A pharmaceutical composition, comprising

20 (a) the polypeptide, recombinant fusion protein, or composition of any one of claims 19-22; and

(b) a pharmaceutically acceptable carrier.

24. An oral care product, comprising the polypeptide, recombinant fusion protein, or composition of any one of claims 19-23.

25 25. The oral care product of claim 24, wherein the dental care product is selected from the group consisting of toothpaste, toothpowders, mouthwash, dental floss, liquid dentifrices, dental tablets, topical gels, troches, chewing gums, dental pastes, gingival massage creams, gargle tablets, lozenges, and food products.

26. An isolated nucleic acid encoding the polypeptide or recombinant fusion protein of  
30 any one of claims 19-21

27. A recombinant expression vector comprising the isolated nucleic acid of claim 26.

28. A recombinant host cell comprising the expression vector of claim 27.

29. A method for treating dental disease, comprising administering to a subject in need thereof an amount effective to treat the dental disease of the polypeptide, recombinant fusion

protein, or composition of any one of claims 19-22, the pharmaceutical composition of claim 23, or the oral care product of claim 24 or 25.

30. The method of claim 29, wherein the dental disorder is selected from the group consisting of periodontitis, tooth erosion, hypersensitivity, bacterial plaque, dental fluorosis, tooth decay, carries, tooth resorption, craniomaxillofacial bone disease, and gingival recession.

