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Supplementary information

Chemically heparinized PEEK via a green method to immobilize bone morphogenetic protein-2 (BMP-2) for enhanced osteogenic activity

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Materials and methods

Chemicals and Materials

Polyetheretherketone (PEEK) sheets were purchased from CS Hyde (Lake Villa, IL, USA). Heparin (a sodium salt from porcine intestinal mucosa, MW 12 kDa) was purchased from Cellsus Ins. (Cincinnati, IA, USA). Ethylcarbodiimide hydrochloride (EDC), 1-hydroxy-benzotriazole hydrate (HOBt), L-cysteine 97%, DL-dithiotreitol (DTT), β-Glycerophosphate disodium salt hydrate, L-ascorbic acid, and Alizarin Red S were purchased from Sigma (St. Louis, MO, USA). A PEEK Cage (Ti Window Lumbar cage) was obtained from Genoss. Co. Ltd. (Suwon-si, Korea). Dexamethasone was purchased from Tokyo Chemical Industry (Tokyo, Japan). Recombinant Human/Murine/Rat BMP-2 and Human/Murine/Rat BMP-2 Standard ABTS ELISA Development Kit were purchased from Peprotech (Rocky Hill, NJ, USA). LabAssay ALP was purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan). (1-Hexadecyl)pyridinium chloride monohydrate (98%), Ellman's reagent, and Micro BCA protein assay kit were purchased from ThermoFisher Scientific (Waltham, MA, USA).3.5kDa MW cutoff dialysis membrane was purchased from Spectrum Laboratories, Inc.(Piscataway, NJ, USA).

Synthesis of thiolated heparin

Thiolated heparin was synthesized using a modification of our previous report.[1] Instead of cysteamine, L-cysteine was used as the source of thiol functionality. Briefly, EDC, HOBt, and an excess amount of L-cysteine were added to a 1 wt% heparin aqueous solution, and the reaction was allowed to continue with stirring overnight at room temperature under pH 6.8. Next, the reaction solution was dialyzed using a 3.5kDa dialysis membrane to remove all small unreacted molecules. After that, a 10-fold molar excess of DTT (mole per COOH of heparin) was added to

break the disulfide bond and obtain free thiol groups. This mixture was then adjusted to pH around 8 and reacted for 12 h before being adjusted to pH 3.5 and undergoing dialysis against a dilute HCl (pH 3.5) solution containing 100 mM NaCl. Finally, the thiolated heparin (Hep-CySH) solution was filtered through a 0.2 μm filter membrane and lyophilized. The degree of thiol modification, which can be varied by adjusting the molar ratios among reactants (Table 1), was determined by the Ellman assay. The ^{1}H-Nuclear Magnetic Resonance (NMR) spectrum of Hep-CySH (DOS 40%) was analyzed using an NMR spectrometer (JNM-ECX400, Jeol, Tokyo, Japan). The thiolated heparin used in this study had a degree of conversion of ~40% from carboxylic acid groups to thiol groups.

Ozone Treatment of PEEK sheet for peroxide introduction

The PEEK sheets were washed with 70% ethanol for 20 min, followed by deionized water for another 20 min in an ultra-sonicator and dried under vacuum before use for all experiments. The PEEK sheet was then treated with ozone generated using dried oxygen gas passed through an ozone generator (Ozone generator LAB-I, Ozonetech) with a pressure of 2 bar and an oxygen flow rate of around 0.3 l/min in a pyrex vessel.

The Peroxide density produced by different durations of ozone treatment was analyzed. The PEEK sheet was ozonized for 5, 15, 30, 60, 75, and 90 min. After that, the peroxides produced on the surface of the ozone-treated PEEK sheet were determined spectrophotometrically by the iodide method.[2] Briefly, the treated sheet was placed in benzene-isopropyl alcohol (1:6 (v/v)) containing potassium iodide and 1ppm ferric chloride and kept at 60 ºC for 10 min. After adding water to stop the reaction, the oxidized iodine was measured as a triiodide anion from the absorbance of the solution at 360 nm with the molar absorptivity of 2.3 x 10^4 l/mol cm. The density of peroxide was expressed in mol of peroxide per cm^2 of the PEEK sheet.

