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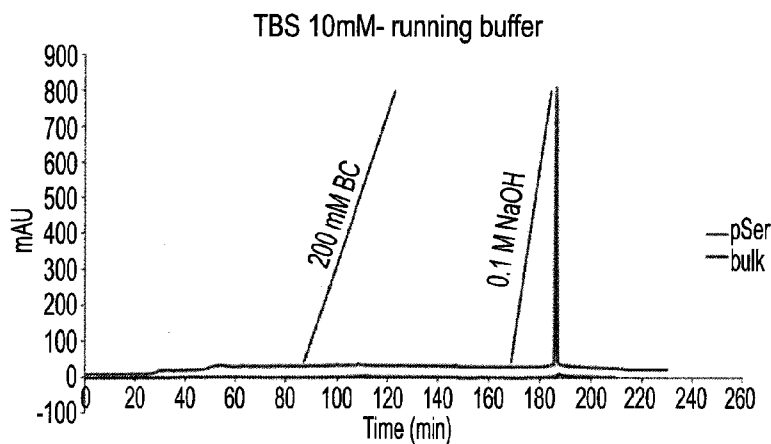


FIG. 7A

(57) Abstract: An isolated peptide is disclosed. The peptide comprises a titanium oxide binding amino acid sequence connected to a heterologous biologically active amino acid sequence via a beta sheet breaker linker, wherein: (i) the titanium oxide binding amino acid sequence is selected to bind coordinatively with titanium oxide; (ii) the titanium oxide binding amino acid sequence is selected to induce a beta sheet structure; and (iii) the titanium oxide binding amino acid sequence binds to titanium oxide with a higher affinity than said biologically active amino acid sequence binds to the titanium oxide under physiological conditions. Use of the peptides and titanium devices comprising same are also disclosed.



## FUNCTIONALIZED TITANIUM BINDING PEPTIDES AND IMPLANTS COATED WITH SAME

### FIELD AND BACKGROUND OF THE INVENTION

5           The present invention, in some embodiments thereof, relates to functionalized titanium binding peptides and, more particularly, but not exclusively, to titanium binding peptides which are capable of promoting bone growth and mineralization.

          Attempts to use titanium for implant fabrication dates to the late 1930's. It was found that titanium was tolerated in cat femurs, as was stainless steel and vitalium (a  
10   CoCrMo alloy). Titanium's lightness and good mechanical and chemical properties are salient features for implant applications. One titanium alloy (Ti6Al4V) is widely used to manufacture implants. The main alloying elements of this alloy are aluminium (5.5 - 6.5%) and vanadium (3.5 - 4.5%). Whilst the strength of the titanium alloys varies from lower than to equal to that of stainless steel, when compared by specific strength  
15   (strength per density), the titanium alloys outperform any other implant material.

          More than 1000 tonnes (2.2 million pounds) of titanium devices of every description and function are implanted in patients worldwide every year, including for bone and joint replacement, dental implants, maxillo and cranio/facial treatments and cardiovascular devices. Light, strong and totally bio-compatible, titanium is one of few  
20   materials that naturally match the requirements for implantation in the human body.

          The natural selection of titanium for implantation is determined by a combination of most favourable characteristics including immunity to corrosion, bio-compatibility, strength, low modulus and density and the capacity for joining with bone and other tissue - osseointegration. The mechanical and physical properties of titanium alloys  
25   combine to provide implants which are highly damage tolerant. The human anatomy naturally limits the shape and allowable volume of implants. The lower modulus of titanium alloys compared to steel is a positive factor in reducing bone resorption. Two further parameters define the usefulness of the implantable alloy, the notch sensitivity, - the ratio of tensile strength in the notched vs. un-notched condition, and the resistance to  
30   crack propagation, or fracture toughness. Titanium scores well in both cases. Typical NS/TS ratios for titanium and its alloys are 1.4 - 1.7 (1.1 is a minimum for an acceptable implant material). Fracture toughness of all high strength implantable alloys is above

50MPam-1/2 with critical crack lengths well above the minimum for detection by standard methods of non-destructive testing.

Titanium (Ti) spontaneously forms an oxide layer up to a thickness of about 2 to 5 nm both in air and in the body, providing corrosion resistance. However, the normal oxide layer of titanium is not sufficiently bioactive to form a direct bond with juxtaposed bone, which may translate into a lack of osseointegration, leading to long-term failure of titanium implants.

In the past, many attempts have been made to improve the surface properties of Ti-based implants; e.g., by modifying Ti topography, chemistry, and surface energy, in order to better integrate into bone. Surface modification techniques include mechanical methods such as sand blasting, chemical methods such as acid etching, and the use of various coatings. A disadvantage of these approaches is that neither the mechanical nor the chemical methods produce highly controllable topological properties, and cell/tissue adherence may be unpredictable or insufficient for practical use. In some cases, the methods may cause formation of surface residuals, which can be interfere with osteoblast (bone forming cell) adherence and function.

Despite progress in modifying metal surfaces to improve tissue and cell adhesion properties, adequate in vivo osseointegration on implant prostheses remains a challenge. Substrates that promote significant bone-tissue interactions with biomaterial surfaces over a period of time would be highly desirable. In order to ensure effective tissue adhesion, and thus clinical success of orthopaedic/dental implants, it is important to develop stable, biocompatible surfaces that enhance osteoblast functions for new bone formation. Additionally, the increasing importance of antimicrobial and other bioactive agents for in vivo implants requires improved materials and more effective means of releasing drugs at selected sites in the body.

Gertler et al [Langmuir, 2010) teach titanium surfaces attached to peptides.

U.S. Patent Application No. 20100015197 teaches amphiphilic peptides and peptide matrices thereof useful in vitro and in situ biomineralization and inducing bone repair.

Meyers et al [Advanced Materials, 2007, 19, 2492-2498] teaches a 20-30 mer peptide that comprises a titanium binding domain and a domain that binds to endothelial cells. The peptides were identified using a genetically engineered peptide library. The

drawback of this approach lies in the panning procedure which uses limited adsorption and desorption conditions. The panning enriches the electrostatically physisorbed peptides, while leaving strongly bound ones unrevealed.

5            SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided an isolated peptide comprising a titanium oxide binding amino acid sequence connected to a heterologous biologically active amino acid sequence via a beta sheet breaker linker, wherein:

10            (i)      the titanium oxide binding amino acid sequence is selected to bind coordinatively with titanium oxide;

              (ii)      the titanium oxide binding amino acid sequence is selected to induce a beta sheet structure; and

              (ii)      the titanium oxide binding amino acid sequence binds to titanium oxide  
15            with a higher affinity than the biologically active amino acid sequence binds to the titanium oxide under physiological conditions.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide encoding the isolated peptides described herein.

              According to an aspect of some embodiments of the present invention there is  
20            provided an article of manufacture comprising a titanium surface and the isolated peptide described herein adsorbed to the surface.

              According to an aspect of some embodiments of the present invention there is  
provided a method of generating or repairing a tissue, the method comprising implanting  
the article of manufacture described herein into a subject in need thereof, thereby  
25            generating or repairing the tissue.

              According to some embodiments of the invention, the isolated peptide comprises naturally occurring amino acids.

              According to some embodiments of the invention, the isolated peptide comprises at least one synthetic amino acid.

30            According to some embodiments of the invention, the isolated peptide comprises at least one D amino acid.

According to some embodiments of the invention, the titanium oxide binding amino acid sequence binds to titanium oxide with at least 2 fold higher affinity than the biologically active amino acid sequence.

5 According to some embodiments of the invention, the beta sheet breaker linker comprises naturally occurring amino acids.

According to some embodiments of the invention, the beta sheet breaker linker comprises synthetic amino acids.

According to some embodiments of the invention, the beta sheet breaker amino linker comprises 2-7 repeating glycine residues.

10 According to some embodiments of the invention, the beta sheet breaker amino acid linker is set forth by Gly-Gly-Gly.

According to some embodiments of the invention, the beta sheet breaker amino linker is a chemical linker.

15 According to some embodiments of the invention, the titanium oxide binding amino acid sequence comprises no more than 7 carboxyl amino acid residues.

According to some embodiments of the invention, the titanium oxide binding amino acid sequence comprises alternating hydrophilic, negatively charged amino acids and hydrophobic amino acids.

20 According to some embodiments of the invention, the alternating hydrophilic and hydrophobic amino acid sequence is not repeated more than 7 times.

According to some embodiments of the invention, at least one of the amino acids of the titanium oxide binding amino acid sequence is phosphorylated.

25 According to some embodiments of the invention, the titanium oxide binding amino acid sequence comprises the sequence Y-X-Y-X, wherein X is any hydrophobic amino acid and Y is selected from the group consisting of glutamic acid, aspartic acid, phosphoserine and L-DOPA.

30 According to some embodiments of the invention, the titanium oxide binding amino acid sequence comprises the sequence Y-X-Y, wherein X is any hydrophobic amino acid and Y is selected from the group consisting of glutamic acid, aspartic acid, phosphoserine and L-DOPA.

According to some embodiments of the invention, the titanium oxide binding amino acid sequence comprises the sequence Y-Y-X-X, wherein X is any hydrophobic

amino acid and Y is selected from the group consisting of glutamic acid, aspartic acid, phosphoserine and L-DOPA.

According to some embodiments of the invention, the X is selected from the group consisting of valine, phenylalanine, leucine, isoleucine and tryptophan.

5 According to some embodiments of the invention, the Y is pSer.

According to some embodiments of the invention, the titanium oxide binding amino acid sequence comprises the sequence pSer-X-pSer-X (SEQ ID NO: 6), wherein X is any hydrophobic amino acid.

10 According to some embodiments of the invention, the titanium oxide binding amino acid sequence comprises the sequence as set forth in SEQ ID NO: 8 or SEQ ID NO: 2.

According to some embodiments of the invention, the C terminus of the titanium oxide binding amino acid sequence is attached to an N terminus of the biologically active amino acid sequence via the linker.

15 According to some embodiments of the invention, the N terminus of the titanium oxide binding amino acid sequence is attached to a C terminus of the biologically active amino acid sequence via the linker.

20 According to some embodiments of the invention, the biologically active amino acid sequence is selected from the group consisting of an osteoinductive or osteoconductive amino acid sequence, a mineralizing amino acid sequence, an anti-inflammatory amino acid sequence, a cell binding amino acid sequence, a cell migration amino acid sequence and an anti-bacterial amino acid sequence.

According to some embodiments of the invention, the osteoinductive or osteoconductive amino acid sequence comprises acidic amino acids.

25 According to some embodiments of the invention, the osteoinductive or osteoconductive amino acid sequence comprises at least 1 glutamic acid residue or aspartic acid residue.

30 According to some embodiments of the invention, the osteoinductive or osteoconductive amino acid sequence comprises 3-15 repeating glutamic acid residues or aspartic acid residues.

According to some embodiments of the invention, the sequence of the osteoinductive or osteoconductive amino acid sequence is set forth in SEQ ID NO: 7.

According to some embodiments of the invention, the osteoinductive or osteoconductive amino acid sequence is selected from the group consisting of a BMP amino acid sequence, an FGF amino acid sequence, a TGF amino acid sequence, an EGF amino acid sequence and a fibrinogen amino acid sequence.

5 According to some embodiments of the invention, the biologically active amino acid sequence is no longer than 50 amino acids.

According to some embodiments of the invention, the peptide is no longer than 50 amino acids.

10 According to some embodiments of the invention, the sequence as set forth in Z-X-Z-X-(Gly)<sub>3</sub>-(Y)<sub>5</sub> (SEQ ID NO: 9) where Z is L-dopa or phosphoserine, where X is any hydrophobic amino acid and Y is aspartic acid or glutamic acid.

According to some embodiments of the invention, the isolated peptide comprises the sequence as set forth in SEQ ID NO: 1 or SEQ ID NO: 12.

15 According to some embodiments of the invention, the isolated peptide comprises the sequence as set forth in SEQ ID NO: 3, 4 or 10.

According to some embodiments of the invention, the isolated peptide comprises the sequence as set forth in SEQ ID NO: 14 or 21.

20 According to some embodiments of the invention, the titanium oxide binding amino acid sequence comprises the sequence as set forth by Pro-pSer-X-pSer-X (SEQ ID NO: 6), where X is a hydrophobic amino acid, the biologically active amino acid sequence is set forth by SEQ ID NO: 7.

According to some embodiments of the invention, the titanium oxide binding amino acid sequence comprises a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 21.

25 According to some embodiments of the invention, when the titanium oxide binding amino acid sequence comprises the sequence as set forth by L-DOPA-X-L-DOPA-X (SEQ ID NO: 5), where X is a hydrophobic amino acid, the biologically active amino acid sequence is set forth by SEQ ID NO: 7.

30 According to some embodiments of the invention, the titanium oxide binding amino acid sequence comprises the sequence PSer-Phe.

According to some embodiments of the invention, the peptide comprises the sequence selected from the group consisting of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18 and SEQ ID NO: 19.

5 According to some embodiments of the invention, the article of manufacture is an implant.

According to some embodiments of the invention, the article of manufacture is a dental implant.

10 According to some embodiments of the invention, the implant is selected from the group consisting of a hip prosthesis, a knee prosthesis, a heart valve and an intravascular stent.

According to some embodiments of the invention, the tissue is selected from the group consisting of bone, cartilage and cardiac tissue.

15 According to some embodiments of the invention, the article of manufacture is for treating a disorder selected from the group consisting of a bone disorder, a cartilage disorder, a cardiac disorder and a dental disorder.

20 Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

## 25 BRIEF DESCRIPTION OF THE DRAWINGS

30 Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.



In the drawings:

FIGs. 1A depicts the functional amino acid in the peptide.

FIG. 1B illustrates how the combination between the peptide and titanium implant will promote calcium-Phosphate mineralization and improve implant-bone  
5 integration.

FIG. 2 is a schematic illustration of how a peptide of embodiments of the invention binds to the titanium surface.

FIG. 3 is a schematic illustration of how the binding was analyzed by chromatography.

FIG. 4 is a schematic illustration of surface-directed mineralization of calcium phosphate. In stage 1, aggregates of calcium-phosphate appear. The clusters approach a surface with chemical functionality. In stage 2, pre-nucleation clusters aggregate near the surface, with loose aggregates still in solution. In stage 3, further aggregation causes densification near the surface. In stage 4, nucleation of amorphous spherical particles  
15 occurs at the surface only.

FIG. 5 is a schematic representation of one of the peptides of the present invention (SEQ ID NO: 1).

FIG. 6 is XPS spectra of Ti slides following 8 hours on a heating plate.

FIGs. 7A-B are chromatograms of PpSer on TiO<sub>2</sub> anatase column. Adsorption of  
20 0.4 mg/ml PpSer (SEQ ID NO: 1) in 10 mM TBS pH 7.4 (A) and 0.8 mg/ml PpSer (SEQ ID NO: 1) in CBS 10 mM pH 7.5 (B), followed by pH 7.4 (0-200 mM) sodium bicarbonate elution gradient, followed by NaOH (0-0.1M) elution gradient.

FIGs. 8A-B are XPS spectra of peptide (SEQ ID NO: 1) diluted in TBS 10 mM to 0.2 mg/ml and bonded to TiO<sub>2</sub> (8A) and XPS spectra of surface b after 40 min in SBF  
25 (8B).

FIG. 9. Calcium and phosphate ion concentration detected in peptide coated samples that were soaked in SBF for 11 days. The samples were withdrawn from SBF after 11 days and immediately washed with ethanol to remove residual solution. The dried samples were then soaked in 0.1 M HCl to dissolve all the mineral and samples of  
30 the acidic solution were measured by ICP for calcium, phosphate and potassium ions (the latter were in negligible concentrations). The figure shows that the samples coated with peptide in presence of BCS exhibited the highest mineralization. Noteworthy, in

these experiments the titanium oxide surface itself induces mineralization too. This result is in accordance with the expected anionic charges on the titanium surface. In addition, phosphate ions from the solution may coordinatively bind to the titanium and induce mineralization. Nevertheless, the peptide coating is expected to improve the binding strength between the mineralized layer and the titanium substrate.

FIGs. 10A-B (A) Chromatograms of PF1, PF2, PF4, PF6 and PF8 on TiO<sub>2</sub> anatase column. Adsorption in 10 mM TBS pH 7.4, followed by pH (7.4-11) elution gradient. The gradient profile represents pH at the end of the column. Inset show the effect of carboxylic groups number on adsorption. (B) CD spectra in the adsorption medium, at 20 °C.

FIGs. 11A-C are schematic illustrations of 'pSer' peptide – SEQ ID NO: 12 (A) and the control peptides 'cpSer' – SEQ ID NO: 13 (B) and β-cpSer – SEQ ID NO: 14 (C).

FIG. 12. CD spectra of pSer, cpSer and β-cpSer peptides. Each of the peptides was dissolved in 10mM TBS (pH7.4) solution to a final concentration of 0.4 mg/ml. The same result was observed for a final peptide concentration of 0.2 mg/ml.

FIG. 13. Attenuated total reflection spectra of pSer, cpSer and β-cpSer peptides dissolved in 10mM TBS (pH=7.4) and dried on ZnSe prism.

FIGs. 14A-B. Chromatograms of pSer, cpSer and β-cpSer peptides on TiO<sub>2</sub> anatase column. The chromatograms presented as peptide adsorption at 280 nm normalized to peptide concentration. Adsorption was performed in 10 mM TBS pH 7.4 followed by sodium bicarbonate (0-200 mM) and NaOH (0-0.1 M) elution gradients. Marked area expanded at the right showing the peptides elution peaks.

FIGs. 15A-C. Adsorption isotherm plots of pSer, cpSer and β-cpSer peptides onto TiO<sub>2</sub> particles, in 10 mM TBS pH=7.4. Continuous line represents the Sips adsorption fitting.

FIG. 16. XPS measurements of TiO<sub>2</sub> surface.

FIGs. 17A-B. Atomic ratio of phosphorus to titanium (A) and atomic ratio of phosphorus and calcium to titanium analyzed by XPS for surfaces coated with pSer, cpSer and β-cpser peptides and incubated in calcium-phosphate solution for 24 hours at 37 °C (B).

## DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to functionalized titanium binding peptides and, more particularly, but not exclusively, to titanium binding peptides which are capable of promoting bone growth and mineralization.

5 Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Titanium is used as a material for coating implants due to its mechanical  
10 properties, good corrosion resistance and biocompatibility based on its chemical properties. However, titanium cannot bond directly to living bone after implantation. Since acidic amino acids have been proposed to be involved in the nucleation of calcium minerals and encourage calcium mineralization and since phosphoserine functional groups are known to have high affinity to oxide surfaces, the present inventors sought to  
15 develop a multi-functional peptidic coating for TiO<sub>2</sub> that would promote mineralization in order to improve implant-osseous integration.

The present inventors deduced rules which govern selection of such peptides. Thus, the peptide should comprise two functional moieties, the first being a titanium oxide binding moiety and the second a biologically functional moiety, wherein the first  
20 moiety has a higher affinity for titanium than the second. The first moiety should have a Beta sheet structure and the first and second moiety should be linked via a Beta sheet breaker linker.

Whilst reducing the present invention to practice, the present inventors synthesized an exemplary peptide - Pro-pSer-Trp-pSer-Trp-(Gly)<sub>3</sub>-(Glu)<sub>5</sub> (SEQ ID NO:  
25 1) and showed that it was capable of being strongly adsorbed to a titanium surface.