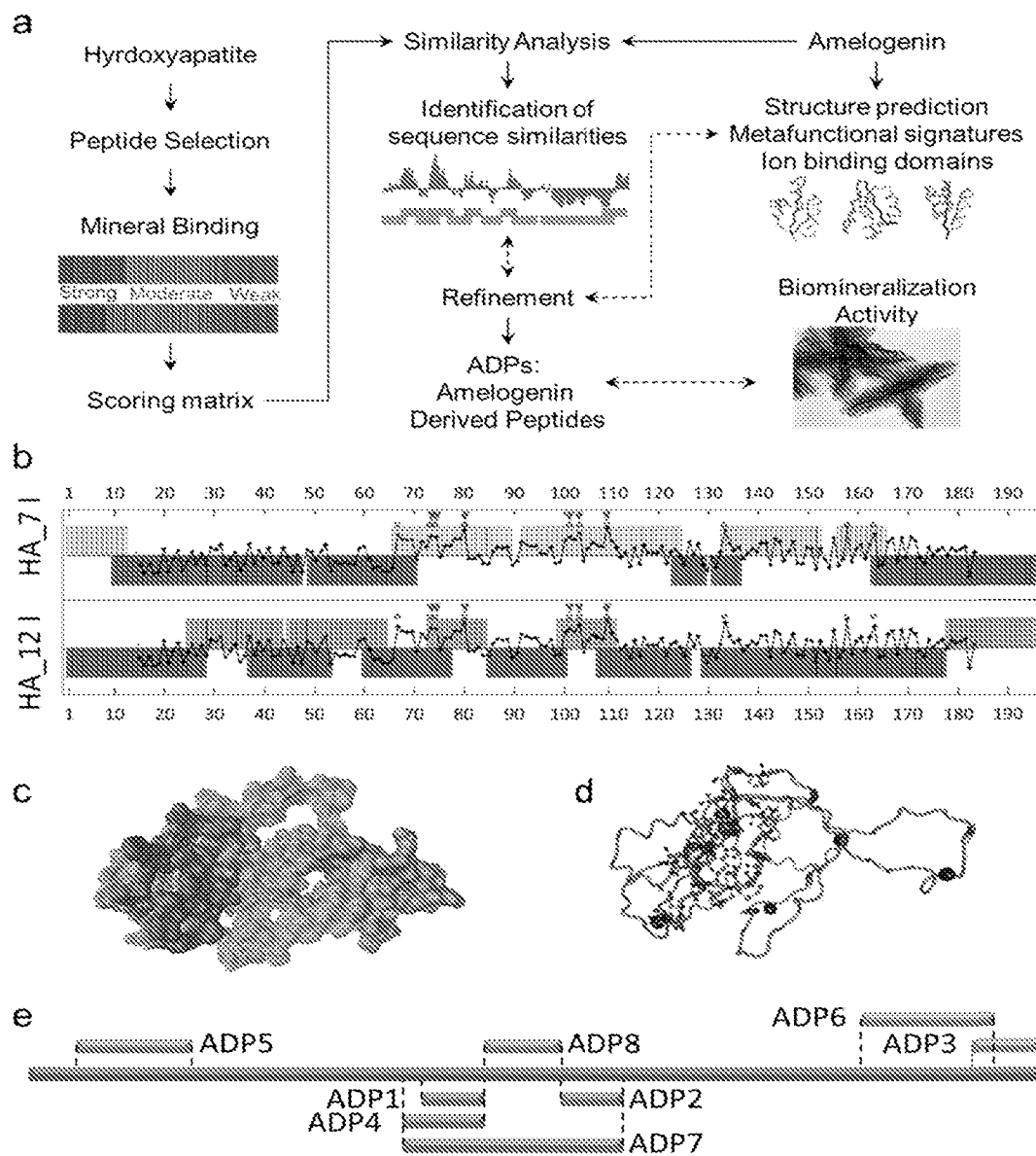


Figure 1

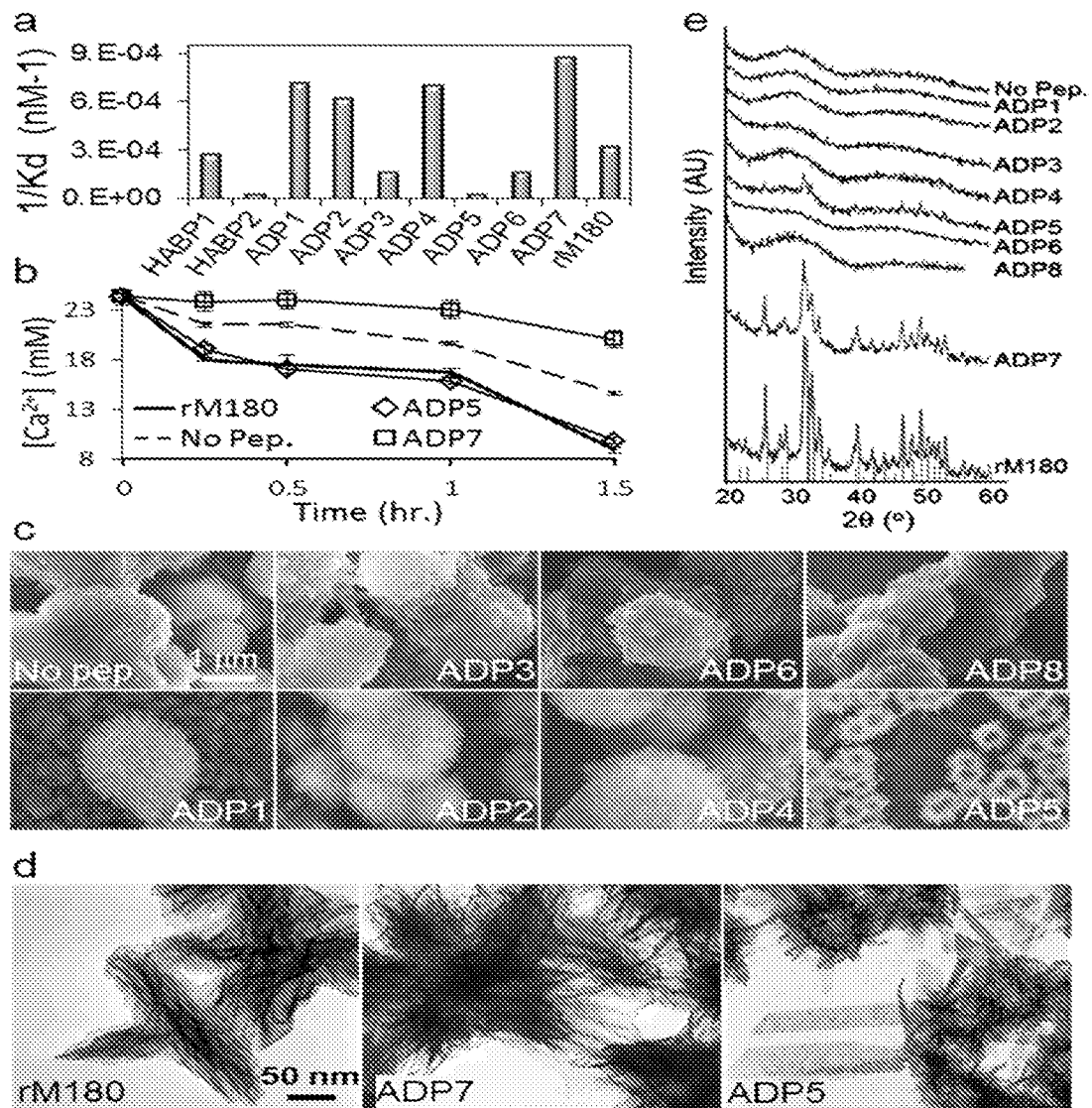