TBO assay

The amount of grafted heparin on the PEEK sheet was determined by the TBO assay.[3] Briefly, the sheet was incubated with 200μL of a 0.005% TBO solution in aqueous 0.01M HCl/0.2 wt% NaCl, under gentle shaking at 37 ºC for 1 h. During this process, the Hep/TBO complex formed on the surface of the sheet, and the sheet was rinsed with distilled water. Next, 300 μL of a 4:1 (v/v) mixture of ethanol and aqueous 0.1 M NaOH was added to dissolve the Hep/TBO complex. The supernatant was transferred into a 96-well plate and the absorbance was measured using a microplate reader at 530 nm. The absorbance was used to calculate the amount of immobilized heparin through the standard curve. The amount of heparin was expressed in μg of heparin per cm^2 of the PEEK sheet. A commercial PEEK lumbar bone cage was processed with the same method as the PEEK sheet, from ozonation to heparinization, and stained with TBO to confirm the heparin grafting.

Surface analyses of hPEEK

The surface chemical composition of the heparin-grafted PEEK sheet was analyzed by X-ray photoelectron spectroscopy (NEXSA, Thermo Fisher Scientific). The spectra were recorded with binding energies from 100 eV to 800 eV. Pristine PEEK (PEEK) and only ozone treatment followed by UV irradiation PEEK (oPEEK) were used as controls.

The surface wettability of PEEK, oPEEK, and heparin-grafted PEEK (hPEEK) was measured by using a contact angle goniometer (Phoenix 300, Surface Electro Optics). All samples were fully dried before measurement. The touch mode was applied with a water droplet volume of 2 μL. An average of three individual samples was taken.

The microstructures of PEEK, hPEEK, PEEK/BMP2, and hPEEK/BMP2 were observed using scanning electron microscopy (SEM) (JSM-7500F, Jeol, Tokyo, Japan). The samples were fully dried after BMP2 loading and coated with Pt to be observed.

Cytotoxicity test of PEEK sheets

Cytotoxicity test for PEEK sheets was carried out according to ISO/EN 10993 Part 5 guidelines.[4] To obtain extraction media, the DMEM medium was incubated with PEEK and hPEEK, respectively, for 24 h at 37 ºC. MG-63 cells were seeded into a 48-well plate at concentration 1 x 10^4 cells/well and incubated for 24 h at 37 ºC. After 24 h incubation, the media was replaced with extraction media, and cells were further incubated for another 24 h. Cell viability was analyzed by CCK-8 assay.

Cell proliferation assay

Alamar blue assay was used to study the proliferation of MG-63 cells on PEEK, BMP-2 loaded PEEK (PEEK/BMP2), hPEEK and BMP-2 loaded heparin-grafted PEEK (hPEEK/BMP2). MG-63 cells were seeded at density 1 x 10^4 cells/mL on PEEK samples in a 48-well plate. After 24 h incubation, the culture media was changed to osteogenic inductive medium which was composed of Dulbecco's modified eagle's medium (DMEM) supplement with 10% FBS, 1% antibiotics-antimycotics, 10 nM dexamethasone, 10mM β-Glycerophosphate disodium salt hydrate, and 50 μg/mL L-ascorbic acid. At predetermined time points, the samples were rinsed with phosphate-buffered saline (PBS) then transferred to a new well plate and treated with Alamar blue reagent for 2 h. Subsequently, the incubation solutions were transferred to a 96-well plate, and fluorescence was measured with a microplate reader (Varioskan LUX, Thermofisher Scientific) using excitation/emission wavelengths of 560/590 nm.

Table S1. Reaction conditions for controlling thiolation degree of heparin

Chemical	Molar Ratio			
	Hep-cySH (10)	**Hep-cySH (20)**	**Hep-cySH (30)**	**Hep-cySH (40)**
Heparin (-COOH)	1	1	1	1
EDC	0.5	1	1.75	3.5
HOBT	0.5	1	1.1	2.2
L-cysteine 97%	2	2	2.2	3.3
Thiolation (%)	13.1 ± 1.3	23.7 ± 1.6	31.8 ± 2.7	42.1 ± 5.0

Numbers are the relative molar ratios among reactants.
Hep-cySH (X): X% degree of thiolation among COOH groups of heparin

