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Supplementary Information

Nanoscale graphene coating on commercially pure titanium for accelerated bone regeneration

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EXPERIMENTAL SECTION

Preparation of CP Ti. CP Ti with a diameter of 8 mm was polished with a sand paper 400P for 3 min, 1200P for 5 min, and 2400P for 10 min. Then, CP Ti was polished with a polishing cloth having aluminum oxide suspension for 20 min and colloidal silica for 60 min. The prepared CP Ti samples were washed by sonication for 10 min in acetone, ethanol, and distilled water in sequence.

RGO coating on CP Ti. CP Ti samples were immersed in a piranha solution at a volume ratio of hydrogen peroxide to sulfuric acid = 1/3 for 1 h. After vigorous washing of piranha solution using DI water more than thrice, the samples were dried in a vacuum desiccator. The samples were immersed in 3% (3-aminopropyl)triethoxysilane (APTES) ethanol solution for 1 h on an orbital shaker. After washing with pure ethanol and distilled water thrice to remove unabsorbed APTES, the samples were dried again in a vaccum desiccator. Graphene oxide (GO) solution at a concentration of 4 mg/mL with a lateral size of 0.3-2 μm was poured on

the APTES functionalized CP Ti. Then, GO covered CP Ti samples were spin-coated at 100 rpm for 5 sec and then at 2,000 rpm for 30 sec to obtain GO-coated CP Ti with a uniform thickness. RGO-CP Ti was obtained after chemical reduction of GO-coated CP Ti under hydrazine vapor at 40 ^{o}C for 24 h. RGO-CP Ti samples were vigorously washed with DI water to remove hydrazine molecules under the surface.

Characterization of RGO-CP Ti. The surface morphology of CP Ti, piranha treated CP Ti (oxidized CP Ti), and RGO-CP Ti were visualized by scanning electron microscopy (SEM). The nano morphology of RGO-CP Ti was analyzed by tapping-mode atomic force microscope (AFM, Veeco Multimode Scanning Probe Microscope with the NanoScope IV Controller, Santa Barbara, CA). Elemental analysis of relative titanium, oxygen, and silicone contents was conducted on CP Ti, oxidized CP Ti, and APTES functionalized CP Ti by energy-dispersive spectroscopy (EDS). Carbon-peak analysis was performed on GO coated CP Ti and RGO-CP Ti by X-ray photoelectron spectroscopy (XPS) (ESCALAB 250, Thermo VG Scientific, West Sussex, UK). Contact angles of CP Ti and RGO-CP Ti were measured after dropping 10 μL DI water with a contact angle analyzer (SmartDrop, Femtofab, Korea). To investigate the interface between graphene and Ti, cross-sectional TEM samples were prepared using a focused ion beam (FIB, Helios NanoLab 450S, FEI Inc., Hillsboro, OR, USA) by conventional lift-off technique. Low energy millings with 5 keV and 1 keV were followed to minimize the surface damage. High resolution transmission electron microscopy (HRTEM) and electron energy loss spectroscopy (EELS) were carried out to characterize the morphology and the chemical composition of the interface region using an aberration-corrected TEM (JEOL 2200FS, JEOL Ltd., Tokyo, Japan) operated at 200 kV.

Drug loading test on RGO-CP Ti. Fifty μL of dexamethasone or ascorbic acid solution at a concentration of 100 μg/mL, or bovine serum albumin (BSA) at a concentration of 1

mg/mL was also loaded on RGO-CP Ti for 1 h. Then, supernatant was harvested to determine the drug loading efficiency. The absorbance of dexamethason and ascorbic acid was measured at 260 nm and 250 nm, respectively. The loading concentration was calculated using a standard curve obtained at various concentrations of dexamethasone and ascorbic acid. The BSA loading concentration was determined using protein concentration measurement using Eppendorf Biospectrophotometer (Eppendorf AG, Hamburg, Germany). All the concentrations were calculated by subtracting the initial concentration with that in the supernatant and then averaged using 5 samples.

Cell viability and attachment tests. Cell viability test was performed using the standard MTT assay after 3 days culture of pre-osteoblast MC3T3-E1 cells at a density of 2×10^4 cells per well in 24 well cell culture plate containing 8 mm RGO-CP Ti and CP Ti samples. Two hundred μL of 5 mg/mL MTT solution was added in each well and incubated in a 37^{o}C CO_2 incubator for 1 h. Then, media was exchanged with DMSO and read at 560 nm using a microplate reader (EMax ® microplate reader, Bucher Biotec AG, Basel, Switzerland). Cell attachment was observed with a Leica TCS-SP5-MP-SMD confocal system (Leica Microsystems Wetzlar, Wetzlar, Germany) after F-actin staining using phalloidin-tetramethylrhodamine B isothiocyanate. Briefly, cells were fixed in 4% formaldehyde for 20 min and washed with fresh PBS thrice for each 5 min. Then, cells were immersed in cold 100% acetone for 5 min and washed with PBS thrice for each 5 min. Cells were permeabilized with 0.1% TRITON X-100 in PBS for 30 min and washed again in PBS. Cells were stained with 50 μg/mL phalloidin solution dissolved in PBS and incubated at room temperature for 40 min. After washing with PBS several times, cells were mounted using a DAPI containing gel mount before fluorescence imaging.