Figure 2



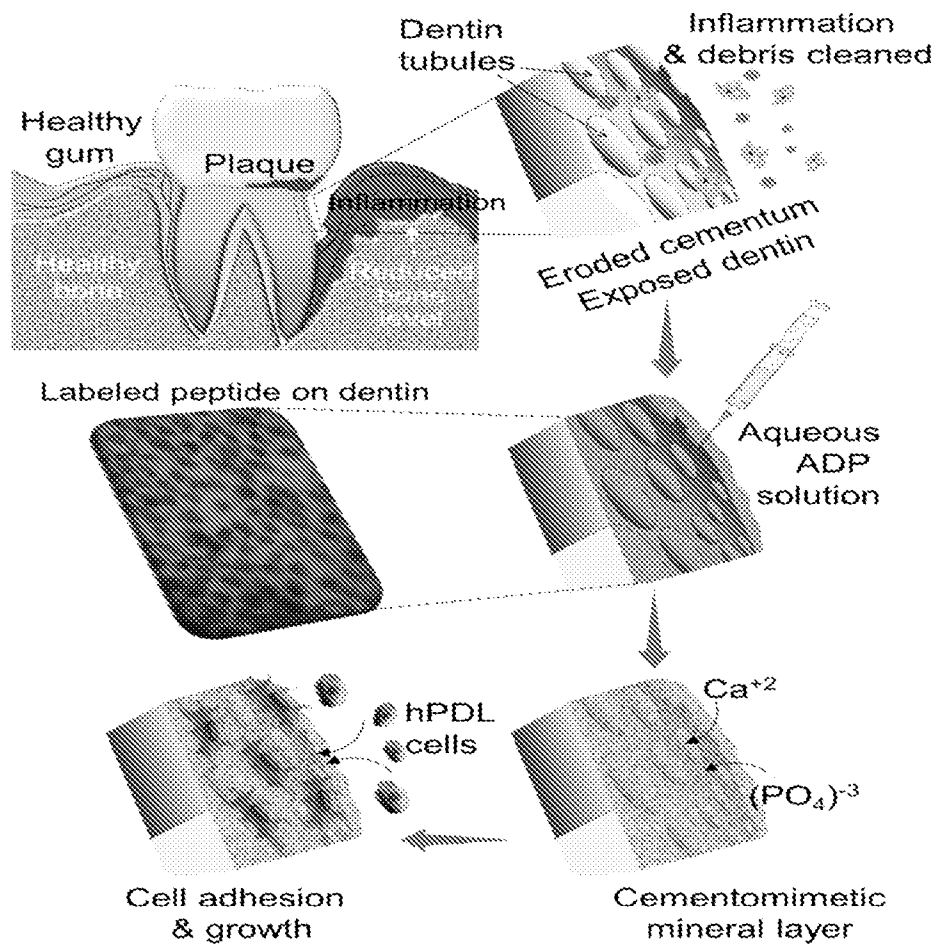


Figure 3