Using sophisticated chromatographical analysis, the present inventors showed that the peptide was attached to the titanium oxide (TiO<sub>2</sub>) surface via its phosphoserine residue (Figure 7A). Further, it was shown that about one third of the peptide connected to carbonate molecules that were on the TiO<sub>2</sub> surface and two-thirds connected to the  
30 TiO<sub>2</sub> surface through pSer residue (Figure 7B). This result was confirmed using X-ray photoelectron spectroscopy (XPS) measurements (Figures 8A-B).

Thus, according to one aspect of the present invention there is provided an isolated peptide comprising a titanium oxide binding amino acid sequence connected to a heterologous biologically active amino acid sequence via a beta sheet breaker linker, wherein:

- 5 (i) the titanium oxide binding amino acid sequence is selected to bind coordinatively with titanium oxide;
- (ii) the titanium oxide binding amino acid sequence is selected to induce a beta sheet structure; and
- (ii) the titanium oxide binding amino acid sequence binds to titanium oxide  
10 with a higher affinity than the biologically active amino acid sequence binds to the titanium oxide under physiological conditions.

The term "peptide" as used herein refers to a polymer of natural or synthetic amino acids, encompassing native peptides (either degradation products, synthetically synthesized polypeptides or recombinant polypeptides) and peptidomimetics (typically,  
15 synthetically synthesized peptides), as well as peptoids and semipeptoids which are polypeptide analogs.

The peptides of this aspect of the present invention are preferably no longer than  
15 amino acids, no longer than 16 amino acids, no longer than 17 amino acids, no longer than 18 amino acids, no longer than 19 amino acids, no longer than 20 amino  
20 acids, no longer than 21 amino acids, no longer than 22 amino acids, no longer than 23 amino acids, no longer than 24 amino acids, no longer than 25 amino acids, no longer than 26 amino acids, no longer than 27 amino acids, no longer than 28 amino acids, no longer than 29 amino acids, no longer than 30 amino acids, no longer than 40 amino acids, no longer than 50 amino acids.

25 According to another embodiment the peptides of this aspect of the present invention are no longer than 100 amino acids, more preferably no longer than 90 amino acids, more preferably no longer than 80 amino acids, more preferably no longer than 70 amino acids, more preferably no longer than 60 amino acids, more preferably no longer than 50 amino acids, more preferably no longer than 40 amino acids, more  
30 preferably no longer than 30 amino acids, and more preferably no longer than 20 amino acids.

As mentioned, the peptides of this aspect of the present invention comprise two functional moieties attached to each other by a linker. Each of these components will be discussed individually below.

***Titanium oxide binding amino acid sequence***

5 The term "titanium" as used herein includes metal titanium, titanium alloy, amorphous titanium dioxide, titanium dioxide anatase crystal, titanium dioxide rutile crystal and titanium dioxide brookite crystal.

It will be appreciated that under atmospheric conditions, the surface of titanium and its alloys become spontaneously covered with a nanometric oxide layer, composed  
10 mainly of TiO<sub>2</sub>. In order to improve corrosion resistance and physiological reaction, this layer can be thickened by applying anodic potential. Thus, the titanium oxide binding peptide may bind titanium or an alloy of titanium.

The titanium oxide binding amino acid sequence preferably binds with titanium oxide with a Km of about 0.1-200 μM, or more preferably, 10-150 μM, 70-120 μM  
15 when tested in 10mM TBS at a pH of about 7.4. According to a particular embodiment, the titanium oxide binding amino acid sequence binds with a Km of about 100 μM when tested in 10mM TBS at a pH of about 7.4.

The titanium oxide binding amino acid sequence is selected such that it binds coordinatively to titanium (and not via electrostatic binding alone).

20 Thus, the present invention contemplates that the titanium oxide binding amino acid sequence comprises at least one of the following groups: a carboxyl group, a phosphate group, a phosphonate group and/or an L-dopa group.

According to one embodiment, the sequence is selected such that it forms a beta sheet secondary structure. Methods of selecting peptides that form a beta sheet  
25 secondary structure are disclosed in U.S. Patent Application No. 20100015197, incorporated herein by reference, as well as the peptides themselves.

Examples of primary amino acid sequences known to form a beta sheet secondary structure include alternating hydrophilic, negatively charged amino acids and hydrophobic amino acids - for example in a sequence X-Y-X-Y, where X is a  
30 hydrophobic amino acid and Y is a hydrophilic negatively charged amino acid.

It will be appreciated that X may not represent the same amino acid along the sequence, but a different hydrophobic amino acid and Y may not represent the same

hydrophilic negatively charged amino acid, but a different one. For example, the sequence may be as set forth leucine-phosphoserine-valine-phosphotyrosine.

According to one embodiment, the core sequence X-Y is not repeated more than 7 times. Thus, the present invention contemplates repeating the X-Y core sequence  
5 twice, three times, four times, five times or six times.

Examples of hydrophobic amino acid include tyrosine, tryptophan, alanine, methionine, phenylalanine, leucine, proline, isoleucine, valine and glycine.

Examples of hydrophilic, negatively charged amino acids include phosphoserine, phosphotyrosine, phosphothreonine, glutamic acid and aspartic acid.

10 Phosphate compounds are utilized for surface modification of various transition metal oxides since they possess high binding strength. Phosphate, which appears as a free ion in physiological medium as well as in post translational modified biomolecules, coordinatively adsorbs to  $TiO_2$  from aqueous media, displacing surface terminal hydroxyl groups, directly to titanium atoms. Further, it was shown that that phosphate  
15 groups bind stronger than carboxyls than amine groups to titanium oxide.

Thus, according to still another embodiment, at least one amino acid of the titanium oxide binding amino acid sequence is phosphorylated. Thus, for example, the present invention contemplates that at least one of the amino acids comprises phosphoserine, phosphothreonine or phosphotyrosine.

20 According to a particular embodiment, the titanium oxide binding amino acid sequence comprises an alternating sequence of acidic amino acids and phosphoserine groups.

The present invention contemplates that the titanium oxide binding amino acid sequence is no longer than 20 amino acids. According to another embodiment, the  
25 titanium oxide binding amino acid sequence is no longer than 15 amino acids. According to still another embodiment, the titanium oxide binding amino acid sequence is no longer than 10 amino acids. According to another embodiment, the titanium oxide binding amino acid sequence is no longer than 5 amino acids.

The titanium oxide binding sequence may be as set forth by Z-X-Z-X, where Z  
30 is phosphoserine or L-dopa, where X is any hydrophobic amino acid, as listed herein above.

According to one embodiment, the first amino acid sequence of the titanium oxide binding sequence is a proline.

Contemplated titanium oxide binding amino acid sequences according to this embodiment are set forth in SEQ ID NOs: 22-26.

5 Thus, according to a particular embodiment the titanium oxide binding sequence is set forth by Pro-Z-X-Z-X (SEQ ID NO: 27), where X is any hydrophobic amino acid, as listed herein above and where Z is phosphoserine or L-dopa.

It will be appreciated that the titanium oxide binding sequence may also be able to bind to additional metals, metal oxides or even ceramics (e.g. magnesium oxide or  
10 calcium phosphate).

Thus, the peptides disclosed herein may also bind cobalt, cobalt alloys, chromium, chromium alloys, tantalum, tantalum alloys, stainless steel, zirconium, zirconium oxide, zirconium dioxide.

***Linker:***

15 As mentioned, the linker of the present invention is a  $\beta$ -sheet breaker linker. Typically, the linker provides a bend or turn at or near the junction between the two functional elements of the peptide. The linker which connects the first element to the second element is preferably a covalent bond (e.g. a peptide bond).

According to a particular embodiment, the conformational constraint is selected  
20 from the group consisting of, a proline or proline mimetic, an N alkylated amino acid, a double bond or triple bond or any other moiety which introduces a rigid bend into the peptide backbone.

In addition to proline, specific examples of moieties which induce suitable conformations include but are not limited to N-methyl amino acids such as sarcosine,  
25 hydroxy proline, anthranilic acid (2-amino benzoic acid) and 7-azabicycloheptane carboxylic acid.

The linker may comprise a single amino acid.

Examples of  $\beta$ -sheet breaker amino acid residues include, but are not limited to proline, glycine, lysine and serine (according to Chou and Fasman (1978) Annu. Rev.  
30 Biochem. 47, 258).

According to another preferred embodiment of this aspect of the present invention, the  $\beta$ -sheet breaker amino acid residue is a synthetic amino acid such as a

C $\alpha$ -methylated amino acid, which conformational constraints are restricted [Balaram, (1999) J. Pept. Res. 54, 195-199]. Unlike natural amino acids, C $\alpha$ -methylated amino acids have a hydrogen atom attached to the C $\alpha$ , which affects widely their sterical properties regarding the  $\phi$  and  $\psi$  angles of the amide bond. Thus, while alanine has a wide range of allowed  $\phi$  and  $\psi$  conformations,  $\alpha$ -aminoisobutyric acid (Aib, see Table 2, below) has limited  $\phi$  and  $\psi$  conformations.

Alternatively, the linker of the present invention may comprise an amino acid sequence. The sequence may be 2, 3, 4, 5, 6 or 7 amino acids long. According to a particular embodiment the linker comprises the sequence (Gly)<sub>2</sub> or (Gly)<sub>3</sub>.

According to another embodiment, the linker is a synthetic linker.

***Biologically active amino acid sequence:***

The biologically active amino acid sequence of the peptide of this aspect of the invention may comprise any biological activity, including, but not limited to a cell migration activity, an antimicrobial activity, an antibacterial activity, a cell homing activity, an osteoinducing (or osteoconducting) activity, an anti-inflammatory activity, a mineralizing activity, anti-adhesive activity, anti-thrombogenic activity.

It will be appreciated that the biologically active amino acid sequence is heterologous to the titanium binding amino acid sequence.

The qualifier "heterologous" when relating to the heterologous biologically active amino acid sequence indicates that the heterologous biologically active amino acid sequence is derived from a protein which does not normally comprise a titanium binding peptide to which it is fused so as to form the full-length peptide.

As used herein, the phrase "osteoinducing activity" refers to an ability to promote, induce, conduct, stimulate, generate, or otherwise effect the production of bone or the repair of bone. The osteoinducing activity may elicit an effect on the repair of the defect in terms of shortening the time required to repair the bone, by improving the overall quality of the repair, where such a repair is improved over situations where there is a lack of osteoinducing activity, or may achieve contemporaneously both shortened repair times and improved bone quality. It is appreciated that osteogenic agents may effect bone production or repair by exploiting endogenous systems, such as by the inhibition of bone resorption.



Osteoinducing peptides may promote bone growth by acting as bone anabolic agents. Osteoinducing peptides may also affect repair of the bone defect by stabilizing the defect to promote healing. The ramifications of using such osteoinducing peptides include increased healing rates, effecting a more rapid new bone ingrowth, improved repair quality, or improved overall quality of the resulting bone.

In one embodiment the peptide is a full length or biologically active fragment of bone morphogenetic proteins (BMPs), such as BMP-2, BMP-4, BMP-6, BMP-7, BMP-9, BMP-12, BMP-13 and BMP-14, chrysalin, osteogenic growth peptide (OGP), bone cell stimulating factor (BCSF), KRX-167, NAP-52, gastric decapeptide, parathyroid hormone (PTH), a fragment of parathyroid hormone, osteopontin, osteocalcin, a fibroblast growth factor (FGF), such as basic fibroblast growth factor (bFGF) and FGF-1, osteoprotegerin ligand (OPGL), platelet-derived growth factor (PDGF), an insulin-like growth factor (IGF), such as IGF-1 and IGF-2, vascular endothelial growth factor (VEGF), transforming growth factor (TGF), such as TGF-alpha and TGF-beta, epidermal growth factor (EGF), growth and differentiation factor (GDF), such as GDF-5, GDF-6, and GDF-7, thyroid-derived chondrocyte stimulation factor (TDCSF), vitronectin, laminin, amelogenin, intergrin, collagen and amelin.

Fibrinogen and biologically active fragments thereof are also known to act as osteoinducing peptides, as well as fibronectin and biologically active fragments thereof (e.g. RGD or RGE).

Acidic amino acid sequences are also known to be osteoinductive. Thus, for example the present invention contemplates a biologically active amino acid sequence comprising at least 3, at least 4 or at least 5 negatively charged acidic amino acids (e.g. (Glu)<sub>5</sub> (SEQ ID NO: 7) or (Asp)<sub>5</sub> (SEQ ID NO: 11).

Preferably, the osteoconductive amino acid sequence comprises 3, 5 or 7 negatively charged acidic amino acids.

According to still another embodiment, the negatively charged acidic amino acid sequence is no longer than 15 amino acids.

According to still another embodiment, the negatively charged acidic amino acid sequence is no longer than 100 amino acids.

Non-thrombogenic or anti-adhesive amino acid sequences include for example tissue plasminogen activator, reteplase, TNK-tPA and glycoprotein IIb/IIIa inhibitors.

Preferably, the biologically active amino acid sequence is no longer than 100 amino acids, more preferably no longer than 90 amino acids, more preferably no longer than 80 amino acids, more preferably no longer than 70 amino acids, more preferably no longer than 60 amino acids, more preferably no longer than 50 amino acids, more preferably no longer than 40 amino acids, more preferably no longer than 30 amino acids, more preferably no longer than 20 amino acids, and even more preferably no longer than 10 amino acids.

It will be appreciated that the titanium oxide binding amino acid sequence binds to titanium oxide with a higher affinity than the biologically active amino acid sequence when measured under identical conditions. Preferably, the titanium oxide binding amino acid sequence binds to titanium oxide with at least 1.2 the affinity than the biologically active amino acid sequence binds to titanium oxide. More preferably, the titanium oxide binding amino acid sequence binds to titanium oxide with at least 1.4 times the affinity than the biologically active amino acid sequence binds to titanium oxide. More preferably, the titanium oxide binding amino acid sequence binds to titanium oxide with at least 1.5 times the affinity than the biologically active amino acid sequence binds to titanium oxide. More preferably, the titanium oxide binding amino acid sequence binds to titanium oxide with at least twice times the affinity than the biologically active amino acid sequence binds to titanium oxide. More preferably, the titanium oxide binding amino acid sequence binds to titanium oxide with at least three times the affinity than the biologically active amino acid sequence binds to titanium oxide. More preferably, the titanium oxide binding amino acid sequence binds to titanium oxide with at least three times the affinity than the biologically active amino acid sequence binds to titanium oxide. More preferably, the titanium oxide binding amino acid sequence binds to titanium oxide with at least four times the affinity than the biologically active amino acid sequence binds to titanium oxide. More preferably, the titanium oxide binding amino acid sequence binds to titanium oxide with at least five times the affinity than the biologically active amino acid sequence binds to titanium oxide. More preferably, the titanium oxide binding amino acid sequence binds to titanium oxide with at least ten times the affinity than the biologically active amino acid sequence binds to titanium oxide.

Selection of which titanium oxide binding amino acid sequence to pair with which biologically active amino acid sequence may be performed by testing the binding of each of the sequences individually to a titanium oxide surface. This may be effected by as indicated by performing dissolution tests (differences in pH ionic strength and hydrophobicity of eluting agents).

Care should also be taken when pairing the functional moieties that the biologically active amino acid sequence does not interfere with the binding of the titanium oxide binding amino acid sequence. Conversely, care should also be taken that the titanium oxide binding amino acid sequence does not interfere with the biological activity of the biologically active amino acid sequence.

Thus, for example a contemplated pair for a peptide of the present invention comprises pSer-Trp-pSer-Trp- (SEQ ID NO: 2) as the titanium oxide binding amino acid sequence and (Glu)<sub>5</sub> (SEQ ID NO: 7) as the biologically active (in this case osteoinducing) activity. Thus a contemplated peptide of the present invention is as set forth in Pro-pSer-Trp-pSer-Trp-(Gly)<sub>3</sub>-(Glu)<sub>5</sub> (SEQ ID NO: 1).

Another contemplated pair for a peptide of the present invention comprises pSer-Phe-pSer- (SEQ ID NO: 3) as the titanium oxide binding amino acid sequence and (Glu)<sub>3</sub> as the biologically active (in this case osteoinducing) activity. Thus another contemplated peptide of the present invention is as set forth in Pro-pSer-Phe-pSer-(Gly)<sub>2</sub>-(Glu)<sub>3</sub> (SEQ ID NO: 4).

Another contemplated pair for a peptide of the present invention comprises L-DOPA-X-L-DOPA-X- (SEQ ID NO: 5) as the titanium oxide binding amino acid sequence and (Glu)<sub>5</sub> (SEQ ID NO: 7) as the biologically active (in this case osteoinducing) activity, where X is any hydrophobic amino acid, as set forth herein above.

The two functional domains of the peptide of the present invention may be connected through any orientation. Thus, for example, the N terminus of the titanium oxide binding amino acid sequence may be connected to the C terminus of the biologically active amino acid sequence. Alternatively, the C terminus of the titanium oxide binding amino acid sequence may be connected to the N terminus of the biologically active amino acid sequence.

As mentioned the sequences of the peptides of this aspect of the present invention may comprise modifications. Such modifications include, but are not limited to N terminus modification, C terminus modification, polypeptide bond modification, including, but not limited to, CH<sub>2</sub>-NH, CH<sub>2</sub>-S, CH<sub>2</sub>-S=O, O=C-NH, CH<sub>2</sub>-O, CH<sub>2</sub>-CH<sub>2</sub>, S=C-NH, CH=CH or CF=CH, backbone modifications, and residue modification. Methods for preparing peptidomimetic compounds are well known in the art and are specified, for example, in Quantitative Drug Design, C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992), which is incorporated by reference as if fully set forth herein. Further details in this respect are provided hereinunder.

Polypeptide bonds (-CO-NH-) within the polypeptide may be substituted, for example, by N-methylated bonds (-N(CH<sub>3</sub>)-CO-), ester bonds (-C(R)H-C-O-O-C(R)-N-), ketomethylen bonds (-CO-CH<sub>2</sub>-),  $\alpha$ -aza bonds (-NH-N(R)-CO-), wherein R is any alkyl, e.g., methyl, carba bonds (-CH<sub>2</sub>-NH-), hydroxyethylene bonds (-CH(OH)-CH<sub>2</sub>-), thioamide bonds (-CS-NH-), olefinic double bonds (-CH=CH-), retro amide bonds (-NH-CO-), polypeptide derivatives (-N(R)-CH<sub>2</sub>-CO-), wherein R is the "normal" side chain, naturally presented on the carbon atom.

These modifications can occur at any of the bonds along the polypeptide chain and even at several (2-3) at the same time.

Natural aromatic amino acids, Trp, Tyr and Phe, may be substituted for synthetic non-natural acid such as Phenylglycine, TIC, naphthylelanine (Nol), ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr.

In addition to the above, the polypeptides of the present invention may also include one or more modified amino acids or one or more non-amino acid monomers (e.g. fatty acids, complex carbohydrates etc).

As used herein in the specification and in the claims section below the term "amino acid" or "amino acids" is understood to include the 20 naturally occurring amino acids; those amino acids often modified post-translationally *in vivo*, including, for example, hydroxyproline, phosphoserine and phosphothreonine; and other unusual amino acids including, but not limited to, 2-amino adipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine. Furthermore, the term "amino acid" includes both D- and L-amino acids (stereoisomers).

Tables 1 and 2 below list naturally occurring amino acids (Table 1) and non-conventional or modified amino acids (Table 2) which can be used with the present invention.