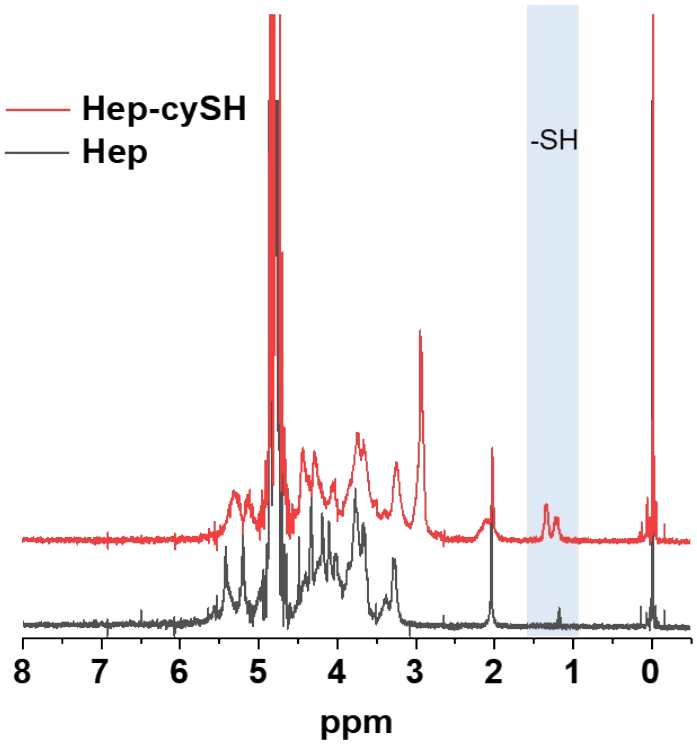

Fig. S1 ^{1}H-NMR spectra of free heparin (Hep) and thiolated heparin (Hep-cySH).

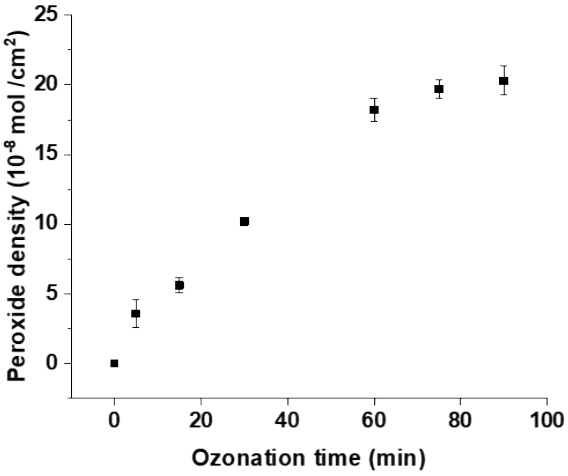

Fig. S2 Generated peroxide density at various ozonation times.