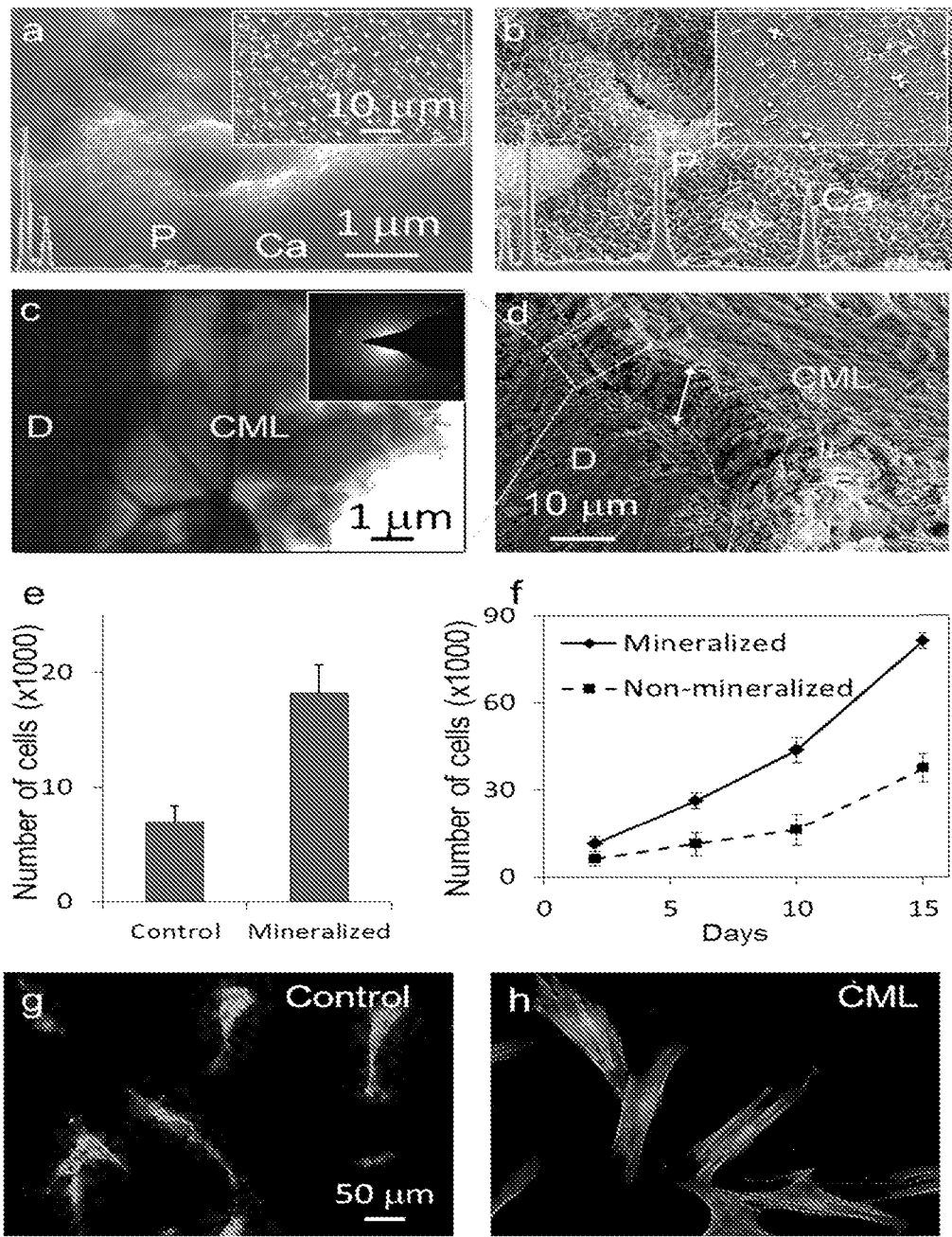


Figure 4

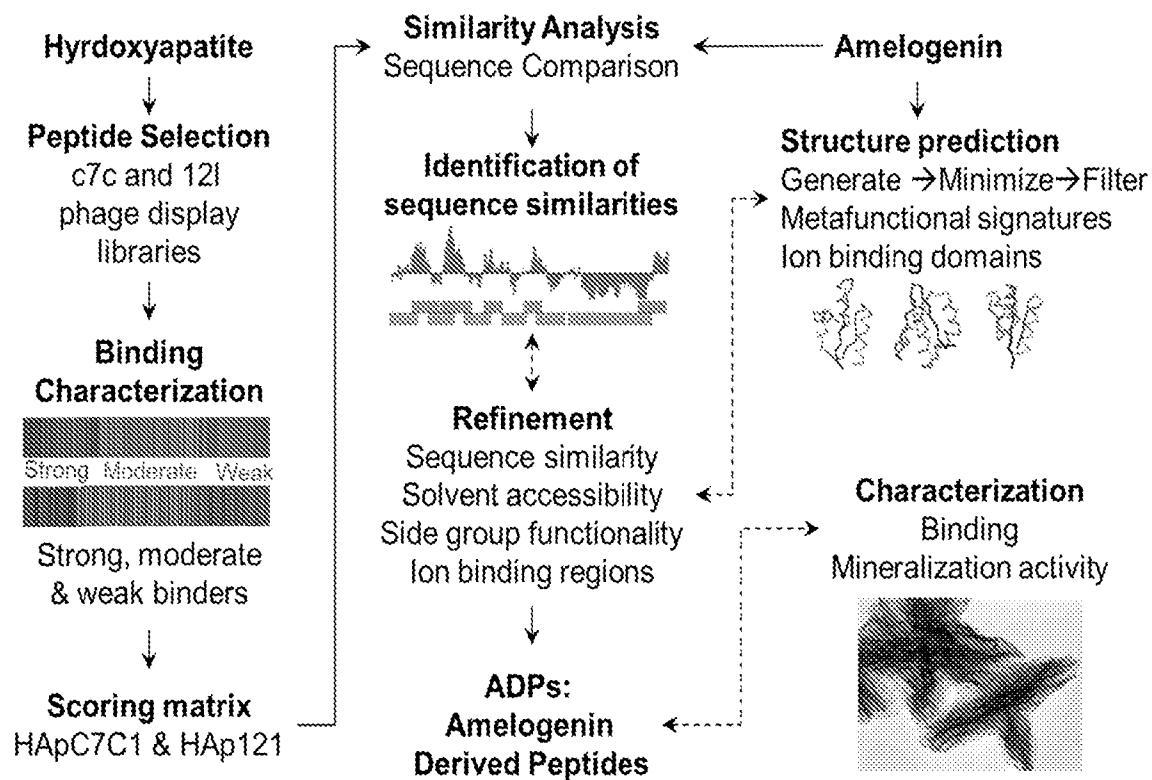


