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# Characterization and *in vitro* biological evaluation of mineral/osteogenic growth peptide nanocomposites synthesized biomimetically on titanium

Cen Chen[a], Xiangdong Kong[a], Sheng-Min Zhang[b], In-Seop Lee[a,c,*]

[a] Bio-X Center, College of Life Sciences, Zhejiang Sci-Tech University, Hangzhou 310018, China
[b] Advanced Biomaterials and Tissue Engineering Center, Huazhong University of Science and Technology, Wuhan 430074, China
[c] Institute of Natural Sciences, Yonsei University, Seoul 120-749, Republic of Korea

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ABSTRACT

Nanocomposite layers of mineral/osteogenic growth peptide (OGP) were synthesized on calcium phosphate coated titanium substrates by immersing in calcium-phosphate buffer solution containing OGP. Peptide incorporated mineral was characterized by determining quantity loaded, effects on mineral morphology and structure. Also, the biological activity was investigated by cell adhesion, proliferation assay, and measurement of alkaline phosphatase (ALP) activity. X-ray photoelectron spectroscopy (XPS) and micro-bicinchoninic acid (BCA) assay revealed that OGP was successfully incorporated with mineral and the amount was increased with immersion time. Incorporated OGP changed the mineral morphology from sharp plate-like shape to more rounded one, and the octacalcium phosphate structure of the mineral was gradually transformed into apatite. With confocal microscopy to examine the incorporation of fluorescently labeled peptide, OGP was evenly distributed throughout mineral layers. Mineral/OGP nanocomposites promoted cell adhesion and proliferation, and also increased ALP activity of mesenchymal stem cells (MSCs). Results presented here indicated that the mineral/OGP nanocomposites formed on titanium substrates had the potential for applications in dental implants.



## 1. Introduction

An ideal dental implant would incorporate osteoinductivity and osteoconductivity into the design of the implant biomaterial [1], as well as biocompatibility [2], matched mechanical properties [3], corrosion resistance [4], and the ability to support the bone ingrowths onto an implant [5]. Titanium (Ti) and its alloy has been widely applied to dental implantology owing to their superior mechanical properties [6], good biocompatibility [7]. As their bone forming ability is less than bioactive ceramics, such as calcium phosphates and bioactive glasses [8], a numerous surface modification techniques have been developed. The topographical and biochemical surface modification has become a key factor in the development of improving implant biocompatibility.

Coprecipitation of osteoinductive biomolecules and osteoconductive calcium phosphate (Ca-P) mineral layer on implants is one approach for biochemical surface modification. An important advantage of this approach is the ability to prepare Ca-P mineral layers under a mild condition, therefore leading to preserve biological activity of biomolecules [9]. In addition, the degradation of precipitated mineral layers *in vivo* should result in the gradual exposure and more controlled release of incorporated biomolecules [10]. In our previous studies, coprecipitation of fibronectin [11] and basic fibroblast growth factor [12] with mineral layer on titanium resulted in improving osseointegration of titanium implants *in vitro* and *in vivo*.

Osteogenic growth peptide (OGP, isoelectric point = 11.4, *Mw* = 1.5 kDa) is a short, linear, endogenous 14-mer growth factor peptide (Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly) present in serum at μmol/L concentrations [13]. As a soluble peptide, OGP regulates earlier proliferation, differentiation, and matrix mineralization of osteoblast lineage cells *in vitro* [14], and enhances fracture healing *in vivo* with administration systemically [15].

For the dental implant application, coprecipitated OGP might be more practical than superficially adsorbed OGP due to increases in peptides loading and longer subsequent releasing. Up-to-date, only a few groups have examined the effectiveness of OGP immobilized

* Corresponding author. Address: Institute of Physics & Applied Physics, and Institute of Natural Sciences, Yonsei University, 134 Shinchon-dong, Seodaemoon-gu, Seoul 120-749, Republic of Korea. Tel.: +82 2 2123 4806; fax: +82 2 313 3537.
*E-mail addresses:* inseop@yonsei.ac.kr, inseop2@gmail.com (I.-S. Lee).

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on dental implant substrate, and covalently grafted OGP increased cell density or proliferation [16–18].

In the present study, OGP was coprecipitated with mineral by immersing modified titanium substrates (mTi) in DPBS containing $CaCl_2$. Peptides presence in nanocomposite, the quantity of peptide incorporated, the morphology and structural features of the mineral with incorporated peptides, and the localization of the peptides were examined. The effects of mineral/OGP nanocomposite layer on mesenchymal stem cells (MSCs) were investigated in terms of adhesion, proliferation, and alkaline phosphatase (ALP) activity.

## 2. Materials and methods

### 2.1. Preparation of modified titanium substrate

Commercially pure titanium discs (grade IV, 10 mm in diameter and 2 mm in thickness) with machined surface were used as substrates. Thin calcium phosphate films with a thickness of 500 nm were deposited by ion-beam assisted deposition (IBAD). The IBAD deposited samples were denoted as mTi. Prior to deposition, the substrates were sonicated in acetone and then deionized water. The details of deposition have been described elsewhere [19,20]. Briefly, evaporants of calcium phosphate were prepared by sintering the powder mixtures of CaO (Cerac, USA) and hydroxyapatite (Alfa, USA) at 1200 °C for 2 h in air. For deposition, an electron beam evaporator (Telemark, USA) and an end-hall type ion gun (Commonwealth Scientific, USA) were employed. Heat treatments after the deposition were performed at 350 °C with the heating rate of 5 °C/min and held for 1 h, and then cooled to room temperature in furnace. The thickness of deposited calcium phosphate film was measured by a surface profiler (Model P-10, Tencor, Santa Clara, CA, USA).