***In vivo* osseointegration assessment on the calvarial bone defect of SD rats.** Calvarial bone defect model with a critical defect size of 8 mm was prepared on 6 week old SD rats using a dental bur. For the fixation of 8 mm round Ti samples, polycaprolactone membrane with a diameter of 10 mm was covered on the bone defect followed by the suture of incised regions. Osseointegration was induced for 8 weeks using 5 SD rats for each group. After 8 weeks, calvarial bone tissues were harvested and fixed in 10% formaldehyde solution.

Synchrotron X-ray imaging of osseointegration. Drug loaded RGO-CP Ti, RGO-CP Ti, or CP Ti inserted in the rat calvarial bone defect region was harvested after 8 weeks. Tissues were dehydrated sequentially by immersing in 30%, 50%, 70%, 80%, 90%, 95% and 100% ethanol for 2 h. Microtomography was performed using Pohang light source 6C BMI beamline at the Pohang Accelerator Laboratory (PAL). The samples were mounted on a motor-controllable stage for 360^{o} 3D imaging and the angularly transmitted light was detected with a CCD camera. Detected images were reconstructed based on x, y, z axis for 3D reconstruction. All the imaging procedures were performed under the instruction of PAL.

Histological analysis of regenerated bone. Dehydrated calvarial bone tissues were immersed in a de-calcification solution for 5 days. Then, inserted Ti samples were removed from the bone defect and the calvarial bone tissues were embedded in paraffin block for micro-tissue slice preparation. Tissues samples were sliced at 20 μm thick using a microtome. Paraffin was removed by xylene and standard H&E staining was performed. Collagen staining was conducted using a picrosirious red stain kit (Polyscience, Inc., Warrington, PA) according to the manufacturer's instruction.

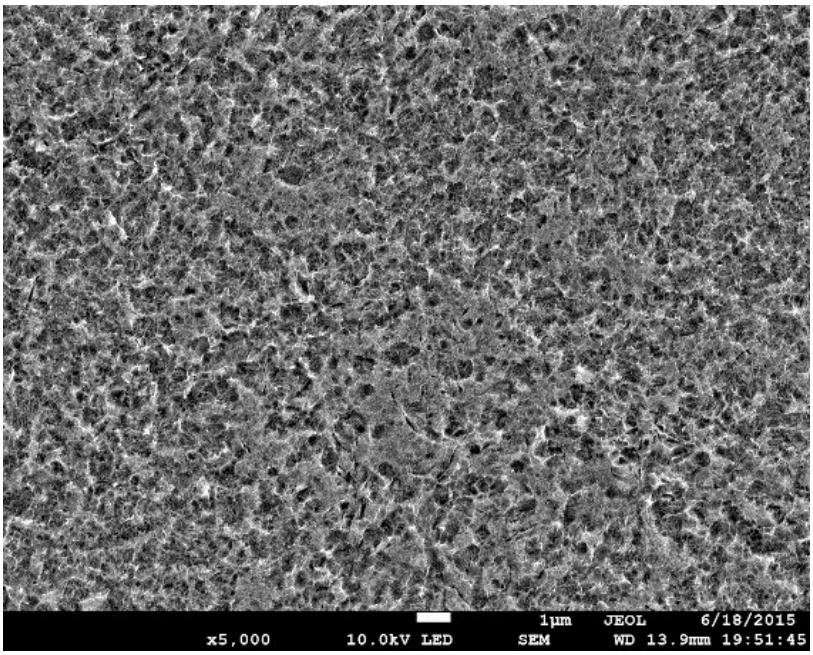

Fig. S1. SEM image of oxidized Ti surface.