Figure 5

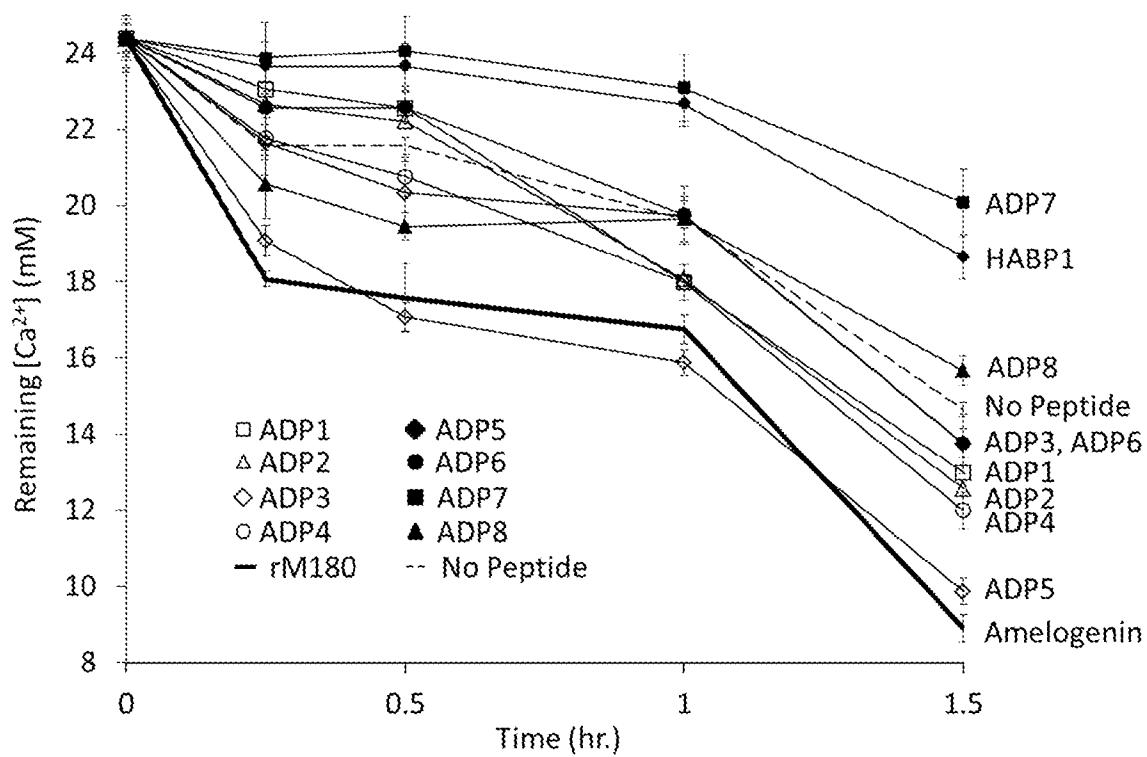


Figure 6