### 2.2. Coprecipitation OGP with mineral on CaP coated titanium

Reagent-grade $CaCl_2$ (100 mg/L) were dissolved in Dulbecco's phosphate buffered saline (Calcium/Magnesium free, Gibco-BRL Life Technologies, USA) to prepare DPBS solution. Synthetic OGP (20 μg/mL, GL Biochem, China) was added to DPBS solution to prepare DPBS containing OGP (DPBSO). Each mTi disk was sterilized in 70% ethanol, and distilled water twice, then place under UV light over night. And then the sterilized mTi was immersed into 1.0 mL of DPBS solution or DPBSO solution at 25 °C for 24 h farthest. Treated samples were then rinsed with distilled water twice and air dried at ambient temperature. All the solutions were sterilized by filtration using a membrane with a pore size of 0.20 μm before use.

### 2.3. Peptides presence in composite

The surfaces of samples immersed in DPBS and DPBSO solution were evaluated by X-ray photoelectron spectroscopy (XPS, PHI-5300 ESCA) with A1 Kα X-rays, with the photoelectron take-off angle being set at 45°.

### 2.4. Amount of peptide incorporated

The total amount of OGP incorporated on the mTi was quantified using a micro-bicinchoninic acid (BCA) assay (Pierce, USA). Briefly, 800 μl aliquots of the initial solution and the solution after immersion of the samples ($n = 3$) were mixed with 800 μl of freshly prepared working solution in 24-well plate and incubated at 60 °C for 1 h. After cooling to room temperature, the solutions were transferred to tubes and read at 562 nm using UV/visible spectrophotometer (TU-1810, Persee, China). The absorbance was then converted to OGP concentration using an albumin standard curve. The incorporated OGP was determined by subtracting the residual OGP from the initially added OGP.

### 2.5. Morphology and structural features of the mineral with incorporated OGP

Mineral morphology with or without incorporated OGP was observed using environmental scanning electron microscope (ESEM, Quanta 200, FEI). After immersion the substrates in DPBS or DPBSO, the samples were examined by X-ray diffraction (XRD, X'Pert PRO, PANalytical B.V.) using Cu Kα radiation to analyze the structural features formed on the surfaces.

### 2.6. Localization of OGP in the mineral/OGP nanocomposites

To visualize cross section of OGP spatial distribution, a ratio of 7:1 of OGP:fluorescein isothiocyanate labeled OGP (FITC-OGP) was used. The peptide was incorporated as previously stated in Section 2.2. The effects of different incubation intervals (15, 30 min, and 1, 3, 6, 9, 12, 24 h) were studied. Each prepared sample was glued on a cover slide. A cover slip was glued on the top of sample and then the sample was viewed using confocal microscope (MRC-1024 MP, BioRad) at an excitation wavelength of 488 nm. Using the Bio-Rad Radiance 2000 LaserSharp imaging program, a series of images was taken in 1 μm intervals through approximately 20 μm using 60× magnification oil immersions. A side depth profile through the thickness of the mineral layer was obtained by stacking the series of images.

### 2.7. Cultivation of MSCs

Sprague-Dawle (SD) rat mesenchymal stem cells (MSCs) (Cyagen Biosciences, China) were grown in a low-glucose Dulbecco's modified eagle's medium (LG-DMEM, Gibco-BRL Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL Life Technologies, USA), and an antibiotic-antimycotic (Gibco-BRL Life Technologies, USA). This mixture was employed as a cell culture medium. The cells were incubated at 37 °C in a fully humidified atmosphere of 5% $CO_2$ in air. The culture medium was changed every 3 d until the cells reached 80–100% confluence. To determine the effects of mineral/OGP on adhesion, proliferation, and ALP activity, cells were seeded on the samples immersed in DPBSO solution for 24 h. For control experiments, MSC cells were seeded on the samples immersed in DPBS solution for 24 h.

### 2.8. Attachment of MSCs

Cells were seeded on mineral/OGP or control samples ($n = 6$) at $3 \times 10^5$ cells/cm$^2$ in culture medium and incubated for 4 h at 37 °C with 5% $CO_2$. After 4 h incubation, all non-adherent cells were removed by washing the samples with phosphate buffer solution (PBS) twice. In the quantitative assay, the protein was extracted for the adhered cells with RIPA lysis buffer (Beyotime, China). The amount of cells adhered was determined from the total protein content measured by BCA assay as previously stated. For actin staining, cells were fixed using a solution of 10% neutral buffered formalin for 10 min. The cells were permeabilised using 0.1% Triton X-100 in PBS. An FITC conjugated phalloidin solution (Beyotime, China) was added at a concentration of 10 μg/mL in the 5% bovine serum albumin/PBS (BSA/PBS) for 1 h at room temperature. And the samples were then washed to remove the phalloidin and observed using a confocal microscope in random fields at 60× magnification oil immersions.