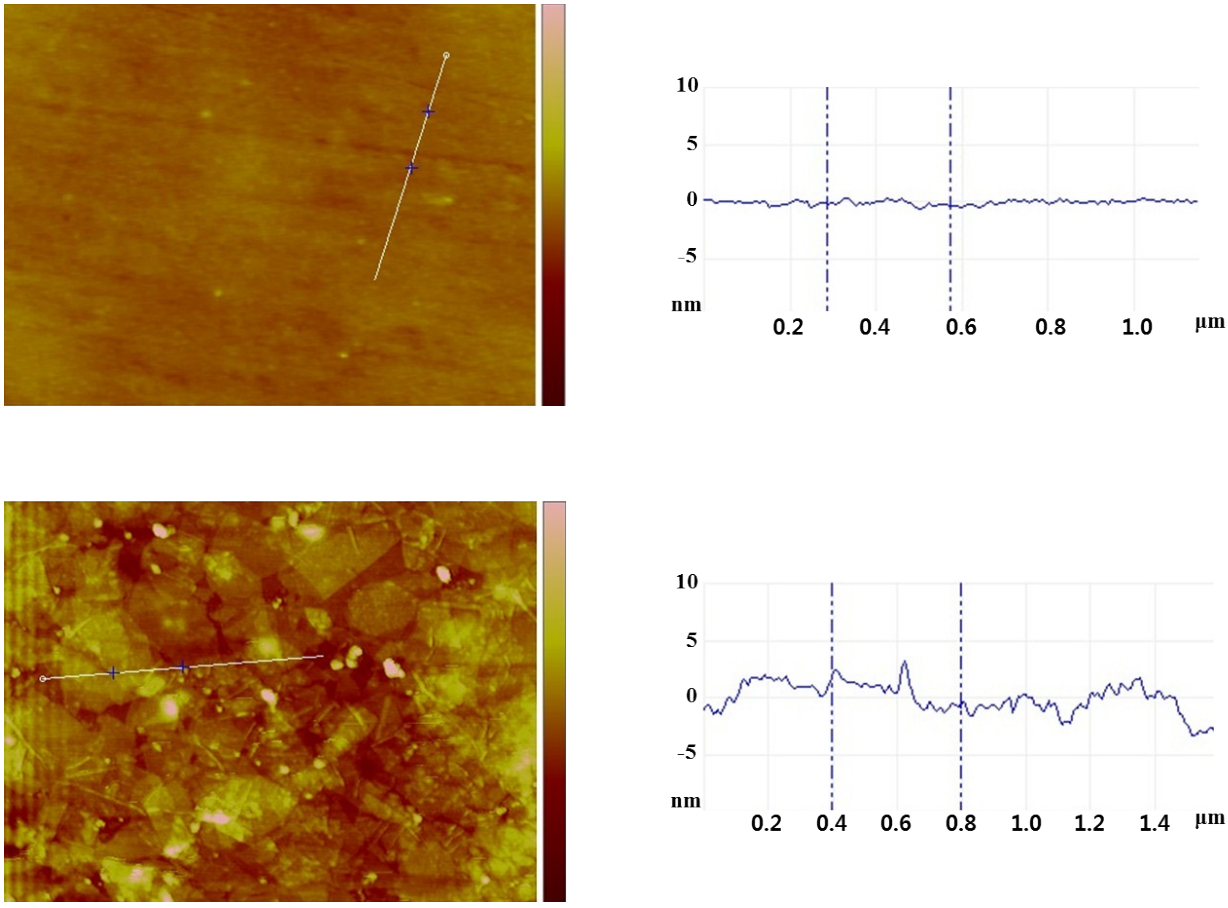

Fig. S2. Cross-sectional AFM images of CP Ti and RGO-CP Ti.

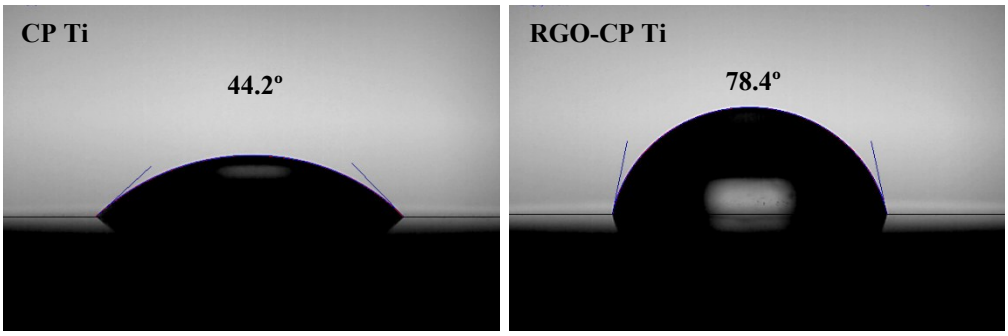

Fig. S3. Contact angle analysis of CP Ti and RGO-CP Ti.

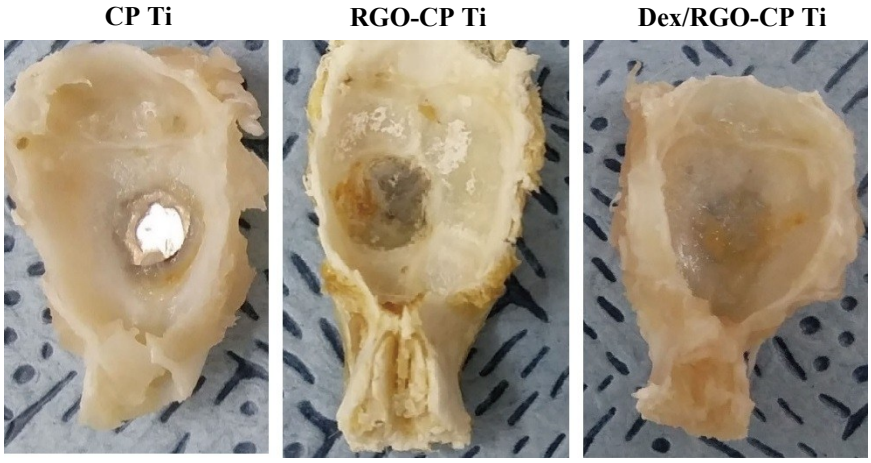

Fig. S4. Photographic images of harvested calvarial bones in SD rats.