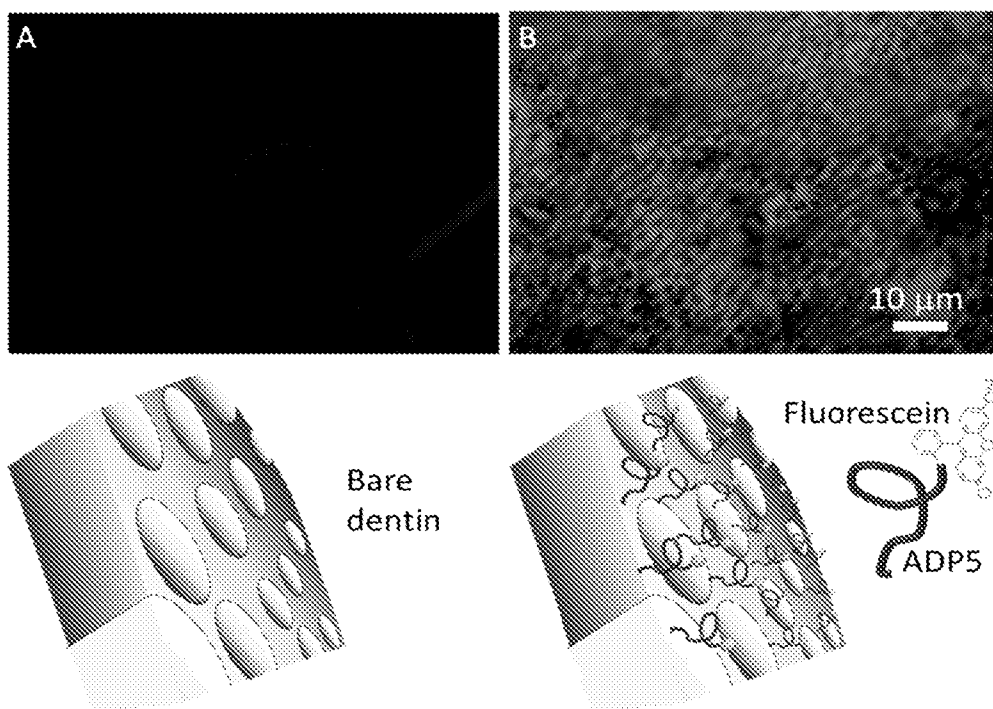
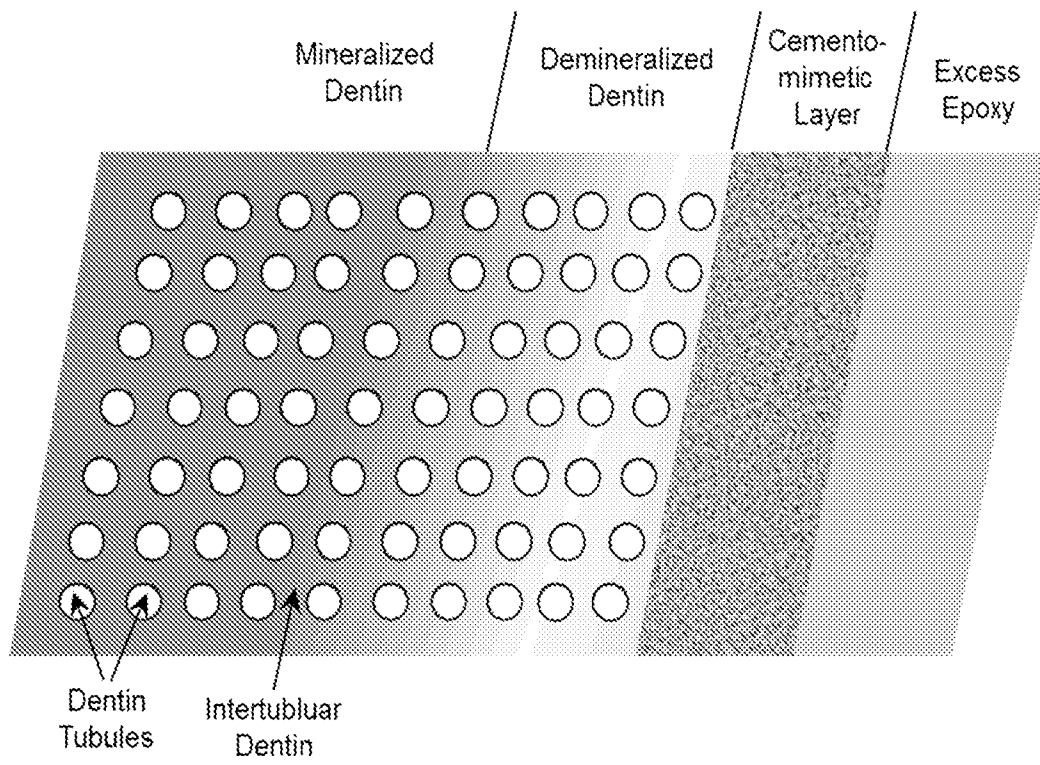