**Table 1**

Amino Acid	Three-Letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
glycine	Gly	G
Histidine	His	H
isoleucine	Ile	I
leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
Valine	Val	V
Any amino acid as above	Xaa	X

5

**Table 2**

Non-conventional amino acid	Code	Non-conventional amino acid	Code
$\alpha$ -aminobutyric acid	Abu	L-N-methylalanine	Nmala
$\alpha$ -amino- $\alpha$ -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
aminoisobutyric acid	Aib	L-N-methylaspartic acid	Nmasp
aminonorbornyl-carboxylate	Norb	L-N-methylcysteine	Nmcys
cyclohexylalanine	Chexa	L-N-methylglutamine	Nmgin
cyclopentylalanine	Cpen	L-N-methylglutamic acid	Nmglu
D-alanine	Dal	L-N-methylhistidine	Nmhis
D-arginine	Darg	L-N-methylisoleucine	Nmile
D-aspartic acid	Dasp	L-N-methylleucine	Nmleu
D-cysteine	Dcys	L-N-methyllysine	Nmlys
D-glutamine	Dgln	L-N-methylmethionine	Nmmet
D-glutamic acid	Dglu	L-N-methylnorleucine	Nmmle
D-histidine	Dhis	L-N-methylnorvaline	Nmnva
D-isoleucine	Dile	L-N-methylornithine	Nmorn
D-leucine	Dleu	L-N-methylphenylalanine	Nmphe
D-lysine	Dlys	L-N-methylproline	Nmpro
D-methionine	Dmet	L-N-methylserine	Nmser
D-ornithine	Dorn	L-N-methylthreonine	Nmthr
		L-N-methyltryptophan	Nmtrp
		L-N-methyltyrosine	Nmtyr

D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	$\alpha$ -methyl-aminoisobutyrate	Maib
D-valine	Dval	$\alpha$ -methyl- $\gamma$ -aminobutyrate	Mgab
D- $\alpha$ -methylalanine	Dmala	$\alpha$ -ethylcyclohexylalanine	Mchexa
D- $\alpha$ -methylarginine	Dmarg	$\alpha$ -methylcyclopentylalanine	Mcpen
D- $\alpha$ -methylasparagine	Dmasn	$\alpha$ -methyl- $\alpha$ -naphthylalanine	Manap
D- $\alpha$ -methylaspartate	Dmasp	$\alpha$ -methylpenicillamine	Mpen
D- $\alpha$ -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D- $\alpha$ -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D- $\alpha$ -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D- $\alpha$ -methylisoleucine	Dmile	N-amino- $\alpha$ -methylbutyrate	Nmaabu
D- $\alpha$ -methylleucine	Dmleu	$\alpha$ -naphthylalanine	Anap
D- $\alpha$ -methyllysine	Dmlys	N-benzylglycine	Nphe
D- $\alpha$ -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D- $\alpha$ -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D- $\alpha$ -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D- $\alpha$ -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D- $\alpha$ -methylserine	Dmser	N-cyclobutylglycine	Ncbut
D- $\alpha$ -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D- $\alpha$ -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D- $\alpha$ -methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
D- $\alpha$ -methylvaline	Dmval	N-cyclododecylglycine	Ncdod
D- $\alpha$ -methylalnine	Dnmala	N-cyclooctylglycine	Ncoct
D- $\alpha$ -methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D- $\alpha$ -methylasparagine	Dnmas	N-cycloundecylglycine	Ncund
D- $\alpha$ -methylaspartate	Dnmas	N-(2,2-diphenylethyl)glycine	Nbhm
D- $\alpha$ -methylcysteine	Dnmcy	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl- $\gamma$ -aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmor	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nva
D-N-methyltyrosine	Dnmtyr	N-methyl- $\alpha$ -naphthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
$\gamma$ -aminobutyric acid	Gabu	N-(p-hydroxyphenyl)glycine	Nhtyr

L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L- $\alpha$ -methylalanine	Mala
L- $\alpha$ -methylarginine	Marg	L- $\alpha$ -methylasparagine	Masn
L- $\alpha$ -methylaspartate	Masp	L- $\alpha$ -methyl- <i>t</i> -butylglycine	Mtbug
L- $\alpha$ -methylcysteine	Mcys	L-methylethylglycine	Metg
L- $\alpha$ thylglutamine	Mgln	L- $\alpha$ -methylglutamate	Mglu
L- $\alpha$ -methylhistidine	Mhis	L- $\alpha$ -methylhomo phenylalanine	Mhphe
L- $\alpha$ -methylisoleucine	Mile	N-(2- methylthioethyl)glycine	Nmet
D-N-methylglutamine	Dnmgln	N-(3- guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolethyl)glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl- $\gamma$ -aminobutyrate	Nmgabu
N- methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmor	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N- methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1- methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2- methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyl- $\alpha$ -naphthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
$\gamma$ -aminobutyric acid	Gabu	N-( <i>p</i> -hydroxyphenyl)glycine	Nhtyr
L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L- $\alpha$ -methylalanine	Mala
L- $\alpha$ -methylarginine	Marg	L- $\alpha$ -methylasparagine	Masn
L- $\alpha$ -methylaspartate	Masp	L- $\alpha$ -methyl- <i>t</i> -butylglycine	Mtbug
L- $\alpha$ -methylcysteine	Mcys	L-methylethylglycine	Metg
L- $\alpha$ -methylglutamine	Mgln	L- $\alpha$ -methylglutamate	Mglu
L- $\alpha$ ethylhistidine	Mhis	L- $\alpha$ - methylhomophenylalanine	Mhphe
L- $\alpha$ thylisoleucine	Mile	N-(2- methylthioethyl)glycine	Nmet
L- $\alpha$ -methylleucine	Mleu	L- $\alpha$ -methyllysine	Mlys
L- $\alpha$ -methylmethionine	Mmet	L- $\alpha$ -methylnorleucine	Mnle
L- $\alpha$ -methylnorvaline	Mnva	L- $\alpha$ -methylornithine	Morn
L- $\alpha$ - methylphenylalanine	Mphe	L- $\alpha$ -methylproline	Mpro
L- $\alpha$ -methylserine	mser	L- $\alpha$ -methylthreonine	Mthr
L- $\alpha$ ethylvaline	Mtrp	L- $\alpha$ -methyltyrosine	Mtyr
L- $\alpha$ -methylleucine	Mval Nnbhm	L-N- methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbonylmethyl-glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbonylmethyl(1)glycine	Nnbhe

1-carboxy-1-(2,2-diphenyl ethylamino)cyclopropane	Nmbc		
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*Table 2 Cont.*

The amino acids of the peptides of the present invention may be substituted either conservatively or non-conservatively.

The term "conservative substitution" as used herein, refers to the replacement of an amino acid present in the native sequence in the peptide with a naturally or non-naturally occurring amino or a peptidomimetics having similar steric properties. Where the side-chain of the native amino acid to be replaced is either polar or hydrophobic, the conservative substitution should be with a naturally occurring amino acid, a non-naturally occurring amino acid or with a peptidomimetic moiety which is also polar or hydrophobic (in addition to having the same steric properties as the side-chain of the replaced amino acid).

As naturally occurring amino acids are typically grouped according to their properties, conservative substitutions by naturally occurring amino acids can be easily determined bearing in mind the fact that in accordance with the invention replacement of charged amino acids by sterically similar non-charged amino acids are considered as conservative substitutions.

For producing conservative substitutions by non-naturally occurring amino acids it is also possible to use amino acid analogs (synthetic amino acids) well known in the art. A peptidomimetic of the naturally occurring amino acid is well documented in the literature known to the skilled practitioner.

When affecting conservative substitutions the substituting amino acid should have the same or a similar functional group in the side chain as the original amino acid.

The phrase "non-conservative substitutions" as used herein refers to replacement of the amino acid as present in the parent sequence by another naturally or non-naturally occurring amino acid, having different electrochemical and/or steric properties. Thus, the side chain of the substituting amino acid can be significantly larger (or smaller) than the side chain of the native amino acid being substituted and/or can have functional groups with significantly different electronic properties than the amino acid being substituted. Examples of non-conservative substitutions of this type include the substitution of phenylalanine or cyclohexylmethyl glycine for alanine, isoleucine for



glycine, or  $-\text{NH}-\text{CH}[(\text{-CH}_2)_5\text{-COOH}]-\text{CO}-$  for aspartic acid. Those non-conservative substitutions which fall under the scope of the present invention are those which still constitute a peptide having anti-bacterial properties.

Thus, the present invention contemplates peptides having a sequence homology  
5 at least 80 % identical, more preferably at least 85 % identical, more preferably at least 90 % identical, more preferably at least 91 % identical, more preferably at least 92 % identical, more preferably at least 93 % identical, more preferably at least 94 % identical, more preferably at least 95 % identical, more preferably at least 96 % identical, more preferably at least 97 % identical, more preferably at least 98 %  
10 identical, more preferably at least 99 % identical to the sequences as set forth in SEQ ID NOs: 1-20 as determined using BlastP software of the National Center of Biotechnology Information (NCBI) using default parameters). In measuring homology between a peptide and a protein of greater size, homology is measured only in the corresponding region; that is, the protein is regarded as only having the same general length as the  
15 peptide, allowing for gaps and insertions.

The N and C termini of the peptides of the present invention may be protected by function groups. Suitable functional groups are described in Green and Wuts, "Protecting Groups in Organic Synthesis", John Wiley and Sons, Chapters 5 and 7, 1991, the teachings of which are incorporated herein by reference.

20 These moieties can be cleaved *in vivo*, either by hydrolysis or enzymatically, inside the cell. Hydroxyl protecting groups include esters, carbonates and carbamate protecting groups. Amine protecting groups include alkoxy and aryloxy carbonyl groups, as described above for N-terminal protecting groups. Carboxylic acid protecting groups include aliphatic, benzylic and aryl esters, as described above for C-terminal protecting  
25 groups. In one embodiment, the carboxylic acid group in the side chain of one or more glutamic acid or aspartic acid residue in a peptide of the present invention is protected, preferably with a methyl, ethyl, benzyl or substituted benzyl ester.

Examples of N-terminal protecting groups include acyl groups ( $-\text{CO}-\text{R}_1$ ) and alkoxy carbonyl or aryloxy carbonyl groups ( $-\text{CO}-\text{O}-\text{R}_1$ ), wherein  $\text{R}_1$  is an aliphatic,  
30 substituted aliphatic, benzyl, substituted benzyl, aromatic or a substituted aromatic group. Specific examples of acyl groups include acetyl, (ethyl)-CO-, n-propyl-CO-, iso-propyl-CO-, n-butyl-CO-, sec-butyl-CO-, t-butyl-CO-, hexyl, lauroyl, palmitoyl,

myristoyl, stearyl, oleoyl phenyl-CO-, substituted phenyl-CO-, benzyl-CO- and (substituted benzyl)-CO-. Examples of alkoxy carbonyl and aryloxy carbonyl groups include CH<sub>3</sub>-O-CO-, (ethyl)-O-CO-, n-propyl-O-CO-, iso-propyl-O-CO-, n-butyl-O-CO-, sec-butyl-O-CO-, t-butyl-O-CO-, phenyl-O-CO-, substituted phenyl-O-CO- and benzyl-O-CO-, (substituted benzyl)-O-CO-. Adamantan, naphthalen, myristoleyl, toluen, biphenyl, cinnamoyl, nitrobenzoy, toluoyl, furoyl, benzoyl, cyclohexane, norbornane, Z-caproic. In order to facilitate the N-acylation, one to four glycine residues can be present in the N-terminus of the molecule.

The carboxyl group at the C-terminus of the compound can be protected, for example, by an amide (i.e., the hydroxyl group at the C-terminus is replaced with -NH<sub>2</sub>, -NHR<sub>2</sub> and -NR<sub>2</sub>R<sub>3</sub>) or ester (i.e. the hydroxyl group at the C-terminus is replaced with -OR<sub>2</sub>). R<sub>2</sub> and R<sub>3</sub> are independently an aliphatic, substituted aliphatic, benzyl, substituted benzyl, aryl or a substituted aryl group. In addition, taken together with the nitrogen atom, R<sub>2</sub> and R<sub>3</sub> can form a C<sub>4</sub> to C<sub>8</sub> heterocyclic ring with from about 0-2 additional heteroatoms such as nitrogen, oxygen or sulfur. Examples of suitable heterocyclic rings include piperidinyl, pyrrolidinyl, morpholino, thiomorpholino or piperazinyl. Examples of C-terminal protecting groups include -NH<sub>2</sub>, -NHCH<sub>3</sub>, -N(CH<sub>3</sub>)<sub>2</sub>, -NH(ethyl), -N(ethyl)<sub>2</sub>, -N(methyl)(ethyl), -NH(benzyl), -N(C<sub>1</sub>-C<sub>4</sub> alkyl)(benzyl), -NH(phenyl), -N(C<sub>1</sub>-C<sub>4</sub> alkyl)(phenyl), -OCH<sub>3</sub>, -O-(ethyl), -O-(n-propyl), -O-(n-butyl), -O-(iso-propyl), -O-(sec-butyl), -O-(t-butyl), -O-benzyl and -O-phenyl.

The peptides of the present invention may also comprise non-amino acid moieties, such as for example, hydrophobic moieties (various linear, branched, cyclic, polycyclic or hetrocyclic hydrocarbons and hydrocarbon derivatives) attached to the peptides; non-peptide penetrating agents; various protecting groups, especially where the compound is linear, which are attached to the compound's terminals to decrease degradation. Chemical (non-amino acid) groups present in the compound may be included in order to improve various physiological properties such as decreased degradation or clearance; decreased repulsion by various cellular pumps, improve immunogenic activities, increased specificity, increased affinity, decreased toxicity and the like.

The peptides of the invention may be linear or cyclic (cyclization may improve stability, or uptake into the microbe). Cyclization may take place by any means known in

the art. Where the compound is composed predominantly of amino acids, cyclization may be via N- to C-terminal, N-terminal to side chain and N-terminal to backbone, C-terminal to side chain, C-terminal to backbone, side chain to backbone and side chain to side chain, as well as backbone to backbone cyclization. Cyclization of the peptide may also take  
5 place through non-amino acid organic moieties comprised in the peptide.

The peptides of the present invention can be biochemically synthesized such as by using standard solid phase techniques. These methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis. Solid phase polypeptide synthesis procedures are well known in the  
10 art and further described by John Morrow Stewart and Janis Dillaha Young, Solid Phase Polypeptide Syntheses (2nd Ed., Pierce Chemical Company, 1984).

Large scale peptide synthesis is described by Andersson Biopolymers 2000;55(3):227-50.

Synthetic peptides can be purified by preparative high performance liquid  
15 chromatography [Creighton T. (1983) Proteins, structures and molecular principles. WH Freeman and Co. N.Y.] and the composition of which can be confirmed via amino acid sequencing.

Recombinant techniques may also be used to generate the peptides of the present invention. To produce a peptide of the present invention using recombinant technology,  
20 a polynucleotide encoding the peptide of the present invention is ligated into a nucleic acid expression vector, which comprises the polynucleotide sequence under the transcriptional control of a cis-regulatory sequence (e.g., promoter sequence) suitable for directing constitutive, tissue specific or inducible transcription of the polypeptides of the present invention in the host cells.

25 In addition to being synthesizable in host cells, the peptides of the present invention can also be synthesized using *in vitro* expression systems. These methods are well known in the art and the components of the system are commercially available.

As mentioned, the peptides of this aspect of the invention may be useful for coating devices such as implants and surgical instruments.

30 According to one embodiment, the surface of the device is coated with titanium or zirconium oxide.

The device may comprise additional metals, metal oxides or other components such as ceramics, including but not limited to cobalt, cobalt alloys, chromium, chromium alloys, tantalum, tantalum alloys, stainless steel, zirconium, zirconium oxide, zirconium dioxide, magnesium oxide and calcium phosphate.

5           The peptides may be attached to the surface of the implant using any method known in the art including spraying, wetting, immersing, dipping, painting, ultrasonic welding, welding, bonding or adhering or otherwise providing a surface with the peptides of the present invention. Typically, the peptides of the present invention self-assemble on the surface.

10           The peptides of the present invention may be attached as monolayers or multiple layers.

          The amount of peptide applied to the surface can vary depending on the desired outcome. Thus, for example, the present invention contemplates adjusting the ability of a device to promote bone growth or osseointegration by varying the surface density of an osseointegrating peptide of the present invention on the device. Increasing the surface  
15           density of the peptide may increase the ability of the device to promote bone growth or osseointegration compared to devices having a lower surface density of peptide. Similarly, decreasing the surface density of the peptide on the device decreases the ability of the device to promote bone growth or osseointegration. By varying the surface  
20           density of the peptide, devices can be tailored to the needs of a specific patient to obtain the amount of bone growth or osseointegration needed by the patient.

          The peptide can be passively adsorbed to the device for example by incubating the implant in 0.4 mg/ml in Tris-buffered saline (TBS) for about 1 hour. Alternatively, the peptide can be suspended in a vehicle and applied to the device. Typically, the  
25           vehicle is a pharmaceutically acceptable vehicle that contains a binding agent, for example a gel or thickener that causes the peptide to adsorb or adhere to the surface of the device.

          It will be appreciated that the surface may be pre-treated prior to coating with the peptide of the present invention using any method known in the art in order to  
30           enhance peptide adsorption or for any other purpose.

An "implant" as used herein refers to any object intended for placement in a human body that is not a living tissue. The implant may be temporary or permanent. The implant is typically a device comprising artificial components.

Titanium implants may be used for a variety of purposes, listed herein below  
5 and the present invention contemplates coating these devices with the peptides of the present invention.

***Bone and Joint Replacement:*** About one million patients worldwide are treated annually for total replacement of arthritic hips and knee joints. The prostheses come in many shapes and sizes. Hip joints normally have a metallic femoral stem and head  
10 which locates into an ultrahigh molecular weight low friction polyethylene socket, both secured in position with polymethyl methacrylate bone cement. Some designs, including cementless joints, use roughened bioactive surfaces (including hydroxyapatite) to stimulate osseointegration, limit resorption and thus increase the implant lifetime for younger recipients. Internal and external bone-fracture fixation provides a further major  
15 application for titanium as spinal fusion devices, pins, bone-plates, screws, intramedullary nails, and external fixators.

According to the teachings of the present invention various types of bones can be formed and/or repaired using the presently described methods, these include without being limited to, ethmoid, frontal, nasal, occipital, parietal, temporal, mandible, maxilla,  
20 zygomatic, cervical vertebra, thoracic vertebra, lumbar vertebra, sacrum, rib, sternum, clavicle, scapula, carpal bones, ilium, ischium, pubis, patella, calcaneus, and tarsal bones. The present invention also contemplates generation of long bones (i.e. bones which are longer than they are wide and grow primarily by elongation of the diaphysis with an epiphysis at the ends of the growing bone). Examples of long bones include  
25 femur, tibia, fibula (i.e. leg bones), humerus, radius, ulna (i.e. arm bones), metacarpal, metatarsal (i.e. hand and feet bones), and the phalanges (i.e. bones of the fingers and toes).

Since the implants of the present invention may be used to generate tissue thereon, they may be used for treating diseases characterized by tissue damage or loss  
30 (e.g. bone or connective tissue loss).

As used herein, the term "treating" refers to inhibiting or arresting the development of a disease, disorder or condition and/or causing the reduction, remission,

or regression of a disease, disorder or condition in an individual suffering from, or diagnosed with, the disease, disorder or condition. Those of skill in the art will be aware of various methodologies and assays which can be used to assess the development of a disease, disorder or condition, and similarly, various methodologies and assays which can be used to assess the reduction, remission or regression of a disease, disorder or condition.