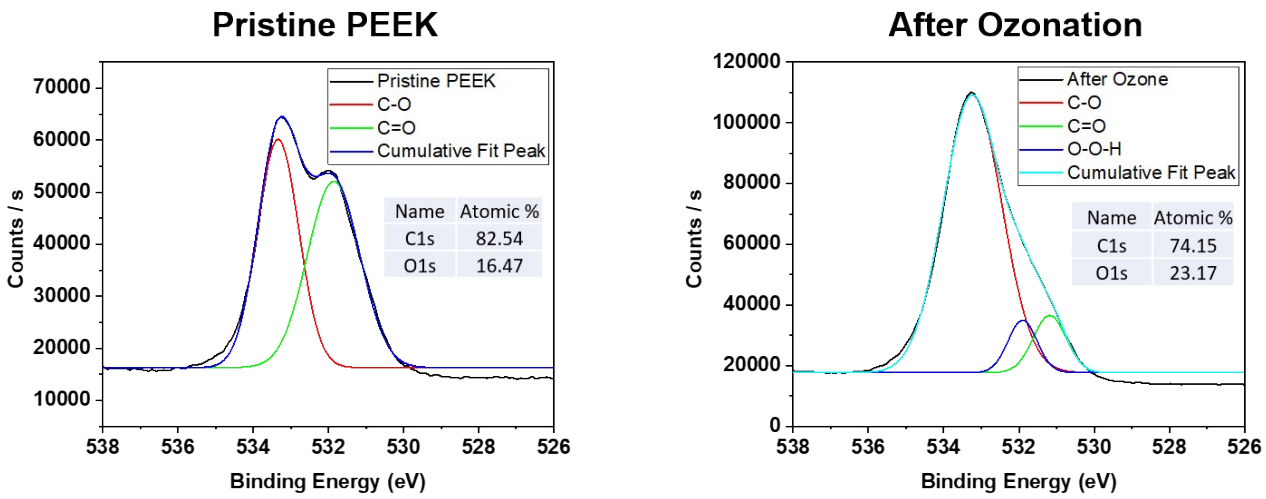

Fig. S3 The O1s core-level spectra of pristine PEEK and PEEK after 60 min ozone treatment. The generation of peroxide group on PEEK after ozone treatment was proved by the appearance of –O-O-H peak as well as the increase of O1s atomic %.

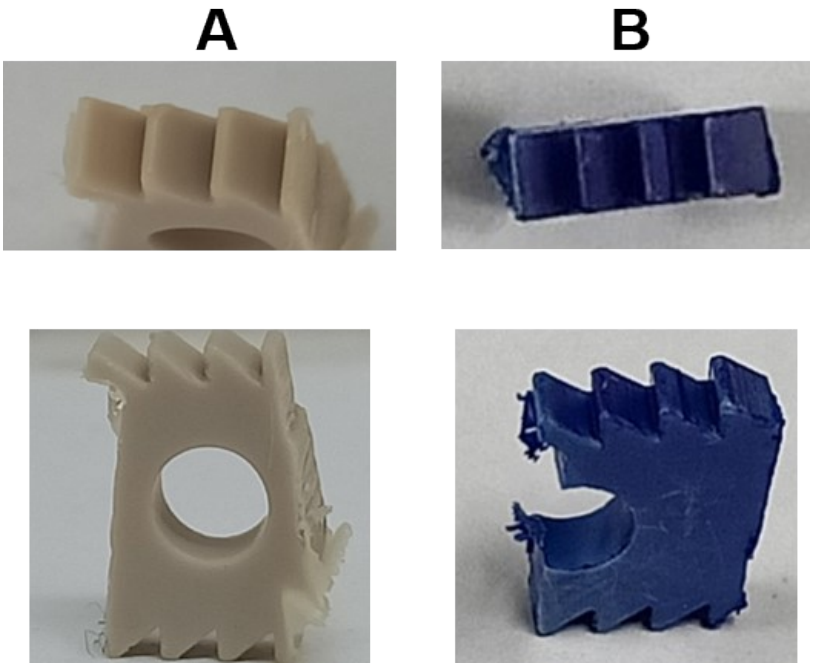

Fig. S4 Toluidine blue O staining of **(A) a** pristine PEEK cage, **(B) the** heparin-grafted PEEK cage.