Figure 7

**Figure 8**

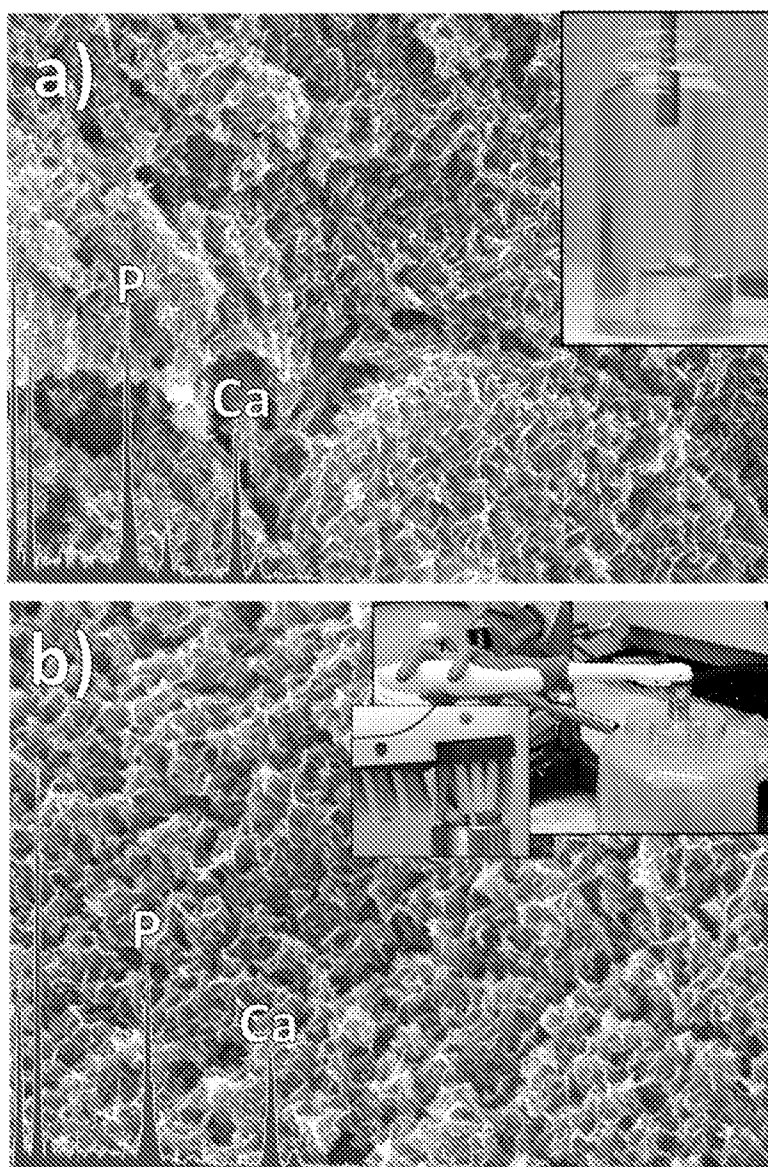


Figure 9

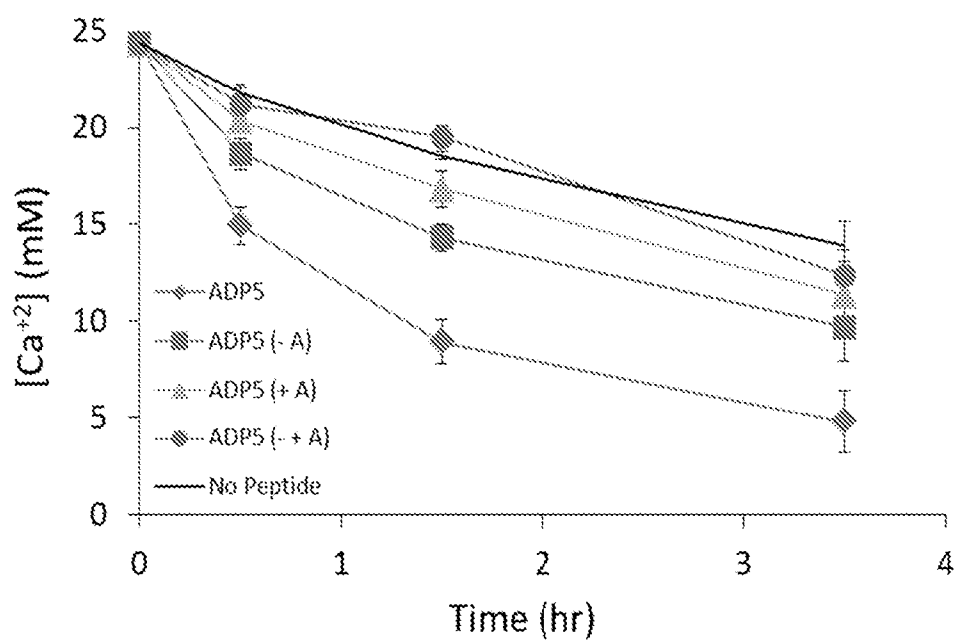


Figure 10



# INTERNATIONAL SEARCH REPORT

International application No  
PCT/US2012/039650

## A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/78 C12N15/62 A61K38/39 C12N5/10  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, COMPENDEX, Sequence Search, EMBASE, FSTA

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                              | Relevant to claim No. |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
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| Y         | WO 00/06734 A1 (UNIV NORTHWESTERN [US];<br>VEIS ARTHUR [US]; NEBGEN DENISE R [US])<br>10 February 2000 (2000-02-10)<br>the whole document<br>-----<br>-/-                                                                                                                                                                                                                       | 19-30                 |