As used herein, the phrase “pathology characterized by bone or connective tissue damage or loss” refers to any disorder, disease or condition exhibiting a bone or connective tissue damage (*i.e.*, non-functioning tissue, cancerous or pre-cancerous tissue, broken tissue, fractured tissue, fibrotic tissue, or ischemic tissue) or a bone or connective tissue loss (e.g., following a trauma, an infectious disease, a genetic disease, and the like) which require tissue regeneration. Examples for disorders or conditions requiring bone or connective tissue regeneration include, but are not limited to, bone cancer, articular cartilage defects, musculoskeletal disorders, including degenerative disc disease and muscular dystrophy, osteoarthritis, osteoporosis, osteogenesis, Paget's disease, bone fractures, and the like.

As used herein, the term “subject” refers to mammals, including humans. Preferably, this term encompasses individuals who suffer from pathologies as described hereinabove.

Preferably the implant is implanted at a ligament, tendon, cartilage, intervertebral disc or bone tissue.

Thus for example, when administration of the implant is for bone regeneration, the scaffold is placed at a desired location in bone in such conditions such as non-union fractures, osteoporosis, of periodontal disease or defect, osteolytic bone disease, post-plastic surgery, post-orthopedic implantation, post neurosurgical surgery that involves calvaria bone removal, in alveolar bone augmentation procedures, for spine fusion and in vertebral fractures.

When the administration of the implant is for generation of tendon/ligament tissue, the implant is placed at a desired location in tendon/ligament following tissue tear due to trauma or inflammatory conditions.

When the administration of the implant is for generation of cartilage tissue, the scaffold is placed at a desired location in cartilage to treat defects due to Rheumatoid Arthritis, Osteoarthritis, trauma, cancer surgery or for cosmetic surgery.

When the administration of the implant is for generation of intervertebral disc tissues including nucleous pulposus and annulus fibrosus, the scaffold is placed at a  
5 desired location of nucleous pulposus degeneration, annulus fibrosus tears, or following nucleotomy or discectomy.

**Dental Implants:** A major change in restorative dental practice worldwide has been possible through the use of titanium implants. A titanium 'root' is introduced into  
10 the jaw bone with time subsequently allowed for osseointegration. The superstructure of the tooth is then built onto the implant to give an effective replacement. Dental implants can be used to treat or repair damaged or missing teeth, and facial bones. In certain embodiments, the dental implant can be entirely for aesthetic purposes. In addition, implants can be use as a filler to augment or form dental tissue as to support the function  
15 of natural tissues (such as teeth or bone) or artificial prosthesis.

**Maxillo and Cranio/facial treatments:** Surgery to repair facial damage using the patients own tissue cannot always obtain the desired results. Artificial parts may be required to restore the ability to speak or eat as well as for cosmetic appearance, to  
20 replace facial features lost through damage or disease. Osseointegrated titanium implants meeting all the requirements of bio-compatibility and strength have made possible unprecedented advances in surgery, for the successful treatment of patients with large defects and hitherto highly problematic conditions.

**Cardiovascular devices** Titanium is regularly used for pacemaker cases and defibrillators, as the carrier structure for replacement heart valves, and for intra-vascular  
25 stents. External Protheses Titanium is suitable for both temporary and long term external fixations and devices as well as for orthotic calipers and artificial limbs, both of which use titanium extensively for its light weight, toughness and corrosion resistance. Thus, for example, the present invention therefore envisions coating vascular stents with the peptides of the present invention. The peptides may serve to repel or attract specific  
30 type of proteins in cells which may affect the cell cycle of endothelial cells in contact with the surface to reduce or prevent restenosis, or general type of implants coated by

the method of the present invention to achieve beneficial effect in the integration of the implant with tissue.

***Surgical Instruments:*** A wide range of surgical instruments are made in titanium. The metal's lightness is a positive aid to reducing any fatigue of the surgeon. Instruments are frequently anodised to provide a non reflecting surface, essential in microsurgical operations, for example in eye surgery. Titanium instruments withstand repeat sterilization without compromise to edge or surface quality, corrosion resistance or strength. Titanium is non magnetic, and there is therefore no threat of damage to small and sensitive implanted electronic devices.

10 As used herein the term "about" refers to  $\pm 10\%$ .

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of" means "including and limited to".

15 The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

20 As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

25 Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies  
30 regardless of the breadth of the range.



As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

### EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed.

(1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected  
5 Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide  
10 Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR  
15 Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are  
20 provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

## EXAMPLE 1

### MATERIALS AND METHODS

25 **Materials:** TiO<sub>2</sub> particles (10 μm, Sachtleben, Duisburg, Germany). Analytical-grade sodium acetate, Trizma base (Tris) and sodium hydroxide (Sigma, St. Louis, MO), sodium chloride, sodium phosphate and hydrochloric acid (Merck, Darmstadt, Germany), and HPLC-grade acetonitrile (Sigma, St. Louis, MO) were used for the chromatographic adsorption and desorption solutions. Water was purified by the Milli-Q  
30 system (Millipore, Bedford, MA) to 18.2 MΩ3 cm.

**Chromatography on TiO<sub>2</sub> surfaces:** as described in Golan Gertler, Gideon Fleminger and Hanna Rapaport. Langmuir 2010, 26(9), 6457–6463, and further

illustrated in Figure 3. Stainless steel (4.6\_50mm) column was packed with TiO<sub>2</sub> anatase (Sachtapore NP 10 μ/100A °, particle size/pore size respectively) suspended in methanol. The packed columns were washed consecutively with water and 0.1 M NaOH to desorb surface contaminants. The columns were then pre-equilibrated with the running buffer. Lyophilized peptides were dissolved in the running buffer (0.2mg/mL) and centrifuged at 5000 g for 5 min prior to injection into the column to remove possible aggregates. The supernatants (100 μL) were injected on to the TiO<sub>2</sub> column at a flow rate of 0.02mL/min to attain 40 min retention time for adsorption. After 65 minutes, the flow rate was increased to 0.5mL/min. Ten millimolar Tris and phosphate buffer solutions, pH 7.4, were used as adsorption (running) media. In addition, these buffers complemented by 150 mM NaCl (TBS, PBS, respectively) were also examined. Elution was carried out by gradients of the following solutions applied consecutively to the column: 1 M NaCl, 80 % acetonitrile, 200 mM sodium phosphate, and 0.1 M NaOH, monitored at 215-280 nm. The chromatograms were obtained at room temperature using 0.2 μm filtered (Millipore, Badford, MA) solutions.

Revised simulated body fluids (SBF) solution was prepared according to Oyane *et. al.*, with the following ion concentrations: Na<sup>+</sup> 142 mM, K<sup>+</sup> 5 mM, Mg<sup>2+</sup> 1.5 mM, Ca<sup>2+</sup> 2.5mM, Cl<sup>-</sup> 103 mM, HCO<sub>3</sub><sup>-</sup> 27 mM, HPO<sub>4</sub><sup>2-</sup> 1 mM SO<sub>4</sub><sup>2-</sup> 0.5 mM.

**TiO<sub>2</sub> surface preparation:** as described in Catherine D.Reyes, Timothy A.Petrie, Kellie L.Burns, Zvi Schwarts and Andres J.Garcia. (2007) *Biomaterials* 28(21): 3228-323.

Surfaces were prepared by Thermal Evaporation- microscope slides were coated with pure 99.9% Ti particles to 40 nm thickness. Oxide layer was promoted by heating the slides for 8 hours on 180 °C.

**Peptide binding to TiO<sub>2</sub> surfaces:** as set forth in Figure 2.

**Peptide:** The peptide Pro-pSer-Trp-pSer-Trp-(Gly)<sub>3</sub>-(Glu)<sub>5</sub> (SEQ ID NO: 1), also referred to as PpSer was synthesized using common automated peptide synthesis techniques. The β-strand peptides were obtained as lyophilized powder at >95% purity level (determined by reversed phase HPLC). The acidic peptides were solubilized using 10mM NaOH solution followed by bath sonication and centrifugation at 5000g for 8 minutes to remove possible aggregates.

A schematic illustration of the peptide is presented in Figure 5.

**RESULTS**

The peptide dissolved in two different running buffer, Tris buffer saline (TBS) and sodium bicarbonate saline (BCS). The affinity of functional groups to TiO<sub>2</sub> from previous works was known to be PO<sub>4</sub><sup>-3</sup> > HCO<sub>3</sub><sup>-</sup> > COO<sup>-</sup>. (The diagonal line in the chromatography is the elution gradient: 0-200mM sodium bicarbonate & 0-0.1M NaOH).

Peptides that bind to TiO<sub>2</sub> only through Glutamic acid functional groups will elute in BC gradient, however peptides that bind to TiO<sub>2</sub> through pSer functional groups will elute in the NaOH gradient.

As illustrated in Figure 7A, the peptide bonded to TiO<sub>2</sub> through pSer functional groups in TBS buffer. The peptide is most likely bound to the surface through phosphate groups.

The idea behind using BCS as running buffer is to block the surface with carbonate ions before the peptide injection. This way the phosphate groups, that have a higher affinity to the surface, can displace the carbonate ion and bond to the TiO<sub>2</sub> surface while the Glu tail stays free- as can be seen from the chromatography results presented in Figure 7B, 2/3 of the peptide was able to displace the carbonate ions. 1/3 of the peptide can't displace the carbonate ions and bonded to them through hydrogen bonds therefore they eluted at BC gradient.

***XPS analysis of peptide on TiO<sub>2</sub> (early stages of mineralization):***

The results are presented in Figures 8A-B and summarized in Table 3, herein below. Shown are the XPS atomic ratios of TiO<sub>2</sub>, peptide diluted in TBS bonded on TiO<sub>2</sub>, peptide diluted in BCS bonded on TiO<sub>2</sub> and all the slides after 40 minutes in SBF1.5 (c).

**Table 3**

Atomic Ratio	TiO <sub>2</sub> *	TBS+ P <sub>pSer</sub> *	BCS+ P <sub>pSer</sub> *	TiO <sub>2</sub> SBF **	TBS+ P <sub>pSer</sub> SBF **	BCS+ P <sub>pSer</sub> SBF **	SBF Results
Ca/P	-----	-----	-----	1.72	0.835	1.80	Adsorption kinetics of Ca <sup>+2</sup> to carbonate ions and to TiO <sub>2</sub> surface through electrostatic interactions is faster than the
Ti/Ca	-----	-----	-----	5.28	28.42	9.67	

							mineralization process
Ti/P	----	23.67	58.52	9.10	23.74	17.45	The P concentration in TBS buffer belongs to the peptide
C/P	-----	<b>29.95</b>	181.28	45.43	36.84	132.06	C concentration increase thanks to SBF solution.

At pH=7.4 TiO<sub>2</sub> surface is negatively charge

\* The peptide dissolved in two different running buffers, TBS and BCS, and bonded to TiO<sub>2</sub> slides. The carbonate-phosphate (C/P) ratio in TBS buffer is the indication of a bound peptide on the surface- the peptide contains 64 C and 2 P – 31:1. In BCS this ratio is higher because C concentration increases thanks to carbonate ions.

\*\* The TiO<sub>2</sub> + peptide complex after being 40 min in SBF (simulated body fluids).

**TBS:** From Ti/P ratio it can be seen that the P belongs to the peptide.

From Ca/P ratio it can be seen that the area on the surface that didn't bind peptide is negatively charge, therefore there is Ca<sup>+2</sup> adsorption to the surface.

C/P ratio is bigger because C concentration increases thanks to SBF solution.

**BCS:** Ti/P ratio becomes smaller because the P concentration is increases thanks to the mineralization process that started.

Ca/P ratio indicates that the Ca<sup>+2</sup> ions adsorb immediately both to TiO<sub>2</sub> surface and to the peptide coated surfaces. Differences point to peptide effect on calcium adsorption; for example Ca/P ratio in sample prepared in TBS buffer is close to 1 as expected by the interactions between 5 carboxyls that would be neutralized on average by 2.5 calcium ions versus the number of phosphate ions in the peptide (2).

***Inductively coupled plasma mass spectrometry (ICP-MS) results***

The results are illustrated in Figure 9 and Table 4 herein below.

***Table 4***

ratio	TBS	CBS	Ti
Ca/P	1.38	1.46	1.38

It can be seen from the ICP results, that surface+ peptide TBS has lower amounts of Ca,P than surface + peptide BCS. Therefore the carbonate ions affects the peptide and from this it can be concluded that the Glu tail can change its position as a response to the surrounding- a proof that the peptide is bi-functional.

***Chromatographic analysis of PF1, PF2, PF4, PF6 and PF8 on TiO<sub>2</sub> anatase column***

The following peptides were analyzed chromatographically on a TiO<sub>2</sub> anatase column - Pro-Asp-(Phe-Asp)<sub>n</sub> n = 1, 2, 4, 6 and 8, (SEQ ID NO: 20) denoted P<sub>F1</sub>, P<sub>F2</sub>, P<sub>F4</sub>, P<sub>F6</sub> and P<sub>F8</sub>. These peptides are known to have a B sheet structure.

**RESULTS**

The results are presented in Figures 10A-B. The results illustrate the effect of peptide length on binding efficiency for peptides designed to assume the beta-sheet structure - thus demonstrating that there is a link between the designed sequence and the efficiency of binding.

**EXAMPLE 2****MATERIALS AND METHODS*****Peptides:***

The peptides Pro-Ser(phos)-Phe-Ser(phos)-Trp-(Gly)<sub>3</sub>-(Glu)<sub>5</sub> denoted 'pSer' (SEQ ID NO: 12) and Pro-pSer-Phe-pSer-Trp-(Gly)<sub>3</sub> denoted 'cpSer' (SEQ ID NO: 13) was synthesized and purified by high performance liquid chromatography (HPLC) to >95% (Cambridge Research Biochemical). The peptide Pro-Ser(phos)-Ser(phos)-Phe-Trp-(Gly)<sub>3</sub> denoted 'β-cpSer', (SEQ ID NO: 14) was synthesized and purified by HPLC to >95% (Caslo). TiO<sub>2</sub> particles (10 μm, Sachtleben, Duisburg, Germany) had a surface area of 62.4 m<sup>2</sup>g<sup>-1</sup>. Unless otherwise specified, all reagents were purchased from Sigma-Aldrich (Rehovot, Israel) and were of the highest available purity. Sodium hydroxide, sodium bicarbonate, potassium phosphate and calcium chloride dehydrate. Tris-buffer saline (TBS) was prepared with Trizma<sup>TM</sup> base (10 mM) and NaCl (154 mM) adjusted to pH 7.50 with 0.1 M HCl. All solutions were prepared with deionized water (DIW) (18.2 MΩ x cm, Direct Q-5 Merck Millipore, Billerica, MA).

***Circular Dichroism (CD) Spectroscopy:***

Circular dichroism (CD) measurements were performed to characterize the secondary structure of peptides in aqueous solution. Spectra in the range of 190–260 nm were recorded at room temperature on a Jasco J-715 spectropolarimeter (Tokyo, Japan), using a 1 mm quartz cuvette. Peptides solutions were prepared by dissolving peptide's lyophilized powders in TBS to concentrations of 0.2 and 0.4 mg ml<sup>-1</sup>. Data are reported as mean molar ellipticity per residue [deg cm<sup>2</sup> dmol<sup>-1</sup>residue<sup>-1</sup>].

***Attenuated total reflection (ATR-FTIR) spectroscopy***

Samples of 1 mg ml<sup>-1</sup> peptides were prepared by dissolving peptide powder into TBS and were deposited on ZnSe 45° trapezoid prism (REFLEX Analytical Corporation) and dried at 37 °C to form a film that is macroscopically thick. The FTIR spectra reported here were all recorded using a Nicolet 6700 FTIR spectrometer (Thermo Scientific) fitted with a narrow-band liquid nitrogen cooled MCT detector. The spectra were recorded in the range from 4000 to 600 cm<sup>-1</sup>, 4-1 resolution and 244 scans. The FTIR data were collected using the OMNIC software. After collection the spectra were corrected for distortion using atmospheric suppression (to minimize infrared absorption by CO<sub>2</sub> and water vapor in the ambient air). A baseline correction function was applied to all spectra. Reference spectra were measured using the bare ZnSe prism.

***Chromatography:***

A stainless steel (4.6x50 mm) column was packed with TiO<sub>2</sub> anatase (Sachtopore NP 10 μ/100 Å, particle size/pore size respectively) suspended in methanol. The packed column was washed consecutively with water and 0.1 M NaOH to desorb surface contaminants. The column was then pre-equilibrated with the loading / running buffer. The chromatographic process was performed on a HPLC system (Agilent Technologies, 1260 Infinity, Germany). Peptides dissolved in the loading/running buffer (0.4 mg/ml) were sonicated for 10 minutes and centrifuged at 145000 rpm for 8 minutes and were injected to the TiO<sub>2</sub> column at a flow rate of 0.02 ml/min to attain ~40 minutes retention time for adsorption. 10mM tris buffered saline pH 7.4 (TBS) was used as adsorption/running media. After 65 minutes, the flow rate was increased to 0.5 ml/min. Elution was carried out by gradients of 200 mM sodium bicarbonate solution pH 7.4 and 0.1 M NaOH. Elution was monitored at 280nm. All solutions were filtered using 0.2 μm filters (Millipore, Badford, MA).

***Adsorption Isotherm:***

The adsorption of all three peptides to TiO<sub>2</sub> particles were measured with peptides solution in a range of concentrations from 0.002 to 0.524 mM in 10 mM TBS solution. TiO<sub>2</sub> particles were suspended in TBS 10 mM, pH 7.4, to a final concentration of 1.8 mg/ml. To each test tube containing 0.5 ml of peptide at different concentrations, 0.5 ml of TiO<sub>2</sub> particles solution was added. The samples were continuously mixed with

a tube rotator for 1 h at room temperature. To precipitate the TiO<sub>2</sub> particles, the test tubes were centrifuged at 5000 rpm for 3 minutes and the absorption of the supernatant was measured at 280 nm using a microplate-reader (BioTek instruments). Peptide concentration was determined based on calibration curve of each peptide prepared in TBS solution. In order to describe the adsorption of molecules on TiO<sub>2</sub> particles surface the sips equation, also known as the Langmuir- Freundlich equation (1), was used:

$$(1) \quad S = \frac{S_{\max} (kC)^{\alpha}}{1 + (kC)^{\alpha}}$$

where S is the amount of adsorbed molecules (mol m<sup>-2</sup>), S<sub>max</sub> is the maximum amount of molecules adsorbed (mol m<sup>-2</sup>), C is their concentration in solution at equilibrium (mM), k is the association coefficient (M<sup>-1</sup>) and α is the constant representing this distribution.<sup>30</sup>

#### ***Preparation of TiO<sub>2</sub> surfaces***

Titanium coated silicon wafers and Borosilicate glass slides (Pgo, Germany) were prepared in the nanofabrication facility of BGU. The titanium (99.995%, Kurt J. Lesker) was evaporated thermally at a pressure equal to 2.8\*10<sup>-7</sup> mbar using a thermal evaporator (Odem LTD, Rehovot, Israel) to thickness of 40 nm. Prior to coating, the substrates were cleaned in acetone and isopropanol. Next, these were subjected to oxygen plasma cleaning for 7 minutes (0.4 mbar of oxygen pressure in chamber). After the coating process, all surfaces were placed on a heating plate at 180 °C for 8 hours<sup>31</sup>. Silicon coated surfaces were cut to 1 X 0.5cm<sup>2</sup> using ADT7100 icing saw. The surfaces were characterized for titanium dioxide (TiO<sub>2</sub>) by XPS. XPS spectra were measured using ESCALAB 250 spectrometer with Al X-ray source and monochromator. General survey and high-resolution spectra of elements were recorded. Calibration of the peaks position was performed according to the position of the C1s line (284.8 eV). The Ar-etching was done under the pressure of 1x10<sup>-8</sup> mbar using the ion source with 3μA 2kV power and the rate of sputtering was 6A/sec.