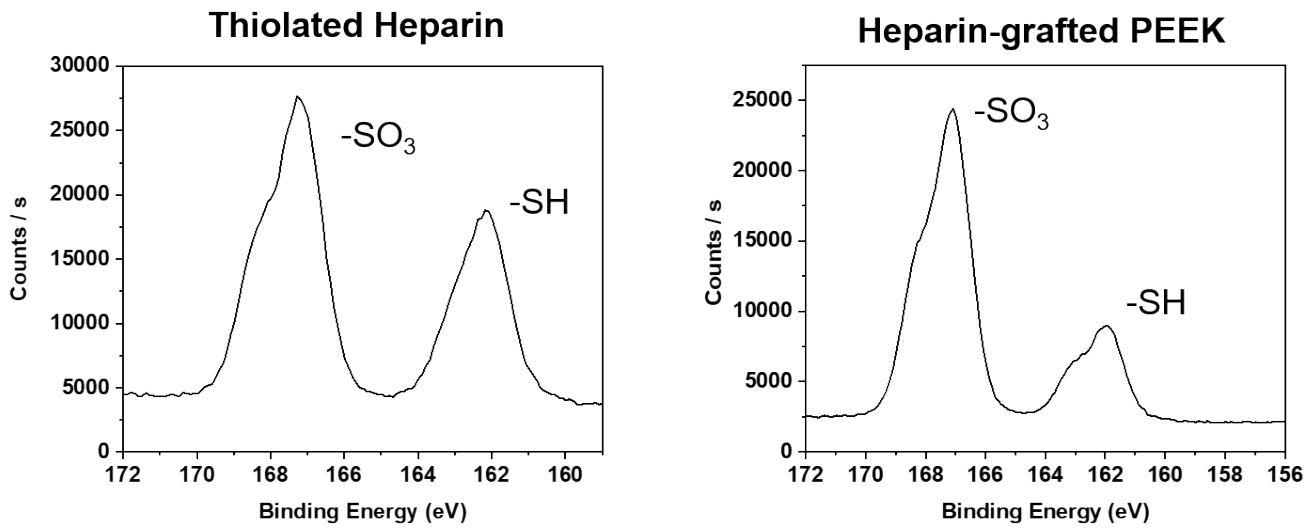

Fig. S5 The S 2p core-level spectra of thiolated heparin and heparin-grafted PEEK. The relative change in the ratio between $–SO_3$ and –SH indicated the chemical conjugation of thiolated heparin.

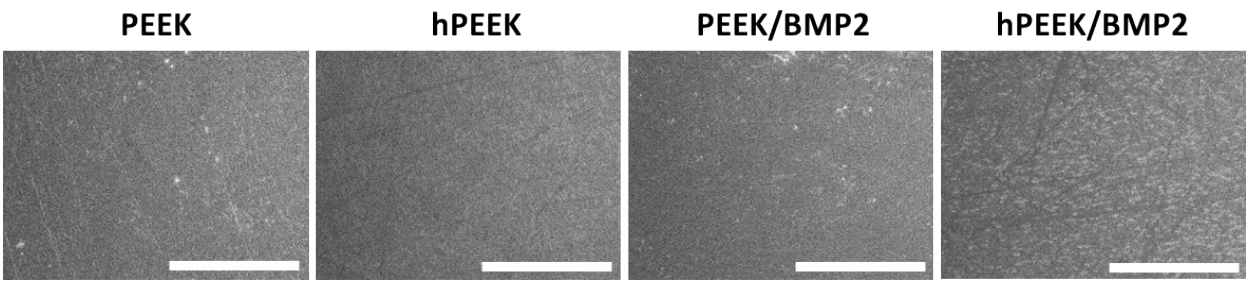

Fig. S6 Scanning electron microscopy (SEM) images of the samples (scale bar = 50 μm).

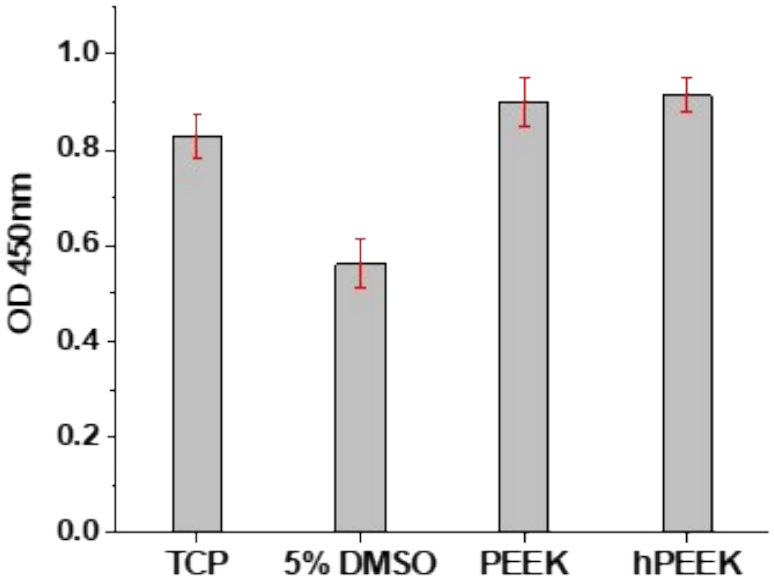

Fig. S7 Cytotoxicity of PEEK and hPEEK against osteoblasts cells (MG-63). 5% DMSO was used as a negative control while TCP was as a positive control.

References

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