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

20 August 2012

Date of mailing of the international search report

11/09/2012

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Authorized officer

Bassias, Ioannis

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/US2012/039650

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                               |                       |
|------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                            | Relevant to claim No. |
| Y                                                    | US 6 677 306 B1 (VEIS ARTHUR [US] ET AL)<br>13 January 2004 (2004-01-13)<br>the whole document<br>-----                                                                                                                                                                                                                                                                       | 19-30                 |
| Y                                                    | WO 2008/021290 A2 (HOMESTEAD CLINICAL CORP<br>[US]; INST SYSTEMS BIOLOGY [US]; HOOD<br>LEROY [U] 21 February 2008 (2008-02-21)<br>the whole document<br>-----                                                                                                                                                                                                                 | 19-30                 |
| A                                                    | DELGADO S ET AL: "Amelogenin, the major<br>protein of tooth enamel: A new<br>phylogenetic marker for ordinal mammal<br>relationships",<br>MOLECULAR PHYLOGENETICS AND EVOLUTION,<br>ORLANDO, FL, US,<br>vol. 47, no. 2, 1 May 2008 (2008-05-01),<br>pages 865-869, XP022622910,<br>ISSN: 1055-7903, DOI:<br>10.1016/J.YMPEV.2008.01.025<br>[retrieved on 2008-02-02]<br>----- | 1-30                  |
| A                                                    | ZHANG H ET AL: "Full length amelogenin<br>binds to cell surface LAMP-1 on tooth<br>root/periodontium associated cells",<br>ARCHIVES OF ORAL BIOLOGY, PERGAMON PRESS,<br>OXFORD, GB,<br>vol. 55, no. 6, 1 June 2010 (2010-06-01),<br>pages 417-425, XP027051299,<br>ISSN: 0003-9969<br>[retrieved on 2010-04-10]<br>-----                                                      | 1-30                  |
| A                                                    | HANSON FONG ET AL: "Enamel Structure<br>Properties Controlled by Engineered<br>Proteins in Transgenic Mice",<br>JOURNAL OF BONE AND MINERAL RESEARCH,<br>vol. 18, no. 11,<br>1 November 2003 (2003-11-01), pages<br>2052-2059, XP55035694,<br>ISSN: 0884-0431, DOI:<br>10.1359/jbmr.2003.18.11.2052<br>-----                                                                  | 1-30                  |
| A,P                                                  | WO 2011/077086 A2 (UCL BUSINESS PLC [GB];<br>DONOS NIKOLAOS [GB]; OLSEN IRWIN [GB];<br>AMIN HAR) 30 June 2011 (2011-06-30)<br>-----                                                                                                                                                                                                                                           | 1-30                  |
| A,P                                                  | WO 2011/073447 A9 (STRAUMANN HOLDING AG<br>[CH]; MOLENBERG AALDERT [CH]; RANEVSKI<br>RUZICA [CH] 29 March 2012 (2012-03-29)<br>-----                                                                                                                                                                                                                                          | 1-30                  |
|                                                      | -/--                                                                                                                                                                                                                                                                                                                                                                          |                       |

# INTERNATIONAL SEARCH REPORT

International application No

PCT/US2012/039650

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                            | Relevant to claim No. |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| T         | <p>GUNGORMUS MUSTAFA ET AL:<br/> "Cementomimetics-constructing a<br/> cementum-like biomineralized microlayer<br/> via amelogenin-derived peptides.",<br/> INTERNATIONAL JOURNAL OF ORAL SCIENCE JUN<br/> 2012 LNKD- DOI:10.1038/IJOS.2012.40<br/> PUBMED:22743342,<br/> vol. 4, no. 2, June 2012 (2012-06), pages<br/> 69-77, XP002682010,<br/> ISSN: 1674-2818</p> <p style="text-align: center;">-----</p> |                       |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2012/039650

| Patent document<br>cited in search report |    | Publication<br>date |      | Patent family<br>member(s) |  | Publication<br>date |
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|                                           |    |                     | WO   | 0006734 A1                 |  | 10-02-2000          |
| -----                                     |    |                     |      |                            |  |                     |
| US 6677306                                | B1 | 13-01-2004          | NONE |                            |  |                     |
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| WO 2008021290                             | A2 | 21-02-2008          | AU   | 2007284651 A1              |  | 21-02-2008          |
|                                           |    |                     | CA   | 2660286 A1                 |  | 21-02-2008          |
|                                           |    |                     | EP   | 2057465 A2                 |  | 13-05-2009          |
|                                           |    |                     | JP   | 2010500568 A               |  | 07-01-2010          |
|                                           |    |                     | US   | 2012087862 A1              |  | 12-04-2012          |
|                                           |    |                     | WO   | 2008021290 A2              |  | 21-02-2008          |
| -----                                     |    |                     |      |                            |  |                     |
| WO 2011077086                             | A2 | 30-06-2011          | NONE |                            |  |                     |
| -----                                     |    |                     |      |                            |  |                     |
| WO 2011073447                             | A9 | 29-03-2012          | NONE |                            |  |                     |
| -----                                     |    |                     |      |                            |  |                     |