#### ***TiO<sub>2</sub> surface coating:***

Surfaces were incubated in TBS solution for 45 min in room temperature, and then the surfaces were incubated in 0.4 mg ml<sup>-1</sup> peptide -TBS 10 mM solution for 1 hr at



room temperature and were dried with nitrogen. Peptide coating were analyzed by XPS.

***Calcium-phosphate adsorption on coated surfaces:***

The ability to absorb calcium-phosphate (Ca-P) ions by the different peptide coatings were measured using Ca-P solution. The solution was prepared at physiological pH and contained  $K_2HPO_4$  (1.0 mM),  $CaCl_2 \cdot 2H_2O$  (2.5 mM), dissolved consecutively in deionized water at 37 °C and buffered at pH ~7.4 using 50 mM Trizma™ base and an appropriate volume of 1M HCl. Coated surfaces were incubated in Ca-P solution at 37 °C for 24 h.

**RESULTS**

The designed bifunctional peptide, ‘pSer’ (Figure 11A) features a binding motif to titanium oxide and a stretch of negatively charged amino acids intended to induce adsorption of calcium to promote calcified mineralization. The titanium oxide binding motif was designed to induce the  $\beta$ -pleated structure with two phosphoserine amino acids decorating the hydrophilic face of this binding region. Trp and Phe residues were chosen to be the alternating hydrophobic amino acids to assist in peptide detection by spectroscopy. This binding motif segment is followed by a linker of three Gly residues and five consecutive negatively charged Glu amino acids. A peptide (Figure 11B) lacking the stretch of the Glu residues, denoted ‘cpSer’, was also studied as a control to assess the effect of the Glu tail on the structure of the peptide and on the calcium adsorption. A third peptide (Figure 11C) lacking the  $\beta$ -pleated structure at the binding motif denoted ‘ $\beta$ -cpSer’, was also studied as a control to the  $\beta$ -pleated structure on the affinity to titanium oxide.

CD measurements provided information on peptide conformation in the solution used for the spontaneous adsorption of the peptides to the titanium oxide. All peptides showed random coil conformation in 10mM TBS buffer at physiological pH over the 0.2-0.4 mg ml<sup>-1</sup> range of concentrations (Figure 12).

Peptides were deposited on ZnSe prism to form a film that is macroscopically thick (see Materials and Methods) and ATR-IR spectra were acquired. These measurements provide information on the conformation the peptides’ tend to assume on a surface by a simple drying process. All three peptides show an adsorption band at 1000-1100 cm<sup>-1</sup> that is associated with the phosphate groups of the phosphoserine amino

acids<sup>32,33</sup>. In addition, peptides show nicely the amide I, II and III peaks at ~1630, ~1540 and 1300 cm<sup>-1</sup> respectively<sup>34</sup>. The spectra show differences in both the amide I and II intensities and width which provide evidences for the differences in their conformations<sup>35</sup>. The three peptides show a peak at 1630 cm<sup>-1</sup> is indicative of peptides in  $\beta$ -sheet conformation. However, the amide I peak profile of pSer and cpSer that induce the  $\beta$ -pleated structure in the anchoring motif appears similar and both also show the amide I weak split at ~1692 cm<sup>-1</sup> which constitutes a strong evidence for the antiparallel  $\beta$ -sheet arrangement<sup>34,36-39</sup>. The amide I peak of peptide  $\beta$ -cpSer however shows a pronounced shoulder at ~1645 cm<sup>-1</sup> that corresponds to helical or random structures indicating that this peptide shows significantly lower propensity compared to the two other peptides, towards  $\beta$ -sheet structure in the dry state. The three spectra show a peak at ~1400 cm<sup>-1</sup> that corresponds to the carboxylate (COO-) symmetric stretching modes in pSer however this peak appears slightly stronger possibly due to the contribution of the Glu-tail motif. All three peptides show the amide II band at ~1545 cm<sup>-1</sup>. In the pSer spectra this peak appears to partially overlap with absorption at ~1520 cm<sup>-1</sup> that is characteristic of  $\beta$ -sheet structure<sup>40</sup>. This latter band that points to the abundance of  $\beta$ -sheet conformation within the film, is also present to a weaker extent in cpSer spectra and is almost absent in  $\beta$ -cpSer in accordance with the design of these peptide sequences. The relatively strong amide II band at ~1520 in pSer suggests that in the macroscopic film the Glu-tail motif is also packed in  $\beta$ -sheet structure. The differences in peptides conformation detected in the macroscopic film generated on the solid support highlight the strong tendency of pSer and cpSer to assume the  $\beta$ -pleated structure.

#### 25 *Adsorption to titanium oxide micron size particles:*

Peptide adsorption and desorption conditions were characterized by HPLC. A column was packed with micrometer size particles of the TiO<sub>2</sub> anatase phase (see Materials and Methods). Tris buffer saline (TBS), pH 7.4, served as a loading buffer. At this pH the oxide surface is negatively charged<sup>41</sup> hence ruling out the possibility of peptide adsorbing to the interface by electrostatic interactions. The salt ions expected to have an electrostatic repulsion screening effect and make negatively charge TiO<sub>2</sub> surface more accessible for the negatively charged peptides to generate coordinative

bonds that require short range interaction distance<sup>42</sup>. The elutions used conditions were consecutive linear gradients of sodium bicarbonate (0–200 mM, pH7.4) and NaOH (0–0.1M). The bicarbonate ions may bind by coordinative bonds to the oxide surface, similar to the way phosphate ions can be bound to the surface. Yet the binding strength of the bicarbonate ions to TiO<sub>2</sub> is weaker than that of phosphate ions<sup>43</sup>. The chromatograms of all peptides carried with sodium bicarbonate (BC) elution followed by the NaOH gradient (Figures 14A-B) showed a main peak during the NaOH gradient, at 85-95 mM. It was previously shown by the present inventors that carboxylic residues doesn't adsorb to TiO<sub>2</sub> under these conditions, therefore these results strongly suggest that all peptides become adsorbed by coordinative bonds to the surface through the phosphates groups of the phosphoserine. The tailing peaks shape of pSer and β-cpSer together with the small eluted volume during the void volume of the column probably indicates on impurity of the peptides' powders or secondary interactions of the peptides with the column. Those secondary interactions may occur as a result of different orientation of the phosphate groups at the TiO<sub>2</sub> binding surface. The minor differences at the elution times between the peptides shows that this method isn't sensitive enough to provide the information about the affinity of each peptide to TiO<sub>2</sub>. Therefore, adsorption isotherm to micro-size titanium oxide particles was performed in order to characterize the extent of interactions between the peptides and the oxide (Figures 15A-C). The data was fitted to Sips equation (eq.1). Sips isotherms is a combined form of Langmuir and Freundlich expressions and it relies on the assumption that there are a distribution of binding energies of the sites of a surface, this distribution is represented by  $\alpha$ . When  $\alpha \rightarrow 1$  Sips equation reduces to Langmuir equation and represents binding of a monolayer with homogenous binding energy and small  $\alpha$  values together with low concentrations reduces Sips equation to Freundlich equation with a potential to saturation at high concentrations (unlike the original Freundlich equation). Therefore, fitting peptides adsorption isotherm data to Sips equation give us an insight into their binding energy distributions. Fitting the collected data to Sips equation (continuous line at Figures 15A-C and Table 5 herein below) yields  $\alpha=0.8$  for pSer peptide and  $\alpha=0.4$  for control peptides, cpSer and β-cpSer. Table 5 contains the Sips equation values for the three peptides and the standard deviation values (SD).

The high  $\alpha$  value for pSer indicated that its adsorption resemble to Langmuir isotherm behavior, monolayer adsorption. However,  $\alpha$  value for cpSer and  $\beta$ -cpSer indicate a wider distribution of binding energies, resemble Freundlich isotherm behavior. Two additional values are obtained from the fitting, the maximum amount of molecules adsorbed to TiO<sub>2</sub> surface ( $S_{\max}$ ) and the association coefficient (k).  $S_{\max}$  values gives information about the nominal area these peptides occupy at the oxide surface. The  $S_{\max}$  values for pSer and cpSer are similar  $(3.33 \pm 0.03) \cdot 10^{-7} \text{ mol m}^{-2}$  and  $(3.93 \pm 0.52) \cdot 10^{-7} \text{ mol m}^{-2}$ , respectively. The inverse of those values provides the nominal area each peptide occupies, the result shows that pSer and cpSer occupy similar area,  $\sim 498 \text{ A}^2 \text{ molec}^{-1}$  and  $\sim 422 \text{ A}^2 \text{ molec}^{-1}$ , respectively. Overall, this result is strong evidence that pSer peptide adsorbing onto TiO<sub>2</sub> only through the binding motif and the Glu tail is free. The freedom of movement of the unbounded Glu tail may be the reason for pSer adsorption isotherm is resemble Langmuir monolayer adsorption. However, this value for  $\beta$ -cpSer is calculated to be  $\sim 221 \text{ A}^2 \text{ molec}^{-1}$ , twice smaller than the other peptides. The reason for that variance can be due to the different sequence of the binding motif, 'pSer- Phe -pSer-Trp' for pSer and cpSer versus 'pSer-pSer-Phe-Trp' for  $\beta$ -cpSer, therefore it can occupy smaller area, also can be seen at the illustration at Figure 11. The k values represent the affinity between peptides and TiO<sub>2</sub>, again, pSer and cpSer show resemblance in their results,  $(21.06 \pm 0.60) \cdot 10^3 \text{ M}^{-1}$  and  $(21.23 \pm 14.49) \cdot 10^3 \text{ M}^{-1}$ , respectively. However, the k value for  $\beta$ -cpSer is significantly lower,  $(3.76 \pm 2.15) \cdot 10^3 \text{ M}^{-1}$ , this result indicates that  $\beta$ -sheet structured binding motif has stronger binding affinity to TiO<sub>2</sub>.

*Table 5*

	<b>pSer</b>	<b>cpSer</b>	<b><math>\beta</math>-cpSer</b>
R <sup>2</sup>	1	0.99793	0.99953
$S_{\max}$ [mol·m <sup>-2</sup> ]	3.33E-7	3.93E-7	7.48E-7
SD	3.23E-9	5.24E-8	0.18E-8
K [M <sup>-1</sup> ]	21.06E3	21.29E3	3.76E3
SD	0.60E3	14.49E3	2.15E3

A	0.79	0.41	0.42
SD	0.01	0.04	0.02

### *Adsorption to titanium oxide surfaces*

Titanium surfaces were prepared by thermal evaporation coating of silicon wafers with 40 nm of pure titanium (see Materials and Methods) and characterized using X-ray photoelectron spectroscopy (XPS). XPS is a surface sensitive technique that detects presence of trace amount of elements on the surface. XPS measurements were performed on the TiO<sub>2</sub> surfaces in order to ensure that the oxidation phase is TiO<sub>2</sub>, a high resolution Ti2p spectrum identified three major peaks (Figure 16). Two of them refer to the state of titanium in the oxide; a peak at 458.3eV was attributed to Ti<sup>4+</sup> 2p<sub>3/2</sub> and a peak at 464eV to Ti<sup>4+</sup> 2p<sub>1/2</sub>. These peak positions consistent with those observed for TiO<sub>2</sub> reference spectra<sup>44,45</sup>. The third peak at 453.5eV was attributed to Ti<sup>0</sup> 2p<sub>3/2</sub> and refers to the state of titanium in the metal; the percentage of this peak was less than 2 % therefore a major area of the surface was in oxide state.

Peptide adsorption to TiO<sub>2</sub> surfaces was detected using XPS, Figure 17A shows the atomic concentration of phosphorous (the only source for it is from the phosphoserine amino acids) to that of titanium obtained by survey scans of peptide coated TiO<sub>2</sub> surfaces. The results show a similar peptide adsorption for all three peptides. This result is consistent with the adsorption isotherm found for pSer and cpSer peptides. However it contradicts the β-cpSer adsorption isotherm result. This phenomenon can be explained by the different surface topography and surface area between TiO<sub>2</sub> particles and TiO<sub>2</sub> surface, adsorption surface area at adsorption isotherm experiment is 1.123 m<sup>2</sup> and the surface area is 10<sup>-4</sup> m<sup>2</sup>. At small surface areas, the difference between peptides' adsorption cannot be observed. In order to examine the influence of the Glu tail on calcified mineralization process, titanium oxide surfaces were coated with pSer peptide or with the control peptides (cpSer or β-cpSer) and incubated for 24 hours inside calcium-phosphate solution (see Materials and Methods). Atomic concentrations of calcium and phosphorus were compared with titanium as measured by XPS. The results in Figure 17B shows that surfaces which were coated with pSer peptide adsorbed significantly higher calcium and phosphate concentration compared to surfaces that were coated with cpSer or β-cpSer peptides. It can be seen

that both of the control peptides adsorbed approximately the same calcium and phosphate concentrations due to the fact that both of them are lacking the Glu tail.

Using HPLC with TiO<sub>2</sub> microparticles column as described herein, it was also found that Trp-Glu (0.4 mg/ml) in TBS (10 mM, pH = 7.4) loading buffer does not adsorb to the oxide. Several solutions of sodium phenyl phosphate dibasic with different concentrations, dissolved in the same TBS, were allowed to adsorb to TiO<sub>2</sub> microparticles (1.8 mg/ml in 1 ml). Table 6, herein below lists the initial concentrations and the difference in concentration of the solution between the initial and the one following adsorption. According to Table 6, the phenyl phosphate adsorbs only at concentrations lower than 0.25 mg/ml probably because at higher concentrations the compound undergoes some aggregation and stays only in the solution. This adsorption behavior is unusual and therefore no binding affinity constants were determined.

**Table 6**

mg/ml in adsorbing solution	1	0.5	0.25	0.125	0.0625
Difference in concentration of the solution between the initial and the final one.	~0	~0	0.008776	0.02083	0.023648

## CONCLUSION

The three peptides were found to adsorb coordinatively to TiO<sub>2</sub> microparticles as evident by the high pH (~12.6) desorption conditions needed in the HPLC chromatograms to elute the peptides. Differences in peptide's affinity to the TiO<sub>2</sub> could not be determined based on the HPLC since the present inventors could not find an elution profile that would widen the range in which the peptides desorb, so to be able to detect with confidence these differences. Therefore adsorption isotherms were performed for each of the peptides. Robust differences were found between the peptides showing that the b-sheet binding motif exhibits higher binding constants compared to the random structure peptide. Based on similar area per molecule occupied by pSer and

cpSer it can be deduced that pSer is bound to the surface through the binding motif while its tail is pointing away from the surface. This result is also supported by the XPS measurements in which it was found that pSer adsorbs more Ca and P ions compared to the two other control peptides.

5           Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

10           All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or  
15           identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

## WHAT IS CLAIMED IS:

1. An isolated peptide comprising a titanium oxide binding amino acid sequence connected to a heterologous biologically active amino acid sequence via a beta sheet breaker linker, wherein:

(i) said titanium oxide binding amino acid sequence is selected to bind coordinatively with titanium oxide;

(ii) said titanium oxide binding amino acid sequence is selected to induce a beta sheet structure; and

(iii) said titanium oxide binding amino acid sequence binds to titanium oxide with a higher affinity than said biologically active amino acid sequence binds to said titanium oxide under physiological conditions.

2. The isolated peptide of claim 1, comprising naturally occurring amino acids.

3. The isolated peptide of claim 1, comprising at least one synthetic amino acid.

4. The isolated peptide of claim 1, comprising at least one D amino acid.

5. The isolated peptide of claim 1, wherein said titanium oxide binding amino acid sequence binds to titanium oxide with at least 2 fold higher affinity than said biologically active amino acid sequence.

6. The isolated peptide of claim 1, wherein said beta sheet breaker linker comprises naturally occurring amino acids.

7. The isolated peptide of claim 1, wherein said beta sheet breaker linker comprises synthetic amino acids.



8. The isolated peptide of claim 1, wherein said beta sheet breaker amino linker comprises 2-7 repeating glycine residues.

9. The isolated peptide of claim 8, wherein said beta sheet breaker amino acid linker is set forth by Gly-Gly-Gly.

10. The isolated peptide of claim 1, wherein said beta sheet breaker amino linker is a chemical linker.

11. The isolated peptide of claim 1, wherein said titanium oxide binding amino acid sequence comprises no more than 7 carboxyl amino acid residues.

12. The isolated peptide of claim 1, wherein said titanium oxide binding amino acid sequence comprises alternating hydrophilic, negatively charged amino acids and hydrophobic amino acids.

13. The isolated peptide of claim 12, wherein said alternating hydrophilic and hydrophobic amino acid sequence is not repeated more than 7 times.

14. The isolated peptide of claim 1, wherein at least one of the amino acids of said titanium oxide binding amino acid sequence is phosphorylated.

15. The isolated peptide of claim 1, wherein said titanium oxide binding amino acid sequence comprises the sequence Y-X-Y-X, wherein X is any hydrophobic amino acid and Y is selected from the group consisting of glutamic acid, aspartic acid, phosphoserine and L-DOPA.

16. The isolated peptide of claim 1, wherein said titanium oxide binding amino acid sequence comprises the sequence Y-X-Y, wherein X is any hydrophobic amino acid and Y is selected from the group consisting of glutamic acid, aspartic acid, phosphoserine and L-DOPA.

17. The isolated peptide of claim 1, wherein said titanium oxide binding amino acid sequence comprises the sequence Y-Y-X-X, wherein X is any hydrophobic amino acid and Y is selected from the group consisting of glutamic acid, aspartic acid, phosphoserine and L-DOPA.

18. The isolated peptide of any of claims 15-17, wherein X is selected from the group consisting of valine, phenylalanine, leucine, isoleucine and tryptophan.

19. The isolated peptide of any one of claims 15-17, wherein Y is phosphoserine (pSer).

20. The isolated peptide of claim 1, wherein said titanium oxide binding amino acid sequence comprises the sequence pSer-X-pSer-X (SEQ ID NO: 6), wherein X is any hydrophobic amino acid.

21. The isolated peptide of claim 20, wherein said titanium oxide binding amino acid sequence comprises the sequence as set forth in SEQ ID NO: 8 or SEQ ID NO: 2.

22. The isolated peptide of claim 1, wherein a C terminus of said titanium oxide binding amino acid sequence is attached to an N terminus of said biologically active amino acid sequence via said linker.

23. The isolated peptide of claim 1, wherein a N terminus of said titanium oxide binding amino acid sequence is attached to a C terminus of said biologically active amino acid sequence via said linker.

24. The isolated peptide of claim 1, wherein said biologically active amino acid sequence is selected from the group consisting of an osteoinductive or osteoconductive amino acid sequence, a mineralizing amino acid sequence, an anti-inflammatory amino acid sequence, a cell binding amino acid sequence, a cell migration amino acid sequence and an anti-bacterial amino acid sequence.

25. The isolated peptide of claim 24, wherein said osteoinductive or osteoconductive amino acid sequence comprises acidic amino acids.

26. The isolated peptide of claim 25, wherein said osteoinductive or osteoconductive amino acid sequence comprises at least 1 glutamic acid residue or aspartic acid residue.

27. The isolated peptide of claim 26, wherein said osteoinductive or osteoconductive amino acid sequence comprises 3-15 repeating glutamic acid residues or aspartic acid residues.

28. The isolated peptide of claim 26, wherein a sequence of said osteoinductive or osteoconductive amino acid sequence is set forth in SEQ ID NO: 7.

29. The isolated peptide of claim 24, wherein said osteoinductive or osteoconductive amino acid sequence is selected from the group consisting of a BMP amino acid sequence, an FGF amino acid sequence, a TGF amino acid sequence, an EGF amino acid sequence and a fibrinogen amino acid sequence.

30. The isolated peptide of claim 1, wherein said biologically active amino acid sequence is no longer than 50 amino acids.

31. The isolated peptide of claim 1, wherein the peptide is no longer than 50 amino acids.

32. The isolated peptide of claim 1, comprising the sequence as set forth in Z-X-Z-X-(Gly)<sub>3</sub>-(Y)<sub>5</sub> (SEQ ID NO: 9) where Z is L-dopa or phosphoserine, where X is any hydrophobic amino acid and Y is aspartic acid or glutamic acid.

33. The isolated peptide of claim 32, comprising the sequence as set forth in SEQ ID NO: 1 or SEQ ID NO: 12.

34. The isolated peptide of claim 16, comprising the sequence as set forth in SEQ ID NO: 3, 4 or 10.

35. The isolated peptide of claim 17, comprising the sequence as set forth in SEQ ID NO: 14 or 21.

36. The isolated peptide of claim 1, wherein when said titanium oxide binding amino acid sequence comprises the sequence as set forth by Pro-pSer-X-pSer-X (SEQ ID NO: 6), where X is a hydrophobic amino acid, said biologically active amino acid sequence is set forth by SEQ ID NO: 7.

37. The isolated peptide of claim 1, wherein said titanium oxide binding amino acid sequence comprises a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 21.

38. The isolated peptide of claim 1, wherein when said titanium oxide binding amino acid sequence comprises the sequence as set forth by L-DOPA-X-L-DOPA-X (SEQ ID NO: 5), where X is a hydrophobic amino acid, said biologically active amino acid sequence is set forth by SEQ ID NO: 7.

39. The isolated peptide of claim 1, wherein said titanium oxide binding amino acid sequence comprises the sequence P-Ser-Phe.

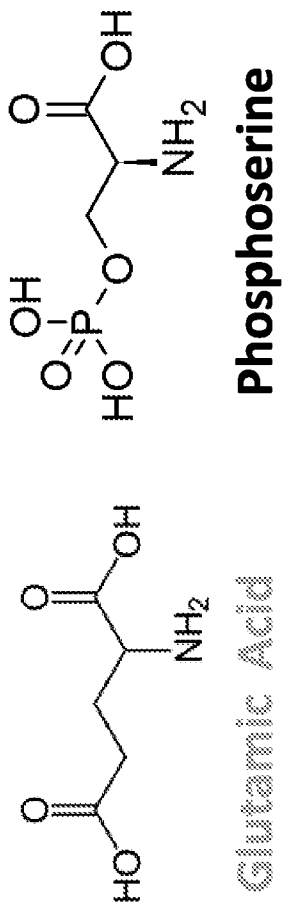
40. The isolated peptide of claim 39, wherein said peptide comprises the sequence selected from the group consisting of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18 and SEQ ID NO: 19.

41. An isolated polynucleotide encoding the isolated peptide of claim 2.

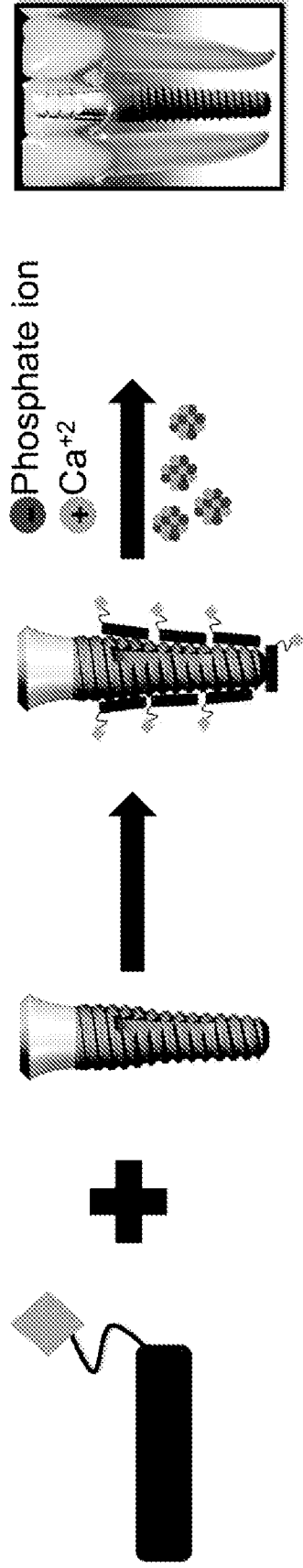
42. An article of manufacture comprising a titanium surface and the isolated peptide of claim 1 adsorbed to said surface.

43. The article of manufacture of claim 42, being an implant.
44. The article of manufacture of claim 43, being a dental implant.
45. The article of manufacture of claim 43, wherein the implant is selected from the group consisting of a hip prosthesis, a knee prosthesis, a heart valve and an intravascular stent.
46. A method of generating or repairing a tissue, the method comprising implanting the article of manufacture of claim 43 into a subject in need thereof, thereby generating or repairing the tissue.
47. The method of claim 46, wherein said tissue is selected from the group consisting of bone, cartilage and cardiac tissue.
48. The article of manufacture of claim 43, for treating a disorder selected from the group consisting of a bone disorder, a cartilage disorder, a cardiac disorder and a dental disorder.

**FIG. 1A**



**FIG. 1B**



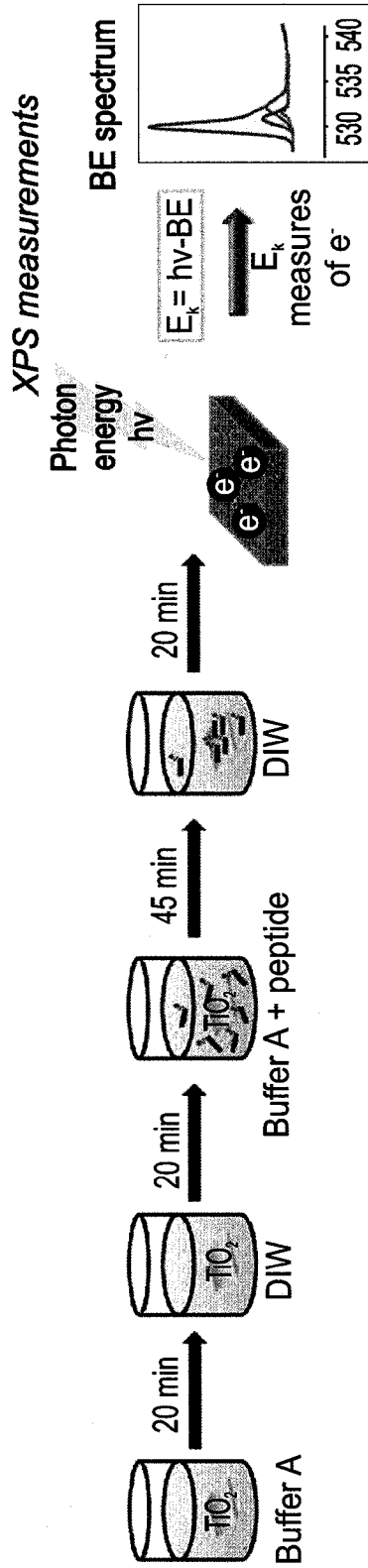


FIG. 2

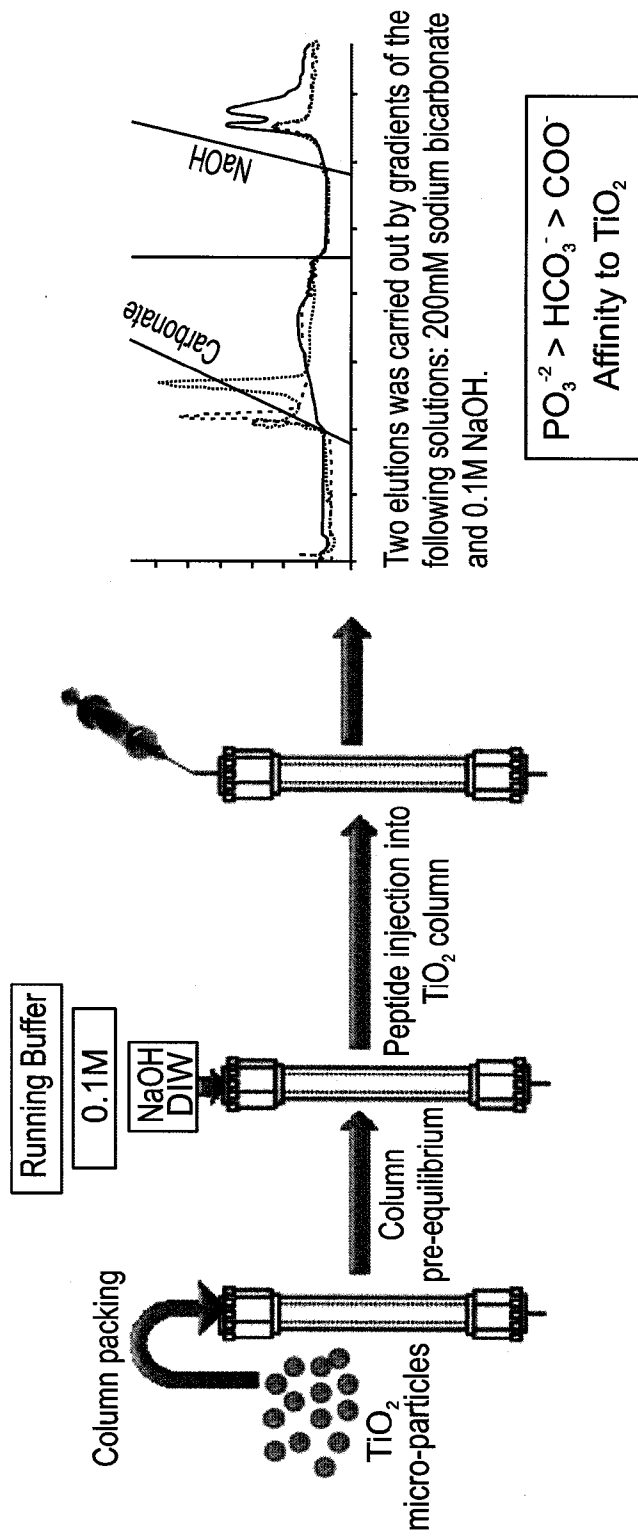
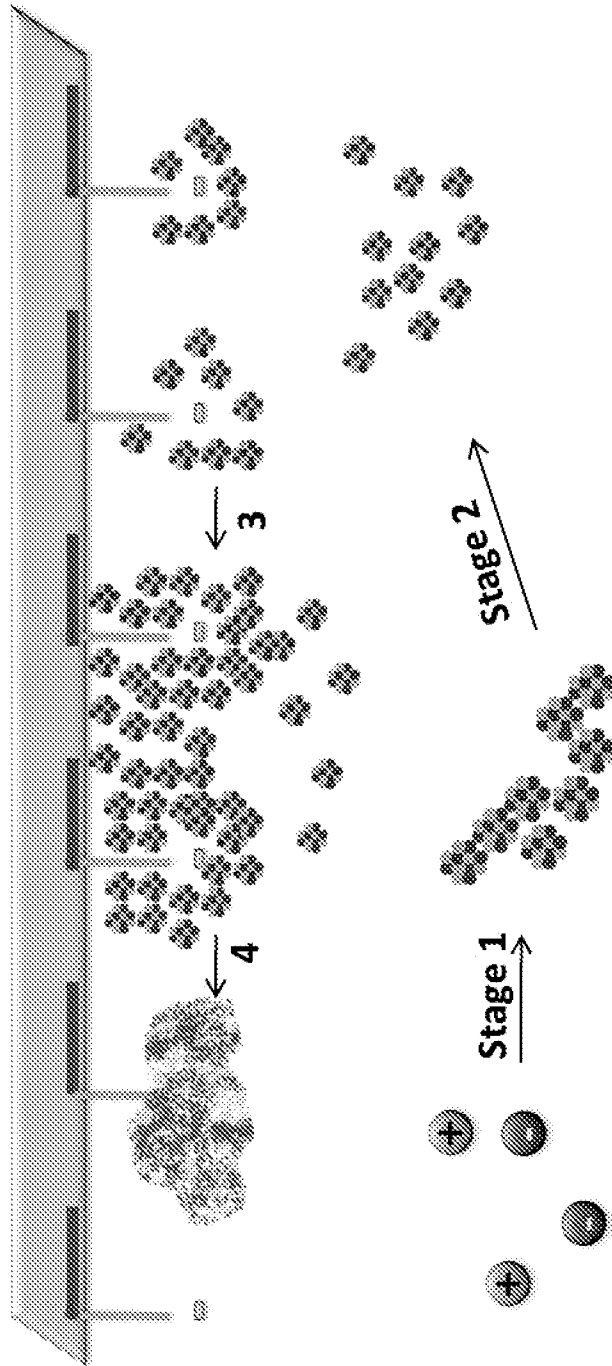


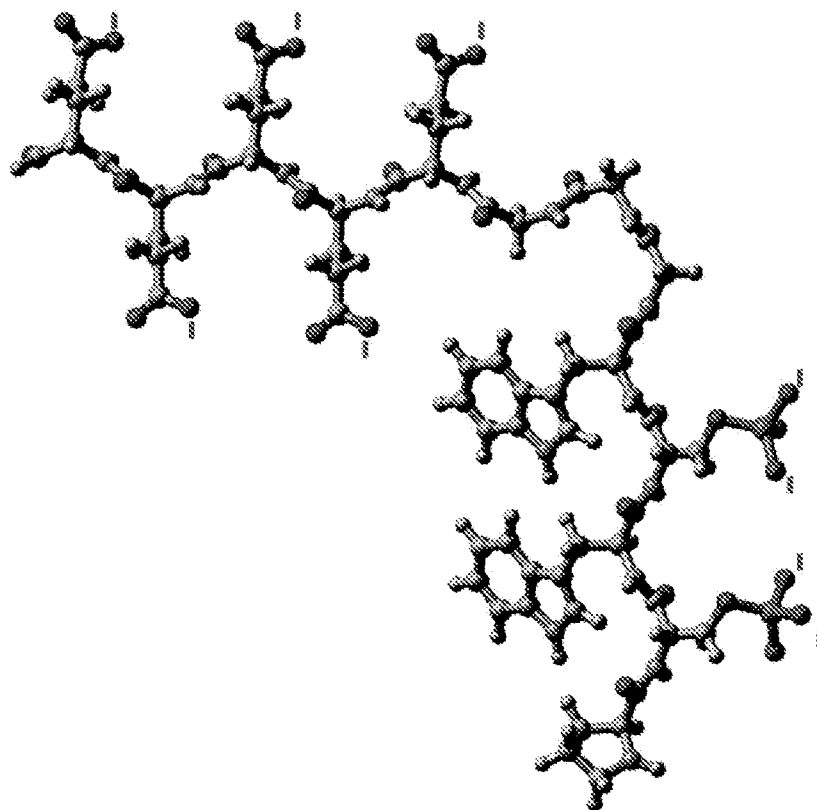
FIG. 3



FIG. 4



**FIG. 5**



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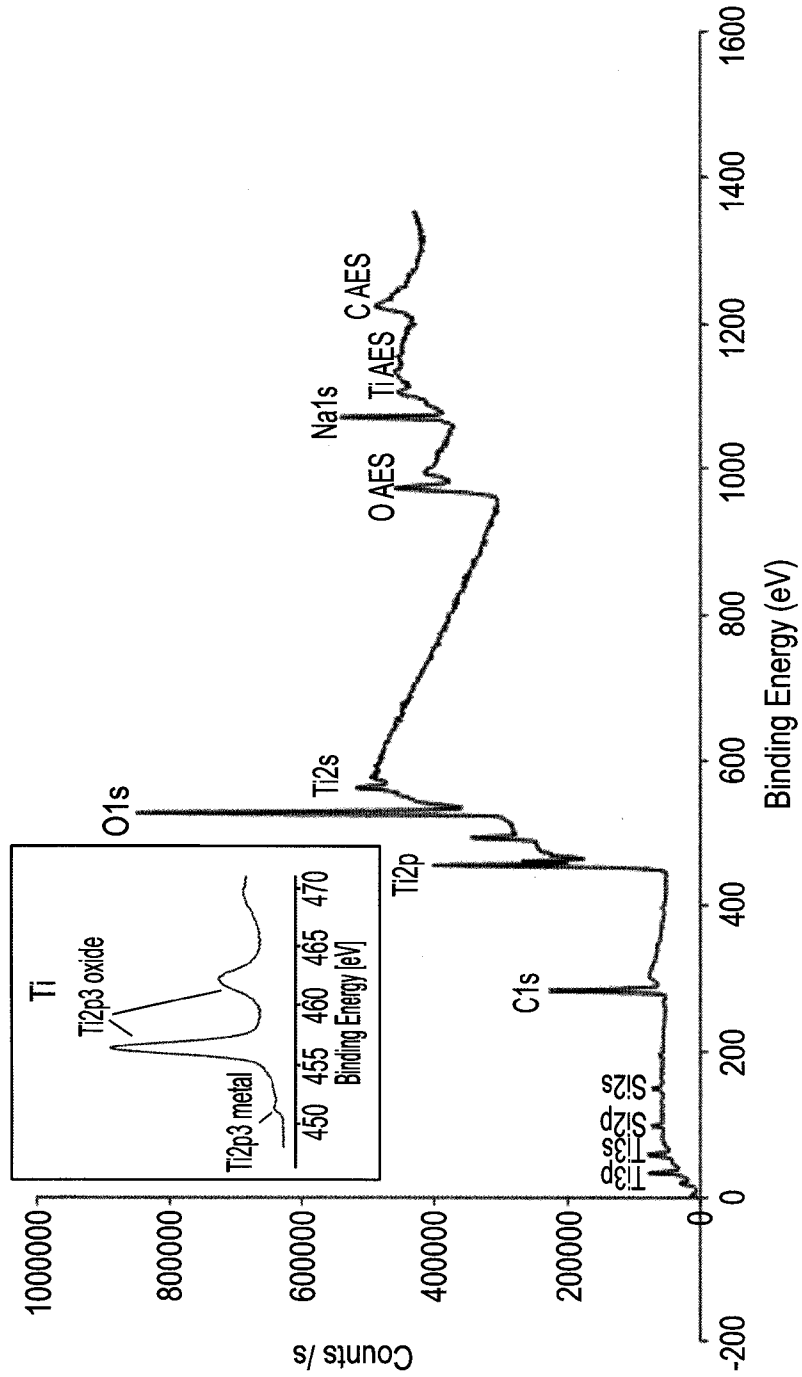


FIG. 6

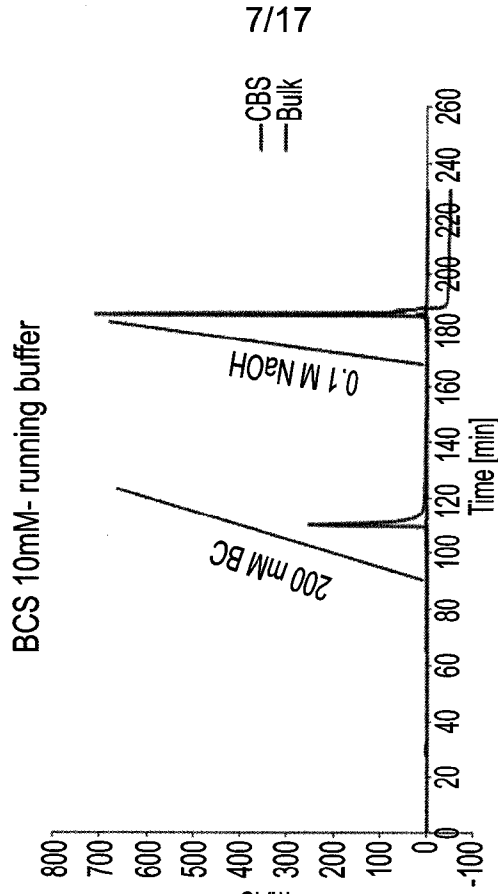


FIG. 7B

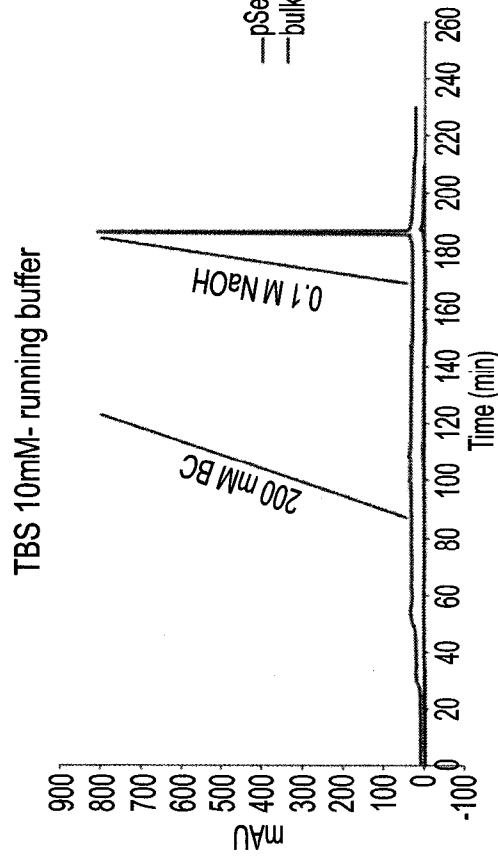


FIG. 7A

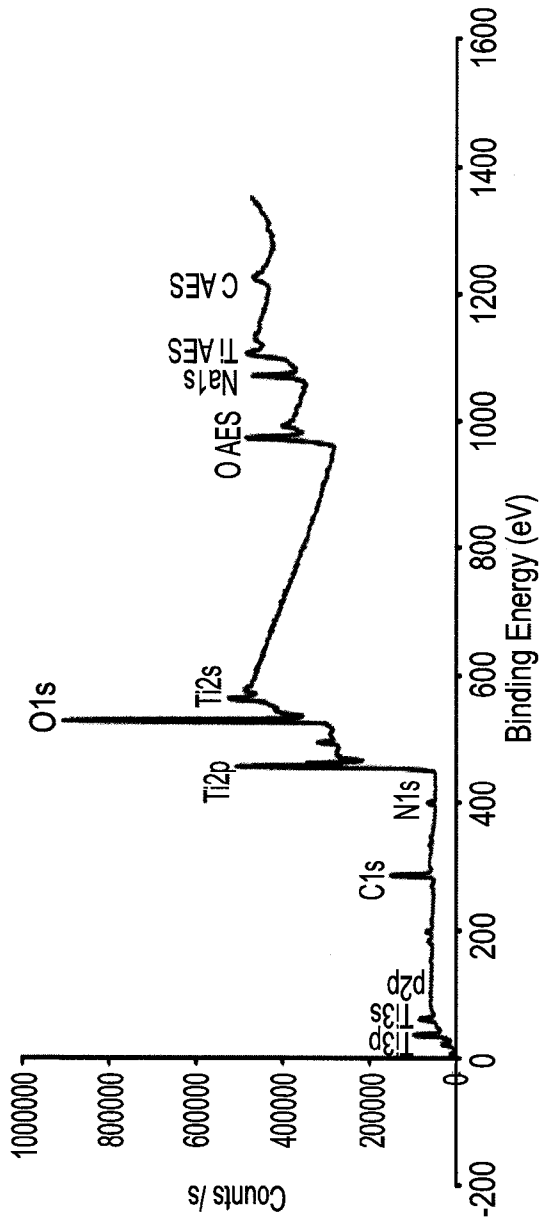


FIG. 8A

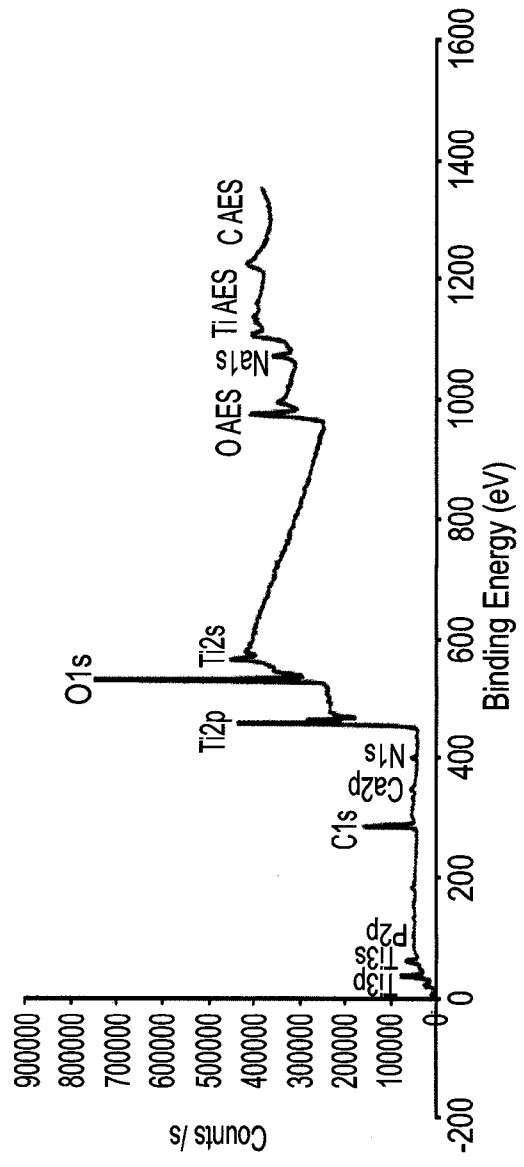
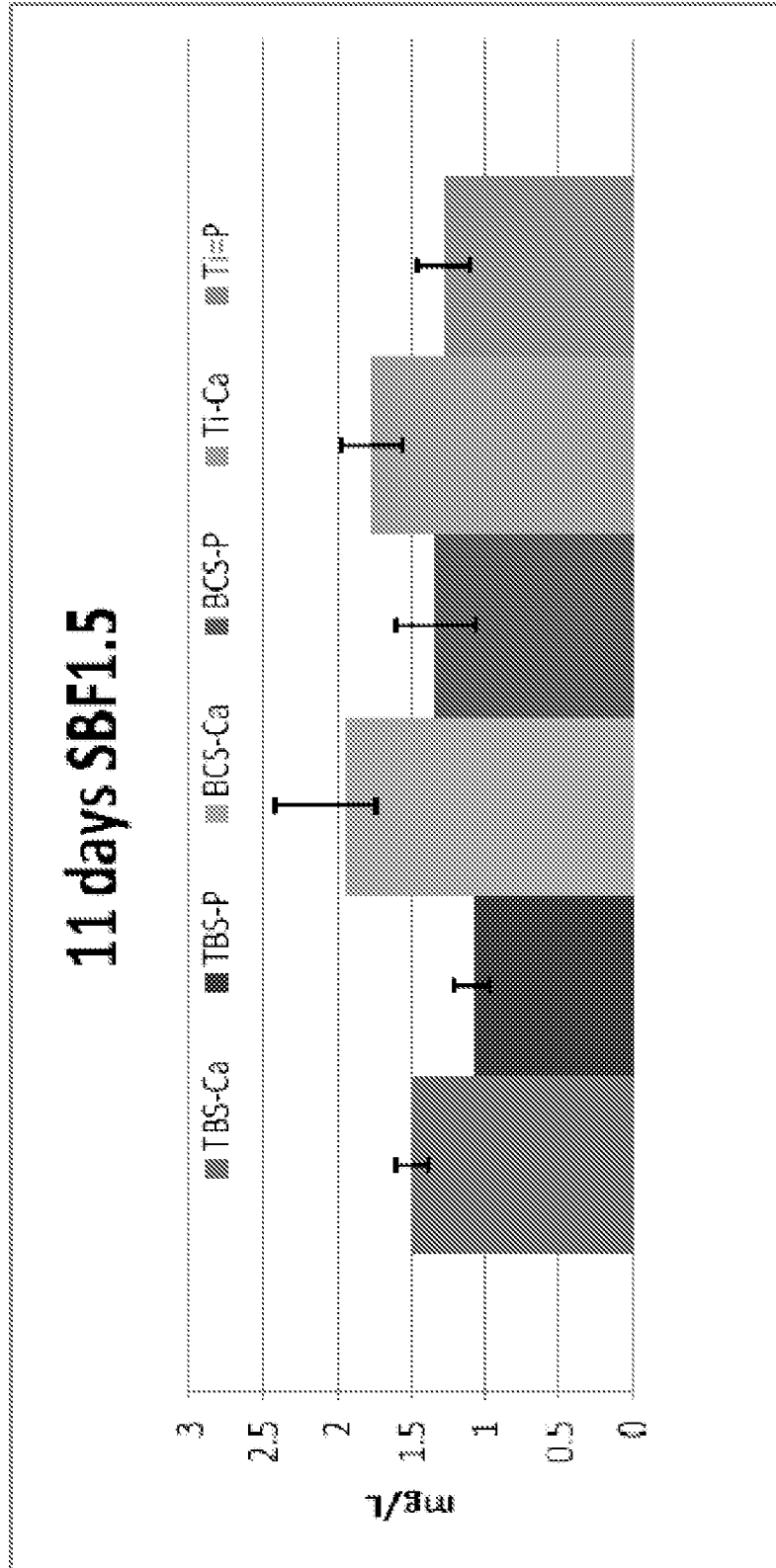


FIG. 8B

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**FIG. 9**



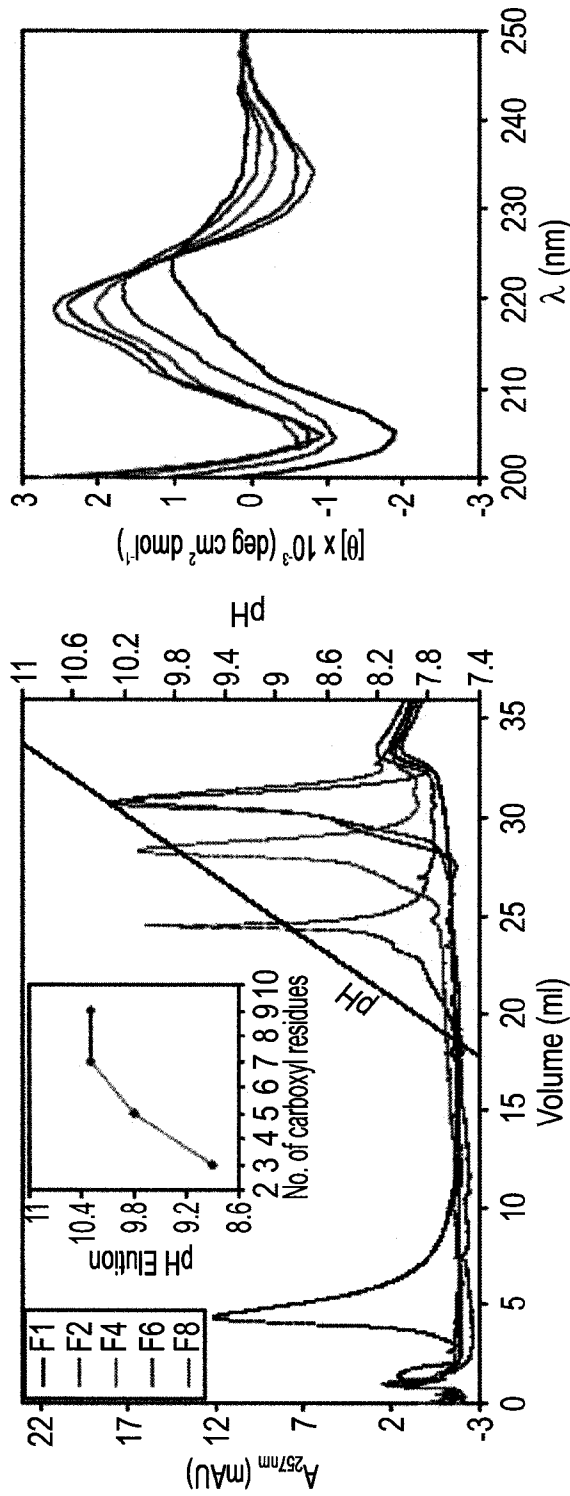
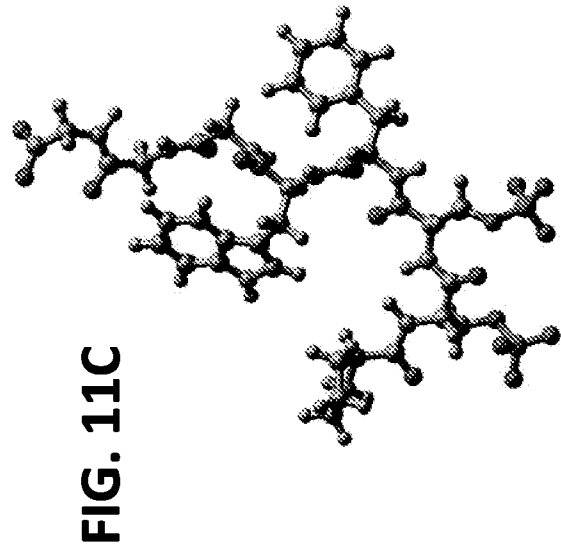
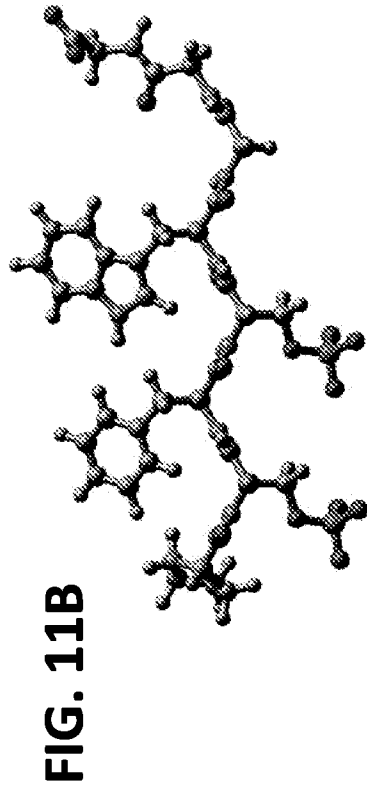
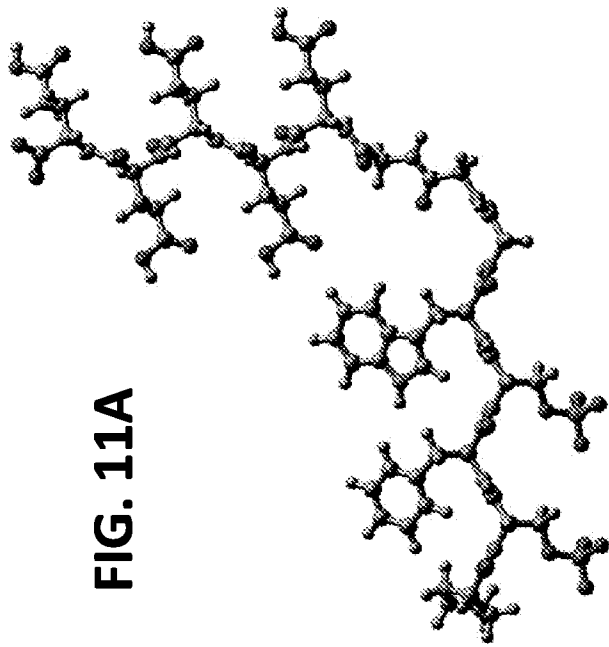


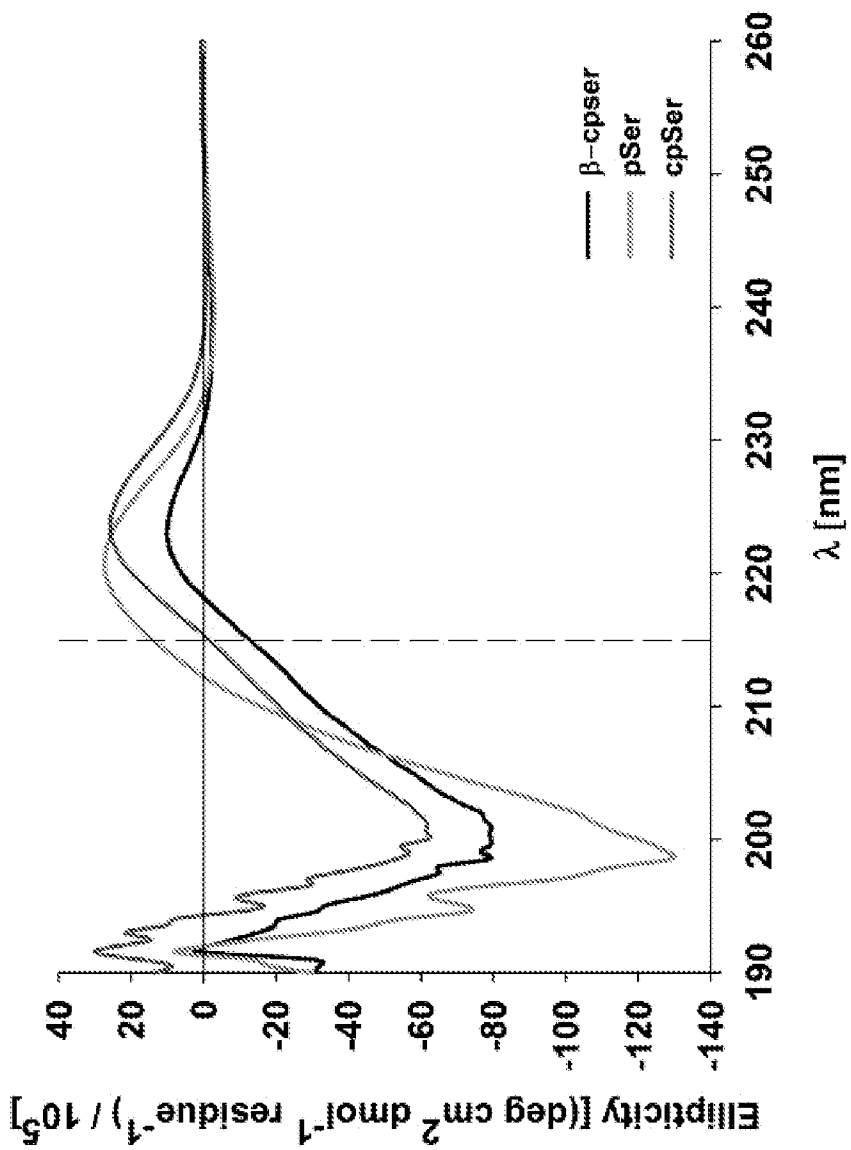
FIG. 10B

FIG. 10A





**FIG. 12**



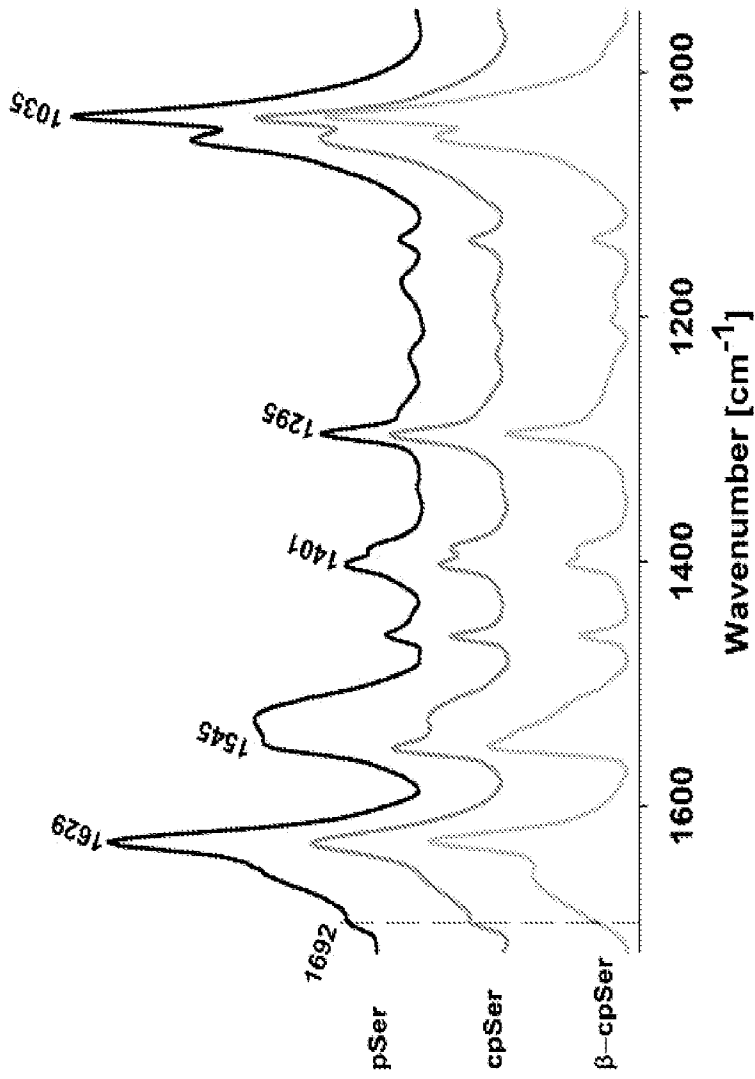


FIG. 13

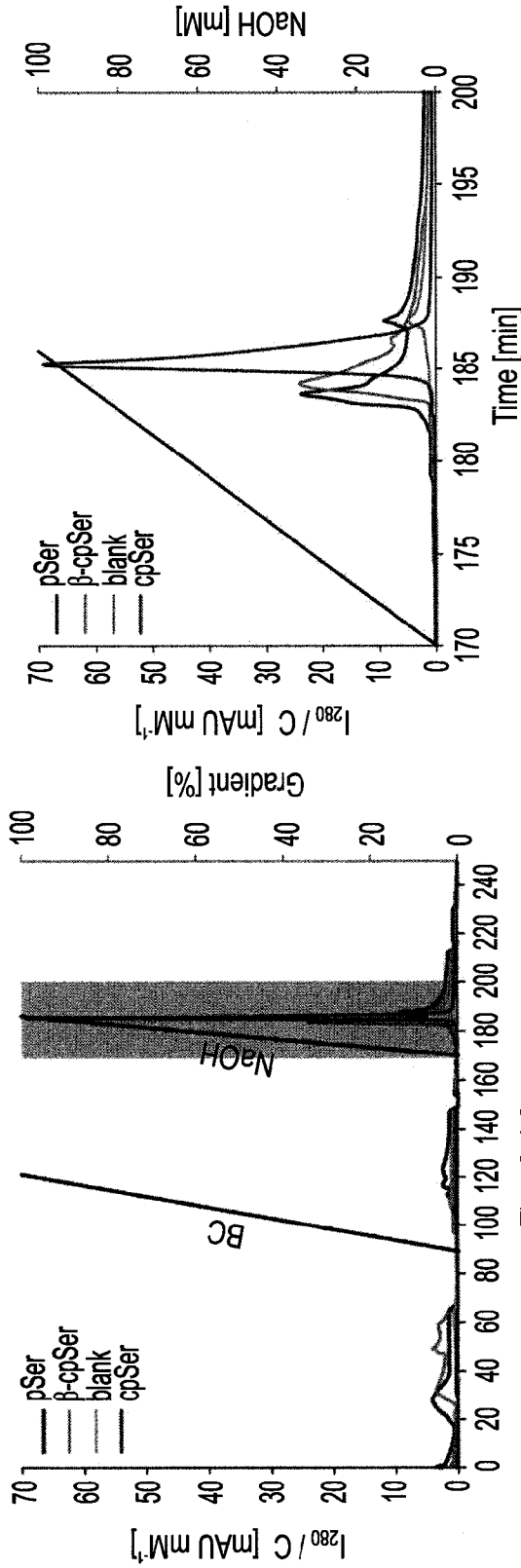


FIG. 14B

FIG. 14A

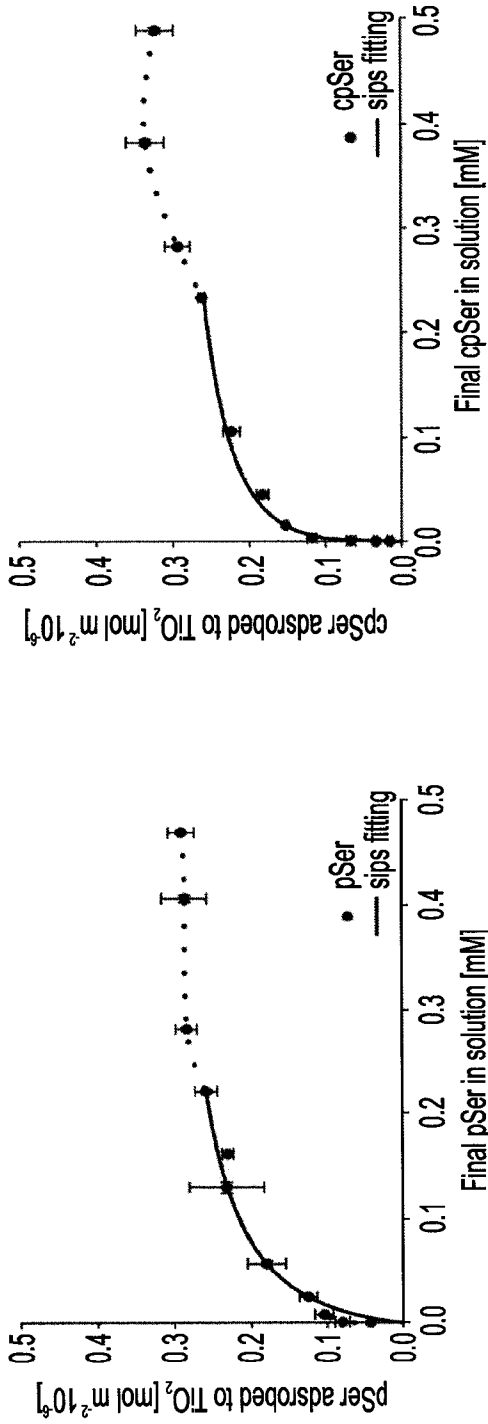


FIG. 15A

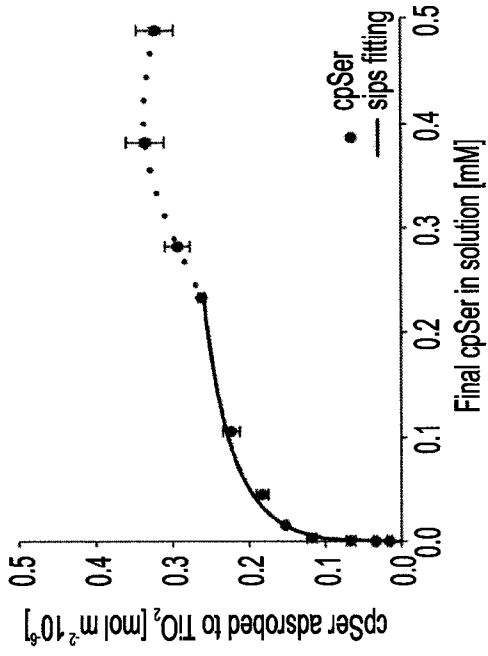


FIG. 15B

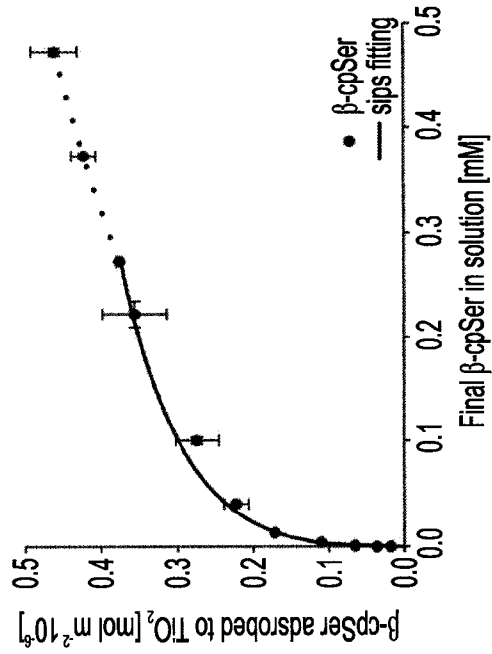
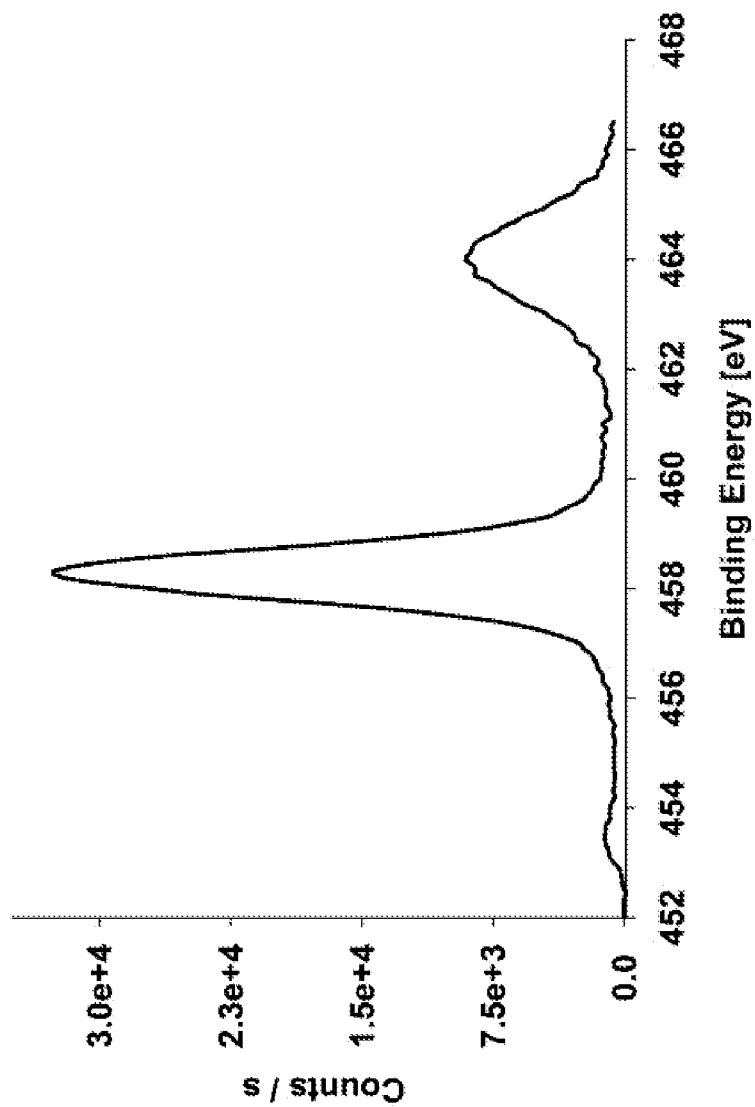
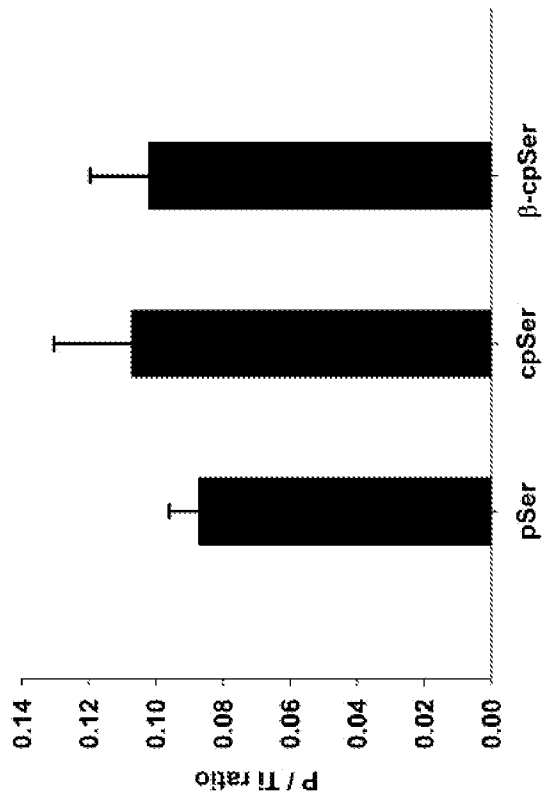


FIG. 15C

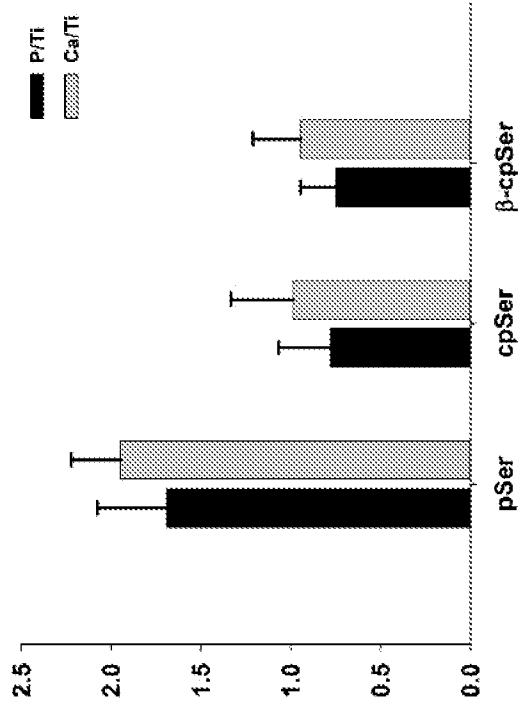
**FIG. 16**



**FIG. 17A**



**FIG. 17B**



INTERNATIONAL SEARCH REPORT

International application No  
PCT/IL2013/050476

A. CLASSIFICATION OF SUBJECT MATTER  
INV. C07K7/08 A61L27/06 A61C8/00  
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)  
A61L A61C A61F C07K  
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, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 661 910 A1 (JAPAN SCIENCE & TECH AGENCY [JP]) 31 May 2006 (2006-05-31)	1,2,5,6, 10,11, 22-26, 30,31, 41-44, 46-48
Y	paragraphs [0005], [0008], [0021], [0032], [0033], [0034], [0039]  -----  -/--	3,4,7, 12-21, 27-29, 32-40,45

Further documents are listed in the continuation of Box C.

See patent family annex.

\* Special categories of cited documents :

<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"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)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"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</p> <p>"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</p> <p>"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</p> <p>"&amp;" document member of the same patent family</p>
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Date of the actual completion of the international search  9 August 2013	Date of mailing of the international search report  22/08/2013
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer  Bonello, Steve

## INTERNATIONAL SEARCH REPORT

International application No

PCT/IL2013/050476

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MEYERS S R ET AL: "Endothelialization of titanium surfaces", ADVANCED MATERIALS, WILEY VCH VERLAG, DE, vol. 19, no. 18, 2 August 2007 (2007-08-02), pages 2492-2498, XP002528796, ISSN: 0935-9648, DOI: 10.1002/ADMA.200700029	1,2,5,6, 10,22, 24,42
Y	page 2492, column 2, paragraph 2 page 2493, column 1, paragraph 2 - column 2, paragraph 2; table 1 page 2495, column 2, paragraph 3 - page 2496, column 1, paragraph 1	3,4,7, 12-21, 27-29, 32-40,45
X	ZREIQAT H ET AL: "Differentiation of human bone-derived cells grown on GRGDSP-peptide bound titanium surfaces", JOURNAL OF BIOMEDICAL MATERIALS RESEARCH, WILEY, NEW YORK, NY, US, vol. 64A, no. 1, 1 January 2003 (2003-01-01), pages 105-113, XP002358271, ISSN: 0021-9304, DOI: 10.1002/JBM.A.10376	1,2,5,6, 8-11,22, 24-26, 30,31, 42,43
Y	page 106, column 1, paragraph 3 - column 2, paragraph 1; table 1 page 110, column 1, paragraph 2 - page 111, column 1, paragraph 1 page 112, column 1, paragraph 1	3,4,7, 12-21, 27-29, 32-40,45
Y	GERTLER GOLAN ET AL: "Characterizing the adsorption of peptides to TiO <sub>2</sub> in aqueous solutions by liquid chromatography.", LANGMUIR : THE ACS JOURNAL OF SURFACES AND COLLOIDS 4 MAY 2010, vol. 26, no. 9, 4 May 2010 (2010-05-04), pages 6457-6463, XP002711087, ISSN: 1520-5827 page 6462, columns 1-2 page 6463	3,4,7, 12-21, 27-29, 32-40,45
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International application No

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Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>RAFFAINI GIUSEPPINA ET AL: "Molecular modelling of protein adsorption on the surface of titanium dioxide polymorphs.", PHILOSOPHICAL TRANSACTIONS. SERIES A, MATHEMATICAL, PHYSICAL, AND ENGINEERING SCIENCES 28 MAR 2012, vol. 370, no. 1963, 28 March 2012 (2012-03-28), pages 1444-1462, XP002711088, ISSN: 1364-503X page 1450, paragraph 1; figure 1 page 1451; figure 2 page 1454, paragraph 2 page 1456 - page 1458 -----</p>	1-48
A	<p>BANEYX ET AL: "Selection and analysis of solid-binding peptides", CURRENT OPINION IN BIOTECHNOLOGY, LONDON, GB, vol. 18, no. 4, 14 September 2007 (2007-09-14), pages 312-317, XP022244964, ISSN: 0958-1669, DOI: 10.1016/J.COPBIO.2007.04.008 page 315, column 1, paragraph 2 -----</p>	1-48

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Information on patent family members

International application No

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